THE UNIVERSITY OF MANITOBA

IDENTIFICATION OF REGIONS OF ADENOVIRUS TYPE 5 E1A ONCOGENE INVOLVED IN METASTATIC SUPPRESSION OF T24 *ras*-TRANSFORMED RAT EMBRYO FIBROBLASTS

by

MOHAMMED ASIM ASHIQUE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF HUMAN GENETICS

WINNIPEG, MANITOBA

MAY 1993

ISBN 0-315-86014-6

Canada

Name _____

Dissertation Abstracts International is arranged by broad, general subject categories. Please select the one subject which most nearly describes the content of your dissertation. Enter the corresponding four-digit code in the spaces provided.

HUMAN GENETICS

SUBJECT TERM

0369

SUBJECT CODE

U·M·I

Subject Categories

THE HUMANITIES AND SOCIAL SCIENCES

COMMUNICATIONS AND THE ARTS

Architecture 0729
Art History 0377
Cinema 0900
Dance 0378
Fine Arts 0357
Information Science 0723
Journalism 0391
Library Science 0399
Mass Communications 0708
Music 0413
Speech Communication 0459
Theater 0465

EDUCATION

General 0515
Administration 0514
Adult and Continuing 0516
Agricultural 0517
Art 0273
Bilingual and Multicultural 0282
Business 0688
Community College 0275
Curriculum and Instruction 0727
Early Childhood 0518
Elementary 0524
Finance 0277
Guidance and Counseling 0519
Health 0680
Higher 0745
History of 0520
Home Economics 0278
Industrial 0521
Language and Literature 0279
Mathematics 0280
Music 0522
Philosophy of 0998
Physical 0523
Psychology 0525
Reading 0535
Religious 0527
Sciences 0714
Secondary 0533
Social Sciences 0534
Sociology of 0340
Special 0529
Teacher Training 0530
Technology 0710
Tests and Measurements 0288
Vocational 0747

LANGUAGE, LITERATURE AND LINGUISTICS

Language
- General 0679
- Ancient 0289
- Linguistics 0290
- Modern 0291

Literature
- General 0401
- Classical 0294
- Comparative 0295
- Medieval 0297
- Modern 0298
- African 0316
- American 0591
- Asian 0305
- Canadian (English) 0352
- Canadian (French) 0355
- English 0593
- Germanic 0311
- Latin American 0312
- Middle Eastern 0315
- Romance 0313
- Slavic and East European 0314

PHILOSOPHY, RELIGION AND THEOLOGY

Philosophy 0422
Religion
- General 0318
- Biblical Studies 0321
- Clergy 0319
- History of 0320
- Philosophy of 0322

Theology 0469

SOCIAL SCIENCES

American Studies 0323
Anthropology
- Archaeology 0324
- Cultural 0326
- Physical 0327

Business Administration
- General 0310
- Accounting 0272
- Banking 0770
- Management 0454
- Marketing 0338

Canadian Studies 0385
Economics
- General 0501
- Agricultural 0503
- Commerce-Business 0505
- Finance 0508
- History 0509
- Labor 0510
- Theory 0511

Folklore 0358
Geography 0366
Gerontology 0351
History
- General 0578
- Ancient 0579
- Medieval 0581
- Modern 0582
- Black 0328
- African 0331
- Asia, Australia and Oceania 0332
- Canadian 0334
- European 0335
- Latin American 0336
- Middle Eastern 0333
- United States 0337

History of Science 0585
Law 0398
Political Science
- General 0615
- International Law and Relations 0616
- Public Administration 0617

Recreation 0814
Social Work 0452
Sociology
- General 0626
- Criminology and Penology 0627
- Demography 0938
- Ethnic and Racial Studies 0631
- Individual and Family Studies 0628
- Industrial and Labor Relations 0629
- Public and Social Welfare 0630
- Social Structure and Development 0700
- Theory and Methods 0344

Transportation 0709
Urban and Regional Planning 0999
Women's Studies 0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES

Agriculture
- General 0473
- Agronomy 0285
- Animal Culture and Nutrition 0475
- Animal Pathology 0476
- Food Science and Technology 0359
- Forestry and Wildlife 0478
- Plant Culture 0479
- Plant Pathology 0480
- Plant Physiology 0817
- Range Management 0777
- Wood Technology 0746

Biology
- General 0306
- Anatomy 0287
- Biostatistics 0308
- Botany 0309
- Cell 0379
- Ecology 0329
- Entomology 0353
- Genetics 0369
- Limnology 0793
- Microbiology 0410
- Molecular 0307
- Neuroscience 0317
- Oceanography 0416
- Physiology 0433
- Radiation 0821
- Veterinary Science 0778
- Zoology 0472

Biophysics
- General 0786
- Medical 0760

EARTH SCIENCES

Biogeochemistry 0425
Geochemistry 0996
Geodesy 0370
Geology 0372
Geophysics 0373
Hydrology 0388
Mineralogy 0411
Paleobotany 0345
Paleoecology 0426
Paleontology 0418
Paleozoology 0985
Palynology 0427
Physical Geography 0368
Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES

Environmental Sciences 0768
Health Sciences
- General 0566
- Audiology 0300
- Chemotherapy 0992
- Dentistry 0567
- Education 0350
- Hospital Management 0769
- Human Development 0758
- Immunology 0982
- Medicine and Surgery 0564
- Mental Health 0347
- Nursing 0569
- Nutrition 0570
- Obstetrics and Gynecology 0380
- Occupational Health and Therapy 0354
- Ophthalmology 0381
- Pathology 0571
- Pharmacology 0419
- Pharmacy 0572
- Physical Therapy 0382
- Public Health 0573
- Radiology 0574
- Recreation 0575
- Speech Pathology 0460
- Toxicology 0383

Home Economics 0386

PHYSICAL SCIENCES

Pure Sciences

Chemistry
- General 0485
- Agricultural 0749
- Analytical 0486
- Biochemistry 0487
- Inorganic 0488
- Nuclear 0738
- Organic 0490
- Pharmaceutical 0491
- Physical 0494
- Polymer 0495
- Radiation 0754

Mathematics 0405
Physics
- General 0605
- Acoustics 0986
- Astronomy and Astrophysics 0606
- Atmospheric Science 0608
- Atomic 0748
- Electronics and Electricity 0607
- Elementary Particles and High Energy 0798
- Fluid and Plasma 0759
- Molecular 0609
- Nuclear 0610
- Optics 0752
- Radiation 0756
- Solid State 0611

Statistics 0463

Applied Sciences

Applied Mechanics 0346
Computer Science 0984
Engineering
- General 0537
- Aerospace 0538
- Agricultural 0539
- Automotive 0540
- Biomedical 0541
- Chemical 0542
- Civil 0543
- Electronics and Electrical 0544
- Heat and Thermodynamics 0348
- Hydraulic 0545
- Industrial 0546
- Marine 0547
- Materials Science 0794
- Mechanical 0548
- Metallurgy 0743
- Mining 0551
- Nuclear 0552
- Packaging 0549
- Petroleum 0765
- Sanitary and Municipal 0554
- System Science 0790

Geotechnology 0428
Operations Research 0796
Plastics Technology 0795
Textile Technology 0994

PSYCHOLOGY

General 0621
Behavioral 0384
Clinical 0622
Developmental 0620
Experimental 0623
Industrial 0624
Personality 0625
Physiological 0989
Psychobiology 0349
Psychometrics 0632
Social 0451

Nom _____

Dissertation Abstracts International est organisé en catégories de sujets. Veuillez s.v.p. choisir le sujet qui décrit le mieux votre thèse et inscrivez le code numérique approprié dans l'espace réservé ci-dessous.

SUJET

CODE DE SUJET

U·M·I

Catégories par sujets

HUMANITÉS ET SCIENCES SOCIALES

COMMUNICATIONS ET LES ARTS

Architecture 0729
Beaux-arts 0357
Bibliothéconomie 0399
Cinéma 0900
Communication verbale 0459
Communications 0708
Danse 0378
Histoire de l'art 0377
Journalisme 0391
Musique 0413
Sciences de l'information 0723
Théâtre 0465

ÉDUCATION

Généralités 515
Administration 0514
Art 0273
Collèges communautaires 0275
Commerce 0688
Économie domestique 0278
Éducation permanente 0516
Éducation préscolaire 0518
Éducation sanitaire 0680
Enseignement agricole 0517
Enseignement bilingue et multiculturel 0282
Enseignement industriel 0521
Enseignement primaire. 0524
Enseignement professionnel 0747
Enseignement religieux 0527
Enseignement secondaire 0533
Enseignement spécial 0529
Enseignement supérieur 0745
Évaluation 0288
Finances 0277
Formation des enseignants 0530
Histoire de l'éducation 0520
Langues et littérature 0279
Lecture 0535
Mathématiques 0280
Musique 0522
Orientation et consultation 0519
Philosophie de l'éducation 0998
Physique 0523
Programmes d'études et enseignement 0727
Psychologie 0525
Sciences 0714
Sciences sociales 0534
Sociologie de l'éducation 0340
Technologie 0710

LANGUE, LITTÉRATURE ET LINGUISTIQUE

Langues
- Généralités 0679
- Anciennes 0289
- Linguistique 0290
- Modernes 0291

Littérature
- Généralités 0401
- Anciennes 0294
- Comparée 0295
- Médiévale 0297
- Moderne 0298
- Africaine 0316
- Américaine 0591
- Anglaise 0593
- Asiatique 0305
- Canadienne (Anglaise) 0352
- Canadienne (Française) 0355
- Germanique 0311
- Latino-américaine 0312
- Moyen-orientale 0315
- Romane 0313
- Slave et est-européenne 0314

PHILOSOPHIE, RELIGION ET THÉOLOGIE

Philosophie 0422
Religion
- Généralités 0318
- Clergé 0319
- Études bibliques 0321
- Histoire des religions 0320
- Philosophie de la religion 0322

Théologie 0469

SCIENCES SOCIALES

Anthropologie
- Archéologie 0324
- Culturelle 0326
- Physique 0327

Droit 0398
Économie
- Généralités 0501
- Commerce-Affaires 0505
- Économie agricole 0503
- Économie du travail 0510
- Finances 0508
- Histoire 0509
- Théorie 0511

Études américaines 0323
Études canadiennes 0385
Études féministes 0453
Folklore 0358
Géographie 0366
Gérontologie 0351
Gestion des affaires
- Généralités 0310
- Administration 0454
- Banques 0770
- Comptabilité 0272
- Marketing 0338

Histoire
- Histoire générale 0578
- Ancienne 0579
- Médiévale 0581
- Moderne 0582
- Histoire des noirs 0328
- Africaine 0331
- Canadienne 0334
- États-Unis 0337
- Européenne 0335
- Moyen-orientale 0333
- Latino-américaine 0336
- Asie, Australie et Océanie 0332

Histoire des sciences 0585
Loisirs 0814
Planification urbaine et régionale 0999
Science politique
- Généralités 0615
- Administration publique 0617
- Droit et relations internationales 0616

Sociologie
- Généralités 0626
- Aide et bien-âtre social 0630
- Criminologie et établissements pénitentiaires 0627
- Démographie 0938
- Études de l' individu et de la famille 0628
- Études des relations interethniques et des relations raciales 0631
- Structure et développement social 0700
- Théorie et méthodes. 0344
- Travail et relations industrielles 0629

Transports 0709
Travail social 0452

SCIENCES ET INGÉNIERIE

SCIENCES BIOLOGIQUES

Agriculture
- Généralités 0473
- Agronomie. 0285
- Alimentation et technologie alimentaire 0359
- Culture 0479
- Élevage et alimentation 0475
- Exploitation des péturages 0777
- Pathologie animale 0476
- Pathologie végétale 0480
- Physiologie végétale 0817
- Sylviculture et faune 0478
- Technologie du bois 0746

Biologie
- Généralités 0306
- Anatomie 0287
- Biologie (Statistiques) 0308
- Biologie moléculaire 0307
- Botanique 0309
- Cellule 0379
- Écologie 0329
- Entomologie 0353
- Génétique 0369
- Limnologie 0793
- Microbiologie 0410
- Neurologie 0317
- Océanographie 0416
- Physiologie 0433
- Radiation 0821
- Science vétérinaire 0778
- Zoologie 0472

Biophysique
- Généralités 0786
- Medicale 0760

SCIENCES DE LA TERRE

Biogéochimie 0425
Géochimie 0996
Géodésie 0370
Géographie physique 0368
Géologie 0372
Géophysique 0373
Hydrologie 0388
Minéralogie 0411
Océanographie physique 0415
Paléobotanique 0345
Paléoécologie 0426
Paléontologie 0418
Paléozoologie 0985
Palynologie 0427

SCIENCES DE LA SANTÉ ET DE L'ENVIRONNEMENT

Économie domestique 0386
Sciences de l'environnement 0768
Sciences de la santé
- Généralités 0566
- Administration des hipitaux 0769
- Alimentation et nutrition 0570
- Audiologie 0300
- Chimiothérapie 0992
- Dentisterie 0567
- Développement humain 0758
- Enseignement 0350
- Immunologie 0982
- Loisirs 0575
- Médecine du travail et thérapie 0354
- Médecine et chirurgie 0564
- Obstétrique et gynécologie 0380
- Ophtalmologie 0381
- Orthophonie 0460
- Pathologie 0571
- Pharmacie 0572
- Pharmacologie 0419
- Physiothérapie 0382
- Radiologie 0574
- Santé mentale 0347
- Santé publique 0573
- Soins infirmiers 0569
- Toxicologie 0383

SCIENCES PHYSIQUES

Sciences Pures

Chimie
- Généralités 0485
- Biochimie 487
- Chimie agricole 0749
- Chimie analytique 0486
- Chimie minérale 0488
- Chimie nucléaire 0738
- Chimie organique 0490
- Chimie pharmaceutique 0491
- Physique 0494
- PolymÇres 0495
- Radiation 0754

Mathématiques 0405
Physique
- Généralités 0605
- Acoustique 0986
- Astronomie et astrophysique 0606
- Electronique et électricité 0607
- Fluides et plasma 0759
- Météorologie 0608
- Optique 0752
- Particules (Physique nucléaire) 0798
- Physique atomique 0748
- Physique de l'état solide 0611
- Physique moléculaire 0609
- Physique nucléaire 0610
- Radiation 0756

Statistiques 0463

Sciences Appliqués Et Technologie

Informatique 0984
Ingénierie
- Généralités 0537
- Agricole 0539
- Automobile 0540
- Biomédicale 0541
- Chaleur et ther modynamique 0348
- Conditionnement (Emballage) 0549
- Génie aérospatial 0538
- Génie chimique 0542
- Génie civil 0543
- Génie électronique et électrique 0544
- Génie industriel 0546
- Génie mécanique 0548
- Génie nucléaire 0552
- Ingénierie des systÇmes 0790
- Mécanique navale 0547
- Métallurgie 0743
- Science des matériaux 0794
- Technique du pétrole 0765
- Technique minière 0551
- Techniques sanitaires et municipales 0554
- Technologie hydraulique 0545

Mécanique appliquée 0346
Géotechnologie 0428
Matières plastiques (Technologie) 0795
Recherche opérationnelle 0796
Textiles et tissus (Technologie) 0794

PSYCHOLOGIE

Généralités 0621
Personnalité 0625
Psychobiologie 0349
Psychologie clinique 0622
Psychologie du comportement 0384
Psychologie du développement 0620
Psychologie expérimentale 0623
Psychologie industrielle 0624
Psychologie physiologique 0989
Psychologie sociale 0451
Psychométrie 0632

IDENTIFICATION OF REGIONS OF ADENOVIRUS TYPE 5 E1A ONCOGENE INVOLVED IN METASTATIC SUPPRESSION OF T24 *ras*-TRANSFORMED RAT EMBRYO FIBROBLASTS

BY

MOHAMMAD ASIM ASHIQUE

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

To a great man, my dear father,

for dedicating his life to his children.

OBJECTIVITY

ACKNOWLEDGEMENTS

I would like to express my appreciation to many individuals with whom I have enjoyed working during the course of this project.

Several individuals have been very helpful in providing both technical and conceptual advice relating to my project. In this regard, I would like to offer a very special thank you to Jackie Damen, Geoff Hicks, Robert Hurta, Shanti Samuel, Nancy Stewart and William Taylor.

The metastasis group has been very beneficial in helping me to maintain direction and focus during the growth of this project. Thanks to Pardeep Bhatia, Feng Deng, William "Billster" Taylor, Ping Yang, Michael Mowat, Jim Wright, and especially to Arnold Greenberg, for constructive criticism and much needed support.

Thank you to my supervisor Dr. Michael Mowat for any and all effort made in the way of this project.

I have received excellent technical assistance from Lenka Jarolim, Mary Sauder, Arthur Chan, and Paul Moreira. I must thank Donna Chornenki who has been very helpful in providing me with assistance in obtaining the literature I have required.

Throughout my stay here I have greatly valued the company of my labmates. Most have been supportive and hard-working, but fun-loving at the same time. A very special thank you to my friend Lenka Jarolim whose intellectually stimulating and good-natured mannerism has been invaluable. Thanks also to

MICB's "metal queen" Nancy "Nanster" Stewart, Wenzheng "Wild Thang" Yang; and a late addition to our group Bruce "Yellow" Schlatter who has been downright cool. Special thanks to my buddy Lisa Andres (a.k.a. Grasshopper) who brought something to our lab that had been sadly missing for a long time, a positive attitude. I will miss you all.

A big thank you to a couple of cool characters, Susan Snusher and Agnes Warkentin for friendly chats and for being encouraging and supportive when others were not.

I would like to thank everyone over at the Department of Human Genetics, especially Michael Carpenter. Other individuals I must thank include Mohamed Abdelhaleem, Bob Choy, Genevieve Curpen, Christine Hall, and Dr. Chidi Ngwu from Ed Harlow's lab for the hybridomas, supernatants, and E1A expression vectors I have required for this work. Thanks to Dr. Lance Liotta and Dr. Frank Graham for the cell lines.

I would like to express my appreciation to all members of my immediate family, Meera Chohan, Winston Duncan and Dennis Smith for their love, understanding, support and friendship.

Most of all, I thank Allah (God) for giving me the strength and patience that I needed to successfully complete this project.

"With your bitch slap rappin' and your cocaine tongue you get nothin' done!"

ABSTRACT

Metastasis is a complex process that results in the spread of tumor cells to secondary sites in the host organism. The expression of several genes that encode proteins with varying roles in normal cellular metabolism has been associated with development of the metastatic phenotype in mammalian cells. Previous work has correlated oncogene expression, including that of activated H-ras (T24 ras), with enhanced metastatic potential. Human adenovirus type 5 E1A is a well documented oncogene that encodes two major mRNA species of 13S and 12S from two exons resulting in the production of polypeptides of 289 and 243 amino acids, respectively. Three regions have been conserved in the larger protein with an internal 46 amino acid region corresponding to conserved domain 3 missing in the smaller protein. E1A positively and negatively regulates transcription of several viral and cellular genes. Transfection of highly metastatic T24 ras-expressing 5R cells with human adenovirus type 5 E1A-expressing vectors results in the suppression of metastasis. Since E1A is a potent oncogene it is paradoxical that it suppresses expression of a progressed phenotype induced by a second oncogene. This work attempted to better understand the suppressive mechanism operating in E1A-expressing cells by mapping the region(s) required for suppression of metastasis in 5R. Transfection of 5R with expression vectors encoding wild-type and mutant E1A polypeptides, and subsequent evaluation of metastatic capabilities of the resulting cell

lines, revealed that sequences located at the N-terminus and those encoded by exon 2 of E1A are involved in this process. In contrast to the findings of previous studies only the 13S mRNA protein product, and not the 12S product, was capable of eliciting the suppressive effect. The fact that conserved domain 3 is an undisputed transcriptional activator suggested that an E1A-responsive cellular gene was inducing the suppressive effect. The N- and C-terminal regions required for maintenance of the suppressive effect also encode transactivation functions. Further investigation revealed elevated T24 ras expression in the highly metastatic E1A-expressing cell lines, while cell lines with low metastatic potentials, including that expressing the wild-type 13S product, exhibited reduced T24 ras expression relative to 5R. Statistical analysis revealed a direct correlation between the level of T24 ras protein, $p21^{ras}$, and the metastatic potential of the cell lines. The results presented here preclude the binding of E1A to the RB tumor suppressor product, $p105^{RB}$, and to other well known cellular polypeptides from involvement in metastatic suppression. They also suggest that transcriptional repression functions of E1A are not involved in down-regulation of T24 ras induced metastasis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

1) Oncogenic transformation of mammalian cells.

Oncogenes were first described as retrovirus-encoded genes with tumorigenic capabilities in host animals. These genes were subsequently found to be dominant mutated forms of normal host genes (proto-oncogenes) that had been transduced by retroviruses. Genetic damage to proto-oncogenes is causally related to the phenomenon of cellular transformation in mammalian cells (Bishop, 1987, 1991). Such mutations are referred to as dominant and result in a gain of function. Most proto-oncogenes encode proteins that are involved in the cascade of events by which growth factors stimulate normal cell division (reviewed by Bishop, 1991).

Oncogenes encode proteins that typically fall into four categories: growth factors (eg. sis), growth factor receptors (erbB, fms, kit), tranducers of growth factor responses (src, ras, raf) and nuclear transcriptional regulators that are involved in growth factor induced gene expression (jun, fos). Generally, the transcription factor type oncogenes accomplish transformation by cooperating with oncogenes from other categories (reviewed by Hunter, 1991). The total number of oncogenes identified to date is about 60 (see Varmus, 1987).

Transformation can also occur by recessive mutation. This involves a class of genes referred to as anti-oncogenes or tumor-suppressor genes and results in a loss of function. Although numerous tumor-suppressor genes are currently known (see Table 1), the most extensively studied ones are the

Table 1. Representative tumor suppressor genes incriminated in human tumors.

Tumor Suppressor Gene	Chromosomal Locus	Neoplasm(s)
RB	13q14	Retinoblastoma; osteosarcoma; carcinoma of breast, bladder and lung
P53	17q12-13.3	Astrocytoma; osteosarcoma; carcinoma breast, colon and lung
WT1	11p13	Wilms' tumor
?	11p15.5	Wilms' tumor
DCC	18q21	Colon carcinoma
NF1	17q11.2	Neurofibromatosis type 1
FAP	5q21-22	Colon carcinoma
MEN-1	11q13	Tumors of parathyroid, pancreas, pituitary and adrenal cortex
?	16q22.1-23.2	Liver carcinoma
?	3p21	Lung carcinoma
?	3p12-14	Kidney carcinoma
?	1p36.1	Neuroblastoma

Question marks are used where no nomenclature has been adopted. Taken from Bishop (1991).

retinoblastoma tumor suppressor (RB) and p53.

Although the functions of RB and p53 are not fully understood it is clear that their protein products play critical roles in regulation of the cell cycle. This is based on the finding that the active levels of both proteins fluctuate in a cell cycle-dependent manner (Bischoff et al., 1990; Weinberg, 1990). Also several DNA tumor viruses (SV40, papillomavirus, polyomavirus and adenovirus) produce proteins that accomplish cell immortalization by targeting these same two proteins (Buchkovich et al., 1990; Weinberg, 1990). Since tumor-suppressor genes are not of specific relevance to the work described here they will not be discussed in any further detail, although a significant amount of in depth information exists in the area (for review see Marshall, 1991a).

Evidence shows that virtually all forms of genetic damage found in human cancers occur spontaneously in cultured cells (Meuth, 1990). Dominant mutations in proto-oncogenes result in sustained or augmented activity, caused by either changes in gene expression, usually overexpression, or mutations within the gene products that result in a loss of control of their biochemical activities. Most, if not all, mutations described so far lead to a change in the level of activity of the oncogene product, as opposed to a change in the function or specificity of the protein such as a change in substrate. Quantitative change as opposed to qualitative change. The three main molecular events leading to conversion of proto-

oncogenes to oncogenes are point mutation, chromosomal translocation and DNA amplification (Bishop, 1991).

Point mutations have been described as the underlying cause of tumorigenesis in many human tumors. Several oncogenes, the ras genes in particular, are activated by this mechanism. The fact that many human tumors were consistently found to contain point mutations in ras played a major role in strengthening the suspicion that mutagenesis plays a role in the genesis of cancer (Barbacid, 1987). Activation of ras by point mutation is discussed in detail later.

Several oncogenes are activated by translocation, including c-abl, bcl-1, and c-myc. Similarily c-abl, erbB2/neu, c-myb, c-myc, L-myc, N-myc, K-ras, and N-ras are amongst the proto-oncogenes activated by amplification (for review see Burck et al., 1988).

Other mechanisms of oncogene activation do exist. These include deletions, rearrangements, and insertions of, or near, oncogenic sites which may or may not involve transposable elements (Bishop, 1991). Table 2 lists some of these mechanisms known to operate in human cancers.

The studies described herein involve the ras oncogene,and no other cellular oncogenes. This fact warrants a concentrated discussion of findings related to the mechanism of transformation by ras, and a more progressed phenotype of ras-transformed cells, metastasis.

Table 2. Mechanisms of activation of proto-oncogenes.

Mechanism	Consequence	Examples
Transduction	Insertion of exons of a proto-oncogene into a retrovirus genome	*src*
Point mutation	Altered sequence and biochemical function of protein product	c-H-*ras*
Amplification	Augmented production of mRNA and protein via increased gene dosage	N-*myc*
Chromosomal translocation	Altered regulation of expression, sometimes with creation of hybrid proteins	Ph^1 (t[22:9] *abl*-*bcr*)
Insertion mutation	Augmented production of mRNA and protein via promoter or enhancer in LTR, sometimes accompanied by truncation or fusion of coding sequences	c-*myc* c-*erb*B
Protein-protein interaction	Stabilization and altered biochemical function	E1A + RB p53 + SV40 T $pp60^{c\text{-}src}$ + Py mT
Rearrangement	Altered regulation of expression and/or changes in protein activity	c-*ret* c-*trk*

Adapted and modified from Varmus (1987) and Bishop (1991).

2) The ras oncogenes.

The central role of *ras* in the pathogenesis of a wide variety of human tumors is well established (Table 3) (reviewed by Bos, 1989). The prevalence of *ras* mutation, as opposed to mutations in other cellular oncogenes, in so many different tissue types remains unexplained (Bishop, 1991). This fact, however, points to a generalized role for *ras* genes in the regulation of proliferation of diverse cell types.

The *ras* oncogene was the first activated human oncogene to be isolated. In 1981, several groups of researchers reported that a transforming factor in the genomic DNA of the T24 human bladder carcinoma cell line could be transformed in NIH 3T3 cells by DNA transfection (Krontiris and Cooper, 1981; Perucho *et al.*, 1981; Shih *et al.*, 1981). Soon after, this transforming gene was cloned by several groups (Goldfarb *et al.*, 1982; Pulciani *et al.*, 1982; Shih and Weinberg, 1982). Sequencing revealed that the cellular oncogenes from various tumors were related to those already known to exist in some RNA tumor viruses. The T24 bladder carcinoma oncogene exhibited homology to the *Harvey-ras* oncogene (termed Ha- or H-*ras*) from Harvey Murine Sarcom Virus (Parada *et al.*, 1982; Santos *et al.*, 1982). A cellular oncogene from some colon and lung carcinomas was found to be homologous to the Kirsten-*ras* oncogene (Ki- or K-*ras*) of the Kirsten Murine Sarcoma Virus (Der *et al.*, 1982). Another member of the *ras* gene family

Table 3. Human tumors exhibiting transforming *ras* genes.

Ras Gene and tumor type	Origin of cells
c-H-*ras*	
Bladder carcinoma	Cell line
Bladder carcinoma	Primary tissue
Lung carcinoma	Cell line
Melanoma	Cell line
Mammary carcinosarcoma	Cell line
Acute myelogenous leukemia	Primary tissue
c-K-*ras*	
Lung carcinoma	Cell line
Lung carcinoma	Primary tissue
Colon carcinoma	Cell line
Colon carcinoma	Primary tissue
Pancreatic carcinoma	Cell line
Gallbladder carcinoma	Cell line
Rhabdomyosarcoma	Cell line
Ovarian carcinoma	Cell line
Ovarian carcinoma	Primary tissue
Gastric carcinoma	Primary tissue
Acute lymphocytic leukemia	Cell line
Acute myelogenous leukemia	Primary tissue
Myelodysplasia	Primary tissue
Renal cell carcinoma	Primary tissue
Bladder carcinoma	Cell line
N-*ras*	
Neuroblastoma	Cell line
Burkitt's lymphoma	Cell line
Fibrosarcoma	Cell line
Rhabdomyosarcoma	Cell line
Promyelocytic leukemia	Cell line
Acute myelogenous leukemia	Primary tissue
Melanoma	Cell line
T cell leukemia	Cell line
Chronic myelogenous leukemia	Primary tissue
Myelodysplasia	Primary tissue

Taken from Burck *et al.* (1988).

was subsequently identified as a mutant transforming gene in human neuroblastoma and rhabdomyosarcoma cell lines that had a sequence closely related to the H- and K-ras genes (Hall et al., 1983; Shimizu et al., 1983; Taparowsky et al., 1983; Brown et al., 1984). This gene was termed N-ras.

3) Ras function.

The three activated ras genes, H-ras, K-ras and N-ras, encode highly similar guanine nucleotide-binding proteins with molecular weights of 21-kDa (Marshall, 1991b). Although the biochemical pathways of normal ras protein (or $p21^{ras}$) function are not fully understood it is clear that, in addition to GTP/GDP binding, $p21^{ras}$ proteins have GTPase activity and are associated with the inner plasma membrane (reviewed by Barbacid, 1987). Furthermore, the biochemical properties and the peptide sequence of $p21^{ras}$ closely resemble those of the heteromeric G proteins involved in signal transduction through transmembrane signalling systems (Gilman et al., 1984; Hurley et al., 1984; Tanabe et al., 1985). These facts, taken together, suggest a function related to growth factor signal transduction from the plasma membrane to the nucleus for the ras gene family. In most, if not all cells, ras represents a nexus for the control of proliferation based on the fact that inactivation of $p21^{ras}$ protein by antibodies blocks the mitogenic action of some growth factors and transformation by

some oncogenes (Mulcahy et al., 1985; Smith et al., 1986).

The molecular mechanism of $p21^{ras}$ activation in signal transduction involves binding to GDP/GTP. $p21^{ras}$ is inactive when present at the cytoplasmic membrane in a GDP-bound state. It becomes activated by exchanging GDP for GTP. The activated ras protein transmits a signal to an effector molecule, such as adenylate cyclase or phosphodiesterase, and later inactivates itself by hydrolysis of GTP to GDP (reviewed by Marshall, 1991a). The GTPase activity of $p21^{ras}$ is stimulated by a second protein called the GTPase activating protein (GAP) (Trahey and McCormick, 1987; reviewed by Bourne et al., 1990). GAP essentially interacts with GTP-bound $p21^{ras}$ and acts as an effector in regulating hydrolysis of GTP in normal cells (see Figure 1). In many tumor cells, mutant ras genes encode structurally altered forms of $p21^{ras}$ that have a reduced ability to hydrolyze GTP when compared to the wild-type ras gene product (Gibbs et al., 1984; McGrath et al., 1984; Sweet et al., 1984). Moreover, these mutant forms of $p21^{ras}$ exhibit a greatly diminished sensitivity to GAP (Trahey and McCormick, 1987; Vogel et al., 1988). Due to their reduced GTPase activity and resistance to GAP, oncogenic forms of $p21^{ras}$ can remain in the activated state for extended periods of time during which the cell is constitutively stimulated with mitogenic signals resulting in a perpetual loss of growth control.

The most common mechanism by which mammalian ras genes

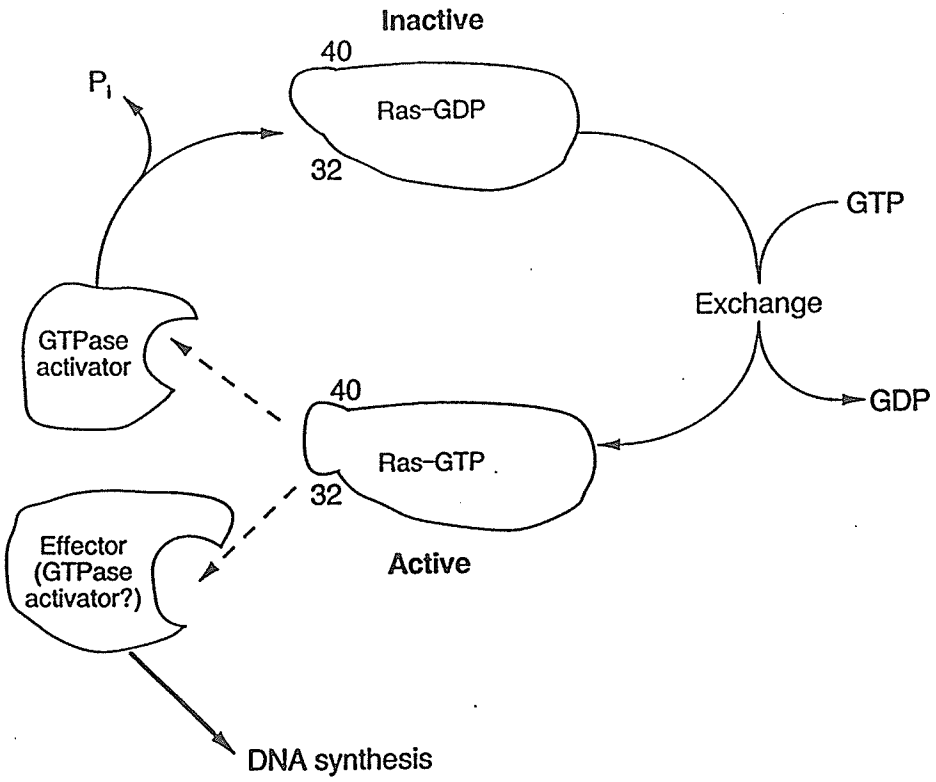


Figure 1. The GDP/GTP cycle of $p21^{ras}$ and signal transduction. In the way the scheme has been drawn, the active GTP form of $p21^{ras}$ interacts through its effector domain with either a regulator GTPase-activating protein or an effector molecule. Interaction with the effector molecule activates a signal transduction pathway that under appropriate conditions can lead to DNA synthesis. It is quite possible that the effector will also be a GTPase-activating protein, so it is not possible to decide at present whether the two known GTPase activators for $p21^{ras}$ (GAP and NF1) are regulators or effectors. (Taken from Marshall, 1991b)

acquire oncogenic properties involves point mutation of specific codons. Naturally occurring mutations have been observed in codons 12 (glycine), 13 (glycine), 59 (alanine), and 61 (glutamine) (Barbacid, 1987). Whereas all cellular ras oncogenes usually carry a single activating mutation retroviral ras oncogenes exhibit two mutations. The H-ras and K-ras oncogenes have both replaced the GLY^{12} and ALA^{59} residues with ARG^{12} and THR^{59} or SER^{12} and THR^{59}, respectively (Dhar et al., 1982; Reddy et al., 1982; Tabin et al., 1982; Taparowsky et al., 1982; Tsuchida et al., 1982) (Table 4). In vitro mutagenesis studies have shown that mutations in codons 63, 116 and 119 can also oncogenically activate ras genes (Fasano et al., 1984; Walter et al., 1986; Sigal et al., 1986).

In biochemical terms, ras can be activated to induce cellular transformation by either qualitative or quantitative mechanisms. Qualitatively, ras can be stuck in an activated state if mutation results in deregulation of GAP-$p21^{ras}$ interaction as described above. In addition, the protein products of mutated transforming alleles of ras genes often exhibit severely impaired GTPase activity (McGrath et al., 1984; Sweet et al., 1984; Temeles et al., 1985). While these qualitative variations on $p21^{ras}$ function are usually the result of activating point mutations involving critical codons, involvement of ras in cellular transformation is not limited to activation by point mutation.

Quantitative changes in ras, such as increased expression

Table 4. *Ras* proto-oncogenes converted to oncogenes by activating point mutations.

Gene	Tumor	Cell type	Lesion
c-H-*ras*	Bladder carcinoma	Human	Gly-12→Val-12
	NMU-induced bladder carcinoma	Rat	Gly-12→Glu-12
	Lung carcinoma	Human	Glu-61→Leu-61
c-K-*ras*	Colon carcinoma	Human	Gly-12→Val-12
	Lung carcinoma	Human	Gly-12→Cys-12
			Gly-12→Arg-12
			Gly-12→Lys-12
			Gln-61→His-61
N-*ras*	Acute Myeloid Leukemia	Human	Gly-12→Asp-12
			Gly-13→Val-13
			Gly-13→Asp-13
	Neuroblastoma	Human	Gln-61→Lys-61
	Melanoma	Human	Gln-61→Lys-61
	Fibrosarcoma	Human	Gln-61→Lys-61
	Lung sarcoma	Human	Gln-61→Arg-61

Adapted from Varmus (1987) and Bishop (1991). See also Varmus (1984).

of normal ras proto-oncogene can also induce certain manifestations of the malignant phenotype. Linkage of normal ras gene to retroviral regulatory elements, long terminal repeats (LTRs), results in malignant transformation of NIH 3T3 cells (Chang et al., 1982; McKay et al., 1986). Similar results have been observed by integration of multiple copies of a DNA clone of the normal H-ras gene (Pulciani et al., 1985). These tumorigenic cells have 30- to 100-fold higher levels of ras expression than either the normal parental cells or cells transformed by ras oncogenes that are activated by a single point mutation.

In human tumors there is no evidence for activation of ras proto-oncogenes by mutations that affect their expression at the transcriptional level (Barbacid, 1987). However, significant amplification of ras (≥ 10 fold) has been observed in many human tumors (Filmus and Buick, 1985; Fujita et al., 1985; Yokota et al., 1986). The overall frequency of ras gene amplification in human tumors is estimated to be no more than 1% (Pulciani et al., 1985; Yokota et al., 1986). Despite these facts quantitative analysis indicates that the level of ras mRNA is 2 to 10 times higher in human neoplasms compared to control tissues (Slamon et al., 1984; Spandidos et al., 1984a, 1984b). Generally the neoplastic characteristics induced by overexpressed ras proto-oncogenes are more limited than those induced by mutated ras genes. A combination of qualitative and quantitative changes in ras usually results in

transformed cells with a more complete spectrum of neoplastic properties (Barbacid, 1987).

In general, cellular ras oncogenes alone cannot transform primary rat embryo cells (Land et al., 1983; Newbold et al., 1983; Ruley, 1983). Efficient transformation of these cells by ras requires the cooperation of one of several nuclear oncogenes including c-myc, N-myc, adenovirus E1A, polyoma large T, or a complementing mutation in the p53 tumor suppressor (see Table 5) (Land et al., 1983; Ruley, 1983; Eliyahu et al., 1984; Parada et al., 1984; Schwab et al., 1985; Weinberg, 1985). In fact, expression of ras oncogenes in rat REF52 cells results in toxicity induced by cell cycle growth arrest at the G_1/S or G_2/M boundary and requires complementation by E1A for cell cycle progression and transformation to occur (Franza et al., 1986; Hirakaw and Ruley, 1988; Hicks et al., 1991).

Activated H-ras (T24/EJ ras) malignantly transforms mammalian cells via DNA-mediated transfections (Varmus, 1984). There is evidence that transformation by T24 ras is dependent upon overexpression of T24 ras. Spandidos and Wilkie (1984) have observed that, when linked to transcriptional enhancers, T24 ras induces complete transformation of early passage NIH-3T3 cells while normal H-ras only induces immortalization. Similarily, Kelekar and Cole (1987) showed that T24 ras immortalized early passage baby rat kidney (BRK) cells could not be morphologically transformed by a long terminal repeat-

Table 5. Oncogene complementation groups.

Group A ("Immortalization")	Group B ("Transformation")
myc family: v-*myc*, c-*myc*, N-*myc*, L-*myc*	*ras* family: H-*ras*, K-*ras*, N-*ras*
Adenovirus E1A	Adenovirus E1B (19-kDa)
Polyoma large T antigen	Polyoma middle T antigen
p53 tumor suppressor mutation	v-*src*
v-*myb*	
SV40 large T antigen	

Taken from Burck *et al.* (1988).

c-*myc* oncogene, but secondary transfection with T24 *ras* produced morphologically transformed colonies that had 20- to 40-fold higher levels of T24 *ras* expression. Other groups have similarily reported a correlation between increased T24 *ras* expression and enhanced malignancy in Balb/3T3 and REF cells (Land *et al.*, 1986; Kovary *et al.*, 1989).

4) Ras and metastasis.

Recently the *ras* oncogene has been found to play a direct role in the modulation of the metastatic phenotype of cells in addition to its well-documented involvement in cellular transformation. NIH 3T3 cells transfected with activated *ras* genes can form metastatic nodules in the lungs of T cell deficient nude mice when injected subcutaneously (Bernstein and Weinberg, 1985; Greig *et al.*, 1985; Muschel *et al.*, 1985; Thorgeirsson *et al.*, 1985). Although *ras* genes are generally unable to transform primary rat embryo cells, Pozzatti *et al.* (1986) have shown that these cells transfected with H-*ras* are not only transformed but exhibit very high metastatic potential upon intravenous tail-vein injection of nude mice.

The influence of *ras* on metastasis appears to be cell-type specific. A study using different mouse cell lines indicates that *ras* oncogenes confer differential metastatic capabilities on cells (Muschel *et al.*, 1985). Also, in contrast to mutated *ras* genes, overexpression of the c-*ras* proto-oncogene does not result in the induction of metastasis in NIH 3T3 cells. Egan *et al.* (1987a) have demonstrated that *ras* plays a direct role in regulating the metastatic propensities of mouse 10T½ cells. That is, the degree of malignancy is *ras*-expression-dependent with cells that express high levels of mutant *ras* exhibiting a relatively higher metastatic potential as determined by intravenous injection. *ras*-mediated transformation is believed to be related to the

mechanism of onset of metastasis, since ras mutations capable of cellular transformation have the potential to elicit full metastatic characteristics on cells (Egan et al., 1989b). This finding supports the conception that both transformation and metastasis are inducible through the aberrant GTPase activity of mutated ras genes.

The mechanism by which ras influences the malignancy of cells likely involves the indirect regulation of expression of metastasis-associated genes. Several genes, such as those encoding extracellular matrix proteins, metalloproteinases, and those regulating growth factor responses are regulated by ras expression (Denhardt et al., 1987; Schwarz et al., 1988, 1990; Gingras et al., 1990). This occurs mainly through activation of the AP-1 transcription factor family by ras (Aoyama and Klemenz, 1993). In addition, several studies indicate that ras-mediated transformation or metastatic progression is dependent upon the occurrence of at least one other complementing cellular mutation (Bishop, 1987; Wright et al., 1990a). Recently, Taylor et al. (1992) have demonstrated that in mouse 10T½ cells transfected with combinations of T24 H-ras, c-myc and a mutant form of p53, the ras/myc/p53 combination produced, in the lowest case, approximately 12 times more metastatic lung nodules than any other single or double gene combination.

5) Molecular mechanisms involved in metastasis.

The term "metastasis" was coined by French physician Joseph Claude Recamier in his 1829 treatise Recherches du Cancer. He was the first to provide biological evidence that metastasis is caused by tumorigenic cells that enter the circulation and colonize in distant tissues and lymph nodes. Today, metastasis is the primary cause of death in cancer patients for whom cancer treatment fails (Liotta, 1992). Despite the fact that metastasis has been recognized as the most critical aspect of cancer for 160 years, the mechanism underlying its development and expression is still not well understood. This is likely due to the fact that the establishment of the metastatic phenotype is very complex, involving the expression of many cellular genes and regulation by both positive and negative factors.

Metastasis is an intricate process involving a cascade of linked sequential steps involving many host-tumor interactions (Fidler et al., 1978; Fidler and Hart, 1982; Liotta et al., 1983; Schirrmacher, 1985; Nicolson, 1988; 1991). Successful metastatic spread of tumor cells requires that they be able to, (1) leave the primary tumor mass, (2) invade the local host tissue, (3) enter the circulation, (4) arrest at a distant vascular bed, (5) extravasate into the target organ interstitium and parenchyma, and (6) proliferate as a secondary colony (Liotta et al., 1991; see Figure 2). Cells

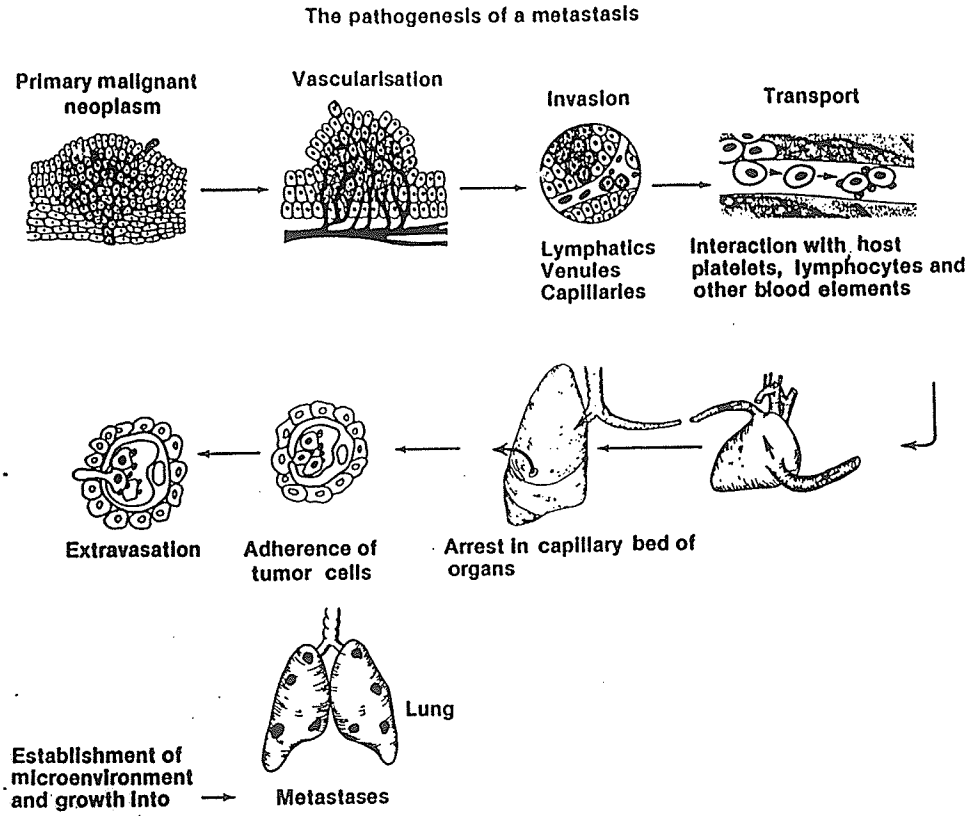


Figure 2. The metastatic process. (Taken from Poste and Fidler, 1980)

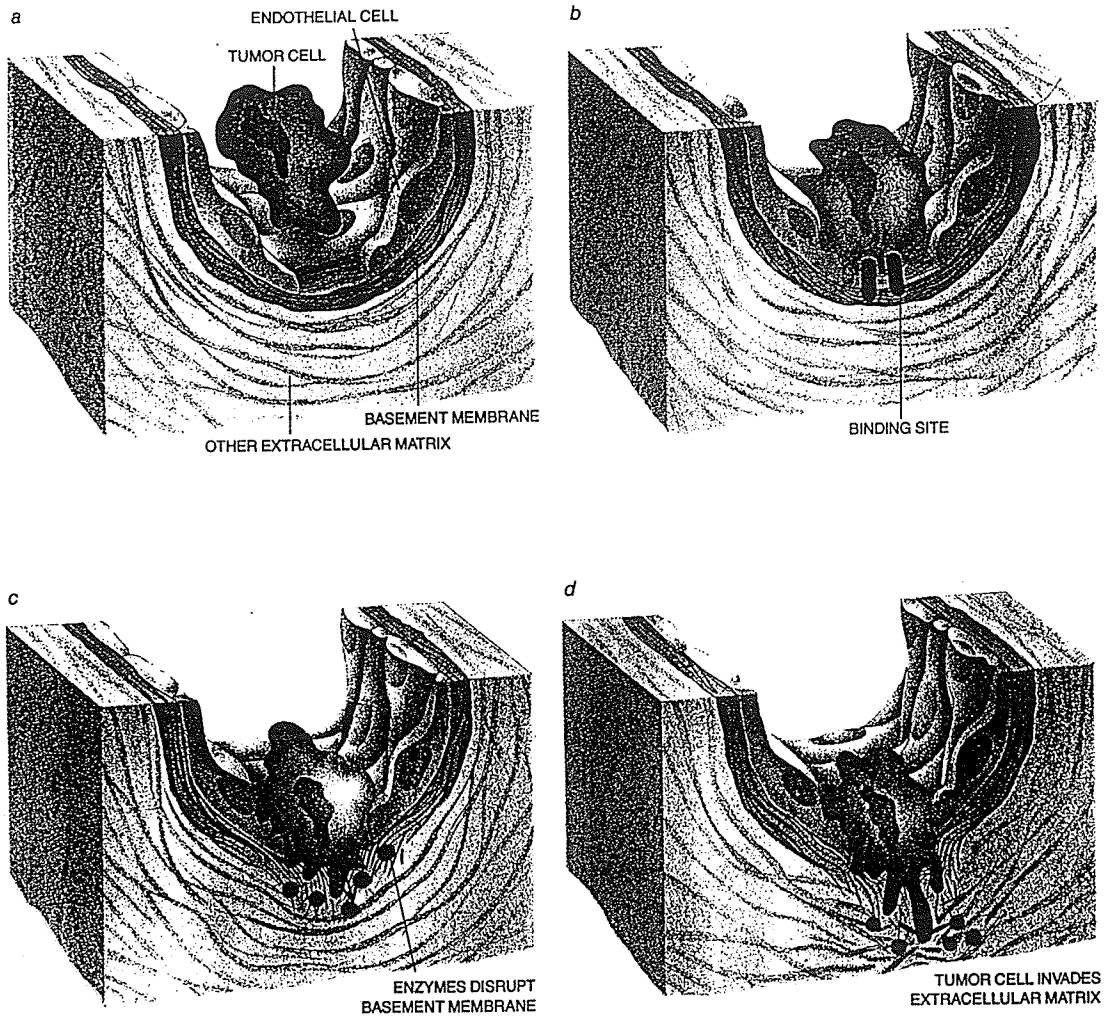
of the primary tumor often enter the circulation by penetrating new blood vessels formed during angiogenesis (Folkman, 1971; Folkman et al., 1989).

The primary tumor mass has been viewed as a heterogenous mass with a small subpopulation of preexisting metastatic tumor cells (Fidler and Hart, 1982). The metastatic subpopulation is short-lived and low in abundance, but dominates the primary tumor mass early in its growth (Kerbel, 1990). The metastatic process is a highly selective one and a very small percentage (< 0.01%) of circulating cells survive to successfully initiate a metastatic colony (Liotta, 1992).

The earliest phase of metastasis is the interaction of tumor cells with the extracellular matrix, or a specialized form of the extracellular matrix called the basement membrane (structure reviewed by Yurchenco and Schittny, 1990). The ability to violate the epithelial basement membrane, a dense matrix of collagen, glycoproteins and proteoglycans, is characteristic of metastatic cells. In fact, a definition of the behavior of metastatic cells is the ability to cross tissue compartment boundaries and interact with different cell types (Barsky et al., 1983; Liotta et al., 1983). The basement membrane surrounding blood vessels provides selective permeability for the transport of proteins and other small molecules but does not contain pores large enough for tumor cells to passively traverse it. Therefore invasion of this structure requires a highly specialized mechanism.

Tumor cell interaction with the basement membrane, or tumor cell invasion, can be separated into three phases including attachment, matrix dissolution, and migration (Liotta et al., 1991; see Figure 3). First, the tumor cell binds to the basement membrane surface by tumor cell surface receptors that recognize basement membrane glycoproteins such as type IV collagen, laminin, and fibronectin (Humphries, et al., 1986; Hynes, 1987; Rao et al., 1989; Aznavoorian et al., 1990). The second step, basement membrane invasion, occurs two to eight hours after attachment at the point of tumor contact. Metastatic tumor cells directly secrete proteolytic enzymes capable of breaking down basement membrane proteins (Gottesman, et al., 1990). This takes place in a highly localized region near the tumor surface where proteinase concentrations outweigh those of natural proteinase inhibitors in the vicinity (Brown, 1990). The third step of basement membrane interaction is locomotion, or motility, of the tumor cell across the basement membrane and stroma through the space created by matrix proteolysis. Locomotion involves pseudopodial protrusion at the leading edge of the migrating cell (Guirguis et al., 1987; Luna et al., 1989). This is a highly regulated process in which the front of the advancing tumor cell surface continues to activate proteases to cleave obstructing matrix protein molecules while the rear of the tumor cell remains firmly attached to the extracellular matrix. Once a tunnel is cleaved in front the proteases are

Figure 3. Tumor cell invasion. Invasion is the complex process that allows tumor cells to escape from the circulation and establish metastases in tissues. (a) As a prelude to invasion, a tumor cell induces the endothelial cells that line the blood vessels to retract, exposing the matrix of proteins called the basement membrane. (b) The tumor cell then attaches to the basement membrane by binding with certain molecules on it. (c) Enzymes secreted by the cell cleave the matrix proteins and cut a hole in the membrane. (d) The tumor cell then moves into the hole while continuing to produce more enzymes that allow it to penetrate the layers of extracellular material beyond the basement membrane and to enter the tissues. (Taken from Liotta, 1992)


a
ENDOTHELIAL CELL
TUMOR CELL
BASEMENT MEMBRANE
OTHER EXTRACELLULAR MATRIX
b
BINDING SITE
c
ENZYMES DISRUPT
BASEMENT MEMBRANE
d
TUMOR CELL INVADES
EXTRACELLULAR MATRIX

switched off so the cell can adhere to the sides of the extracellular matrix, release any attachments at the rear of the cell, and propel itself further forward (Liotta, 1992). Certain tumor cell cytokines, termed "autocrine motility factors" (AMFs) or "scatter factors" (SFs), are believed to be involved in random motility of the tumor cell during invasion (Liotta et al., 1986; Guirguis et al., 1987; Gherardi et al., 1989). These are thought to act through receptor activated G proteins. Studies further examining this mechanism have described inhibition of AMF-mediated motility by pertussis toxin (Stracke et al., 1987).

A number of studies have observed increased expression of metalloproteinases in metastatic cell lines and linked this finding to expression of metastatic phenotype, or more specifically, tumor cell invasion (Liotta et al., 1980; Goldfarb and Liotta, 1986; Matrisian et al., 1986; Mignatti et al., 1986; Wilhelm et al., 1987; Ostrowski et al., 1988; Matrisian and Bowden, 1990; Levy et al., 1991). The members of the matrix metalloproteinase family have the following characteristics: (1) they exhibit proteinase activity involved in degradation of at least one extracellular matrix component (see Figure 4), (2) they contain a zinc ion and are inhibited by chelating agents, (3) they are secreted in a latent form and require activation for proteolytic activity, (4) they are inhibited by specific tissue inhibitors of metalloproteinases, and (5) they have some homologous amino acid sequences

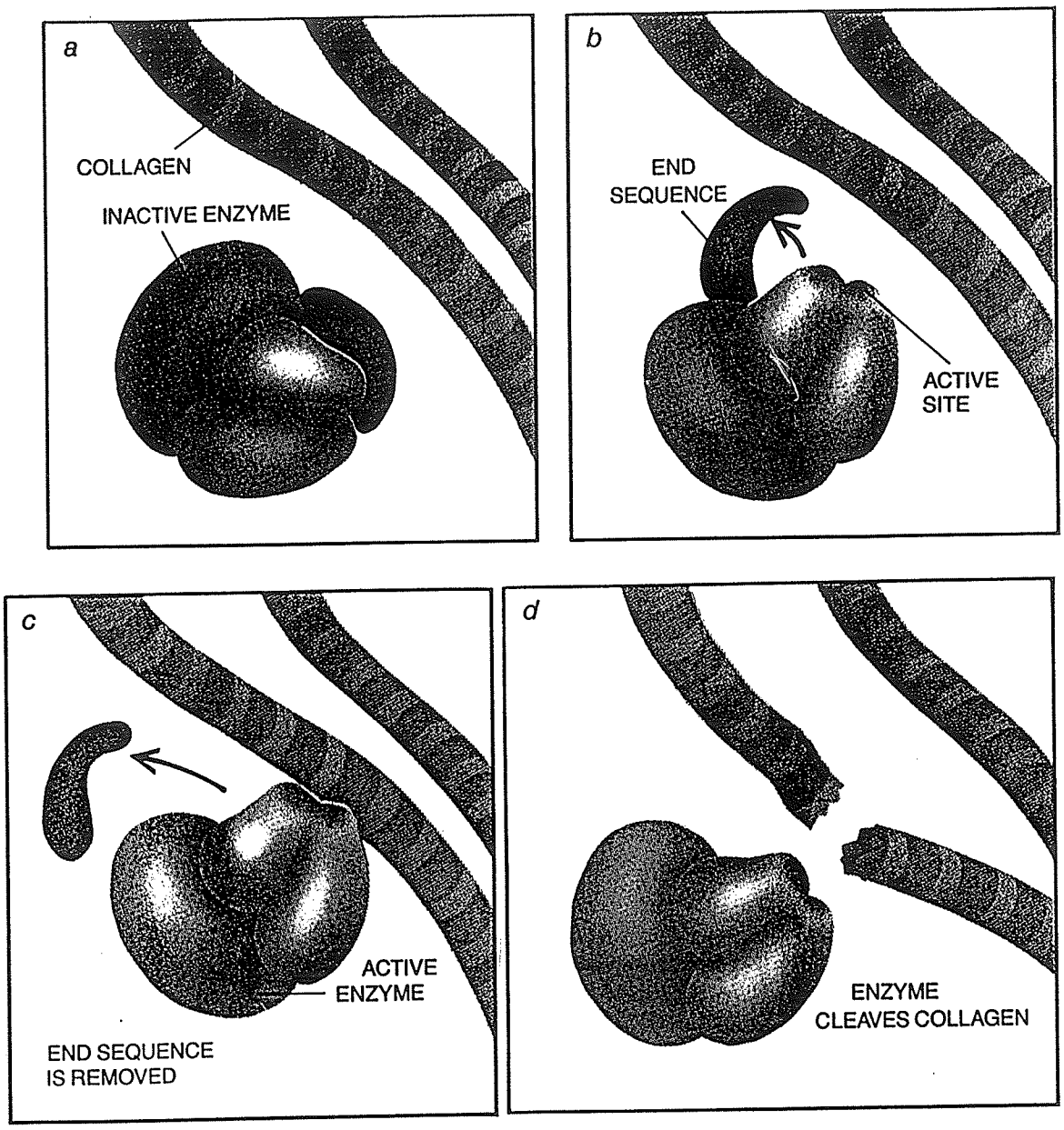


Figure 4. Metalloproteinase activation and role in the development of metastasis. Metalloproteinases are enzymes secreted by tumor cells that play a critical role in the invasion process. (a) Initially, metalloproteinases are inactive because one end of the molecule obstructs the active site of the enzyme. (b) When the enzyme is needed, however, the end sequence is pulled aside and clipped off. (c) With its active site clear, a metalloproteinase bound to a collagen molecule in the extracellular matrix (d) can cleave the protein into fragments. (Taken from Liotta, 1992)

(reviewed by Matrisian, 1990). The metalloproteinase family includes varying forms of interstitial collagen (collagenase I), collagenase IV, and stromelysin.

It is clear that regulated expression of metalloproteinases is a normal part of cellular metabolism. They are involved in a number of normal cellular processes such as proliferation, differentiation, and angiogenesis (Burger, 1970; Liotta, et al., 1991). The observation of increased metalloproteinase gene expression in metastatic cells, however, does not necessarily imply uncontrolled, constitutive expression of these enzymes. Rather, the carefully regulated manner in which cells invade basement membranes is suggestive of a mechanism involving highly organized proteolysis, both spacially and temporally. The actual functions or level of proteolytic activity in invading tumor cells is likely similar to that of normal cells, except that the former couple proteolysis with motility to achieve invasion in a way that normal cells cannot (Liotta et al., 1991).

In addition to the metalloproteinase family, other classes of degradative enzymes have been associated with metastatic progression. These include heparanases, and serine- and thiol-dependent proteinases (Wang et al., 1980; Recklies et al., 1982; Sloane and Honn, 1984; Mignatti et al., 1986; Nakajima et al., 1988; Reich et al., 1988; Nakajima et al., 1989, 1991; Schwarz et al., 1990). As well, several

groups have correlated expression and activity of two cysteine proteinases, cathepsin L and cathepsin B, with metastatic potential of tumor cell lines (Sloane et al., 1981; Denhardt et al., 1987; Rozhin et al., 1989; Yagel et al., 1989; Sloane et al., 1990; Chambers et al., 1992). The observations of several studies are in agreement with the concept proposed by Mignatti et al. (1986) that a cascade involving many of these proteolytic enzymes underlies the invasive process (Thorgeirsson et al., 1982; Wang and Stearns, 1988; Mignatti et al., 1989).

Expression of type IV collagenase, however, appears to be especially important in tumor cell invasion (reviewed by Stetler-Stevenson, 1990). Several studies have discovered a positive correlation between these two events (Liotta et al., 1980; Turpeenniemi-Hujanen et al., 1985; Nakajima et al., 1987, 1989). Augmented collagenase IV activity is associated with induction of metastatic characteristics in tumor cells (Muschel et al., 1985; Garbisa et al., 1987; Bonfil et al., 1989; Ura et al., 1989). Almost all progressed and invasive colon, gastric, and breast cancers show high levels of collagenase IV expression (D'Errico et al., 1991). Moreover, in vitro experiments show that the use of agents that specifically inhibit collagenase IV activity or block its secretion results in a loss of tumor cell invasion (Wang et al., 1980; Reich et al., 1988). Down-regulation of collagenase IV activity by retinoic acid treatment of human

metastatic tumor cells has also been associated with a loss of invasive capabilities (Nakajima et al., 1989). In contrast, normal nontumorigenic, nonmetastatic cells, as well as cells from benign tumors express low levels of 72-kDa collagenase IV (Monteagudo et al., 1990). These findings suggest that there is a central and direct relationship between collagenase IV expression and tumor invasion in a wide variety of cell types.

The malignant phenotype is also regulated by several proteins, or protein families, that have been traditionally viewed as structural or adhesive proteins. These are molecules that, unlike proteases, do not degrade extracellular matrix components, but regulate the interaction between the cell and the extracellular matrix.

The integrins are cell surface adhesion molecules that bind to extracellular matrix components, mainly fibronectin (Hynes, 1987; Ruoslahti, 1988). These molecules are transmembrane glycoproteins that, by virtue of their structure, are able to bridge the extracellular matrix to the intracellular cytoskeleton (for review of structure-function relationships of integrins see Humphries et al., 1993). Signal transduction by integrins is associated with an intracellular tyrosine kinase activity (Kornberg et al., 1991). Moreover, this activity is regulated by both cellular adhesion and oncogenic transformation (Guan and Shalloway, 1992). In addition to their role in cell migration, cell adhesion and lymphocyte homing, integrins are now known to

play a role in regulation of the metastatic phenotype (reviewed in Ruoslahti and Giancotti, 1993). Since the integrins are involved in direct interaction with components of the extracellular matrix, it is not surprising that evidence is now emerging which suggests that they regulate metastasis through regulation of extracellular matrix invasion (for review see Chammas and Brentani, 1991).

The hyaluronan (HA) receptors, including RHAMM and CD44, are another class of proteins which have been associated with metastatic progression. Like the integrins, these molecules also interact with the intracellular cytoskeleton and play roles in cell adhesion and cell migration (for review see Underhill, 1992; Turley, 1992). HA is a glycosaminoglycan that has been implicated in cell locomotion (for review see Turley, 1992). It is a chemoattractant that positively or negatively regulates motility depending upon its concentration (Turley et al., 1991). Partin et al. (1988) and Turley et al. (1991) have shown evidence of H-ras induced locomotion and the latter group has correlated this increased locomotion with autocrine production of HA. Furthermore, when HA interaction was inhibited by the addition of a competetive ligand, H-ras induced motility was inhibited.

RHAMM, Receptor for HA Mediated Motility, is a 58-kDa glycoprotein that not only binds HA but also forms a bridge between HA and a transmembrane docking protein ("connectin") to form a complex referred to as HARC (Turley et al., 1987,

1991). Turley et al. (1991, 1993) have also shown induction of RHAMM expression by mutant ras and inhibition of HA effected locomotion by the addition of RHAMM-specific antibodies in ras transformed cells. This suggests that HA mediated motility in ras transformed cells is dependent upon RHAMM function. Therefore, RHAMM is believed to be an important factor in cellular invasion and metastasis.

CD44 is also an HA-binding glycoprotein but is a transmembrane protein and is therefore different and distinct from RHAMM. Its functions and biochemical activities include lymphocyte homing, binding of collagen, laminin, and fibronectin, HA-dependent adhesion and HA degradation (for review on CD44 see Underhill, 1992). Interestingly, high levels of CD44 have been found in several types of carcinomas, gliomas and many non-Hodgkin's lymphomas (Stamenkovic et al., 1989; Horst et al., 1990; Kuppner et al., 1992). In addition, several groups have observed that expression of certain CD44 splice variants is directly related to the invasive and metastatic potentials of tumor cells (Gunthert et al., 1991; Sy et al., 1991). This points to a direct role for CD44 in metastastic progression in various cell types.

6) Factors positively regulating metastasis.

Plasminogen activators, especially urokinase-type plasminogen activator (uPA), have been linked with metastatic progression of tumor cells (Dano et al., 1985; Reich et al.,

1987; Sappino et al., 1987). Plasminogen activator is actually a serine protease that converts plasminogen, a ubiquitous extracellular zymogen, into the trypsin-like protease plasmin. Plasmin degrades interstitial glycoproteins such as fibronectin and laminin and converts procollagenase into active collagenase forms (Axelrod et al., 1989). Therefore plasminogen activators play a central role in activation of extracellular matrix-related proteinases. Axelrod et al. (1989) have shown that overexpression of urokinase-type plasminogen activator in H-ras-transformed NIH 3T3 cells enhances invasion and experimental metastasis induction. Additionally, functional inactivation of uPA using anti-uPA antibodies blocks human tumor cell invasion in the chick chorioallantoic membrane assay as well as murine melanoma cell metastasis following tail-vein injection (Ossowski and Reich, 1983; Estreicher et al., 1989). Serine proteinase inhibitors also block tumor cell invasion in a similar manner (Mignatti et al., 1986).

Cell adhesion proteins such as fibronectin and laminin, have also been identified as having a role in the movement of malignant and metastatic cells (McCarthy et al., 1985; Juliano, 1987). More precisely, the fragments of the extracellular matrix molecules that are produced by the action of tumor-derived enzymes act as chemotactic factors for metastatic tumor cells.

The expression of several oncogenes has been associated

with metastatic progression. The role of ras in this context is well documented (Muschel et al., 1985; Garbisa et al., 1987; Nicolson, 1987; Hill et al., 1988; Greenberg et al., 1989; Ura et al., 1989; Theodurescu et al., 1991). Additionally, several other cytoplasmic oncogenes including serine/threonine kinases v-mos and v-raf, tyrosine kinases v-src, v-fes and v-fms, as well as mutant p53 can induce metastasis in specific cell types (Egan et al., 1987b; Pohl et al., 1988; Greenberg et al., 1989). Oncogenes involved in expression of the metastatic phenotype are likely involved in a pathway affecting proteinase production. This has been observed in the case of ras which influences tumor cells to overexpress collagenase IV, stromelysin, cathepsin L and AMF (Thorgeirsson et al., 1985; Liotta et al., 1986; Denhardt et al., 1987; Garbisa et al., 1987; Mason et al., 1987; Collier et al., 1988; Ura et al., 1989).

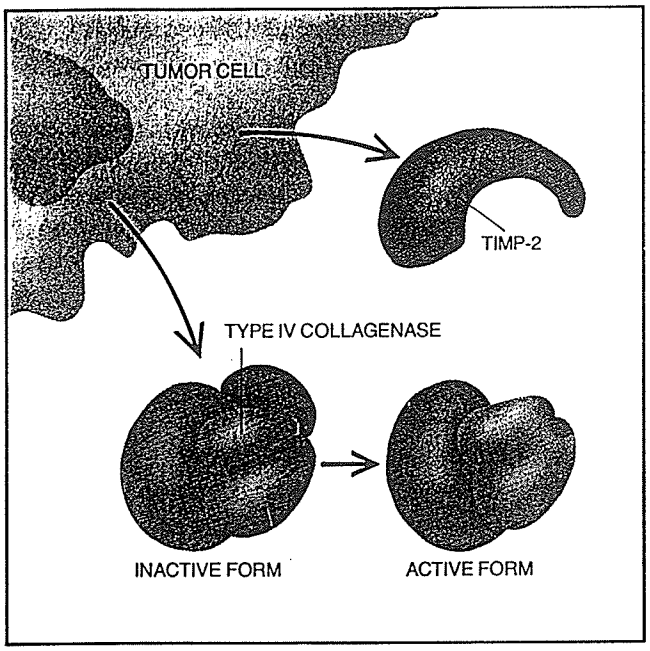
Other classes of genes and factors such as tumor promoting phorbol esters (TPA), and growth factors such as epidermal growth factor (EGF) and fibroblast growth factor (FGF) enhance expression of transin, a rat homologue of human stromelysin (Liotta et al., 1991). Some studies suggest that the nuclear c-fos oncogene, a transcription factor, is involved in modulation of stromelysin levels by growth factors (Kerr et al., 1988; Matrisian and Bowden, 1990). Another growth factor, platelet derived growth factor (PDGF), which operates through ras pathway activation, and TPA, both induce

cathepsin L expression (Liotta et al., 1991; Chambers et al., 1992).

7) Factors negatively regulating metastasis.

As suggested earlier, progression to the metastatic phenotype is under strict control of both positive and negative factors and requires a careful regulation of these molecules (Liotta et al., 1991; Matrisian, 1990). Generally, the action of any specific, or nonspecific, proteinase inhibitor results in decreased levels of tumor cell invasion and metastasis. This has been reported in work involving cathepsin L/B, serine proteinase, and plasminogen activator inhibitors as well as for tissue inhibitors of metalloproteinases (TIMPs) (Carmichael et al., 1986; Mignatti et al., 1986; Levin and Santell, 1987; Yagel et al., 1989). Such natural proteinase inhibitors essentially function as metastatic suppressor proteins that inhibit tumor cell invasion of basement membranes (see Figure 5).

Although many growth factors are associated with enhanced proteinase production and elevated invasion and metastasis, this is not always the case. Transforming growth factor-ß (TGF-ß) exhibits opposite regulation in relation to metalloproteinase and TIMP production. In cultured fibroblast cells TGF-ß induces TIMP expression and represses growth factor induction of stromelysin and collagenases (Edwards et al., 1987). Also, TGF-ß counteracts metastasis induction by



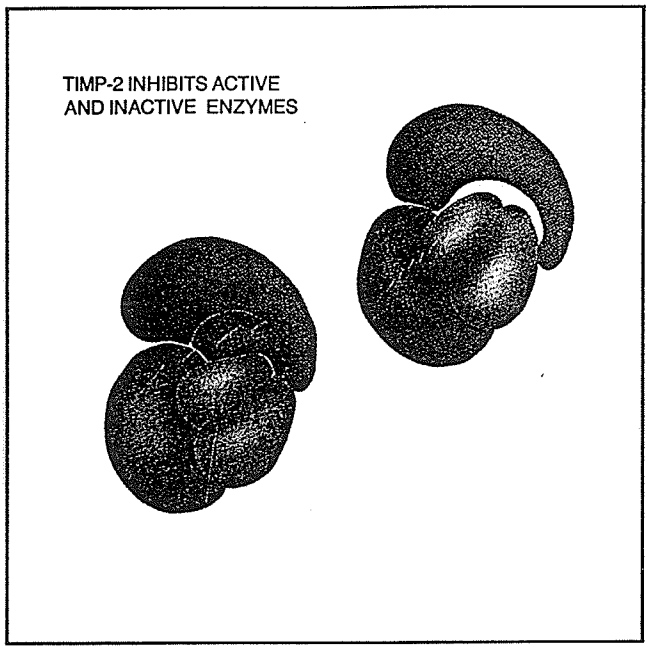


Figure 5. Negative regulation of metastasis by proteinase inhibitors. Tissue inhibitors of metalloproteinases (TIMPs) are proteins secreted by cells that suppress invasion by preventing the breakdown of collagen. One member of the TIMP family, TIMP-2, is particularly effective because it binds with both the active and inactive forms of the metalloproteinase enzyme called type IV collagenase (taken from Liotta, 1992). See Matrisian (1990) for review of TIMPs.

basic fibroblast growth factor (bFGF) by inhibiting proteolytic and angiogenic properties of endothelial cells in vitro (Pepper et al., 1990).

For several years people have hypothesized the existence of metastasis-suppressor genes. This idea gained substantial support following two seperate studies in the mid-1980's which showed that fusion of normal cells with metastatic cells resulted in tumorigenic but nonmetastatic cells (Sidebottom and Clark, 1983; Turpeenniemi-Hujanen et al., 1985). Further analysis showed that mRNA levels of a gene called nm23 were reduced 10-fold in five of seven murine melanoma cell lines that were highly metastatic compared to the other two lines of low metastatic potential (Steeg et al., 1988a). Similar relationships were observed using nm23 mRNA and protein evaluation of human infiltrating ductal breast carcinomas (Bevilacqua et al., 1989). The nm23 gene is highly homologous to the Drosophila awd (abnormal wing differentiation) gene and its protein product appears to be a nucleoside diphosphate kinase (Dearolf et al., 1988a, 1988b; Rosengard et al., 1989; Liotta and Steeg, 1990; Liotta et al., 1991). Steeg et al. (1988b) have shown that suppressed metastatic potential correlates with enhanced nm23 production in ras plus adenovirus E1A-transfected cells but not in cells transfected with ras alone. E1A-mediated suppression of metastasis has also been reported by Pozzatti et al. (1986, 1988) and is further discussed herein.

8) Human adenoviruses and the E1A oncogene.

The human adenoviruses were the first viruses of human origin shown to be oncogenic. Trentin et al. (1962) first found that human adenovirus serotype 12 (Ad12) was capable of inducing tumor formation in newborn hamsters. This finding was subsequently confirmed by several other groups for additional adenovirus serotypes (Huebner et al., 1962; Rabson et al., 1964; Huebner et al., 1965; Pereira et al., 1965). Later the adenovirus serotypes were subdivided into five categories (groups A to E) based on their oncogenic properties (Table 6) (Huebner, 1967). The three major groups and their most extensively studied serotypes are the highly (Ad12), weakly (Ad3/Ad7) and nononcogenic (Ad2/Ad5) adenoviruses. These are also referred to as subgroup A, subgroup B and subgroup C, respectively.

The first in vitro demonstration of adenovirus-mediated oncogenesis involved Ad12 transformation of newborn hamster kidney cells (McBride and Weiner, 1964). In 1967, it was shown that even nononcogenic adenoviruses can transform cells in vitro, provided that the calcium concentration in the growth medium is reduced (Freeman et al., 1967). Today there are at least 37 known human adenovirus serotypes (Burck et al., 1988). Although not all serotypes induce tumors most, if not all, are able to induce oncogenic transformation of mammalian cells in culture and the resulting transformed

Table 6. Subgroups and properties of the human adenoviruses.

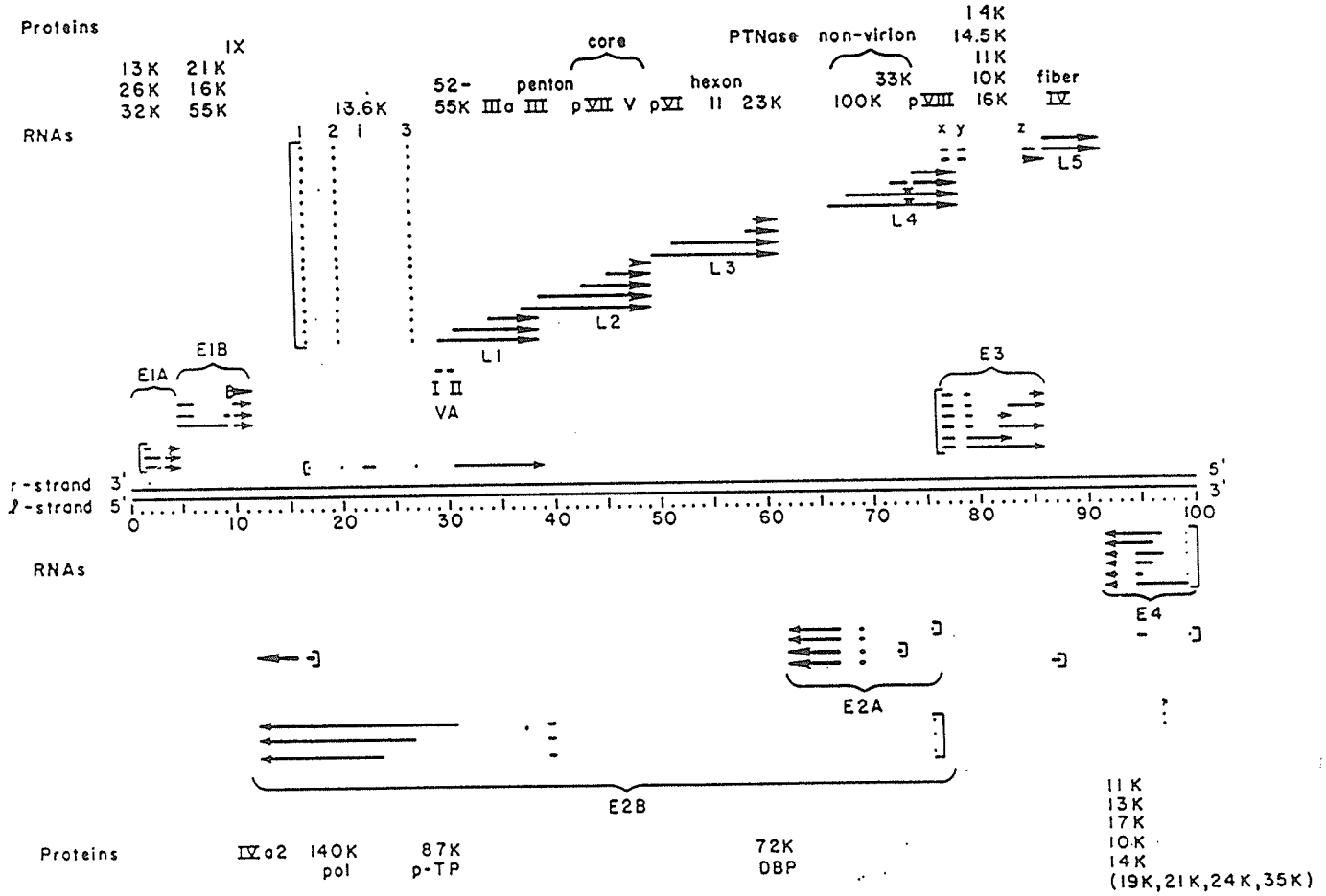
Group	Serotype	Cell Transformation	Animal tumors
A	12, 18, 31	+	High incidence of tumors in newborn hamsters
B	3, 7, 11, 14, 16, 21	+	Low incidence of tumors in newborn hamsters
C	1, 2, 5, 6	+	None
D	8, 9, 10, 13, 15, 17, 19, 20, 22-30	+	Mammary fibroadenomas in rats
E	4	+	None

Taken from Burck *et al.* (1988).

cell lines are frequently able to cause tumors in rodents, especially if the animals are immunologically deficient (Tooze, 1981; Casto, 1968; McAllister and MacPherson, 1968; Gallimore, 1972; Gallimore, 1974; Cook and Lewis, 1979). The most extensively characterized of the adenovirus serotypes are Ad2 and Ad5, and to a lesser extent Ad12.

The genetic structure of the adenovirus consists of 36 kb double stranded DNA (Figure 6) (Pettersson and Roberts, 1986). The genome is divided into early and late genes. Four blocks of genes in the early region are expressed early in the virus growth cycle. The first of these to be expressed is the early region one (E1) which plays a key role in regulating the expression of other viral genes (Jones and Shenk, 1979; Sharp, 1984). This region is located within the left approximately 11% (4 kb) of the genome and is divided into separate transcriptional units identified as early region 1A (E1A) and early region 1B (E1B). E1A spans from 0% to 4.5% and E1B from 4.6% to 11.2% (Figure 7). Studies have shown E1 to be sufficient and primarily responsible for cell transformation and tumor induction by Ad2 (Graham, 1974; Flint et al., 1976). This has been confirmed for a number of other serotypes (Mak, 1979). In fact, the Hind III-G fragment comprising the leftmost 7.8% (2800 base pairs) of the adenovirus genome is sufficient to induce full transformation of primary rat embryo fibroblasts (REFs) in vitro (Graham, 1975).

Figure 6. Genetic organization of the adenovirus genome. Organization of the adenovirus 2 genome, showing locations of the early and late transcriptional units and sizes of mRNA transcripts (taken from Pettersson and Roberts, 1986). See Figures 8 and 9 for updated E1A mRNA transcript sizes.


Proteins
IX
13K 21K
26K 16K
32K 55K
13.6K
52-
55K IIIa III pVII V pVI II 23K
core
penton
hexon
PTNase
non-virion
33K
100K pVIII
14K
14.5K
11K
10K 16K
fiber
IV
RNAs
1 2 1 3
x y
z
L5
L4
L3
L2
L1
E1B
E1A
I II
VA
E3
r-strand 3'
l-strand 5'
5'
3'
0 10 20 30 40 50 60 70 80 90 100
RNAs
E4
E2A
E2B
Proteins
IVa2 140K 87K
pol p-TP
72K
DBP
11K
13K
17K
10K
14K
(19K,21K,24K,35K)

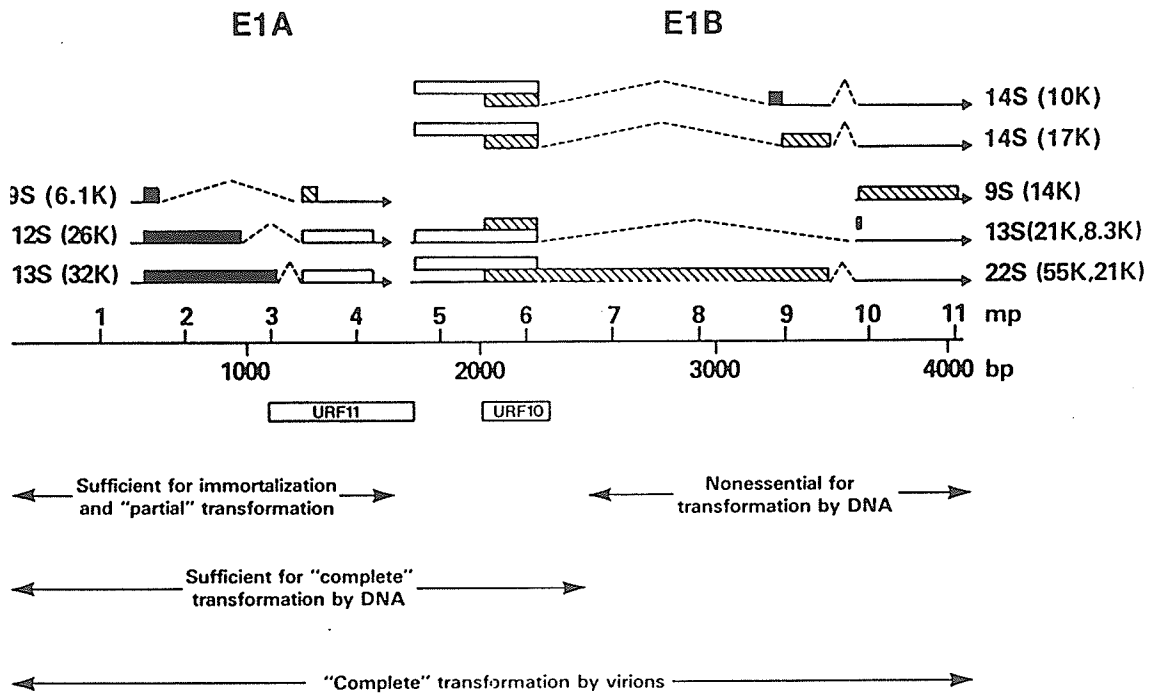


Figure 7. Structure of early region 1 (E1). Data are taken from studies on adenovirus serotypes 2 and 5 (taken from Branton et al. (1985). See Figure 8 for updated Ad2/5 E1A mRNA transcript sizes.

E1A is responsible for most of the transforming activities of E1. Introduction of E1A alone into cultured primary cells results in partial transformation (Houweling et al., 1980). This not true for E1B (van den Elsen et al., 1983). The E1A containing HpaI-E fragment (0-4.5%) of Ad5 is sufficient for production of immortalized cells. However, rather than having the epitheloid morphology characteristic of adenovirus-transformed cells, cells transformed by E1A alone are more fibroblastic, do not clone in soft agar and generally are nontumorigenic. Although E1A expression significantly affects the growth properties of cells, full transformation requires the expression of at least the left half of the E1B gene (Graham, 1975). Independent studies by Ruley (1983) and Land et al. (1983) have shown that other oncogenes such as ras and polyoma virus middle T antigen can substitute for E1B and complement E1A to give rise to fully transformed, tumorigenic cells. A recent study has reported that the gli oncogene, which is amplified in human glioblastomas and encodes a zinc-finger protein believed to be DNA-binding transcription factor, can also complement E1A to fully transform primary cells (Kinzler et al., 1987; 1988; Ruppert et al., 1991).

The E1A mRNA products of Ad2/5 are well characterized. They are transcribed from two exons and are formed by differential splicing from a common precursor mRNA. Five

different mRNA species are produced (Figure 8). Their relative levels are regulated according to the course of infection. At early times after infection two mRNAs, designated 13S and 12S, are synthesized. These mRNAs have been extensively characterized by both RNA analysis and cDNA cloning (Berk and Sharp, 1978; Kitchingham and Westphal, 1980). Late after infection, a third mRNA (9S) accumulates (Spector et al., 1978; Chow et al., 1979). Since the three mRNAs are derived from a common precursor by differential splicing they possess the same 5′ and 3′ termini, share the same splice acceptor site, but differ in their splice donor sites (Perricaudet et al., 1979). While both larger mRNAs are translated in the same reading frame, the reading frame of the 9S mRNA is altered in the second exon due to the structure of the splice junction (Virtanen and Pettersson, 1983). Recent studies of Ad2- and Ad5-infected HeLa cells demonstrated two additional minor mRNA species of 11S and 10S (Stephens and Harlow, 1987). They differ from the 12S and 13S forms in that one more intron near the 5′ end of the primary transcript is removed. Both smaller mRNAs use the same translational reading frame as the 13S and 12S mRNAs. The functions of the gene products of the 10S and 11S mRNAs are unknown.

Transcription of Ad12 E1A leads also to 13S and 12S mRNAs (Perricaudet et al., 1980; Sawada and Fujinaga, 1980). Brockmann et al. (1990) recently analyzed the heterogeneity of Ad12 E1A mRNAs in infected cells and transformed lines by

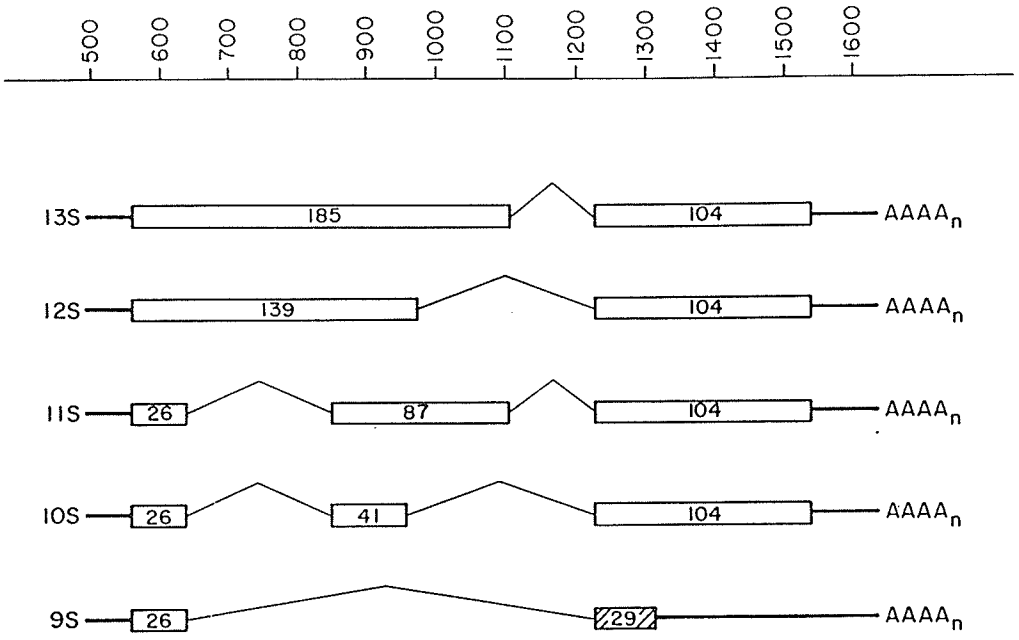


Figure 8. Structures of adenovirus type 5 E1A mRNA transcripts. (Taken from Stephens and Harlow, 1987)

using cDNA polymerase chain reaction (PCR). They were able to clone and characterize four novel cDNAs corresponding to 11S, 10S, 9.5S and 9S mRNAs (Figure 9).

The 12S and 13S mRNAs are most important for E1A functional studies. cDNA cloning in combination with sequence analysis has made it possible to deduce that the protein products of the 12S and 13S mRNAs are completely overlapping. They are 243 amino acids (243R) and 289 amino acids (289R) in length and differ by an internal stretch of 46 amino acids which is almost completely absent in the smaller protein (Perricaudet et al., 1979). Both bring about tumorigenic transformation in cooperation with E1B, ras or polyoma middle T antigen. The 243R protein is required for virus production in growth-arrested permissive cells but is not responsible for the activation of other adenoviral promoters (Montell et al., 1984; Spindler et al., 1985). Activation of other adenoviral genes is required for productive infection in human cells and is regulated by the 289R protein (Stephens and Harlow, 1987). The predicted secondary structures of both the 289R and 243R proteins are shown in Figure 10.

When the protein products of the various E1A mRNAs are separated on one-dimensional SDS-polyacrylamide gels, the proteins migrate with relative molecular weights of 35-kDa to 58-kDa (Branton et al. 1985). When these proteins are further resolved on two-dimensional gels, E1A proteins can be separated into an extremely heterogenous group of polypeptides

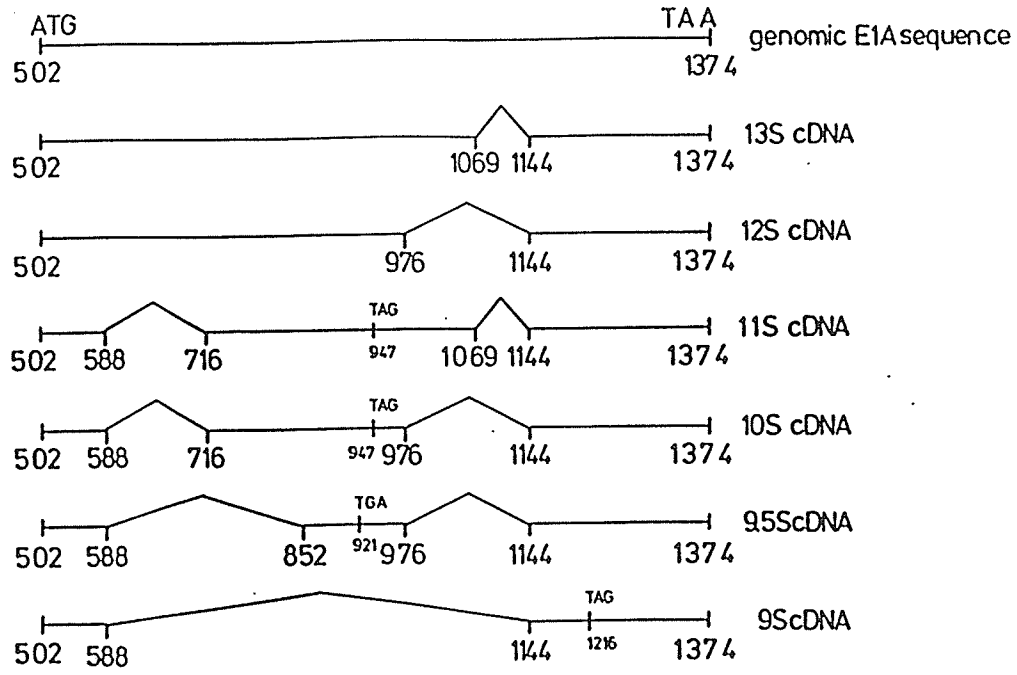


Figure 9. Structures of adenovirus type 12 E1A cDNAs. The start codon (first nucleotide 502) and the first nucleotide of the respective stop codons are indicated. The large numbers below the lines represent the first or last nucleotide of the respective exon. (Taken from Brockmann et al. (1990)

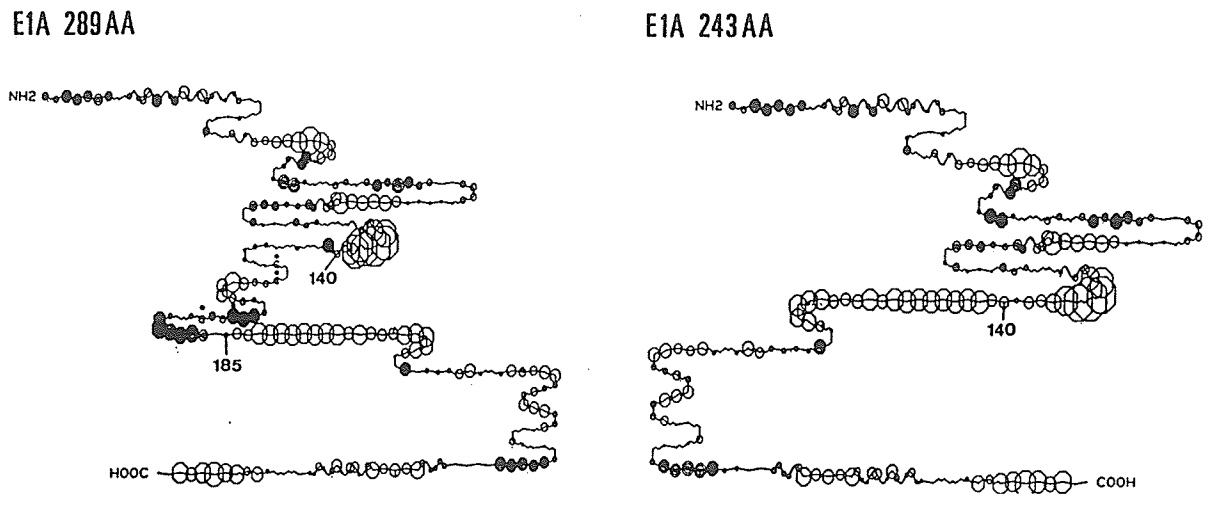


Figure 10. Secondary structure of E1A proteins. Predicted secondary structure and hydrophilicity of the wild type adenovirus 5 289R and 243R E1A proteins. Open circles indicate hydrophilic regions, and filled circles indicate hydrophobic areas. The radius of a circle is proportional to the average hydrophilicity/hydrophobicity calculated for that residue and the next five residues. Numbers correspond to amino acid residues from the amino terminus. (Taken from Glenn and Ricciardi, 1985)

(Harter and Lewis, 1978; Halbert et al., 1979; Esche et al., 1980; Smart et al., 1981; Rowe et al., 1983; Yee et al., 1983; Spindler et al., 1984; Harlow et al., 1985). The heterogeneity is due to both translation of different E1A mRNAs and to extensive posttranslational modification (Yee and Branton, 1985; Tsukamoto et al., 1986; Stephens et al., 1986). E1A polypeptides are phosphoproteins that are localized in the nucleus (for reviews see Graham, 1984; and Berk, 1986).

E1A proteins contain three distinct regions strongly conserved among adenovirus subgroups and species (Figure 11) (van Ormondt et al., 1980; Kimelman et al., 1985). Conserved region 1 (CR1, amino acids 40 to 80) and conserved region 2 (CR2, amino acids 121-139) are entirely within exon 1, while conserved region 3 (CR3, amino acids 140-188) is entirely within exon 1 except for the last 3 amino acids. Exon 1 encodes amino acids 1-185, while exon 2 encodes amino acids 186-289 in the 289R protein.

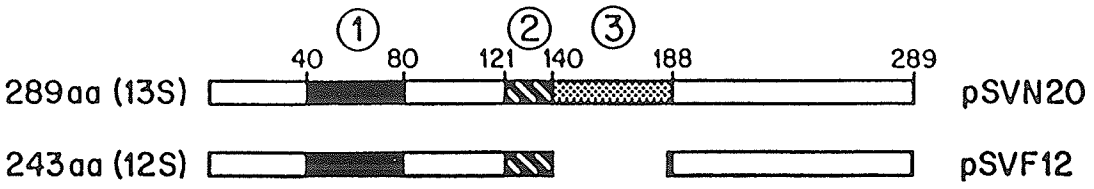


Figure 11. Localization of E1A conserved regions. Adenovirus E1A 13S and 12S mRNA products. The regions which are highly conserved among the adenovirus serotypes are boxed and indicated as domains 1, 2, and 3. (Taken from Velcich and Ziff, 1988)

9) Transcriptional activation by E1A.

The discovery of E1A as an oncogene sparked curiosity as to the mechanism by which it induces cellular transformation. As discussed earlier most oncogenes, cellular or viral, fall into the category of growth factors, membrane receptors, signal transduction pathway components or transcriptional regulators (Bishop, 1985, 1991). Transformed cells almost invariably exhibit a loss of growth control at some level. During investigation of the mechanism of E1A-mediated transformation the fact that E1A is localized to the nucleus was very suggestive of a mechanism in which it would stimulate transformation through the regulation of gene expression at the molecular level.

In fact, E1A is a transcription factor and is associated with transactivation of a large number of viral and cellular genes. Not surprisingly, one of the conserved domains, CR3, is believed to be responsible for this function. The evidence for this comes from studies in which the 13S mRNA, but not the 12S mRNA, is necessary for activation of gene expression (Montell et al., 1982). However, some studies have detected transactivating activity related to the 12S product (Leff et al., 1984; Ferguson et al., 1985).

A variety of mechanisms have been proposed for gene activation by the 13S mRNA of E1A (Flint and Shenk, 1984). Conserved domain 3 contains sequences consistent with that of a zinc-finger binding domain which are required for

transactivation by E1A (Figure 12) (Ricciardi et al., 1981; Montell et al., 1982; Glenn and Ricciardi, 1985; Webster et al., 1991). However, no direct binding of E1A to promoter sequences has yet been demonstrated (Berk, 1986; Chatterjee et al., 1988). The 289R protein cannot bind to single stranded DNA or RNA. It can bind to double-stranded DNA in a sequence independent manner that does not involve CR3, but rather involves C-terminal amino acids 201-216 (Chatterjee et al., 1988; Zu et al., 1992). It has been shown that mutation of the DNA-binding region that renders the protein defective in DNA binding does not affect transcriptional regulation by E1A, therefore the DNA binding activity of E1A is not associated with transcriptional activation (Zu et al., 1992).

Rather, E1A appears to indirectly activate transcription by forming or inducing formation of a transcriptional complex involving cellular transcription factors. Recent studies have shown that E1A is capable of stimulating transcription from promoters transcribed by RNA Polymerase II and RNA Polymerase III suggesting that it may be positively regulating gene expression by activating transcription factors through protein-protein interaction (Berger and Folk, 1985; Gaynor et al., 1985; Hoeffler and Roeder, 1985; Spangler et al., 1987). Alternately, it may be binding a repressor protein(s)study by Datta et al. (1991) revealed that E1A transactivates

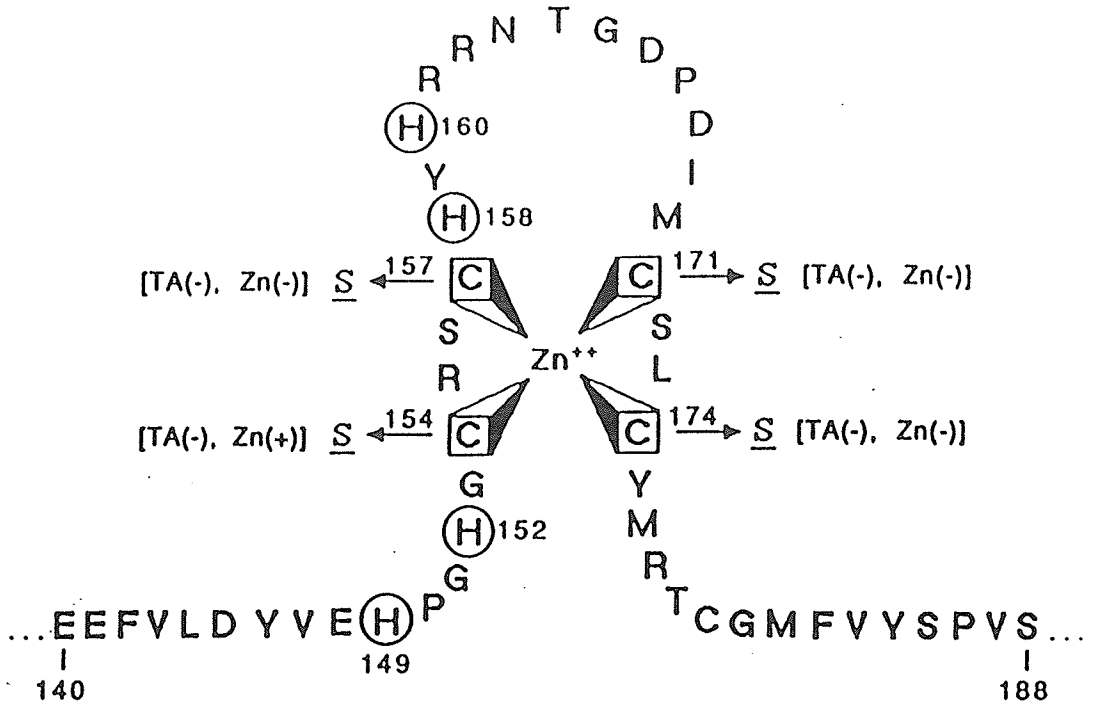


Figure 12. Conserved region 3 (CR3) of E1A contains sequences encoding a zinc finger structure. Shown is a schematic representation of the E1A zinc (Zn^{+2}) finger structure and the effects of individual Cys → Ser substitutions in the transactivating region of adenovirus 5 E1A (residues 140-188, or CR3). All four Cys→Ser mutants fail to transactivate, TA(-), and fail to bind zinc, Zn(-), except for C154S, which binds zinc, Zn(+). Zn^{+2} in the wild-type protein is bound by the highlighted cysteines whereas in C154S Zn^{+2} is bound by two cysteines and two histidines. This coordination by the C154S protein probably involves two of the four circled histidines and Cys-171 and Cys-174. (Taken from Webster et al., 1991)

the adeno-associated VA1 RNA gene transcribed by RNA Polymerase III, by activating TFIIIC, the rate-limiting factor of class III transcription. The data suggest that E1A does not directly bind TFIIIC and that it is not part of the transcription complex. It is believed to indirectly activate TFIIIC by promoting its phosphoryltion and thereby stimulating transcription by RNA Polymerase III (Cromlish and Roeder, 1989; Hoeffler et al., 1989). Similarily E1A-mediated activation of RNA Polymerase II function is now known to involve TFIID, a TATA-binding protein (Wu et al., 1987). A recent study examining synergy between HIV-1 tat, a potent transactivator, and E1A has revealed that elevated transcription from the HIV-LTR in cells expressing both proteins is due to stabilization of transcriptional elongation (Laspia et al., 1990). This finding stands against the traditional belief that E1A-mediated transactivation works at the level of initiation of transcription (Nevins, 1981).

Experiments carried out with inhibitors of protein synthesis suggest that E1A proteins inactivate or counteract a short-lived cellular inhibitor of transcription (Berk et al., 1979; Nevins, 1981; Katze et al., 1981; Shaw and Ziff, 1982; Katze et al., 1983). This inhibitor is now believed to be the product of the cellular retinoblastoma susceptibility gene, RB (Bagchi et al., 1991). The mechanism of this transactivational process is described later.

Recently it has been found that the sequence 5'-ACGTCA-3'

which occurs in most of the viral early promoters and binds activating transcription factor (ATF), is one of the elements that can mediate E1A transactivation (Hurst and Jones, 1987; Hardy and Shenk, 1988; Leza and Hearing, 1988; Lin and Green, 1988; Sassone-Corsi, 1988). The identical sequence is found in several cellular genes whose expression is regulated by the level of cyclic AMP (cAMP) which binds a nuclear protein termed cAMP-responsive element (CRE)-binding protein (CREBP) (Deutsch et al., 1988; Fink et al., 1988; Sassone-Corsi et al., 1988; Berkowitz et al., 1989; Fisch et al., 1989). The transcriptional activation of CRE-containing promoters by E1A is mediated by a protein called CREB-BP1 (Maekawa et al., 1991). A 12-O-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE) represents a promoter target of the protein kinase C signal transduction pathway and binds the nuclear transcription factor AP-1 (jun/fos) (Angel et al., 1987; Lee et al., 1988). Interestingly, the sequence of a TRE differs from that of a CRE by only one nucleotide (TRE is TGACTCA; CRE is TGACGTCA) and both elements have a homologous palindromic sequence. Furthermore, it has been shown that crosstalk in signal transduction is detectable at the transcriptional level and that E1A activates gene transcription by both CREB/ATF and AP-1 (Sassone-Corsi et al., 1990; Flint and Shenk, 1990; Shenk and Flint, 1991). Both E1A and cAMP induce expression of transcription factor AP-1 (Muller et al., 1989). Thus, E1A might modulate a specific

signal transduction pathway normally induced by activation of protein kinase C.

Yet another factor, E4F, is believed to have a role in E1A transactivation. There is a significant stimulation of E4F DNA-binding activity after adenovirus infection that is dependent on E1A function, and the kinetics of E4F activation parallels the kinetics of E4 transcription activation (Raychauduri, 1987). Although E4F and ATF recognize the same site, they can be distinguished both physically and functionally (Rooney et al., 1990). Thus, the interaction of E4F with the E4 promoter correlates with the E1A-dependent transactivation of E4 transcription.

Although the 12S E1A transcript is usually only associated with transcriptional repression, mutant viruses that express the E1A 12S product without the 13S product can activate expression of a number of cellular genes including proliferating cell nuclear antigen (PCNA), $p34^{cdc2}$, heat shock protein 70 (HSP70) and the adenovirus E2 gene (Simon et al., 1987; Draetta et al., 1988; Fahnestock and Lewis, 1989; Jelsma et al., 1989; Bagchi et al., 1990; Kaddurah-Daouk et al., 1990).

E1A transactivation of the heat shock promoter is known to occur through the binding of E1A directly to CBF (CCAAT-binding factor), a protein that regulates HSP70 expression by binding to the CCAAT element at position -70 relative to the transcription initiation site (Lum et al., 1992). There is

some evidence to suggest the existence of a cellular E1A-like activity in cells such as the F9 teratocarcinoma-derived embryonal carcinoma cell line, which expresses the heat shock gene at high levels and is also capable of expressing early adenovirus genes in the absence of E1A (Imperiale et al., 1984; La Thangue and Rigby, 1987; Boeuf et al., 1990).

A mechanism for activation of transcription via the cellular E2F transcription factor by the E1A 12S product, acting alone or in concert with the viral E4 product, has recently been established (Hardy, 1989; Huang and Hearing, 1989). Moreover, this activity is dependent on E1A sequences within CR1 and CR2, therefore this mechanism may also be applicable to the larger 13S mRNA product (Bagchi et al., 1990). The 12S mRNA product is thought to activate gene expression by a mechanism similar to that of the human papillomavirus (HPV) type 16 E7 protein. This belief is further substantiated by significant sequence homology between E7 and E1A conserved regions 1 and 2 (Phelps et al., 1991). Table 7 presents a list of viral and cellular genes that are transcriptionally activated by E1A.

10) Enhancer repression by E1A.

The first report of enhancer repression by E1A was in microinjection experiments (Rossini, 1983). Microinjection of a mutant E1A construct expressing only the smaller 243R protein repressed expression of the adenovirus single-stranded

Table 7. Viral and cellular genes that are transcriptionally activated by adenovirus E1A.

Gene	Reference
Viral:	
E1A	Nevins (1981)
E1B, E2*, E3, E4	Nevins et al. (1979)
HTLV-1 LTR	Chen et al. (1985)
HIV LTR	Nabel et al. (1988)
Cellular:	
c-fos, c-myc	Sassone-Corsi and Borrelli (1987)
c-jun, junB	de Groot et al. (1991)
p53	Braithewaite et al.(1991)
ß-globin	Green et al. (1983)
epsilon-globin	Treisman et al. (1983)
ß-tubulin	Stein and Ziff (1984)
preproinsulin	Gaynor et al. (1984)
HSP70*	Kao and Nevins (1983) Simon et al. (1987)
PCNA*	Jelsma et al. (1989)
$p34^{cdc2*}$	Draetta et al. (1988)

All information refers to Ad2/5.
'*' indicates transactivation of the respective gene by 12S forms of E1A.

DNA-binding protein (E2A) from its late-phase specific promoter (Guilfoyle et al., 1985). This was the first report that E1A could differentially activate and repress transcription of the same gene.

Subsequently, Borrelli et al. (1984) showed that E1A-mediated repression operated in the promoter region of the E1A gene itself and involved the enhancer element as the target. E1A-mediated repression of the E1A region was also shown to exist in the context of replicating plasmids in COS cells (Smith et al., 1985). This study proposed an interesting model for E1A autoregulation during viral infection in attempting to explain the positive and negative regulation of the E1A gene by E1A protein products (Figure 13).

E1A proteins also repress activation of transcription of genes from other viral species. These include the SV40, human immunodeficiency virus (HIV) and polyoma enhancers (Borrelli et al., 1984; Velcich and Ziff, 1985; Ventura et al., 1990). When E1A genes were cotransfected into HeLa cells with the early SV40 transcription unit, production of early SV40 mRNA was diminished. It was determined that this repression was not mediated by SV40 large T-antigen since it was also observed when the promoter was recombined to other structural genes. Two observations suggest that the E1A-mediated repression operates through a repression of the SV40 72 base-pair repeat enhancer element. Firstly, the repression was

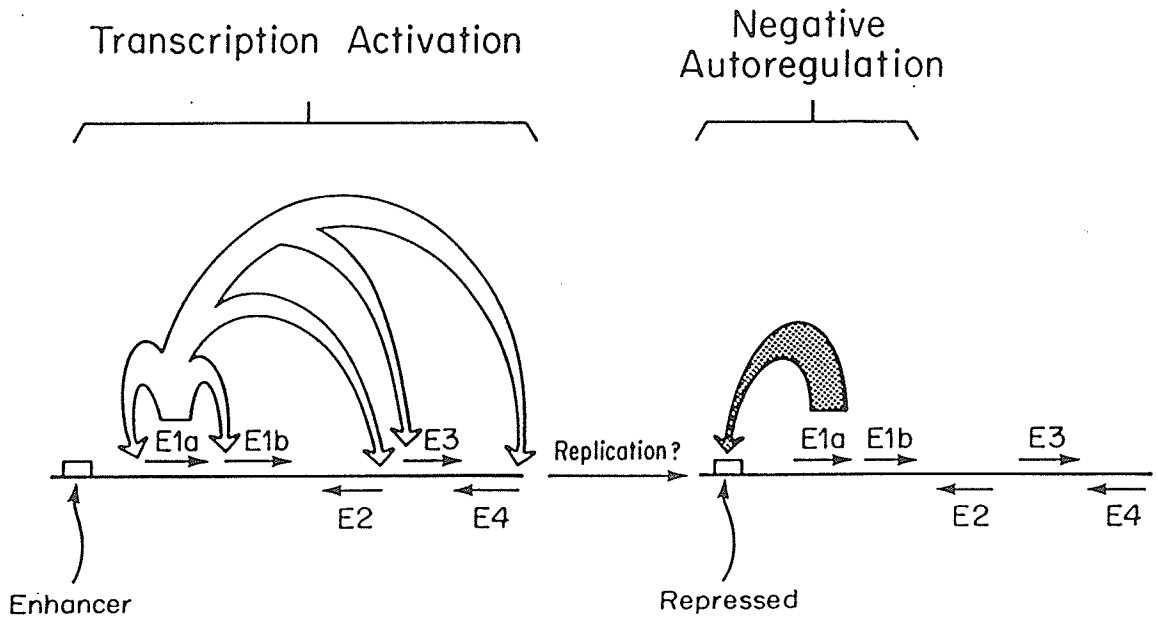


Figure 13. Transcriptional autoregulation by adenovirus E1A. Hypothetical model for positive and negative regulation of the E1A gene by E1A protein products during viral infection. (Taken from Smith et al., 1986)

also observed for recombinant genes in which the 72 base pair repeat enhanced transcription from heterologous promoter regions. Secondly, when clones of the 72 base pair repeat region were added to the transfection the E1A repression was reversed (Borrelli, 1984). This latter observation further suggested that the E1A-mediated repression of SV40 enhancer function resulted from the binding of an inhibiting factor to the enhancer DNA sequence. According to this model, the cotransfected 72 base pair repeat sequence competed for binding the proposed repressor, thereby derepressing the test gene and reversing the E1A-mediated repression (Borrelli, 1984).

A very interesting type of repression was discovered by van der Eb and his colleagues while studying possible differences between nononcogenic (Ad5) and oncogenic (Ad12) adenoviruses (Bernards et al., 1983; Schrier et al., 1983). They observed that the major histocompatibility complex (MHC) class-I antigens were repressed in cells transformed by Ad12 but were not repressed in Ad5-transformed cells. Further studies based on the construction of hybrid genomes showed that repression was associated with region E1A. This effect is only detected in primary cells and is not observed in continuous cell lines. Since nononcogenic adenoviruses fail to block class I expression it has been proposed that highly oncogenic adenoviruses are tumorigenic because they evade the immune surveillance of the host cell (Bernards et al., 1983;

Schrier et al., 1983).

E1A represses transcription of a number of growth factor-inducible protease genes. These include the JE, stromelysin, interstitial collagenase (collagenase I) and collagenase IV genes (Garbisa et al., 1987; Offringa et al., 1988; Timmers et al., 1989; Frisch et al., 1990; Offringa et al., 1990). As discussed earlier, secreted metalloproteinases which degrade extracellular matrix macromolecules have been implicated in the invasion of tumor cells through basement membranes during metastasis (reviewed in Liotta, 1986). Since regulated expression of these genes is required for normal cellular division and proliferative growth it is conceivable that regulatory changes, therefore, would influence the metastatic potential of affected cells.

Recently Hara et al. (1988) have shown that another metastasis-related gene, fibronectin, is also down-regulated by E1A in quiescent rat 3Y1 cells. This effect is elicited through induction of an E1A-responsive negative factor called $G_{10}BP$, which binds to two GC box sequences upstream of the fibronectin gene, AGGGGGGGGG and GGGGGGGCGGG (Nakamura et al., 1992). The second of these sequences overlaps a recognition site of transcription factor Sp1 (GGGCGG). Therefore, transcriptional repression of fibronectin in E1A expressing cells is believed to involve displacement of Sp1 by $G_{10}BP$ and other related factors (Nakamura et al., 1992). Promoter sequences of c-myc, α-actin, and H-ras contain

binding sites for both $G_{10}BP$ and Sp1. While transcriptional repression of c-myc and α-actin has been observed in E1A expressing cells, there is no such evidence in the literature for H-ras expression.

Table 10 provides a comprehensive list of the viral and cellular genes which are known to be repressed by E1A.

11) Interaction of E1A proteins with cellular polypeptides.

While several functions of the E1A proteins are known, the precise mechanism by which E1A polypeptides act to affect cellular characteristics has remained unclear. It is clear that E1A affects cellular protein concentrations and function by influencing their production at the DNA or transcriptional level. Shortly after the production of monoclonal antibodies by Harlow et al. (1985) and Yee and Branton (1985) it became clear that E1A may alter the normal properties of mammalian cells by forming protein-protein complexes with cellular proteins.

Harlow et al. (1986) were among the first to demonstrate direct association of E1A with cellular polypeptides. Extracts from adenovirus-transformed human embryonic kidney 293 cells immunoprecipitated with monoclonal antibodies for E1A proteins coprecipitate with a series of proteins with relative molecular weights of 28-, 40-, 50-, 60-, 80-, 90-,

Table 8. Viral and cellular genes whose expression is down-regulated by E1A-mediated enhancer repression.

Gene	Reference
Viral:	
E1A	Borrelli et al. (1984)
E2A	Rossini (1983)
SV40 enhancer	Velcich and Ziff (1985)
polyoma enhancer	Borrelli et al. (1984)
HIV LTR	Ventura et al. (1990)
Hepatitis B virus (HBV) enhancer 2	Chen et al. (1992)
Cellular:	
Oncogenes	
c-myc	Timmers et al. (1988)
erbB/neu	Yu et al. (1990)
junB	Kitabayashi et al. (1991)
Immune-related	
MHC class I (Ad12)	Schrier et al. (1983)
IgG heavy chain	Hen et al. (1985)
IgH enhancer	Lillie et al. (1986)
ISRE (interferon-stimulated response element)	Kalvakolanu et al. (1991) Gutch and Reich (1991)
interleukin-6 (IL-6)	Janaswami et al. (1992)
Metastasis-related	
collagenase I	Frisch et al. (1990)
collagenase IV	Frisch et al. (1990)
urokinase	Frisch et al. (1990)
plasminogen activator	Young et al. (1989)
JE	Timmers et al. (1988)
stromelysin	Offringa et al. (1988)
fibronectin	Hara et al. (1988)
Muscle	
MyoD1	Enkemann et al. (1990)
myogenin	Enkemann et al. (1990)
troponin	Enkemann et al. (1990)
α-actin	Enkemann et al. (1990)
muscle creatine kinase	Enkemann et al. (1990)
myosin heavy chain	Webster et al. (1988)
Other	
insulin	Stein and Ziff (1987)

All information refers to Ad2/5 unless otherwise indicated.

110-, 130- and 300-kDa. The two most abundant of these polypeptides are the 110-kDa (p110) and the 300-kDa (p300) proteins. Both the 12S and 13S E1A protein products bind to the 110-kDa and 300-kDa species. In addition, these complexes are found in adenovirus-transformed and adenovirus-infected cells (Harlow et al., 1986). The 110-kDa band is better resolved as a doublet of a 105-kDa and a 107-kDa polypeptide (Yee and Branton, 1985). It seems that these protein complexes mediate at least some of the physiological alterations induced by E1A, as any mutation that destroys binding of E1A to the p300, p105 or p107, also inactivates the ability of E1A to cooperate with ras in transforming baby-rat kidney cells (Whyte et al., 1988a).

The 105-kDa protein has been shown to be the product of the retinoblastoma susceptibility (RB) gene (Whyte et al., 1988b, Egan et al., 1989a). The RB gene is a well studied tumor-suppressor, inactivation of which is often associated with the appearance of retinoblastomas and certain soft-tissue sarcomas (reviewed by Murphee and Benedict, 1984). This interaction is the first demonstration of a physical interaction between an oncogene and an anti-oncogene (Whyte et al., 1988b).

The RB gene product ($p105^{RB}$) is the first negative cell cycle regulator to be identified (Friend et al., 1986). Although this negative regulation by RB was implied in the general model of carcinogenesis proposed by Knudson (1971) and

Comings (1973) it was not confirmed until recently. RB encodes a nuclear phosphoprotein which is associated with DNA binding activity (Lee et al., 1987). Modification of $p105^{RB}$ protein by phosphorylation or dephosphorylation occurs in a cell cycle-dependent manner and regulates progression of cells to the S phase of the cell cycle (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1989a; Mihara et al., 1989). During quiescence (G_0) and G_1, the $p105^{RB}$ is found in a hypophosphorylated state. As cells progress to the S phase it becomes hyperphosphorylated, and following mitosis (M) it returns to the hypophosphorylated state (Ludlow et al., 1990). The state of phosphorylation of $p105^{RB}$ is believed to be controlled by the cell cycle regulating kinase $p34^{cdc2}$. A complex involving direct association of $p105^{RB}$ with $p34^{cdc2}$ has been shown to facilitate phosphorylation of sites on the RB product in vitro similar to those phosphorylated in vivo (Lin et al., 1991; Hu et al., 1992). E1A proteins are also phosphorylated by the $p34^{cdc2}$ protein kinase in vitro, and are phosphorylated at the highest levels in vivo in mitotic cells which express maximal levels of $p34^{cdc2}$ kinase activity (Dumont and Branton, 1992).

In addition to the adenovirus E1A protein, transforming oncoproteins from other DNA tumor viruses bind to $p105^{RB}$ to form protein complexes. These include SV40 large T antigen and human papillomavirus-16 E7 protein (DeCaprio et al., 1989b; Dyson et al., 1989b). SV40 large T antigen binds to

the hypophosphorylated form of RB (Ludlow et al., 1989). This is likely true for E1A and E7 as well since the regions of these proteins that are involved in binding $p105^{RB}$ exhibit significant sequence homology (Figure 14) (Stabel et al., 1985; Figge et al., 1988; Phelps et al., 1988; Dyson et al., 1992). Dyson et al. (1990) have shown that this sequence homology also exists in several different genera of polyomaviruses, all of which form complexes with the $p105^{RB}$ protein through the large T antigens. Moreover, this region has been conserved during evolution in all four viral species (Moran et al., 1988; Phelps et al., 1988; Munger et al., 1989; Dyson et al., 1990).

$p105^{RB}$ is believed to have a role in the regulation of transcription of genes involved in cell cycle regulation and also plays a role in transcriptional activation by 12S E1A. The mechanism of $p105^{RB}$-mediated transcriptional regulation involves the transcription factor E2F. E2F is a cellular transcription factor which is induced, in the presence of E1A, to transactivate the viral E2 promoter (Kovesdi et al., 1986a; 1986b). E2F is present in uninfected cells but the concentration of the active factor is low and apparently limits transcription. In the presence of E1A there is a large increase in the level of active E2F, as measured by DNA-binding, which closely correlates with activation of E2 transcription (Kovesdi et al., 1987; Reichel et al., 1987; Reichel et al., 1988). Furthermore, the activation of E2F

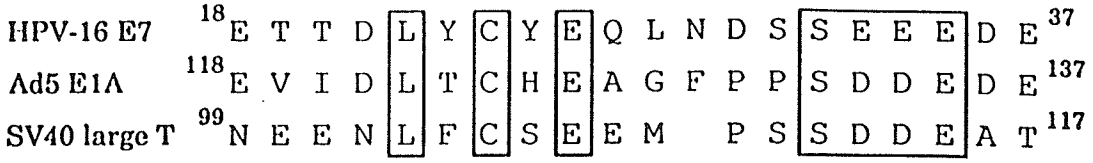


Figure 14. Amino acid sequence similarity among HPV-16 E7, Ad5 E1A, and SV40 TAg. (Taken from Pietenpol et al., 1990)

does not require new protein synthesis suggesting that the E2F protein is already present and E1A activates it at the protein level (Reichel et al., 1988). A similar factor involved in E1A-inducible transcription of the adenovirus E4 gene, termed E4F, is different from E2F (Raychaudhuri et al., 1987). Expression of the E4 gene along side E1A results in a stimulation of DNA binding activity and induction of cooperative binding of E2F mediated by the E4 gene product (Babiss, 1989; Reichel et al., 1989; Raychaudhuri et al., 1990). Further studies have shown a direct protein level interaction of E4 with E2F (Marton et al., 1990; Neill et al., 1990).

It has recently been demonstrated that the mechanism by which E1A activates E2F involves dissociation of a heteromeric protein complex involving the E2F transcription factor (Bagchi et al., 1990). This complex involves another element termed E2F inhibitory protein (E2F-I). The binding of the E2F-I complex to E2F specifically inhibits DNA binding activity of E2F and thereby inhibits or inactivates transcriptional activation (Raychaudhuri et al., 1991). One of the components of the E2F-I complex is the $p105^{RB}$ protein (Bagchi et al., 1991; Chellappan et al., 1991). $p105^{RB}$, therefore, essentially represses E2F-mediated transcription by directly binding to and inactivating E2F (Arroyo and Raychaudhuri, 1992). Thus, it is currently a widely accepted belief that the mechanism by which E1A activates E2F is to bind $p105^{RB}$ and thereby release

E2F proteins to freely form transcriptional complexes at specific DNA sequences. An alternate mechanism is possible based on the finding that activation of E2F is dependent on the presence of a phosphorylation function (Bagchi et al., 1991). Also, Wang et al. (1991) have shown that E1A induces phosphorylation of $p105^{RB}$ independently of direct association between E1A and $p105^{RB}$. It is possible then, that this phosphorylation process may hinder E2F-$p105^{RB}$ binding and thereby free or activate E2F in E1A transformed cells.

The $p105^{RB}$ protein also binds to another cellular transcription factor, DRTF1, originally defined in F9 cells (La Thangue and Rigby, 1987). DRTF1 binds to the same sequence as E2F (La Thangue, 1990). It is involved in transcriptional activation of a number of cellular genes that encode proteins involved in cell cycle progression including DHFR and DNA polymerase alpha (Blake and Azizkhan, 1989; Hiebert et al., 1991; Pearson et al., 1991). Two recent studies show that E1A prevents $p105^{RB}$ from binding to, and inactivating DRTF1, thereby allowing cell cycle progression (Bandara and La Thangue, 1991; Zamanian and La Thangue, 1992). Thus, it is clear that RB regulates the cell cycle, at least in part by regulating expression of cell cycle regulated genes and E1A acts to preclude RB-mediated cell cycle control in transformed cells by interfering with the normal function of $p105^{RB}$ (Howe and Bayley, 1992). This represents a novel mechanism for transcriptional activation by E1A (Bagchi et

al., 1990; Howe and Bayley, 1992).

The identity of the 300-kDa protein is not yet known. The ability of E1A to bind p300, however, is believed to be related to its enhancer repression function. The evidence for this comes from studies in which E1A mutants unable to bind p300 are unable to repress insulin enhancer-stimulated transcription and also do not induce cellular DNA synthesis in quiescent cells as seen with wild-type E1A proteins (Stein et al., 1990). Also, the ability of E1A proteins to suppress differentiation of murine myoblasts correlates with their binding to p300 (Mymryk et al., 1992). It is known that p300 is a ubiquitously expressed nuclear phosphoprotein that is actively phosphorylated in all phases of the cell cycle but shows an additional phosphatase-sensitive modification specific to M-phase enriched cell populations (Yaciuk and Moran, 1991). In addition, p300 has an intrinsic DNA-binding activity and shows a preferential affinity for specific DNA sequences similar to those of a series of enhancer elements that are recognized by NF-kB, and a second related transcription factor H2TF1 (Rikitake and Moran, 1992). Interestingly, the kappa light chain enhancer, SV40 enhancer, IL-6 enhancer, and the HIV long terminal repeat, all of which are E1A regulated, contain motifs structurally and functionally related to NF-kB and H2TF1 (Sen and Baltimore, 1986; Nomiyama et al., 1987; and Nabel and Baltimore, 1987; Janaswami et al., 1992).

The 60-kDa protein that interacts with E1A to form a stable protein complex has been identified as human cyclin A (Pines and Hunter, 1990; Giordano et al., 1989). The cyclins are regulatory subunits that complex with kinases to regulate many of the important steps in cell cycle progression. The best characterized of the cyclin-containing complexes is the association of cyclin B with the $p34^{cdc2}$ kinase (for reviews see Murray and Kirschner, 1989, and Nurse, 1990). Cyclin A binds independently to two kinases, associating with $p34^{cdc2}$ or a related protein p33 (Giordano et al., 1989; Pines and Hunter, 1990; Giordano et al., 1991a). In adenovirus-transformed cells, E1A seems to associate with the p33/cyclin A but not with $p34^{cdc2}$/cyclin A (Kleinberger and Shenk, 1991). p33 is the protein product of the human cyclin-dependent kinase 2 (cdk2) gene (Tsai et al., 1991). $p33^{cdk2}$ shares 65% homology with $p34^{cdc2}$ (Lee and Nurse, 1987). The two polypeptides are related but distinct. Herrmann et al. (1991) have found that an E1A-associated serine/threonine protein kinase activity, presumably that of $p33^{cdk2}$, is cell cycle regulated, being most active in S and G2/M. $p33^{cdk2}$ can phosphorylate two E1A-associated proteins, p107 and p130, as well as histone H1 added as an exogenous substrate. Therefore, the $p105^{RB}$-phosphorylation activity related to E2F-$p105^{RB}$ dissociation and E2F activation that was referred to earlier is likely mediated by the $p33^{cdk2}$/cyclin A complex.

The cellular protein p107 is believed to be related to,

but different from $p105^{RB}$ based on several functional similarities between the two. Both proteins bind E1A proteins and this interaction occurs at the same E1A peptide region (Harlow et al., 1986; Whyte et al., 1988a). A similar interaction has been observed involving SV40 large T antigen (Dyson et al., 1989a; Ewen et al., 1989). cDNA cloning and sequencing of the gene encoding p107 has revealed significant sequence homology with RB including the region corresponding to the "pocket" domain implicated as E1A/T binding region (Ewen et al., 1991). Furthermore, both p107 and $p105^{RB}$ pockets can bind specifically to the same set of cellular proteins in cell-free assays (Kaelin et al., 1991; Ewen et al., 1992; Faha et al., 1992). This includes the cellular E2F protein. p107, however, does not react with some $p105^{RB}$-specific antibodies.

Binding of p107 and $p105^{RB}$ to E2F appears to be cell cycle regulated (Shirodkar et al., 1992). E2F associates with a hypophosphorylated form of $p105^{RB}$ found primarily in G_1 cells (Chellappan et al., 1991). The E2F-$p105^{RB}$ complex dissociates near the G_1/S boundary, releasing free E2F to activate cell proliferation-related transcription (Mudryj et al., 1990; Bandara and La Thangue, 1991). During S phase, E2F forms a second protein complex that involves cyclin A, $p33^{cdk2}$, and p107 which appears to replace $p105^{RB}$ (Mudryj et al., 1991; Cao et al., 1992; Shirodkar et al., 1992). These observations suggest that p107 and $p105^{RB}$ cooperate in the regulation of E2F activity, with each affecting different stages of the cell

cycle (Figure 15). Since E1A binds $p105^{RB}$ and the cyclin A/$p33^{cdk2}$/p107 complex, it affects cellular proliferation at two levels. Firstly, by binding $p105^{RB}$, E1A precludes E2F from interaction with $p105^{RB}$ and thereby activates E2F-mediated transcription before the desired cell stage. Also, the binding of E1A to the second complex circumvents E2F regulation through phosphorylation, or interaction with a phosphorylation-related complex. These facts clarify the complex interaction of E1A with these cellular polypeptides and put into perspective the mechanism by which E1A overrides cell cycle controls and accomplishes cellular transformation through protein binding activities.

Figure 16 shows a map of the regions of E1A involved in cellular protein binding.

12) Mapping of functional domains in E1A.

Expression of E1A in mammalian cells results in a number of cellular effects in addition to those already described. Many of these are likely related to the transformed phenotype exhibited by E1A-transformed cells, as well as E1A-mediated metastatic suppression which is discussed later. By using mutant E1A genes containing point mutations or deletions at specific sites or regions researchers have been able to map the several different transformation-related functions of E1A to specific regions of the gene. Since this study involves a

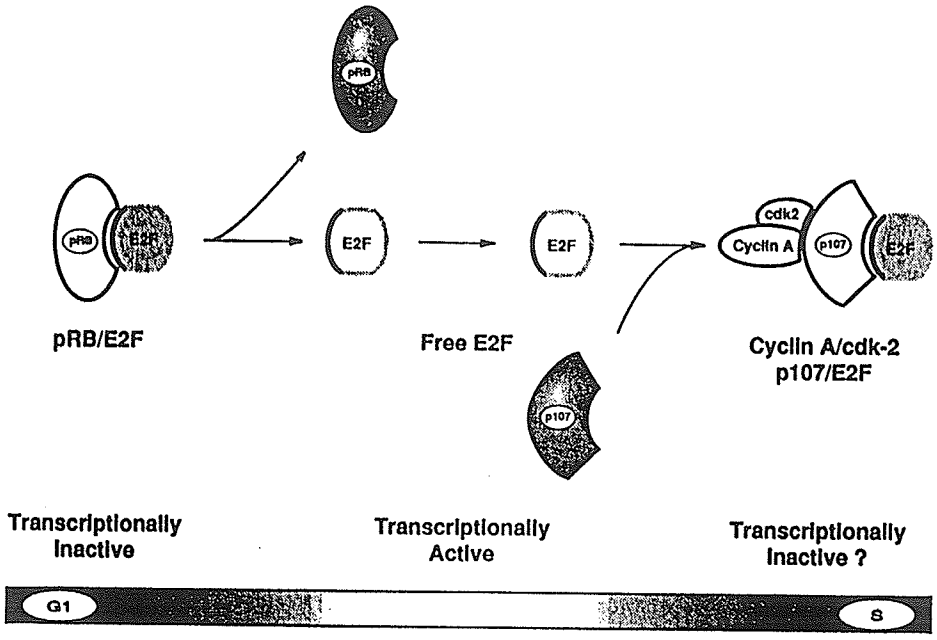


Figure 15. Regulation of E2F activity by $p105^{RB}$ and p107 involves cell cycle-specific complex formation. E2F complex formation is cell cycle-dependent so as to provide strict control of E2F-mediated transcriptional activation of proliferation-associated genes. Cyclin A (p60), $p33^{cdk2}$, p107 and $p105^{RB}$, all of which bind E1A, are involved in regulation of E2F. p107 essentially replaces $p105^{RB}$ in S phase so as to involve proteins capable of phosphorylation activity. In E1A expressing cell lines E1A replaces the E2F protein in both complexes and thereby constitutively activates E2F and deregulates expression of cell cycle progression-related genes leading to a transformed cell phenotype. (Figure taken from Cao et al., 1992)

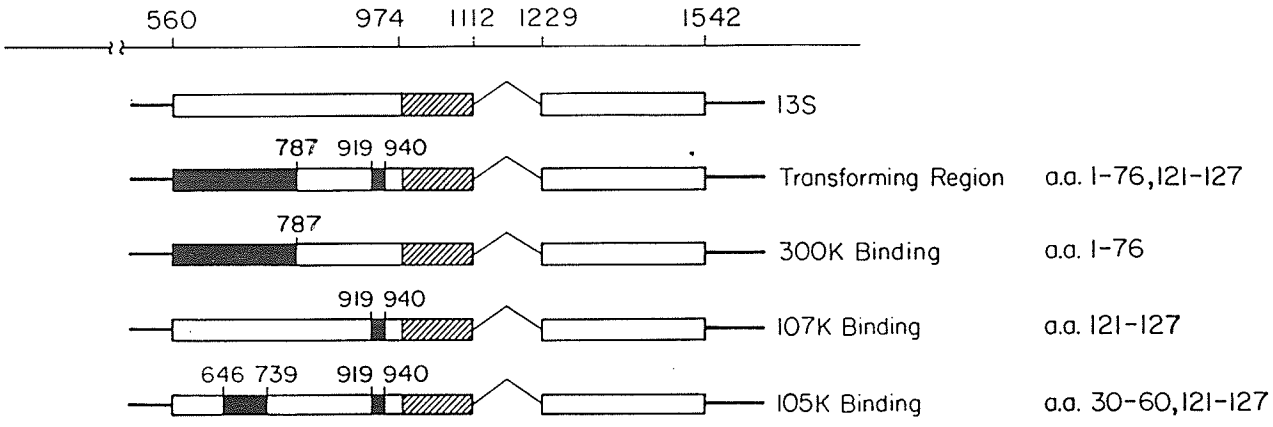


Figure 16. Comparison of regions of E1A involved in transformation and regions involved in binding to cellular proteins. Schematic representations of the E1A region highlighting the sequences required for transformation and the regions required for binding to each of the p300, p107, and $p105^{RB}$ cellular proteins. The boxed regions represent protein coding regions. The hatched region represents the protein coding sequences that are unique to the 13S mRNA. The solid regions repesent sequences required for the respective functions stated on the right. Numbers refer to nucleotide positions. The corresponding amino acid sequences for each region shown in black are at the right. (Taken from Whyte et al., 1989)

similar approach to address the modulation of metastatic potential of mammalian cells by E1A, it is important to understand what other functions of E1A may be affected by the introduction of such selective mutation into E1A.

The 13S E1A protein product, as mentioned earlier, is the polypeptide most active in transactivation functions. In fact, the 46 amino acid region comprising CR3 is capable of acting independently of other E1A sequences. Introduction of this small peptide alone into cells results in transcriptional activation of E1A-responsive promoter regions, making it the smallest known transcription factor (Lillie et al., 1987). Transactivation capabilities of the 12S product are very limited.

Results involving mutations in CR1 and CR2 suggest that these regions are necessary for transcriptional repression (Lillie et al., 1987; Schneider et al., 1987). The repression of growth factor-inducible genes JE, stromelysin and c-myc by E1A is dependent only on CR1 and not CR2 (van Dam et al., 1989). Mutation from 125 to 127 in CR2 weakens repression of heavy-chain and polyoma enhancers (Kuppuswamy and Chinnadurai, 1987). Moreover, mutations of regions outside CR1 and CR2 also significantly influence E1A-mediated enhancer repression. Deletion of an N-terminal region (2 to 36) that does not include CR1 results in loss of repression of insulin enhancer-stimulated activity (Stein et al., 1990). Mutations in N-terminal regions encoded by exon 1, and C-terminal regions

encoded by exon 2 of E1A, inhibit repression of the polyoma enhancer (Velcich and Ziff, 1988). Jelsma et al. (1989) have shown that CR1, CR2 and both the N-terminal and C-terminal regions of E1A are all important in maintenance of the enhancer repression function of E1A and that mutation in any of these regions reduces or abolishes this function. Therefore essentially all regions of E1A, except CR3, have some impact on the repression of viral and cellular genes.

The Ad5 12S E1A protein product induces cellular DNA synthesis and proliferation in primary epithelial cells in both the presence and absence of serum (Quinlan and Grodzicker, 1987). The induction of DNA synthesis has been shown for both 13S and 12S E1A proteins and is closely related to the processes of immortalization and transformation in cooperation with ras (Braithewaite et al., 1983; Bellett et al., 1985; Stabel et al., 1985; Kaczamarek et al., 1986; Nakajima et al., 1987; Zerler et al., 1987).

Cell cycle activation alone, however, is not sufficient for immortalization functions of E1A (Quinlan and Douglas, 1992). DNA synthesis and cellular proliferation are regulated by different regions of the E1A protein. A number of studies using E1A deletion mutants have implicated CR1 as being involved in induction of DNA synthesis (Lillie et al., 1987; Zerler et al., 1987; Smith and Ziff, 1988). Mutation of sequences in CR2 does not affect host cell DNA synthesis induction (Moran and Zerler, 1988). However, single point

mutations within CR2 abolishes the activity which induces proliferation of primary epithelial baby rat kidney (BRK) cells (Lillie et al., 1986; Moran et al., 1986; Schneider et al., 1987). Loss of conserved region 1 is often associated with loss of both DNA synthesis and proliferation. This is likely because during the induction of proliferation in quiescent cells CR1 is primarily important for induction of DNA synthesis, whereas CR2 is required for further cell cycle progression (Moran and Zerler, 1988).

Induction of DNA synthesis is likely related to the activation of PCNA by the 12S and 13S protein product of E1A (Zerler et al., 1987; Jelsma et al.,1989). PCNA, a protein of approximately 35-kDa, is associated with DNA replication and cell proliferation in both normal and transformed cells (Celis et al., 1984; Mathews et al., 1984; Bravo, 1986). In addition, binding of E1A to p300 and $p105^{RB}$ correlates with DNA synthesis induction (Howe et al., 1990; Stein et al., 1990). These two proteins appear to regulate DNA synthesis based on the observation that there are very low levels of cellular DNA synthesis in cells expressing E1A mutants defective in direct protein interaction with either protein. As discussed earlier p300 is associated with E1A-mediated repression in transformed cells. Its involvement in DNA synthesis suggests it may regulate cell cycle related genes. As previously discussed, the role of $p105^{RB}$ as a cell cycle regulator is well documented.

Oncogenic transformation has generally been seen as a multistep process, with cellular immortalization being the first step (Weinberg, 1985). Kuppuswamy et al. (1988) have shown that an E1A protein mutated at amino acids 47-50 is defective in immortalization of primary BRK cells, but can cooperate with T24-ras in oncogenic transformation. This confirms that, in the case of E1A, immortalization and oncogenic cooperation are separate functions but suggests that immortalization is not a prerequisite for oncogenic transformation by T24-ras.

Although the presence of conserved domain 3 correlates strongly with transcriptional activation of cellular genes, the various transformation-related activities of E1A are independent of this domain as both the 243R and 289R polypeptides are capable of establishing cells in culture and cooperating with ras to induce cellular transformation (Carlock and Jones, 1981; Haley et al., 1984; Ricciardi et al., 1981; Zerler et al., 1985; 1986; Schneider et al., 1987; Bautista et al., 1991).

Several studies have suggested that oncogenic cooperation by E1A is linked to the enhancer repression of cellular genes (Lillie et al., 1986; Lillie et al., 1987; Schneider et al., 1987). Cells expressing E1A proteins that are defective for enhancer repression functions are also unable to induce full transformation in these studies. However, evaluation of transformation and repression functions using E1A mutations

outside of CR1 and CR2 indicate that transformation is not based solely on enhancer repression properties of E1A. Some mutants retain their ability to cooperate with ras in transformation of cells but lack the enhancer repression activity (Velcich and Ziff, 1988). Alternately, some transformation-defective mutants of E1A exhibit wild-type repression (Kuppuswamy and Chinnadurai, 1987; Jelsma et al., 1989). Therefore transformation by E1A appears to involve a repression function in concert with some other function, but repression alone is not sufficient to induce cooperation with ras. Thus, E1A ras cooperation activity is separate from both its positive and negative transcriptional regulatory functions.

The ability of E1A to transform cells in cooperation with ras has been mapped by various groups to a region in CR2. Moran et al. (1986) have shown that deletion of amino acids 121-150 significantly impairs ras cooperation with E1A. Moreover, single amino acid substitution at positions 124 and 135 have similar results. Lillie et al. (1986) have demonstrated loss of transformation by single amino acid substitutions at positions 126 and 131. Similar results have been attained using insertional mutation of region 125 to 127 (Kuppuswamy and Chinnadurai, 1987).

Schneider et al. (1987) have suggested that CR1 may also be involved in a function associated with cellular transformation. This suggestion arises from studies in which

individual deletion mutants from amino acid positions 121 to 136 and 41 to 62 both fail to cooperate with ras. In fact, transformation by E1A is associated with its ability to bind to p300, p107 and p105 cellular polypeptides (Figure 16) (Whyte et al., 1989). Interaction with p300 and both p105 and p107 is necessary for transformation by E1A (Egan et al., 1989a). Note that sequences from both CR1 and CR2 are involved in the binding to these proteins.

The production of transformed foci in a ras cooperation assay is regulated by sequences within CR1 and CR2. Expression of both domains is required, and focus formation occurs even when the two domains are introduced into cells on seperate plasmids (Moran and Zerler, 1988; Smith and Ziff, 1988).

Quinlan and Grodzicker (1986) have described the induction of an epithelial cell growth factor in E1A 12S transformed cells. This growth factor is produced also during infection with an adenovirus variant which stimulates proliferation of nonestablished epithelial cells (Quinlan et al., 1987). This growth factor is induced by E1A in F9 teratocarcinoma cells and is required for immortalization of primary BRK epithelial cells (Quinlan et al., 1988; Subramanian et al., 1988; Quinlan, 1989; Quinlan and Douglas, 1992). Amino acid sequences of E1A required for induction of this growth factor have been mapped to regions near the N- and C-termini of the 12S protein. Regions spanning from amino

acids 1 to 13 at the N-terminus and 208 to 236 at the C-terminus are necessary for growth factor production and immortalization (Quinlan et al., 1988; Quinlan and Douglas, 1992). Subramanian et al. (1988) have found that individual E1A proteins mutated at amino acid regions 18 to 20 and 125 to 127 are both defective in transformation and growth factor induction suggesting that the two phenomena are related.

Expression of E1A renders NIH 3T3 cells susceptible to tumor necrosis factor-α (TNF-α) cytolysis, whereas expression of a variety of other oncogenes including v-src, c-src, H-ras, c-myc and polyomavirus middle and large T, does not (Chen et al., 1987; Duerksen-Hughes et al., 1989). TNF-α, a product of activated macrophages, is cytostatic and cytotoxic for a variety of transformed cell lines and has antiviral as well as antineoplastic properties (Old, 1985; Sugarman et al., 1985; Koff and Fann, 1986). Enhanced TNF-α sensitivity may partially or fully account for the fact that E1A induces sensitivity to macrophage and natural killer cell-mediated cytotoxicity (Cook et al., 1989a; Cook et al., 1989b; Routes and Cook, 1989). Vanhaesebroeck et al. (1990) have shown that modulation of TNF-α sensitivity by E1A depends on the cell type under study and does not correlate with E1A expression. The mechanism by which E1A induces the cytotoxic action of TNF-α is independent of other biological functions of E1A including ras cooperation, immortalization, induction of DNA synthesis and transcriptional repression (Ames et al., 1990).

The region responsible for this function has been mapped to conserved region 1 from amino acids 36 to 60 (Duerksen-Hughes et al., 1991).

Upon viral infection cells are known to induce an immune response that targets specific epitopes of viral proteins. The cellular immune response to viral infection can be divided into two components, an early-appearing major histocompatibility complex (MHC)- unrestricted response mediated by natural killer (NK) cells and macrophages and a later-appearing, MHC-restricted response mediated by cytotoxic T lymphocytes (CTLs) (Doherty and Zinkernagel, 1977; Rager-Zisman and Bloom, 1982; Welsh, 1986). The specific cellular immune system response to adenovirus-infected and -transformed cells has been studied. Immunization of rodents with either Ad2/5 or Ad12 induces virus-specific, protective immunity to transplantation of tumorigenic cells transformed by adenovirus of the same group, a property attributed to tumor-specific transplantation antigens (Sjogren et al., 1967). The results of recent studies indicate that these adenovirus-specific transplantation antigens are encoded by the E1A gene (Sawada et al., 1986). More specifically, studies in both mice and rats show that immunization with Ad5 E1A-transformed cells can induce a class I-restricted CTL response directed against epitopes encoded by the E1A gene (Bellgrau et al., 1988; Kast et al., 1989). Preliminary mapping studies suggest that the E1A CTL epitopes are encoded within two regions in exon 1,

while a region in exon 2 is required for CTL induction. Urbanelli et al. (1991) have found that a C-terminal domain of E1A is required for induction of CTLs and tumor specific transplantation immunity. Meanwhile, Routes et al. (1991) have attained results suggesting that two regions encoded by exon 1, from amino acids 22 to 83, and 112 to 138, encode for the immunodominant epitopes of anti-adenovirus type 5 CTLs.

Transformation by the E1A oncogene has definite affects on cellular differentiation processes. The 12S E1A protein, but not the 13S protein, induces expression of the endo A differentiation marker in F9 cells (Velcich and Ziff, 1989). In addition, Quinlan et al. (1989) has found that cellular differentiation and proliferation are induced by E1A in F9 cells. These cells exhibit morphological alterations and express the surface antigens, SSEA-1 and SSEA-3, which are characteristic of differentiated cells. In contrast to this other groups have associated E1A expression to a repression of differentiation. Expression of myc and E1A in PC12 rat pheochromocytoma cells blocks morphological differentiation and causes nerve growth factor (NGF) to stimulate rather than inhibit cell proliferation (Maruyama et al., 1987). E1A-mediated suppression of myogenic differentiation has also been reported (Webster et al., 1988). Moreover, expression of E1A in terminally differentiated nonproliferating F9 cells yields a high frequency of colonies of dividing cells (Weigel et al., 1990). These cells proliferate in the presence of retinoic

acid and lose the fully differentiated phenotype as characterized by the loss of expression of a series of differentiation specific genes. These findings, taken together, suggest that the differentiation process, as defined by differentiation markers, can be reversed by expression of E1A.

Nuclear proteins have nuclear localization signals that direct and facilitate movement of the translated polypeptide from the cytoplasm to the nucleus (Dingwall et al., 1982; Hall et al., 1984; Goldfarb et al., 1986). E1A contains a nuclear localization signal near the C-terminus (Krippl et al., 1985). Just the last five amino acids of E1A are sufficient to direct rapid nuclear accumulation of E1A (Lyons et al., 1987). The small pentameric sequence of E1A, LYS-ARG-PRO-ARG-PRO, aside from its high content of basic amino acids, is not strikingly similar to the nuclear localization signals of nuclear proteins from other DNA viruses such as SV40 large T antigen, PRO-LYS-LYS-LYS-ARG-LYS-VAL, and polyoma virus large T antigen, which has two sequences, PRO-LYS-LYS-ALA-ARG-GLU-ASP and VAL-SER-ARG-LYS-ARG-PRO-ARG (Richardson et al., 1986).

13) E1A as a transformation and metastasis suppressor.

E1A has been conventionally considered as a dominant oncogene since it transforms cells in vitro and many of the resulting cell lines induce tumor formation in vivo. Recent observations of E1A-transfected lines suggest that in addition

to its well known transforming activities, E1A encodes for functions which suppress transformation, tumorigenesis and metastasis. E1A therefore, fulfills the definition of both a dominant oncogene and a tumor- and metastasis-suppressor (Chinnadurai, 1992).

Yu et al. (1991) have found that introduction of Ad5 E1A into mouse NIH 3T3 cells transformed by the neu oncogene causes reversion to the normal cell phenotype as judged by conventional transformation parameters such as cell morphology, contact inhibition, growth in soft agar and tumorigenesis in nude mice. It is known that E1A represses the activity of the neu promoter (Yu et al., 1990). It appears, at least in this case, that the enhancer repression function of E1A may account for the E1A-mediated reversion of the transformed phenotype. Similar results have been seen using a diverse group of established human tumor cell lines. Frisch (1991) has demonstrated that cell lines such as HeLa, HT1080 (fibrosarcoma) and A2058 (melanoma) can be converted to a non- or less tumorigenic state by stable expression of Ad2/5 E1A.

E1A expression also suppresses metastatic progression of tumor cells. Pozzatti et al. (1986) have observed that rat embryo fibroblasts (REF) transformed by activated ras (T24 ras) and E1A are substantially less metastatic than cells transformed by ras alone. This is not true where Ad12 E1A is used in place of Ad2 (Pozzatti et al., 1988). Furthermore,

introduction of genomic E1A DNA or cDNAs coding for either the 12S or 13S E1A protein product into highly metastatic rodent or human cells reduces the metastatic potential of these cells as quantitated by tail-vein injection of these cell lines in nude mice (Pozzatti et al., 1988; Frisch et al., 1990). It has been observed that in stably transfected cells there may be a correlation between reduced metastatic potential and a reduction in the levels of metastasis-associated metalloproteinases such as stromelysin, collagenase I and collagenase IV (Garbisa et al., 1987; Frisch et al., 1990; Offringa et al., 1990).

In addition to E1A-mediated repression of secreted proteases, E1A activates expression of the cellular nm23 gene (Steeg et al., 1988b). nm23 expression has been previously associated with low metastatic potential in rodent model systems and is therefore believed to be a metastasis-suppressor (Steeg et al., 1988b). Thus, it is possible that both the positive and negative transcriptional regulatory functions of E1A could contribute to the reduction of metastatic potential in tumor cells.

Although the mechanism by which E1A suppresses transformation has not yet been elucidated, mapping studies have suggested regions of the protein that may be important in carrying out these functions. Subramanian et al. (1989) have found that in E1A-T24 ras cooperation experiments that E1A mutants lacking the C-terminal 61 or 67 amino acids induce

rapidly growing tumors in syngeneic rats and athymic mice, whereas cells transformed by the wild-type 12S E1A product and ras are not tumorigenic and can only induce slowly growing tumors. In addition the E1A mutants have a much higher metastatic potential compared to wild-type E1A. Similarily, Douglas et al. (1991) have also found that a region encoded by exon 2 suppresses transformation by the 12S E1A in cooperation with ras. A deletion of the region from nucleotides 1437 to 1488 (corresponding to amino acids 207-224 in the 243R E1A protein, or 253-270 in the 289R protein) results in an increased number of foci which appear earlier in the assay compared to cells containing wild-type E1A. This region is outside of the nuclear localization signal, therefore subcellular localization of E1A does not affect its cotransforming ability.

Invasion of basement membranes is one of the basic characteristics of highly metastatic cells and is one of the earliest stages in the metastatic process (Liotta, 1992). Linder et al. (1992) have used a series of exon 2 mutants to map the region responsible for invasive properties of rat embryo fibroblasts cotransformed with ras and 13S E1A. The results indicate that a region from amino acids 223 to 246 is responsible for reduced cellular invasion of reconstituted basal membranes. That is, mutants in this region exhibited enhanced invasive properties compared to other mutants and wild-type E1A, implicating this region in suppression of

invasion. Moreover, inability of proteins containing mutations in this region to invade correlates with a defect in down-regulation of stromelysin expression which may account for the loss of invasive ability of these mutants.

14) Current objectives.

Based on the findings of Pozzatti et al. (1986, 1988) that the 12S and 13S Ad2 E1A products suppress the metastatic potential of highly metastatic, T24/EJ ras-transfected, 5R rat embryo fibroblast cells, I am interested in understanding the mechanism by which this effect is elicited. It is paradoxical that a potent oncogene is able to suppress a transformation-associated characteristic. As described earlier, transformation and metastasis appear to be associated phenomena in ras-transformed cells (Egan et al., 1989a). Earlier studies at our institute have shown enhanced transformation and metastatic potential with increased ras expression (Egan et al., 1987a; Taylor et al., 1992). Hence, the suppressive effect of E1A on metastasis is both surprising and intriguing. The inability of Ad12 E1A to lower metastatic potential in these same cells simply adds to the mystery.

The approach to understanding the underlying mechanism involves addressing the problem in three seperate stages. The first would be to identify a region of the E1A protein that is involved in suppression. The second would be to identify a specific function associated with that region, such as

transcriptional regulatory activities, including transactivation and enhancer repression, or cellular protein interaction. Since many of the biochemical activities of E1A have already been mapped, the location of the 'suppressor region' would suggest what function was involved. Thirdly, an attempt would be made to identify a specific gene or gene product, the presence or absence of which, would be associated with metastatic suppression.

A large number of plasmids expressing wild-type (243R and 289R) and mutant E1A proteins were obtained. Mutations of all regions, including N-terminus, CR1, CR2, CR3, and C-terminus were used so as to represent defects in all biochemically defined areas of E1A. These were transfected into highly metastatic 5R cells. It was expected that all cell lines expressing mutant E1As, and both wild-type proteins, would suppress metastasis in in vivo metastasis assays involving tail-vein injection of nude mice; and that one, maybe two, lines expressing mutations in the 'suppressor region' would fail to suppress metastasis. Thereby the critical region would be mapped. After hypothesizing as to the biochemical activity of E1A that is involved in suppression, based on the location of the region, screening for variable expression of metastasis-associated genes, including ras, could be carried out to relate metastatic suppression to activation/deactivation of some gene or protein.

By utilizing the outlined agenda I was able to deduce

that sequences located at the N-terminus, CR3, as well as C-terminal sequences encoded by exon 2, encode biochemical functions necessary for suppression of metastasis by E1A. In contrast to previous reports, I found that only the 289R E1A, and not the 243R E1A, is capable of metastatic suppression. Furthermore, mutations in CR1 and CR2 had no effect on suppression. Therefore these regions and functions associated with them can be precluded from involvement in the mechanism of metastatic suppression.

All mutant E1As used in this study have previously been characterized for positive and negative transcriptional activities. All of the mutant E1A proteins that are theoretically defective in transcriptional activation were found to be defective in metastatic suppression, suggesting that transactivation functions of E1A are related to suppression of metastasis. This idea is further supported by the finding that, without exception, cells expressing mutant E1A proteins that are theoretically functional in transactivation activities exhibited suppressed metastatic potentials comparable to wild-type (289R) levels. Furthermore, there was a correlation of activated H-ras expression with metastatic potential. Highly metastatic cell lines exhibited elevated T24/EJ ras levels, in some cases higher than the 5R parental cells, whereas cell lines with low metastatic potentials exhibited low levels of T24/EJ ras compared to 5R. This effect was seen at the protein level and

it is not known at this time whether E1A affects $p21^{ras}$ protein levels at the transcriptional or post-transcriptional level. However, the fact that the presence of regions encoding transcriptional regulatory activity of E1A is associated with $p21^{ras}$ levels suggests that E1A may directly or indirectly affect transcription of the ras oncogene.

MATERIALS AND METHODS

1) Plasmids.

Wild-type E1A expression vectors pSVN20 and pSVF12, and mutant E1A expression vectors pSVXL174, pSVXL105, pSVXL132, pSVXL124 and pSVXL214 were obtained from E.B. Ziff. Wild-type vector pSVE1a (4.6 kb) has been previously described (Velcich and Ziff, 1985). It contains a clone of nucleotides 1 to 1834 of genomic adenovirus type 5 DNA inserted between the Eco RI and Pst I sites of the pBR322-derived vector pSVOd. pSVOd does not contain the pBR322 sequences inhibitory to replication in mammalian cells, carries both AMP^R and TET^R markers and has unique Pst I, EcoR I, Hind III, Bam HI and Sal I sites. It contains the SV40 origin of replication but no enhancer sequences (Mellon et al., 1981). pSVE1a does not contain the AMP^R marker due to the insertion of the Eco RI-Pst I E1A fragment (Smith et al., 1985). Wild-type E1A vectors pSVN20 and pSVF12 are the intronless forms of pSVE1a, reconstructed with sequences from the 12S and 13S cDNAs, respectively. Mutated forms of the 13S vector were constructed by Xho I linker insertion mutagenesis as described by Smith et al. (1985). All of the above-mentioned wild-type and mutant vectors have been used previously in transcriptional studies by Velcich and Ziff (1988). Figure 17 illustrates the polypeptides resulting from expression of these plasmids.

E1A vectors containing mutations in CR2 were obtained

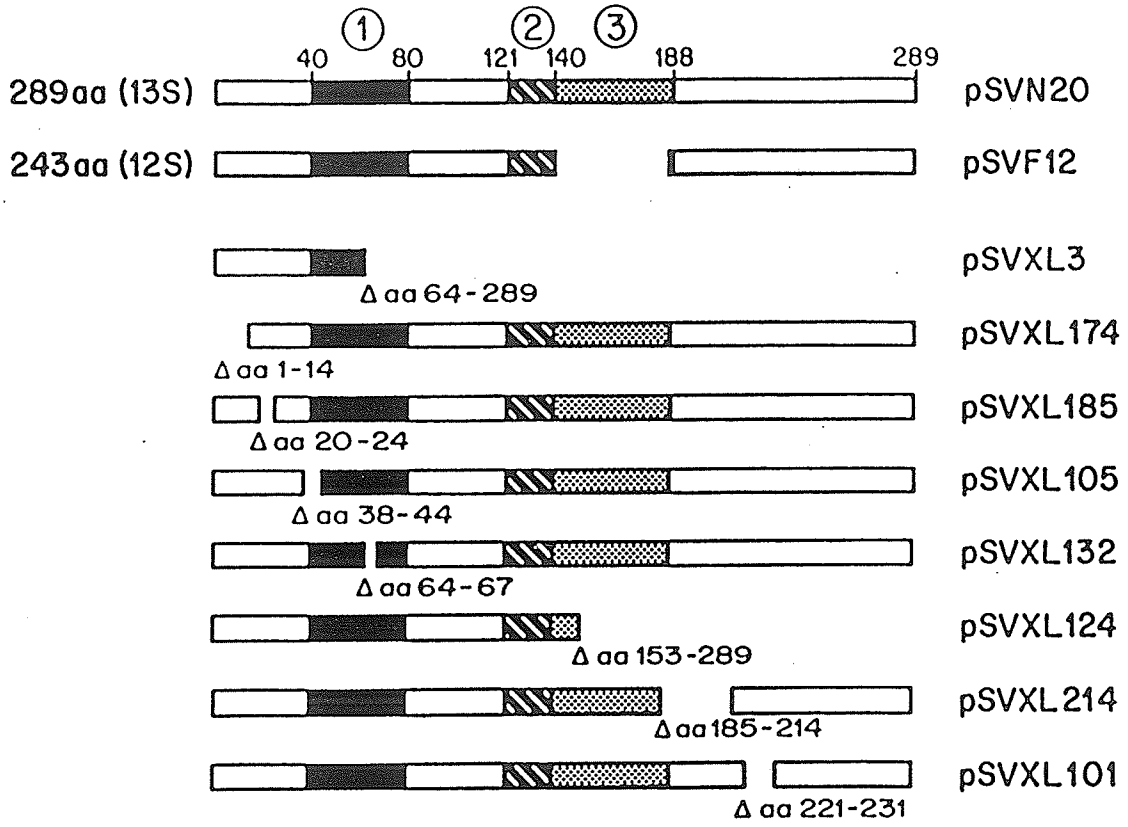


Figure 17. Predicted E1A polypeptide structures encoded by wild-type and mutant E1A vectors. The 289R wild-type protein encoded by the 13S mRNA is represented at the top. The regions which are highly conserved among the adenovirus serotypes are boxed and indicated as domains 1, 2, and, 3. Below are illustrated the predicted protein structures encoded by the 12S cDNA and the 13S cDNA E1A mutant vectors studied in this report, except for pSVXL3, pSVXL185, and pSVXL101 which were not used. Also marked are the wild-type amino acid residues in each mutant. (Taken from Velcich and Ziff, 1988)

Plasmid pGC212 contains nucleotides 310 to 2798 of the Ad2 genomic DNA (Chinnadurai, 1983). This includes E1A and E1B. The E1B region has been removed by digestion with Sst I and Hind III to remove nucleotides 1767 to 2798. The resulting vector was partially or completely linearized with various endonucleases specific for CR2 sequences and blunt ended either by filling in at the 5′ overhang or by removing the 3′ overhang with T4 DNA polymerase. EcoR I linkers of appropriate lengths were added by linker tailing. The resulting mutant plasmids contain in-frame insertions, deletions and substitution mutations. The two mutants used in this study are 120-1 (mutation at amino acids 120 and 121) and 130-3 (mutation from amino acids 130 to 133). Figure 18 illustrates the genetic structure of these mutations.

In DNA transfection experiments the Hygromycin B transferase gene was used as a selectable marker. The plasmid pY3, which contains the hygromycin B transferase gene, confers resistance to hygromycin B (hmB), an aminocyclitol antibiotic which inhibits protein synthesis in prokaryotes and eukaryotes (Gonzalez et al., 1978; Blochlinger and Diggelman, 1984). The pY3 plasmid was obtained from E. Ruley and used in co-transfections with wild-type and mutant E1A gene plasmids.

2) Cell lines and culture conditions.

The highly metastatic cell line, 5R, was obtained from R.

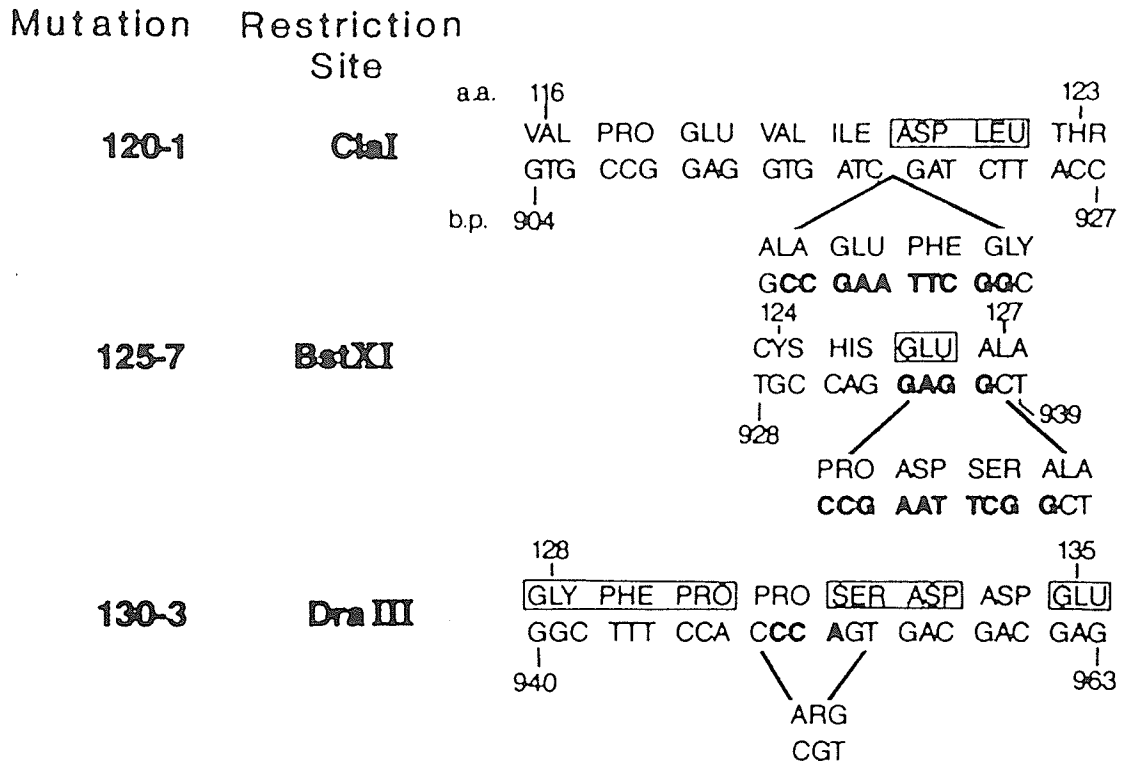


Figure 18. Illustration of adenovirus E1A conserved domain 2 mutations. Numbers indicate base pair (b.p.) and amino acid (a.a.) positions. The amino acid sequences conserved (Kimelman et al., 1985) among various human adenoviruses (Ad2, 5, 7, 12) and simian adenovirus 7 (SA7) are boxed. The nucleotide sequences deleted from the wild-type sequences and added in mutant sequences are shown in bold letters. Mutant 125-7 was not used in this study. (Taken from Kuppuswamy and Chinnadurai, 1987)

Pozzatti. 5R is a primary rat embryo fibroblast (REF) cell line that expresses the transfected plasmid pEJ, which encodes the activated T24/EJ form of c-H-ras-I gene, and demonstates a high metastatic potential in vivo (Pozzatti et al., 1988).

The 293 cell line is an adenovirus type 5-transformed cell line (Graham et al., 1977). It is the most commonly used positive control cell line in E1A expression experiments, by virtue of the fact that it overexpresses the E1A proteins. It was obtained from Dr. F.L. Graham (McMaster University).

All cells were routinely maintained in culture on the surface of plastic tissue culture plates (Becton Dickinson Labware; Corning Glass Works) in alpha-minimal essential medium (α-mem; Flow Laboratories, Inc.) supplemented with 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Inc.). All tissue culture procedures were performed in a laminar flow hood (The Baker Company, Inc. model NCB-6) to maintain sterility of the cell cultures. Cultures were incubated under controlled conditions of 5% CO_2 and 100% humidity at 37°C. All solutions involved in tissue culture maintainance, including the culture medium, were stored at 4°C.

Successfully transfected lines, and lines being selected during transfection, were maintained under similar conditions plus 300 ug/ml Hygromycin B (Sigma Chemical Co.). The concentration of Hygromycin B that is appropriate for selection is cell line dependent. In this study the ideal concentration was determined for 5R cells by conducting a test

experiment in which concentrations of 100 ug/ml, 200 ug/ml, 300 ug/ml and 400 ug/ml Hygromycin B were used to select for 5R cells cotransfected with pY3 and E1A plasmid DNA. The ideal concentration was arbitrarily determined to be that which resulted in an average of approximately 20 to 40 resistant colonies per 150 mm plate.

3) Subculture and long term storage of cell lines.

Phosphate buffered saline (PBS):

8.00 g NaCl
0.20 g KCl
1.44 g Na_2HPO_4
0.24 g KH_2PO_4, per litre.

Every effort was made to prevent all cell lines from becoming confluent while in culture. Subculture, or passaging, of cell lines was performed by first washing the culture plate with sterile phosphate buffered saline (PBS) solution. Approximately 1 ml of trypsin solution, 0.05% trypsin and 0.02% EDTA (Flow Laboratories, Inc.), was added to each 150 mm culture dish to detach the cells from the plate. After 2 to 4 minutes the cells were removed by washing with, and suspending the cells in, 5-10 ml of PBS. The solution was transferred to a fresh 15 ml centrifuge tube (Corning, Inc.) and centrifuged at 1,500g for 5 minutes at 4°C. The supernatant was aspirated off and the cells were resuspended in 5-10 ml PBS. The cells were replated at a 1 in 10 dilution on a fresh plate with fresh culture media.

For long term storage all cell lines were washed with PBS and suspended in a freezing solution containing 50% (v/v) FBS, 38% α-mem and 12% dimethylsulfoxide (DMSO, BDH Chemicals) at a concentration of approximately 10^7 cells/ml in 1 ml cryotube freezing vials (Simport Plastics Ltd.). Cells were slowly cooled to -70°C and maintained at that temperature. To recover frozen cells, the vial was rapidly warmed to 37°C. The thawed cells were added to 1 ml of normal culture medium (at 37°C) in a fresh culture dish. The volume was doubled every 5 minutes using culture medium for a total of 4 cycles. This was done to allow the cells to slowly adapt to the change in osmotic pressure and thereby maximize cell recovery. The culture was incubated at 37°C overnight before any further manipulation of the cells was attempted.

3.1) Cell counting procedure.

The cell culture to be counted was trypsinized as described above. After centrifugation the cells were resuspended in 5 ml of fresh PBS. A 50 ul aliquot of the cell suspension was added to a fresh polypropylene tube (Falcon 2063). A 50 ul sample of 0.1% Eosin Yellowish (Fisher Scientific Co.)/PBS solution was added to the tube and mixed with the cell suspension. The cell density was determined using a Reichert Bright-Line Hemacytometer and an Olympus-Tokyo microscope. The following formula was used:

$$(A/B) \times (2 \times 10^4) = C.$$

Where, A = number of cells in 'B' fields,
B = number of visual fields counted,
and C = cell density (cells/ml).

The required number of cells were aliquoted based on the cell density of the suspension.

4) Plasmid DNA Transfection.

5R cells were plated at 5×10^5 cells/60mm plate on day 1. On day 2, the culture medium was replaced with 4 ml of fresh medium. 20 ug of E1A plasmid DNA was transfected into the cells by using the calcium phosphate precipitation procedure (Graham and van der Eb, 1973; Corsaro and Pearson, 1981). E1A plasmid DNA and pY3 plasmid was added at a 20:1 ratio to maximize the probability of E1A incorporation in drug resistant colonies. A DNA-$CaCl_2$ solution was prepared containing total DNA at 50 ug/ml and $CaCl_2$ at 250mM and was added dropwise, with simultaneous bubbling, to an equal volume of HEPES buffer containing 50mM HEPES-NaOH, pH 7.1, 250 mM NaCl, and 1.5 mM Na_2HPO_4-NaH_2PO_2, pH 7.0 (Miller et al., 1986). The mixture was allowed to sit for 30 minutes at room temperature to allow for precipitate formation. 0.5 ml of precipitate was added to each plate of cells and incubated for 10 to 16 hours after which the precipitate was removed and

fresh medium was added. On day 3, the cells were passaged and plated onto large 150 mm plates. On day 4, the normal growth medium was replaced with selection medium containing 300 ug/ml Hygromycin B. The cells were incubated for 14 to 16 days with fresh culture medium being added every 4 days.

4.1) Cloning of transfected cells.

Plates were washed gently with sterile PBS solution to remove floating or loosely attached cells. Selected colonies were cloned by trypsinization using metal cloning cylinders sealed to the culture plates by sterilized petroleum jelly. Colonies that were in doubt due to the presence of other colonies in close proximity were subcloned by the same method and subsequently cloned. Approximately 20 colonies were selected for each transfection (each plasmid) and treated as independent cell lines. 5 vials of each clonal cell line were frozen at -70°C for further analysis.

5) Cellular DNA and RNA analysis.

5.1) Spectrophotometric determination of the amount of DNA and RNA.

Dilutions of 1 in 100 for DNA and a 1 in 500 for RNA, to a final volume of 1 ml, were used in spectrophotometric readings. For quantitating the amount of DNA or RNA, readings

were taken on a spectrophotometer (LKB Biochrom Ultrospec 4050) at wavelengths of 260 nm and 280 nm. The reading at 260 nm was used in the calculation of the concentration of nucleic acid in the sample. Contamination with phenol or protein can be determined from the OD_{260}/OD_{280} ratio (see Sambrook et al., 1989, for all details). Since contamination affects accuracy of nucleic acid quantitation, samples not meeting this purity criterium were generally not used until the contamination was removed by further extraction.

5.2) Nick translation and purification of labelled DNA probe.

Selected DNA probe was nick translated using the Amersham nick translation kit (N.5000) and ^{a-32}P labelled dCTP (ICN Biomedicals, Inc.). The procedure outlined in the kit was followed with an expected specific activity of at least 2 X 10^8 cpm/ug.

After incubating the probe for 1.5-2.0 hours at 15°C the probe was purified using a Sephadex G-50 column, in a 500 ul microfuge tube plugged with sterile glass wool. The sephadex G-50 solution was suspended in buffer containing 150 mM NaCl, 10 mM EDTA, 0.1% SDS, and 50 mM Tris-HCl, pH 7.5. The labelled DNA was eluted with 1X Tris-EDTA (TE), pH 8.0, using volumes of 75 ul, 100 ul and another 100 ul to purify the labelled DNA fragments from free, unincorporated nucleotides

in the reaction buffer. The equivalent of 1 ul of purified probe was put into a polyethylene scintillation vial and counted on a Beckman LS 7800/9800 scintillation counter to determine radioactivity of the DNA probe solution. The value (in cpm/ul) was used to calculate specific activity and to determine the quantity of DNA probe solution to be added to each hybridization experiment. Generally a probe concentration of 2 X 10^6 cpm/ml, or a total of 2 X 10^7 cpms per 10 ml hybridization solution, was used.

5.3) Isolation of genomic DNA from mammalian cells.

The procedure used for isolation of genomic DNA from all cell lines is based on the techniques published by Blin and Stafford (1976), with minor modifications. One 150 mm plate was used for each cell line. The monolayer was washed once with cold PBS (4°C). Using a policeman, the cells were scraped into about 10 ml of PBS, transferred into a 15 ml polystyrene centrifuge tube and centrifuged at 1500g for 5 minutes at 4°C. The cells were resuspended in 10 ml cold PBS and centrifuged once again to remove all traces of culture medium. The cell pellet was resuspended in about 1 ml TE (pH 8.0). 5 ml of extraction buffer was added and the solution was incubated for 1 hour at 37°C. Pancreatic RNAase was added to a final concentration of 100 ug/ml, mixed well with a glass rod and incubated at 37°C for 2 hours. Then proteinase K was

added to a final concentration of 100 ug/ml, mixed well and incubated for 3 hours at 50°C with occasional swirling. An equal volume of TE-equilibrated phenol was added and the two phases were gently mixed by slowly turning the tube end over end for 1 hour. The two phases were separated by centrifugation at 5,000g for 10 minutes at room temperature. The viscous aqueous phase was transferred to a clean centrifuge tube and the phenol extraction was repeated. The aqueous phase was then extracted with an equal volume of chloroform overnight. The aqueous phase was again separated by centrifugation and dialysed in three changes of 4 litres of TE (pH 8.0) overnight or until the OD_{270} of the dialysate was less than 0.05. The OD of the sample was then measured and the DNA sample was only used if the ratio of OD_{260} to OD_{280} was greater than 1.75. DNA samples were stored at 4°C.

5.4) Restriction enzyme digestion of DNA samples.

Each sample of genomic DNA to be analyzed for amplification and E1A gene copy number was first digested with a restriction enzyme having a single recognition site on the plasmid with which the cell line was transfected but no recognition sequences within the E1A gene itself. For cell lines transfected with wild-type and pSVXL series plasmids the most appropriate restriction enzyme was determined to be Hind III. Meanwhile EcoR I was chosen for 120-1- and 130-3-

transfected clones based on the proximity of the restriction site to the E1A insertion site on the plasmid.

20 ug of genomic DNA was digested with the appropriate restriction enzyme (Gibco BRL) at a concentration of 2 units per ug DNA in the reaction buffer accompanying the enzyme diluted to a concentration of 1X in the final reaction volume. The reaction was performed in a sterile microfuge tube and was carried out overnight in a 37°C drybath. The reaction was stopped by placing the tube(s) on ice.

5.5) Electrophoresis of DNA through gels.

6X DNA-gel loading buffer:

0.25%	Bromophenol blue
0.25%	Xylene cyanol FF
15.00%	Ficoll (type 400; Pharmacia)

DNA-gel running buffer (1X TBE):

10.8 g	Tris base
5.5 g	Boric acid
10.0 ml	1.0 M EDTA, pH 8.0, per litre.

Agarose (Sigma Chemical Co.) was melted in hot DNA gel-running buffer to a final concentration of 0.7% agarose. The solution was cooled to 60°C and ethidium bromide (Sigma Chemical Co.) was added (from a stock solution of 10 mg/ml) to a final concentration of 0.5 ug/ml. The solution was poured into a plastic gel tray and cooled for 1 hour. Meanwhile the Lambda-Hind III digest marker DNA (Gibco BRL) sample was prepared using reaction-buffer conditions identical to the DNA

samples. This was incubated at 65°C for 15 minutes to ensure that there was no interaction of the individual DNA fragments. The restriction enzyme digested DNA samples were mixed with a sufficient quantity of 6X DNA gel-loading buffer to give a final concentration of 1X in the loaded sample. The marker sample was treated in the same way.

When the gel had solidified it was placed in an electrophoresis chamber (Bio-Rad DNA Sub Cell) containing DNA gel-running buffer. The DNA samples were loaded into the wells, marker lane first, and the gel was run at 5 V/cm for 15 minutes on a Bio-Rad Model 250/2.5 Power Supply. The voltage was reduced and the DNA was electrophoresed at 3-4 V/cm.

When the dye front had migrated at least 12 cm the gel was placed on a transilluminator (Ultra-Violet Products, Inc.) alongside a metric ruler and a photograph of the gel was taken using a Polaroid MP 4 Land camera. The photograph was used to evaluate restriction fragment sizes in subsequent steps and to estimate degree of DNA digestion.

5.6) Southern transfer and hybridization of probe DNA.

5.6.1) Southern transfer of DNA.

The following is based on the principles outlined by Southern (1975) with modifications as described in Sambrook et al., 1989. After electrophoresis, unused areas of the gel

were cut away and the top right corner was cut off in order to orient the gel during succeeding operations. The gel was soaked in 0.2 N HCl for 15 minutes. The DNA was then denatured by soaking the gel in several volumes of denaturation solution (1.5 M NaCl, 0.5 N NaOH) for 45 minutes with gentle shaking. The gel was briefly rinsed in double distilled water (DDW) and neutralized by soaking for 30 minutes in several volumes of neutralization solution (1 M Tris pH 7.4, 1.5 M NaCl) with gentle shaking. The solution was changed and the gel was soaked for another 20 minutes.

The gel was removed from the neutralization solution and placed upside down on a piece of wet Whatman 3MM chromatography paper large enough to contact the transfer buffer (10X SSC or SSPE) in a plastic blotting tray. The top right corner of the gel was cut to orient the gel during succeeding operations. A piece of 0.45 um pore size nitrocellulose (Schleicher and Schuell, Inc.) 1 mm longer than the gel on each side was soaked in double distilled water for 5 minutes. A corner was cut to match that of the gel and it was placed on top of the gel. Two pieces of Whatman 3MM paper the same size as the filter were soaked in DDW for 5 minutes and placed on top of the nitrocellulose. Throughout this procedure air bubbles were smoothed out using a glass rod to ensure that all contacts were direct and flush. Strips of Parafilm (American Can Co.) were cut and placed around the gel to prevent short-circuiting. A stack of paper towels about 7

cm high, cut to approximately the same size as the gel, were placed on top of the 3MM papers. A glass plate was put on top of the towels and weighed down with a 1.0 kg weight. The transfer was allowed to proceed for 12-18 hours.

When the transfer was complete the filter was soaked in 2X SSC/SSPE for 5 minutes to remove any pieces of agarose sticking to the filter. The filter was placed on a piece of 3MM paper and air dried for 30-45 minutes. It was then placed between two pieces of 3MM paper and baked for 2 hours at 80°C in a vacuum oven (Bio-Rad Model 583 Gel Dryer).

In some cases nylon membrane (Nytran, Schleicher and Schuell, Inc.) was used and transfer was carried out in essentially the same manner as outlined above.

5.6.2) Hybridization and autoradiography.

8 ml of prehybridization solution was transferred to a 15 ml polystyrene tube and heated to 65°C in a water-bath. Meanwhile 1 ml of salmon sperm DNA (10 mg/ml, Sigma Chemical Co.) was heated to 100°C and boiled for 10 minutes. The salmon sperm was added to the prehybridization solution along with 1 ml of 50% dextran sulfate solution (Sigma Chemical Co.).

The complete prehybridization solution was added to the filter which had been placed in a plastic bag sealed on three sides using a Quik-Seal bag sealer (National Instrument Co.,

Inc.). After removal of all air bubbles in the solution with a pasteur pipette the top of the bag was sealed and it was incubated for 2 hours at 42°C. Meanwhile the radioactive probe was prepared. A corner of the bag was cut using a razor blade and the boiled probe was added to a pool of hybridization solution. All air bubbles were again removed and the bag was sealed. The bag was incubated at 42°C and hybridization was allowed to proceed overnight.

The following day the hybridization fluid was drained from the bag and the filter was washed briefly in washing solution 1 (2X SSC/SSPE, 0.1% SDS) to remove any nonhybridizing radioactivity. The washing solution was discarded and fresh solution was added. The filter was rinsed for 15-30 minutes at room temperature with gentle agitation on a water bath shaker. The solution was discarded and fresh washing solution 2 (0.05X SSC/SSPE/ 0.1% SDS) (at 65°C) was added. The filter was washed in a 65°C water bath twice for 30 minutes each with gentle shaking.

The filter was air-dried on paper towel to remove excess liquids. It was not allowed to completely dry. Autoradiography was used to analyze the filter. It was placed in a plastic bag and exposed to Scientific Imaging Film (Eastman Kodak Company) with an intensifying screen (Dupont Cronex-Lightning Plus FE) in a metal x-ray cassette (Picker X-ray) at -70°C for 2-4 days. The film was then developed in an x-ray developer and examined.

5.7) Isolation of total RNA from mammalian cells.

All materials and solutions utilized in the isolation of RNA were treated with the RNAase inhibitor diethyl pyrocarbonate (DEPC, Sigma Chemical Co.) as outlined in Sambrook et al. (1989).

The method used to isolate total RNA from parental and transfected cell lines is based on the procedure described by Stallcup and Washington (1983). For each cell line one subconfluent 150 mm plate was washed twice with ice-cold PBS. Plates were stored on ice until all monolayers were washed. 3 ml of 10 mM EDTA (pH 8.0), 0.5% SDS was added to each plate and using a policeman the cells were scraped into a 15 ml disposable polypropylene tube. Each plate was rinsed with 3 ml of 0.1 M sodium acetate (pH 5.2), 10 mM EDTA (pH 8.0) and the solution was transferred to the tube containing the cell lysate. After adding 6 ml of phenol (equilibrated with water) the contents were mixed by shaking the tube for 2 minutes at room temperature. The phases were separated by centrifugation at 5,000 rpm for 10 minutes at 4°C in a Damon/IEC Division DPR 6000 centrifuge. Using a sterile pipette, the upper aqueous phase was transferred to a fresh tube containing 440 ul of ice-cold 1 M Tris-HCl (pH 8.0) and 180 ul of 5 M NaCl. After addition of 2 volumes of ice-cold ethanol the tube was mixed and stored for 30 minutes at -20°C. The RNA was pelleted by centrifugation at 5000 rpm for 10 minutes at 4°C. The

supernatant was well drained and the RNA was redissolved in 200 ul of ice-cold TE (pH 8.0). The solution was transferred to a sterile 1.5 ml microfuge tube containing 4 ul of 5 M NaCl and 500 ul of ice-cold ethanol. The RNA was again collected by centrifugation at 12,000g for 5 minutes at 4°C in a Brinkmann eppendorf microfuge 5415. The supernatant was discarded and the tube was left open to allow the last traces of ethanol to evaporate. The RNA was redissolved in 30-50 ul of DEPC-treated water and stored at -20°C.

To further purify the RNA preparations, the following procedure was used to remove contaminating oligodeoxyribonucleotides. Instead of redissolving the RNA in DEPC-treated water as described above, 200 ul of 3 M sodium acetate (pH 5.2) was added. The suspension was centrifuged at 12,000g for 10 minutes at room temperature in a microfuge. The supernatant was discarded, and the pellet was redissolved in 200 ul of TE (pH 7.6). 20 ul of 3 M sodium acetate (pH 5.2) was added. The solution was mixed well, and 550 ul of ice-cold ethanol was added. After chilling the solution on ice for 30 minutes the RNA was recovered by centrifugation at 12,000g for 10 minutes at 4°C. The pellet was washed twice with ice-cold 70% ethanol and, after allowing the pellet to air dry to remove residual ethanol, it was resuspended in 30-50 ul DEPC-treated water and stored at -20°C.

Large scale total RNA isolation involved the procedures outlined in Sambrook et al. (1989) pages 7.19-7.22.

5.8) Electrophoresis of RNA through formaldehyde gels.

Formaldehyde gel-loading buffer :

50 %	Glycerol
1 mM	EDTA (pH 8.0)
0.25%	Bromophenol blue
0.25%	Xylene cyanol FF

5X Formaldehyde gel-running buffer :

0.1 M	MOPS (pH 7.0)
40 mM	Sodium acetate
5 mM	EDTA (pH 8.0)

RNA sample buffer :

13%	5X Formaldehyde gel-running buffer
22%	Formaldehyde (Mallinckrodt, Inc.)
65%	Formamide (Sigma Chemical Co.)

RNA gel-staining solution :

0.5 ug/ml	Ethidium bromide (Sigma Chemical Co.)
100 mM	Ammonium acetate (Fisher Scientific)

The formaldehyde gel was prepared by melting agarose (Sigma Chemical Co.) in boiling water to a concentration of 1 g/100 ml, cooling it to 60°C, and adding 5X formaldehyde gel-running buffer and formaldehyde to give final concentrations of 1X and 2.2 M, respectively. The gel was cooled for at least 1 hour in a chemical hood.

20 to 30 ug of RNA, made up to 4.5 ul with DEPC-treated water, was mixed with 15.5 ul of freshly made RNA sample buffer in a fresh, sterile microfuge tube. The samples were incubated for 15 minutes at 55°C, chilled on ice for 3 to 5

minutes and centrifuged for 5 seconds to deposit all of the fluid in the bottom of the tube. 2 ul of sterile, DEPC-treated formaldehyde gel-loading buffer was added to each sample.

All gels were run in a Bio-Rad DNA Sub Cell using a Bio-Rad Model 250/2.5 power supply. Before loading the samples, the gel was prerun for 5 minutes at 5 V/cm in 1X formaldehyde gel-running buffer. The samples were loaded into the lanes of the gel and it was run at 5 V/cm for 15 minutes, and then at 3-4 V/cm. An outside lane was loaded with an arbitrary RNA preparation to serve as a marker lane. When the gel electrophoresis was complete (when the bromophenol blue marker had migrated approximately 12 cm) the marker lane was cut off and stained with formaldehyde gel-staining solution for 30-60 minutes. The 18S and 28S ribosomal RNA (rRNA) bands were visualized using a transilluminator (Ultraviolet Products Inc.), the migration distances were recorded and used as reference molecular-weight markers for other samples of the corresponding gel.

5.9) Northern blotting and hybridization procedures.

The procedure used for blotting RNA from formaldehyde gels to nitrocellulose or nylon (Nytran) are identical to that for Southern hybridization involving DNA gels. However, since RNA is single stranded, there is no need for acid treatment, alkaline denaturation, and neutralization of the gel before

blotting onto the filter.

6) Analysis of ras protein expression by Western blotting and hybridization.

6.1) Protein extraction.

Lysis buffer:

50.0	mM	HEPES, pH 7.0
250.0	mM	NaCl
0.1%		Nonidet P-40 (NP-40)

On the day before cell lysis the appropriate cell lines were plated on 10 cm tissue culture plates at 1 X 10^6 cells per plate. The cells were incubated overnight. The following day the medium was removed, the plates were washed twice with cold PBS and the plates were placed on a tray of ice. 750 ul of lysis buffer was added to each plate and the cells were incubated, with occasional rocking, for a half hour on ice. The cell lysates were removed using a rubber policeman, transferred to a 1.5 ml eppindorf tube, centrifuged at 12,000 rpm for 5 minutes, and the supernatant was transferred to a fresh eppindorf tube. All lysates were stored at -20°C and were kept on ice during further handling.

6.2) SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

12.5% Polyacrylamide resolving gel:

16.9	ml	DDW
30.4	ml	Acrylamide solution (30 g Acrylamide:0.8 g BIS)
27.0	ml	Tris, pH 8.8
360.0	ul	20% SDS
240.0	ul	10% Ammonium persulfate
40.0	ul	TEMED

5% Polyacrylamide stacking gel:

17.0	ml	DDW
4.2	ml	Acrylamide solution
3.1	ml	Tris, pH 6.8
125.0	ul	20% SDS
125.0	ul	10% Ammonium persulfate
25.0	ul	TEMED

4X Sample buffer:

200.0	mM	Tris-Cl, pH 6.8
400.0	mM	Dithiothreitol
8.0%		SDS
0.4%		Bromophenol blue
40.0%		Glycerol

5X SDS-PAGE running buffer:

125.00	mM	Tris
1.25	M	Glycine, pH 8.3
0.5 %		SDS

Procedures used for protein analysis were based on those outlined in Harlow and Lane (1988). The protein samples (lysates) were run using a 5% stacking gel with a 12.5% resolving gel. The resolving gel was poured first using a Biorad protein gel apparatus. All ingredients were mixed in a beaker except for the ammonium persulfate and the TEMED which were added simultaneously just before the gel solution was added to the apparatus. It was covered with about 2 ml of

0.1% SDS and polymerization was allowed to proceed for about 45 min. The 0.1% SDS, which formed a separate layer at the top, was removed. Next, the ingredients of the stacking gel were mixed and similarly added to the gel apparatus with a 15 well Teflon comb. After 1 hour the comb was removed, the apparatus was placed in a running tank, the top chamber was filled with 1X SDS and the bottom of the tank was filled with 2 litres of 0.5X SDS solution.

50 ug of protein was used per cell line to be examined. Protein concentrations in the lysate were determined using a standard Biorad protein assay. The appropriate volume of protein was mixed with the required volume of 4X sample buffer and the samples were loaded onto the gel. The first lane was designated as the marker lane and 10 ul of prestained SDS-PAGE molecular weight standard (Biorad) was loaded. The gel was run using a constant current of 35 A for about 5 hours or until the dye front had reached the end of the gel.

6.3) Western blotting of electrophoresed proteins.

Blotting buffer:

25	mM	Tris, pH 8.3
192	mM	Glycine
20%		Methanol

20X Tris buffered saline (TBS) stock:

121.1 g	Tris
120.0 g	NaCl, to pH 7.6 and 1 litre

Tween Tris buffered saline (TTBS):

TBS with 0.05% Tween-20 (Sigma)

Developing solution:

45.0	ml	Borate buffer (0.93 g boric acid/l, pH 9.5)
5.0	ml	0.1% NBT (p-nitroblue tetrazolium chloride)
0.5	ml	5 mg/ml BCIP (5-bromo-4-chloro-3-indolylphosphate) in diethylformamide
100.0	ul	2M $MgCl_2$ (added just before developing)

The gel was placed in transfer/blotting buffer to equilibrate for 1 hour at 4°C. Meanwhile one piece of nitrocellulose equivalent to the gel-size and six pieces of Whatman 3MM paper were cut and soaked in fresh blotting buffer. The gel and nitrocellulose were sandwiched between three pieces of 3MM paper on each side and loaded onto a transfer assembly. The tank was filled with blotting buffer and the apparatus was run for 1.5 hours at 120 V, and then 100 V for the final 30 minutes, with constant stirring of the buffer at 4°C. The blotted filter was air-dried, placed between two pieces of 3MM paper and stored in a plastic bag at 4°C until it was developed.

Before developing, the membrane was wet in TBS for 5 minutes. Then, 100 ml of blocking solution (3% gelatin, Difco Laboratories) was added and it was incubated with gentle shaking for 45 minutes. The filter was washed once with TTBS for five minutes and incubated overnight at room temperature in 5 ml of antibody buffer (1% gelatin) with a 1:100 dilution

of PAb*ras*10 (Dupont),a *ras* pan-specific monoclonal antibody, for $p21^{ras}$ analysis. The following day the filter was washed twice for five minutes in TTBS. Then, 50 ml of antibody buffer was added with 16.7 ul of anti-mouse IgG alkaline phosphatase conjugated secondary antibody (Sigma) and incubated with shaking for 1 hour. The filter was again washed twice in TTBS and twice in borate buffer. Meanwhile the developing solution was prepared in a foil wrapped flask. After the final wash, the blot was developed by incubation in the developing solution, in a foil-covered tray to protect the light-sensitive solution, for 45 to 90 minutes. The reaction was stopped with several washes of distilled water.

7) Measuring in vitro invasion properties of cells.

To evaluate the invasive potential of transfected and parental cell lines, *in vitro* invasion assays were performed. On day one 3 X 10^4 cells were plated and incubated under normal cell culture conditions. It was expected that by day 2 the cell number would approximately double.

On day 2 the collagen gel was prepared in a 25 cm^2 tissue culture flask (Corning, Inc.). 1 ml of 5X d-MEM was mixed with 1.5 ml of sterile double distilled water and 2.5 ml of Vitrogen collagen I solution (Collagen corp.) in a sterile polystyrene tube. The solution was transferred to a sterile 25 cm^2 tissue culture flask and the gel solution was allowed

to polymerize for at least 20 minutes at 37°C. Meanwhile the cell culture to be examined was trypsinized and centrifuged for 5 minutes at 1,500g and 4°C. The cell pellet was resuspended in sterile PBS, recentrifuged and then suspended in 5 ml of culture medium. The cell suspension was added to the surface of the collagen gel in the flask. The flask was incubated under normal culture conditions.

At exact 2 hour intervals the culture flask was removed from the incubator and examined for cell invasion. A measurement was taken at time 0 before beginning the incubation. To characterize the invasive ability of the cells, three separate fields of cells were selected and examined under a microscope (Olympus-Tokyo) at 100X magnification. For each field, the number of cells at each of five different levels was counted. Level 1 was focused at the top of the gel and the number of cells was counted. level 2 was focused at about 5 um below the surface of the gel. In this way 5 different levels were used, where level 5 was approaching the bottom of the gel. By counting the number of cells at each level I was able to characterize the percentage of cells that had invaded, and to what degree, at any specific time point. The same fields were not necessarily used in each time point measurement. Three different fields were measured at each time point to account for variation and to get data that was most representative of the invasive characteristics of the cell lines. All samples were examined at time points

of 0, 2, 4, 6, 8, 10, 12, and 24 hours.

Statistical analysis was done on the results obtained from the invasion assays as described on the following page.

8) Measuring in vivo metastatic potential of cell lines.

Bouin's solution :

15 parts	picric acid (saturated)
4 parts	40% formaldehyde
1 part	glacial acetic acid

To evaluate the metastatic characteristics of cell lines involved in this study the cell lines were injected into nude mice and the metastatic potential was determined from the number of tumor nodules observed in the lungs of sacrificed animals. The procedure used was identical to that of Pozzatti et al. (1986).

On day 1, 1 X 10^6 cells of each cell line to be examined were plated on 150 mm tissue culture plates and incubated under normal conditions overnight. On day 2, the cells were removed from the plate by trypsinization, centrifuged at 1,500g for 5 minutes and resuspended in 10 ml PBS. The centrifugation was repeated and the cells were resuspended in 5 ml PBS and put on ice. The cell density was determined using the cell counting procedure described earlier. The cell density was adjusted to 2.5 X 10^5 cells/ml for a total volume of 1.2 ml.

5 nude mice were injected with each cell line to be

examined in order to account for normal variation. Each mouse was tail-vein injected with 0.2 ml (5 X 10^4 cells). The tumors were given 14-16 days to develop. Thereafter, the mice were sacrificed and the lung were inflated by tracheal injection of Bouin's solution. The lungs were removed and stored in marked scintillation vials in Bouin's solution. The number of tumor nodules were subsequently counted for each lung using a Wild Heerbrugg M3 dissecting microscope and the data was compiled for all of the different cell lines that were used. Statistical analysis was done on all of the resulting data.

9) Quantitation and statistical analysis.

Variation in the *in vitro* invasion and *in vivo* experimental metastasis assays amongst cell lines expressing the same E1A plasmid was estimated by calculating the standard error (SE) based on standard deviation values (see Chase, 1967).

To make it easier to compare the metastatic potentials of cell lines expressing different E1A plasmids, a percent metastasis value was calculated for each cell line as follows:

$$\% \text{ MET}_X = \frac{MT_X - MT_{base}}{MT_{5R} - MT_{base}} \times 100$$

where, $\% \text{ MET}_X$ = % metastasis of cell line X
MT_X = mean tumor formation by X
MT_{base} = mean tumor formation by the baseline control cell line(s) (N20 series)
MT_{5R} = mean tumor formation by 5R.

The relationship between transcriptional activities of E1A and metastatic suppression was examined using the following t-test:

$$t_{n-2,\,a} = \frac{r(n-2)^{1/2}}{(1-r^2)^{1/2}}$$

where, n = number of variates
n-2 = degrees of freedom
α = represents degree of certainty
r = correlation coefficient.

RESULTS

1) Expression of E1A genes in transfected cells.

A series of different wild-type and mutant E1A expression vectors were transfected into the metastatic rat embryo fibroblast 5R line to determine which regions of E1A are involved in suppression of T24 *ras*-mediated metastasis. Introduction of the different E1A expression vectors resulted in the growth of many (>40) colonies except in the case of pSVN20 transfection. Transfection of 5R cells with pSVN20 resulted in the growth of only one cell colony. The transfection was repeated twice and a total of approximately 20 colonies resulted. These results support the observation of others that expression of wild-type E1A can be toxic in some cells (Lowe and Ruley, 1993).

5R cells transfected with E1A expression vectors were screened for E1A expression using northern hybridization. Two or, where possible, three E1A expressing clones from each transfection were chosen for further analysis. The nomenclature developed for these lines and the description of the respective E1A polypeptides expressed by each are listed in Table 9. Figures 19-21 illustrate the expression of the 13S and/or 12S E1A mRNAs in these cell lines.

An intense effort was made to show expression of E1A polypeptides in the aforementioned cell lines without any success. This does not necessarily imply a lack of E1A protein expression because other investigators have also found that while E1A expression can be shown in adenovirus *infected*

Figure 19. Expression of E1A mRNA species in E1A transfected cells. Northern blot analysis showing expression of E1A mRNA. The first lane, 293, shows expression of E1A in 293 cells (positive control), while the 5R lane shows the negative control profile of 5R cells. Other lanes illustrate E1A expression in E1A transfected lines N20-11, N20-20, F12-1, F12-8, F12-13, 120-1, 120-4, and 120-5. The arrows indicate the positions of the unspliced (un), 13S and 12S E1A mRNA bands.

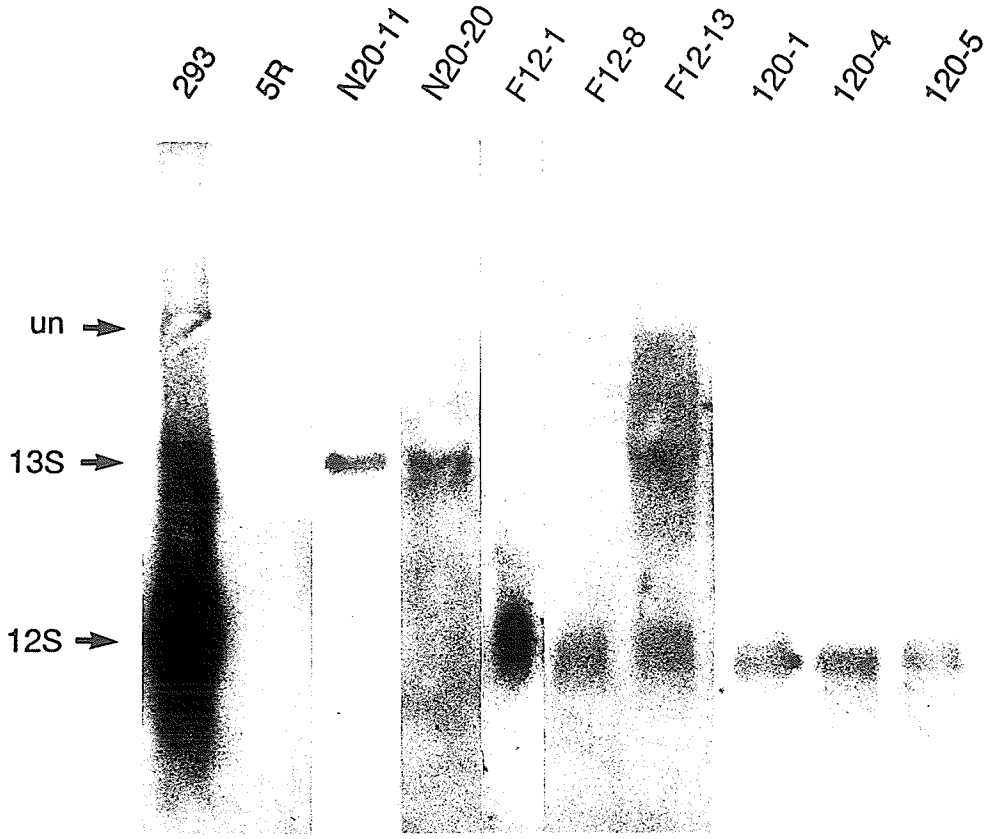

293
5R
N20-11
N20-20
F12-1
F12-8
F12-13
120-1
120-4
120-5
un
13S
12S

Figure 20. Expression of E1A mRNA species in E1A transfected cells. Northern blot analysis showing expression of E1A mRNA. The first lane, 293, shows expression of E1A in 293 cells (positive control), while the 5R lane shows the negative control profile of 5R cells. Other lanes illustrate E1A expression in E1A transfected lines 130-2, 130-9, 130-10, 132-16, 132-18, 132-20, 105-4, 105-8, and 105-18. Arrows indicate the positions of the unspliced (un), 13S, and 12S E1A mRNA bands. The shifting of the positions of the bands between lanes is due to changes in E1A mRNA size caused by deletion mutation of the transfected E1A gene.

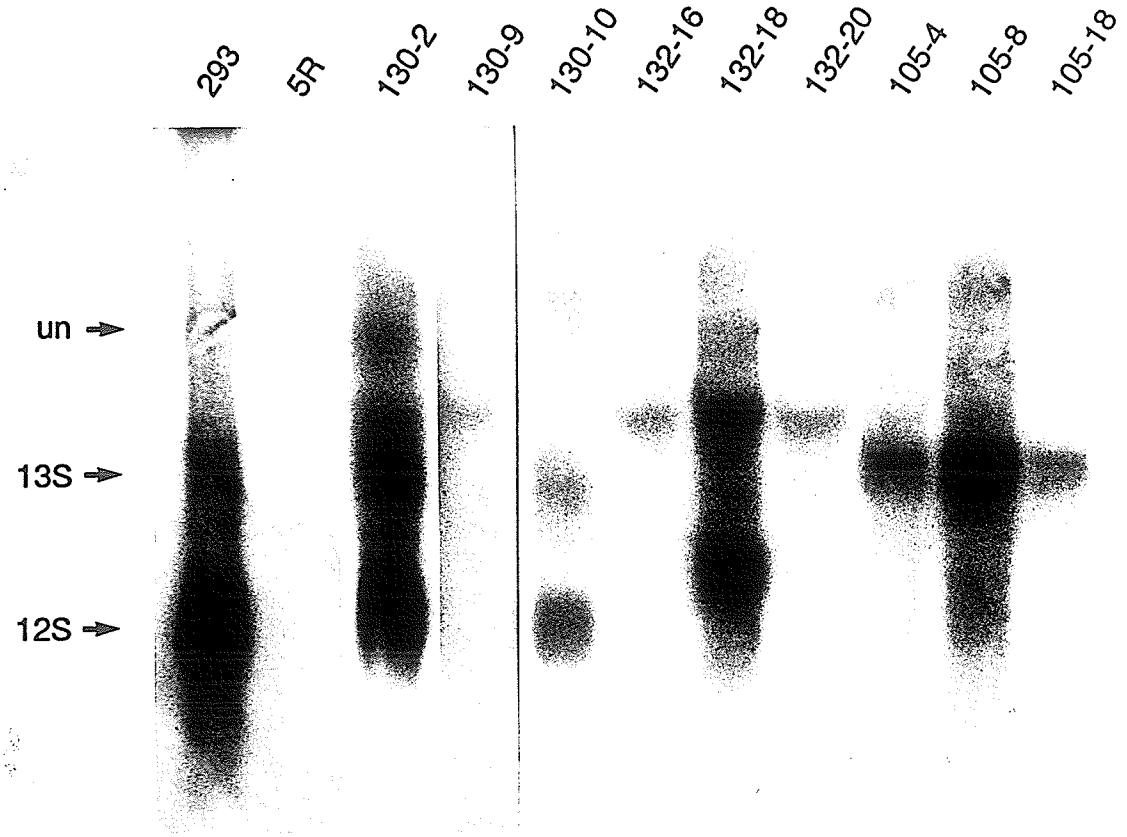

293
5R
130-2
130-9
130-10
132-16
132-18
132-20
105-4
105-8
105-18
un
13S
12S

Figure 21. Expression of E1A mRNA species in E1A transfected cells. Northern blot analysis showing expression of E1A mRNA. The first lane, 293, shows expression of E1A in 293 cells (positive control), while the 5R lane shows the negative control profile of 5R cells. Other lanes illustrate E1A expression in E1A transfected lines 174-9, 174-14, 214-3, 214-9, 214-14, 124-6, 124-12, and 124-20. The arrows indicate the positions of the unspliced (un), 13S and the 12S E1A mRNA bands. The shifting of the positions of the bands between lanes is due to changes in E1A mRNA size caused by mutation deletion of the transfected E1A gene.

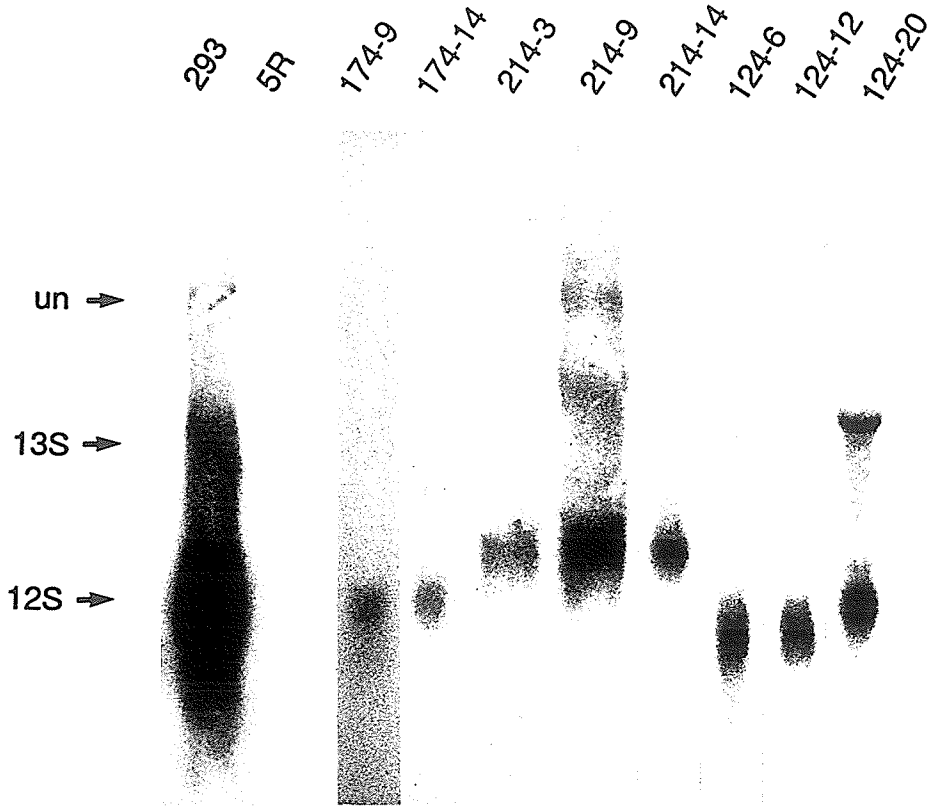

293
5R
174-9
174-14
214-3
214-9
214-14
124-6
124-12
124-20
un
13S
12S

Table 9. Nomenclature used in reference to 5R cells transfected with E1A expression vectors.

Cell line	Plasmid	E1A mutation[a]
N20-9	pSVN20	13S wild-type
N20-11	"	"
N20-20	"	"
F12-1	pSVF12	12S wild-type
F12-8	"	"
F12-13	"	"
120-1	pGC212	ins. at 120-1
120-4	(modified)	"
120-5	"	"
130-2	pGC212	ins. at 130-3
130-9	(modified)	"
130-10	"	"
105-4	pSVXL105	▲ 38-44
105-8	"	"
105-18	"	"
132-16	pSVXL132	▲ 64-67
132-18	"	"
132-20	"	"
124-6	pSVXL124	▲ 159-289
124-12	"	"
124-20	"	"
174-9	pSVXL174	▲ 1-14
174-14	"	"
214-3	pSVXL214	▲ 185-214
214-9	"	"
214-14	"	"

'a' - numbers refer to amino acid residue positions.
'ins.' - refers to insertion mutation.
'▲' - denotes deletion mutation.

cells, it is often difficult to demonstrate in E1A transfected cells (E. Ruley and E. Harlow, personal communication).

2) In vitro invasion assays of E1A transfected cell lines.

In vitro invasion assays were used to evaluate the ability of E1A expressing lines to invade a collagen-based gel so as to provide an initial characterization of metastatic properties of the cell lines. The invasive capabilities of the cell lines are outlined in Tables 10-37 and summarized in Table 38.

The parental cell line, 5R, exhibited a high level of invasion. After 24 hours 33.3% of the cells had begun to invade the collagen gel. REF52 cells were used as negative controls and had a low-to-moderate invasion level of 13.5%. REF52 cells are likely not an accurate choice for use as a negative control. The reasons for this are discussed in depth in the Discussion. Cell lines expressing both the 289R and the 243R wild-type E1A proteins exhibited low levels of invasion indicating a suppression of invasion by E1A in these lines. Similar results were obtained for most cell lines expressing mutant E1A genes except for the N-terminal mutant lines 174-9 and 174-14 which had percent invasion values of 18.3% and 24.8%, respectively. The 124 series cell lines which contain a partial deletion of conserved region 3 and complete deletion of exon 2 exhibited levels of invasion approaching, and in some cases exceeding, those of the

Table 10. Invasive potential of parental 5R cells.

Time (hrs)	Cells per level					% Invasion ± SE
	1	2	3	4	5	
0	146	0	0	0	0	0
2	143	10	0	0	0	7.0 ± 5.7
4	143	9	0	0	0	6.3 ± 2.5
6	139	11	0	0	0	7.9 ± 3.8
8	133	9	0	0	0	6.8 ± 4.0
10	126	15	1	0	0	12.7 ±10.5
12	160	19	0	0	0	11.9 ± 2.4
22	292	38	13	8	0	20.2 ± 2.2
24	210	59	11	0	0	33.3 ± 7.7

Invasion assays involved measurement of the number of cells penetrating a collagen I-based gel at the various time points listed. "Level" refers to the number of cells penetrating various depths of the gel, where 5 is the deepest.

Table 11. Invasive potential of REF52 cells.

Time (hrs)	Cells per level					% Invasion ± SE
	1	2	3	4	5	
0	119	0	0	0	0	0
2	110	0	0	0	0	0
4	103	1	0	0	0	< 1.0
6	135	1	0	0	0	< 1.0
8	117	0	0	0	0	0
10	135	9	0	0	0	6.7 ± 1.6
12	136	4	0	0	0	2.9 ± 0.5
22	141	14	5	0	0	13.5 ± 2.8
24	110	9	2	0	0	10.0 ± 3.9

Table 12. Invasive potential of N20-9 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	141	0	0	0	0	0
2	149	2	0	0	0	1.3 ± 1.6
4	159	5	0	0	0	3.1 ± 0.7
6	175	11	0	0	0	6.3 ± 2.8
8	251	9	0	0	0	3.6 ± 1.3
10	219	6	0	0	0	2.7 ± 0.5
12	238	3	0	0	0	1.3 ± 0.7
24	330	7	0	0	0	2.1 ± 1.0

Table 13. Invasive potential of N20-11 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	93	0	0	0	0	0
2	90	0	0	0	0	0
4	112	2	0	0	0	1.8 ± 2.1
6	109	0	0	0	0	0
8	119	3	0	0	0	2.5 ± 1.2
10	148	4	0	0	0	2.7 ± 2.7
12	131	4	0	0	0	3.1 ± 1.3
24	251	1	0	0	0	< 1.0

Table 14. Invasive potential of N20-20 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	110	0	0	0	0	0
2	124	5	0	0	0	4.0 ± 2.2
4	116	0	0	0	0	0
6	119	5	0	0	0	4.2 ± 2.3
8	151	0	0	0	0	0
10	152	0	0	0	0	0
12	163	1	0	0	0	< 1.0
24	283	0	0	0	0	0

Table 15. Invasive potential of F12-1 cells.

Time (hrs)	Cells per level					% Invasion ± SE
	1	2	3	4	5	
0	121	0	0	0	0	0
2	124	10	0	0	0	8.1 ± 0.5
4	138	8	0	0	0	5.8 ± 2.5
6	208	6	0	0	0	2.9 ± 1.3
8	224	1	0	0	0	< 1.0
10	261	8	0	0	0	3.1 ± 1.3
12	264	4	0	0	0	1.5 ± 1.3
24	385	5	1	0	0	1.6 ± 1.1

Table 16. Invasive potential of F12-8 cells.

Time (hrs)	Cells per level					% Invasion ± SE
	1	2	3	4	5	
0	132	0	0	0	0	0
2	144	3	0	0	0	2.1 ± 1.0
4	170	9	0	0	0	5.3 ± 2.1
6	200	1	0	0	0	< 1.0
8	198	0	0	0	0	0
10	212	4	0	0	0	1.9 ± 1.6
12	320	5	0	0	0	1.6 ± 1.1
24	434	6	0	0	0	1.4 ± 0.6

Table 17. Invasive potential of F12-13 cells.

Time (hrs)	Cells per level					% Invasion ± SE
	1	2	3	4	5	
0	124	0	0	0	0	0
2	133	4	0	0	0	3.0 ± 1.8
4	161	4	0	0	0	2.5 ± 1.1
6	193	1	0	0	0	< 1.0
8	192	4	2	0	0	3.1 ± 1.3
10	177	3	0	0	0	1.7 ± 1.2
12	232	3	0	0	0	1.3 ± 0.6
24	389	8	0	0	0	2.1 ± 0.9

Table 18. Invasive potential of 120-1 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	320	0	0	0	0	0
2	353	6	0	0	0	1.7 ± 1.0
4	273	5	0	0	0	1.8 ± 1.6
6	357	4	0	0	0	1.1 ± 0.8
8	249	5	0	0	0	2.0 ± 1.1
10	330	4	0	0	0	1.2 ± 0.9
12	400	7	0	0	0	1.8 ± 0.5
24	563	7	0	0	0	1.2 ± 0.6

Table 19. Invasive potential of 120-4 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	250	0	0	0	0	0
2	235	0	0	0	0	0
4	302	0	0	0	0	0
6	251	3	0	0	0	1.2 ± 0.6
8	369	1	0	0	0	< 1.0
10	291	7	0	0	0	2.4 ± 0.6
12	369	17	0	0	0	4.6 ± 0.6
24	570	16	0	0	0	2.8 ± 0.5

Table 20. Invasive potential of 120-5 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	243	0	0	0	0	0
2	254	0	0	0	0	0
4	259	1	0	0	0	< 1.0
6	300	5	0	0	0	1.7 ± 0.6
8	372	3	0	0	0	< 1.0
10	331	5	0	0	0	1.5 ± 0.9
12	390	6	0	0	0	1.5 ± 0.6
24	692	26	0	0	0	3.8 ± 0.8

Table 21. Invasive potential of 130-2 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	282	0	0	0	0	0
2	293	0	0	0	0	0
4	319	5	0	0	0	1.6 ± 0.9
6	312	3	0	0	0	< 1.0
8	393	3	0	0	0	< 1.0
10	496	0	0	0	0	0
12	443	12	0	0	0	2.7 ± 0.1
24	582	6	0	0	0	1.0 ± 0.2

Table 22. Invasive potential of 130-9 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	313	0	0	0	0	0
2	306	0	0	0	0	0
4	348	1	0	0	0	< 1.0
6	323	0	0	0	0	0
8	447	3	0	0	0	< 1.0
10	403	0	0	0	0	0
12	514	2	0	0	0	< 1.0
24	646	2	0	0	0	< 1.0

Table 23. Invasive potential of 130-10 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	202	0	0	0	0	0
2	217	3	0	0	0	1.4 ± 1.2
4	217	2	0	0	0	< 1.0
6	226	2	0	0	0	< 1.0
8	291	7	0	0	0	2.4 ± 1.5
10	245	6	0	0	0	2.4 ± 1.3
12	273	6	0	0	0	2.2 ± 1.2
24	363	5	0	0	0	1.4 ± 0.5

Table 24. Invasive potential of 105-4 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	113	0	0	0	0	0
2	110	8	0	0	0	7.3 ± 1.8
4	146	6	0	0	0	4.1 ± 0.8
6	142	4	0	0	0	2.8 ± 0.5
8	220	14	0	0	0	6.4 ± 0.8
10	188	17	0	0	0	9.0 ± 2.7
12	205	7	0	0	0	3.4 ± 1.4
24	330	12	0	0	0	3.6 ± 1.2

Table 25. Invasive potential of 105-8 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	92	0	0	0	0	0
2	96	6	0	0	0	6.3 ± 1.2
4	113	15	0	0	0	13.3 ± 3.1
6	127	11	0	0	0	8.7 ± 2.5
8	161	15	0	0	0	9.3 ± 2.1
10	174	22	0	0	0	12.6 ± 1.7
12	182	17	0	0	0	9.3 ± 3.3
24	313	24	0	0	0	7.7 ± 1.2

Table 26. Invasive potential of 105-18 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	118	0	0	0	0	0
2	126	4	0	0	0	3.2 ± 2.0
4	111	10	0	0	0	9.0 ± 1.6
6	153	5	0	0	0	3.3 ± 0.4
8	172	9	0	0	0	5.2 ± 0.2
10	203	6	0	0	0	3.0 ± 2.0
12	214	4	0	0	0	1.9 ± 0.2
24	267	15	0	0	0	5.6 ± 1.8

Table 27. Invasive potential of 132-16 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	309	0	0	0	0	0
2	322	0	0	0	0	0
4	364	5	0	0	0	1.4 ± 1.2
6	454	0	0	0	0	0
8	528	0	0	0	0	0
10	456	1	0	0	0	< 1.0
12	564	5	0	0	0	< 1.0
24	1080	2	0	0	0	< 1.0

Table 28. Invasive potential of 132-18 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	352	0	0	0	0	0
2	386	0	0	0	0	0
4	477	2	0	0	0	< 1.0
6	562	2	0	0	0	< 1.0
8	641	7	0	0	0	1.1 ± 1.4
10	660	4	0	0	0	< 1.0
12	810	3	0	0	0	< 1.0
24	1068	3	0	0	0	< 1.0

Table 29. Invasive potential of 132-20 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	254	0	0	0	0	0
2	252	4	0	0	0	1.6 ± 1.3
4	273	5	0	0	0	1.8 ± 0.5
6	301	4	0	0	0	1.3 ± 1.4
8	329	3	0	0	0	< 1.0
10	369	6	0	0	0	1.6 ± 0.9
12	368	3	1	0	0	< 1.0
24	548	0	0	0	0	0

Table 30. Invasive potential of 124-6 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	226	0	0	0	0	0
2	254	27	0	0	0	10.6 ± 1.5
4	333	62	0	0	0	18.6 ± 3.3
6	267	26	0	0	0	9.7 ± 0.3
8	427	38	0	0	0	8.9 ± 1.3
10	508	104	0	0	0	20.5 ± 3.3
12	531	187	1	0	0	35.4 ± 4.0
24	585	179	0	0	0	30.6 ± 1.7

Table 31. Invasive potential of 124-12 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	168	0	0	0	0	0
2	181	23	0	0	0	12.7 ± 1.8
4	188	39	0	0	0	20.7 ± 6.8
6	263	8	0	0	0	3.0 ± 2.5
8	342	62	0	0	0	18.1 ± 1.6
10	335	80	0	0	0	23.9 ± 5.3
12	403	126	0	0	0	31.3 ± 2.5
24	523	138	0	0	0	26.4 ± 2.4

Table 32. Invasive potential of 124-20 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	177	0	0	0	0	0
2	206	24	0	0	0	11.7 ± 1.0
4	190	27	0	0	0	14.2 ± 2.5
6	285	18	0	0	0	6.3 ± 1.6
8	340	46	0	0	0	13.5 ± 1.4
10	368	89	0	0	0	24.2 ± 4.1
12	463	117	0	0	0	25.3 ± 0.6
24	546	94	0	0	0	17.2 ± 1.3

Table 33. Invasive potential of 174-9 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	129	0	0	0	0	0
2	120	18	0	0	0	15.0 ± 4.8
4	93	17	0	0	0	18.3 ± 3.7
6	134	9	0	0	0	6.7 ± 0.5
8	129	4	0	0	0	3.1 ± 1.4
10	136	12	0	0	0	8.8 ± 1.9
12	144	18	0	0	0	12.5 ± 1.7
24	231	29	0	0	0	12.6 ± 4.7

Table 34. Invasive potential of 174-14 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	112	0	0	0	0	0
2	78	8	0	0	0	10.3 ± 4.3
4	184	12	0	0	0	6.5 ± 2.0
6	184	16	0	0	0	8.7 ± 4.8
8	219	19	0	0	0	8.7 ± 0.8
10	294	26	0	0	0	8.8 ± 1.8
12	231	26	3	0	0	12.6 ± 2.8
24	400	99	0	0	0	24.8 ± 2.2

Table 35. Invasive potential of 214-3 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	93	0	0	0	0	0
2	90	3	0	0	0	3.3 ± 2.3
4	110	0	0	0	0	0
6	158	3	0	0	0	1.9 ± 1.1
8	161	4	0	0	0	2.5 ± 1.2
10	202	17	3	0	0	10.0 ± 0.6
12	212	16	0	0	0	7.5 ± 2.3
24	395	18	0	0	0	4.6 ± 0.7

Table 36. Invasive potential of 214-9 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	115	0	0	0	0	0
2	119	3	0	0	0	2.5 ± 1.0
4	143	2	0	0	0	1.4 ± 1.7
6	204	5	0	0	0	2.5 ± 1.4
8	256	5	0	0	0	2.0 ± 1.9
10	255	20	1	0	0	8.3 ± 3.6
12	269	10	0	0	0	3.7 ± 0.8
24	343	14	0	0	0	4.1 ± 0.9

Table 37. Invasive potential of 214-14 cells.

Time (hrs)	Cells per level 1	2	3	4	5	% Invasion ± SE
0	131	0	0	0	0	0
2	128	5	0	0	0	3.9 ± 1.7
4	126	7	0	0	0	5.6 ± 2.8
6	157	6	0	0	0	3.9 ± 1.7
8	174	2	0	0	0	1.1 ± 0.9
10	168	1	0	0	0	< 1.0
12	197	12	0	0	0	6.1 ± 1.4
24	272	12	0	0	0	4.4 ± 0.6

Table 38. A comparison of the invasive capabilities of cell lines expressing different wild-type and mutant E1A genes.

Cell line	E1A mutation[a]	% Maximum Invasion	Mean	Standard[b] Deviation
5R	Parental (pos. control)	33.3		-
N20-9	13S wild-type	3.1	4.5	0.77
N20-11	"	4.2		
N20-20	"	6.3		
F12-1	12S wild-type	8.1	5.5	1.18
F12-8	"	5.3		
F12-13	"	3.1		
120-1	ins. at 120-121	2.0	3.5	0.63
120-4	(CR2)	4.6		
120-5	"	3.8		
130-2	ins. at 130-133	2.7	2.0	0.51
130-9	"	1.0		
130-10	"	2.4		
105-4	▲ 38-44 (CR1)	9.0	10.4	1.17
105-8	"	13.3		
105-18	"	9.0		
132-16	▲ 64-67 (CR1)	1.4	1.4	0.17
132-18	"	1.1		
132-20	"	1.8		
124-6	▲ 153-289	35.4	30.1	2.40
124-12	(CR3 & C-terminus	31.3		
124-20	"	25.3		
174-9	▲ 1-14	18.3	21.6	1.77
174-14	(N-terminus)	24.8		
214-3	▲ 185-214	10.0	8.1	0.92
214-9	(C-terminus)	8.3		
214-14	"	6.1		

'a' - numbers indicate amino acid residue positions.
'b' - standard deviations refer to deviation of maximum % invasion values between clonal cell lines expressing the same mutant, or wild-type E1A gene form.

parental cell line. Cell lines 124-6, 124-12, and 124-20 had invasion values of 35.4%, 31.3%, and 25.3%. No other transfected cell lines, with the exception of the 174 series, even approached values of this magnitude.

3) In vivo experimental metastasis assays.

The cell lines were then used in in vivo metastasis assays using tail-vein injection of immunodeficient nude mice. The experiment was carried out by way of two separate assays. Since the conditions were regulated for cell lines within an assay group, and not necessarily between cell lines used in different assays, it must be emphasized that comparisons of levels of metastasis should be made only between control and test lines used in the same assay. The results of the metastasis assays are outlined in Table 39. Values over 300 were considered too numerous to count and are designated as >300. To make for easier comparison of metastatic potential between cell lines the tumor formation values were converted to, as expressed as, percent metastasis values (Table 42). 100% metastasis was taken as a value of >300 while the 0% value was taken from the base-line tumor formation values of the N20 series lines. Percent metastasis values of test cell lines are therefore relative indicators of the degree of metastatic capabilities of cells as they compare to minimal and maximal limits defined by the assay.

The parental 5R cell line was used in the first assay but

Table 39. The *in vivo* metastatic potentials of cell lines expressing different wild-type and mutant E1A genes.

Cell line	E1A mutation[a]	Number of mice	Mean tumors ± SE
Assay 1:			
5R	parental line (positive control)	4	>300.0
N20-11	13S wild-type	5	7.0 ± 3.5
N20-20	"	6	14.2 ± 5.9
124-6	▲ 153-289	5	140.6 ± 23.6
124-12	(CR3 & C-term.)	5	172.7 ± 15.8
124-20	"	5	>300.0
174-9	▲ 1-14	4	69.7 ± 5.4
174-14	(N-terminus)	5	106.7 ± 14.2
214-3	▲ 185-214	6	>300.0
214-9	(C-terminus)	5	96.4 ± 16.3
Assay 2:			
N20-11	13S wild-type	3	74.0 ± 2.1
F12-1	12S wild-type	4	258.8 ± 35.7
F12-13	"	4	231.0 ± 35.2
120-4	ins. at 120-121	5	31.8 ± 4.0
120-5	(CR2)	4	85.0 ± 10.9
130-2	ins. at 130-133	5	94.6 ± 16.5
130-9	(CR2)	5	42.2 ± 6.8
130-10	"	5	17.8 ± 2.8
105-4	▲ 38-44 (CR1)	5	137.8 ± 26.6
105-8	"	3	64.3 ± 8.3
105-18	"	5	84.8 ± 6.4
132-16	▲ 64-67 (CR1)	5	53.6 ± 17.5
132-18	"	6	8.5 ± 1.8
132-20	"	5	37.6 ± 9.4
214-14	▲ 185-214 (C-terminus)	5	112.8 ± 28.8

'a' - numbers indicate amino acid residue positions.

Table 40. Percent metastasis values for cell lines assayed for metastatic potential and overall % metastasis values for cell lines expressing the same mutant E1A gene.

Cell line	% Metastasis ± SE	Cell line series	Overall % metastasis ± SE[a]
Assay 1:			
5R	100	5R	100
N20-11	0	N20	0
N20-20	0		
124-6	45 ± 7.6	124	67 ± 13.7
124-12	56 ± 5.0		
124-20	100		
174-9	20 ± 2.6	174	27
174-14	33 ± 4.4		
214-3	100	214	49 ± 21.1
214-9	30 ± 2.1		
Assay 2:			
N20-11	0		see above[b]
F12-1	82 ± 11.3	F12	76
F12-13	70 ± 10.7		
120-4	0	120	3
120-5	5 ± 0.7		
130-2	11 ± 1.9	130	4 ± 3.0
130-9	0		
130-10	0		
105-4	28 ± 5.4	105	12 ± 6.9
105-8	0		
105-18	7 ± 0.5		
132-16	0	132	0 ± 0
132-18	0		
132-20	0		
214-14	17 ± 4.3		see above

'a' - The overall % metastasis value represents the mean of % metastasis values cell lines expressing the same E1A mutant and the SE measure variation between these lines.
'b' - N20-11 was repeated in the second assay and 214-14 was run separately from the other two 214 series clones. Their overall % metastasis values are presented above.

not the second. As expected, 5R cells induced maximally high levels of metastatic lung nodules (>300). In assay 1 the N20-11 and N20-20 cell lines exhibited very low metastatic potentials, averaging 7.0 and 14.2 metastatic nodules, respectively. The 174-9 and 174-14 lines exhibited low to moderate levels of metastasis, 69.7 and 106.7 tumors, corresponding to 20% and 33% metastasis, respectively. The three 124 series cell lines showed a consistently high range of metastatic tumor nodule formation, 45%, 56%, and 100% metastasis, consistent with the potency observed in the invasion assays. The two 214 series cell lines expressing E1A polypeptides with deletions in exon 2 showed high, but variable, metastatic potentials of 100% and 30% metastatic lung tumors.

In assay 2, the negative control value for N20-11 cells was 74.0 mean tumors, corresponding to the 0% value for the second assay. The only cell lines that exhibited high metastatic potentials in this group were F12-1 and F12-13. These lines had tumor values of 258.8 and 231.0, or 82% and 70% metastasis, respectively, indicating that the internal 46 amino acid sequence lacking in the smaller protein may be important. All other cell lines had low levels of lung tumor nodule formation.

4) Analysis of T24 $p21^{ras}$ expression and correlation with metastatic potential.

All cell lines were examined for expression of T24/EJ ras protein, T24 $p21^{ras}$. Most cell lines were examined at least twice and the results were consistent. It was observed that there was differential expression of T24 $p21^{ras}$ depending on the type of E1A protein expressed by the cell line. Furthermore, there was a direct correlation between T24 $p21^{ras}$ expression and metastatic potential. Figure 22 shows that T24 $p21^{ras}$ expression in a series of metastatic cell lines is equivalent to, or higher than, that in the parental 5R line. In contrast, T24 $p21^{ras}$ levels in N20-11 and N20-20 were strikingly lower than in these lines. The differences in T24 $p21^{ras}$ levels were confirmed by densitometry. Figure 23 illustrates the correlation between the metastatic potentials of these cell lines and expression of T24 $p21^{ras}$.

Figure 24 shows that decreased T24 $p21^{ras}$ levels were not unique to the N20 series cell lines, but other nonmetastatic cell lines, including the 120, 130, 105, 132, also exhibited reduced $p21^{ras}$ expression relative to 5R. Also, for visual contrast purposes protein lysates from a series of metastatic and nonmetastatic cell lines were assayed for $p21^{ras}$ expression along side one another. Figure 26 shows that T24 $p21^{ras}$ levels in nonmetastatic cell lines were higher than the endogenous levels in REF52 cells but lower than in 5R and other

metastatic cell lines. It is interesting to note that ras was expressed at higher levels in some metastatic E1A-expressing cell lines than in 5R cells. Cell lines examined for T24 $p21^{ras}$ expression in Figures 24 and 26 also showed a correlation between T24 $p21^{ras}$ levels and metastatic capability and this is illustrated in Figures 25 and 27, respectively.

It is possible to differentiate between expression of endogenous H-ras and the transfected T24/EJ ras. Increased expression of T24/EJ ras in REF cells, including REF52 cells, typically results in a reduction or complete loss of the endogenous H-ras. Normal endogenous H-ras in REF52 cells is visualized on Western blots as a doublet (processed and unprocessed forms) (Figure 26, REF52 lane), and increased expression of T24/EJ ras is seen as a thick band running between them (Hicks et al., 1991). The formation of a doublet can also be seen in several lanes of nonmetastatic cell lines in Figure 24.

Figure 22. Differential expression of $p21^{ras}$ in E1A expressing cell lines. Western blot analysis showing E1A dependent variation in $p21^{ras}$ levels. All cell lines analyzed, except N20-11 and N20-20, exhibited moderate to high metastatic potentials and ras expression correlates with these values. Consistent with this correlation, nonmetastatic cell lines N20-11 and N20-20 exhibited significant reduction in relative ras expression. The PRO-6 cell line expresses a transfected mutant p53 gene (Hicks et al., 1991) and was examined with the intention of using it as a negative control which expresses basal levels of ras. However, analysis revealed that $p21^{ras}$ levels were elevated in this cell line and it was therefore not valid as a representative negative control.

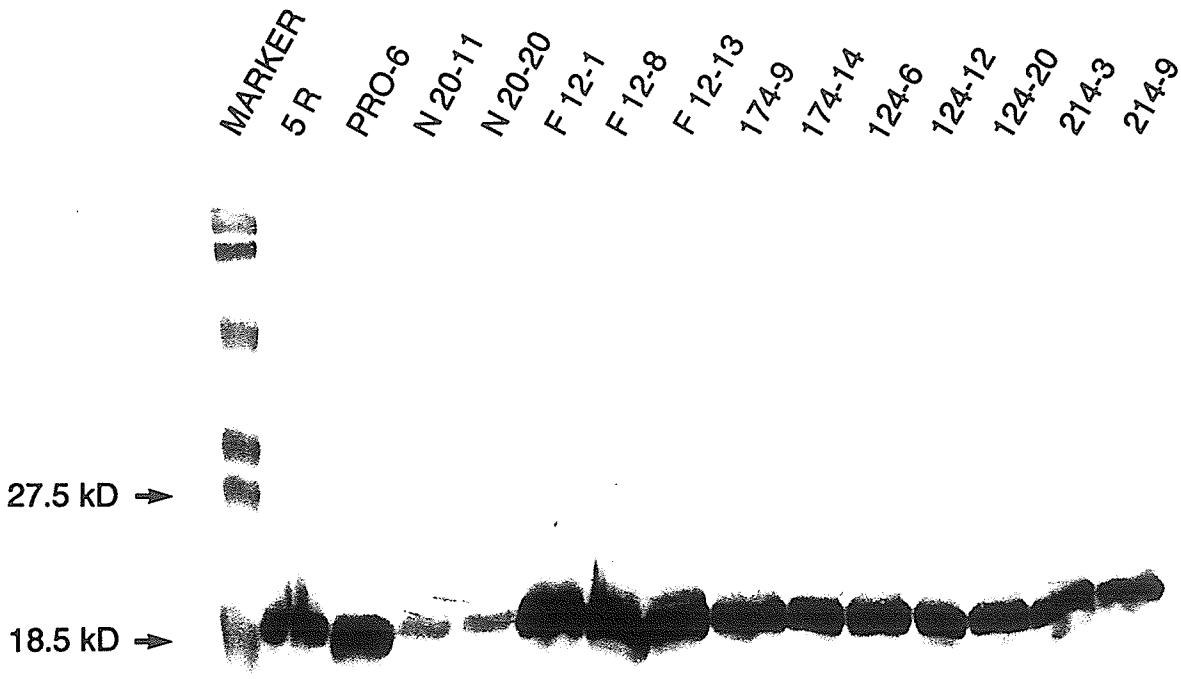

MARKER
5R
PRO-6
N20-11
N20-20
F12-1
F12-8
F12-13
174-9
174-14
124-6
124-12
124-20
214-3
214-9
27.5 kD
18.5 kD

Figure 23. Linear regression analysis of correlation between T24 $p21^{ras}$ expression and metastatic potential in E1A expressing cell lines. T24 $p21^{ras}$ levels were quantitated by densitometry and plotted against the metastatic potentials of relevant cell lines represented in Figure 22. T-test analysis of the correlation coefficient (r = .56361) quantitatively confirmed the correlation that was qualitatively apparent upon examination of the data. The t-test value for 10 degrees of freedom, df, is 2.1576 compared to a critical value $t_{df=10,a=.05}$= 1.812 (Fisher and Yates, 1974). Therefore there is greater than 95% probability that there is a linear relationship between ras expression and metastatic potential in these cell lines.

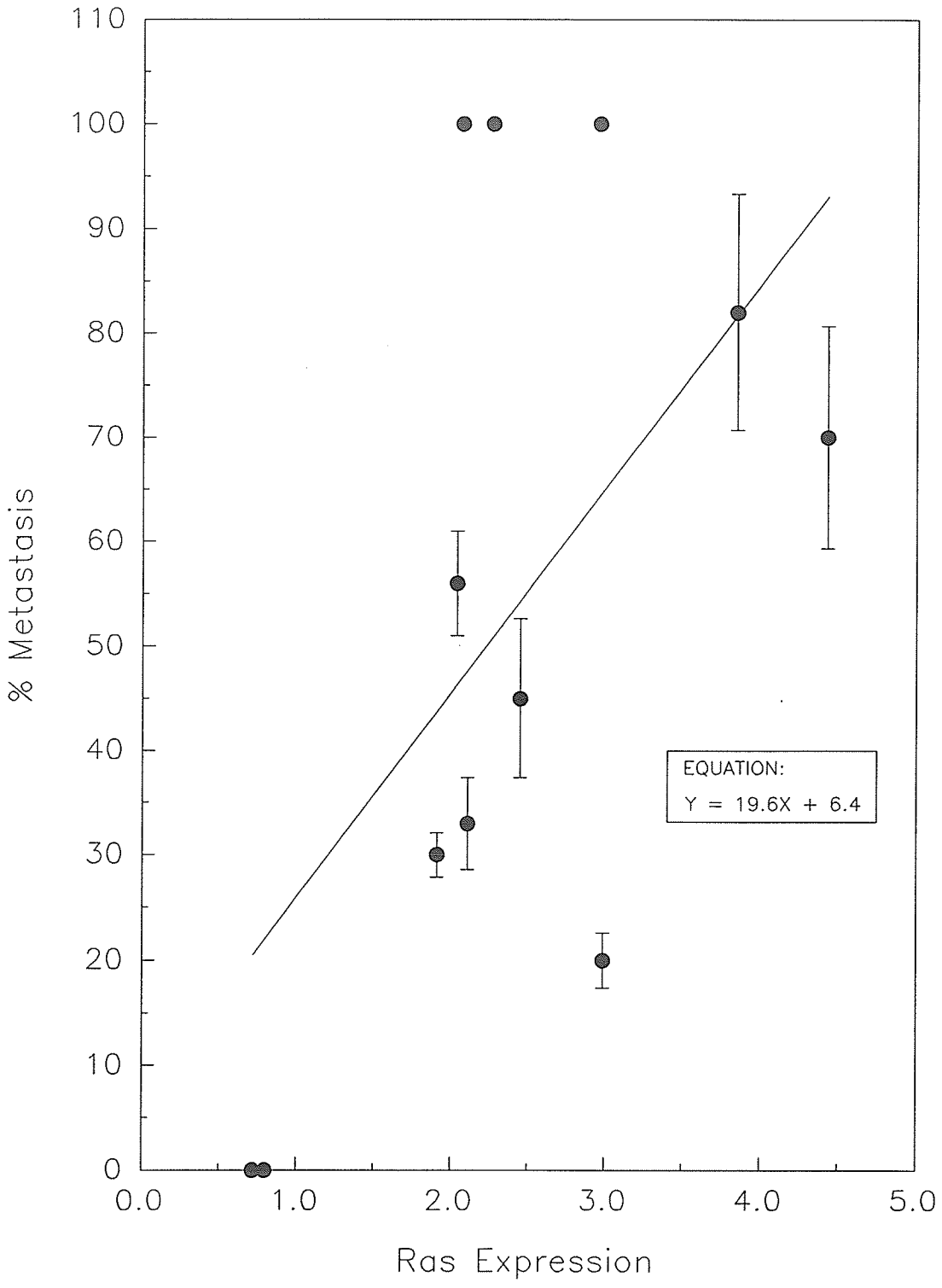

110
100
90
80
70
60
50
40
30
20
10
0
% Metastasis
0.0
1.0
2.0
3.0
4.0
5.0
Ras Expression
EQUATION:
Y = 19.6X + 6.4

Figure 24. Differential expression of $p21^{ras}$ in E1A expressing cell lines. Western blot analysis showing E1A dependent variation in $p21^{ras}$ levels. Expression in the 5R and the F12 series lines was higher than in the nonmetastatic 120, 130, 105 and 132 series lines. The latter lines exhibited the doublet profile characteristic of normal ras expression.

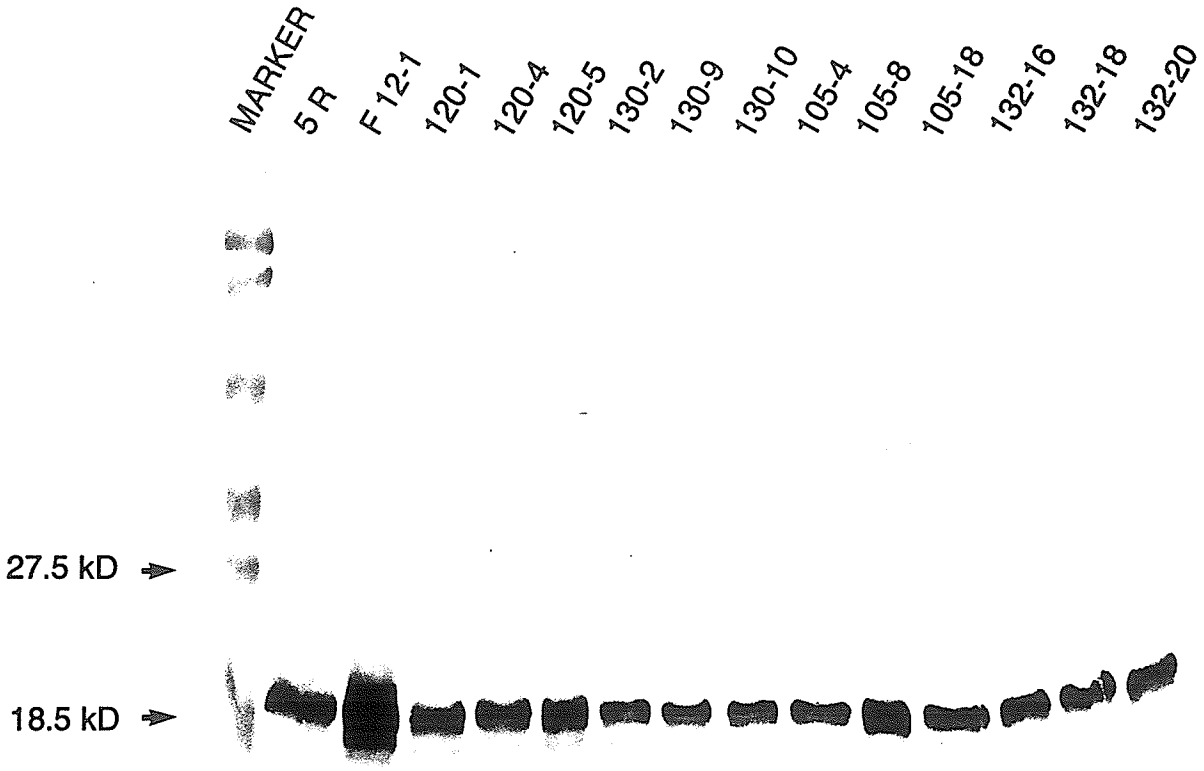

MARKER
5 R
F 12-1
120-1
120-4
120-5
130-2
130-9
130-10
105-4
105-8
105-18
132-16
132-18
132-20
27.5 kD
18.5 kD

Figure 25. Linear regression analysis of correlation between T24 $p21^{ras}$ expression and metastatic potential in E1A expressing cell lines. T24 $p21^{ras}$ levels were quantitated by densitometry and plotted against the metastatic potentials of relevant cell lines represented in Figure 24. T-test analysis of the correlation coefficient (r = .79588) quantitatively confirmed the correlation that was qualitatively apparent upon examination of the data. The t-test value for df=11, is 4.36 compared to a critical value $t_{df=11,a=.005}$= 3.106 (Fisher and Yates, 1974). Therefore there is greater than 99.5% probability that there is a linear relationship between ras expression and metastatic potential in these cell lines.

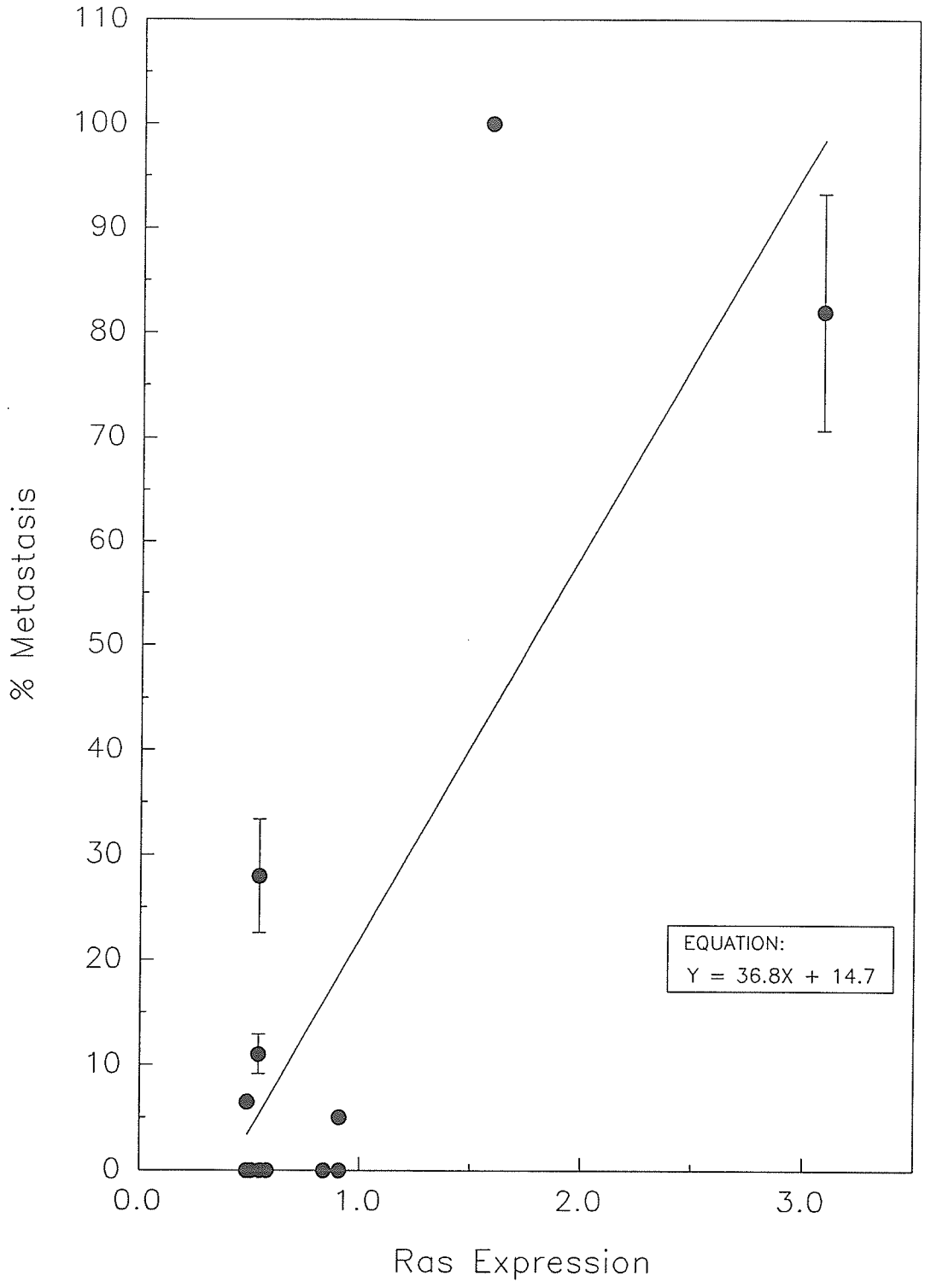

110
100
90
80
70
60
50
40
30
20
10
0
% Metastasis
0.0
1.0
2.0
3.0
Ras Expression
EQUATION:
Y = 36.8X + 14.7

Figure 26. Differential expression of $p21^{ras}$ in E1A expressing cell lines. Western blot analysis showing E1A dependent variation in $p21^{ras}$ levels. Lanes were loaded with a variety of metastatic and nonmetastatic cell lines to demonstrate the contrasting differences in ras expression. REF52 lane shows the intensity of endogenous expression of normal ras as well as the normal doublet formation.

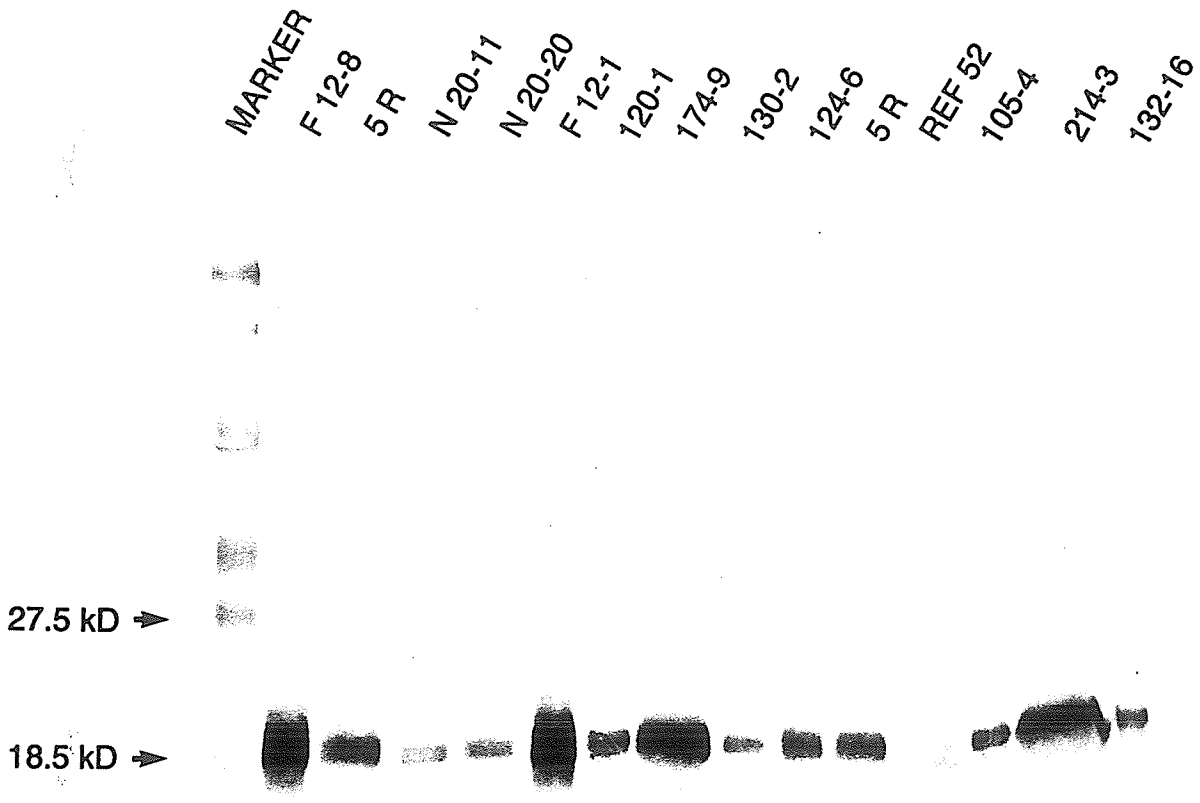
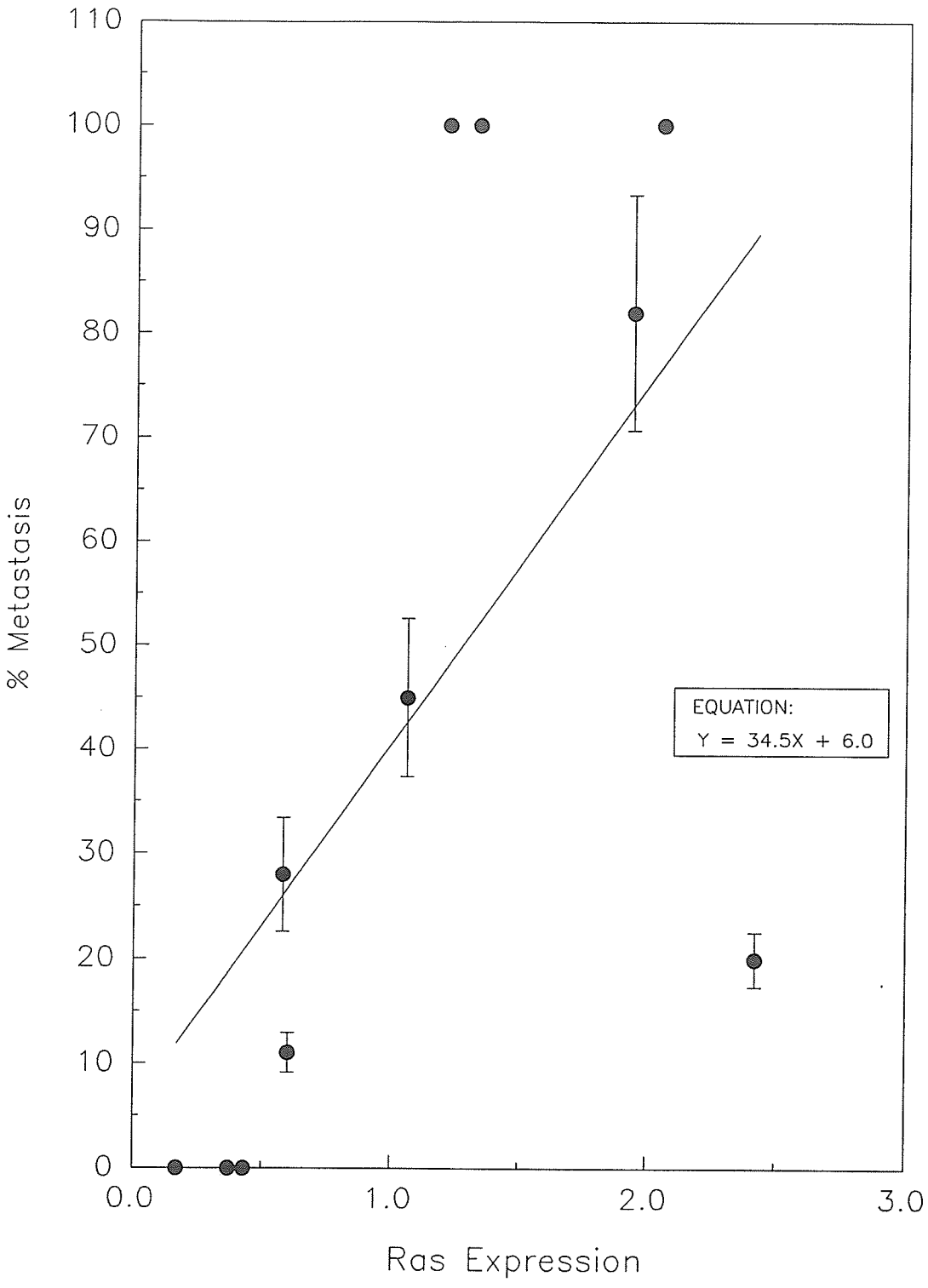

MARKER
F 12-8
5 R
N 20-11
N 20-20
F 12-1
120-1
174-9
130-2
124-6
5 R
REF 52
105-4
214-3
132-16
27.5 kD
18.5 kD

Figure 27. Linear regression analysis of correlation between T24 $p21^{ras}$ expression and metastatic potential in E1A expressing cell lines. T24 $p21^{ras}$ levels were quantitated by densitometry and plotted against the metastatic potentials of relevant cell lines represented in Figure 26. T-test analysis of the correlation coefficient (r = .60936) quantitatively confirmed the correlation that was qualitatively apparent upon examination of the data. The t-test value for df=9, is 2.31 compared to a critical value $t_{df=9,a=.025}$= 2.262 (Fisher and Yates, 1974). Therefore there is greater than 97.5% probability that there is a linear relationship between ras expression and metastatic potential in these cell lines.


110
100
90
80
70
60
50
40
30
20
10
0
% Metastasis
0.0
1.0
2.0
3.0
Ras Expression
EQUATION:
Y = 34.5X + 6.0

5) Analysis of T24 ras gene copy number.

T24 ras gene copy number was examined in the cell lines under study to determine whether differences in $p21^{ras}$ levels were due to variation in gene expression or gene copy number. Figures 28-A, 29-A and 30-A show southern analysis of ras. Figures 28-B, 29-B, and 30-B illustrate the GAPDH loading controls. Although the figures are of poor clarity it appears that differences in band strength are likely due to loading differences and not due to increased ras copy number. Densitometry was used to quantitatively varify this. This suggests that the differential T24 $p21^{ras}$ levels that were observed in the relevant cell lines may not be attributable to gene amplification, but rather that expression of ras may be regulated at transcription, or at some point thereafter.

Figure 28. Analysis of ras copy number in E1A expressing cell lines. (A) Southern blot analysis of ras gene copy number. Genomic DNA extracts from the illustrated cell lines were digested with Hind III, except for 130-2 which was digested with Eco RI. Although there were visual differences in band intensities of individual cell lines, GAPDH loading controls (B) showed that variation was due to differential loading.

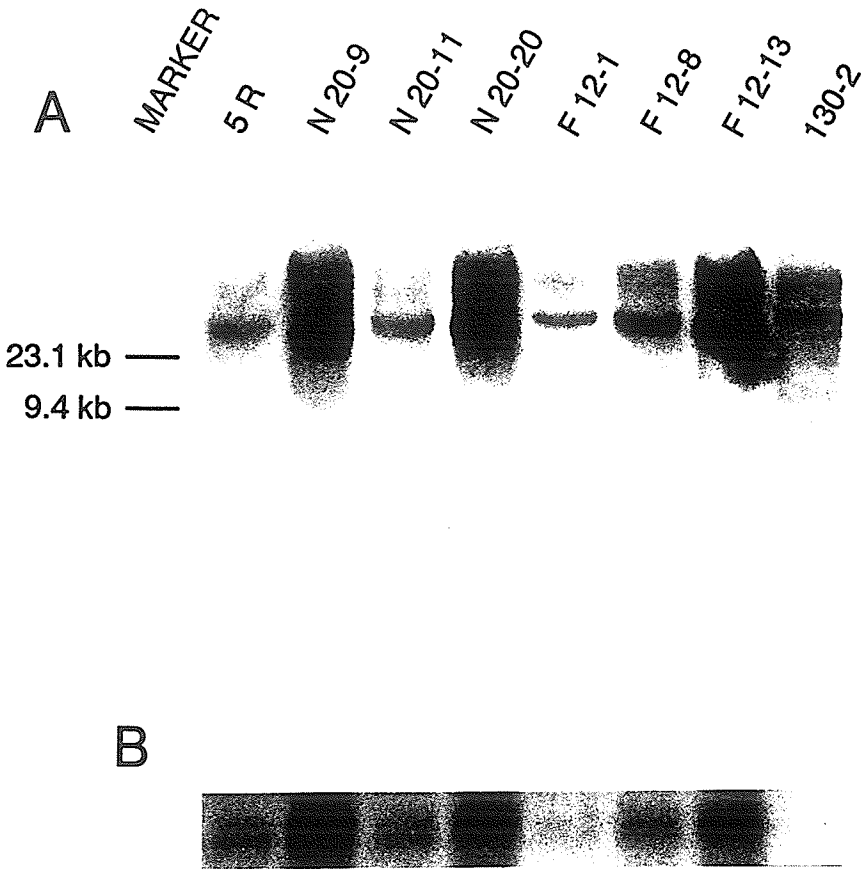

A
MARKER
5 R
N 20-9
N 20-11
N 20-20
F 12-1
F 12-8
F 12-13
130-2
23.1 kb
9.4 kb
B

Figure 29. Analysis of ras copy number in E1A expressing cell lines. (A) Southern blot analysis of ras copy number. Genomic DNA extracts from the illustrated cell lines were digested with Hind III, except for the 120 and 130 series cell lines which were digested with Eco RI. Although there were visual differences in band intensities of individual cell lines, GAPDH loading controls (B) showed that variation was due to differential loading.

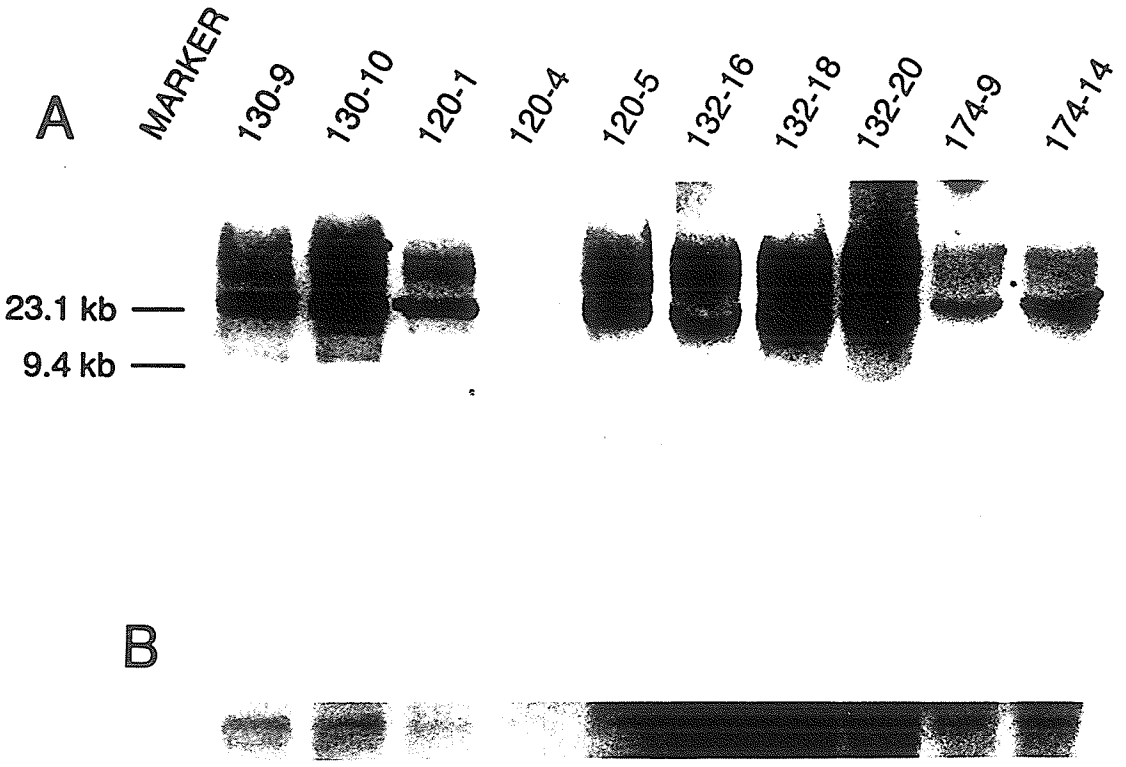
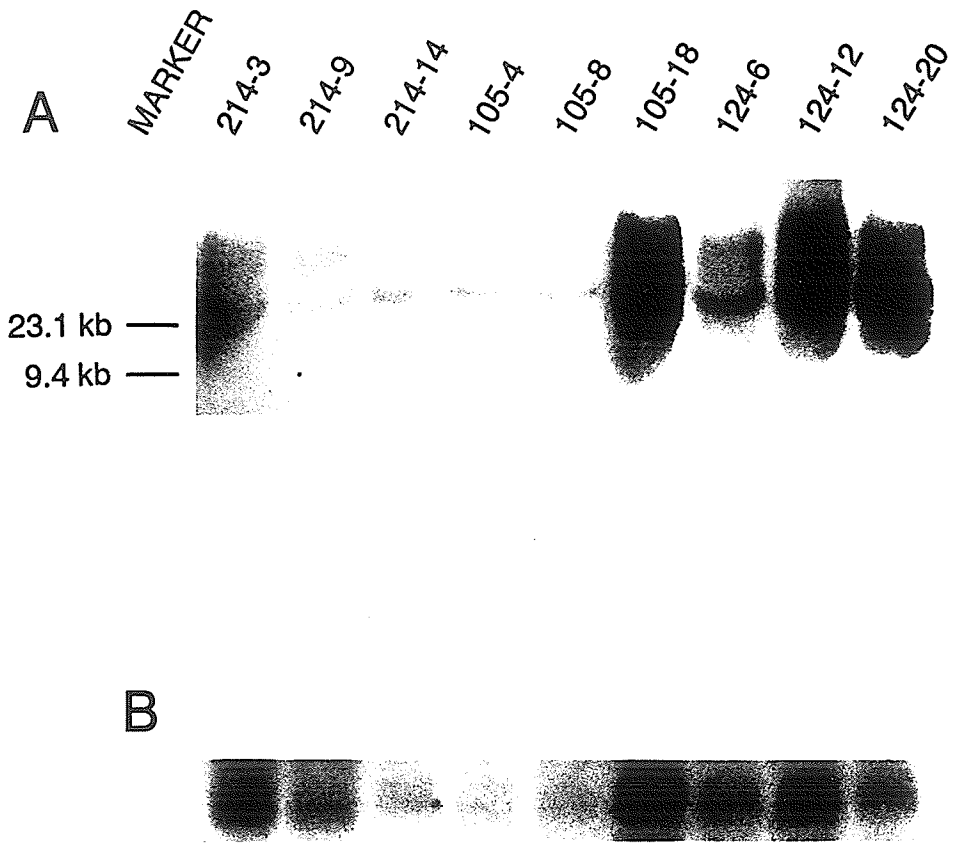

A
MARKER
130-9
130-10
120-1
120-4
120-5
132-16
132-18
132-20
174-9
174-14
23.1 kb
9.4 kb
B

Figure 30. Analysis of ras copy number in E1A expressing cell lines. (A) Southern blot analysis of ras copy number. Genomic DNA extracts from the illustrated cell lines were digested with Hind III. Although there were visual differences in band intensities of individual cell lines, GAPDH loading controls (B) showed that variation was due to differential loading.


A
MARKER
214-3
214-9
214-14
105-4
105-8
105-18
124-6
124-12
124-20
23.1 kb
9.4 kb
B

DISCUSSION

1) Amino acid sequences encoded by both exon 1 and exon 2 of E1A are involved in suppression of metastasis.

Based on the in vitro invasion and in vivo metastasis data there is sufficient reason to believe that sequences within conserved region 3 and those encoded by exon 2 are important in suppression of ras-mediated metastasis in rat embryo fibroblast cells. N-terminal sequences also appear to be important. Both of the above mentioned procedures produced results that were consistent with each other, with the exception of differences in the F12 series mutants. The data from the in vitro invasion assays suggest that the 243R E1A protein can suppress invasion as well as the 289R protein, implying that the 46 amino acid region (CR3) that distinguishes the two proteins is not involved in suppression of collagen invasion. In contrast, the in vivo experimental metastasis assays reveal that the absence of CR3 in the smaller protein results in loss of metastatic suppression, thereby implicating CR3 in this function.

With regard to this apparent contradiction it must be emphasized that the invasion assays done in this study were not intended for use as indicators of metastatic potential on their own. The reasons for this are that, (1) tumor cell invasion is only one aspect of metastatic progression and does not take into account external pressures operating in the host organism that ultimately dictate whether a cell survives to form a secondary cell colony, and (2) the extracellular matrix

is a complex structure consisting of several different proteins in specific proportions. Therefore, any effort to evaluate true invasive capability of a cell line must involve an attempt to mimic the actual conditions of the extracellular matrix. Finally, (3) the invasion assays done in this study involved a collagen I-based gel and therefore the results can only be used as measurements of collagenase I activity. The level of collagenase I activity alone is not an accurate indicator of invasive capability because, as discussed earlier, the expression of several other proteases is associated with tumor cell invasion. Therefore, the data from the invasion assays are valuable as supplementary data but should not be perceived as independently interpretable results in reference to metastatic potential.

Theoretically, differences in values between the invasion assays and metastasis assays can be explained by the fact that they represent different steps in the metastatic cascade. The invasion assays measure the ability of the cell to successfully complete the earlier steps related to invasion of the extracellular matrix. Meanwhile, the metastasis assays used in this project involved tail-vein injection which measures the second half of the metastatic cascade. For these reasons the two assays are essentially complementary and the data obtained from each should not be compared.

The validity of the controls used in the invasion assays must also be examined. Although the parental 5R cell line is

an accurate positive control, the use of the REF52 cell line as a negative control may be questioned by some. REF52 is an immortalized cell line that has been used previously in transformation experiments. This cell line is similar to primary rodent cells in that it cannot be transformed by the ras oncogene alone but can be transformed by ras in cooperation with E1A (Franza et al., 1986). Despite the suitability of REF52 cells in E1A transformation experiments, the fact that it is an established cell line may disqualify it for use as a negative control in invasion experiments since it may contain cellular changes that influence invasion. It was used because it was the only rat embryo fibroblast cell line available that closely resembles primary cells. The best negative control would have been to use the closest ancestor of the 5R cell line before it was transfected with H-ras. Since 5R arose from ras-transfection of primary REF cells this is not possible. Therefore, since a true negative control was not available, N20 series values obtained in metastatic analysis were represented as indicators of negative control values. It must be emphasized however, that N20 values are more indicative of base line values than actual negative control values.

In relation to the experimental metastasis assays conducted in this study it has been suggested by some investigators that tail-vein injection of nude mice is not an accurate indicator of metastatic potential and that

subcutaneous injection yields data closer to actual values. Nevertheless, tail-vein injections provide numbers very similar to those achieved by subcutaneous injection and therefore the former procedure is currently widely accepted as a reasonable experimental indicator of metastatic potential (Talmidge and Fidler, 1982; Fidler, 1984; 1986).

Metastasis assays were carried out in two different experiments. Negative (basal) controls were used in both assays. The negative control values show variation between experiments. For this reason it is important that comparisons of tumor formation between cell lines only be made between lines used in the same experiment. Also, tumor formation values of test cell lines should be judged in relation to negative control values of the corresponding assay. Percent metastasis calculations have been adjusted to account for differing negative control values between assays so as to facilitate comparisons between all cell lines.

The objectives of this project were to identify a region involved in metastatic suppression and to associate that with a biochemical activity of E1A such as transcriptional activation, enhancer repression or cellular protein binding. A number of possible regions and their respective functions were eliminated on the basis of our findings. For example, conserved region 1 which is involved in enhancer repression of some genes, including c-myc, JE, and stromelysin, and is partly involved in p300 binding (van Dam et al., 1989), is not

involved in suppression of the metastatic phenotype. The 105 and 132 series mutants which express E1A proteins with CR1 deletion mutations showed strong metastatic suppression very close to levels of N20 series suppression. Cell lines expressing the 105 series E1A mutants had percent metastasis values of 28%, 0%, and 7%, an average of 12%. Cell lines expressing the 132 series mutants all had values of 0% since none of them exceeded the value of the N20 base-line control.

Since enhancer repression essentially involves most regions of E1A and is not strictly mappable to CR1 it is not possible to completely eliminate it from involvement in suppression of metastasis based on the results of these cell lines alone. Velcich and Ziff (1988) have examined the ability of these two E1A mutants to repress the polyoma enhancer and found that while the 105 mutant showed nearly wild-type repression the 132 mutant showed only intermediate repression. If enhancer repression was involved in suppression of metastasis the results of this study should have indicated enhanced metastatic potential in the 132 series cell lines. This was not observed and, in fact, the 105 series cell lines had slightly higher numbers of lung tumors. Therefore, although the possibility that metastatic suppression may require enhancer repression still exists it appears, based on the results of the 105 and 132 series cell lines, that enhancer repression may be expendable. This is further supported by the observation that the 12S expressing

F12 series cell lines did not suppress metastasis.

Based on the results of the 105 and 132 series cell lines it is possible to eliminate p300 binding from involvement in metastatic suppression. It has previously been shown that these two mutants exhibit reduced transformation frequencies in cooperation with H-ras (Velcich and Ziff, 1988). While the 105 mutants maintain fairly high transformation frequencies of 74.2% of wild-type levels, the 132 mutants are almost completely defective in transformation with 8.8% of wild-type activity. Since Velcich and Ziff (1988) have demonstrated that E1A ras cooperation activity is separate from its positive and negative transcription regulatory functions this decrease in transformation cannot be due to either of these functions. Moreover, Whyte et al. (1989) have related cooperative transformation by E1A to binding of p300 through amino acids 1-76, which includes sequences within CR1. Therefore, the deficiency in transformation by the 105 and especially the 132 mutants must be due to loss of p300 binding. Since the corresponding cell lines in this study continue to suppress metastasis, p300 binding is not important for metastatic suppression by E1A.

The use of CR2 insertion mutations in E1A indicate that this region is not involved in suppression of metastasis. The 120 series mutants express an E1A protein with only 25% of wild-type transformation frequency in cooperation with E1B and about 60% in cooperation with H-ras in baby rat kidney (BRK)

cells (Kuppuswamy and Chinnadurai, 1987). This is likely attributable to diminished $p105^{RB}$ binding. The 130 series mutants have about 50% wild-type transformation frequency in cooperation with E1B and about 35% in H-ras cooperation assays. Cell lines expressing 120 and 130 class E1A proteins had average percent metastasis values of 3% and 4%, respectively. Therefore, binding to $p105^{RB}$ is not necessary for metastatic suppression by E1A.

2) Transcriptional activation functions of E1A are associated with metastatic suppression.

The data strongly suggest that transcriptional activation by E1A may have a role in suppression of metastasis. All cell lines used in this study that express E1A genes with mutations in regions that had been previously associated with transcriptional activation functions exhibit enhanced metastasis.

Until recently, it was a widely accepted fact that CR3 of E1A was the "transactivation domain". No other regions had been shown to have a role in transcriptional activation of gene expression. Several very recent studies, however, have identified a number of other regions that have either crucial or auxiliary roles in transactivation of some genes by E1A. There is a very striking correlation between absence of these regions in mutant E1A-expressing cell lines and the loss, or

partial loss, of metastatic suppression in this study. For example, Kraus et al. (1992) have recently reported that N-terminal sequences of the 12S E1A product are necessary for transactivation of the HSP70 promoter. Deletion of amino acids 2-36 in this study resulted in complete loss of HSP70 induction, while additional results suggested that loss of amino acids 38-51, near the N-terminal of CR1, could produce a reduction to about 33% HSP70 transactivation. The transactivation mediated by N-terminal sequences of E1A is different from E2F-dependent transactivation since previous studies have shown that this same N-terminal mutant can activate E2F-dependent transcription (Raychaudhuri et al., 1991). This N-terminal-mediated transactivation is dependent on the TATAA element and has been termed TATAA-dependent transactivation (Kraus et al., 1992).

The 174 series cell lines 174-9 and 174-14, which contain E1A deletions of amino acids 1-14, both showed moderately high levels of invasion. In addition, the levels of metastatic lung tumor formation, 20% and 33%, were substantially higher than the basal control values of the N20-11 and N20-20 lines. This indicates a partial loss or a diminished capability of these mutant E1A protein species to suppress metastasis and suggests that defective transcriptional activation by these mutants may account for this. Velcich and Ziff (1988) have previously shown that cells expressing 174 mutants continue to exhibit high levels of polyoma enhancer repression.

Therefore, decreased metastatic suppression by E1A in the 174 series cell lines is not attributable to loss of repression and correlates with the loss of sequences required for gene activation.

The 214 series cell lines express an E1A protein with a deletion of amino acids 185-214. Amino acid 185 is the last amino acid of both exon 1 and of the 46 amino acid unique sequence of the 13S E1A product. Previous studies have shown that a mutation in this region can result in loss of transactivation activity by E1A (Glenn and Ricciardi, 1985; Lillie et al., 1986; Schneider et al., 1987). Velcich and Ziff (1988) have shown that this holds true in reference to E2 promoter transactivation in cells that express the 214 E1A mutation. In my study three different 214 series cell lines were examined for metastatic potential. 214-3 and 214-9 were examined in the first assay. They produced mean percent metastasis values of 100% and 30%, respectively. 214-14 had a percent metastasis value of 17% in the second assay. There is considerable variation in values between these three cell lines. Nevertheless, both 214-9 and 214-14 had low-to-moderate values compared to their respective controls, and 214-3 had maximal tumor formation. I suggest that this is a consequence of inactivation of positive transcriptional functions of E1A in these cells.

In a recent study Bondesson et al. (1992) have reported the discovery of two auxiliary regions in exon 2 of E1A that

are involved in transactivation. These have been termed auxiliary region 1 (AR1) and auxiliary region 2 (AR2) and are located at amino acid residues 193-221 and 221-246, respectively. These regions cooperate with CR3 to produce a potent transactivator protein. AR1 and AR2 cannot act as activation domains on their own. The effects of AR1 and AR2 are not additive and only one of the two regions is sufficient to cooperate with CR3 to activate transcription. However, removal of both elements reduces CR3 transactivation of the E4 promoter to 5% of the wild-type protein level. Interestingly, although promoter elements responsive to ATF and E4F have very similar sequences, E1A transactivation of ATF is not AR1- or AR2-dependent.

Cells expressing E1A proteins containing the 124 mutation have been shown to be completely inactive in transactivation functions (Velcich and Ziff, 1988). The E1A proteins expressed by these lines contain deletions of amino acids 153-289. The deleted region includes part of CR3 and both AR elements. Consistent with the direct relationship between transactivation and metastatic suppression seen in the 174 and 214 series cell lines, E1A mutant cell lines 124-6, 124-12, and 124-20 all exhibited high metastatic potentials with an overall mean of 67% metastasis.

Previous work by Pozzatti et al. (1988) produced data suggesting that both major E1A protein species could effectively suppress metastasis in 5R cells. In fact, they

found that suppression by the 12S E1A mRNA product was stronger than that by the 13S mRNA product. The smaller protein reduced metastatic potential 126-fold compared to parental cells, while the larger protein reduced metastatic potential by only 10-fold. In contrast, my study shows that the 13S product exhibits similar suppression efficiencies (12.3-fold), but the 12S product fails to show significantly decreased levels of metastatic suppression; 1.2-fold, based on an average of 245 tumors compared to 300 for parental 5R cells.

Although there is no obvious explanation for this discrepancy, it is possible that it may be due to transfection of different E1A-expressing plasmids. Pozzatti's group used plasmids pE1a-12S and pE1a-13S, while I used pSVF12 and pSVN20 for expression of the 12S and 13S E1A forms, respectively. Both pairs of plasmids contain cDNAs of the 12S and 13S E1As and have been used in previously published work comparing activities of the two resulting protein products (Gilardi and Perricaudet, 1983; Velcich and Ziff, 1985; 1988). One apparent difference in the plasmids is that the pE1a plasmids express Ad2 E1As while the PSVF12/pSVN20 plasmids express Ad5 E1As. As discussed earlier the Ad2 and Ad5 E1A genes are considered to be interchangeable based on extensive sequence similarity. Nevertheless, it is possible that metastatic suppression by E1A may be sensitive to minor sequence differences between the two, resulting in the observed

discrepancy in metastatic influence of 12S E1A forms. Such differences have been observed between Ad2 and Ad12, although they were more easily justified since the sequences of E1A genes of these two serotypes are more divergent (Pozzatti et al., 1988).

An alternative explanation exists for the contrasting observations regarding 12S E1A-mediated metastatic suppression. While both studies confirmed E1A expression using northern hybridization analysis this expression may have been turned off subsequently. As E1A induces apotoptic cell death there is selective pressure against expression of E1A in some cells (Lowe and Ruley, 1993). Thus, while it is possible that this may have occurred in my 12S E1A-expressing cell lines, it is unlikely for three major reasons. Firstly, suppression of cellular invasion in the in vitro invasion assays suggests that E1A expression was sustained in the F12 series lines. Secondly, not only was E1A expression confirmed for the F12 series lines following transfection (data not shown) but it was reaffirmed following the in vitro invasion and in vivo experimental metastasis assays as shown in Figure 19. Finally, as the 13S E1A induces apoptosis to essentially the same degree as 12S E1A, the same selective pressure should exist within cell lines expressing both forms of E1A. If such a selection were present, anomalies consistent with lack of E1A expression should be apparent in the N20 series cell lines. Based on the data from both experimental assays and

the northern blotting there is no indication of this. Therefore it can be concluded that while there still may be selective pressure against E1A expression in 5R cells, there is sufficient reason to believe that it is not strong enough to be phenotypically expressed.

The discussion E1A expression has thus far been conducted in absolute terms. That is, we have been if the E1A genes are or are not being expressed. However, a point must be made with regards to the relative levels of E1A expressed in the cells lines. The importance of the level of oncogene expression in metastasis has been previously reported for oncogenes including ras (Egan et al., 1987; Taylor et al., 1992). There is no reason to believe that differences in expression levels would not affect metastatic potentials in E1A expressing lines. Since relative E1A expression levels were not quantitatited by either Pozzatti's group or myself, it is possible that there are differences in E1A expression and that these differences are responsible for the observed differences in metastatic potentials for cell lines expressing similar E1A genes. Similarily, it is possible that metastatic potentials of cell lines used in this study are affected by differential E1A expression.

The implication from the results reported by Pozzatti's group was that the transactivation function of E1A, as it relates to the presence of CR3, is dispensable in relation to metastatic suppression. Consequently, for several years

individuals interested in understanding the mechanism of metastatic suppression in E1A-expressing cells have dismissed transactivation as a biochemical activity of E1A that could be important in this respect. In addition, the fact that E1A could repress expression of several metastasis-related proteases, such as collagenase I, collagenase IV, and stromelysin, gave credence to the dogma that the ability of E1A to repress transcription from enhancer elements of specific genes was likely important in establishment of the nonmetastatic phenotype in malignant tumor cells upon introduction of E1A. In agreement with this, Garbisa et al. (1987) observed induction of type IV collagenase activity and the metastatic phenotype in cells transfected with c-H-ras but not c-H-ras plus E1A. Also, Frisch et al. (1990) related inhibition of metastasis in several E1A-transfected human tumor cell lines to repressed expression of the secreted proteases, type IV collagenase, interstitial collagenase, and urokinase. While these studies associated repression of specific genes with loss of the metastatic phenotype in E1A expressing cells, they did not directly address the mechanism by which this effect is elicited.

The results of this study suggest that transcriptional activation functions of E1A are important in maintenance of metastatic suppression while transcriptional repression functions are not. All mutant E1A plasmids used in this study have been previously analyzed for transactivation capabilities

of the E2 promoter (Kuppuswamy and Chinnadurai, 1987; Velcich and Ziff, 1988). The only cell lines that were defective in transactivation of E2 were those expressing F12, 124, 174, and 214 series mutations of E1A, while the remainder exhibited levels of transactivation comparable to the wild-type levels of the 13S E1A mRNA product (N20 series) (Table 41). Of the transactivation defective cell lines 174 showed the highest transactivation activity at 28% of wild-type levels. All others were much lower. When comparing this transactivation data with the metastasis data presented in this study there was a striking association between transactivation positive cell lines and the suppression of metastasis, while all cell lines expressing E1A mutants that were shown to be transactivation negative exhibited enhanced metastatic capabilities. Even the relative level of metastatic suppression corresponded with transactivation efficiency, as the cell lines expressing the E1A mutant with the highest transactivation capabilities (174 series) had the highest metastatic suppression of the four E1A mutant forms defective in metastatic suppression.

The relationship between transcriptional activation of the E1A mutants, as presented in Table 41, and metastatic suppression can be examined by plotting these two activities and conducting a linear regression analysis. Figure 31 shows that there is in fact a close linear inverse correlation between functional transactivation activity and metastatic

potential. This statistically varifies that the transcriptional activation function of E1A is involved in the suppression of T24 ras-mediated metastasis. Meanwhile, a similar comparison between enhancer repression function of E1A and metastatic capability reveals a linear but relatively weaker correlation (Figure 32). The apparent involvement of enhancer repression functions in metastatic suppression is likely due to the fact that in C-terminal regions of E1A, the positive and negative transcriptional activities overlap. Therefore, while the enhancer repression function is probably not important, the presence of intact transactivational domains of E1A shows a striking correlation and is closely associated with negative regulation of the metastatic phenotype.

Table 41. Previously determined transcriptional properties of mutant E1A plasmids expressed in E1A transfected lines and relationship to metastatic capability[a]

Plasmid	Cell line series	Transcription[b] Activation	Repression	% Metastasis
Neg. control	(no E1A)	<0.1	1.00	100
Pos. control	(genomic)	24.9	0.12	-
pSVN20 (13S)	N20	16.8	0.13	0
pSVF12 (12S)	F12	<0.1	0.07	76
pSVXL105	105	18.6	0.26	12
pSVXL132	132	15.5	0.60	0
pSVXL124	124	1.8	0.90	67
pSVXL174	174	6.9	0.24	27
pSVXL214	214	3.0	0.85	49

'a' - The table represents transcriptional characteristics as previously determined by Velcich and Ziff (1988) as well as the corresponding metastatic characteristics determined by data presented in the results of this study.
'b' - Transactivation was measured by quantitating % cells positive for DNA-binding protein (DBP). Repression values were determined by quantitation of polyoma enhancer promoted ß-globin mRNA production (northern blotting) in transfected cells.

Figure 31. Linear regression analysis of correlation between transactivation function of E1A and suppression of metastasis. The transactivation and metastasis data presented in Table 41 were used to create a plot. Linear regression of the points and calculation of the correlation coefficient (r = 0.91958) statistically varified an inverse relationship between transactivation, as it relates to the presented data, and metastasis. The t-test value for df=6 was determined to be 5.73 compared to the critical value $t_{df=6,a=.005}$= 3.71 (Fisher and Yates, 1974). Therefore, it can be said with greater than 99.5% certainty that transactivation functions of E1A are involved in negative regulation of the metastatic phenotype in T24-ras-transformed cells.

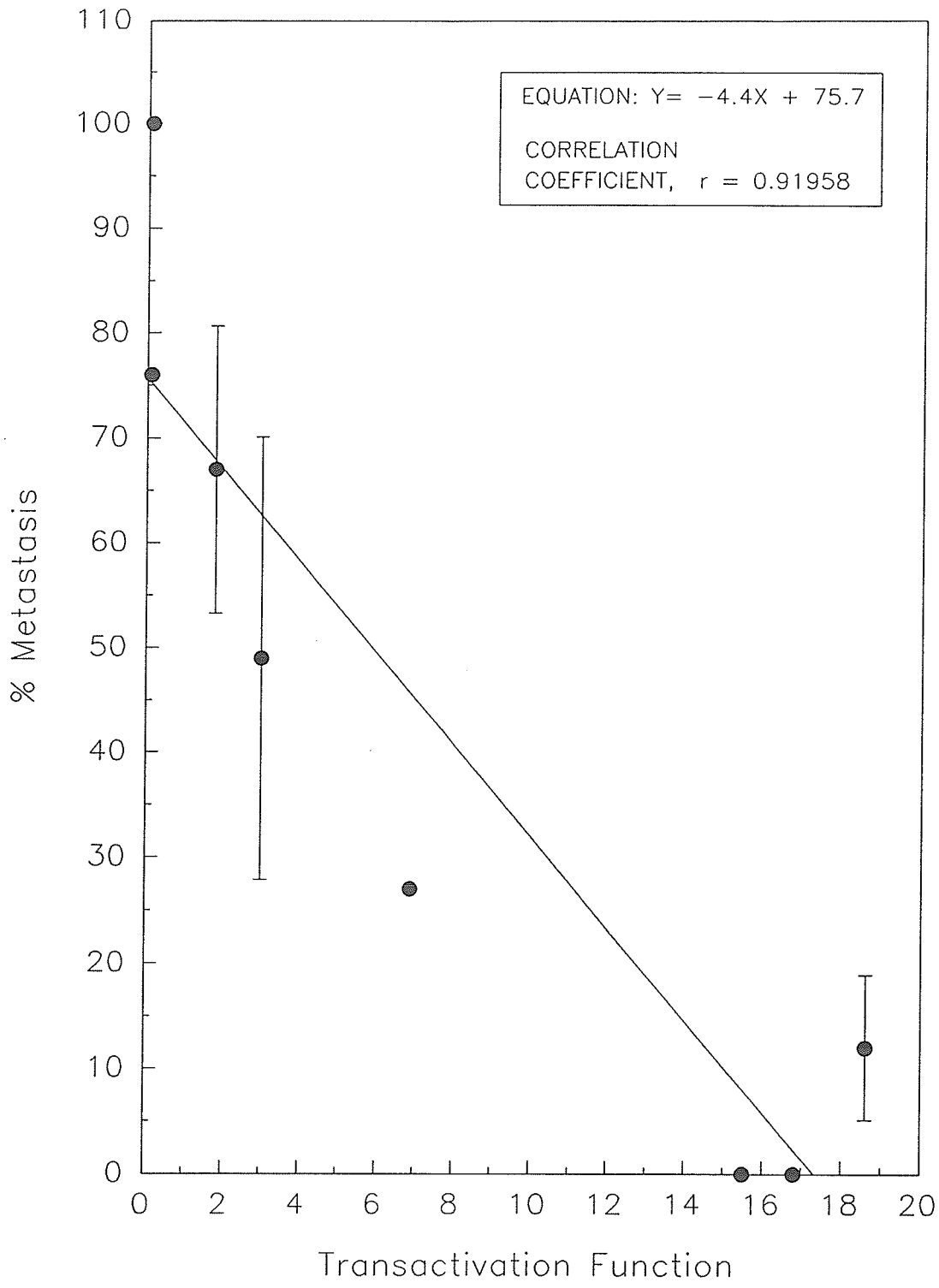
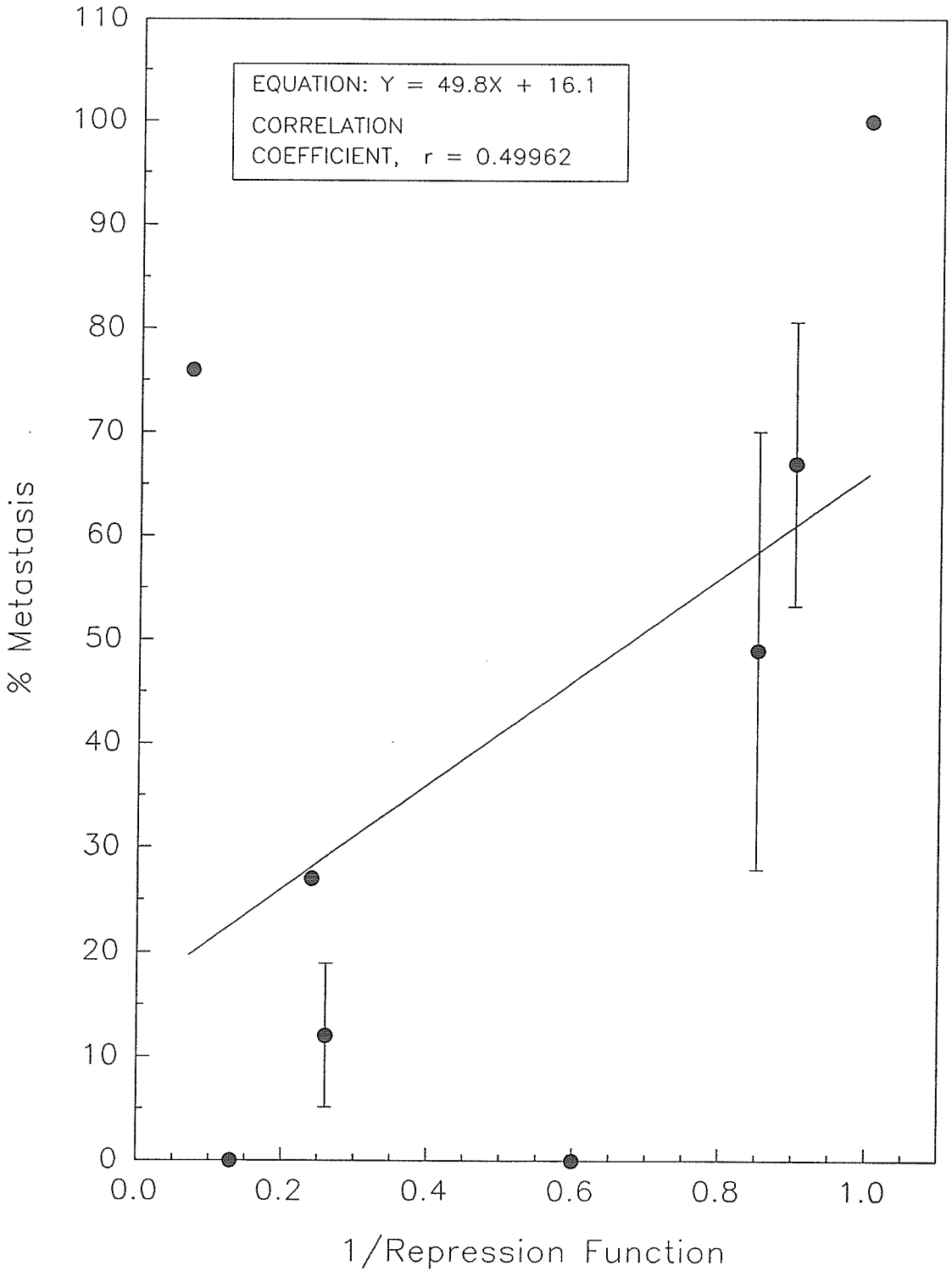

EQUATION: Y= −4.4X + 75.7
CORRELATION
COEFFICIENT, r = 0.91958
% Metastasis
110
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
Transactivation Function

Figure 32. Linear regression analysis of correlation between enhancer repression function of E1A and suppression of metastasis. The enhancer repression and metastasis data presented in Table 41 were used to create a plot. Linear regression of the points and calculation of the correlation coefficient (r = 0.49962) statistically varified an inverse relationship between transactivation, as it relates to the presented data, and metastasis. The t-test value for df=6 was determined to be 1.413 compared to the critical values $t_{df=6,a=.10}$= 1.44 and $t_{df=6,a=.25}$= 0.718 (Fisher and Yates, 1974). Therefore, it can be said with greater than 75% certainty that enhancer repression functions of E1A are involved in negative regulation of the metastatic phenotype in T24-ras-transformed cells. Therefore, there is a statistically weaker relationship between transcriptional repression functions of E1A, as compared to transactivation, and suppression of metastasis.


EQUATION: Y = 49.8X + 16.1
CORRELATION
COEFFICIENT, r = 0.49962
% Metastasis
1/Repression Function
0
10
20
30
40
50
60
70
80
90
100
110
0.0
0.2
0.4
0.6
0.8
1.0

3) Correlation between metastatic capability and T24 $p21^{ras}$ levels in E1A expressing cells.

The preceeding information leads us to question what kind of pathway involving the transactivational capabilities of E1A could facilitate metastatic suppression. From this other questions arise such as, (1) How does all of this data relating transactivation with metastatic suppression fit in with previous data relating metastatic suppression with repression of protease gene expression? (2) Could there be a metastasis suppressor gene?

The results of some recent studies, when taken together, suggest a paradoxical mechanism in which the transactivation function of E1A is necessary for repression of transcription of some proteases (Subramanian et al., 1989; Engel et al., 1992; Linder et al., 1992). In agreement with the findings presented here, these studies also found that regions in exon 2 of E1A are required for metastatic suppression in ras-transformed cells. Subramanian et al. (1989) found that mutants of the 243R E1A protein lacking the C-terminal 61 or 67 amino acids lost the ability to suppress metastasis in ras-transformed primary baby rat kidney (BRK) cells. The fact that they observed metastatic suppression by the wild-type 243R protein is different from my observation but may be explained by the fact that they used primary BRK whereas my study used an established REF cell line. Nevertheless our

findings that regions of exon 2 are important are consistent.

Reports by researchers in Sweden recently produced further evidence of E1A exon 2 involvement in metastatic suppression (Engel et al., 1992; Linder et al., 1992). They showed that deletion of essentially all of exon 2 of E1A (amino acids 193-289) was sufficient to elevate levels of invasion and that this effect was elicited mainly through amino acids 193-245. They also showed that the biochemical mechanism of this effect involved transcriptional repression of stromelysin-1 by a region corresponding to AR2 in the wild-type protein, and that loss of this region resulted in elevated stromelysin-1 mRNA levels and enhanced invasive capabilities of REFs.

It is very interesting that a sequence of amino acids that has been associated with transcriptional activation is also required for repression of a protease gene! This suggests that the positive and negative transcriptional functions of E1A are linked, at least in relation to the specific gene involved in these studies. This may involve a scenario whereby E1A transactivates a gene whose protein product plays a role in repression of protease gene expression. It is clear from these studies that a transactivation domain of E1A is required for both repression of protease gene expression and for suppression of tumor cell invasion, a metastasis-related phenomenon. This associates transactivation with metastatic suppression, which is in

agreement with my results, and also explains the findings of others that repression of metalloproteinase expression is required for suppression of metastasis by E1A (Garbisa et al., 1987; Frisch et al., 1990).

When Velcich and Ziff (1988) examined the positive and negative transcriptional regulatory activities of three exon 2 mutants, 124, 214, and a third not used in this study, 101 which contains a deletion of amino acids 221-231 of E1A, they also found a correlation between transactivation and enhancer repression functions of E1A. Both 124 and 214 were defective in transactivation of the E2 promoter and also in repression of the polyoma enhancer. Meanwhile the 101 mutants were functionally active in both functions. This correlation was not observed in E1A genes with mutations outside of exon 2. For example, 174 and F12 series cell lines, which are partially and fully defective in transactivation, respectively, showed strong repression of the polyoma enhancer. This suggests that the relationship between positive and negative transcriptional regulation of genes may not be applicable to all transactivation mechanisms involving E1A.

To elaborate on this point, it is clear that there are several different mechanisms by which E1A can transactivate gene expression involving different regions of the protein. To summarize this briefly, Kraus et al. (1992) have shown that N-terminal regions of E1A are involved in TATAA-dependent

transactivation by the 12S E1A mRNA product. This is different from the E2F-dependent transactivation mechanism involving amino acids 38-73 (CR1) and 124-135 (CR2) of the 12S E1A product (Raychaudhuri et al., 1991). A third mechanism involves AR1 and AR2-dependent transactivation mediated by CR3 of the 13S E1A mRNA product as in the case of E4 activation (Bondesson et al., 1992). Finally, there is the AR1- and AR2-independent mechanism involving CR3 which operates in the case of ATF-mediated transcriptional activation (Bondesson et al., 1992). It must be emphasized that these different mechanisms were deciphered only recently and that there is a possibility that more may be found following further investigation. I suggest that since these different mechanisms are selectively and specifically involved in the activation of transcription, not all are related to enhancer repression and metastatic progression. For example, although the deletion of N-terminal sequences of E1A resulted in somewhat enhanced levels of metastasis, TATAA-dependent transactivation is probably not as important in metastatic suppression as the AR1- and AR2-dependent CR3-mediated transactivational mechanism since E1A proteins missing CR3 and regions in exon 2 showed the strongest correlation with enhanced metastatic potential in this study, and with the metastatic phenotype and metalloproteinase gene expression in other studies (Subramanian et al., 1989; Engel et al., 1992). The fact that CR1 and CR2 mutation of E1A did not affect metastatic

potential precludes the E2F-dependent mechanism from involvement in metastatic suppression. This is further supported by the finding that the 120 series cell lines expressing E1A genes encoding proteins expected to be deficient in binding to $p105^{RB}$ did not affect metastatic suppression. It must be noted however that CR1, like AR2, is necessary for transcriptional repression of stromelysin and therefore the AR1- and AR2-dependent and E2F-dependent transactivation mechanisms as they relate to transcriptional repression may somehow be related (van Dam et al., 1989).

The relationship between transcriptional activation and both enhancer repression and metastatic suppression observed in this study is very intriguing and leads us to question what kind of gene may be activated by E1A that is capable of producing such drastic effects on cellular characteristics. Since the metastatic phenotype of 5R cells is induced by T24 ras the candidate gene product must operate along the ras pathway and possess biochemical properties allowing it to counteract the biochemical effects of T24 ras. A strong candidate gene is the cellular nm23 gene originally identified by Steeg et al. (1988a). nm23 is highly expressed in nonmetastatic cells while being weakly expressed in metastatic lines and is therefore considered to be a metastasis suppressor gene. Furthermore, in ras plus E1A-transfected nonmetastatic cell lines the activation of nm23 has been associated with expression of E1A suggesting that E1A

positively regulates expression of nm23 (Steeg et al., 1988b). Additional studies have revealed that the protein product of nm23 is a nucleoside diphosphate kinase (Rosengard et al., 1989; Liotta and Steeg, 1990). Since normal c-ras is a nucleoside triphosphate phosphatase (GTPase) and transforms cells by virtue of augmented GTPase activity in cells expressing mutant ras genes, expression of a nucleoside diphosphate kinase would reverse the effects of ras in signal transduction. Functionally, nm23 acts as an antagonist to ras function. Expression of H-ras is associated with expression of several proliferation-associated genes through the PDGF pathway including stromelysin, which are also important in malignant progression of tumor cells (Diaz-Meco et al., 1991). It is therefore conceivable that E1A-mediated nm23 expression results in the reversal of many ras-induced characteristics including metalloproteinase expression and enhanced metastatic potential. This also provides a logical explanation for the fact that transactivation domains of E1A are required for metalloproteinase gene repression. Thus, although nm23 expression was not examined in this study, it would be a strong candidate for investigation of possible correlation with metastatic potentials in the metastatic and nonmetastatic mutant E1A cell lines used in this study.

The results presented here show that T24 $p21^{ras}$ expression is repressed, relative to 5R cells, in the nonmetastatic E1A-expressing cell lines. Therefore, although the above scenario

involving nm23 is still possible it appears more likely that T24 $p21^{ras}$ expression, and not T24 $p21^{ras}$ activity, is regulated in these cell lines. All cell lines that exhibited low metastatic potentials expressed T24 $p21^{ras}$ at significantly reduced levels. T24 $p21^{ras}$ expression in N20-11 and N20-20 cells, which expressed the wild-type 289R protein and exhibited very weak metastatic potentials, were reduced approximately 5-fold compared to 5R cells. Cell lines expressing E1A mutants that showed low metastatic capabilities had reduced (2-3-fold) T24 $p21^{ras}$ levels but not as drastic as those of N20 series lines which were reduced as much as 5-6-fold.

In contrast, F12-1, F12-8, and F12-13 cells, which express the wild-type 243R protein and exhibit high metastatic capabilities, express T24 $p21^{ras}$ at a 2-fold increase compared to 5R cells. Expression of T24 $p21^{ras}$ in other metastatic cell lines, including the 174, 214, and 124 series lines, is comparable to parental levels. These results are in agreement with previous observations by our group of enhanced metastatic potentials in cells expressing elevated levels of T24 ras (Egan et al., 1987a; Taylor et al., 1992).

The implicit regulation of ras by E1A is not unprecidented. Earlier work by another group demonstrated that transfection of E1A into T24 ras-transfected cells affected T24 $p21^{ras}$ expression. Franza et al. (1986) examined the transforming capabilities of T24 ras in REF52 cells and

found that T24 ras alone could not stably transform the cells but that it could collaborate with E1A to give rise to morphologically transformed cells. Further, they observed that T24 $p21^{ras}$ levels in the E1A-transfected cell lines were ten-fold higher than in the untransformed T24 ras-transfected lines. This implies positive regulation of T24 ras expression by E1A which is opposite from the observations presented here. The reasons for this apparent discrepancy are unclear at this point. Nevertheless, there is significance in the fact that there is a correlation between E1A expression and changes in T24 $p21^{ras}$, whether they be positive or negative. While the aforementioned study examined morphological transformation capacities there is no evidence in the literature that ras levels are differentially regulated in response to E1A in metastasis studies. In this respect the findings presented here are novel.

Southern analysis of the E1A expressing cell lines suggests that ras is not amplified. However, it must be pointed out that resolution and clarity of the figures presenting this data is somewhat weak and the data is considered questionable by some. Ras gene amplification should be examined more carefully to confirm that there are no differences in ras copy number.

Based on the data presented here it is not possible to determine the level at which T24 ras expression is regulated in these cell lines. However, there are several observations

that suggest that T24 ras may be regulated at the transcriptional level and that this effect could be elicited through the transcriptional regulatory activities of E1A. Firstly, there is an inverse correlation between the expression of T24 ras and the presence of transactivation-related E1A protein domains in my study. That is, possible loss of transactivation functions results in increased ras expression. Similarily, as discussed earlier (p. 196), regions encoded by exon 2 appear to overlap with regard to positive and negative transcriptional functions and loss of AR2 is associated with enhanced stromelysin expression. Although the mechanism of such reversed gene regulation is not understood it may be due to derepression of these genes, perhaps by a failure of transactivation-defective mutants to induce transcription of a gene whose product plays a role in transcriptional repression. In this way both H-ras and stromelysin may be up-regulated by E1A mutants defective in transactivation functions. In addition, there may be a causal relationship between expression of H-ras and stromelysin as Diaz-Meco et al. (1991) have shown that ras and other factors that operate along the ras pathway, including PDGF and phosphatidylcholine-hydrolyzing phospholipase C, transcriptionally activate the stromelysin promoter.

Secondly, Nakamura et al. (1992) have shown that transcriptional repression of the fibronectin gene by E1A is due to induction of an E1A-responsive negative factor called

G_{10} binding protein (G_{10}BP). G_{10}BP recognizes the sequences AGGGGGGGGG/AGGGGCGGGGG/GGGGGGGCGGG. In the fibronectin promoter two of these sequences overlap the Sp1 transcription factor binding sequence GGGCGG. Since G_{10}BP has a stronger affinity for GC boxes than Sp1, the latter is displaced and repression of fibronectin transcription results. Recognition sequences for both of these factors are present in the H-ras promoter. It is therefore possible, and perhaps even likely, that negative regulation of H-ras by E1A occurs at the level of transcription. This would also explain the correlation between intact transactivational domains and down-regulation of H-ras since these regions may be required for induction of G_{10}BP or some related protein.

Alternatively, it is possible that T24 $p21^{ras}$ levels are regulated post-transcriptionally. E1A may affect stability and/or translation of T24 ras mRNA, or stability of the translated protein product. Of these three scenarios the latter is most likely. This is based on the finding by Lowe and Ruley (1993) that E1A stabilizes the protein encoded by the p53 tumor suppressor gene. Based on my data, if E1A were affecting T24 $p21^{ras}$ stability, it would destabilize it. Nevertheless, the fact that it plays a role in modulating protein half-life values suggests that E1A may perhaps oppositely affect T24 $p21^{ras}$ and p53 half-life values and that this activity could be regulated by specific domains. Similar 'opposite' activities (positive and negative), as discussed

earlier (p. 196), have been observed in the case of transcriptional regulation by E1A.

4) Future considerations.

Paradoxical behavior of E1A has also been observed in transformation studies. The 289R E1A polypeptide contains domains that positively and negatively affect morphological transformation. While the oncogenic affects of E1A, and the regions responsible for induction of these affects, are well documented several groups have reported anti-oncogenic affects of E1A in cooperative T24 ras transformation studies involving a wide variety of cell types (Frisch, 1991; Yu et al., 1991; Chinnadurai, 1992). Subramanian et al. (1989) and Douglas et al. (1992) have both determined that a region of the 243R protein encoded by exon 2 is responsible for this transformation suppressor activity. Recently the former group has shown that C-terminal sequences that are important for the negative modulation of T24-ras mediated transformation, tumorigenesis and metastasis bind to a 48-kDa cellular protein (Boyd et al., 1993). This phosphoprotein has been termed C-terminal binding protein (CtBP). The level of phosphorylation of CtBP appears to be regulated during the cell cycle, suggesting that it may play a role in cellular proliferation. The C-terminal 67 amino acids, as discussed earlier, are important in binding to CtBP and negatively affecting transformation-related functions of T24-ras. However, amino

acids 225-238 are especially important as they include the direct binding region (Boyd et al., 1993). While this is the only region that has been identified to date it is likely that other regions will be implicated in having a role in transformation suppression because investigators have only recently begun examining tumor suppressor functions encoded by E1A.

It is possible that transformation suppression and modulation of protein expression by E1A may operate through a mechanism where tumor suppressor protein(s) (p53) are stabilized, and oncoprotein(s), such as $p21^{ras}$ perhaps, are destabilized. By affecting the protein concentrations of factors directly involved in tumorigenesis E1A may be capable of further modulating the tumorigenic effects already elicited by the p300 and RB-binding transformation domains. Although there are prelimary data that are consistent with such a hypothesis further work is needed to provide results that would either preclude or support such a mechanism. This would explain the concurrent change in both protein concentrations and the transformation/metastatic potential of cells expressing E1A and T24-ras.

Margaret Quinlan's research group has described induction of an epithelial cell growth factor in 12S E1A transformed cells (Quinlan and Grodzicker, 1986). Since induction of this growth factor by E1A is associated with cellular immortalization, ras cooperation, and proliferation it plays

a direct role in E1A-mediated transformation (Quinlan et al., 1987; 1988; Quinlan, 1989). The identity of the growth factor is not known. TGF-α and EGF have been ruled out as possible candidates (Quinlan et al., 1987). The factor is part of a high molecular weight complex and is induced by an autocrine mechanism. In addition, it is believed to be an attachment factor based on observations that it facilitates adhesion of cultured mammalian cells (Quinlan et al., 1987). These characterizations are based on observations in epithelial cells including hepatocytes, BRK cells, and F9 cells.

Regions involved in induction of the growth factor include amino acid regions 1-13, 18-20, 125-127, and 208-236 in the 12S E1A product (Quinlan et al., 1988; Subramanian et al., 1988; Quinlan and Douglas, 1992). Two of these regions, the N-terminal region (1-13) and the C-terminal region (208-236) were involved in this study. The 174 series cell lines expressed an E1A product that contained a deletion of the first region, while the 124 series cell lines expressed E1A lacking the second region. It must be pointed out that these were 13S E1A products and not 12S. Nevertheless, individual deletions of both regions resulted in defective metastatic suppression by E1A. The other two regions (18-20, 125-127) were not involved in this study therefore no correlation can be made between the loss of these regions and elevated metastatic potentials. Nevertheless, based on the results of the 174 and 124 series mutants, growth factor induction could

be involved in metastatic suppression by E1A.

Pozzatti et al. (1988) have shown that the Ad12 E1A protein is not capable of inducing the metastatic suppression that has been shown for Ad5 E1A. Since Ad12 E1A differs from Ad5 E1A in the mRNA transcript sizes, and the final protein products of the two larger mRNAs are smaller (235R and 266R), it is possible that regions responsible for suppression of metastasis are spliced out in Ad12 E1A. Also, sequence differences in the final E1A products may result in loss of this function in Ad12. Analysis of ras inducibility in Ad12 E1A transfections of 5R cells could possibly confirm this process as the determining factor in regulating the differential metastatic influences of E1A proteins of the two serotypes.

In closing, although I have identified some regions of E1A involved in negative regulation of metastatic potential in T24 ras-transformed REF cells, it is unlikely that all metastatic cell lines are biochemically defective in identical areas. That is, the cause of loss of metastatic suppression likely differs from one class of cell line to another depending on the type of E1A mutant expressed by each. This is because E1A can target multiple steps in the metastatic cascade and the reduced metastatic potentials in wild-type E1A transfected cells are the result of cumulative changes in cellular gene expression. Transcriptional activation, and perhaps a related transcriptional repression activity, is

responsible for metastatic suppression based on the results presented here. However, it is clear that E1A-mediated transcriptional activation itself operates through several different mechanisms. Therefore, different E1A mutants may lose the ability to suppress metastasis through related but different mechanisms depending on the specific gene(s) or protein(s) that is affected by mutation in that specific region of E1A. Thus, although the results presented here are conclusive with respect to regions involved in metastatic suppression and associated repression of ras, further elaboration and interpretation of these findings must be carried out with caution for E1A is the most intricate and complex oncogene identified to date. Further investigation and characterization of the cell lines developed here will help scientists to better understand the process of metastatic progression as it relates to the H-ras and E1A oncogenes.

REFERENCES

1) Alitalo, K. and M. Schwab. 1986. Oncogene amplification in tumor cells. **Adv. Cancer Res. 47:** 235-282.

2) Ames, R.S., B. Holskin, M. Mitcho, D. Shalloway and M.-J. Chen. 1990. Induction of sensitivity to the cytotoxic action of tumor necrosis factor alpha by adenovirus E1A is independent of transformation and transcriptional activation. **J. Virol. 64:** 4115-4122.

3) Angel, P., M. Imagawa, R. Chui, B. Stein, R.J. Imbra, H.J. Rahmsdorf, C. Jonet, P. Herrlich and M. Karin. 1987. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. **Cell 49:** 729-739.

4) Aoyama, A. and R. Klemenz. 1993. Oncogene-mediated effects on cellular gene expression. **Critic. Rev. Oncogenesis 4:** 53-94.

5) Arroyo, M. and P. Raychaudhuri. 1992. Retinoblastoma-repression of E2F dependent transcription depends on the ability of the retinoblastoma protein to interact with E2F and is abrogated by adenovirus E1A oncoprotein. **Nucleic Acids Res. 20:** 5947-5954.

6) Axelrod, J.H., R. Reich and R. Mishkin. 1989. Expression of human recombinant plasminogen activators enhances invasion and experimental metastasis of H-ras-transformed NIH 3T3 cells. **Mol. Cell. Biol. 9:** 2133-2141.

7) Aznavoorian, S., M.L. Stracke, H. Krutzsch, E. Schiffmann and L.A. Liotta. 1990. Signal transduction for chemotaxis and haptotaxis by matrix molecules in tumor cells. **J. Cell Biol. 110:** 1427-1438.

8) Babiss, L.E. 1989. The cellular transcription factor E2F requires viral E1A and E4 gene products for increased DNA-binding activity and functions to stimulate adenovirus E2A gene expression. **J. Virol. 63:** 2709-2717.

9) Bagchi, S., P. Raychaudhuri and J.R. Nevins. 1989. Phosphorylation-dependent activation of the adenovirus-inducible E2F transcription factor in a cell-free system. **Proc. Natl. Acad. Sci. USA 86:** 4352-4356.

10) Bagchi, S., P. Raychaudhuri and J.R. Nevins. 1990. Adenovirus E1A proteins can dissociate heteromeric complexes involving E2F transcription factor: a novel mechanism for E1A trans-activation. **Cell 62:** 659-669.

11) Bagchi, S., R. Weinmann and P. Raychaudhuri. 1991. The

retinoblastoma protein copurifies with E2F-I, an E1A-regulated inhibitor of the transcription factor E2F. **Cell 65:** 1063-1072.

12) Bandara, L.R. and N.B. La Thangue. 1991. Adenovirus E1A prevents the retinoblastoma product from complexing with a cellular transcription factor. **Nature 351:** 494-497.

13) Barbacid, M. 1987. Ras genes. **Annu. Rev. Biochem. 56:** 779-827.

15) Barsky, S.H., G.P. Seigal, F. Jannotta and L. Liotta. 1983. Loss of basement membrane components by invasive tumors but not by their benign counterparts. **Lab. Invest. 49:** 140-149.

16) Bautista, D.S., M. Hitt, J. McCrory and F.L. Graham. 1991. Isolation and characterization of insertion mutants in E1A of adenovirus type 5. **Virology 182:** 578-596.

17) Bellett, A.J.D., P. Li, E.T. David, E.J. Mackey, A.W. Braithewaite and J.R. Cutt. 1985. Control functions of adenovirus transformation region E1A gene products in rat and human cells. **Mol. Cell. Biol. 5:** 1933-1939.

18) Bellgrau, D., T.A. Walker and J.L. Cook. 1988. Recognition of adenovirus E1A gene products on immortalized cell surfaces by cytotoxic T lymphocytes. **J. Virol. 62:** 1513-1519.

19) Berger, S.L. and W.R. Folk. 1985. Differential activation of RNA polymerase III-transcribed genes by the polyomavirus enhancer and the adenovirus E1A gene products. **Nucleic Acids Res. 13:** 1413-1428.

20) Berk, A.J. and P.A. Sharp. 1978. Structure of the adenovirus 2 early mRNAs. **Cell 14:** 695-711.

21) Berk, A.J., F. Lee, T. Harrison, J. Williams and P.A. Sharp. 1979. Pre-early Ad5 gene product regulates synthesis of early viral mRNAs. **Cell 17:** 935-944.

22) Berk, A.J. 1986a. Adenovirus promoters and E1A transactivation. **Ann. Rev. Genet. 20:** 45-79.

23) Berk, A.J. 1986b. Functions of adenovirus E1A. **Cancer Surveys 5:** 367-387.

24) Berkowitz, L., K. Riabowol and M.Z. Gilman. 1989. Multiple sequence elements of a single functional class are required for cyclic AMP responsiveness of the mouse

c-fos promoter. **Mol. Cell. Biol. 9:** 4272-4281.

25) Bernards, R., P.I. Schrier, A. Houweling, J.L. Bos, A.J. van der Eb, M. Zijlstra and C.J.M. Melief. 1983. Tumorigenicity of cells transfomed by adenovirus type 12 by evasion of T-cell immunity. **Nature 305:** 776-779.

26) Berstein S.C., and R.A. Weinberg. 1985. Expression of the metastatic phenotype in cells transfected with human metastatic tumor DNA. **Proc. Natl. Acad. Sci. USA 82:** 1726-1730.

27) Bevilacqua, G., M.E. Sobel, L.A. Liotta and P.S. Steeg. 1989. Association of low nm23 RNA levels in human primary infiltrating ductal breast carcinomas with lymph node involvement and other histopathological indicators of high metastatic potential. **Cancer Res. 49:** 5185-5190.

28) Bischoff, J.R., P.N. Friedman, D.K. Marshak, C. Prives and D. Beach. 1990. Human p53 is phosphorylated by p60-cdc2 and cyclin B-cdc2. **Proc. Natl. Acad. Sci. USA 87:** 4766-4770.

29) Bishop, J.M. 1985. Viral oncogenes. **Cell 42:** 23-38.

30) Bishop, J.M. 1987. The molecular genetics of cancer. **Science 235:** 305-311.

31) Bishop, J.M. 1991. Molecular themes in oncogenesis. **Cell 64:** 235-248.

32) Blake, M.C. and J.C. Azizkhan. 1989. Transcription factor E2F is required for efficient expression of the hamster dihydrofolate reductase gene in vitro and in vivo. **Mol. Cell. Biol. 9:** 4994-5002.

33) Blin, N. and D.W. Stafford. 1976. A general method for isolation of high molecular weight DNA from eukaryotes. **Nucleic Acids Res. 3:** 2303-2308.

34) Blochlinger, K. and H. Diggelman. 1984. Hygromycin B phosphotransferase as a selectable marker for DNA transfer experiments with higher eucaryotic cells. **Mol. Cell. Biol. 4:** 2929-2931.

35) Boeuf, H., P. Jansen-Durr and C. Kedinger. 1990. E1a-mediated transactivation of the adenovirus EIIa early promoter is restricted in undifferentiated F9 cells. **Oncogene 5:** 691-699.

36) Bondesson, M., C. Svensson, S. Linder and G. Akusjarvi.

1992. The carboxy-terminal exon of the adenovirus E1A protein is required for E4F-dependent transcription activation. **EMBO J. 11:** 3347-3354.

37) Bonfil, D.R., R.R. Reddel, H. Ura, R. Reich, .Fridman, C.C. Harris and A.J.P. Klein-Szanto. 1989. Invasive and metastatic potential of a v-Ha-ras-transformed human bronchial epithelial cell line. **J. Natl. Cancer Inst. 81:** 587-594.

38) Borrelli, E., R. Hen and P. Chambon. 1984. Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. **Nature 312:** 608-612.

39) Bos, J.L. 1989. ras oncogenes in human cancer: A review. **Cancer Res. 49:** 4682-4689.

40) Bourne, H.R., D.A. Sanders and F. McCormick. 1987. The GTPase superfamily. I. A conserved switch for diverse cell functions. **Nature 238:** 125-131.

41) Boyd, J.M., T. Subramanian, U. Schaeper, M. La Regina, S. Bayley and G. Chinnadurai. 1993. A region in the C-terminus of adenovirus 2/5 E1A protein is required for association with a cellular phosphoprotein and important for the negative modulation of T24-ras mediated transformation, tumorigenesis and metastasis. **EMBO J. 12:** 469-478.

42) Braithewaite, A.W., B.F. Cheetham, P. Li, C.R. Parish, L.K. Waldron-Stevens and A.J.D. Bellett. 1983. Adenovirus-induced alterations of the cell growth cycle: a requirement for expression of E1A but not E1B. **J. Virol. 45:** 192-199.

43) Braithwaite, A.W., C. Nelson, A. Skulimowski, J. McGovern, D. Pigotti and J. Jenkins. 1990. Transactivation of the p53 oncogene by E1A gene products. **Virol. 177:** 595-605.

44) Branton, P.E., S.T. Bayley and F.L. Graham. 1985. Transformation by human adenoviruses. **Biochem. Biophys. Acta. 780:** 67-94.

45) Bravo, R. 1986. Synthesis of the nuclear protein cyclin (PCNA) and its relationship with DNA replication. **Exp. Cell Res. 163:** 287-293.

46) Brockmann D., B. Tries and H. Esche. 1990. Isolation and characterization of novel adenovirus type 12 E1A mRNAs by cDNA PCR techniques. **Virol. 179:** 585-590.

47) Brown, P.D., A.T. Levy, I. Margulies, L. Liotta and W.G. Stetler-Stevenson. 1990. Independent expression and cellular processing of the 72-kDa type IV collagenase and interstitial collagenase in human tumorigenic cell lines. **Cancer Res. 50:** 6184-6191.

48) Brown, R., C.J. Marshall, S.G. Pennie, and A. Hall. 1984. Mechanism of activation of an N-ras gene in the human fibrosarcoma cell line HT1080. **EMBO J. 3:** 1321-1326.

49) Buchkovich, K., L.A. Duffy and E. Harlow. 1989. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle . **Cell 58:** 1097-1105.

50) Buchkovich, K., N. Dyson, P. Whyte and E. Harlow. 1990. Cellular proteins that are targets for transformation by DNA tumor viruses. **Ciba Found. Symp. 150:** 262-271.

51) Burck, K.B., E.T. Liu and J.W. Larrick. 1988. Oncogenes: An Introduction to the Concept of Cancer Genes. Springer-Verlag, New York. pp. 98-132.

52) Burger, M.M. 1970. Proteolytic enzymes initiating cell division and escape from contact inhibition of growth. **Nature 227:** 170-171.

53) Cao, L., B. Faha, M. Dembski, L.-H. Tsai, E. Harlow and N. Dyson. 1992. Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. **Nature 355:** 176-179.

54) Carlock, L.R. and N.C. Jones. 1981. Transformation-defective mutant of adenovirus type 5 containing a single altered E1A mRNA species. **J. Virol. 40:** 657-664.

55) Carmichael, D.F., A. Sommer, R.C. Thompson, D.C. Anderson, C.G. Smith, H.G. Welgus and G.P. Stricklin. 1986. Primary structure and cDNA cloning of human fibroblast collagenase inhibitor. **Proc. Natl. Acad. Sci. USA 83:** 2407-2411.

56) Casto, B. 1968. **J. Virol. 2:** 368-383.

57) Celis, J.E., R. Bravo, P.M. Larson and S.J. Fey. 1984. Cyclin: a nuclear protein whose level correlates directly with the proliferative state of normal as well as transformed cells. **Leukemia Res. 8:** 143-157.

58) Chambers, A.F., R. Colella, D.T. Denhardt and S.M. Wilson. 1992. Increased expression of cathepsins L and B and decreased activity of their inhibitors in

metastatic, ras-transformed NIH 3T3 cells. **Mol. Carcinogenesis 5:** 238-245.

59) Chammas, R. and R. Brentani. 1991. Integrins and metastasis: an overview. **Tumor Biol. 12:** 309-320.

60) Chang, E.H., M.E. Furth, E.M. Scolnick and D.R. Lowy. 1982. Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. **Nature 297:** 479-483.

61) Chase, C.I. 1967. Elementary Statistical Procedures. McGraw-Hill, Inc. Toronto. pp 64-67.

62) Chatterjee, P.K., M. Bruner, S.J. Flint and M.L. Harter. 1988. DNA-binding properties of an adenovirus 289R E1A protein. **EMBO J. 7:** 835-841.

63) Chellappan, S.P., S. Hiebert, M. Mudryj, J.M. Horowitz and J.R. Nevins. 1991. The E2F transcription factor is a cellular target for the RB protein. **Cell 65:** 1053-1061.

64) Chen, I.S.Y., A.J. Caan, N.P. Shah and R.B. Gaynor. 1985. Functional relationship between the HTLV-IIx and the adenovirus E1A proteins in transcriptonal activation. **Science 230:** 570-573.

65) Chen, M.-J., B. Holskin, J. Strickler, J. Gorniak, M.A. Clark, P.J. Johnson, M. Mitcho and D. Shalloway. 1987. Induction by E1A oncogene expression of cellular susceptibility to lysis by TNF. **Nature 330:** 581-583.

66) Chen, P.L., P. Scully, J.Y. Shew, J.Y. Wang and W.H. Lee. 1989. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. **Cell 58:** 1193-1198.

67) Chen, S.-T., H. Su and J.-K. Yee. 1992. Repression of liver-specific hepatitis B virus enhancer 2 activity by adenovirus E1A proteins. **J. Virol. 66:** 7452-7460.

68) Chin, J.R., G. Murphy and Z. Werb. 1985. Stromelysin, a connective tissue-degrading metalloendopeptidase secreted by stimulated rabbit synovial fibroblasts in parallel with collagenase. Biosynthesis, isolation, characterization and substrates. **J. Biol. Chem. 260:** 12367-12376.

69) Chinnadurai, G. 1983. Adenovirus 2 1p$^+$ locus codes for a 19 kd tumor antigen that plays an essential role in cell transformation. **Cell 33:** 759-766.

70) Chinnadurai, G. 1992. Adenovirus E1A as a tumor-suppressor. **Oncogene 7:** 1251-1255.

71) Chow, L.T., T.R. Broker and J.T. Lewis. 1979. Complex splicing patterns of RNAs from the early regions of adenovirus-2. **J. Mol. Biol. 134:** 265-303.

72) Collier, I.E., S.M. Wilhelm, A.Z. Eisen, B.L. Marmer, G.A. Grant, J.L. Seltzer, A. Kronberger, C.S. He, E.A. Bauer and G.I. Goldberg. 1988. H-ras oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloproteinase capable of degrading basement membrane collagen. **J. Biol. Chem. 263:** 6579-6587.

73) Comings, D.E. 1973. A general theory of carcinogenesis. **Proc. Natl. Acad. Sci. USA 70:** 3324-3328.

74) Cook, J.L. and A.M.J. Lewis. 1979. Host response to adenovirus 2-transformed hamster embryo cells. **Cancer Res. 39:** 1455-1461.

75) Cook, J.L., D.L. May, B.A. Wilson, B. Holskin, M.-J. Chen, D. Shalloway and T.A. Walker. 1989a. Role of tumor necrosis factor-α in E1A oncogene-induced susceptibility of neoplastic cells to lysis by natural killer cells and activated macrophages. **J. Immunol. 142:** 4527-4534.

76) Cook, J.L., D.L. May, B.A. Wilson and T.A. Walker. 1989b. Differential induction of cytolytic susceptibility by E1A, myc, and ras oncogenes in immortalized cells. **J. Virol. 63:** 3408-3415.

77) Corsaro, C.M. and M.L. Pearson. 1981. Enhancing the efficiency of DNA-mediated gene transfer in mammalian cells. **Som. Cell Genet. 7:** 603-616.

78) Cromlish, J.A. and R.G. Roeder. 1989. Human transcription factor IIIC (TFIIIC). Purification, polypeptide structure, and the involvement of thiol groups in specific DNA binding. **J. Biol. Chem. 264:** 18100-18109.

79) D'Errico, A., S. Garbisa, L.A. Liotta, V. Castronovo, W.G. Stetler-Stevenson and W.G. Griogioni. 1991. Augmentation of type IV collagenase, laminin receptor, and Ki67 proliferation antigen associated with human colon, gastric and breast carcinoma progression. **Mod. Pathol. 4:** 239-246.

80) Dano, K., P.A. Andreasen, P.A. Grondahl-Hansen, P.

Kristensen, L.S. Neilsen and L. Skriver. 1985. Plasminogen activators, tissue degradation and cancer. **Adv. Cancer Res. 44:** 139-166.

81) Datta, S., C.-J. Soong, D.M. Wang and M.L. Harter. 1991. A purified adenovirus 289-amino-acid E1A protein activates RNA polymerase III transcription in vitro and alters transcription factor TFIIIC. **J. Virol. 65:** 5297-5304.

82) de Groot, R., N. Foulkes, M. Mulder, W. Kruijer and P. Sassone-Corsi. 1991. Positive regulation of jun/AP-1 by E1A. **Mol. Cell. Biol. 11:** 192-201.

83) De Klein, A., A. Guerts van Kessel, G. Grosveld, C.R. Bartram, A. Hagenmeijer, D. Bootsma, N.K. Spurr, N. Heisterkamp, J. Groffen and J.R. Stephenson. 1982. A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelogenous leukemia. **Nature 300:** 765-767.

84) Dearolf, C.R., E. Hersperger and A. Shearn. 1988a. Developmental consequences of awd^{b3}, a cell-autonomous lethal mutation of Drosophila induced by hybrid dysgenesis. **Dev. Biol. 129:** 159-168.

85) Dearolf, C.R., N. Tripoulas, J. Biggs and A. Shearn. 1988b. Molecular consequences of awd^{b3}, a cell-autonomous lethal mutation of Drosophila induced by hybrid dysgenesis. **Dev. Biol. 129:** 169-178.

86) DeCaprio, J.A., J.W. Ludlow, J. Figge, J.Y. Shew, C.M. Huang, W.H. Lee, E. Marsilio, E. Paucha and D.M. Livingston. 1988. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. **Cell 54:** 275-283.

87) DeCaprio, J.A., J.W. Ludlow, D. Lynch, Y. Furukawa, J. Griffin, H. Piwnica-Worms, C.M. Huang and D.M. Livingston. 1989. The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. **Cell 58:** 1085-1095.

88) Der, C.J., T.G. Krontiris, and G. Cooper. 1982. Transforming genes of human bladder and lung carcinoma cell lines are homologous to the ras genes of Harvey and Kirsten sarcoma viruses. **Proc. Natl. Acad. Sci. USA 79:** 3637-3640.

89) Dernhardt, D.T., A.H. Greenberg, S.E. Egan, R.T. Hamilton and J.A. Wright. 1987. Cysteine proteinase cathepsin L expression correlates closely with the metastatic

potential of H-ras transformed murine fibroblasts. **Oncogene 2:** 55-59.

90) Deutsch, P.J., J.L. Hoeffler, J. Jameson, J.C. Lin and J.F. Habener. 1988. Structural determinants for trancriptional activation by cAMP-responsive DNA elements. **J. Biol. Chem. 263:** 18466-18472.

91) Dhar, R., R,W. Ellis, T.Y. Shih, S. Oroszlan, B. Shapiro, J. Maizel, D. Lowy, and E. Scolnick. 1982. Nucleotide sequence of the p21 transforming protein of Harvey murine sarcoma virus. **Science 217:** 934-936.

92) Diaz-Meco, M.T., I. Dominguez, L. Sanz, M.M. Municio, E. Berra, M.E. Cornet, A.G. de Herreros, T. Johansen and J. Moscat. 1992. Phospholipase C-mediated hydrolysis of phosphatidylcholine is a target of transforming growth factor ß1 inhibitory signals. **Mol. Cell. Biol. 12:** 302-308.

93) Diaz-Meco, M.T., S. Quinones, M.M. Municio, L. Sanz, D. Bernal, E. Cabrero, J. Saus and J. Moscat. 1991. Protein kinase C-independent expression of stromelysin by platelet-derived growth factor, ras oncogene, and phosphatidylcholine-hydrolizing phospholipase C. **J. Biol. Chem. 266:** 22597-22602.

94) Dingwall, C., S.V. Sharnick and R.A. Laskey. 1982. A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. **Cell 30:** 449-458.

95) Douglas, J.L., S. Gopalakrishnan and M.P. Quinlan. 1991. Modulation of transformation of primary epithelial cells by the second exon of the Ad5 E1A12S gene. **Oncogene 6:** 2093-2103.

96) Draetta, G., D. Beach and E. Moran. 1988. Synthesis of p34, the mammalian homolog of the yeast $cdc2^{+}$/CDC28 protein kinase, is stimulated during adenovirus-induced proliferation of primary baby rat kidney cells. **Oncogene 2:** 553-557.

97) Drobetsky, E. and M. Meuth. 1983. Increased mutational rates in chinese hamster ovary cells serially selected for drug resistance. **Mol. Cell. Biol. 3:** 1882-1885.

98) Duerksen-Hughes, P.J., W.S.M. Wold and L.R. Gooding. 1989. Adenovirus E1A renders infected cells sensitive to cytolysis by tumor necrosis factor. **J. Immunol. 143:** 4193-4200.

99) Duerksen-Hughes, P.J., T.W. Hermiston, W.S.M. Wold and

L.R. Gooding. 1991. The amino-terminal portion of CD1 of the adenovirus E1A proteins is required to induce susceptibility to tumor necrosis factor cytolysis in adenovirus-infected mouse cells. **J. Virol. 65:** 1236-1244.

100) Dumont, D.J. and P.E. Branton. 1992. Phosphorylation of adenovirus E1A proteins by the $p34^{cdc2}$ protein kinase. **Virology 189:** 111-120.

101) Dyson, N., R. Bernards, S.H. Friend, L.R. Gooding, J.A. Hassell, E.O. Major, J.M. Pipas, T. Vandyke and E. Harlow. 1990. Large T antigens of polyomaviruses are able to form complexes with the retinoblastoma protein. **J. Virol. 64:** 1353-1356.

102) Dyson, N., K. Buchkovich, P. Whyte and E. Harlow. 1989a. The cellular 107K protein that binds to adenovirus E1A also associates with large T antigens of SV40 and JC virus. **Cell 58:** 249-255.

103) Dyson, N., P. Guida, K. Munger and E. Harlow. 1992. Homologous sequences in adenovirus E1A and human papillomavirus E7 proteins mediate interaction with the same set of cellular proteins. **J. Virol. 66:** 6893-6902.

104) Dyson, N., P.M. Howley, K. Munger and E. Harlow. 1989b. The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. **Science 242:** 934-937.

105) Edwards, D.R., G. Murphy, J.R. Reynolds, S.E. Whitman, A.J.P. Docherty, P. Angel and J.K. Heath. 1987. Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibition. **EMBO J. 6:** 1899-1904.

106) Egan, C., S.T. Bayley and P.E. Branton. 1989a. Binding of the RB protein to E1a products is required for adenovirus transformation. **Oncogene 4:** 383-388.

107) Egan, S.E., J.J. Broere, L. Jarolim, J.A. Wright and A.H. Greenberg. 1989b. Co-regulation of metastatic and transforming activity of normal and mutant ras genes. **Int. J. Cancer 43:** 443-448.

108) Egan, S.E., G.A. McClarty, L. Jarolim, J.A. Wright, I. Spiro, G. Hager and A.H. Greenberg. 1987a. Expression of H-ras correlates with metastatic potential: evidence for direct regulation of the metastatic phenotype in $10T\frac{1}{2}$ and NIH 3T3 cells. **Mol. Cell. Biol. 7:** 830-837.

109) Egan, S.E., J.A. Wright, L. Jarolim, K. Yanagihara, R.H. Bassim and A.H. Greenberg. 1987b. Transformation by oncogenes encoding protein kinases induces the metastatic phenotype. **Science 238:** 202-205.

110) Eliyahu, D., A. Raz, P. Gruss, D. Givol and M. Oren. 1984. Participation of p53 cellular tumor antigen in transformation of normal embryonic cells. **Nature 312:** 646-649.

111) Engel, G., P. Popowicz, H. Marshall, G. Norling, C. Svensson, G. Auer, G. Akusjarvi and S. Linder. 1992. Elevated stromelysin-1 and reduced collagenase-IV expression in invasive rat embryo fibroblasts expressing E1A deletion mutants + T24-H-ras. **Int. J. Cancer 51:** 761-766.

112) Enkemann, S.A., S.F. Konieczny and E.J. Taparowsky. 1990. Adenovirus 5 E1A represses muscle-specific enhancers and inhibits expression of the myogenic regulatory factor genes, MyoD1 and Myogenin. **Cell Growth and Differentiation 1:** 375-382.

113) Erickson, J., A. Ar-rushdi, H.L. Drwinga, P.C. Nowell and C.M. Croce. 1983. Transcriptional activation of the translocated c-myc oncogene in Burkitt lymphoma. **Proc. Natl. Acad. Sci. USA 80:** 820-824.

114) Esche, H., M.B. Mathews and J.B. Lewis. 1980. Proteins and messenger RNAs of the transforming region of wild-type and mutant adenoviruses. **J. Mol. Biol. 142:** 399-417.

115) Estreicher, A., A. Wohlwend, D. Belin, W.-D. Schleuning and J.D. Vassalli. 1989. Characterization of the cellular binding site for the urokinse-type plasminogen activator. **J. Biol. Chem. 264:** 1180-1189.

116) Ewen, M.E., B. Faha, E. Harlow and D.M. Livingston. 1992. Interaction of p107 with cyclin A independent of complex formation with viral oncoproteins. **Science 255:** 85-87.

117) Ewen, M.E., J.W. Ludlow, E. Marsilio, J.A. DeCaprio, R.C. Millikan, S.H. Cheng, E. Paucha and D.M. Livingston. 1989. An N-terminal transformation-governing sequence of SV40 large T antigen contributes to the binding of both $p110^{Rb}$ and a second cellular protein, p120. **Cell 58:** 257-267.

118) Ewen, M.E., Y. Xing, J.B. Lawrence and D.M. Livingston. 1991. Molecular cloning, chromosomal mapping, and

expression of the cDNA for p107, a retinoblastoma gene product-related protein. **Cell 66:** 1155-1164.

119) Faha, B., M.E. Ewen, L.-H. Tsai, D.M. Livingston and E. Harlow. 1992. Interaction between human cyclin A and adenovirus E1A-associated p107 protein. **Science 255:** 87-90.

120) Fahnestock, M.L. and J.B. Lewis. 1989. Genetic dissection of the transactivating domain of the E1A 289R protein of adenovirus type 2. **J. Virol. 63:** 1495-1504.

121) Fasano, O., T. Aldrich, F. Tamanoi, E. Taparowsky, M. Furth and M. Wigler. 1984. Analysis of the transforming potential of the human H-ras gene by random mutagenesis. **Proc. Natl. Acad. Sci. USA 81:** 4008-4012.

122) Ferguson, B., B. Krippl, O. Andrisani, N. Jones, H. Westphal and M. Rosenberg. 1985. E1A 13S and 12S mRNA products made in Escherichia coli both function as nucleus-localized transcription activators but do not directly bind DNA. **Mol. Cell. Biol. 5:** 2653-2661.

123) Fidler, I.J. and I.R. Hart. 1982. Biologic diversity in in metastatic neoplasms-origins and implications. **Science 217:** 998-1001.

124) Fidler, I.J. 1984. The evolution of biological heterogeneity in metastatic neoplasms. In, Nicolson, G.L. and L. Milas, eds. Cancer invasion and metastasis: Biological and therapeutic aspects. Raven Press, New York, pp. 5-27.

125) Fidler, I.J. 1986. Rationale and methods for the use of nude mice to stduy the biology and therapy of human cancer metastasis. **Cancer and Metastasis Rev. 5:** 29-49.

126) Figge, J., T. Webster, T.F. Smith and E. Paucha. 1988. Prediction of similar transforming regions in simian virus 40 large T, adenovirus E1A and myc oncoproteins. **J. Virol. 62:** 1814-1818.

127) Filmus, J.E. and R.N. Buick. 1985. Stability of c-K-ras amplification during progression in a patient with adenocarcinoma of the ovary. **Cancer Res. 45:** 4468-4472.

128) Fink, F.S., M. Verhave, S. Kasper, T. Tsukada, G. Mandel and R.G. Goodman. 1988. The CGTCA sequence motif is essential for biological activity of the vasoactive intestinal peptide gene cAMP-regulated enhancer. **Proc. Natl. Acad. Sci. USA 85:** 6662-6666.

129) Fisch, T., M.R. Prywes and R. Roeder. 1989. Multiple sequence elements in the c-fos promoter mediate induction by cAMP. **Genes Dev. 3:** 198-211.

130) Fisher and Yates. 1974.

131) Flint, S.J., J. Sambrook, J. Williams, and P.A. Sharp. 1976. Viral nucleic acid sequences in transformed cells IV. A study of the sequences of adenovirus 5 DNA and RNA in four lines of adenovirus 5-transformed rodent cells using specific fragments of the viral genome. **Virol. 72:** 456-470.

132) Flint, J. and T. Shenk. 1989. Adenovirus E1A protein paradigm viral transactivator. **Annu. Rev. Genet. 23:** 141-161.

133) Folkman, J. 1971. Tumor angiogenesis: therapeutic implications. **New Engl. J. Med. 285:** 1182-1186.

134) Folkman, J., K. Watson, D. Ingber and D. Hanahan. 1989. Induction of angiogenesis during the transition from hyperplasia to neoplasia. **Nature 339:** 58-61.

135) Franza, B.R., K. Maruyama, J.I. Garrels and H.E. Ruley. 1986. In vitro establishment is not a sufficient prerequisite for transformation by activated ras oncogene. **Cell 44:** 409-418.

136) Freeman, A.E., P.H. Black, E.A. Vanderpool, P.H. Henry, J.B. Austin and R.J. Huebner. 1967. Transformation of primary rat emryo fibroblast cells by adenovirus type 2. **Proc. Natl. Acad. Sci. USA 58:** 1205-1212.

137) Friend, S.H., R. Bernards, S. Rogelj, R.A. Weinberg, J.M. Rapaport, D.M. Albert and T.P. Dryja. 1986. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. **Nature 323:** 743-646.

138) Frisch, S.M. 1991. Antioncogenic effect of adenovirus E1A in human tumor cells. **Proc. Natl. Acad. Sci. USA 88:** 9077-9081.

139) Frisch, S.M., R. Reich, I.E. Collier, L.T. Genrich, G. Martin and G.I. Goldberg. 1990. Adenovirus E1A represses protease gene expression and inhibits metastasis of human tumor cells. **Oncogene 5:** 75-83.

140) Fujita, J., S.K. Srivastava, M.H. Kraus, J.S. Rhim, S.R. Tronick and S.A. Aaronson. 1985. Frequency of molecular alterations affecting ras proto-oncogene in human urinary tract tumors. **Proc. Natl. Acad. Sci. USA 82:** 3849-3853.

141) Gallimore, P.H. 1972. Tumor production in immunosuppressed rats with cells transformed in vitro by adenovirus type 2. **J. Gen. Virol. 16:** 99-102.

142) Gallimore, P.H. 1974. Viral DNA in transformed cells II. A study of the sequences of adenovirus 2 DNA in nine lines of transformed rat cell using specific fragments of the viral genome. **J. Mol. Biol. 89:** 49-72.

143) Garbisa, S., R. Pozzatti, R.J. Muschel, U. Saffiotti, M. Ballin, R.H. Goldfarb, G. Khoury and L.A. Liotta. 1987. Secretion of type IV collagenolytic protease and metastatic phenotype: Induction by transfection with c-Ha-ras but not c-Ha-ras plus Ad2-E1A. **Cancer Res. 47:** 1523-1528.

144) Gaynor, R.B., D. Hillman and A.J. Berk. 1984. Adenovirus early region 1A activates transcription of a non-viral gene introduced into mammalian cells by infection or transfection. **Proc. Natl. Acad. Sci. USA 81:** 1193-1197.

145) Gaynor, R.B. and A.J. Berk. 1985. Cis-acting induction of adenovirus transcription. **Cell 33:** 683-693.

146) Gherardi, E., J. Gray, M. Stroker, M. Perryman and R. Furlong. 1989. Purification of scatter factor , a fibroblast-derived basic protein that modulates epithelial interactions and movement. **Proc. Natl. Acad. Sci. USA 86:** 5844-5848.

147) Gilardi, P. and M. Perricaudet. 1984. The E4 transcriptional unit of Ad2: far upstream sequences are required for its transactivation by E1A. **Nucleic Acids Res. 12:** 7877-7888.

148) Gilman, A.G. 1984. G proteins and the dual control of adenylate cyclase. **Cell 36:** 577-579.

149) Gingras, M.C., L. Jarolim, J. Finch, G.T. Bowden, J.A. Wright and A.H. Greenberg. 1990. Transient alterations in the expression of proteases and extracellular matrix genes during metastatic lung colonization by H-ras-transformed 10T½ fibroblasts. **Cancer Res. 50:** 4061-4066.

150) Giordano, A., P. Whyte, E. Harlow, B.R. Franza, Jr., D. Beach and G. Draetta. 1989. A 60K cdc2-associated polypeptide complexes with the E1A proteins in adenovirus-infected cells. **Cell 58:** 981-990.

151) Giordano, A., J.H. Lee, J.A. Scheppler, C. Herrmann, E. Harlow, U. Deuschle, D. Beach and B.R. Franza, Jr.

1991a. Cell cycle regulation of histone H1 kinase activity associated with the adenoviral protein E1A. **Science 253:** 1271-1275.

152) Giordiano, A., C. McCall, P. Whyte and B.R. Franza. Jr. 1991b. Human cyclin A and the retinoblastoma protein interact with similar but distinguishable sequences in the adenovirus E1A gene products. **Oncogene 6:** 481-485.

153) Giulotto, E., C. Knight and G.R. Stark. 1987. Hamster cells with increased rate of DNA amplification is a new phenotype. **Cell 48:** 837-845.

154) Glenn, G.M. and R.P. Ricciardi. 1985. Adenovirus 5 early region 1A host range mutants hr3, hr4, and hr5 contain point mutations which generate single amino acid substitutions. **J. Virol. 56:** 66-74.

155) Goldberg, G.I., S.M. Wilhelm, A. Kronberger, E.A. Bauer, G.A. Grant and A.Z. Eisen. 1986. Human fibroblast collagenase. Complete primary structure and homology to an oncogene transformation-induced rat protein. **J. Biol. Chem. 261:** 6600-6605.

156) Goldfarb, D.S., J. Gariepy, G. Schoolnik and R.D. Kornberg. 1986. Synthetic peptides as nuclear localization signals. **Nature 322:** 641-644.

157) Goldfarb, M., K. Shimizu, M. Perucho and M. Wigler. 1982. Isolation and preliminary characterization of a human transforming oncogene from T24 bladder carcinoma cells. **Nature 296:** 404-409.

158) Goldfarb, R.H. and L.A. Liotta. 1986. Proteolytic enzymes in cancer invasion. **Sem. Thromb. Hemostasis 12:** 294-307.

159) Gonzalez, A., A. Jimenez, D. Valquez and J.E. Davies. 1978. Studies on the mode of action of hygromycin B, an inhibitor of translocation in eukaryotes. **Biochim. Biophys. Acta 521:** 459-469.

160) Gottesman, M. 1990. The role of proteases in cancer. **Sem. Cancer Biol. 1:** 97-160.

161) Graham, F.L. 1984. Transformation by and oncogenicity of human adenoviruses. In, H.S. Ginsberg, ed; The Adenoviruses, pp. 339-398. Plenum Press, New York.

162) Graham, F.L., P.S. Abrahams, C. Mulder, H.J. Heijneker, S.O. Warnaar, F.A.J. deVries, W. Fiers and A.J. van der Eb. 1975. Studies on in vitro transformation by DNA and

DNA fragments of human adenoviruses and simian virus 40. **Cold Spring Harbor Symp. Quant. Biol. 39:** 637-650.

163) Graham, F.L., J. Smiley, W.C. Russell and R. Nairn. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. **J. Gen. Virol. 36:** 59-72.

164) Graham, F.L. and A.J. van der Eb. 1973. Transformation of rat cells by DNA of human adenovirus 5. **Virol. 52:** 456-467.

165) Graham, F.L., A.J. van der Eb and H.L. Heijneker. 1974. Size and location of the transforming region of human adenovirus type 5 DNA. **Nature 251:** 687-691.

166) Green, M.R., R. Treisman and T. Maniatis. 1983. Transcriptional activation of cloned human ß-globin genes by viral immediate-early gene products. **Cell 35:** 137-148.

167) Greenberg, A.H., S.E. Egan and J.A. Wright. 1989. Oncogenes and metastatic progression. **Invasion Metastasis 9:** 360-378.

168) Greig, R.G., T.P. Koestler, D.C. Trainer, S.P. Corwin, L. Miles, T. Klein, R. Sweet, S. Yokoyama and G. Poste. 1985. Tumorigenic and metastatic properties of "normal" and ras-transfected NIH/3T3 cells. **Proc. Natl. Acad. Sci. USA 82:** 3698-3710.

169) Guan, J.L. and D. Shalloway. 1992. Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. **Nature 358:** 690-692.

170) Guilfoyle, R.A., W.P. Osheroff and M. Rossini. 1985. Two functions encoded by adenovirus early region 1A are responsible for the activation and repression of the DNA-binding protein gene. **EMBO J. 4:** 707-713.

171) Guirguis, R., I. Margulies, G. Taraboletti, E. Schiffman and L.A. Liotta. 1987. Cytokine-induced pseudopodial protrusion is coupled to tumour cell migration. **Nature 329:** 261-263.

172) Gunthert, U., M. Hoffman, W. Rudy, S. Reber, M. Zoller, I. Hausmann, S. Matzku, A. Wenzel, H. Ponta and P. Herrich. 1991. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. **Cell 65:** 13-24.

173) Gutch, M.J. and N.C. Reich. 1991. Repression of the interferon signal transduction pathway by the adenovirus E1A oncogene. **Proc. Natl. Acad. Sci. USA 88:** 7913-7917.

174) Halbert, D.N., D.J. Spector and H.J. Raskas. 1979. In vitro translation products specified by the transforming region of adenovirus type 2. **J. Virol 31:** 621-629.

175) Haley, K.P., G. Overhauser, L.E. Babiss, H.S. Ginsberg and N.C. Jones. 1984. Transformation properties of type 5 adenovirus mutants that differentially express the E1A gene products. **Proc. Natl. Acad. Sci. USA 81:** 5734-5738.

176) Hall, A., C.J. Marshall, N. Spurr and R.A. Weiss. 1983. Identification of transforming gene in two human sarcoma cell lines is a new member of the ras gene family located on chromosome one. **Nature 304:** 396-400.

177) Hall, M.N., L. Hereford and I. Herskowitz. 1984. Targetting of E. coli ß-galactosidase to the nucleus in yeast. **Cell 36:** 1057-1065.

178) Haluska, F.G., Y. Tsuyimoto and C.M. Croce. 1987. Oncogene activation by chromosome translocation in human malignancies. **Annu. Rev. Genet. 21:** 321-347.

179) Hara, E., S. Nakada, K. Takehana, T. Nakijima, T. Lino and K. Oda. 1988. Molecular cloning and characterization of cellular genes whose expression is repressed by the adenovirus E1A gene products and growth factors in quiescent rat cells. **Gene 70:** 97-106.

180) Hardy, S. and T. Shenk. 1988. Adenoviral control regions activated by E1A and the cAMP response element bind to the same factor. **Proc. Natl. Acad. Sci. USA 85:** 4171-4175.

181) Hardy, S., D.A. Engel and T. Shenk. 1989. An adenovirus early region 4 gene product is required for induction of the infection-specific form of cellular E2F activity. **Genes Dev. 3:** 1062-1074.

182) Harlow, E., B. Franza and C. Schley. 1985. Monoclonal antibodies specific for adenovirus early region 1A proteins: extensive heterogeneity in early region 1A products. **J. Virol. 55:** 533-546.

183) Harlow, E. and D. Lane. 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

184) Harlow, E., P. Whyte, B.R. Franza, Jr. and C. Schley.

1986. Association of adenovirus early-region 1A proteins with cellular polypeptides. **Mol. Cell. Biol. 6:** 1579-1589.

185) Harter, M.L. and J.B. Lewis. 1978. Adenovirus type 2 early proteins synthesized in vitro and in vivo: Identification in infected cells of the 38,000 to 50,000 molecular weight protein encoded by the left end of the adenovirus type 2 genome. **J. Virol. 26:** 736-749.

186) Hen, R., E. Borrelli and P. Chambon. 1985. Repression of the immunoglobulin heavy chain enhancer by the adenovirus-2 E1A products. **Science 230:** 1391-1394.

187) Herrmann, C.H., L.-K. Su and E. Harlow. 1991. Adenovirus E1A is associated with a serine/threonine protein kinase. **J. Virol. 65:** 5848-5859.

188) Hicks, G.G., S.E. Egan, A.H. Greenberg and M. Mowat. 1991. Mutant p53 tumor suppressor alleles release ras-induced cell cycle growth arrest. **Mol. Cell. Biol. 11:** 1344-1352.

189) Hiebert, S.W., M. Blake, J. Azizkhan and J.R. Nevins. 1991. Role of E2F transcription factor in E1A-mediated transactivation of cellular genes. **J. Virol. 65:** 3547-3552.

190) Hill, S.A., A. Wilson and A.F. Chambers. 1988. Clonal heterogeneity, experimental metastatic ability, and p21 expression in H-ras-transformed NIH 3T3 cells. **J. Natl. Cancer Inst. 80:** 484-490.

191) Hirakawa, T. and H.E. Ruley. 1988. Rescue of cells from ras oncogene-induced growth arrest by a second complementing oncogene. **Proc. Natl. Acad. Sci. USA 85:** 1519-1523.

192) Hoeffler, W.K. and R.G. Roeder. 1985. Enhancement of RNA polymerase III tanscription by the E1A gene product of adenovirus. **Cell 41:** 955-963.

193) Hoeffler, W.K., R. Kovelman and R.G. Roeder. 1988. Activation of transcription factor IIIC by the adenovirus E1A protein. **Cell 53:** 907-920.

194) Horst, E., C.J.L.M. Meijer, T. Radaszkiewicz, G.J. Ossekoppele, J.H.J.M. Van Krieken and S.T. Pals. 1990. Adhesion molecules in the prognosis of diffuse large-cell lymphoma: Expression of a lymphocyte homing receptor (CD44), LFA-1 (CD11a/18), and ICAM-1 (CD54). **Leukemia 4:** 595-599.

195) Houweling, A., P.J. van den Elsen and A. J. van der Eb. 1980. Partial transformation of primary rat cells by the leftmost 4.5% fragment of adenovirus 5 DNA. **Virol. 105:** 537-550.

196) Howe, J.A. and S.T. Bayley. 1992. Effects of Ad5 E1A mutant viruses on the cell cycle in relation to the binding of cellular proteins including the retinoblastoma protein and cyclin A. **Virology 186:** 15-24.

197) Howe, J.A., J.S. Mymryk, C. Egan, P.E. Branton and S.T. Bayley. 1990. Retinoblastoma growth suppressor and a 300-kDa protein appear to regulate cellular DNA synthesis. **Proc. Natl. Acad. Sci. USA 87:** 5883-5887.

198) Hu, Q., J.A. Lees, K.J. Buchkovich and E. Harlow. The retinoblastoma protein physically associates with the human cdc2 kinase. **Mol. Cell. Biol. 12:** 971-980.

199) Huang, M.M. and P.J. Hearing. 1989. Adenovirus early region 4 encodes two gene products with redundant effects in lytic infection. **J. Virol. 62:** 2605-2615.

200) Huebner, R.J., W.P. Rowe and W.T. Lane. 1962. Oncogenic effects in hamsters of human adenovirus types 12 and 18. **Proc. Natl. Acad. Sci. USA 48:** 2051-2058.

201) Huebner R.J., M.J. Casey, R.M. Chanock and K. Schell. 1965. Tumors induced in hamsters by a strain of adenovirus type 3: Sharing of tumor antigens and "neoantigens" with those produced by adenovirus type 7 tumors. **Proc. Natl. Acad. Sci. USA 54:** 381-388.

202) Huebner, R.J. 1967. Adenovirus-directed tumor and T antigens. **Perspect. Virol. 5:** 147.

203) Humphries, M.J., A.P. Mould and D.S. Tuckwell. 1993. Dynamic aspects of adhesion receptor function - integrins both twist and shout. **Bioessays 15:** 391-397.

204) Humphries, M.J., K. Olden and K.M. Yamada. 1986. A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. **Science 233:** 467-470.

205) Hunter, T. 1991. Cooperation between oncogenes. **Cell 64:** 249-270.

206) Hurley, J.B., M.I. Simon, D.B. Teplow, J.D. Robishaw and A.G. Gilman. 1984. Homologies between signal transducing G proteins and ras gene products. **Science 226:** 860-862.

207) Hurst, H.C. and N.C. Jones. 1987. Identification of factors that interact with the E1A-inducible adenovirus E3 promoter. **Genes Dev. 1:** 1131-1146.

208) Hynes, R.O. 1987. Integrins: a family of cell surface receptors. **Cell 48:** 549-554.

209) Imperiale, M.J., H.-T. Kao, L.T. Feldman, J.R. Nevins and S. Strickland. 1984. Common control of the heat shock gene and early adenovirus genes: evidence for a cellular E1A-like activity. **Mol. Cell. Biol. 4:** 867-874.

210) Janaswami, P.M., D.V.R. Kalvakolanu, Y. Zhang and G.C. Sen. 1992. Transcriptional repression of interleukin-6 gene by adenoviral E1A proteins. **J. Biol. Chem. 267:** 24886-24891.

211) Jelsma, T.N., J.A. Howe, J.S. Mymryck, C.M. Evelegh, N.F.A. Cunniff and S.T. Bayley. 1989. Sequences in E1A proteins of human adenovirus 5 required for cell transformation, repression of a transcriptional enhancer, and induction of proliferating cell nuclear antigen. **Virol 171:** 120-130.

212) Jones N. and T. Shenk. 1979. An adenovirus type 5 early region function regulates expression of other early viral genes. **Proc. Natl. Acad. Sci USA 76:** 3665-3669.

213) Juliano, R.L. 1987. Membrane receptors for extracellular matrix molecules: relationship to cell adhesion and tumor metastasis. **Biochem. Biophys. Acta. 907:** 261-278.

214) Kaczmarek, L., B. Ferguson, M. Rosenberg and R. Baserga. 1986. Induction of cellular DNA synthesis by purified adenovirus E1A proteins. **Virol. 152:** 1-10.

215) Kaddurah-Daouk, R., L.W. Lillie, G.H. Daouk, M.R. Green, R. Kingston and P. Schimmel. 1990. Induction of a cellular enzyme for energy metabolism by transforming domains of adenovirus E1A. **Mol. Cell. Biol. 10:** 1476-1483.

216) Kaelin, W.G., Jr., D.C. Pallas, J.A. DeCaprio, F.J. Kaye and D.M. Livingston. 1991. Identification of cellular proteins that can interact specifically with the T/E1A-binding region of the retinoblastoma gene product. **Cell 64:** 521-532.

217) Kalderon, D., B.A. Oostra, B.A. Ely and A.E. Smith. 1984. Sequence requirements for nuclear localization of SV40 large-T antigen. **Nature 311:** 33-38.

218) Kalvakolanu, D.V.R., S.K. Bandyopadhyay, M.L. Harter and G.C. Sen. 1991. Inhibition of interferon-inducible gene expression by adenovirus E1A proteins: block in transcriptional complex formation. **Proc. Natl. Acad. Sci. USA 88:** 7459-7463.

219) Kao, H.T. and J.R. Nevins. 1983. Transcriptional activation and subsequent control of the human heat shock gene during adenovirus infection. **Mol. Cell. Biol. 3:** 2058-2065.

220) Kast, W.M., R. Offringa, P.J. Peters, A.C. Voordouw, R.H. Meleon, A.J. van der Eb and C.J.M. Melief. 1989. Eradication of adenovirus E1-induced tumors by E1A-specific cytotoxic T lymphocytes. **Cell 59:** 603-614.

221) Katze, M.G., H. Persson and L. Philipson. 1981. **Mol. Cell. Biol. 1:** 807-813.

222) Katze, M.G., H. Persson, B.M. Johansson and L. Philipson. 1983. Control of adenovirus gene expression: cellular gene products restrict expression of adenovirus host range mutants in nonpermissive cells. **J. Virol. 46:** 50-59.

223) Kelekar, A. and M.D. Cole. 1987. Immortalization by c-myc, H-ras, and E1A oncogenes induces differential cellular gene expression and growth factor responses. **Mol. Cell. Biol. 7:** 3899-3907.

224) Kerbel, R.S. 1990. Growth dominance of the metastatic cancer cell: cellular and molecular aspects. **Adv. Cancer Res. 55:** 87-131.

225) Kerr, L.D., J.T. Holt and L.M. Matrisian. 1988. Growth factors regulate transin gene expression by c-fos-dependent and c-fos-independent pathways. **Science 242:** 1424-1427.

226) Kimelman, D., J.S. Miller, D. Porter and B.E. Roberts. 1985. E1A regions of the human adenoviruses and of the highly oncogenic simian adenovirus 7 are closely related. **J. Virol. 53:** 399-409.

227) Kinzler, K.W., S.H. Bigner, D.D. Bigner, J.M. Trent, M.L. Law, S.J. O'Brien, A.J. Wong and B. Vogelstein. 1987. Identification of an amplified, highly expressed gene in a human glioma. **Science 236:** 70-73.

228) Kinzler, K.W., J.M. Ruppert, S.H. Bigner and B. Vogelstein. 1988. The gli gene is a member of the Kruppel family of zinc-finger proteins. **Nature 332:** 371-

374.

229) Kitabayashi, I., R. Chiu, G. Gachelin and K. Yokoyama. 1991. E1A dependent up-regulation of c-jun/AP-1 activity. **Nucleic Acids Res. 19:** 649-655.

230) Kitchingman, G.R. and H. Westphal. 1980. The structure of adenovirus 2 early nuclear and cytoplasmic RNAs. **J. Mol. Biol. 137:** 23-48.

231) Kleinberger, T. and T. Shenk. 1991. A protein kinase present in a complex with adenovirus E1A proteins. **Proc. Natl. Acad. Sci. USA 88:** 11143-11147.

232) Koff, W.C. and A.V. Fann. 1986. Human tumor necrosis factor-alpha kills herpesvirus-infected but not normal cells. **lymphokine Res. 5:** 215-221.

233) Kovary, K., M.C.S. Armelin and H.A. Armelin. 1989. Ha-Ras-1 oncogene dosage differentially affects Balb/3T3 cells' growth factor requirement and tumorigenicity. **Oncogene Research 4:** 55-64.

234) Kovesdi, I., R. Reichel and J.R. Nevins. 1986a. Identification of a cellular transcription factor involved in E1A transactivation. **Cell 45:** 219-228.

235) Kovesdi, I., R. Reichel and J.R. Nevins. 1986b. E1A transcription induction: Enhanced binding of a factor to upstream promoter sequences. **Science 231:** 719-722.

236) Kovesdi, I., R. Reichel and J.R. Nevins. 1987. Role of an adenovirus E2 promoter binding factor in E1A-mediated coordinate gene control. **Proc. Natl. Acad. Sci. USA 84:** 2180-2184.

237) Knudson, A.G.J. 1971. Mutation and cancer: statistical study of retinoblastoma. **Proc. Natl. Acad. Sci. USA 68:** 820-823.

238) Kornberg, L.J., H.S. Earp, C.E. Turner, C. Prockop and R.L. Juliano. 1991. Signal transduction by integrins: increased protein tyrosine phosphorylation caused by clustering of ß1 integrins. **Proc. Natl. Acad. Sci. USA 88:** 8392-8396.

239) Kraus, V.B., E. Moran and J.R. Nevins. 1992. Promoter-specific trans-activation by the adenovirus $E1A_{12S}$ product involves separate E1A domains. **Mol. Cell. Biol. 12:** 4391-4399.

240) Krippl, B., B. Ferguson, N. Jones, M. Rosenberg and H.

Westphal. 1985. Mapping of functional domains in adenovirus E1A proteins. **Proc. Natl. Acad. Sci. USA 82:** 7480-7484.

241) Krontiris, T.G. and G.M. Cooper. 1981. Transforming activity of human tumor DNAs. **Proc. Natl. Acad. Sci. USA 78:** 1181-1184.

242) Kuppner, M.C., E.V. Meir, T. Gauthier, M.-F. Hamou and N. De Tribolet. 1992. Differential expression of the CD44 molecule in human brain tumours. **Int. J. Cancer 50:** 572-577.

243) Kuppuswamy, M.N. and G. Chinnadurai. 1987. Relationship between the transforming and transcriptional regulatory functions of adenovirus 2 E1A oncogene. **Virol. 159:** 31-38.

244) Kuppuswamy, M., T. Subramanian and G. Chinnadurai. 1988. Separation of immortalization and T24-ras cooperative functions of adenovirus E1A. **Oncogene 2:** 613-615.

245) La Thangue, N. and P.W.J. Rigby. 1987. An adenovirus E1A-like transcription factor is regulated during the differentiation of murine embryonal carcinoma stem cells. **Cell 49:** 507-513.

246) Land, H., A.. Chen, J.P. Morgenstern, L.F. Parada and R.A. Weinberg. 1986. Behavior of myc and ras oncogenes in transformation of rat embryo fibroblasts. **Mol. Cell. Biol. 6:** 1917-1925.

247) Land, H., L.F. Parada and R.A. Weinberg. 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. **Nature 304:** 596-602.

248) Laspia, M.F., A.P. Rice and M.B. Mathews. 1990. Synergy between HIV-1 Tat and adenovirus E1A is principally due to stabilization of transcriptional elongation. **Genes Dev. 4:** 2397-2408.

249) Lee, W., P. Mitchell and R. Tjian. 1987. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. **Cell 49:** 741-752.

250) Lee, M.G. and P. Nurse. 1987. Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2. **Nature 327:** 31-35.

251) Lee, W.E., J.Y. Shew, F.D. Hong, T.W. Sery, L.A. Donoso, L.J. Young, R. Bookstein and E.Y. Lee. 1987. The

retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity. **Nature 329:** 642-645.

252) Leff, T., R. Elkaim, C.R. Goding, P. Jalinot, P. Sassone-Corsi, M. Perricaudet, C. Kedinger and P. Chambon. 1984. Individual products of the adenovirus 12S and 13S mRNAs stimulate viral E1A and E3 expression at the transcriptional level. **Proc. Natl. Acad. Sci. USA 81:** 4381-4385.

253) Levin, E.G. and L. Santell. 1987. Association of a plasminogen activator inhibitor (PA-I) with the growth substratum and membrane of human endothelial cells. **J. Cell. Biol. 105:** 2543-2549.

254) Levy, A.T., V. Cioce, M.E. Sobel, S. Garbisa, W.F. Griogioni, L.A. Liotta and W.G. Stetler-Stevenson. 1991. Increased expression of the M_r 72,000 type IV collagenase in human colonic adenocarcinoma. **Cancer Res. 51:** 439-444.

255) Leza, M.A. and P. Hearing. 1988. Cellular transcription factor binds to adenovirus early region promoters and to a cyclic AMP response element. **J. Virol. 62:** 3003-3013.

256) Lillie, J.W., M. Green and M.R. Green. 1986. An adenovirus E1a protein region required for transformation and transcriptional repression. **Cell 46:** 1043-1051.

257) Lillie, J.W., P.M. Lowenstein, M.R. Green and M. Green. 1987. Functional domains of adenovirus type 5 E1A proteins. **Cell 50:** 1091-1100.

258) Lin, Y. and M.R. Green. 1988. Interaction of a common cellular transcription factor, ATF, with regulatory elements in both E1A- and cyclic AMP-inducible promoters. **Proc. Natl. Acad. Sci. USA 85:** 3396-3400.

259) Lin, B. T.-Y., S. Gruenwald, A.O. Morla, W.-H. Lee and J.Y.J. Wang. 1991. Retinoblastoma cancer suppressor gene product is a substrate of the cell cycle regulator cdc2 kinase. **EMBO J. 10:** 857-864.

260) Linder, S., P. Popowicz, C. Svensson, H. Marshall, M. Bondesson and G. Akusjarvi. 1992. Enhanced invasive properties of rat embryo fibroblasts transformed by adenovirus E1A mutants with deletions in the carboxy-terminal exon. **Oncogene 7:** 439-443.

261) Liotta, L.A. 1992. Cancer cell invasion and metastasis. **Scientific American 266:** 54-63.

262) Liotta, L.A., R. Mandler, G. Murano, D.A. Katz, R.K. Gordon, P.K. Chiang and E. Schiffmann. 1986. Tumor cell autocrine motility factor. **Proc. Natl. Acad. Sci. USA 83:** 3302-3306.

263) Liotta, L.A., C.N. Rao and S.H. Barsky. 1983. Tumor invasion and extracellular matrix. **Lab. Invest. 49:** 636-649.

264) Liotta, L.A., C.N. Rao and U.M. Wewer. 1986. Biochemical interactions of tumor cells with the basement membrane. **Ann. Rev. Biochem. 55:** 1037-1057.

265) Liotta, L. and P. Steeg. 1990. Clues to the funtion of nm23 and Awd proteins in development, signal transduction, and tumor metastasis provided by studies of Dictyostelium discoideum. **J. Natl. Cancer Inst. 82:** 1170-1172.

266) Liotta, L.A., P.S. Steeg and W.G. Stetler-Stevenson. 1991. Cancer metastasis and angiogenesis: An imbalance of positive and negative regulation. **Cell 64:** 327-336.

267) Liotta, L.A. and W. Stetler-Stevenson. 1989. Metalloproteinases and malignant conversion: Does correlation imply causality? **J. Natl. Cancer Inst. 81:** 556-557.

268) Liotta, L.A., K. Tryggvason, S. Garbisa, I. Hart, C.M. Foltz and S. Shafie. 1980. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. **Nature 284:** 67-68.

269) Lowe, S.W. and H.E. Ruley. 1993. Stabilization of the p53 tumor suppressor is induced by adenovirus-5 E1A and accompanies apotosis. **Genes Dev. 7:** 535-545.

270) Ludlow, J.W., J.A. DeCaprio, C.-M. Huang, W.-H. Lee, E. Paucha and D.M. Livingston. 1989. SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. **Cell 56:** 57-65.

271) Ludlow, J.W., J. Shon, J.M. Pipas, D.M. Livingston and J.A. DeCaprio. 1990. The retinoblastoma susceptibility gene product undergoes cell cycle-dependent dephosphorylation and binding to and release from SV40 large T. **Cell 60:** 387-396.

272) Lum, L.S.Y., S. Hsu, M. Vaewhongs and B. Wu. 1992. The hsp70 gene CCAAT-binding factor mediates transcriptional activation by the adenovirus E1A protein. **Mol. Cell.**

Biol. 12: 2599-2605.

273) Luna, E.J., L.J. Wuestehube, H.M. Ingalls and C.P. Chia. 1989. The Dictyostelium discoideum plasma membrane: a model system for the study of actin-membrane interactions. **Adv. Cell. Biol. 3:** 1-33.

274) Lyons, R.H., B.Q. Ferguson and M. Rosenberg. 1987. Pentapeptide nuclear localization signal in adenovirus E1A. **Mol. Cell. Biol. 7:** 2451-2456.

275) Macartney. H.W. and H. Tschesche. 1983. Latent and active human polymorphonuclear leukocyte collagenases. Isolation, purification and characterization. **Eur. J. Biochem. 130:** 71-78.

276) Maekawa, T., S. Matsuda, J. Fujisawa, M. Yoshida and S. Ishii. 1991. Cyclic AMP response element-binding protein, CRE-BP1, mediates the E1A-induced but not the Tax-induced trans-activation. **Oncogene 6:** 627-632.

277) Mak, S., I, Mak, J.R. Smiley and F.L. Graham. 1979. Tumorigenicity and viral gene expression in rat cells transformed by Ad12 virus or by the EcoR1 fragment of Ad 12 DNA. **Virol. 98:** 456-460.

278) Marshall, C.J. 1991a. Tumor suppressor genes. **Cell 64:** 313-326.

279) Marshall, C.J. 1991b. How does $p21^{ras}$ transform cells? **Trends Genet. 7:** 91-95.

280) Marton, M.J., S.B. Baim, D.A. Ornelles and T. Shenk. 1990. The adenovirus E4 17-kilodalton protein complexes with the cellular transcription factor E2F, altering its DNA-binding properties and stimulating E1A-independent accumulation of E2 mRNA. **J. Virol. 64:** 2345-2359.

281) Maruyama, K., S.C. Schiavi, W. Huse, G.L. Johnson and H.E. Ruley. 1987. myc and E1A oncogenes alter the responses of PC12 cells to nerve growth factor and block differentiation. **Oncogene 1:** 361-367.

282) Mason, R.W., S. Gal and M.M. Gottesman. 1987. The identification of the major excreted protein (MEP) from a transformed mouse fibroblast cell line as a catalytically active precursor form of cathepsin L. **Biochem J. 248:** 449-454.

283) Mathews, M.B., R.M. Berstein, B.R. Franza and J.I. Garrels. 1984. Identity of the proliferating cell nuclear antigen and cyclin. **Nature 309:** 374-376.

284) Matrisian, L.M. 1990. Metalloproteinases and their inhibitors in matrix remodelling. **Trends Genet. 6:** 121-125.

285) Matrisian, L.M. and T. Bowden. 1990. Stromelysin/transin and tumor progression. **Sem. Cancer Biol. 1:** 107-115.

286) Matrisian, L.M., G.T. Bowden, P. Krieg, G. Furstenberger, J.P. Briand, P. Leroy and R. Breathnach. 1986. The mRNA coding for the secreted protein transin is expressed more abundantly in malignant than benign tumors. **Proc. Natl. Acad. Sci. USA 83:** 9413-9417.

287) McAllister, R.M. and I. MacPherson. 1968. Transformation of a hamster cell line by adenovirus type 12. **J. Gen. Virol. 2:** 99-106.

288) McBride, W.D. and A. Weiner. 1964. In vitro transformation of hamster kidney cells by human adenovirus type 12. **Proc. Soc. Exp. Biol. Med. 115:** 870-874.

289) McCarthy, J.B., M.L. Basara, S.L. Palm, D.F. Sas and L.T. Furcht. 1985. The role of cell adhesion proteins-laminin and fibronectin-in the movement of malignant and metastatic cancer cells. **Cancer Metas. Rev. 4:** 125-152.

290) McKay, I.A., C.J. Marshall, C. Cales and A. Hall. 1986. Transformation and stimulation of DNA synthesis in NIH-3T3 cells are a titrable function of normal p21N-ras expression. **EMBO J. 5:** 2617-2621.

291) Mellon, P., V. Parker, Y. Gluzman and T. Maniatis. 1981. Identification of DNA sequences required for transcription of the human α_1-globin gene in a new SV40 host-vector system. **Cell 27:** 279-288.

292) Meuth, M. 1990. The structure of mutation in mammalian cells. **Biochem. Biophys. Acta. 1032:** 1-17.

293) Mignatti, P., E. Robbins and D.B. Rifkin. 1986. Tumor invasion through the amniotic membrane: requirement for a proteinase cascade. **Cell 47:** 487-498.

294) Mignatti, P., R. Tsuboi, E. Robbins and D.B. Rifkin. 1989. In vitro angiogenesis on the human amniotic membrane: requirement for basic fibroblast growth factor-induced proteinases. **J. Cell Biol. 108:** 671-682.

295) Mihara, K., X.R. Cao, A. Yen, S. Chandler, B. Driscoll, A.L. Murphee, A.T. Ang and Y.K. Fung. 1989. Cell cycle-

dependent regulation of phosphorylation of the human retinoblastoma gene product. **Science 246:** 1300-1303.

296) Miller, A.D., D.R. Trauber and C. Buttimore. 1986. Factors involved in production of helper virus-free retrovirus vectors. **Som. Cell. Mol. Genet. 12:** 175-183.

297) Monteagudo, C., M. Merino, J. San-Juan, L. Liotta and W. Stetler-Stevenson. 1990. Immunohistologic distribution of type IV collagenase in normal, benign and malignant breast tissue. **Am. J. Pathol. 136:** 585-592.

298) Montell, C., E.F. Fisher, M.H. Caruthers and A.J. Berk. 1982. Resolving the functions of overlapping viral genes by site-specific mutagenesis at a mRNA splice site. **Nature 295:** 380-384.

299) Montell, C., G. Courtois, C.Y. Eng and A.J. Berk. 1984. Complete transformation by adenovirus 2 requires both E1A proteins. **Cell 36:** 951-961.

300) Moran, E. 1988. A region of SV40 large TAg can substitute for a transforming domain of the adenovirus E1A products. **Nature 334:** 168-170.

301) Moran, E. and M.B. Mathews. 1987. Multiple functional domains in the adenovirus E1A gene. **Cell 48:** 177-178.

302) Moran, E., B. Zerler, T.M. Harrison and M.B. Mathews. 1986. Identification of seperate domains in the adenovirus E1A gene for immortalization activity and the activation of virus early genes. **Mol. Cell. Biol. 6:** 3470-3480.

303) Moran, B. and B. Zerler. 1988. Interaction between cell growth-regulating domains in the products of the adenovirus E1A oncogene. **Mol. Cell. Biol. 8:** 1756-1764.

304) Mudryj, M., S.H. Devoto, S.W. Hiebert, T. Hunter, J. Pines and J.R. Nevins. 1991. Cell cycle regulation of the E2F transcription factor involves an interaction with cyclin A. **Cell 65:** 1243-1253.

305) Mudryj, M., S.W. Hiebert and J.R. Nevins. 1990. A role for the adenovirus inducible E2F transcription factor in a proliferation dependent signal pathway. **EMBO J. 9:** 2179-2184.

306) Mulcahy, L.S., M.R. Smith and D.W. Stacey. 1985. Requirements for ras proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. **Nature 313:** 241-243.

307) Muller, U., M.P. Roberts, D.A. Engle, W. Doerfler and T. Shenk. Induction of transcription factor AP-1 by adenovirus E1A protein and cAMP. **Genes Dev. 3:** 1991-2002.

308) Munger, K., B.A. Werness, N. Dyson, W.C. Phelps, E. Harlow and P.M. Howley. 1989. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. **EMBO J. 8:** 4099-4105.

309) Murphee, A.L. and W.F. Benedict. 1984. Retinoblastoma: clues to human oncogenesis. **Science 223:** 1028-1033.

310) Murray, A.W. and M.W. Kirschner. 1989. Dominoes and clocks: the union of two views on the cell cycle. **Science 246:** 614-621.

311) Muschel, R.J., J.E. Williams, D.R. Lowy and L.A. Liotta. 1985. Harvey ras induction of metastatic potential depends on oncogene activation and the type of recipient cell. **Am. J. Pathol. 121:** 1-8.

312) Mymryk, J.S., R.W.H. Lee and S.T. Bayley. Ability of adenovirus 5 E1A proteins to suppress differentiation of BC_3H1 myoblasts correlates with their binding to a 300 kDa cellular protein. **Mol. Biol. Cell 3:** 1107-1115.

313) Nabel, G. and D. Baltimore. 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. **Nature 326:** 711-713.

314) Nabel, G.L., S.A. Rice, D.M. Knipe and D. Baltimore. 1988. Alternative mechanisms for activation of human immunodeficiency virus enhancer in T cells. **Science 239:** 1299-1301.

315) Nakajima, M., T. Irimura and G.L. Nicolson. 1988. Heparanases and tumor metastasis. **J. Cell. Biochem. 36:** 157-167.

316) Nakajima, M., D. Lotan, M.M. Baig, R.M. Carralero, W.R. Wood, M.J.C. Hendrix and R. Lotan. 1989. Inhibition of retinoic acid of type IV collagenolysis and invasion through reconstituted basement membrane by metastatic rat adenocarcinoma cells. **Cancer Res. 49:** 1698-1706.

317) Nakajima, M., K. Morikawa, A. Fabra, C.D. Bucana and I.J. Fidler. 1990. Influence of organ environment on extracellular degradative activity and metastasis of human colon carcinoma cells. **J. Natl. Cancer Inst. 82:** 1890-1898.

318) Nakajima, M., D. Welch, P.N. Belloni and G.L. Nicholson. 1987. Degradation of basement membrane type IV collagen and lung subendothelial matrix by rat mammary adenocarcinoma cell clones of differing metastatic potentials. **Cancer Res. 47:** 4869-4876.

319) Nakajima, T., M. Masuda-Murata, E. Hara and K. Oda. 1987. Induction of cell cycle progression by adenovirus E1A gene 13S- and 12S-mRNA products in qiuescent rat cells. **Mol. Cell. Biol. 7:** 3846-3852.

320) Nakamura, T., T. Nakajima, S. Tsunoda, S. Nakada, K. Oda, H. Tsurui and A. Wada. 1992. Induction of E1A-responsive negative factors for transcription of the fibronectin gene in adenovirus E1-transformed rat cells. **J. Virol. 66:** 6436-6450.

321) Neill, S.D., C. Hemstrom, A. Virtanen and J.R. Nevins. 1990. An adenovirus E4 gene product trans-activates E2 transcription and stimulates binding through a direct association with E2F. **Proc. Natl. Acad. Sci. USA 87:** 2008-2012.

322) Nevins, J.R., H.W. Ginsberg, J.M. Blanchard, M.C. Wilson and J.E. Darnell. 1979. Regulation of the primary expression of the early adenovirus transcription units. **J. Virol. 32:** 727-733.

323) Nevins, J.R. 1981. Mechanism of activation of early viral transcription by the adenovirus early gene product. **Cell 26:** 213-220.

324) Newbold, R.F. and R.W. Overell. 1983. Fibroblast immortality is a prerequisite for transformation by EJ c-Ha-ras oncogene. **Nature 304:** 648-651.

325) Nicolson, J. and J.R. Nevins. 1991. Distinct DNA targets for trans-activation by HTLV-1 tax and adenovirus E1A. **Virology 182:** 156-167.

326) Nicolson, G.L. 1987. Tumor cell instability, diversification and progression to the metastatic phenotype: from oncogene to oncofetal expression. **Cancer Res. 47:** 1473-1478.

327) Nicolson, G.L. 1988. Cancer metastasis: tumor cell and host organ properties important in metastasis to specific secondary sites. **Biochim. Biophys. Acta 948:** 175-224.

328) Nicolson, G.L. 1991. Molecular mechanisms of cancer metastasis: tumor and host properties and the role of oncogenes and suppressor genes. **Curr. Opinion Oncol. 3:**

75-92.

329) Nicolson, R., G. Murphy and R. Breathnach. 1989. Human and rat malignant-tumor-associated mRNAs encode stromelysin-like metalloproteinases. **Biochem. 28:** 5195-5203.

330) Nielsch, U., R. Pine, S.G. Zimmer and L.E. Babiss. 1992. Induced expression of the endogenous beta interferon gene in adenovirus type 5-transformed rat fibroblasts. **J. Virol. 66:** 1884-1890.

331) Nomiyama, H., C. Fromental, J.H. Xiao and P. Chambon. 1987. Cell specific activity of the constituent elements of the simian virus 40 enhancer. **Proc. Natl. Acad. Sci. USA 84:** 7881-7885.

332) Nurse, P. 1990. Universal control mechanism regulating onset of M-phase. **Nature 344:** 503-508.

333) Offringa, R., A.M.M. Smits, A. Houweling, J.L. Bos and A.J. van der Eb. 1988. Similar effects of adenovirus E1A and glucocorticoid hormones on the expression of the metalloprotease stromelysin. **Nucleic Acids Res. 16:** 10973-10984.

334) Offringa, R., S. Gobel, H. van Dam, M. Timmers, A. Smits, R. Zwart, B. Stein, J.L. Bos, A.J. van der Eb and P. Herrlich. 1990. A novel function of the transforming domain of E1A: Repression of AP-1 activity. **Cell 62:** 527-538.

335) Old, L.J. 1985. Tumor necrosis factor (TNF). **Science 230:** 630-632.

336) Ossowski, L. and E. Reich. 1983. Antibodies to plasminogen activator inhibit human tumor metastasis. **Cell 35:** 611-619.

337) Ostrowski, L.E., J. Rinch, P. Kreig and L. Matrisian. 1988. Expression pattern of a gene for a secreted metalloproteinase during the late phases of tumor progression. **Mol. Carcinogenesis 1:** 13-19.

338) Parada, L.F., H. Land, R.A. Weinberg, D. Wolf and V. Rotter. 1984. Cooperation between gene encoding p53 tumor antigen and ras in cellular transformation. **Nature 312:** 646-649.

339) Parada, L.F., C.J. Tabin, C. Shih and R.A. Weinberg. 1982. Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus ras gene. **Nature 297:** 474-478.

340) Partin, A.W., J.T. Isaacs, B. Trieger and D.S. Coffey. 1988. Early cell motility chamges associated with an increase in metastatic ability in rat prostatic cancer cells transfected with the Harvey ras oncogene. **Cancer Res. 48:** 6050-6053.

341) Pearson, B.E., H.P. Nasheuer and T.S.-F. Wang. 1991. Human DNA polymerase α gene: Sequences controlling expression in cycling and serum-stimulated cells. **Mol. Cell. Biol. 11:** 2081-2095.

342) Pepper, M.S., D. Belin, R. Montesano, L. Orci and J.-D. Vassalli. 1990. Transforming growth factor-β_1 modulates basic fibroblast growth factor-induced proteolytic and angiogenic properties of endothelial cells in vitro. **J. Cell. Biol. 111:** 743-755.

343) Pereira, M.S., H.G. Pereira and S.K. Clarke. 1965. **Lancet i:** 21-23.

344) Perricaudet, M., G. Akusjarvi, A. Virtanen and U. Pettersson. 1979. Structure of two spliced mRNAs from the transforming region of the human subgroup C adenoviruses. **Nature 281:** 694-696.

345) Perricaudet, M., J.M. Le Moullec, P. Tiollais and U. Pettersson. 1980. Structure of two adenovirus type 12 transforming polypeptides and their evolutionary implications. **Nature 288:** 174-176.

346) Perucho, M., M. Goldfarb, K. Shimizu, C. Lama, J. Fogh and M. Wigler. 1981. Human tumor-derived cell lines contain common and different transforming genes. **Cell 27:** 467-476.

347) Pettersson, U. and R.J. Roberts. 1986. Adenovirus gene expression and regulation: a historical perspective. In, Botchan, M., T. Grodzicker and P.A. Sharp, eds. Cancer cells/4. DNA Tumor Viruses: Control of gene expression and replication., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

348) Phelps, W.C., S. Bagchi, J.A. Barnes, P. Raychaudhuri, V. Kraus, K. Munger, P.M. Howley and J.R. Nevins. 1991. Analysis of transactivation by human papillomavirus type 16 E7 and adenovirus 12S E1A suggests a common mechanism. **J. Virol. 65:** 6922-6930.

349) Phelps, W.C., C.L. Lee, K. Munger and P.M. Howley. 1988. The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. **Cell 53:** 539-547.

350) Pientenpol, J.A., R.W. Stein, E. Moran, P. Yaciuk, R. Schlegel, R.M. Lyons, M.R. Pittelkow, K. Munger, P.M. Howley and H.L. Moses. 1990. TGF-ß1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. **Cell 61:** 777-785.

351) Pines, J. and T. Hunter. 1990. Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. **Nature 346:** 760-763.

352) Pohl, J., N. Goldfinger, A. Rader-Pohl, V. Rotter and V. Schirrmacher. 1988. p53 increases experimental metastatic capacity of murine carcinoma cells. **Mol. Cell. Biol. 8:** 2078-2081.

353) Poste, G. and I.J. Fidler. 1980. The pathogenesis of cancer metastasis. **Nature 283:** 139-145.

354) Pozzatti, R., R. Muschel, J. Williams, R. Padmanabhan, B. Howard, L.A. Liotta and G. Khoury. 1986. Primary rat embryo cells transformed by one or two oncogenes show different metastatic potentials. **Science 232:** 223-227.

355) Pozzatti, R., M. McCormick, M.A. Thompson, and G. Khoury. 1988. The E1A gene of adenovirus type 2 reduces the metastatic potential of ras-transformed rat embryo cells. **Mol. Cell. Biol. 8:** 2984-2988.

356) Pulciani, S., E. Santos, A.V. Lauver, K.L. Long, K.C. Robbins and M. Barbacid. 1982. Oncogenes in human tumor cell lines: Molecular cloning of a transforming gene from human bladder carcinoma cells. **Proc. Natl. Acad. Sci. USA 79:** 2845-2849.

357) Pulciani, S., E. Santos, L.K. Long, V. Sorrentino and M. Barbacid. 1985. Ras gene amplification and malignant transformation. **Mol. Cell. Biol. 5:** 2836-2841.

358) Quantin, B., G. Murphy and R. Breathnach. 1989. Pump-1 cDNA codes for a protein with characteristics similar to those of classical collagenase family members. **Biochem. 28:** 5327-5333.

359) Quinlan, M.P. 1989. The Ad5 12S growth factor induces F9 cell proliferation and differentiation. **Oncogene 4:** 1051-1055.

360) Quinlan, M.P. and J.L. Douglas. 1992. Immortalization of primary epithelial cells requires first- and second-exon functions of adenovirus type 5 12S. **J. Virol. 66:** 2020-2030.

361) Quinlan, M.P. and T. Grodzicker. 1986. Production of a cell proliferation factor by baby rat kidney cells infected with adenovirus type-5 12S virus. **Cancer Cells 4:** 327-337.

362) Quinlan, M.P. and T. Grodzicker. 1987. Adenovirus E1A 12S protein induces DNA synthesis and proliferation in primary epithelial cells in both the presence and absence of serum. **J. Virol. 61:** 673-682.

363) Quinlan, M.P., N. Sullivan and T. Grodzicker. 1987. Growth factor(s) produced during infection with an adenovirus variant stimulates proliferation of nonestablished epithelial cells. **Proc. Natl. Acad. Sci. USA 84:** 3283-3287.

364) Quinlan, M.P., P. Whyte and T. Grodzicker. 1988. Growth factor induction by the adenovirus type 5 E1A 12S protein is required for immortalization of primary epithelial cells. **Mol. Cell. Biol. 8:** 3191-3203.

365) Quinlan, M.P. 1989. The Ad5 12S growth factor induces F9 cell proliferation and differentiation. **Oncogene 4:** 1051-1055.

366) Rabson A.S., R.L. Kirschstein and P.J. Paul. 1964. **J. Natl. Cancer Inst. 32:** 77-82.

367) Rager-Zisman, B. and B.R. Bloom. 1982. Natural killer cells in resistance to virus-infected cells. **Springer Semin. Immunopathol. 4:** 397-414.

368) Rao, C., V. Castronovo, M.C. Schmitt, U. Wewer, A. Claysmith, L.A. Liotta and M. Sobel. 1989. Evidence for a precursor of the high-affinity metastasis associated murine laminin receptor. **Biochem. 28:** 7476-7486.

369) Raychaudhuri, P., S. Bagchi, S.D. Neill and J.R. Nevins. 1990. Activation of the E2F transcription factor in adenovirus-infected cells involves E1A-dependent stimulation of cooperative binding mediated by an E4 gene product. **J. Virol. 64:** 2702-2710.

370) Raychaudhuri, P., R. Rooney and J.R. Nevins. 1987. Idenification of an E1A-inducible cellular factor that interacts with regulatory sequences within the adenovirus E4 promoter. **EMBO J. 6:** 4073-4081.

371) Recklies, A.D., A.R. Poole and J.S. Mort. 1982. A cysteine proteinase secreted from human breast tumors is immunologically related to cathepsin B. **Biochem. J. 207:** 633 636.

372) Reddy, E., R. Reynolds, E. Santos and M. Barbacid. 1982. A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. **Nature 300:** 149-152.

373) Reich, R., E. Thompson, Y. Iwamoto, G.R. Martin, J.R. Deason, G.C. Fuller and R. Miskin. 1988. Effects of inhibitors of plasminogen activator, serine proteinases, and collagenase IV on the invasion of basement membranes by metastatic cells. **Cancer Res. 48:** 3307-3312.

374) Reichel, R., I. Kovesdi and J.R. Nevins. 1987. Developmental control of a promoter-specific factor that is also regulated by the E1A gene product. **Cell 48:** 501-516.

375) Reichel, R., I. Kovesdi and J.R. Nevins. 1988. Activation of a preexisting cellular factor as a basis for adenovirus E1A-mediated transcription control. **Proc. Natl. Acad. Sci. USA 85:** 387-390.

376) Reichel, R., S.D. Neill, I. Kovesdi, M.C. Simon, P. Raychaudhuri and J.R. Nevins. 1989. The adenovirus E4 gene, in addition to the E1A gene, is important for transactivation of E2 transcription and for E2F activation. **J. Virol. 63:** 3643-3650.

377) Ricciardi, R.P., R.L. Jones, C.L. Cepko, P.A. Sharp and B.E. Roberts. 1981. Expression of early adenovirus genes requires a viral encoded acidic polypeptide. **Proc. Natl. Acad. Sci. USA 78:** 6121-6125.

378) Richardson, W.D., B.L. Roberts and A.E. Smith. 1986. Nuclear location signals in polyoma virus large-T. **Cell 44:** 77-85.

379) Rikitake, Y. and E. Moran. 1992. DNA-binding properties of the E1A-associated 300-kilodalton protein. **Mol. Cell. Biol. 12:** 2826-2836.

380) Roberts, B.E., J.S. Miller, D. Kimelman, C.L. Cepko, I.R. Lemischka and C.R. Mulligan. 1985. Individual adenovirus type 5 early region 1A gene products elicit distinct alterations of cellular morphology and gene expression. **J. Virol. 56:** 404-413.

381) Rooney, R.J., P. Raychaudhuri and J.R. Nevins. 1990. E4F and ATF, two transcription factors that recognize the same site, can be distinguished both physically and functionally: a role for E4F in E1A <u>trans</u> activation. **Mol. Cell. Biol. 10:** 5138-5149.

382) Rosengard, A.M., C.H.C. Krutzsch, A. Shearn, J.R. Biggs, E. Barker, I.M.K. Margulies, C.R. King L.A. Liotta and P.S. Steeg. 1989. Reduced Nm23/Awd protein in tumour metastasis and aberrant Drosophila development. **Nature 342:** 177-180.

383) Rossini, M. 1983. The role of adenovirus early 1A in the regulation of early regions 2A and 1B expression. **Virol 131:** 49-58.

384) Routes, J.M., D. Bellgrau, W.J. McGrory, D.S. Bautista, F.L. Graham and J.L. Cook. 1991. Anti-adenovirus type 5 cytotoxic T lymphocytes: Immunodominant epitopes are encoded by the E1A gene. **J. Virol. 65:** 1450-1457.

385) Routes, J.M. and J.L. Cook. 1989. Adenovirus persistence in man. Defective E1A product targetting of infected cells for elimination by natural killer cells. **J. Immunol. 142:** 4022-4026.

386) Rowe, D.T., S. Yee, J. Otis, F.L. Graham and P.E. Branton. 1983. Characterization of human adenovirus type 5 early region 1A polypeptides using antitumor sera and antiserum specific for the carboxy terminus. **Virol. 127:** 253-271.

387) Rozhin, J., R.L. Wade, K.V. Honn and B.F. Sloane. 1989. Membrane-associated cathepsin L: A role in metastasis of melanomas. **Biochem. Biophys. Res. Commun. 164:** 556-561.

388) Ruley, H.E. 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. **Nature 304:** 602-606.

Ruoslahti, E. 1988. Fibronectin and its receptors. **Annu. Rev. Biochem. 57:** 375-413.

389) Ruoslahti, E. and F.G. Giancotti. 1989. Integrins and tumor cell dissemination. **Cancer Cells 1:** 119-126.

390) Ruppert, M.J., B. Vogelstein and K.W. Kinzler. 1991. The zinc finger protein GLI transforms primary cells in cooperation with adenovirus E1A. **Mol. Cell. Biol. 11:** 1724-1728.

391) Saito, H., H.C. Hayday, K. Wiman, W.S. Hayward and S. Tonegawa. Activation of the c-myc gene by translocation: A model for translational control. **Proc. Natl. Acad. Sci. USA 80:** 7476-7480.

392) Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual, second ed. Cold

Spring Harbor Laboratory Press, Cold Spring Harbor.

393) Santos, E., S.R. Tronick, S.A. Aaronson, S. Pulciani and M. Barbacid. 1982. T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALB- and Harvey-MSV transforming genes. **Nature 298:** 343-347.

394) Sappino, A.-P., N. Busso, D. Belin and J-.D. Vassali. 1987. Increase of urokinase type plasminogen activator gene expression in human lung and breast carcinomas. **Cancer Res. 47:** 4043-4046.

395) Sassone-Corsi, P. and E. Borrelli. 1987. Promoter transactivation of protooncogenes c-fos and c-myc, but not c-Ha-ras, by products of adenovirus early region 1A. **Proc. Natl. Acad. Sci. USA 84:** 6430-6433.

396) Sassone-Corsi, P. 1988. Cyclic AMP induction of early adenovirus promoters involves sequences required for E1A trans-activation. **Proc. Natl. Acad. Sci. USA 85:** 7192-7196.

397) Sassone-Corsi, P., J. Visvader, L. Ferland, P. Mellon and I.M. Verma. 1988. Induction of proto-oncogene for transcription through the adenylate cyclase pathway: characterization of a c-AMP responsive element. **Genes Dev. 2:** 1529-1538.

398) Sassone-Corsi, P., L.J. Ransone and I.M. Verma. 1990. Cross-talk in signal transduction: TPA-inducible factor jun/AP-1 activates cAMP-responsive enhancer elements. **Oncogene 5:** 427-431.

399) Sawada, Y. and K. Fujinaga. 1980. Mapping of the adenovirus 12 mRNA's transcribed from the transforming region. **J. Virol. 36:** 639-651.

400) Sawada, Y., D. Urbanelli, J. Raskova, T.E. Shenk and K. Raska, Jr. 1986. Adenovirus tumor-specific transplantation antigen is a function of the E1A early region. **J. Exp. Med. 163:** 563-572.

401) Schirrmacher, V. 1985. Experimental approaches, theoretical concepts, and impacts for treatment strategies. **Adv. Cancer Res. 43:** 1-32.

402) Schneider, J.F., F. Fisher, C.R. Goding and N.C. Jones. 1987. Mutational analysis of the adenovirus E1A gene: The role of transcriptional regulation in transformation. **EMBO J. 6:** 2053-2060.

403) Schrier, P.I., R. Bernards, R.T.M.J. Vaessen, A. Houweling and A.J. van der Eb. 1983. Expression of class I major histocompatibilty antigens switched off by highly oncogenic adenovirus 12 in transformed rat cells. **Nature 305:** 771-775.

404) Schwab, M., H.E. Varmus and J.M. Bishop. 1985. Human c-myc gene contributes to neoplastic transformation of mammalian cells in culture. **Nature 316:** 160-162.

405) Schwarz, L.C., M.-C. Gingras, G. Goldberg, A.H. Greenberg and J.A. Wright. 1988. Loss of growth factor dependence and conversion of transforming growth factor-ß$_1$ inhibition to stimulation in metastatic H-ras-transformed murine fibroblasts. **Cancer Res. 48:** 6999-7003.

406) Schwarz, L.C., T. Inoue, T. Irimura, J.E. Damen, A.H. Greenberg and J.A. Wright. 1990. Relationships between heparanase activity and increasing metastatic potential of fibroblasts transfected with various oncogenes. **Cancer Lett. 51:** 187-192.

407) Schwarz, L.C., J.A. Wright, M.-C. Gingras, P. Kondaiah, D. Danielpour, M. Pimental, M.B. Sporn and A.H. Greenberg. 1990. Aberrant TGF-ß production and regulation in metastatic malignancy. **Growth Factors 3:** 115-127.

408) Sen, R. and D. Baltimore. 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. **Cell 46:** 705-716.

409) Sharp, P.A. 1984. In, H.S. Ginsberg, ed; The Adenoviruses, Plenum Press, New York. pp. 173-204.

410) Shaw, A.R. and E.B. Ziff. 1982. Selective inhibition of adenovirus type 2 early region II and III transcription by an anisomycin block of protein synthesis. **Mol. Cell. Biol. 2:** 789-799.

411) Shenk, T. and J. Flint. 1991. Transcriptional and transforming activities of the adenovirus E1A proteins. **Adv. Cancer Res. 57:** 47-85.

412) Shih, C., L.C. Padhy, M. Murray and R.A. Weinberg. 1981. Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. **Nature 290:** 261-264.

413) Shih, C. and R.A. Weinberg. 1982. Isolation of a transforming sequence from a human bladder carcinoma cell line. **Cell 29:** 161-169.

414) Shimizu, K., M. Goldfarb and Y. Suard. 1983. Three human oncogenes are related to the viral ras oncogenes. **Proc. Natl. Acad. Sci. USA 80:** 2112-2116.

415) Shirodkar, S., M. Ewen, J.A. DeCaprio, J. Morgen, D.M. Livingston and T. Chittenden. 1992. The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-dependent manner. **Cell 68:** 157-166.

416) Sidebottom, E. and S.R. Clark. 1983. Cell fusion segregates progressive growth from metastasis. **Br. J. Cancer 47:** 399-405.

417) Sigal, I.S., J.B. Gibbs, J.S. D'Alonzo, G.L. Temeles, B.S. Wolansky, S.H. Socher and E.M. Scolnick. 1986. Mutant ras-encoded proteins with altered nucleotide binding exert dominant biological effects. **Proc. Natl. Acad. Sci. USA 83:** 952-956.

418) Simon, M., K. Kitchner, H.T. Kao, L. Weber, R. Voellmy, N. Heintz and J.R. Nevins. 1987. Selective induction of human heat shock gene transcription by the adenovirus E1A gene products, including the 12S E1A product. **Mol. Cell. Biol. 7:** 2884-2890.

419) Sjogren, H.O., J. Minowada and J. Ankerst. 1967. Specific transplantation antigens of mouse sarcomas induced by adenovirus type 12. **J. Exp. Med. 125:** 689-701.

420) Slamon, D.J., J.B. deKernion, I.M. Verma and M.J. Cline. 1984. Expression of cellular oncogenes in human malignancies. **Science 224:** 256-262.

421) Sloane, B.F., J.R. Dunn and K.V. Honn. 1981. Lysosomal cathepsin B: Correlation with metastatic potential. **Science 212:** 1151-1153.

422) Sloane, B.F. and K.V. Honn. 1984. Cysteine proteinases and metastasis. **Cancer Metastasis Rev 3:** 249-263.

423) Sloane, B.F., K. Moin, E. Krepela and J. Rozhin. 1990. Cathepsin B and its endogenous inhibitors: The role in tumor malignancy. **Cancer Metastasis Rev. 9:** 333-352.

424) Smart, J.E., J.B. Lewis, M.B. Mathews, M.L. Harter and C.W Andersson. 1981. Adenovirus type 2 early proteins: assignment of the early region 1A proteins synthesized in vivo and in vitro to specific mRNAs. **Virol. 112:** 703-713.

425) Smith, D.H., D.M. Kegler and E.B. Ziff. 1985. Vector expression of adenovirus type 5 E1A proteins: Evidence for E1A autoregulation. **Mol. Cell. Biol. 5:** 2684-2696.

426) Smith, D.H., A. Velcich, D. Kegler and E. Ziff. 1986. Transcriptional control by the adenovirus type-5 E1A proteins. In, Botchan, M., T. Grodzicker and P.A. Sharp, eds; Cancer cells/4. DNA Tumor Viruses: Control of gene expression and replication, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

427) Smith, D.H. and E.B. Ziff. 1988. The amino-terminal region of adenovirus serotype 5 E1A protein performs two seperate functions when expressed in primary baby rat kidney cells. **Mol. Cell. Biol. 8:** 3882-3890.

428) Smith, M.R., S.J. DeGudicibus and D.W. Stacey. 1986. Requirement for c-ras proteins during viral oncogene transformation. **Nature 320:** 540-543.

429) Southern, E.M. 1975. Detection of specific sequences among DNA fragments seperated by gel electrophoresis. **J. Mol. Biol. 98:** 503-517.

430) Spandidos, D.A. and N.M. Wilkie. 1984. Malignant transformation of early passage rodent cells by a single mutated human oncogene. **Nature 310:** 469-475.

431) Spandidos, D.A. and N.J. Agnantis. 1984. Human malignant tumors of the breast as compared to their respective normal tissue, have elevated expression of the Harvey ras oncogene. **Anticancer Res. 4:** 269-272.

432) Spandidos, D.A. and I.B. Kerr. 1984. Elevated expression of the human ras oncogene family in premalignant and malignant tumors of the colorectum. **Br. J. Cancer 49:** 681-688.

433) Spangler, R., M. Bruner, B. Dalie and M.L. Harter. 1987. Activation of adenovirus promoters by the adenovirus E1A protein in cell-free extracts. **Science 237:** 1044-1046.

434) Spector, D.J., M. McGregor and H.J. Raska. 1978. Regulation of the appearance of cytoplasmic RNAs from region 1 of the adenovirus 2 genome. **J. Mol. Biol. 126:** 395-414.

435) Spindler, K.R., D.S.E. Rosser and A.J. Berk. 1984. Analysis of adenovirus transforming proteins from early region 1A and 1B with antisera to inducible fusion antigens produced in Escherichia coli. **J. Virol. 49:** 132-141.

436) Spindler, K.R., C.Y. Eng and A.J. Berk. 1985. An adenovirus early region 1A protein is required for maximal viral DNA replication in growth-arrested human cells. **J. Virol. 53:** 742-750.

437) Stabel, S., P. Argos and L. Phillipson. 1985. The release of growth arrest by microinjection of adenovirus E1A DNA. **EMBO J. 4:** 2329-2336.

438) Stallcup, M.R. and L.D. Washington. 1983. Region-specific initiation of mouse mammary tumor virus RNA synthesis by endogenous RNA polymerase II in preparations of cell nuclei. **J. Biol. Chem. 258:** 2802-2807.

439) Stamenkovic, I., M. Amiot, J.M. Pesando and B. Seed. 1989. A lympocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. **Cell 56:** 1057-1062.

440) Steeg, P.S., G. Bevilacqua, L. Kopper, U.P. Thorgeirsson, J.E. Talmadge, L.A. Liotta and M.E. Sobel. 1988a. Evidence for a novel gene associated with low tumor metastatic potential. **J. Natl. Canc. Inst. 80:** 200-204.

441) Steeg, P.S., G. Bevilacqua, R. Pozzatti, L.A. Liotta and M.E. Sobel. 1988b. Altered expression of NM23, a gene associated with low tumor metastatic potential, during adenovirus 2 E1A inhibition of experimental metastasis. **Cancer Res. 48:** 6550-6554.

442) Stein, R.W. and E.B. Ziff. 1984. HeLa cell ß-tubulin gene transcription is stimulated by adenovurus 5 in parallel with viral early genes by an E1A-dependent mechanism. **Mol. Cell. Biol. 4:** 2792-2801.

443) Stein, R.W. and E.B. Ziff. 1987. Repression of insulin gene expression by adenovirus type 5 E1A proteins. **Mol. Cell. Biol. 7:** 1164-1170.

444) Stein, R.W., M. Corrigan, P. Yaciuk, J. Whelan and E. Moran. 1990. Analysis of E1A-mediated growth regulation functions: Binding of the 300-kilodalton cellular product correlates with E1A enhancer repression function and DNA synthesis-inducing activity. **J. Virol. 64:** 4421-4427.

445) Stephens, C., B.R. Franza Jr., C. Schley. and E. Harlow. 1986. Heterogeneity of adenovirus E1A proteins is due to post-translational modification of the primary translation products of the 12S and 13S mRNAs. **Cancer cells 4:** 429-434.

446) Stephens, C. and E. Harlow. 1987. Differential splicing

yields novel adenovirus 5 E1A mRNAs that encode 30 kd and 35 kd proteins. **EMBO J. 6:** 2027-2035.

447) Stetler-Stevenson, W.G. 1990. Type IV collagenases in tumor invasion and metastasis. **Cancer Metastasis Rev. 9:** 289-303.

448) Stracke, M.L., R. Guirguis, L.A. Liotta and E. Schiffmann. 1987. Pertussis toxin inhibits stimulated motility independently of the adenylate cyclase pathway in human melanoma cells. **Biochem. Biol. Res. Comm. 146:** 339-345.

449) Subramanian, T., M. Kuppuswamy, R.J. Nasr and G. Chinnadurai. 1988. An N-terminal region of adenovirus E1A essential for transformation and induction of an epithelial cell growth factor. **Oncogene 2:** 105-112.

450) Subramanian, T., M. La Regina and G. Chinnadurai. 1989. Enhance ras oncogene mediated cell transformation and tumorigenesis by adenovirus 2 mutants lacking the C-terminal region of E1A protein. **Oncogene 4:** 415-420.

451) Sugarman, B.J., B.B. Aggarwal, P.E. Hass, I.S. Figari, M.A. Palladino, Jr. and H.M. Sheppard. 1985. Recombinant human tumor necrosis factor-α: effects on proliferation of normal and transformed cells in vitro. **Science 230:** 943-945.

452) Sy, M.S., Y.J. Guo and I. Stamenkovic. 1991. Distinct effects of two CD44 isoforms on tumor growth in vivo. **J. Exp. Med. 174:** 859-866.

453) Tabin, C.J., S.M. Bradley, C.I. Bargmann, and R. Weinberg. 1982. Mechanism of activation of a human oncogene. **Nature 300:** 143-149.

454) Takahashi, M., S. Ueda and T. Ogino. 1966. Enhancement of the thymidine kinase activity of human embryonic kidney cells and newborn hamster kidney cells by infection with human adenoviruses types 5 and 12. **Virology 30:** 742-743.

455) Talmadge, J.E. and I.J. Fidler. 1982. Cancer metastasis is selective or random depending on the present tumor population. **Nature 27:** 593-594.

456) Tanabe, T., T. Nukada, Y. Nishikawa, K. Sugimoto, H. Suzuki, H. Takahashi, M. Noda, T. Haga, A. Ichiyama, K. Kangawa, N. Minamino, H. Matsuo and S. Numa. 1985. Primary structure of the α-subunit of transducin and its relationship to ras proteins. **Nature 315:** 242-245.

457) Taparowsky, E., K. Shimizu, M. Goldfarb and M. Wigler. 1983. Structure and activation of the human N-ras gene. **Cell 34:** 581.

458) Taparowsky, E., Y. Suard, O. Fasano, K. Shimizu, M. Goldfarb, and M. Wigler. 1982. Activation of the T24 bladder carcinoma transforming gene is linked to a single amino acid change. **Nature 300:** 762-765.

459) Taylor, W.R., S.E. Egan, M. Mowat, A.H. Greenberg and J.A. Wright. 1992. Evidence for synergistic interactions between ras, myc, and a mutant form of p53 in cellular transformation and tumor dissemination. **Oncogene 7:** 1383-1390.

460) Templeton, N.S., P.D. Brown, A.T. Levy, I.M.K. Margulies, L.A. Liotta and W.G. Stetler-Stevenson. 1990. Cloning and characterization of human tumor cell interstitial collagenase. **Cancer Res. 50:** 5431-5437.

461) Theodorescu, D., I. Cornil, B. Fernandez and R.S. Kerbel. 1990. Overexpression of normal and mutated forms of H-ras induce orthotopic bladder invasion in a human transitional cell carcinoma. **Proc. Natl. Acad. Sci. USA 87:** 9047-9051.

462) Thorgeirsson, U.P., T. Turpeenniemi-Hujanen, J.E. Williams, E.H. Westin, C.A. Heilman, J.E. Talmadge and L.A. Liotta. 1985. NIH/3T3 cells transfected with human tumor DNA containg activated ras oncogenes express the metastatic phenotype in nude mice. **Mol. Cell. Biol. 5:** 259-262.

463) Timmers, H.T.M., D. DeWit, J.L. Bos and A.J. van der Eb. 1988. E1A products of adenoviruses reduce the expression of cellular proliferation-associated genes. **Oncogene Res. 3:** 67-76.

464) Timmers, H.T.M., H. van Dam, G.J. Pronk, J.L. Bos and A.J. van der Eb. 1989. Adenovirus E1A represses transcription of the cellular JE gene. **J. Virol. 63:** 1470-1473.

465) Tooze, J. 1981. DNA Tumor Viruses. Cold Spring Harbor Labortory, Cold Spring Harbor, New York.

466) Trahey, M. and F. McCormick. 1987. A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. **Science 238:** 542-545.

467) Treisman, R., M.R. Green and T. Maniatis. 1983. cis and trans activation of globin gene transcription in

transient assays. **Proc. Natl. Acad. Sci. USA 80:** 7428-7432.

468) Tremblay, M.L., C.J. Glade, G.E. Gerber and P.E. Branton. 1988. Identification of the phosphorylation sites in early region 1A proteins of adenovirus type 5 by amino acid sequencing of peptide fragments. **J. Biol. Chem. 263:** 6375-6383.

469) Trentin, J.J., Y. Yabe and G. Taylor. 1962. The quest for human cancer viruses. **Science 137:** 835-841.

470) Tsai, L.-H., E. Harlow and M. Meyerson. 1991. Isolation of the human cdk2 gene that encodes the cyclin A- and adenovirus E1A-associated p33 kinase. **Nature 353:** 174-177.

471) Tsuchida, M., E. Ohtsubo and T. Ryder. 1982. Nucleotide sequence of the oncogene encoding the p21 transforming protein of Kirsten Murine Sarcoma Virus. **Science 217:** 937-939.

472) Tsukamoto, A.S., A. Ponticelli, A.J. Berk and R.B. Gaynor. 1986. Genetic mapping of a major site of phosphorylation in adenovirus type 2 E1A proteins. **J. Virol. 59:** 14-22.

473) Turley, E.A. 1992. Hyaluronan and cell locomotion. **Cancer Metas. Rev. 11:** 21-30.

474) Turley, E.A., L. Austen, K. Vandeligt and C. Clary. 1991. Hyaluronan and a cell-associated hyaluronan binding protein regulate the locomotion of ras-transformed cells. **J. Cell Biol. 112:** 1041-1047.

475) Turley, E.A., D. Moore, L.J. Hayden. 1987. Characterization of hyaluronate binding proteins isolated from 3T3 and murine sarcoma virus transformed 3T3 cells. **Biochemistry 26:** 2997-3005.

476) Turpeenniemi-Hujanen, T., U.P. Thorgeirsson, I.R. Hart, S. Grant and L.A. Liotta. 1985. Expression of collagenase IV (basement membrane collagenase) activity in murine tumor cell hybrids that differ in metastatic potential. **J. Natl. Cancer Inst. 75:** 99-108.

477) Ura, H., R.D. Bonfil, R. Reich, R. Reddel, A. Pfeifer, C.C. Harris and A.J.P. Klein. 1989. Expression of type IV collagenase and procollagenase genes and its correlation with tumorigenic, invasive, and metastatic abilities of oncogene-transformed human bronchial epithelial cells. **Cancer Res. 49:** 4615-4621.

478) Urbanelli, D., Y. Sawada, J. Raskova, N.C. Jones, T. Shenk and K. Raska, Jr. 1991. C-terminal domain of the adenovirus E1A oncogene product is required for induction of cytotoxic T lymphocytes and tumor-specific transplantation immunity. **Virol. 173:** 607-614.

479) van Dam, H., R. Offringa, A.M.M. Smits, J.L. Bos, N.C. Jones and A.J. van der Eb. 1989. The repression of the growth factor-inducible genes JE, c-myc and stromelysin by adenovirus E1A is mediated by conserved region 1. **Oncogene 4:** 1207-1212.

480) van den Elsen, P.J., A. Houweling and A.J. van der Eb. 1983. Expression of region E1B of human adenoviruses in the absence of region E1A is not sufficient for complete transformation. **Virology 128:** 377-390.

481) van Ormondt, H., J. Maat and R. Dijkema. 1980. Comparison of nucleotide sequences of the early E1A regions for subgroups A, B and C of human adenoviruses. **Gene 12:** 63-76.

482) Vanhaesebroeck, B., H.T.M. Timmers, G.J. Pronk, F. van Roy, A.J. van der Eb and W. Fiers. 1990. Modulation of cellular susceptibilty to the cytotoxic/cytostatic action of tumor necrosis factor by adenovirus E1 gene expression is cell type-dependent. **Virology 176:** 362-368.

483) Varmus, H.E. 1984. The molecular genetics of cellular oncogenes. **Ann. Rev. Genetics 18:** 553-612.

484) Varmus, H. 1987. Cellular and viral oncogenes. In, Stamatoyannopoulos, Neinhuis, Leder, Majerus, eds; The Molecular Basis of Blood Diseases. W.B. Saunders Co., Toronto.

485) Velcich, A. and E.B. Ziff. 1985. Adenovirus E1A proteins repress transcription from the SV40 early promoter. **Cell 40:** 705-716.

486) Velcich, A. and E.B. Ziff. 1988. Adenovirus E1A ras cooperation activity is separate from its positive and negative transcription regulatory elements. **Mol. Cell. Biol. 8:** 2177-2183.

487) Velcich, A., and E.B. Ziff. 1989. The adenovirus-5 12S E1A protein, but not the 13S, induces expression of the endo A differentiation marker in F9 cells. **Oncogene 4:** 707-713.

488) Ventura, A.M., M.Q. Arens, A. Srinivasan and G. Chinnadurai. 1990. Silencing of human immunodeficiency

virus long terminal repeat expression by an adenovirus E1A mutant. **Proc. Natl. Acad. Sci. USA 87:** 1310-1314.

489) Virtanen, A. and U. Pettersson. 1983. The molecular structure of the 9S mRNA from early region 1A of adenovirus serotype 2. **J. Mol. Biol. 165:** 496-499.

490) Wang, B.S., G.A. McLoughlin, J.P. Richie and J.A. Mannick. 1980. Correlation of the production of plasminogen activator with tumor metastasis in B16 mouse melanoma cell lines. **Cancer Res. 40:** 288-292.

491) Wang, H.-G.H. Wang, G. Draetta and E. Moran. 1991. E1A induces phosphorylation of the retinoblastoma protein independently of direct physical association between the E1A and retinoblastoma products. **Mol. Cell. Biol. 11:** 4253-4265.

492) Wang, M. and M.E. Stearns. 1988. Blocking of collagenase secretion by estamustine during <u>in vitro</u> tumor cell invasion. **Cancer Res 48:** 6262-6271.

493) Webster, K.A., G.E.O. Muscat and L. Kedes. 1988. Adenovirus E1A products suppress myogenic differentiation and inhibit transcription from muscle-specific promoters. **Nature 332:** 553-557.

494) Webster, L.C., K. Zhang, B. Chance, I. Ayene, J.S. Culp, W.-J. Huang, F.Y.-H. Wu and R.P. Ricciardi. 1991. Conversion of the E1A Cys_4 zinc finger to a nonfunctional $His_2,Cys2$ zinc finger by a single point mutation. **Proc. Natl. Acad. Sci. USA 88:** 9989-9993.

495) Weigel, R.J., S.H. Devoto and J.R. Nevins. 1990. Adenovirus 12S E1A gene represses differentiation of F9 teratocarcinoma cells. **Proc. Natl. Acad. Sci. USA 85:** 9878-9882.

496) Weinberg, R.A. 1985. The action of oncogenes in the cytoplasm and nucleus. **Science 230:** 770-776.

497) Weinberg, R.A. 1990. The retinoblastoma gene and cell growth control. **Trends Biochem. Sci. 15:** 199-202.

498) Welsh, R.M. 1986. Regulation of virus infections by natural killer cells. **Nat. Immun. Cell Growth Regul. 5:** 169-199.

499) Whyte, P., H.E. Ruley and E. Harlow. 1988a. Two regions of the adenovirus early region 1A proteins are required for transformation. **J. Virol. 62:** 257-265.

500) Whyte, P, K.J. Buchkovich, J.M. Horowitz, S.H. Friend, M. Raybuck, R.A. Weinberg and E. Harlow. 1988b. Association between an oncogene and an anti-oncogene: The adenovirus E1A proteins bind to the retinoblastoma gene product. **Nature 334:** 124-129.

501) Whyte, P., N.M. Williamson and E. Harlow. 1989. Cellular targets for transformation by the adenovirus E1A proteins. **Cell 56:** 67-75.

502) Wilhelm, S.M., I.E. Collier, A. Kronberg, A.Z. Eisen, B.L. Marmer, G.A. Grant, E.A. Bauer and G.I. Goldberg. 1987. Human skin fibroblast stromelysin: structure, glycosylation, substrate specificity, and differential expression in normal and tumorigenic cells. **Proc. Natl. Acad. Sci. USA 84:** 6725-6729.

503) Wilhelm, S.M., I.E. Collier, B.L. Marmer, A.Z. Eisen, G.A. Grant and G.I. Goldberg. 1989. SV40-transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages. **J. Biol. Chem. 264:** 17213-17221.

504) Wright, J.A., S.E. Egan and A.H. Greenberg. 1990a. Genetic regulation of metastatic progression. **Anticancer Res. 10:** 1247-1256.

505) Wu, L., D.S.E. Rosser, M.C. Schmidt and A. Berk. 1987. A TATA box implicated in E1A transcriptional activation of a simple adenovirus 2 promoter. **Nature 326:** 512-515.

506) Yaciuk, R. and E. Moran. 1991. Analysis with specific polyclonal antiserum indicates that the E1A-associated 300-kilodalton product is a stable nuclear phosphoprotein that undergoes cell cycle phase-specific modification. **Mol. Cell. Biol. 11:** 5389-5397.

507) Yagel, S., A.H. Warner, H.N. Nellans, P.K. Lala, C. Waghorne and D.T. Denhardt. 1989. Suppression by cathepsin L inhibitors of the invasion of amnion membranes by murine cancer cells. **Cancer Res. 49:** 3553-3557.

508) Yee, S., D.T. Rowe, M.L. Tremblay, M. McDermott and P.E. Branton. 1983. Identification of human adenovirus early region 1 products by using antisera against synthetic peptides correponding to the predicted carboxy termini. **J. Virol. 46:** 1003-1013.

509) Yee, S. and P.E. Branton. 1985. Analysis of multiple forms of human adenovirus type 5 E1A polypeptides using an anti-peptide antisera specific for the amino terminus.

Virol. 146: 315-322.

510) Yee, S. and P.E. Branton. 1985. Detection of cellular polypeptides associated with the human adenovirus type 5 early region 1A polypeptides. **Virol. 147:** 142-153.

511) Yokota, J., Y. Tsunetsugu-Yokota, H. Battifora, C. LeFevre and M.J. Cline. 1986. Alterations of myc, myb and Ha-ras proto-oncogenes in cancers are frequent and show clinical correlation. **Science 231:** 261-265.

512) Young, K.S., R. Weigel, S. Heibert and J.R. Nevins. 1989. Adenovirus E1A-mediated negative control of genes activated during F9 differentiation. **Mol. Cell. Biol. 9:** 3109-3113.

513) Yu, D., J.-I. Hamada, H. Zhang, G.L. Nicolson and M.-C. Hung. 1992. Mechanisms of c-erbB2/neu oncogene-induced metastasis and repression of metastatic properties by adenovirus 5 E1A products. **Oncogene 7:** 2263-2270.

514) Yu, D., K. Scorsone and M.-C. Hung. 1991. Adenovirus type 5 gene products act as transformation suppressors of the neu oncogene. **Mol. Cell. Biol. 11:** 1745-1750.

515) Yu, D., T.-C. Suen, D.-H. Yan, L.-S. Chang and M.-C. Hung. 1990. Transcriptional repression of the neu protooncogene by the adenovirus 5 E1A gene products. **Proc. Natl. Acad. Sci. USA 87:** 4499-4503.

516) Yunis, J.J. 1986. Chromosomal rearrangements, genes, and fragile sites in cancer: clinical and biological implications. In, V.T. De Vita, Jr., S. Hellman and S.A. Rosenberg, eds; Important Advances in Oncology. J.B. Lippincott Co., Philadelphia. pp. 93-128.

517) Yuchenco, P.D. and J.C. Schittny. 1990. Molecular architecture of basement membranes. **FASEB J. 4:** 1577-1590.

518) Zamanian, M. and N.B. La Thangue. 1992. Adenovirus E1A prevents the retinoblastoma gene product from repressing the activity of a cellular transcription factor. **EMBO J. 11:** 2603-2610.

519) Zerler, B., B. Moran, K. Maruyama, J. Moomaw, T. Grodzicker and H.E. Ruley. 1986. Analysis of adenovirus E1A coding sequences which enable ras and pmt oncogenes to transform cultured primary cells. **Mol. Cell. Biol. 6:** 887-899.

520) Zerler, B., R.J. Roberts, M.B. Mathews and E. Moran.

1987. Different functional domains of the adenovirus E1A gene are involved in regulation of host cell cycle products. **Mol. Cell. Biol. 7:** 821-829.

521) Zu, Y.-L., Y. Takamatsu, M.-J. Zhao, T. Maekawa, H. Handa and S. Iishi. 1992. Transcriptional regulation by a point mutation of adenovirus-2 E1A product lacking DNA binding activity. **J. Biol. Chem. 267:** 20181-20187.