Identification of Nuclear Matrix Proteins Crosslinked to DNA by cis-DDP *in situ* in Human Breast Cancer Cell Lines

by

Virginia Spencer

A Thesis Submitted to the Faculty of Graduate Studies

The University of Manitoba

In Partial Fulfilment of the Requirements for the

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THE UNIVERSITY OF MANITOBA

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List of Abbreviations

A	adenine
Å	angstrom
A_{260}	absorbance at 260 nanometers
AB	arginine butyrate
apo	apolipoprotein
bp	base pair
CAPs	2-[cyclohexylamino]-1-propanesulfonic acid
cis-DDP	cis-diamminedichloroplatinum(II)
CSK	cytoskeleton
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-*N,N,N'*-tetraacetic acid
ER	estrogen receptor
FBS	fetal bovine serum
IF	intermediate filament
HAP	hydroxyapatite
hnRNA	heterogeneous nuclear ribonucleic acid
hnRNP	heterogeneous nuclear ribonucleoprotein

kb	kilobase
kDa	kilodalton
LIS	lithium diiodosalicylate
MAR	matrix attachment region
mA	milliamps
mM	millimolar
nM	nanomolar
NM	nuclear matrix
NM-IF	nuclear matrix-intermediate filament
NS	nuclear scaffold
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
RNase	ribonuclease
SAR	scaffold attachment region
SDS	sodium dodecyl sulfate
T	thymine
TBS	tris-buffered saline
µg	microgram
µM	mirometer
V	voltage

Abstract

The nuclear matrix (NM) plays an important role in the structural and functional organization of the cell nucleus. Human breast cancer is a disease that is thought to progress from a well-differentiated, hormone-dependent state to a poorly-differentiated, hormone-independent state. NM protein composition, and the interaction of NM proteins with intermediate filament proteins varies over human breast cancer progression, suggesting that NM proteins may be prognostic markers of cancer progression. Cell lines representing each stage of human breast cancer progression were treated with cis-diamminedichloroplatinum(II) to crosslink proteins to nuclear DNA *in situ*, and the DNA-bound proteins were isolated. Two-dimensional profiles of DNA-crosslinked proteins from the cell lines investigated showed a remarkable similarity to one another as well as to each cell line's respective NM profile. However, differences in the types of DNA-crosslinked proteins were observed between cell lines representative of each stage of disease progression, thus adding to the importance of the NM, and, more specifically, NM-DNA interactions in breast cancer development. As well, the association of some DNA-crosslinked proteins such as cytokeratins 8, 18 and 19 was dependent on estrogen in a hormone-dependent human breast cancer cell line, but not in a hormone-independent human breast cancer cell line. Thus, the acquisition of a hormone-independent phenotype may interfere with the ability of estrogen to regulate intermediate filament-nuclear DNA interactions in human breast cancer cells. These findings suggest that some of the various cellular events leading to malignancy in human breast cancer involve the NM and the cytoskeleton (CSK). Furthermore, the characterization of NM proteins and proteins crosslinked to DNA by cis-DDP *in situ*, which are primarily NM proteins, are two complementary approaches to identify NM proteins that are informative in cancer diagnosis.

Introduction

The nucleus is a complex, highly organized organelle capable of performing a multitude of cellular functions. In past years, a great deal of information has emerged defining the individual structural components of the nucleus such as the nucleosomes, chromatin loops, nuclear envelope and nuclear lamina. Despite this wealth of knowledge, researchers have yet to assemble a picture illustrating how the interactions between these components form a functioning nucleus. What is known, however, is that *in vivo* the genomic DNA is organized into nucleosomes which assemble into 30 nm filaments (Felsenfeld and McGhee, 1986; McGee et al., 1983; Simpson, 1978). These filaments are then folded into negatively supercoiled loop domains which appear to be anchored at their bases to proteins belonging to the skeletal framework of the nucleus called the nuclear matrix (Cook and Brazell, 1976; Paulson and Laemmli, 1977; Vogelstein et al., 1980; Gerdes et al., 1994). The loops range in size from 5 to 200 kb pairs in length (Jackson et al., 1990). The constraint of DNA into these supercoiled units by the NM is believed to structurally organize and regulate DNA replication and transcription (Cook, 1989), thereby influencing the transcriptional activity of DNA domains. Processes such as transcription (Mattern et al., 1996; Mirkovitch et al., 1984; Pienta et al., 1991; Jackson and Cook, 1985; Ciejek et al., 1983), DNA replication (McCready et al., 1980; Berezney and Buchholtz, 1981; Replogle and Pienta, 1996), and RNA processing and transport (Schroder et al., 1987; Zeitlin et al., 1987; Mattern et al., 1996) have been found associated with the NM. Moreover, Mirkovitch and colleagues (1984) have proposed that the NM brings the promoter and upstream regulatory elements of active genes physically close together, forming a subcompartment rich in DNA-binding

sequences able to associate with regulatory factors and nuclear enzymes such as RNA polymerase. Such evidence implies that the NM has a role in gene expression.

The Nuclear Matrix

By definition, the NM is the structure that remains after the treatment of interphase nuclei with nucleases to digest the genomic DNA, and high salt extraction to remove chromatin and loosely bound proteins (Mattern et al., 1996). This structure is composed of three main regions: a surrounding residual nuclear envelope or nuclear lamina, residual nucleoli and an extensive fibrogranular internal matrix (Berezney, 1991; Fey et al., 1986). As much as 98.4% of the NM is composed of high molecular weight, nonhistone proteins (Berezney and Coffey, 1974), many of which remain unidentified to date. Some of these proteins are common among different cell types, while others are tissue- and cell-type specific, reflecting the state of differentiation and transformation (Fey and Penman, 1988; Dworetzky et al., 1990; Chen et al., 1996; Samuel et al., 1997; Mattern et al., 1996; Mattern et al., 1997; Stuurman et al., 1990).

NM proteins identified to date include nuclear lamins A (Nakayasu and Berezney, 1991), B (Stuurman et al., 1990), and C (Nakayasu and Berezney, 1991), nucleolar protein B23 (Stuurman et al., 1990; Mattern et al., 1996 and 1997; Nakayasu and Berezney, 1991), nuclear matrins (Nakayasu and Berezney, 1991), the nuclear-mitotic apparatus protein (Zeng et al., 1994) and various heterogeneous nuclear ribonucleoproteins (hnRNPs) (Holzmann et al., 1997; Mattern et al., 1996 and 1997). The actual composition of the NM appears to vary according to the method of isolation and the degree of scrutiny used when comparing two-

dimensional NM protein profiles of different cells (Stuurman et al., 1990). According to Fey and Penman (1988), only those proteins detected exclusively in the NM fraction are designated as true NM proteins. In addition, any protein requiring intact DNA or RNA for its association with the NM is not considered part of this structure (Fey et al., 1986). In this study, NM proteins were isolated by treating nuclei with DNase I, 0.25 M ammonium sulfate, and RNase A followed by extraction of the remaining intermediate filaments (IFs) using an IF disassembly and assembly process. Proteins such as actin, vimentin, vimentin-related proteins and ribonucleoproteins (RNP) complexes were not considered NM since they were found in other cellular fractions (Fey and Penman, 1988). The remaining proteins unique to only the NM were generally present in low abundance. This creates the possibility that these proteins were actually present in other cellular fractions but were masked by the more abundant proteins (Fey and Penman, 1988). Also, the use of high salt to isolate the NM might strip away true NM proteins, causing them to appear in other cellular fractions (Capco et al., 1982).

Besides these problems, a major concern with this protocol is the selective removal of IF proteins and heterogeneous nuclear ribonuclear RNA (hnRNA) and RNPs. Evidence from various studies suggests that hnRNP proteins may also be present in the NM (Nakayasu and Berezney ,1991; Mattern et al., 1996; Mattern et al., 1997). The exact function of hnRNPs is unclear but they are probably involved in many aspects of RNA metabolism (Dreyfuss et al., 1993). Even though Fey and Penman (1988) believed that hnRNPs are not part of the NM, they were still unable to completely remove these proteins from the NM. This suggests that either the hnRNPs were so tightly associated to the NM that they could not

be efficiently removed or that the NM does indeed contain a small portion of hnRNPs. In addition, stripping of hnRNPs from the matrix by RNase treatment distorted NM shape, thereby suggesting a role of hnRNPs in organizing the nuclear interior (Fey et al., 1986). The protein, hnRNPK, has been shown to have all the properties of a transcription factor (Michelotti et al., 1996). The functional properties of hnRNPK in RNA processing and transcription, and the observations that RNA processing and transcription are associated with the NM (Schroder et al., 1987; Mirkovitch et al., 1984; Pienta et al., 1991; Jackson and Cook, 1985; Ciejek et al., 1983), further support the hypothesis that hnRNPs are part of the NM. Moreover, NM-IF isolation from nuclei stabilized with sodium tetrathionate in the absence of RNase A provided internal matrix structures that were predominantly composed of hnRNP proteins (Mattern et al., 1996 and 1997).

As well, RNA may be an important constituent of the NM. As much as 70% of hnRNA remained associated with the NM after treatment of nuclei with DNase and salt extraction (Fey et al., 1986). Digestion of NMs with RNaseA markedly distorted the NM shape and was only 97% efficient for removing NM-associated hnRNA and complexed proteins (Fey et al., 1986). This indicates that nuclear RNA must play an important role in matrix organization (Fey et al., 1986).

Several observations suggest that IF proteins may be part of the NM: (1) Fey et al. (1984) showed a stable association of IFs with the NM; (2) addition of a IF disassembly-assembly step to the NM-IF isolation procedure could not completely remove all IF proteins (Fey and Penman, 1988); (3) the crosslinking agent, cis-diamminedichloroplatinum(II) (cis-DDP), which crosslinks DNA preferentially to associated NM proteins (Ferraro et al., 1992),

crosslinks actin (Miller and Costa, 1989) and cytokeratins to DNA *in situ* (Ward et al., 1984; Olinksi et al., 1987); (4) intermediate filament-like structures have been observed in the NM (He et al., 1990; Nickerson et al., 1992); (5) antibodies directed against lamin A have localized to NM fibres (Hozak et al., 1995). Furthermore, because of the intimate association of the IFs with the NM, removal of IFs causes parts of the NM to be removed (Capco et al., 1982).

Even if the IFs are not part of the NM, evidence exists showing that these proteins influence NM structure (Maniotis et al., 1997). IFs containing vimentin, desmins, keratins or neurofilament proteins, along with actin-containing microfilaments, and tubulin-containing microtubules make up the CSK of eukaryotic cells (Ingber, 1993). The actin-associated proteins of the CSK bind to specific transmembrane receptors for extracellular matrix (ECM) components, thereby physically connecting this structure with the ECM (Turner and Burridge, 1991). Based on evidence from many studies, the CSK also appears to extend as far into the nucleus as the NM. Immunogold staining of cytoskeletal and NM proteins in resinless electron microscopy sections displays 10 nm intermediate filaments that stretch between the nuclear lamina and the cell periphery (Nickerson et al., 1990). Transmission electron micrographs (Fey et al., 1984) along with *in vitro* binding studies (Wang et al., 1996b; Ward et al., 1984) show an association of IFs with the NM and nuclear DNA, respectively. In addition, cytokeratins are preferentially crosslinked to DNA by cis-DDP (Ward et al., 1984).

The purpose of the linkage between the nucleus, CSK, and the ECM might be to stabilize nuclear form and integrate cell and nuclear structure (Ingber, 1994). The CSK is thought to be organized as a tensegrity network (Wang et al., 1993). According to Ingber

(1994), a tensegrity network consists of a series of isolated, compression-resistant struts that can be physically altered by connections with a continuous series of tension elements. To maintain its stability, this network must generate an internal tension. In cells, structural stability depends on mechanical tension generated within the contractile microfilaments of the CSK. This tension is then transmitted across transmembrane receptors on to the ECM which, in turn, resists the added force and causes a force balance. Application of a mechanical stress to a cell offsets the force balance and causes a number of coordinated events to occur in the CSK and all extended areas without disruption or loss of tensional integrity (Ingber, 1994). Therefore, pressure exerted on to the surrounding ECM can be transmitted to the CSK.

A consequence of this force imbalance could be global rearrangements in the CSK (Ingber, 1994) which may alter the interactions of molecules physically linked to this structure (Hill, 1981). In support of this stress-induced CSK rearrangement is the observation that pulling integrins by micromanipulating microbeads bound to the cell surface causes cytoskeletal filaments to reorient, nuclear shape to become distorted and nucleoli to redistribute along the axis of the applied tension (Maniotis et al., 1997).

Alterations in CSK organization have also been induced in cells transformed with the *ras* oncogene (Pienta and Coffey, 1992). The presence of the *ras* oncogene may stimulate the RAS signal transduction pathway which, in turn, would activate transcription factors such as AP-1 and ETS (Karin and Hunter, 1995). This activation may lead to the altered expression of cytokeratins 8 and 19 (Pankov et al., 1994).

Thus, changes in CSK organization resulting from either physical or chemical changes within a cell may impact NM architecture by causing rearrangements in NM proteins. These

rearrangements could relieve the constraints that prohibit DNA from unwinding (Ingber et al., 1994). As a result, transcriptionally inactive domains may become decondensed into active ones. If the tensegrity model accurately explains a cell's architecture then IFs may play an important role in gene expression by influencing NM structure, and, therefore, chromatin loop organization.

Features of M/SARs

The loop model for chromatin organization predicts a non-random folding of the chromatin fiber (Gasser and Laemmli, 1986a) into loops of approximately 5-200 kb (Jackson et al., 1990) which are attached at their bases to the nonhistone NM proteins (Cockerill and Garrard, 1986a; Mirkovitch et al., 1984). Active genes appear to be under torsional stress (Villeponteau et al., 1984). Thus, the periodic attachment of these genes to the NM maintains the torsional stress (Levy-Wilson and Fortier, 1989). When the histone octamer is removed, DNA is released as negatively supercoiled loops (Vogelstein et al., 1980). Each loop is commonly thought to represent an independent unit of transcription and replication that is insulated from the regulatory influences of neighbouring loop domains (Kalos and Fournier, 1995). The sequences thought to be responsible for the formation of these loops are termed matrix- or scaffold- attachment regions (MARs or SARs) (Mirkovitch et al., 1984; Cockerill and Garrard, 1986a). Thus, the discovery of MARs/SARs has been an important step towards further understanding the functional and structural aspects of chromatin-loop organization.

MARS/SARs often consist of at least 200 bp of A/T-rich DNA (Cockerill and Garrard, 1986a; Kas et al., 1989), have topoisomerase II sites (Yu et al., 1994; Adachi et al., 1989), and contain one or more copies of the sequence AATATTTT or similar sequences (Cockerill and Garrard, 1986a). However, not all MARs are A/T rich (Boulikas, 1995; Levy-Wilson and Fortier, 1989) or contain topoisomerase II sites (Levy-Wilson and Fortier, 1989). In addition, some MARs contain nucleation sites that become stably base-unpaired under negative superhelical tension (Bode et al., 1992; Kohwi-Shigematsu and Kohwi, 1990). Such a feature may be important in MAR function.

Structural analysis shows that MAR sequences contain features such as single-strandedness (Luderus et al., 1994), double-strandedness (Tsutsui et al., 1988; Probst and Herzog, 1985; Luderus e al., 1994), a narrow minor groove resulting from oligo (dA)-oligo(dT) tracts (Kas et al., 1989; Luderus et al., 1994), and bending (Homberger, 1989). MARs have been found in different locations of various genes. They have been found to cohabit with promoters (Stein et al., 1991), and origins of replication (Dijkwel and Hamlin, 1988), and they have been found adjacent to enhancers (Cockerill and Garrard, 1986a). Although they do not share an extensive homology (Levy-Wilson and Fortier, 1989), MAR sequences from different types of cells and from different species have all been shown to compete with each other for NM attachment sites in competitive MAR binding assays (Cockerill and Garrard, 1986b). As a result, the mechanism responsible for MAR binding to the NM appears to be evolutionarily conserved. One exception to this conservation is the 5' proximal human apolipoprotein (apo) B MAR, a sequence that associates with the NM only in cells expressing the apoB gene (Levy-Wilson and Fortier, 1989).

The exact mechanisms responsible for MAR DNA associations with the NM are uncertain. In interphase nuclei, the majority of DNA loop attachment sites have been localized by cytochemical and biochemical assays to the internal NM and the remaining are present in the peripheral nuclear lamina region (Zini et al., 1989; Phi-Van and Stratling, 1988; Izaurralde et al., 1988). The large variety of proteins composing the NM would suggest that there are diverse modes of recognition for DNA structures or sequences (Kay and Bode, 1994).

As was previously mentioned, most MARs contain A-T rich regions (Cockerill and Garrard, 1986a; Kas et al., 1989). These regions are believed to facilitate DNA unwinding catalyzed by helicases, torsional strain, or single-stranded DNA-binding proteins (Bode et al., 1996). Loop formation resulting when MARs bind to the matrix may induce local unwinding of A+T rich sequences. These unwound sequences could then bind to the matrix and reinforce the matrix interaction (Mielke et al., 1990). The unwinding of MARs may be stabilized in the form of a plectonemic DNA structure of B-type, double-stranded, knotted, negative superhelical (Bode et al., 1996) or cruciform (Hsieh and Wang, 1975; Lilley, 1980) character. Thus it is possible that MARs may assume some kind of landmark structure that allows them to associate with the NM (Probst and Herzog, 1985; Tsutsui et al., 1988).

Alternatively, MAR recognition sites may in fact be the single-stranded DNA segments resulting from MAR unwinding events (Bode et al., 1996). The observation that supercoiled, single-stranded, and double-stranded DNA all compete for the same NM DNA-binding sites (Mielke et al., 1990; Tsutsui et al., 1988) combined with the knowledge that many DNA binding NM proteins preferentially bind single-stranded DNA sites (Luderus et

al., 1994; Michelotti et al., 1996; Probst and Herzog, 1985; Kay and Bode, 1994) supports the theory that a single-stranded character may be important for MAR function. Furthermore, this theory is strengthened by the findings that increases in transcriptional activity attributed to MARs are correlated with the ability of MARs to undergo separation into single-strands under negative superhelical tension (Bode et al., 1992). However, single-strandedness does not apply to all MAR sequences since one-half of NM DNA binding sites are inaccessible to single-stranded DNA (Kay and Bode, 1994), and since DNA- binding NM proteins SAF-A (Romig et al., 1992), SATB1 (Dickinson et al., 1992) and ARBP (von Kries et al., 1991) do not recognize single-stranded DNA.

Regardless of the recognition mode, MARs bind to the NM in a manner that is saturable, and of high affinity (Luderus et al., 1994). Once bound, the stable base unpairing of these sequences under torsional strain is believed to relieve a transcriptional unit of this strain (Bode et al., 1992). The thermodynamic energy from the relieved strain could be transmitted to other regions of the domain and generate negative supercoils at these remote sites. By relieving negative superhelicity, MARs may open chromatin domains (Zhao et al., 1993) to the transcriptional machinery, and, therefore, augment transcriptional rates of stably transfected genes (Allen et al., 1993; Bode et al., 1995). Despite this function, MARs are unable to alter transcriptional rates in transient transfection assays (Kalos and Fournier, 1995; Wang et al., 1996a) . Because of this, there is a clear distinction between MAR and enhancer sequences. In addition, other studies provide evidence that contradicts the ability of MARs to augment transcription. For instance, McKnight and colleagues (1992) observed no increase in expression when a gene for whey acidic protein (WAP) and the chicken lysozyme

MAR were cointegrated into a transgenic mouse. Similarly, the apoB MAR did not enhance the expression of a transgene containing the WAP promoter, the late gene SV40 intron, the bovine growth hormone cDNA and the SV40 late gene terminator (Attal et al., 1996).

The inability of these MARs to enhance transcription in both studies may be a result of transgene copy number. Dorer and Henikoff (1994) have suggested that the presence of multiple transgene copies in mammalian cells leads to pairing of the repeat copies. These copies fold together and produce structures that are recognized by proteins specific to heterochromatin. The recognition of these folded gene copies by heterochromatin-specific proteins leads to the formation of heterochromatin, and, therefore, gene silencing. This event may explain the inability of MARs to enhance transgene expression in the study by McKnight and colleagues (1992), because the cells analyzed in this study contained multiple copies of a MAR-transgene construct. Furthermore, Attal et al. (1996) have consistently observed a higher level of expression of low copy number transgenes than high copy number transgenes.

As was previously stated, chromatin is believed to be organized into loop domains in interphase cells by MAR-NM associations (Gerdes et al., 1994; Cockerill and Garrard, 1986a; Mirkovitch et al., 1984), and each loop domain represents one transcription unit (Cook, 1989). This arrangement may provide a mechanism for isolating transcription units from the influence of neighbouring enhancers and silencers. MARs may act as the domain boundaries that partition chromosomes into independently regulated units (Kalos and Fournier, 1995; McKnight et al., 1992). On the other hand, evidence from other studies contradicts the theory that MARs isolate transcription domains from neighbouring DNA sequences. Variable reporter gene expression was observed in constructs containing the 5' distal and proximal apo

B MARs, the apo B second intron enhancer, and the apo B promoter that were stably transfected into mice (Brooks et al., 1994; Wang et al., 1996a). Despite the inability of MARs to completely insulate transgenes from position effects, higher levels of expression were generally seen for constructs containing MARs as opposed to those lacking MARs (Brooks et al., 1994; Wang et al., 1996a). Thus, instead of increasing the level of transgene expression in an individual cell, MARs may increase the percentage of cells expressing a transgene, and this increase will contribute to the overall elevated expression levels.

One explanation for the ability of MARs to increase gene expression could be that MARs support the targeting of enhancers to the NM and provide their proximity to transcribed units (Boulikas, 1995; Yu et al., 1994). Once the enhancer has been brought closer to the NM, the enhancer may increase the probability that a domain will achieve a stable and active transcriptional state (Walters et al., 1995). In support of the theory that MARs work together with enhancers to increase the probability of transgene expression are the findings that certain enhancers are tightly associated with MARs (Gasser and Laemmli, 1986b). Moreover, the β-globin MAR by itself has little effect on transcription of a reporter gene in transformants but when linked to an enhancer, this construct confers high level transgene expression (Yu et al., 1994). Similarly, the transcriptional activation of the immunoglobulin μ heavy chain locus gene during normal lymphoid development also requires a synergistic collaboration between the enhancer and flanking MARs (Forrester et al., 1994). Thus, MARs may bring proximal functional sequences closer to the NM and closer to the appropriate *trans*-acting factors in order to facilitate transcription (Dijkwel, and Hamlin, 1996; Bode et al., 1996).

M/SAR Isolation Techniques

Numerous *in vitro* studies have been performed to determine the nature of MARs and their NM attachment sites (Mirkovitch et al., 1984; Cockerill and Garrard, 1986a; Gasser and Laemmli, 1986a; Izaurralde et al., 1988). In one very significant study for MAR research, the NM was isolated by treatment of nuclei with high salt (2 M NaCl) and DNaseI and then reconstituted with specific DNA sequences in the presence of competitor DNA. By definition, a specific DNA fragment that was preferentially retained by nuclear matrices after competition contained an *in vitro* MAR (Cockerill and Garrard, 1986a).

In another important MAR/SAR study, the nuclear scaffold (NS), which is essentially the same as the NM except it is isolated by treatment of nuclei with a mild LIS detergent and restriction enzymes, was extracted and its associated DNA sequences isolated. By definition, a DNA fragment that remained preferentially associated with the NS after restriction endonuclease digest contained an *in vitro* SAR (Mirkovitch et al., 1984). The terms SAR and MAR are synonymous. MAR-binding proteins identified to date are: mutant p53 (Deppert, 1996; Muller et al., 1996), sp120 (Tsutsui et al., 1993), NMP1/YY1 (Guo et al., 1995), SATB1 (Cunningham et al., 1994; Dickinson et al., 1992), attachment region binding protein (ARBP) (von Kries et al., 1991), scaffold attachment factor A (SAF-A) (Romig et al., 1992), scaffold attachment factor B (SAF-B) (Renz and Fackelmayer, 1996), nucleolin (Dickinson and Kohwi-Shigematsu, 1995), p114 (Yanagisawa et al., 1996), topoisomerase II (Adachi et al., 1989), histone H1 (Izaurralde et al., 1989; Ivanchenko and Avramova, 1992) , lamins A (Luderus et al., 1994), and B (Luderus et al., 1992), and actin (Ivanchenko and

Avramova, 1992). Mutant p53 and p114 are of particular importance for cancer research since they are found only in a subset of breast cancer cells.

Although the use of these *in vitro* studies is widespread, there are various aspects about the methods of NM/NS isolation that create uncertainty in the identification of MARs and MAR-binding proteins. For instance, treatment of nuclei with high salt may severely disturb chromatin structure, possibly causing the DNA to slide or rearrange itself along the matrix attachment sites (Mirkovitch et al., 1984). In addition to altering chromatin structure, high salt treatment also appears to remove some NM proteins and cause significant shrinkage of the matrix structure (He et al., 1990). The high salt might also induce precipitation of the transcription complexes onto the matrix, thereby enriching the isolated NM fraction with artefactual transcription-dependent active genes (Mirkovitch et al., 1984).

Due to the potential DNA loop rearrangement problems resulting from high salt treatment, an alternative NM isolation procedure was developed (Mirkovitch et al., 1984). In this protocol, the NS was extracted with LIS in an attempt to avoid significant alterations in NM morphology, and, therefore, changes in loop structure. However, LIS failed to adequately separate chromatin proteins from the matrix (Fey et al., 1986). In addition, this detergent depleted the NS preparation of RNPs (Fey et al., 1986). The act of stripping these proteins from the NM would open up new matrix binding sites along the DNA. Therefore, DNA sequences that are normally prevented from binding to the NM *in vitro* would be able to associate with this structure. Such an occurrence could lead to inaccurate identification of MAR DNA-NM protein complexes. As a result, this alternative approach may be inadequate for the biochemical analysis of S/MARs.

All previously mentioned protocols expose cells to hypertonic conditions that may in some way alter NM morphology. Jackson and colleagues (1988) developed an alternative approach that allowed them to study chromatin loop-domain organization within cells in a near-physiological buffer with reduced risk of artefact formation. In this method, cells were encapsulated in agarose microbeads that form a protective coat permeable to protein complexes as large as 1.5×10^8 Da, and not larger skeletal elements or chromosomal DNA. Thus, the chromatin within the beads was protected from shearing forces, accessible to enzymes, and inhibited from aggregating in physiological buffer. Aggregation of chromatin may influence accessibility of enzymes, salts and detergents to the structures targeted for study. After encapsulation, the cells were lysed with Triton X-100. Then the chromatin was digested with restriction enzymes, and placed under voltage for up to 5 hours to remove any DNA not associated with the scaffold (Jackson et al., 1985; Jackson et al., 1988; Hempel and Stratling, 1996).

Even though this electroelution protocol has the distinct advantage of allowing MAR identification in physiological conditions (Jackson and Cook, 1985), it has the disadvantages of incomplete chromatin extraction (He et al., 1990), and solubilization of essential nuclear components with Triton X-100 (Jackson and Cook, 1985; Jackson et al., 1988). In addition, the buffer used may have been physiological but compounds that are normal nuclear constituents which function in chromatin packaging were omitted due to their potential deleterious effects on the cell (Hempel and Stratling, 1996). As well, whether the application of an electrical current to cells over a period of up to 5 hours (Jackson and Cook, 1985;

Jackson et al., 1988) caused loop rearrangements by stripping MAR-DNA from NM proteins still remains to be determined.

The identification of MARs and their NM binding sites becomes more complicated when comparing the results of the NM and NS isolation procedures with those of electroelution. In a study by Eggert and Jack (1991), a chromatin fragment containing the *Drosophila ftz* SAR was eluted from the nucleus as readily as a fragment lacking this SAR. Moreover, stably transfecting this SAR into transgenic flies did not have any significant effects on the chromatin structure. Similar results were obtained by Hempel and Stratling (1996) who found that both the 5' chicken lysozyme MAR and the *Drosophila* Kc histone SAR were electroeluted to the same extent as bulk chromatin. These findings further strengthen the loop rearrangement hypothesis proposed to result from high salt extraction protocol (Mirkovitch et al., 1984), as well as the theory that LIS extraction causes artefactual binding of proteins to the matrix (Fey et al., 1986).

The numerous disadvantages resulting from the three previously mentioned procedures for studying DNA-NM interactions create the need for an alternative approach. A more reliable method would be to isolate the MAR DNA bound to its respective NM protein *in situ*. Thus, problems of artefact formation resulting from loop rearrangements and protein stripping would be avoided. The *in situ* isolation of these NM-MAR DNA complexes can be accomplished according to a method developed by Ferraro et al. (1992) where living cells are treated with cis-DDP, an agent that has been shown to preferentially crosslink NM proteins to MAR DNA (Ferraro et al., 1995). This method has the distinct advantage of

allowing matrix protein-DNA complexes to be easily isolated. Once isolated, the crosslink stabilizing these complexes can be easily reversed.

Properties of *cis*-DDP

cis-diamminedichloroplatinum (II) (cis-DDP) is a heavy metal complex containing a central atom of platinum surrounded by two chloride atoms and two ammonia molecules in the cis position (Johnson et al., 1980) (Figure 1). This compound is very electrophilic, and, therefore, capable of reacting with electron-rich nucleic acids and proteins. In both cases, cis-DDP binding is thought to be controlled by the rate of hydrolysis *via* nucleophilic substitution by solvent water (LeRoy and Thompson, 1989; Dedon and Borch, 1987). The resulting hydrolyzed form may then bind to DNA, proteins or sulphur-containing compounds and by some unknown mechanism produce long-lived covalent DNA-DNA and DNA-protein crosslinks (Ward et al., 1984; Miller and Costa, 1989).

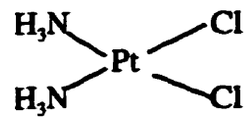

Figure 1. Formula of cis-DDP

In the case of nucleic acid binding, cis-DDP has been shown by x-ray photoelectron spectroscopy to bind to DNA at the C-6 carbonyl oxygen and the N-7 electron-rich sites of the guanine nucleotide base through its two chlorine atoms (Millard et al.,. 1975). As well, this agent has been shown in many studies to produce intrastrand DNA crosslinks. Such an interaction is thought to occur between the C-6 carbonyl oxygen or the N-7 of the guanine

base and the C-2 carbonyl oxygen of thymine (Morris and Gale, 1973). The close proximity of these electrophilic areas to one another in DNA, and the knowledge that cis-DDP interacts with electron-rich substrates provides evidence supporting this action (Morris and Gale, 1973).

In terms of the binding of cis-DDP to proteins, this agent has been shown to react with methionine, histidine and cysteine (Pattanaik et al., 1992), and to have a low propensity for generating protein-protein crosslinks (Lippard and Hoeschele, 1979). Once hydrolyzed, cis-DDP is thought to bind to proteins in two steps. First it binds to a protein possibly through the sulfhydryl group at a relatively fast pace (Yotsuyanagi et al., 1991; Chen et al., 1994). This binding then causes changes to occur in the conformation of the affected protein which expose more cis-DDP binding sites (Yotsuyanagi et al., 1991), and ultimately produce a crosslink that is stable to SDS, urea, and RNase A treatment (Miller and Costa, 1989).

A large volume of evidence explains the interaction of this platinum agent with DNA and proteins individually, but little is known about the mechanism of cis-DDP protein-DNA crosslinking. The observation that sulfhydryl reducing agents reverse this interaction suggests that sulfhydryl linkages may be important (Ward et al., 1984; Costa, 1991).

For reasons unknown, cis-DDP appears to preferentially crosslink DNA to nonhistone nuclear proteins (Banjar et al., 1984; Ward et al., 1984; Miller and Costa, 1990; Lippard and Hoeschele, 1979; Filipski et al., 1983). Using electron microscopy, Khan and Sadler (1978) have shown that this agent primarily targets the nucleolus and inner side of the nuclear double membrane. Proteins readily crosslinked to DNA by cis-DDP include nuclear lamins (Ferraro et al. 1996), actin (Miller and Costa, 1989), cytokeratins (Ward et al., 1984; Wedrychowski

et al., 1986) and NM proteins (Ferraro et al., 1992). In fact, previous studies show that the most abundant crosslinked proteins are components of the NM (Ferraro et al., 1995; Costa, 1991). Histones are crosslinked only when the concentration of cis-DDP is high (Foka and Paoletti, 1986; Lippard and Hoeschele, 1979) (i.e greater than 2 mM) and the incubation period exceeds 7 hours (Ward et al., 1984). In one study, core histones and histone H1 were observed in crosslinked samples isolated from cells treated with 1 mM cis-DDP for 2 hours, however, the amount of histones crosslinked represented only a fraction of the crosslinked proteins even though histones are more abundant than any other nuclear protein (Ward et al., 1984). Similarly, Ward and colleagues (1984) observed that histone H1 had a decreased crosslinking efficiency compared to cytokeratins.

In an attempt to understand the reason for cis-DDP's preference for NM proteins, Bubley et al. (1996) showed that fibroblasts incubated in 150 μM cis-DDP for 1 hour had a 6 fold increase in adduct formation within NM-associated DNA compared to total cellular DNA. These findings are supported by results of previous studies which show cis-DDP to preferentially bind internucleosomal DNA (Morris and Gale, 1973; Hayes and Scovell, 1991). Despite this evidence, binding of cis-DDP to NM-associated DNA was unexpected since DNA sequences associated with the NM are usually A-T rich (Gasser and Laemmli, 1987), and cis-DDP preferentially binds to guanine residues (Pinto and Lippard, 1985). As a result, crosslinking of MAR DNA to NM proteins by cis-DDP might be a function of the MAR DNA conformation (Bubley et al., 1996).

In addition, treatment of fibroblasts with arginine butyrate (AB) resulted in a concentration-dependent increase in cis-DDP-DNA adduct formation compared to cells

treated only with cis-DDP (Bubley et al., 1996). Although AB affects many cellular processes, this agent primarily inhibits histone deacetylase (Boffa et al., 1978). This principal action results in the hyperacetylation of the lysines in histones H2A, H2B, H3 and H4 (Boffa et al., 1978; Schlake et al., 1994) which possibly gives the DNA an open conformation for interactions with transcription factors (Schlake et al., 1994). *cis*-DDP may also then bind to this altered DNA. Thus the increase in cis-DDP binding to this artificially opened DNA conformation provides further evidence to suggest that chromatin configuration has a significant effect on cis-DDP adduct formation (Bubley et al., 1996).

Another factor that may be implicated in cis-DDP adduct formation is the presence of NM proteins bound to the DNA. In a study performed by Hoffmann et al. (1991), the number and distribution of drug-DNA lesions was different when DNA was exposed to cis-DDP in the presence or absence of an *Esherichia coli* single-stranded DNA-binding (SSB) protein. DNA treated with cis-DDP in the presence of the SSB protein showed twice as many cis-DDP adducts compared to DNA treated in the absence of protein (Pinto and Lippard, 1985). Moreover, the majority of DNA sequences that bound to cis-DDP were already identified as putative sites for cis-DDP-single-stranded DNA interactions. As a result, when associating with DNA, the SSB protein may have altered the distribution of DNA in such a way as to expose additional cis-DDP DNA binding sites. Thus, the presence of the SSB may have facilitated the interaction of cis-DDP to regions of DNA not normally accessible to this drug in its absence.

Alternatively, the SSB may have had the ability to melt DNA hairpin structures, thereby allowing cis-DDP to bind to DNA sequences that were otherwise less accessible

(Hoffmann et al., 1991). In this study, no evidence was presented to suggest that cis-DDP crosslinks DNA to the SSB protein. However, the possibility exists that the binding of this agent to DNA and the subsequent alterations in DNA distribution or structure caused by this interaction, may precede further interactions between cis-DDP and the SSB protein. This mechanism of cis-DDP protein-DNA crosslinking may apply to other single-stranded DNA binding proteins such as hnRNPK, a NM protein involved in transcription (Michelotti et al., 1996) and DNA replication (Alberts, 1990).

Evidence that cis-DDP crosslinks linker DNA predominantly to non-histone proteins of NM origin (Bubley et al., 1996) and the observations that many crosslinked proteins are associated with matrix associated/attachment regions (MARs), suggests that this agent can be used to isolate MARs. By definition, a MAR is any region of DNA associated with the NM (Cockerill and Garrard, 1986a; Mirkovitch et al., 1984). MARs have been identified in DNA sequences of cis-DDP crosslinked protein-DNA complexes (Ferraro et al., 1996). In addition, MAR DNA prepared by the LIS detergent extraction protocol of Mirkovitch et al. (1984) recognises and binds to proteins crosslinked to DNA by cis-DDP (Ferraro et al., 1995).

Breast Cancer

Breast cancer is the leading cause of death for women between 40 and 55 years of age in North America. Despite its high prevalence, the pathogenesis of this disease remains unclear (Kuller, 1995; Ernster et al., 1996). A current model of the evolution of this disease is that breast epithelial cells undergo a series of poorly understood genetic changes that result

in the progression from hyperplasia, dysplasia, *in situ* carcinoma, invasive carcinoma and finally metastatic carcinoma (Allred et al., 1993). The development of this disease relies, in part, on the presence of estradiol (Henderson et al., 1988), as well as on the presence of other growth factors and peptide hormones (Lippman and Dickson, 1989). In initial stages, human breast cancer cells are generally well differentiated, require estrogen for growth, and respond to anti-estrogen therapy. However, as the cancer progresses, breast cancer cells become poorly differentiated, and no longer require estrogen for growth. At this stage, other unknown growth control processes are believed to take over the mitogenic function of estrogen. In addition, late stage breast cancer cells are resistant to anti-estrogen therapy (Raymond and Leong, 1989a, 1989b; Clarke et al., 1990; Thompson et al., 1992).

A current model of breast cancer hormonal progression stipulates a correlation between the loss of the ER and the development of a more aggressive phenotype (Clarke et al., 1990). The loss of estrogen receptor (ER) expression has been suggested to occur through outgrowth of a receptor negative tumour cell population, or through the formation of variant estrogen receptors (Murphy et al., 1993). However, when passaged continuously in the presence or absence of estrogen, ER+ breast cancer cells do not display a change in ER phenotype. ER- breast cancer cell lines also have not been found to alter their ER phenotype in culture (Robertson, 1996). Moreover, the ER+ MCF-7 human breast cancer cell line has been shown to develop an endocrine-resistant phenotype without loss of ER (Murphy et al., 1990). Such evidence argues against a phenotypic drift of the ER from a positive to a negative state during breast cancer evolution to hormone-independence. This suggests that the progression of breast cancer cells to a hormone-independent state may not require the loss

of the ER, and that other cellular events are most likely involved in the development of this late stage phenotype.

Even though the mechanisms leading to breast cancer hormonal progression are not understood, a general belief is that the genetic events responsible for the development of a hormone-independent phenotype may involve activation of dominant oncogenes and inactivation of dominant tumor-suppressor genes. Such a theory is supported by evidence that the increased expression of the oncogene c-*erb*-B2/HER-2 is correlated with a resistance to endocrine therapy (Wright et al., 1992). In addition, insertion of the v-H-*ras* oncogene into MCF-7 cells resulted in cells capable of producing tumors in the absence of estrogen (Kasid et al., 1985).

The mechanism by which oncogenes may help confer hormone-independence on breast cancer cells is unknown. However, products from *src, lck, ras* and *raf* oncogenes may stimulate the RAS signal transduction pathway (Bortner et al., 1993). The stimulation of this pathway may activate AP-1 and ETS transcription factors and alter the expression of certain cytokeratins (i.e. cK8 , cK18) (Pankov et al., 1994). Since cytokeratins are part of the CSK, alterations in their expression could influence the structure of the CSK, and, therefore, the structure of the entire cell. Changing the cell structure could alter protein-protein and protein-DNA interactions. Such alterations could lead to the constitutive expression of proteins such as growth factors, which may, by some mechanism, override the mitogenic control of estrogen on breast cancer cells.

Considerable evidence exists showing an altered nuclear shape to be a hallmark of transformation (Pienta et al., 1989; Replogle and Pienta, 1996; Getzenberg et al., 1991). For

instance, nuclei from breast tumor cells appear enlarged with a marked variation in shape while normal breast epithelial cells display nuclei that are smaller, and more round or oval (Pienta and Coffey, 1991). In addition, evidence shows that tumor cells from node-positive breast cancer patients have a sharp increase in nuclear area compared to those from node-negative patients. This suggests that there is a correlation between nuclear area and metastatic potential (Pienta and Coffey, 1991; Komitowski and Janson, 1990; Komitowski et al., 1993).

The observation that nuclear shape is altered by transformation combined with the knowledge that nuclear shape is determined at least in part by the NM (Replogle and Pienta, 1996) suggests that alterations in NM profiles may be indicative of transformation and more specifically, the state of transformation (Getzenberg et al., 1991; Pienta et al., 1989). This theory is supported by observations that normal and cancerous cells show differences in NM protein profiles (Pienta and Coffey, 1992; Dworetzky et al., 1990; Getzenberg et al., 1991; Khanuja et al., 1993; Partin et al., 1993; Getzenberg et al., 1996). Furthermore, breast cancer cells representing different stages of disease progression also display slight differences in NM profiles (Samuel et al., 1997).

Along with changes in the protein composition of the NM, Taylor and colleagues (1991) have observed a significant increase in the average size of DNA loops associated with the NM in human fibroblasts either stimulated into cell division, or transformed with the SV40 T-antigen. As well, increases in the sizes of NM-associated loops were evident in human fibrosarcoma, lung carcinoma, and cervical carcinoma cell lines compared to normal human fibroblasts. From this evidence, a suggestion was made that transformation may induce

changes in DNA structure by altering the extent to which DNA within a particular replicon is supercoiled. Since studies have shown differences in NM profiles between normal and tumor cells (Partin et al., 1993; and Getzenberg et al., 1991; Getzenberg et al., 1996), alterations in NM composition could be responsible for changes in DNA topology. Based on this evidence, the NM may be an important prognostic marker in the pathogenesis of breast cancer.

A protein or group of proteins is considered a prognostic marker when its presence is correlated with other known prognostic factors or when its presence is correlated with survival and disease-free survival. The significance of a prognostic marker is determined by its correlation with other known prognostic factors. Prognostic or diagnostic NM proteins may be easily identified in the blood and urine of cancer patients, thus eliminating the need for invasive tissue collection. Using urine samples from patients, this approach has already been successfully used in a clinical setting to detect urothelial cancer (Miyanaga et al., 1997). In fact, the diagnostic use of a NM protein unique to this cancer provided more sensitivity for disease identification than that of other diagnostic methods such as cytoscopy or voided-urine cytology.

Alternatively, topological DNA changes after transformation may instead result from alterations in CSK organization induced by the transforming agent. In support of this theory is the observation that gene expression changes without apparent changes in NM composition when the CSK is disrupted with cytochalasin D (Macoska et al., 1994; Zambetti et al., 1991). Furthermore, changes in CSK organization have also been induced upon transformation of Kirsten kidney cells with the ras oncogene (Pienta and Coffey, 1992). Genes particularly

influenced during breast cancer progression appear to be those that encode intermediate filaments.

In breast tumor cells, the principal intermediate filament proteins are cK8, cK18, and cK19, whereas normal breast epithelial cells predominantly express cytokeratins cK4, cK5, cK6, cK14 and cK17 (Trask et al., 1990). Based on this differential expression, and observations that cK8 and cK18 expression in human melanoma cell lines leads to an increase in invasive and metastatic properties of these cells (Hendrix et al., 1992), cK8 and cK18 may be considered late markers of carcinoma progression. Besides cytokeratins, the intermediate filament protein vimentin may also have prognostic importance since it is expressed by some hormone-independent breast cancer cell lines and not in hormone-dependent cell lines (Sommers et al., 1989).

The NM plays an important role in the structural and functional organization of the cell nucleus. A large amount of evidence exists to suggest that the NM is associated with IFs (Fey et al., 1984; Fey and Penman, 1988; Ferraro et al., 1992; Miller and Costa, 1989; Ward et al., 1984; Olinkski et al., 1987; He et al., 1990; Nickerson et al., 1992; Hozak et al., 1995). Since IFs are components of the CSK, and the CSK is associated with the ECM (Ingber, 1994), any extracellular force or chemical signal that alters the CSK structure will, in turn, alter the structure of the NM. Since active genes are associated with the NM, alterations in the arrangement of NM proteins may alter the association of NM proteins with DNA and relieve some of the constraints that prohibit DNA from unwinding (Ingber et al., 1994). Such an event could alter gene expression.

Hypothesis and Approach

In a previous study, changes were observed between the two dimensional NM protein profiles of cell lines thought to represent different stages in breast cancer progression (Samuel et al., 1997). The NM proteins obtained for this comparison were isolated using the 2M NaCl high salt extraction procedure previously mentioned. This method, however, is prone to artefact formation. Since the agent, cis-DDP, has been shown to crosslink predominantly NM proteins to DNA, the hypothesis of this experiment is that cis-DDP crosslinking can be used as a complementary approach to the high salt extraction procedure for identifying putative NM proteins that may be informative markers in cancer diagnosis.

To identify changes in DNA-NM protein interactions during breast cancer progression, DNA-binding proteins from several hormone-dependent and hormone-independent cell lines will be isolated using the crosslinking agent cis-DDP, and analyzed by two-dimensional electrophoresis. To confirm that the majority of these DNA-binding proteins are NM proteins, the two-dimensional profiles of DNA-binding proteins and NM proteins from the same cell line will be compared. Proceeding this, the hormone-dependent breast cancer cell line, T-47D5, will be grown under normal, estrogen-deplete, and estrogen- replete conditions, and the hormone-independent cell line, T5-PRF, will be grown under estrogen-deplete conditions. The DNA-binding NM proteins will be isolated from the three T-47D5 treatments and the T5-PRF cell line using cis-DDP and analyzed by two-dimensional electrophoresis. Differences in NM protein levels over the three T-47D5 treatments and the T5-PRF cell line will be assessed to determine if estrogen influences the association of NM proteins with DNA.

Materials and Methods

Cell Lines and Cell Culture

To identify changes in DNA-binding protein profiles throughout breast cancer progression, we studied hormone-dependent (MCF-7, T-47D, ZR-75), ER+, hormone-independent (T5-PRF) and ER-, hormone-independent (MDA-MB-231, MDA-MB-468, BT-20) human breast cancer cell lines. MCF-10A1, a spontaneously immortalized cell line from a reduction mammoplasty breast tissue sample (Tait et al., 1990) was used as a control. With the exception of T5-PRF, MCF-10A1 and MDA-MB-468, all cell lines were maintained at 37°C (humidified atmosphere, 5% CO_2/95% Air) on 150 x 20 mm tissue culture dishes (Nunc) in culture medium containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% (v/v) L-glutamine, 1% (v/v) glucose, 1% penicillin/streptomycin and 5% (v/v) fetal bovine serum (FBS) (Gibco, Grand Island, New York). MCF-10A1 was maintained in DMEM supplemented with 5% horse serum (Gibco), 1% (v/v) glutamine, 1% penicillin/streptomycin, 30% glucose, 1 nM hydrocortisone (Sigma, St. Louis, Missouri) cholera toxin (100 µg/l) (Sigma), insulin (10 mg/l) (Sigma) , epidermal growth factor (20 µg/l) (Upstate Biotech, Lake Placid, New York) in a $C0_2$-free environment. The MDA-MB-468 cell line was maintained in L-15 (Leibovitz) (Gibco) culture medium supplemented with 10% FBS and 1% penicillin / streptomycin in a $C0_2$-free environment. At a confluence of approximately 90%, cells were removed from the plates with a rubber policeman and frozen as pellets containing 1 x 10^7 cells at -70°C.

To determine the effect of estrogen on cytokeratin-DNA interactions T-47D5 and T5-PRF cell lines were used. Both cell lines have been described previously (Watts et al., 1992; Coutts et al., 1996). The cell line T-47D5 is ER+ and hormone-dependent. This cell line was maintained in DMEM supplemented with 1% (v/v) L-glutamine, 1% glucose, 1% penicillin/streptomycin and 5% (v/v) FBS. T5-PRF was developed by passaging T-47D5 cells for at least 60 times in DMEM-Phenol Red Free (PRF) (Sigma) supplemented with 5% (v/v) twice charcoal stripped FBS, glucose, L-glutamine, and penicillin/streptomycin as previously mentioned (5% BS). Cells were routinely passaged at 70-80% confluence. T-47D5 cells acutely depleted of estrogen were grown in 5% BS for one passage. T-47D5 cells acutely depleted and then repleted of estrogen were grown for one passage in 5% BS followed by one passage in 5% BS containing 10 nM estradiol in an ethanol vehicle. All cells were passaged at 70-80% confluence using Earle's EDTA solution. 1 x 10^7 cells were harvested at approximately 80% confluence using a rubber policeman, and cell pellets were stored at -70°C.

Cis-DDP Crosslinking

Cis-DDP crosslinking was performed according to the procedure of Ferraro et al. (1991). Except for the initial 4 centrifugation steps, all steps in this protocol were performed on ice unless otherwise mentioned. All solutions used in this protocol contained 1 mM phenylmethylsulfonyl fluoride (PMSF). 1 x 10^7 cells were washed a total of three times in 30 ml of Hanks buffer and centrifuged at 50 x g for 8 min at room temperature in between each wash.

Cells were then resuspended in a fresh solution of either 1 mM or 3 mM cis-DDP solubilized in Hanks buffer containing 137 mM NaAc instead of NaCl. NaCl was excluded from this solution in order to prevent the chloride ions from competing with the cellular proteins for cis-DDP and, therefore, impair the crosslinking reaction (Lippard, 1982). The suspension (10^6 cells/ml) was incubated at 37°C for 2 hours with constant shaking. The length of incubation and the use of a 1 mM concentration of cis-DDP was based on crosslinking conditions used in previous studies (Ferraro et al., 1991; Ferraro et al., 1992; Ferraro et al., 1995; Ferraro et al., 1996). In these studies, a cis-DDP concentration of 1 mM and an incubation time of 2 hours appeared to be sufficient for optimal DNA-protein crosslinking without significant crosslinking of histones. However, since these studies did not use breast cancer cells, a crosslinking experiment using a 3 mM cis-DDP concentration was also performed to determine if a 1 mM cis-DDP concentration was high enough to produce the same two dimensional pattern of DNA-crosslinked NM proteins.

Following treatment, the cells were centrifuged at 50 x g for 15 min at room temperature, and the cell pellet was resuspended in 10 ml of lysis buffer (5 M urea, 2 M guanidine-hydrochloride, 2 M NaCl, 200 mM potassium phosphate buffer, pH 7.5). The cell lysate was then combined with hydroxyapatite (HAP: Bio-Rad, California) for 1 hour at 4°C with continuous mixing. HAP was used to isolate all cellular DNA in the cell lysate including DNA crosslinked to proteins by cis-DDP. Therefore, the HAP was used to indirectly separate the proteins crosslinked to DNA from all other non-crosslinked proteins in the cell lysate. One gram of HAP was used for every 4 mg of DNA in the cell lysate since this ratio has been shown in a previous study conducted within our lab to bind all cellular DNA with

approximately 100% efficiency (Li et al., 1993). To determine the amount of hydroxyapatite to combine with the cell lysate, 10 µl of cell lysate was aliquoted into 990 µl of lysis buffer and the A_{260} reading was recorded. This reading was applied to the following equation to determine the total mg of DNA present in the cell lysate:

A_{260} x 50 µg/ml x 100 x 10 ml / 1000 µg per mg of DNA = total mg of DNA

One A_{260} unit represents 50 µg of DNA per ml of cell lysate, thus the absorbance reading was first multiplied by 50 and then by the dilution factor (100) to determine the µg of DNA in 1 ml of cell lysate. The resulting value was multiplied by the total volume of cell lysate (10 ml) to determine the total µg of DNA in the cell lysate and then divided by 1000 to convert this value to mg. The total mg of DNA was divided by 4 to determine the amount of HAP to use. After incubating the HAP with the cell lysate for 1 hour, the resin was isolated by centrifuging the cell lysate-HAP slurry at 3000 x g for 5 min at 4°C. The resin was washed three times with 20 ml of lysis buffer. The HAP was then resuspended in 10 ml of lysis buffer containing 1 M thiourea instead of 5 M urea in order to reverse the crosslink between the DNA and the NM proteins. The slurry was incubated at 4°C on an orbitron for 2 hours. Following incubation, the thiourea solution containing the DNA-binding proteins was separated from the HAP by centrifugation at 10000 x g for 20 min at 4°C. This solution was then dialysed for 24 hours at room temperature against 5 changes of double distilled water (400 ml per sample per change). After dialysis, the solution was lyophilized and the resulting proteins resuspended in 100 µl of 8 M urea. The protein concentration for each sample was determined using the Bio-Rad Protein Assay (Bio-Rad) with bovine serum albumin (BSA) as a standard. Samples were stored at -20°C and analyzed by either one-dimensional or two-

dimensional gel electrophoresis. Three separate crosslinking experiments were performed for each cell line studied.

Nuclear Matrix Isolation

Nuclear matrices were isolated according to a procedure previously reported (Samuel et al., 1997). Cell pellets containing 1 x 10^7 cells were resuspended in 10 ml of ice-cold TNM buffer (100 mM NaCl, 300 mM sucrose, 10 mM Tris, pH 8.0, 2 mM $MgCl_2$, 1% (v/v) thiodiglycol) with 1 mM PMSF. The cell suspension was homogenized 5 times with a Teflon pestle on ice. Following homogenization, cells were incubated on ice for 5 min. A final concentration of 0.5% (v/v) Triton X-100 was added to release lipids and soluble proteins while nuclei remain intact. The suspension of nuclei was passed three times through a 18 gauge needle and collected by centrifugation at 1000 x g for 10 min at 4°C. The nuclei were again resuspended in ice-cold TNM buffer with 1 mM PMSF, homogenized and pelleted as before. The nuclei were resuspended to a concentration of 20 A_{260}/ml in cold DIG (50 mM NaCl, 300 mM sucrose, 10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 1% (v/v) thiodiglycol, 0.5% (v/v) Triton X-100) and digested with DNase I at a concentration of 168 units/ml for 20 min at room temperature. Ammonium sulfate (final concentration of 0.25 M) was added with stirring to facilitate chromatin removal, and the NM (NM-IF) pellet was obtained by centrifugation at 9600 x g for 10 min at 4°C. The NM-IF pellet contains NM associated with IFs. This pellet was resuspended in ice-cold DIG with 1 mM PMSF, extracted by adding 4 M NaCl to a final concentration of 2 M, and incubated on ice for 30 min. The sample was centrifuged at 9600 x g for 10 min at 4°C. The NaCl extracted NM pellet (NM2-IF) was

again resuspended in ice-cold DIG, extracted with 2 M NaCl and centrifuged as before. The NM2-IF was resuspended in Disassembly Buffer (8 M urea, 20 mM 2[N-morpholino] ethane sulfonic acid, pH 6.6, 1 mM EGTA, 1 mM PMSF, 0.1 mM $MgCl_2$, 1% (v/v) β-mercaptoethanol) and dialysed overnight at room temperature against 2 liters of Assembly Buffer (0.15 M KCl, 25 mM imidazole, pH 7.1, 5 mM $MgCl_2$, 2 mM DTT, 0.125 M EGTA, 0.2 mM PMSF). Dialysis allowed the urea to be removed and the IFs to reassemble. The IFs were removed by ultracentrifugation at 150,000 x g for 90 min. The resulting supernatant containing NM proteins was removed carefully and lyophilized. Lyophilized samples were resuspended in 8 M urea, aliquoted and frozen at -20°C. Before two-dimensional gel electrophoresis, protein concentrations were determined using the Bio-Rad Assay with BSA as a standard.

Quantification of Cytokeratin Levels in Crosslinked Samples

To find the relative levels of the cytokeratins in the cis-DDP crosslinked protein preparation from various cells, the proteins were resolved on 12% SDS (sodium dodecyl sulfate) gels according to the method of Laemmli (1970). Gels were then stained with 0.04% Serva Blue G (Serva Biochemicals, Westbury, NY), and quantification of cytokeratin levels was performed on the stained gel using scanning densitometry and Quantity One™ version 2.7 software (PDI, Kingston Station, New York). Initially, a linear range of protein staining versus the protein load was established by loading increasing amounts of NM-IF proteins on a SDS gel. Before determining the relative amount of cytokeratin in each preparation, we

checked that the amount of cytokeratins in a preparation was within the linear range. The amount of cytokeratin per μg of protein was then calculated.

Two-Dimensional Gel Electrophoresis

Two dimensional gel electrophoresis was performed on the isolated proteins according to the method of O'Farrell (1975). Separation of proteins by their isoelectric point was performed by loading 80 μg of protein sample on to isoelectric focusing tube gels (1.5 mm x 18 cm) containing 2% pre-blended ampholines of isoelectric point (pI) values of 3.5 - 9.5 and 5 - 8 (Pharmacia BioTech, Uppsala, Sweden). The gels were electrophoresed at 400 V, 400 mA for 16 hours and then 800 V, 400 mA for 2 hours. After electrophoresis, the gels were placed in a sample reducing buffer containing 3 % (w/v) SDS, 1.5 % (w/v) DTT, 0.07 % (w/v) Tris-HCl, pH 6.7, 0.01 % (w/v) and Bromophenol Blue for 20 min at room temperature. Following the incubation, the tube gels were layered on to SDS - 8% PAGE resolving gels prepared and ran according to the method of Doucet and Trifaro (1988). Electrophoresis was performed at a constant amperage of 50 mA per gel for 2.65 hours at room temperature. The molecular mass and pI of the sample proteins was determined using two-dimensional SDS-PAGE standards (Bio-Rad) and carbamylated carbonic anhydrase (Pharmacia Biotech). Gels were stained with silver using the Pharmacia Silver Stain kit and then dried between sheets of gel drying film (Promega Corp.) at room temperature. Stained gels were scanned using a PDI 325OE densitometer (PDI, Huntington Station, N.Y.) and the data was analyzed with Image Master software (Pharmacia Biotech).

Western Blot Analysis

Western blots were prepared by running DNA-crosslinked samples (30 μg) on a 12% SDS gel according to the method of Laemmli (1970) at room temperature for 45 min at 200 V. Proteins were then transferred from the SDS gel on to a nitrocellulose membrane (Bio-Rad) in the presence of CAPs transfer buffer (25 mM of 3-[cyclohexylamino]-1-propanesulfonic acid, pH 10, 20% methanol (v/v)). The protein transfer took place overnight at 30 V and 4°C. Following transfer, the membrane was incubated for 1 hour at room temperature in a blocking solution containing 5% skim milk and 0.2% Tween-20 in 1X Tris-Buffered Saline (TBS: 100 mM Tris-Cl, pH 7.5, 0.9% NaCl). The membrane was then washed two times in 1X TBS containing 0.2% Tween-20, and immunochemically stained with the human anti-vimentin antibody (Monosan, Uden, The Netherlands), and the goat anti-mouse antibody linked to horseradish peroxidase (Sigma) using the ECL (enhanced chemiluminescence) detection system (Amersham Life Science Inc., Arlington Heights, Illinois).

Results

Cell Lines and Cell Culture

The human breast cancer cell lines used for investigating changes in the composition of DNA-binding proteins over breast cancer progression were T-47D, MCF-7, ZR-75, BT-20, MDA-MB-231, MDA-MB-468, and T5-PRF. In addition, the cell line MCF-10A1, a non-tumorigenic human breast epithelial cell line arising from spontaneous immortalization of breast epithelial cells obtained from a reduction mammoplasty, was chosen as the closest representative to normal breast epithelial cells. The characteristics of these cell lines are shown in Table 1. According to the major characteristics observed in early and late stages of malignant progression in human breast cancer (Table 2), the cell lines T-47D, MCF-7 and ZR-75 mimic the phenotype of early stage breast cancer while the cell lines T5-PRF, BT-20, MDA-MB-231 and MDA-MB-468 mimic late stage breast cancer. All these cell lines were cultured by Dr. Shanti Samuel.

The human breast cancer cell lines used for investigating the effects of estrogen on cytokeratins levels within human breast cancer cells were the T-47D5 and T5-PRF cell lines. The T-47D5 cell line was originally thought to be a subclone of T-47D cells, but was later identified by DNA fingerprinting as an MCF-7-related cell line (Watts et al., 1992). These two cell lines were grown and hormonally treated by Ms. Amanda Coutts.

Table 1. Major characteristics of human breast cancer cell lines, as well as a human breast epithelial cell line.

Cell Line	Tumor Type[α]	Tissue Source[β]	ER Status[γ]	Estrogen Dependence	Estrogen Responsiveness		Antiestrogen Sensitivity	Invasive Capacity[ζ]	Metastatic Capacity[κ]	
T-47D	IDC	PE	+ +[μ]	+[μ]		+[μ]	+	+		
T-47D5	IDC	PE	+[ν]	+[ν]	+[ν]		+[ν]	ND	ND	
MCF-7	IDC	PE	+ +[ρ]	+[ρ]		+[σ]	++	++		
ZR-75	IDC	Ascites	+ +[ς]	+ω		+[τ]	+	+		
T5-PRF	IDC	PE	+ -[ν]		-[ν]		+[ν]	ND	ND	
MDA-MB-231	AC	PE	- -[ρ]		-[ρ]		-[έ]	+++++	+++++	
MDA-MB-468	AC	PE	- -[χ]		-[ψ]		-[έ]	+	+++	
BT-20	IDC[λ]	OT[λ]	-[ς] -[ς]		-[ς]		+[ή]	ND[λ]	ND	
MCF-10A1	NT[Ω]	RM[Ω]	-[Ω]	-[Ω]	-[Ω]		ND	ND	ND	

α Unless otherwise specified, the histopathological diagnosis of the cell lines was determined by Thompson et al. (1992): IDC, infiltrating ductal carcinoma; AC, adenocarcinoma; NT, non-tumorigenic.

β Unless otherwise specified, tissue source was obtained from Thompson et al. (1992): PE, pleural effusion; OT, original breast tumor; RD, reduction mammoplasty.

γ Unless otherwise specified, ER status was determined by Sommers et al., 1989.

ζ Activity of each cell line in Boyden chamber chemoinvasion assay. Activity is graded as % MDA-MB-231: +, 0-20%; ++, 20-40%; +++, 40-60%; ++++, 60-80%; ++++, >80%. ND, not determined. Performed by Thompson et al. (1992).

κ Activiy in Boyden chamber chemotaxis toward fibroblast-conditioned medium. Activity is graded as % MDA-MB-231: +, 0-20%; ++, 20-40%; +++, 40-60%; ++++, 60-80%; ++++, >80%. ND, not determined. Performed by Thompson et al. (1992).

λ Although this cell lines was not included in Boyden chamber chemoinvasion assays, BT-20 has been shown in a previous study to have a higher grade of infiltrative growth into precultured heart fragments than the MCF-7 human breast cancer cell line (Mandeville et al., 1987).

μ Determined by Reddel et al. (1984).

ν Determined by Coutts et al. (1996).

ρ Determined by Seibert et al. (1983).

σ Determined by Sutherland et al. (1983).

ς Determined by Engel and Young (1978).

τ Determined by Lippman et al. (1976).

χ Determined by Armstrong et al. (1992).

ψ Determined by Kenney et al. (1993).

Ω Determined by Soule et al. (1990)

ω Determined by Engel et al. (1978)

έ Determined by Toi et al. (1992)

ή Determined by Reddel et al. (1985); however, the cell line BT-20 was much less sensitive to antiestrogen than the T-47D, MCF-7 and ZR-75 cells lines.

Table 2. Major characteristics of early and late stage breast cancer (Clarke et al., 1990).

Early	Late
Hormone-Dependent	Hormone-Independent
Estrogen responsive	Estrogen nonresponsive
Poorly Invasive	Highly invasive
Poorly metastatic	Highly metastatic
Antiestrogen-sensitive	Antiestrogen-insensitive

Crosslinking of DNA to proteins by cis-DDP *in situ* in breast cancer cell lines

DNA-binding protein profiles from hormone-dependent (T-47D, MCF-7, ZR-75) and hormone-independent (T5-PRF, MDA-MB-231, MDA-MB-468, BT-20) human breast cancer cell lines were analyzed to identify alterations in the patterns of DNA-binding proteins over breast cancer progression from a hormone-dependent to a hormone-independent stage. A non-tumorigenic human breast epithelial cell line arising from spontaneous immortalization of breast epithelial cells obtained from a reduction mammoplasty was chosen as the closest representative to normal breast epithelia. In order to obtain DNA-binding protein profiles, each cell line was treated with 1 mM cis-DDP in an environment with a physiological pH and temperature (37°C), and a very low Cl^- ion (4 mM) concentration, and proteins crosslinked to DNA *in situ* were isolated. This cis-DDP treatment crosslinked proteins bound to DNA *in situ*. As a result, a low Cl^- ion concentration of the crosslinking buffer was required since the hydrolysis of cis-DDP into its active DNA-binding (Dedon and Borch, 1987) and protein-binding (LeRoy and Thompson, 1989) form is significantly inhibited in high chloride

environments (Chu, 1994). Proceeding isolation, the proteins crosslinked to DNA in each cell line were resolved by two dimension gel electrophoresis, and the resulting gels were stained with silver. The silver stained two-dimensional gels were then scanned using a densitometer and analyzed using an Image Master System (Pharmacia BioTech). Crosslinking experiments were performed three times for each cell line. Only proteins that consistently appeared in all three crosslinked profiles of a cell line were considered representative DNA-binding proteins.

Analysis of proteins crosslinked to DNA by cis-DDP *in situ* in breast cancer cell lines

Identification of common DNA-crosslinked proteins

Once the DNA-binding protein profile of each cell line was identified, a comparison was made among the DNA-binding protein profiles of the human breast epithelial cell line (Figure 2), and the 3 hormone-dependent (Figure 3) and 4 hormone-independent (Figure 4) human breast cancer cell lines previously mentioned. From this comparison, 4 distinct clusters (A, B, C, D of Figure 5) were identified as common to the profiles of proteins-crosslinked to DNA by cis-DDP in all eight cell lines. Thus, all profiles of proteins crosslinked to DNA by cis-DDP were compared using clusters A, B, C, and D as alignment markers. The molecular weight and isoelectric point coordinates of each cluster are listed in Table 3. These 4 clusters account for the majority of proteins that were abundantly crosslinked to DNA. Thus, human breast epithelial, and human breast cancer cells display a large similarity in their DNA-binding protein profiles.

Table 3. Molecular weight range and isoelectric point range of four DNA-crosslinked protein clusters common to pseudo-normal breast epithelial and breast cancer cell lines.

Protein Cluster	MW range (kDa) of proteins within cluster	pI range of proteins within cluster
A	57	5.4 - 5.5
B	59	5.0 - 5.25
C	66 - 71	5.25 - 5.45
D	38.5 - 54	4.6 - 5.4

A comparison of the isoelectric point and molecular weight coordinates of the prominent commonly crosslinked proteins with the coordinates of proteins identified in previous studies shows that the transcription factor hnRNPK was crosslinked to DNA to the same extent in all the cell lines studied. Lamins A and C were also evident in all crosslinked preparations when higher loads (80 μg) of protein were used (Figures 2, 3, 4).

In addition to lamins A and C, and hnRNPK, cytokeratins 8 (54 kDa, pI 5.4), 18 (45 kDa, pI 5.3), and 19 (41 kDa, pI 4.9) also appeared as prominent proteins in the profiles of proteins crosslinked to DNA *in situ* by cis-DDP for the cell lines studied (Figures 2, 3, 4). These 3 cytokeratins were identified in a previous study as abundant proteins in the NM-IF fraction from T-47D5 hormone dependent breast cancer cells by microsequencing polypeptides excised from a two dimension gel (Coutts et al., 1996). Therefore, this analysis provided the precise locations of these three cytokeratins in the two dimension crosslinked protein profiles. Furthermore, cK8, cK18 and cK19 in the NM-IF preparation comigrated with the three polypeptides p54, p45, and p41 of the cis-DDP DNA-crosslinked protein preparation resolved on two dimension gels (data not shown).

Identification of differences in DNA-crosslinked protein patterns of the various breast cancer cell lines studied

Despite the large degree of similarity in the DNA-binding protein profiles amongst the cell lines, differences were observed between DNA-crosslinked preparations of the pseudo-normal breast epithelial, and the hormone-dependent and hormone-independent breast cancer cell lines (Figures 2, 3, 4). These differences are summarized in Table 4. To confirm that these differences were not due to loading error, the protein representing hnRNPK in the two-dimensional profiles of all the cell lines studied was used as an internal loading control. This protein appeared to be crosslinked to DNA to a similar extent in all the cell lines used.

In the pseudo-normal breast epithelial cell line, MCF-10A1, two DNA-binding proteins (BC1: 36.5 kDa, pI 4.3; BC2: 52 kDa, pI 5.9) were present at higher levels than those observed in cis-DDP preparations of the other cell lines (Figure 2). In addition, DNA-crosslinked protein profiles of hormone-dependent T-47D and MCF-7 breast cancer cell lines showed an increase in abundance of proteins within cluster D compared to the crosslinked profiles of other cell lines (Figure 3). This increase was particularly noticeable for proteins within the molecular weight range of 48 to 53 kDa, and the isoelectric point range of 5.2 - 5.4. A comparison of the isoelectric point and molecular weight coordinates of these proteins with those of IF proteins isolated from the human breast cancer cell lines MDA-MB-231 indicated that a significant portion of the cluster D proteins may be IF proteins. Within this cluster are three proteins (BC3: 47 kDa, pI 5.25, BC4: 46 kDa, pI 5.2, BC5: 43 kDa, pI 5.1) found predominantly in the T-47D and MCF-7 cell lines that are either absent or barely detectable in the DNA-crosslinked preparations of the other cell lines.

Table 4. cis-DDP DNA-crosslinked proteins of hormone-dependent and hormone-independent human breast cancer lines.

	Cell Line							
Protein Name	T-47D	MCF-7	ZR-75	T5-PRF	MDA-MB-231	MDA-MB-468	BT20	MCF10A1
BC1	-	-	-	-	-	-	-	+
BC2	-	-	-	-	-	-	-	+
BC3	+	+	-	+(L)	-	-	-	-
BC4	+	+	-	+(L)	-	-	-	-
BC5	+	+	-	-	-	-	+(L)	-
BC6	-	-	-	-	+	+	+	-
BC7	-	+(L)	-	+(L)	-	+	+	-
BC8	-	+(L)	-	-	+	+	-	-
BC9	+(L)	-	-	-	+	+	-	-
BC10	-	-	-	+(L)	+	-	-	-
BC11	-	-	-	-	-	-	+	-
BC12	-	-	-	-	+	-	-	-
BC13	+(L)	+(L)	+(L)	-	+	+	+	+(L)
BC14	-	-	-	-	+	-	-	-

+/- Indicates the presence (+) or absence (-) of a DNA-binding protein from a two-dimensional profile of DNA-crosslinked proteins.

+(L) Signifies a lower abundance of this protein when compared to levels seen in other cell lines. Abundance was determined using hnRNPK as a loading control.

Similar to the hormone-dependent breast cancer and pseudo-normal breast epithelial cell lines, the hormone-independent breast cancer cell lines also displayed DNA-binding proteins that were either particularly abundant or exclusive to a hormone-independent breast

protein (BC6: 41 kDa, pI 4.65) was most abundant in the cis-DDP crosslinked protein preparations of the 4 hormone-independent cell lines, while one other DNA-binding protein (BC7: 46 kDa, pI 4.9) was significantly more abundant in DNA-crosslinked protein preparations of the BT-20 and MDA-MB-468 cell lines compared to the other cell lines studied. Likewise, three DNA-binding proteins (BC8: 110 kDa, pI 4.7; BC9: 44.5 kDa, pI 4.1; BC10: 34 kDa, pI 4.55) were found most abundantly in DNA-crosslinked protein preparations of both the MDA-MB-231 and MDA-MB-468 cell lines compared to the other cell lines studied. In addition, the BT-20 cell line displayed a cluster of DNA-binding proteins (BC11: 79 kDa, pI 5.2-5.4) that was not detected in DNA-crosslinked protein preparations of any other cell line studied. Furthermore, one protein bearing similar coordinates to proteins within MDA-MB-231 IF preparations (BC12: 57 kDa, pI 5.0) was most abundant in the MDA-MB-231 cell line.

Western blot analysis of a two dimensional profile of MDA-MB-231 proteins crosslinked to DNA by cis-DDP using a vimentin antibody produced a signal at the same molecular weight and isoelectric point coordinates as the BC12 protein. However, the strength of this signal was not equivalent to the intensity of the BC12 protein when stained with silver, suggesting that either vimentin is co-migrating with a protein of a similar molecular weight and isoelectric point or that the association of vimentin with DNA changes the shape of vimentin in a way that hides the epitope recognized by the antibody. Besides BC12, two other proteins were identified most abundantly (BC13: 46 kDa, pI 4.6; BC14: 44.5 kDa, pI 4.65) in the DNA-crosslinked protein preparations of the MDA-MB-231 cell line when compared to the crosslinked preparations of the other cell lines studied. Like

BC12, these proteins also have molecular weight and isoelectric point coordinates that were similar to proteins within MDA-MB-231 IF preparations.

Comparison of NM protein and cis-DDP DNA-crosslinked protein two-dimensional patterns

After two-dimensional profiles of proteins crosslinked to DNA by cis-DDP *in situ* in the cell lines were compared to one another, the two-dimensional profile of cis-DDP DNA-crosslinked proteins from the T-47D cell line was compared to a two-dimensional profile of NM proteins from this same cell line (Figure 6). The preparation and analysis of all NM profiles presented in this paper was performed by Dr. Shanti Samuel in a previous study (Samuel et al., 1997), while any comparision of the NM profiles with DNA-crosslinked profiles was performed by myself. The comparison of the T-47D NM and DNA-crosslinked protein profiles showed a large similarity in the composition of both preparations. The NM preparation displayed the four clusters of abundant proteins observed in the DNA-crosslinked protein preparations from this study (Figure 5). Since the 4 alignment clusters were not as evident at lower loads (40 μg) in the crosslinked preparation, a protein designated as NMP1 (46 kDa; pI, 5.0) also served as a point of reference for comparing the two-dimensional profiles of NM and DNA-crosslinked protein preparations.

In addition to the four clusters, the proteins hnRNPK, lamins A and C, and cytokeratins 8, 18 and 19, which were prominent proteins in DNA-crosslinked preparations, also appeared to be prominent NM proteins in the T-47D cell line. Similar observations were made when comparing the NM profiles of the other cell lines used in this study with their respective DNA-crosslinked pattern (Figures 2, 3, 4, 7, 8). Furthermore, this comparison

showed that the majority of proteins (BC3, BC4, BC5, BC6, BC10, BC11, BC12, BC13, BC14) with a differential abundance between hormone-dependent and hormone-independent breast cancer cell lines were also identified in the NM profiles of the respective cell line from which they were isolated. Table 5 summarizes the presence or absence of DNA-binding proteins with a differential abundance between the 3 hormone-dependent and 3 of the 4 hormone-independent breast cancer cell lines studied. The large similarity between NM and cis-DDP DNA-crosslinked protein profiles suggests that most of the abundant proteins crosslinked to DNA with cis-DDP are NM proteins.

Despite the large similarity between the NM and DNA-crosslinked protein profiles of the T-47D cell line, several proteins that were abundant NM proteins in these cells did not appear in the profile of proteins crosslinked to DNA by cis-DDP in T-47D cells (NMP2: 49kDa, pI 5.0; NMP3:55 kDa, pI 5.45; NMP4: 52 kDa, pI 5.5) (Figure 6). In addition, when comparing the two-dimensional profiles of DNA-crosslinked proteins in the other cell lines with NM profiles of their respective cell line, many DNA-crosslinked proteins were either barely detectable or absent from NM preparations (Figures 2, 3, 4, 7, 8). For instance, the protein BC5 was easily identifiable in the MCF-7 DNA-crosslinked protein preparation, but could not be detected in the MCF-7 NM profile. As well, other proteins (BC1, BC2, BC7, BC8, BC9) crosslinked to DNA by cis-DDP could not be detected in the NM preparations of the cell lines from which they were isolated.

Table 5. Status of cis-DDP DNA-crosslinked proteins of hormone-dependent and hormone-independent breast cancer cell lines in the NM profiles of 7 of the 8 cell lines studied.

	Cell Line						
Protein Name	T-47D	MCF-7	ZR-75	T5-PRF	MDA-MB-231	BT20	MCF10A1
BC1	-	-	-	-	-	-	-
BC2	-	-	-	-	-	-	-
BC3	+	+	-	+	+	+	+
BC4	+	+	-	+	-	+	+
BC5	+	-	-	-	-	-	+
BC6	-	-	-	-	+	-	+(L)
BC7	-	-	-	-	-	-	-
BC8	-	-	-	-	+	-	-
BC9	-	-	-	-	-	-	-
BC10	-	-	-	-	+	-	-
BC11	-	+	-	+	+	+	+
BC12	-	-	-	+(L)	+	-	-
BC13	-	-	-	-	+	-	-
BC14	+(L)	+(L)	-	-	+	-	+

+/- Indicates the presence (+) or absence (-) of a DNA-binding protein from a two-dimensional profile of DNA-crosslinked proteins.

+(L) Signifies a lower abundance of this protein when compared to levels seen in other cell lines. Abundance was determined using hnRNPK as a loading control.

In a previous study, Dr. Shanti Samuel compared the two-dimensional profiles of NM proteins of the following cell lines: MCF-10A1, T-47D, MCF-7, ZR-75, T5-PRF, MDA-MB-231, and BT-20 (Samuel et al., 1997). As a result of this analysis, 5 NMPs designated as NMBC 1, 2, 3, 4 and 5 were found most abundantly in the 3 hormone-dependent (T-47D, MCF-7, ZR-75) and the T5-PRF, hormone-independent human breast cancer cell lines (Figure 7) as well as in hormone-dependent breast tumors, while a NMP designated NMBC 6 was most abundant in hormone-independent human breast cancer cell lines (MDA-MB-231, BT-20) (Figure 8) and hormone-independent breast tumors (Samuel et al., 1997). Thus, while comparing Dr. Samuel's NM profiles of these breast cancer cell lines with their respective DNA-crosslinked protein profiles, the presence or absence of these 6 proteins within DNA-crosslinked protein patterns was noted. Two dimension profiles of proteins crosslinked to DNA by cis-DDP displayed NMBC1 (57 kDa, pI 5.5) and NMBC2 (65 kDa, pI 5.15) as prominent DNA-binding proteins in the all the cell lines studied including MCF-10A1, whereas NMBC3 (40 kDa, pI 5.4), NMBC4 (41 kDa, pI 5.3), NMBC5 (39 kDa, pI 5.5), and NMBC6 (52 kDa, pI 5.7) could not be detected.

In addition to Dr. Samuel's identification of 6 NM proteins with potential prognostic significance in breast cancer, Khanuja and colleagues (1993) identified three NM proteins (X, Y, Z) that were specific to malignant breast tissue. Of these three proteins, however, Dr. Shanti Samuel and colleagues could only identify protein Z in the NM preparations of hormone-dependent and hormone-independent breast cancer cell lines (except for T5-PRF), and hormone-dependent and hormone-independent breast tumors. Similarly, proteins X and Y could not be detected in DNA crosslinked protein preparations of the cell lines studied,

while protein Z was present at levels just above detection in all breast cancer cell lines except for ZR-75, T5-PRF, and BT-20. However, in addition to the breast cancer cell lines, protein Z was also identified in the DNA-crosslinked protein preparations of the pseudo-normal breast epithelial cell line, MCF-10A1.

Estrogen regulation of cytokeratin-DNA interactions in breast cancer cells

A previous study showed that the levels of cytokeratins in the NM-IF fraction of T-47D5 cells were altered when cells were grown in the absence of estrogen (Coutts et al., 1996). Since cytokeratins are some of the prominent proteins crosslinked to DNA by cis-DDP, this agent was used to determine whether the interaction of cytokeratins with DNA *in situ* was regulated by estrogens. T-47D5 cells grown in the absence of estrogen for one passage were treated with cis-DDP and the crosslinked proteins from these cells along with crosslinked proteins from T-47D5 cells cultured in the presence of estrogens were electrophoretically resolved on one dimension SDS gels (Figure 9) and two dimension gels (Figure 10). Comparison of these electrophoretic patterns showed that the abundance of the cytokeratins 8, 18, and 19 bound to DNA in cells grown in the absence of estrogens was reduced to levels 0.60± 0.08, 0.64 ± 0.02, and 0.59 ± 0.11 (n=5, where three experiments were performed and two of these three experiments were repeated), respectively, of the T-47D5 cells grown with estrogen in complete media. When these estrogen-starved T-47D5 cells were passaged into 5% BS supplemented with 10 nM estrogen for 1 passage (72 hours), the amount of cytokeratins 8, 18, and 19 bound to DNA in these cells rebounded to levels higher than those found in T-47D5 cells cultured in the presence of estrogens (1.95 ± 0.22,

1.59 ± 0.22, and 1.61 ± 0.17, respectively) (n=5, where three experiments were performed and two of these three experiments were repeated).

The NM-IF fraction of long term chronic estrogen depleted T5-PRF (ER positive, hormone-independent) breast cancer cells contained elevated levels of cytokeratins 8, 18 and 19 compared to the parent T-47D5 cell line cultured in the presence of estrogens (Coutts et al., 1996). The cytokeratin 8, 18 and 19 levels in the preparation of proteins crosslinked to DNA by cis-DDP in T5-PRF cells were greater than those in preparations from T-47D5 cells cultured with estrogens (2.87 ± 0.9, 2.0 ± 0.41, and 1.93 ± 0.16, n=5, respectively).

The proteins shown in Figures 9 and 10 were from cells crosslinked with 1 mM and 3 mM cis-DDP, respectively. Scanning densitometry of SDS-PAGE gels containing DNA-crosslinked protein samples obtained by treatment of the T-47D5 and T5-PRF cell lines with 3 mM cis-DDP showed levels of cytokeratins 8, 18 and 19 in the three T-47D5 estrogen treatments as well as in the T5-PRF cell line that were similar to cytokeratin levels obtained by crosslinking cells with 1 mM cis-DDP. Thus, the relative abundance of these three cytokeratins in the crosslinked protein preparations from T-47D5 cells cultured with or without estrogens and from T5-PRF cells remained the same as the relative abundance of cytokeratins observed in cells treated with 1 mM cis-DDP.

In addition to cytokeratins 8, 18 and 19, changes in protein levels were also observed for proteins in cluster D that were immediately surrounding the three cytokeratins (Figure 10). This indicated that proteins other than cytokeratins may also be estrogen-regulated. However, the identity of these proteins remains unknown.

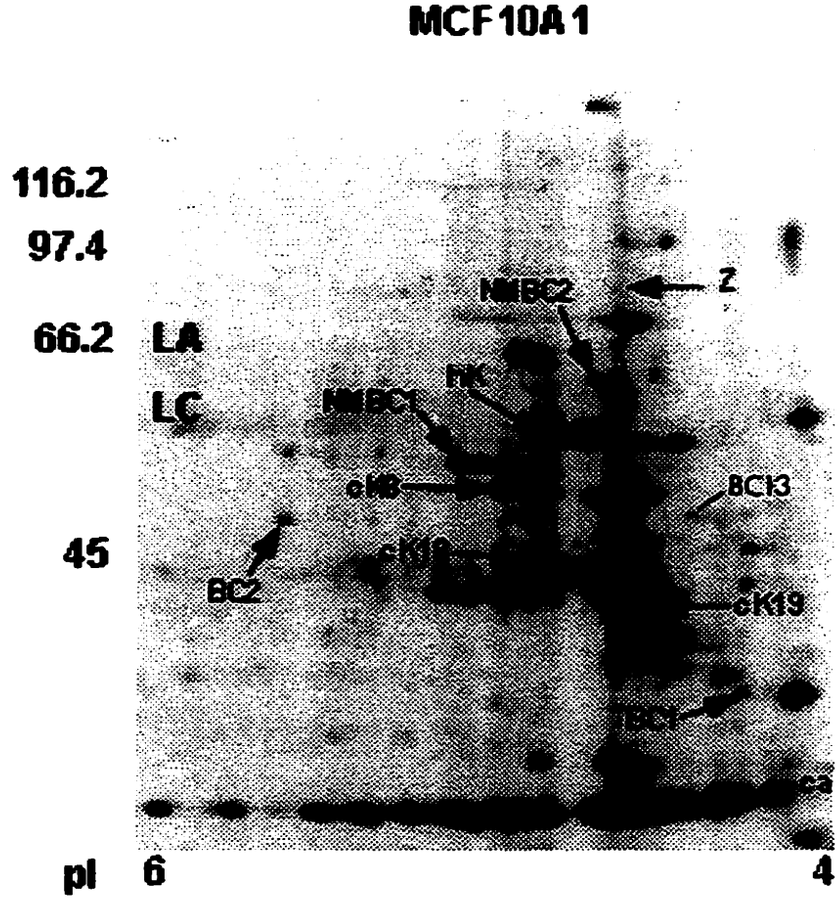

Figure 2. Proteins crosslinked to DNA by cis-DDP *in situ* from the human breast epithelial cell line MCF-10A1. Eighty μg of proteins crosslinked to DNA with 1 mM cis-DDP were electrophoretically resolved on two dimension gels. The gels were stained with silver. The position of the carbamylated forms of carbonic anhydrase is indicated by ca. The position of the molecular mass standards (kDa) is shown to the left of the two dimension gel patterns. LA and LC show the position of lamin A and C, respectively. cK8, cK18 and cK19 identify cytokeratins 8, 18 an 19, respectively. hK designates the position of transcription factor hnRNPK. NMBC1 and NMBC2 represent proteins found at higher levels in NM preparations of hormone-dependent breast cancer cell lines in a previous study (Samuel et al., 1997). Protein Z identifies the location of a NM protein previously found to be specific to malignant tissue (Khanuja et al., 1993). BC1 and BC2 represent proteins found only in MCF-10A1 cis-DDP preparations of proteins crosslinked to DNA *in situ*. BC13 represents a protein most abundant in cis-DDP DNA-crosslinked preparations of hormone-independent human breast cancer cell lines.

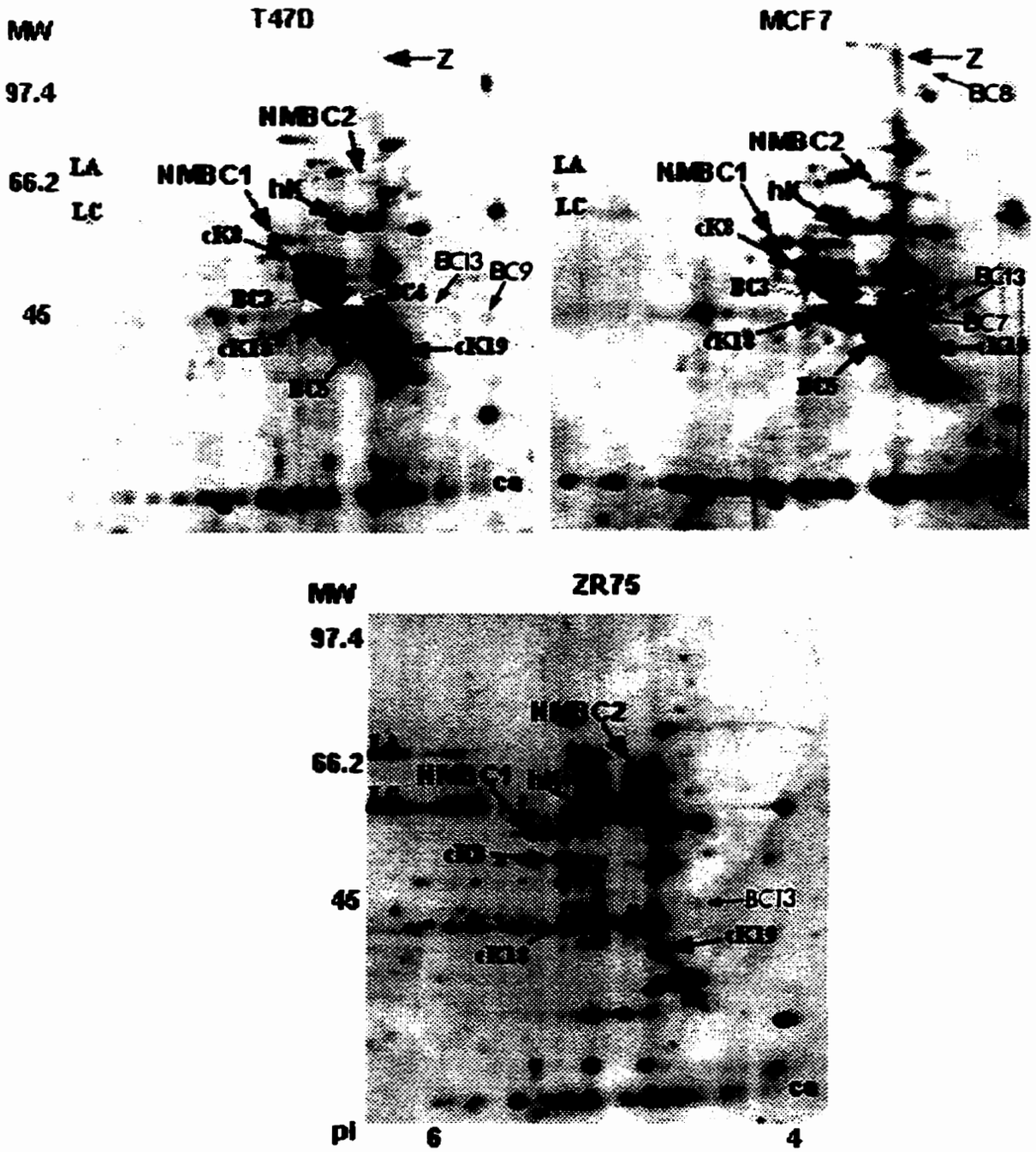

Figure 3. Proteins crosslinked to DNA by cis-DDP *in situ* from hormone-dependent breast cancer cell lines. Eighty μg of proteins crosslinked to DNA with 1 mM cis-DDP were electrophoretically resolved on two dimension gels. The gels were stained with silver. The position of the carbamylated forms of carbonic anhydrase is indicated by ca. The position of the molecular mass standards (kDa) is shown to the left of the two dimension gel patterns. LA and LC show the position of lamin A and C, respectively. cK8, cK18 and cK19 identify cytokeratins 8, 18 and 19, respectively. hK designates the position of transcription factor hnRNPK. NMBC1 and NMBC2 represent proteins found at higher levels in NM preparations of hormone-dependent breast cancer cell lines in a previous study (Samuel et al., 1997). Protein Z identifies the location of a NM protein previously found to be specific to malignant tissue (Khanuja et al., 1993). BC3, BC4, and BC5 represent proteins found predominantly in hormone-dependent cis-DDP preparations of proteins crosslinked to DNA *in situ* compared to crosslinked preparations of the other cell lines studied. BC7, BC8, BC9, BC10 and BC13 represent proteins found most abundantly in DNA-crosslinked preparations of hormone-independent human breast cancer cell lines.

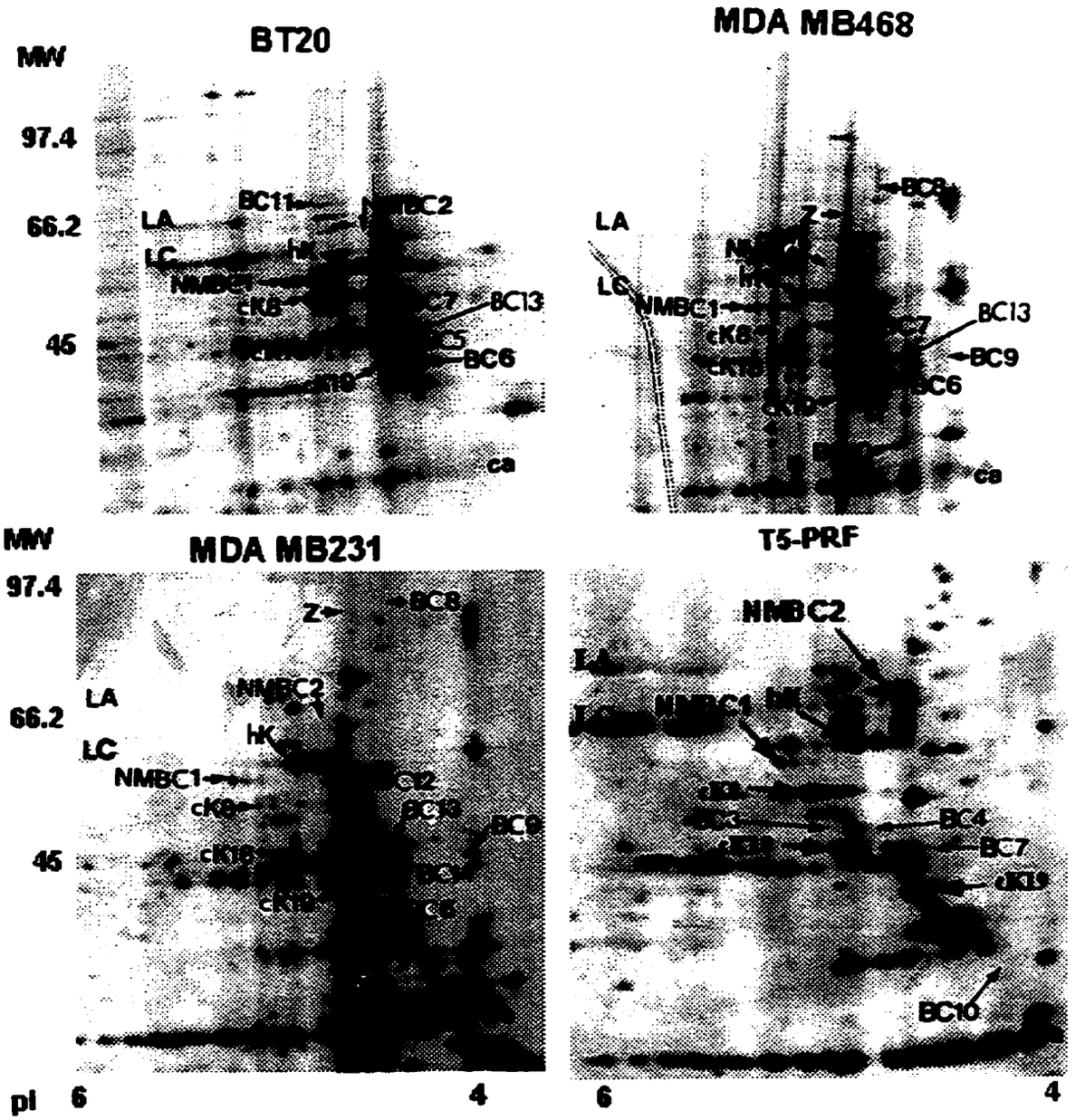

Figure 4. Proteins crosslinked to DNA by cis-DDP *in situ* from hormone-independent breast cancer cell lines. Eighty µg of proteins crosslinked to DNA with 1 mM cis-DDP were electrophoretically resolved on two dimension gels. The gels were stained with silver. The position of the carbamylated forms of carbonic anhydrase is indicated by ca. The position of the molecular mass standards (kDa) is shown to the left of the two dimension gel patterns. LA and LC show the position of lamin A and C, respectively. cK8, cK18 and cK19 identify cytokeratins 8, 18 and 19, respectively. hK designates the position of transcription factor hnRNPK. NMBC1 and NMBC2 represent proteins found at higher levels in NM preparations of hormone-dependent breast cancer cell lines in a previous study (Samuel et al., 1997). Protein Z identifies the location of a NM protein previously found to be specific to malignant tissue (Khanuja et al., 1993). BC3 and BC4 represent proteins found predominantly in hormone-dependent cis-DDP preparations of proteins crosslinked to DNA *in situ* compared to crosslinked preparations of the other cell lines studied. BC6 represents a DNA-crosslinked protein found at higher levels in three of the four hormone-independent breast cancer cell lines studied. BC7 represents a DNA-crosslinked protein that is most abundantly found in BT20 and MDA- MB-468 breast cancer cell lines. BC8, BC9, and BC10 represent DNA-crosslinked proteins that are found most abundantly in the MDA- MB-468 and MDA-MB-231 breast cancer cell lines. BC11 represents a DNA-crosslinked protein found most abundantly in cis-DDP protein preparations of theBT20 breast cancer cell line. BC12. BC13 and BC14 represent DNA-binding proteins found most abundantly in the MDA- MB-231 breast cancer cell line. BC5 represents a DNA-crosslinked protein found most abundantly in hormone-dependent human breast cancer cell lines.

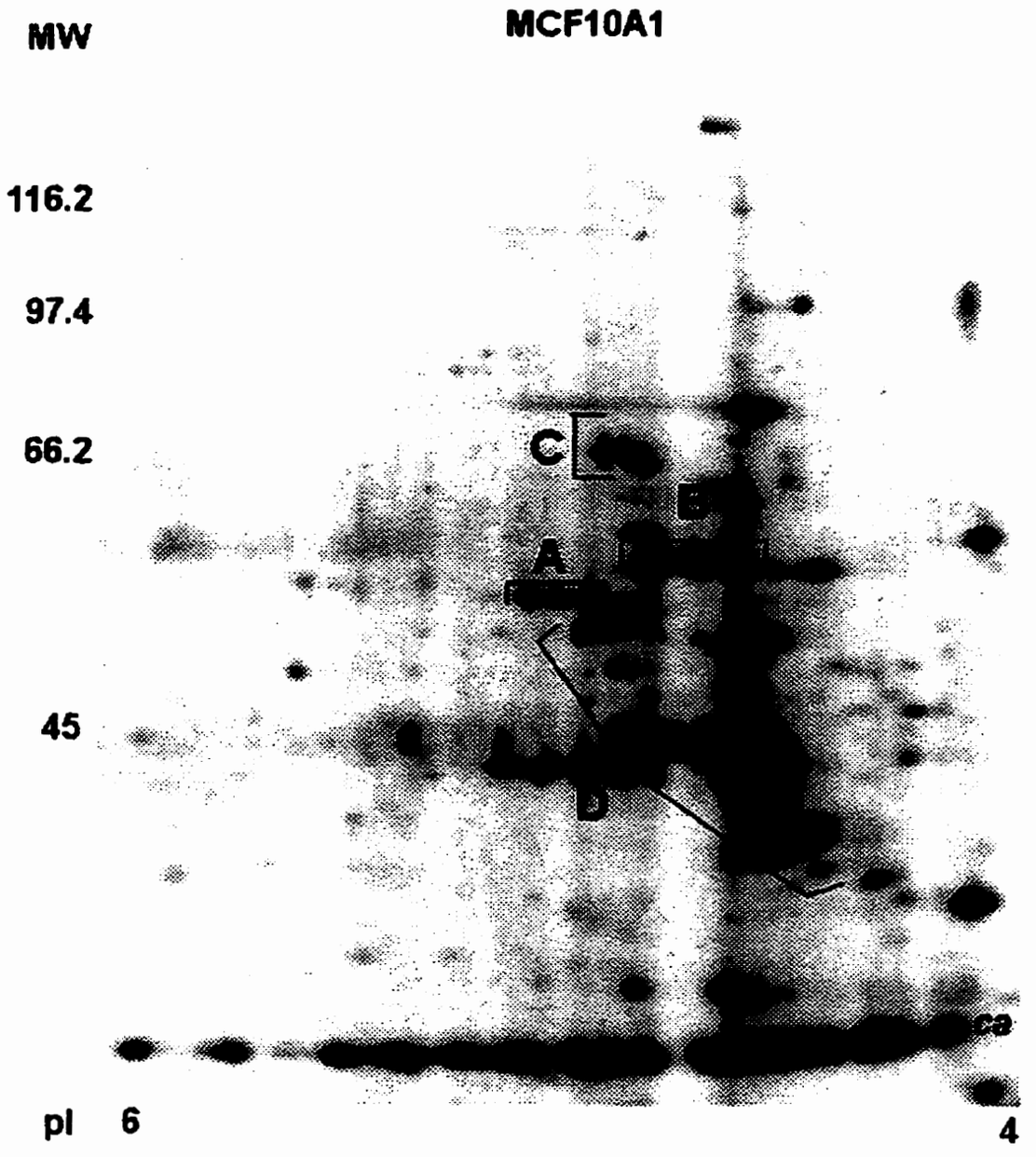

Figure 5. Proteins crosslinked to DNA by cis-DDP *in situ* that are common to breast epithelial and breast cancer cell lines. Eighty μg of proteins crosslinked to DNA with 1 mM cis-DDP from a pseudo-normal breast epithelial cell line were electrophoretically resolved on two dimension gels. The gels were stained with silver. The position of the carbamylated forms of carbonic anhydrase is indicated by ca. The position of the molecular mass standards (kDa) is shown to the left of the two dimension gel patterns. A, B, C and D designate protein clusters commonly seen in the profiles of proteins bound to DNA by cis-DDP *in situ* for the eight cell lines.

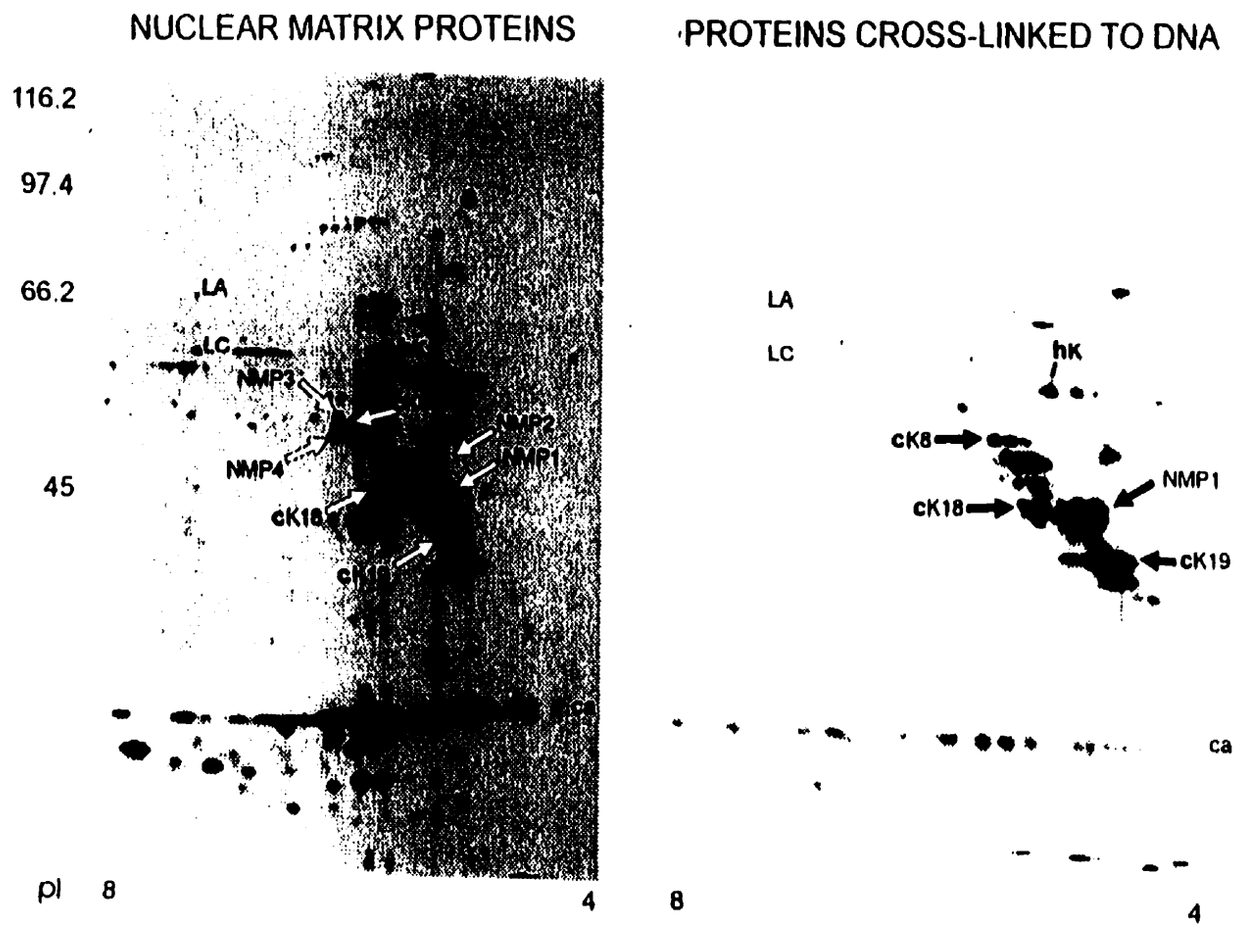

Figure 6. T47D human breast cancer nuclear matrix proteins and proteins crosslinked to DNA by cis-DDP *in situ*. Forty µg of NM proteins and 40 µg of proteins crosslinked to DNA with 1 mM cis-DDP were electrophoretically resolved on two dimension gels. The gels were stained with silver. The position of the carbamylated forms of carbonic anhydrase is indicated by ca. The position of the molecular weight standards (in thousands) is shown to the left of the two dimension gel patterns. LA and LC show the position of lamin A and C, respectively. NMP1 is a nuclear matrix protein found in both preparations. cK8, cK18 and cK19 identify cytokeratins 8, 18 and 19, respectively.

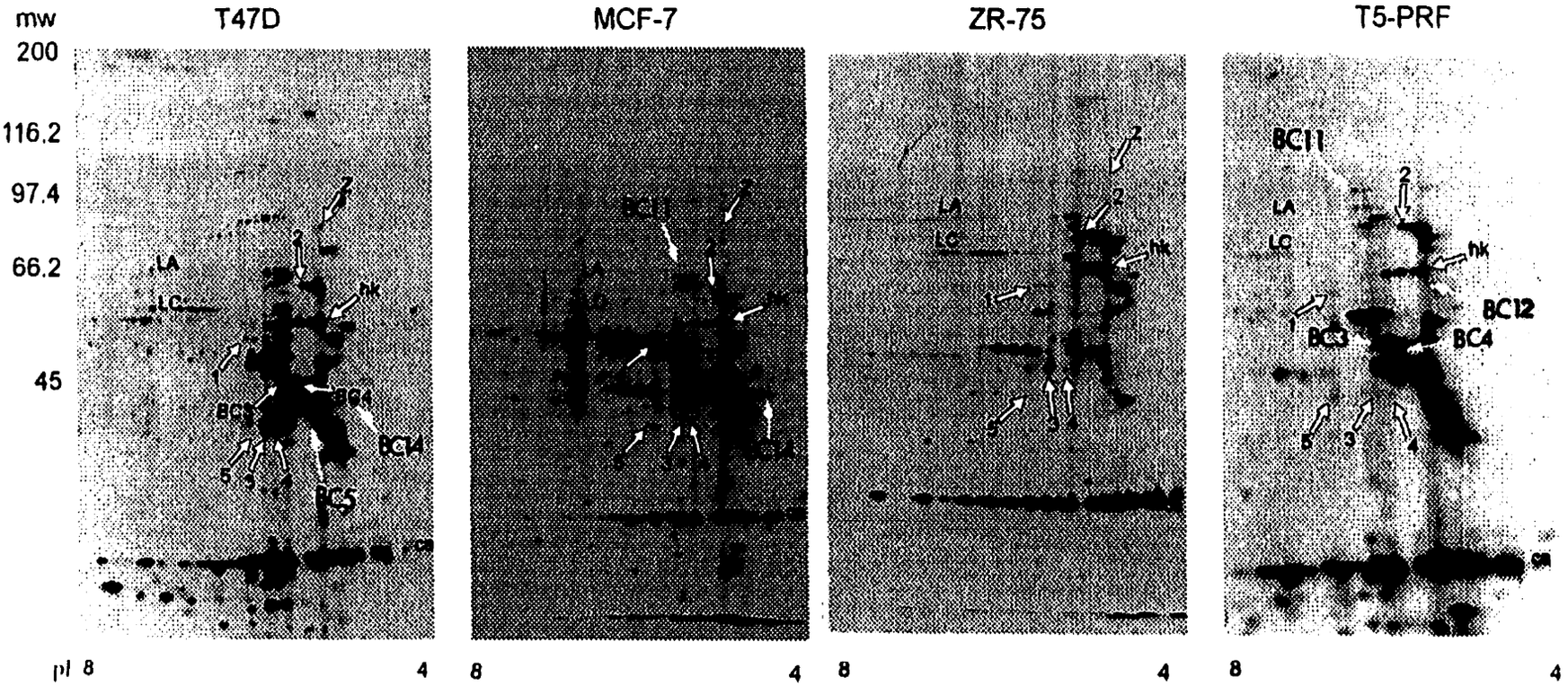

Figure 7. Nuclear matrix profiles of hormone-dependent breast cancer cell lines. Prepared by Dr. Shanti Samuel. Forty µg of protein was electrophoretically resolved on two dimension gels. The gels were stained with silver. The position of the carbamylated forms of carbonic anhydrase is indicated by ca. The position of the molecular mass standards (kDa) is shown on the left side of each two dimension gel pattern. LA and LC show the position of lamin A and C, respectively. The white arrows show the location of five nuclear matrix proteins (NMBC 1-5) identified by Samuel et al. (1997) as more abundant to the hormone-dependent breast cancer cell lines. NMBC-Z identifies the location of a NM protein previously found to be specific to malignant tissue (Khanuja et al., 1993). The location of hnRNPK is indicated as hK. BC3, BC4, and BC5 designate proteins most abundantly found in the cis-DDP preparations of proteins crosslinked to DNA in hormone-dependent human breast cancer cell lines. BC11, BC12, and BC14 represent proteins found most abundantly in cis-DDP DNA-crosslinked preparations of hormone-independent human breast cancer cell lines.

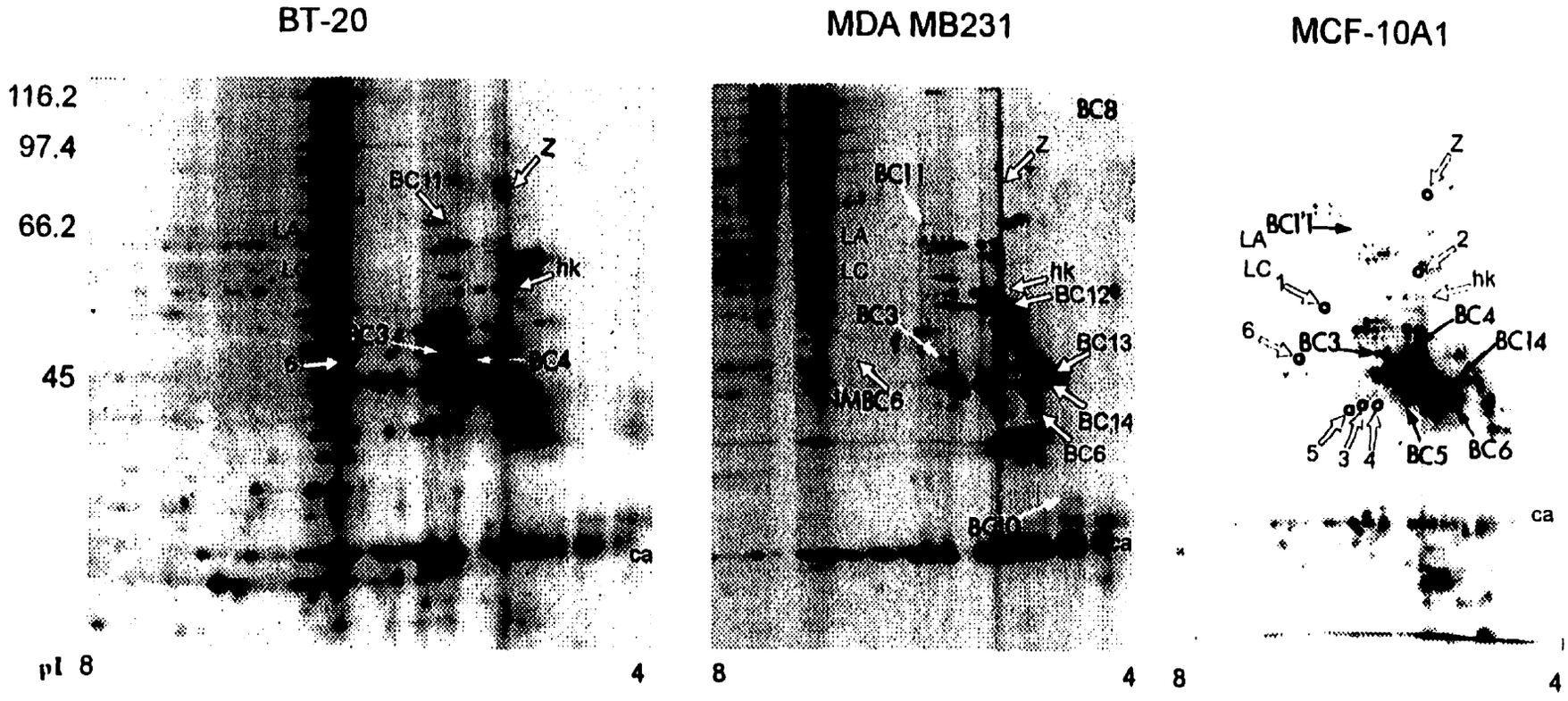

Figure 8. Nuclear matrix profiles of hormone-independent human breast cancer cell lines and a pseudo-normal breast epithelial cell line. Forty µg of protein was electrophoretically resolved on two dimension gels. Prepared by Dr. Shanti Samuel. The gels were stained with silver. The position of the carbamylated forms of carbonic anhydrase is indicated by ca. The position of the molecular mass standards (kDa) is shown on the left side of each two-dimension gel pattern. LA and LC show the position of lamin A and C, respectively. The location of the transcription factor hnRNPK is indicated by hK. The white arrows show the location of NMBC-6, a NM protein identified by Dr. Shanti Samuel as more abundant in hormone-independent breast cancer cell lines. Protein Z identifies the location of a NM protein previously found to be specific to malignant tissue (Khanuja et al., 1993). The circles show the absence of NMBCs 1-6, and NMBC-Z in the normal breast epithelial cell line. BC3, BC4, and BC5 represent proteins found predominantly in cis-DDP DNA-crosslinked preparations of hormone-dependent human breast cancer cell lines. BC6, BC8, BC10, BC11, BC12, BC13, and BC14 represent proteins most abundantly found in cis-DDP DNA-crosslinked protein preparations of hormone-independent human breast cancer cell lines.

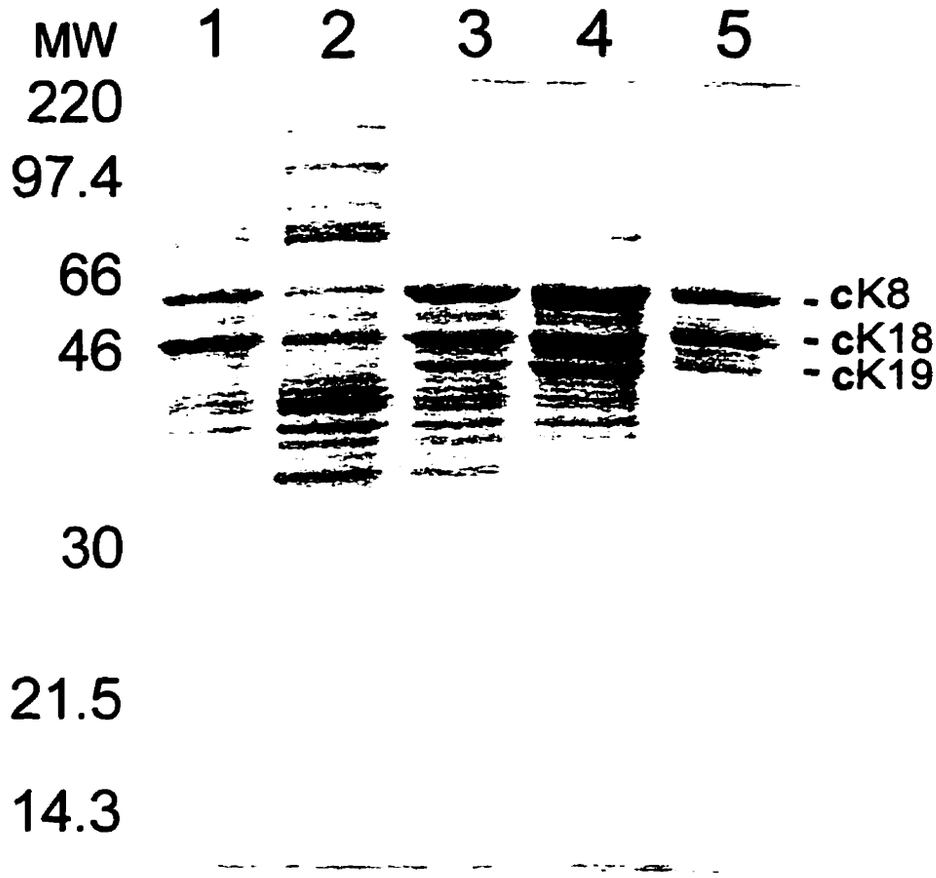

Figure 9. Cytokeratins crosslinked to DNA by cis-DDP in T-47D5 and T5-PRF human breast cancer cells in estrogen-replete and estrogen-deplete conditions. Cells were crosslinked with 1 mM cis-DDP, and 10 µg of protein crosslinked to DNA was electrophoretically resolved on a SDS gel (lane 1-4). The gel was stained with Serva Blue. Lane 1, T-47D5 cells cultured in the presence of estrogen. Lane 2, T-47D5 cells grown without estrogen for one passage. Lane 3, T-47D5 cells grown in the absence of estrogen for one passage and then cultured in the presence of estrogen for one passage. Lane 4, T5-PRF cells cultured in the absence of estrogen. Lane 5, 10 µg of protein isolated from NM-IF fraction of T5-PRF cells. The position of the molecular weight standards (in thousands) is shown on the left side of the gel. cK8, cK18, and cK19 identify the cytokeratins 8, 18 and 19.

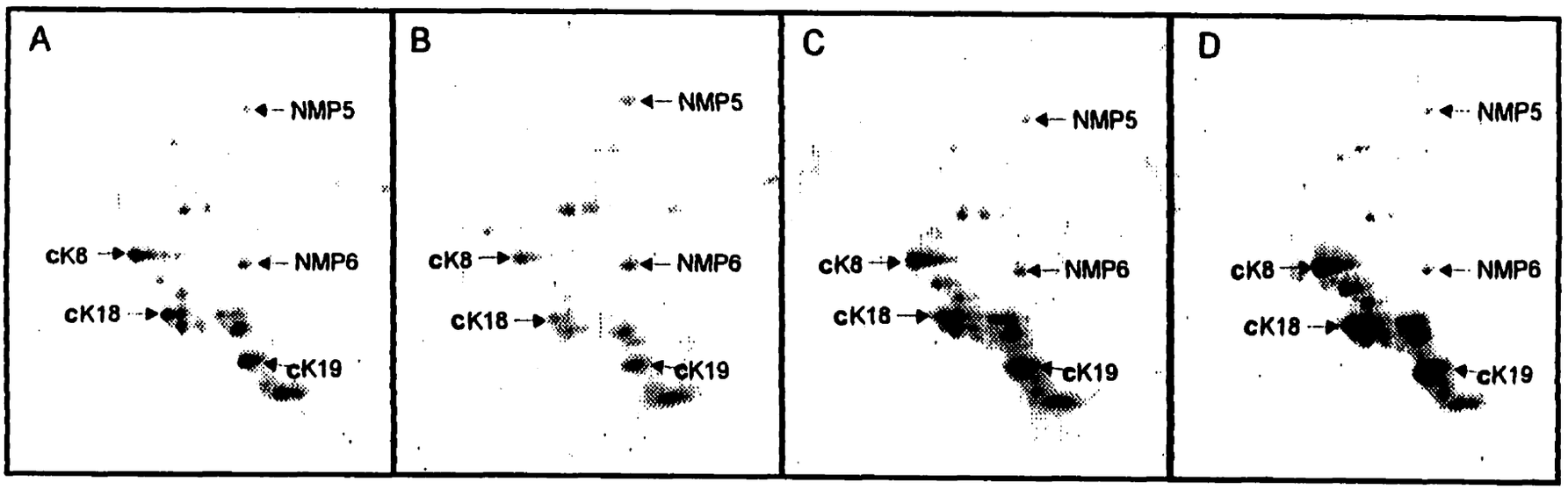

Figure 10. Two-dimensional gel patterns of proteins crosslinked to DNA by 3 mM cis-DDP in T-47D5 and T5-PRF human breast cancer cells in estrogen-replete and estrogen-deplete conditions. Twenty μg of protein crosslinked to DNA was electrophoretically resolved on two dimension gels. The gels were stained with silver. Panel A, T-47D5 cells cultured in the presence of estrogen. Panel B, T-47D5 cells grown without estrogen for one passage. Panel C, T-47D5 cells were grown in the absence of estrogen for one passage and then cultured in the presence of estrogen for one passage. Panel D, T5-PRF cells cultured in the absence of estrogen. cK8, cK18 and cK19 identify the cytokeratins 8, 18, and 19. Two NM proteins used as internal standards are shown as NMP5 and NMP6.

Discussion

At present the majority of evidence used to elucidate structural and functional aspects of DNA loop organization has been derived from *in vitro* techniques. These techniques, however, are prone to artefact formation since they involve NM extraction protocols that may strip proteins away from DNA, cause sliding of DNA over NM attachment points, or cause the exchange of proteins bound to DNA. Such events could alter or eliminate original DNA-protein interactions, therefore, causing the misidentification of NM proteins and DNA sequences involved in loop formation. An alternative *in situ* approach whereby NM protein-DNA interactions can be identified in intact cells or nuclei before physically extracting the NM or DNA from these entities would be ideal for studying DNA loop organization because it would eliminate the problems of potential artefact formation inherent to current *in vitro* techniques. Such an approach can be accomplished through the use of cis-DDP, a heavy metal complex shown to crosslink DNA predominantly to NM proteins in intact cells and nuclei (Ferraro et al., 1995; Costa, 1991). Indeed, the preference of this agent for NM proteins associated with DNA was further validated when a comparison of a NM protein profile with a cis-DDP DNA-crosslinked protein profile performed in this investigation showed that the majority of proteins crosslinked to DNA *in situ* in T-47D cells were present in the NM of the same cell line. Moreover, this agent appeared to have some specificity for particular NM proteins since a small subset of proteins (NMP2, NMP4, NMP5), presumably proteins not bound to DNA, were not detected in the cis-DDP DNA-crosslinked protein preparation.

Despite the preference of cis-DDP for NM proteins, this agent still crosslinked proteins not detected in NM preparations to DNA (eg. BC1, BC2, BC5, BC7, BC8, BC9, BC10). However, some of these proteins may have been NM proteins weakly associated with the NM that were accidentally removed by the high salt treatment used in NM isolation. In addition, the protocol used for NM isolation involved the selective removal of IF proteins by a disassembly and reassembly and/or precipitation step. Since a portion of IF proteins are tightly associated with the NM (Fey and Penman, 1988), it is possible that IF removal during NM isolation may also lead to the removal of some NM proteins. In addition, some of the DNA-crosslinked proteins not appearing in NM preparations may be IF proteins. As a result, there is uncertainty in identifying a DNA-crosslinked protein as a NM protein based on molecular mass and isoelectric point data obtained from two-dimensional patterns of NM preparations. An additional approach that could confirm the identity of cis-DDP DNA-crosslinked proteins as NM proteins would be to immunostain the DNA-crosslinked protein in question with a fluorescent antibody *in situ* and to determine if this protein is exclusively localized to the NM.

The preferential crosslinking of cis-DDP to NM proteins would, therefore, suggest that the NM contains IF proteins. It is possible that some DNA-crosslinked proteins not found in NM preparations may not be NM proteins, and are, instead, nuclear proteins simply bound to DNA. Therefore, caution must be exercised when using the cis-DDP crosslinking technique to identify NM proteins associated to DNA.

Analysis of the two dimensional patterns of proteins crosslinked to DNA *in situ* by cis-DDP showed that most proteins crosslinked to DNA by cis-DDP were common to the cell

lines used in this study. In addition, the extent of crosslinking with 1 mM cis-DDP appeared to be optimal since the levels of cytokeratins within each T-47D5 treatment and within the T5-PRF cell line did not change when the cis-DDP concentration was increased to 3 mM.

Proteins identified as common to the cell lines studied were the transcription factor hnRNPK, and lamins A and C. The protein hnRNPK is an internal matrix protein (Mattern et al., 1996 and 1997) which functions as a transcription factor that binds to specific single-stranded regions of DNA (Michelotti et al., 1996). Since hnRNPK is from the NM, any DNA sequence associated with this protein would be considered a MAR. Furthermore, nuclear lamins A and C have been identified in NM preparations (Nakayasu and Berezney, 1991), and lamin A has been shown to specifically bind single-stranded MAR DNA sequences *in vitro* (Luderus et al., 1994). The crosslinking of nuclear lamins (especially lamin C) has also been observed in Novikoff hepatoma cells incubated in 2 mM cis-DDP for 8 hours. The presence of these 3 proteins in all cis-DDP preparations is consistent with the findings of Turano and colleagues which show that MAR sequences are well represented in DNA fragments crosslinked to NM proteins by cis-DDP *in situ* (Ferraro et al., 1995 and 1996).

In addition to hnRNPK and lamins A and C, cK8, cK18 and cK19 also appeared in the common subset of NM proteins crosslinked to DNA *in situ* by cis-DDP in all cell lines. The identification of these three cytokeratins in human breast cancer cells agrees with findings that immortalized cells have increased levels of cK18 and tumor cells have increased levels of cK8, cK18 and cK19 compared to normal epithelial cells (Trask et al., 1990). In addition, the crosslinking of these 3 cytokeratins to DNA agrees with results of Hnilica and colleagues who found the principal proteins crosslinked to DNA by cis-DDP *in situ* in Novikoff

hepatoma nuclei to be cytokeratins p39 (cK19), p49 (cK18) and p56 (cK8) (Olinski et al., 1987; Ward et al., 1984).

Another IF protein identified in cis-DDP DNA-crosslinked protein preparations in the MDA-MB-231 ER- human breast cancer cell line was vimentin. The identification of this protein as a DNA-binding protein agrees with the findings of a previous study that showed vimentin to selectively bind DNA sequences *in vitro* (Wang et al., 1996b). In this study, the DNA sequences binding to vimentin had sequences characteristic of MARs, sequences recognized by transcription factors, or sequences whose structural properties are important in recombination and gene expression. However, the crosslinking of proteins to DNA in human breast cancer cells appears to be the first case where the association of vimentin with DNA was shown *in situ*. The identification of this IF protein as a DNA-binding protein provides further evidence of an association of cytoskeletal IFs with the nucleus. Furthermore, the crosslinking of cytokeratins and vimentin to DNA by cis-DDP, a short distance cross-linker (approximately 4 Å) that predominantly crosslinks DNA to NM proteins, strongly suggests that some or all types of IF proteins are NM proteins, or that cytoskeletal IFs are able to penetrate the nucleus in close proximity to DNA (Olinski et al., 1987; Ward et al., 1984).

The presence of cytoskeletal IFs in the NM is a concept that is continually argued by various researchers. Fey and Penman (1988) argue that proteins present in the NM as well as other cellular fractions are not NM proteins. Thus, their definition of the NM excludes cytoskeletal IF proteins such as vimentin, actin, and RNP complexes. By creating this definition of the NM, Fey and Penman fail to consider the possibility that particular proteins

such as cytoskeletal IF proteins can be divided into two subsets: one subset that exists in the cytosolic fraction of a cell, and one subset that exists in the nucleus. Such a theory is feasible since the NM extraction protocol used by Fey and Penman (1988) was unable to completely remove cytoskeletal IF proteins from NM preparations. Thus, IFs are either tightly associated to the NM or they are actually components of the NM.

IF proteins help compose part of the CSK of eukaryotic cells (Ingber, 1993). The CSK is believed to be organized as a tensegrity network (Wang et al., 1993) that provides a cell with structural stability by maintaining a force balance between mechanical tension generated from within the CSK and from the ECM (Ingber, 1994). Application of a mechanical stress to a cell is believed to offset the force balance, causing global rearrangements in the CSK (Ingber, 1994), and, therefore, changes in IF protein organization. Such an effect could alter gene expression by rearranging IF proteins bound to DNA, and, therefore, altering chromatin loop organization. This theory further explains the suggestion of Getzenberg and Coffey (1990) that differences in the distribution of genes throughout the nucleus result from changes in the nuclear structure responsible for controlling nuclear organization. The involvement of the CSK in chromatin loop organization would be consistent with the observation that alterations in actin filaments by cytochalasin D inhibit synthesis of most proteins in mouse mammary epithelial cells in a concentration-dependent manner (Seely and Aggeler, 1991). However, such an inhibitory effect may instead be a result of the ability of cytochalasin to induce alterations in the stability of the association of mRNA with the cytoskeleton (Macoska et al., 1994).

Changes in CSK (and IF) organization may also occur through alterations in a cell's chemical composition. Products from oncogenes such as *src*, *lck*, *ras* and *raf*, may stimulate the RAS signal transduction pathway (Bortner et al., 1993). Once stimulated, the Ras signal transduction pathway may activate AP-1 and ETS transcription factors through the phosphorylating activity of kinases (Karin and Hunter, 1995). This activation may lead to the persistent expression of particular cytokeratins such as cK8 and cK18 (Pankov et al., 1994), proteins which form part of the CSK. The aberrant expression of cytokeratin genes in various carcinoma cell lines is believed to be related to the presence of abnormal cytokeratin filamentous structures often found associated with malignant transformation (Moll et al., 1982).

In addition to oncogene products, hormones such as estrogen also appear to alter CSK organization. Treatment of MCF-7 breast cancer cells with physiological concentrations of estradiol modified the cell surface by covering it with numerous microvilli. Moreover, this hormone transformed 40-50% of these MCF-7 cells into secretory cells with secretory granules (Vic et al., 1982). However, both these effects were not observed in hormone-independent cell line (BT-20). In another study, Marchisio and colleagues showed that treatment of estrogen-starved MCF-7 breast cancer cells with estrogen causes the formation of a network of keratin fibers along with rearrangements in the actin microfilaments and keratin IFs. Such effects occurred independently of the ability of estrogen to induce cell proliferation (Sapino et al., 1986).

Based on observations that rat vaginal epithelium treated with estrogen displayed an increase in the synthesis of cytokeratins (Kronenberg and Clark, 1985), it is quite possible that

the estrogen-induced CSK alterations observed in the two previously mentioned studies on the MCF-7 cell line may have proceeded through the ability of this hormone to alter cytokeratin levels. The effect of hormones on cytokeratin levels has also been observed in a previous study where androgen, but not estrogen, repressed the levels of cK8 and cK18 in the rat ventral prostate while anti-androgenic compounds had opposite effects (Hsieh et al., 1992). Moreover, Coutts et al. (1996) showed that the levels of cK8, cK18 and cK19 present within NM-associated IFs were dramatically reduced in an ER+, hormone-dependent cell line (T-47D5) grown in acutely estrogen-depleted conditions. Treatment of these estrogen-starved cells with estrogen restored the cytokeratins levels. However, cells chronically depleted of estrogen over expressed all 3 cytokeratins compared to the T-47D5 parent grown in the presence of estrogen. Thus, all 3 cytokeratins were estrogen regulated in hormone-dependent T-47D5 human breast cancer cells, and this estrogen regulation was lost once these cells obtained a hormone-independent phenotype.

Taking the study by Coutts et al. (1996) one step forward, proteins from T-47D5 cells grown under the same conditions were crosslinked to DNA *in situ* by cis-DDP in order to determine if changes in the ability of estrogen to effect the levels of cK8, cK18 and cK19 bound to DNA occur during progression from hormone-dependence to hormone-independence. The observed decrease in levels of DNA-crosslinked cK8, cK18 and cK19 in T-47D5 cells cultured in estrogen deplete conditions for 1 passage combined with the dramatic increase upon passaging these cells into estrogen-replete conditions shows that the interaction of cytokeratins and DNA is regulated by estrogen in hormone-dependent breast cancer cells. This observation implies that a reorganization of nuclear DNA has occurred in

these cells when estrogens are removed.

In contrast, the ER positive human breast cancer cell line, T5-PRF, that has acquired the ability to grow normally in a medium greatly depleted of estrogens had higher amounts of cytokeratins associated with the NM and DNA than did the parent cell line cultured with estrogen. Furthermore, Coutts et al. (1996) showed that treatment of the T5-PRF cell line with estrogen does not lead to further up-regulation of these cytokeratins (Coutts et al., 1996).

The elevation of DNA-binding cytokeratin levels in the T5-PRF cell line suggests that acquisition of a hormone-independent phenotype may, in part, result from an increase in the production of DNA-binding cytokeratins and/or from changes in the CSK structure that increase cytokeratin-DNA interactions. These events, however, may not be involved in ER-, hormone-independent breast cancer cells since cell lines with these phenotypes (eg. MDA-MB-231 and HBL100) do not display an upregulation of cytokeratin levels in the presence or absence of estrogen (Coutts et al., 1996). Instead, the cell lines, MDA-MB-231 and HBL100, display vimentin as a prominent IF. This suggests that the mechanisms responsible for hormone-independence in ER+ cell lines most likely differ from those in ER- cell lines. However, despite the different mechanisms involved for the different cell lines, the acquisition of a hormone-independent phenotype most likely involves alterations in the cell structure through changes in the cytoskeletal IF component of the CSK and the NM.

These findings are consistent with those of Vic and colleagues (1982) who observed no effects of physiological concentrations of estrogen on the CSK of hormone-independent cells. More specifically, the T5-PRF cell line has apparently lost the capacity of estrogen to

regulate cytokeratin interaction with nuclear DNA: the organization of nuclear DNA mediated by cytokeratins in later stage breast cancer is maintained regardless of whether estrogen is present or absent. Similar changes in levels of other proteins crosslinked to DNA within the 38.5-54 kDa range and within a pI range of 4.6-5.4 were also observed in both T-47D5 treatments and in the T5-PRF cell line, however the identity of these proteins remains to be determined.

How the T5-PRF cell line manages to express elevated levels of these estrogen-regulated proteins in estrogen-deplete conditions still needs to be determined. In one of the current models of breast cancer development, the effect of estrogen on disease progression is believed to be mediated through the estrogen receptor (ER) (Landers and Spelsberg, 1992). However, the elevated expression of cK8, cK18 and cK19 in estrogen-deplete conditions suggests that, during disease progression, human breast cancer cells develop the ability to activate the ER through pathways other than those involving interactions between estrogen and the ER. In support of this theory is the observation that estrogen-independence in breast cancer cells is associated with changes in the expression of estrogen-regulated genes (Brunner et al., 1993). Alternatively, estrogen-independence may be a result of the ability of the ER to be activated in a ligand-independent manner (Aronica and Katzenellenbogen, 1993). However, the possibility exists that, instead of having ligand-independent mechanisms of ER activation, the breast cancer cell may become supersensitive to estrogen, and, therefore, only require minute amounts of this hormone for growth.

The observation that estrogen regulates the expression of DNA-binding cytokeratins only in hormone-dependent cell lines is important in understanding breast cancer development

since the progression of breast epithelial cells to malignancy is accompanied with increased expression of cK8, cK18 and cK19 (Trask et al., 1990). The presence of cytokeratins only in epithelial cells (Osborn and Weber, 1982) along with their differential expression over breast cancer progression (Trask et al., 1990) makes cytokeratins one of the classical diagnostic markers for breast cancer.

While cytokeratins may be markers of malignancy, the IF protein vimentin may be a marker for breast cancer progression. Vimentin was detected only in DNA-crosslinked protein preparations of the ER-, hormone-independent breast cancer cell line MDA-MB-231. These results agree with the findings of other studies (Thompson et al., 1992; Sommers et al., 1989). However, the results of another study by Regenass and colleagues (1987) showed a heterogeneous expression of vimentin in ER+ and ER- breast cancer cell lines, as well as in normal primary human mammary cultures. Because of this, it was speculated that the heterogeneity of vimentin expression over the various breast cancer cell lines was a cause of the tissue environment and culture conditions (Curschellas et al., 1987). In another study, vimentin was only expressed in solid metastatic and primary epithelial tumors originating from human serous cavity fluids when these cells were shed into body cavities (Ramaekers et al., 1983). Thus, metastasis was associated with vimentin expression in epithelial cells. As a result, vimentin expression was considered as a process occurring *in situ* when cells lose contact with neighbouring cells or with solid tissue during metastasis (Ramaekers et al., 1983).

Despite these two studies, the expression of vimentin has been associated with various features of aggressive tumors such as the absence of hormone receptors (Thompson et al.,

1992), increased tumor cell proliferation (Thompson et al., 1992; Seshadri et al., 1996), increased tumorigenicity (Hendrix et al., 1997) , p53 expression (Seshadri et al., 1996), and hormone-independence (Sommers et al., 1989; Curschellas et al., 1987). Moreover, *in vitro* studies show that over expression of vimentin in the ER+, hormone-dependent MCF-7 cell line, a vimentin-negative cell line representative of early breast cancer development, causes enhanced cell motility, and increased invasiveness without changing the cell's metastatic potential (Hendrix et al., 1997). During this conversion to a more invasive phenotype, the transfected MCF-7 cells displayed shifts in the abundance of different integrin subunits, as well as an increased migration to laminin (Hendrix et al., 1997), a potent ECM signalling site with many different biological activities (Kobota et al., 1992). Since integrins interact with IFs (Quaranta and Jones, 1991), and since IFs are believed to influence nuclear structure by transmission of ECM signals (Ingber, 1994), vimentin may influence the cell's interpretation of ECM signals by altering the shape, spreading and migration of breast cancer cells in such a way that provides vimentin-positive cells with a selective advantage over cells not expressing this IF protein (Hendrix et al., 1997). However, the inability of vimentin alone to increase the metastatic potential of MCF-7 cells to levels observed in the vimentin-positive cell line MDA-MB-231 suggests that the presence of vimentin and cytokeratin IF proteins alone is not sufficient to induce the metastatic phenotype (Hendrix et al., 1997). Therefore, other cellular events together with vimentin and cytokeratin expression must be responsible for the aggressive behaviour of the MDA-MB-231 cell line.

Thus, the initiation of breast cancer from a normal epithelial stage followed by its progression from a hormone-dependent to a hormone-independent stage involves a series of

cellular events that may include changes in the expression of IF proteins such as cK8, cK18, and cK19, and vimentin. This is further supported by the observation that, in addition to expressing vimentin, the hormone-independent breast cancer cell line, MDA-MB-231, expressed two DNA-binding proteins in cis-DDP crosslinked preparations (BC13 and BC14) that were either absent or barely detectable in all other cell lines which had molecular masses and isoelectric points similar to proteins found in MDA-MB-231 IF preparations. At present, these two proteins have not been identified. Whether changes in IF composition initiate and/or are a direct consequence of events involved in hormonal progression of breast cancer remains to be determined.

Along with changes in IF protein expression, the expression of NM proteins in breast cancer cells may also serve as a marker of disease progression. Studies on prostate (Partin et al., 1993; Getzenberg et al., 1991), bladder (Getzenberg et al., 1996), colon (Keesee et al., 1994), and breast (Khanuja et al., 1993) cancer have all shown differences in NM composition between cells or tissue in a normal and a diseased state. For instance, a recent study identified 3 proteins (X, Y, and Z) as being specific to malignant breast tissue (Khanuja et al., 1993). Furthermore, Dr. Shanti Samuel and colleagues (1997) identified 5 NM proteins (NMBC1, NMBC2, NMBC3, NMBC4, NMBC5) that were detectable in hormone-dependent breast cancer cells and tumors, and 1 protein (NMBC6) that was only detectable in hormone-independent breast cancer cells and tumors.

Since cis-DDP preferentially crosslinks DNA to NM proteins (Ferraro et al., 1995; Costa, 1991), this agent was used to identify DNA-binding protein markers of breast cancer progression. In preparations of proteins crosslinked to DNA by cis-DDP *in situ* in this study,

protein Z, as well as NMBC 1 and NMBC2 were identified in all cell lines except T5-PRF and BT-20. This would suggest that, in cis-DDP DNA-binding protein preparations, these three NM proteins have no diagnostic or prognostic significance for breast cancer development. However, the presence of NMBC1 and NMBC2 in the pseudo-normal MCF-10A1 DNA-crosslinked protein preparation could be a consequence of changes in gene expression incurred by spontaneous immortalization. Thus, the analysis of two-dimensional profiles of DNA-crosslinked proteins in normal breast epithelial cell lines needs to be performed in order to confirm the prognostic significance of these two proteins in cis-DDP DNA-crosslinked protein preparations. The four other potential prognostic markers for breast cancer that were identified by Dr. Samuel et al. (1997) were not observed in preparations of proteins crosslinked to DNA by cis-DDP, therefore, indicating that these proteins do not bind to DNA at significant levels for detection.

A comparison of cis-DDP DNA-binding protein preparations identified several proteins that may be of either diagnostic or prognostic importance in breast cancer development. Hormone-dependent human breast cancer cell lines displayed three prominent proteins that were either absent or barely detectable in DNA-crosslinked preparations of hormone-independent breast cancer and pseudo-normal breast epithelial cell lines (BC3, BC4, BC5). In addition, hormone-independent human breast cancer cell lines displayed several DNA-binding proteins that were most abundant in cells with a hormone-independent breast cancer cell phenotype. Of these hormone-independent DNA-binding proteins, only one (BC6: 41 kDa, pI 4.65) was identified most abundantly in three of the four hormone-independent breast cancer cell lines, while four were most prominent in only two of the three hormone-

independent cell lines compared to the other cell lines studied (BC7, BC8, BC9, BC10). Other cis-DDP-isolated DNA-binding proteins were found most abundantly in the hormone-independent breast cancer cell lines BT-20 and MDA-MB-231 (BC11, BC12, BC13, BC14), as well as the pseudo-normal breast epithelial cell line, MCF-10A1 (BC1, BC2). However, the detection of these proteins in only one of the seven cell lines studied creates difficulty in discerning whether these proteins were present due to cell-type specific differences or hormonal growth requirements.

As was just previously stated, a higher level of abundance of the DNA-crosslinked proteins, BC6-BC14, was observed in three of the four hormone-independent breast cancer cell lines compared to the three hormone-dependent and the pseudo-normal breast epithelial cell lines. However, most of these proteins were not detectable in the hormone-independent cell line, T5-PRF. Such an observation suggests that either the DNA-crosslinked proteins, BC6-BC14, are only expressed in breast cancer cells with a hormone-independent phenotype, or, as was previously stated earlier in this paper, that different mechanisms are taking place in ER+ and ER- cell lines to give both these cell types a hormone-independent phenotype. Alternatively, the absence of these proteins from the T5-PRF cell line could be a result of the cell-type specific differences between the T5-PRF cell line and the three ER- cell lines studied.

Despite the higher level expression of the DNA-crosslinked proteins, BC6-BC14, in hormone-independent breast cancer cell lines when compared to hormone-dependent cell lines, the prognostic importance of these proteins still remains questionable. These proteins may also be found in other cancer cell lines, and may not be indicative of breast cancer. To address this concern, further analysis needs to be performed on the two-dimensional cis-DDP-

crosslinked profiles of other cancer cell lines. This problem may also present itself for the three proteins (BC3, BC4, BC5) found most abundantly in the hormone-dependent cell lines studied.

In addition to this problem, the use of cell lines rather than tumor samples to identify DNA-binding proteins of potential prognostic importance is also a problem. Cells grown in culture are not exposed to the same factors as cells grown *in vivo*. Therefore, the differences observed among the various cell lines studied may not be representative of the differences that would be observed *in vivo*. As a result, further analysis on cis-DDP DNA-crosslinked proteins from tumor samples of various phenotypes needs to be performed followed by clinical trials on breast cancer patients.

When comparing the two-dimensional profiles of DNA-crosslinked protein preparations with NM preparations, several DNA-binding proteins with a potential prognostic value in cis-DDP preparations of breast cancer cell lines thought to represent different stages in the hormonal progression model showed no potential prognostic importance in the NM profiles of the same cell lines (BC3, BC4, BC5, BC6, BC11). Therefore, while a NM protein may be present throughout breast cancer development, it may only bind to DNA and, by some unknown mechanism, influence gene expression at specific disease stages. However, the presence of some of these putative prognostic DNA-binding proteins was not always restricted to a particular breast cancer cell or breast epithelial cell phenotype, indicating that the change in gene expression for some DNA-binding proteins throughout breast cancer development may be less extreme than for others.

One important cellular component that may be implicated in these changes in DNA-binding protein composition throughout the different stages of breast cancer development is the CSK. The CSK is believed to help stabilize nuclear structure (Ingber, 1994). Thus, changes in the nuclear structure may affect CSK organization and induce changes in the expression of some DNA-binding proteins over breast cancer progression. The influence of changes in nuclear shape on the DNA-binding NM protein composition is most likely mediated through the IF component of the CSK (Ingber, 1994). Since IFs have been shown to influence NM structure (Maniotis et al., 1997), structural changes in the CSK induced by alterations in nuclear structure may influence the shape of the NM, and, therefore, the chromatin loop organization. Alternatively, structural changes in the CSK induced by the ECM may, in turn, cause changes in the NM structure which could lead to changes in the nuclear structure. The end result of either scenario would be a rearrangement in chromatin loop organization. Alterations in chromatin loop organization could then lead to changes in the association of certain NM proteins with DNA, as well as changes in gene expression. The observation that the distribution of proteins with molecular weight and isoelectric point coordinates similar to those of IF proteins was different between hormone-dependent and hormone-independent human breast cancer cell lines provides evidence to suggest the involvement of the CSK and the NM in the differential expression of DNA-binding proteins during breast cancer progression.

Although cis-DDP predominately crosslinked NM proteins to DNA, several of these potentially prognostic DNA-binding proteins could not be detected in NM preparations of the same cell type from which they were obtained. Despite the possibility that cis-DDP may

crosslink some proteins not found in the NM, the use of this agent appears to be ideal for identifying DNA-binding NM proteins. Previous studies have primarily used *in vitro* techniques for isolating NM proteins (Samuel et al., 1997; Fey and Penman, 1988; Mirkovitch et al., 1984). However, these studies have been performed under non-physiological conditions and have involved NM isolation protocols that may strip NM proteins from the NM (Samuel et al., 1997; Fey and Penman, 1988) or inadequately separate chromatin from the NS (Mirkovitch et al., 1984). The use of cis-DDP to isolate NM protein-DNA complexes allows the isolation of NM proteins *in situ* under physiological conditions. Thus, the problems associated with *in vitro* techniques are avoided. As a result, the characterization of proteins crosslinked to DNA by cis-DDP *in situ*, which are primarily NM proteins, can be used as a complementary approach to the current NM isolation protocol for identifying NM proteins of putative diagnostic or prognostic significance in cancer development.

In summary, the hypothesis of this study was that cis-DDP crosslinking can be used as a complementary approach to the high salt NM extraction protocol currently used for identifying putative NM proteins that may be infomative biomarkers for cancer diagnosis. To prove this hypothesis, DNA-binding proteins from several hormone-dependent and hormone-independent cell lines were isolated using the crosslinking agent cis-DDP, and analyzed by two-dimensional electrophoresis. Following this, the DNA-crosslinked proteins were putatively identified as NM proteins by comparing the two-dimensional profiles of DNA-binding proteins and NM proteins from the same cell line. The ability of estrogen to influence the association of DNA-binding NM proteins with DNA was then studied using the hormone-dependent breast cancer cell line, T-47D5, grown under normal, estrogen-deplete, and

estrogen-replete conditions, and the hormone-independent cell line, T5-PRF, grown under estrogen-deplete conditions. DNA-binding NM proteins were crosslinked to DNA using cis-DDP from the three T-47D5 treatments and the T5-PRF cell line and then analyzed by two-dimensional electrophoresis. Differences in NM protein levels over the three T-47D5 treatments and the T5-PRF cell line were assessed.

The results of this study combined with those of previous studies (Ferraro et al., 1995; Costa, 1991) show that cis-DDP preferentially crosslinks NM proteins to DNA in intact cells. However, the identity of these cis-DDP DNA-crosslinked proteins as NM proteins needs to be confirmed by immunoflourescence to determine the location of these proteins in the cell.

In addition to displaying a large similarity to NM preparations, the majority of cis-DDP DNA-crosslinked proteins extracted from various human breast cancer cell lines were similar. However, differences in the types of DNA-crosslinked proteins were still observed between cell lines thought to represent different stages in the breast cancer progression model. These differences demonstrate that NM proteins, and, more specifically, NM-DNA interactions may be of importance in cancer prognosis.

Despite these findings, additional cis-DDP DNA-crosslinking studies must be performed on breast epithelial and breast tumor samples to determine if NM proteins and NM-DNA interactions have a prognostic value *in vivo*. DNA-crosslinking studies using cis-DDP must also be performed on other cancer cell lines to determine the specificity of these NM proteins and NM-DNA interactions in breast cancer cells. Once determined, proteins of a potential prognostic value can then be further studied in clinical trials in order to accurately assess their meaning in breast cancer development.

In addition to observing changes in patterns of DNA-binding proteins between hormone-dependent and hormone-independent breast cancer cell lines, the behaviour of some DNA-crosslinked NM proteins such as cK8, cK18 and cK19 appeared to vary with hormone growth requirements. The association of cK8, cK18 and cK19 with DNA was dependent on estrogen in a hormone-dependent human breast cancer cell line, but not in a hormone-independent human breast cancer cell line. The ability of estrogen to influence cytokeratin-DNA interactions in a hormone-dependent and not in a hormone-independent breast cancer cell line suggests that, during the hormonal progression of breast cancer to a more advanced, hormone-independent stage, alternative mechanisms develop that allow the cell to undergo changes in structure and DNA organization without hormonal regulation.

These findings combined with the large abundance of vimentin in the human breast cancer cell line MDA-MB-231 are consistent with the idea that some of the various cellular events leading to malignancy in breast cancer may involve changes in cell structure that may lead to changes in protein expression through alterations in DNA organization.

The ability of cis-DDP to crosslink cytokeratins to DNA *in situ* suggested that cytokeratins are NM proteins associated with nuclear DNA. Similar observations have also been shown by Hnilica and colleagues (Wedrychowski et al., 1986; Olinski et al., 1987). This finding was important since the current protocol for NM isolation attempts to remove IF proteins; thus, cytokeratins present in NM preparations will be those that escaped reassembly and/or precipitation steps performed to remove IF proteins. These observations argue that informative NM proteins may be lost during the preparation of NM proteins. As a result, the characterization of NM proteins and proteins crosslinked to DNA by cis-DDP

in situ, which are primarily NM proteins, are two complementary approaches that can be used to identify putative NM proteins that may be informative markers in cancer diagnosis.

References

Adachi, Y., Kas, E., & Laemmli, U.K. (1989). Preferential, cooperative binding of DNA topoisomerase II to scaffold-associated regions. *EMBO J.* **8**, 3997-4006.

Alberts, B.M. (1990). Recipes for replication. *Nature, Lond.* **346**, 514-515.

Allen, G.C., Hall, G.E. Jr., Childs, L.C., Weissinger, A.K., Spiker, S., & Thompson, W.F. (1993). Scaffold attachment regions increase reporter gene expression in stably transformed plant cells. *Plant Cell* **5**, 603-613.

Allred, D.C., O'Connell, P., & Fuqua, S.A.W. (1993). Biomarkers in early breast neoplasia. *J. Cell. Biochem.* **17G**, 125-131.

Armstrong, D.K., Isaacs, J.T., Ottaviano, Y.L., & Davidson, N.E. (1992). Programmed cell death in an estrogen-independent human breast cancer cell line, MDA-MB-468. *Cancer Res.* **52**, 3418-3424.

Aronica, S.M., & Katzenellenbogen, B.S. (1993). Stimulation of estrogen receptor mediated transcription and alteration in the phosphorylation state of the rat uterine estrogen receptor by estrogen, cyclic adenosine monophosphate, and insulin-like growth factor-1. *Mol. Endocrinol.* **7**, 743-752.

Attal, J., Cajero-Juarez, M., Petitclerc, D., Theron, M.C., Stinnakre, M.G., Bearzotti, M., Kann, G., & Houdebine, L.M. (1996). The effect of matrix attached regions (MAR) and specialized chromatin structure (SC) on the expression of gene constructs in cultured cells and in transgenic mice. *Mol. Biol. Rep.* **22**, 37-46.

Banjar, Z.M., Hnilica, L.S., Briggs, R.C., Stein, J., & Stein, G. (1984). *cis-* and *trans-*diamminedichloroplatinum(II)-mediated cross-linking of chromosomal non-histone proteins to DNA in HeLa cells. *Biochemistry* **23**, 1921-1926.

Berezney, R. & Coffey, D.S. (1974). Identification of a nuclear matrix protein. *Biochem. Biophys. Res. Commun.* **60**, 1410-1417.

Berezney, R. (1991). The nuclear matrix: A heuristic model for investigating genomic organization and function in the cell nucleus. *J. Cell. Biochem.* **47**, 109-123.

Berezney. R., & Buchholtz, L.A. (1981). Dynamic association of replicating DNA fragments with the nuclear matrix of regenerating liver. *Exp. Cell Res.* **132**, 1-13.

Bode, J., Kohwi, Y., Dickinson, L., Joh, T., Klehr, D., Mielke, C., & Kohwi-Shigematsu, T. (1992). Biological significance of unwinding capability of nuclear matrix-associating DNAs. *Science* **255,** 195-197.

Bode, J., Schlake, T., Rios-Ramirez, M., Mielke, C., Stengert, M., Kay, V., & Klehr-Wirth, D. (1995). Scaffold/matrix-attached regions (S/MARs): structural properties creating transcriptionally active loci. In Jeon, K.W., Berezney, R. (ed): "Structural and Functional Organization of the Nuclear Matrix - International Review of Cytology", Vol. 162A, Chap.8. Orlando: Academic Press, pp. 389-453.

Bode, J., Stengert-Iber, M., Kay, V., Schlake, T., & Dietz-Pfeilstetter, A. (1996). Scaffold/matrix-attached regions: Topological switches with multiple regulatory functions. *Crit. Rev. Euk. Gene Exp.* **6,** 115-138.

Boffa, L.C., Vidali, G., Mann, R.S., & Allfrey, V.G. (1978). Suppression of histone deacetylation *in vivo* and *in vitro* by sodium butyrate. *J. Biol. Chem.* **253,** 3364-3366.

Bortner, D.M., Langer, S.J., & Ostrowski, M.C. (1993). Non-nuclear oncogenes and the regulation of gene expression in transformed cells. *Crit. Rev. Oncog.* **4,** 137-160.

Boulikas, T. (1995). How enhancers work: Juxtapositioning of DNA control elements by synergistic interaction of MARs (review-hypothesis). *Int. J. Oncol.* **6,** 1313-1318.

Brooks, A.R., Nagy, B.P., Taylor, S., Simonet, W.S., Taylor, J.M., & Levy-Wilson, B. (1994). Sequences containing the second-intron enhancer are essential for transcription of the human apolipoprotein B gene in the livers of transgenic mice. *Mol. Cell. Biol.* **14,** 2243-2256.

Bubley, G.J., Xu, J., Kupiec, N., Sanders, D., Foss, F., O'Brien, M., Emi, Y., Teicher, B.A., & Patierno, S.R. (1996). Effect of DNA conformation on cisplatin adduct formation. *Biochem. Pharmacol.* **51,** 717-721.

Capco, D.G., Wan, K.M. & Penman, S. (1982). The nuclear matrix: Three-dimensional architecture and protein composition. *Cell* **29,** 847-858.

Chen, D., Ohta, N., Ukai, M., Masuda, M., & Yotsuyanagi, T. (1994). Binding and aggregation of human γ-globulin by *cis*-diamminedichloroplatinum (II) through disulphide bond. *Biol. Pharm. Bull.* **17,** 1561-1566.

Chen, H.Y., Sun, J.M., Hendzel, M.J., Rattner, J.B., & Davie, J.R. (1996). Changes in the nuclear matrix of chicken erythrocytes that accompany maturation. *Biochem. J.* **320,** 257-265.

Chu, G. (1994). Cellular responses to cisplatin: The role of DNA-binding proteins and DNA repair. *J. Biol. Chem.* **269**, 787-790.

Ciejek, E.M., Tsai, M.J., & O'Malley, B.W. (1983). Actively transcribed genes are associated with the nuclear matrix. *Nature, Lond.* **306**, 607-609.

Clarke, R., Dickson, R.B., & Brunner, N. (1990). The process of malignant progression in human breast cancer. *Ann. Oncol.* **1**, 401-497.

Cockerill, P.N., & Garrard, W.T. (1986a). Chromosomal loop anchorage of the kappa immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. *Cell* **44**, 273-282.

Cockerill, P.N., & Garrard, W.T. (1986b). Chromosomal loop anchorage sites appear to be evolutionarily conserved. *FEBS Lett.* **204**, 5-7.

Cook, P.A. & Brazell, I.A. (1976). Characterization of nuclear structures containing superhelical DNA. *J. Cell Sci.* **22**, 303-324.

Cook, P.R. (1989). The nuceloskeleton and the topology of transcription. *Eur. J. Biochem.* **185**, 487-501.

Costa, M.. (1991). DNA-protein complexes induced by chromate and other carcinogens. *Environ. Hlth. Perspect.* **92**, 45-52.

Coutts, A.S., Davie, J.R., Dotzlaw, H., & Murphy, L.C. (1996). Estrogen regulation of nuclear matrix-intermediate filament proteins in human breast cancer cells. *J. Cell. Biochem.* **63**, 174-184.

Cunningham, J.M., Purucker, M.E., Jane, S.M., Safer, B., Vanin, E.F., Ney, P.A., Lowrey, C.H., & Nienhuis, A.W. (1994). The regulatory element 3' to the ^ γ-globin gene binds to the nuclear matrix and interacts with special A-T-rich binding protein 1 (SATB1), an SAR/MAR-associating region DNA binding protein. *Blood* **84**, 1298- 1308.

Curschellas, E., Matter, A., & Regenass, U. (1987). Immunolocalization of cytoskeletal elements in human mammary epithelial cells. *Eur. J. Cancer Clin. Oncol.* **23**, 1517-1527.

Dedon, P.C., & Borch, R.F. (1987). Characterization of the reactions of platinum antitumor agents with biologic and nonbiologic sulfur-containing nucleophiles. *Biochem. Pharmacol.* **36**, 1955-1964.

Deppert, W. (1996). Binding of MAR-DNA elements by mutant p53: possible implications for its oncogenic functions. *J. Cell. Biochem.* **62**, 172-180.

Dickinson, L.A., & Kohwi-Shigematsu, T. (1995). Nucleolin is a matrix-attachment region DNA-binding protein that specifically recognizes a region with high base-unpairing potential. *Mol. Cell. Biol.* **15**, 456-465.

Dickinson, L.A., Joh, T., Kohwi, Y., & Kohwi-Shigematsu, T. (1992). A tissue-specific MAS/SAR DNA-binding protein with unusual binding site recognition. *Cell* **70**, 631-645.

Dijkwel, P.A., & Hamlin, J.L. (1996). Sequence and context effects on origin function in mammalian cells. *J. Cell. Biochem.* **62**, 210-222.

Dijkwel, P.A., & Hamlin, J.L. (1988). Matrix attachment regions are positioned near replication initiation sites, genes and an interamplicon junction in the amplified dihydrofolate reductase domain of Chinese hamster ovary cells. *Mol. Cell. Biol.* **8**, 5398-5409.

Dorer, D.R., & Henikoff, S. (1994). Expansions of transgene repeats cause heterochromatin formation and gene silencing in *Drosophila*. *Cell* **77**, 993-1002.

Doucet, J.P., & Trifaro, J.M. (1988). A discontinuous and highly porous sodium dodecyl sulfate-polyacrylamide slab gel system of high resolution. *Anal. Biochem.* **168**, 265-271.

Dreyfuss, G., Matunis, M.J., Pinol-Roma, S., & Burd, C.G. (1993). hnRNP proteins and the biogenesis of mRNA. *Annu. Rev. Biochem.* **62**, 289-321.

Dworetzky, S.I., Fey, E.G., Penman, S., Lian, J.B, Stein, J.L, & Stein, G.S. (1990). Progressive changes in the protein composition of the nuclear matrix during rat osteoblast differentiation. *Proc. Natl. Acad. Sci. USA* **87**, 4605-4609.

Eggert, H., & Jack, R. (1991). An ectopic copy of the Drosophila ftz associated SAR neither reorganizes local chromatin structure nor hinders elution of a chromatin fragment from isolated nuclei. *EMBO J.* **10**, 1237-1243.

Engel, L. W., & Young, N.A. (1978). Human breast carcinoma cells in continuous culture: A review. *Cancer Res.* **38**, 4327-4339.

Engel, L.W., Young, N.A., Tralka, T.S., Lippman, M.E., O'Brien, S.J., & Joyce, M. J. (1978). Establishment and characterization of three new continuous cell lines derived from human breast carcinomas. *Cancer Res.* **38**, 3352-3364.

Ernster, V.L., Barclay, J., Kerlikowske, K., Grady, D., & Henderson, C. (1996). Incidence of and treatment for ductal carcinoma *in situ* of the breast. *JAMA* **275**, 913-918.

Felsenfeld, G., & McGhee, J.D. (1986). Structure of the 30 nm chromatin fiber. *Cell* **44**, 375-377.

Ferraro, A., Grandi, P., Eufemi, M., Altieri, F., Cervoni, L., & Turano, C. (1991). The presence of N-glycosylated proteins in cell nuclei. *Biochem. Biophys. Res. Commun.* **178**, 1365-1370.

Ferraro, A., Grandi, P., Eufemi, M., Altieri, F., & Turano, C. (1992). Crosslinking of nuclear proteins to DNA by *cis*-diamminedichloroplatinum in intact cells: Involvement of nuclear matrix proteins. *FEBS Lett.* **307**, 383-385.

Ferraro, A., Eufemi, M., Cervoni, L., Altieri, F., & Turano. (1995). DNA-nuclear matrix interactions analyzed by cross-linking reactions in intact nuclei from avian liver. *Acta. Biochim. Pol.* **42**, 145-152.

Ferraro, A., Cervoni, L., Eufemi, M., Altieri, F., & Turano, C. (1996). Comparison of DNA-protein interactions in intact nuclei from avian liver and erythrocytes: A cross-linking study. *J. Cell. Biochem.* **62**, 495-505.

Fey, E.G., Wan, K.M., & Penman, S. (1984). Epithelial cytoskeletal framework and nuclear-matrix-intermediate filament scaffold: Three-dimensional organization and protein composition. *J. Cell Biol.* **98**, 1973-1984.

Fey, E.G., Krochmalnic, G., & Penman, S. (1986). The nonchromatin substructures of the nucleus: The ribonucleoprotein (RNP)-containing and RNP-depleted matrices analyzed by sequential fractionation and resinless section electron microscopy. *J. Cell Biol.* **102**, 1654-1665.

Fey, E.G. & Penman, S. (1988). Nuclear matrix proteins reflect cell type of origin in cultured human cells. *Proc. Natl. Acad. Sci. USA* **85**, 121-125.

Filipski, J., Kohn, K.W., & Bonner, W.M. (1983). Differential crosslinking of histones and non-histones in nuclei by cis-Pt(II). *FEBS Lett.* **152**, 105-108.

Foka, M., & Paoletti, J. (1986). Interaction of *cis*-diamminedichloroplatinum(II) to chromatin: Specificity of the drug distribution. *Biochem. Pharmacol.* **35**, 3283-3291.

Forrester, W.C., van Genderen, C., Jenuwein, T., & Grosschedl, R. (1994). Dependence of enhancer-mediated transcription of the immunoglobulin μ gene on nuclear matrix attachment regions. *Science* **265**, 1221-1225.

Gasser, S.M., & Laemmli, U.K. (1986a). The organisation of chromatin loops: characterization of a scaffold attachment site. *EMBO J.* **5**, 511-518.

Gasser, S.M., & Laemmli, U.K. (1986b). Cohabitation of scaffold-binding regions with upstream/enhancer elements of three developmentally regulated genes of *D. melanogaster*. *Cell* **46**, 521-530.

Gasser, S.M., & Laemmli, U.K. (1987). A glimpse at chromosomal order. *Trends Genet.* **3**, 16-22.

Gerdes, M.G., Carter, K., Moen, P.T., & Lawrence, J.B. (1994). Dynamic changes in the higher-level chromatin organization of specific sequences revealed by *in situ* hybridization in nuclear halos. *J. Cell Biol.* **126**, 289-304.

Getzenberg, R.H., & Coffey, D.S. (1990). Tissue specificity of the hormonal response in sex accessory tissues is associated with nuclear matrix protein patterns. *Mol. Endocrinol.* **4**, 1336-1342.

Getzenberg, R.H., Pienta, K.J., Huang, E.Y.W., & Coffey, D.S. (1991). Identification of nuclear matrix in the cancer and normal rat prostate. *Cancer Res.* **51**, 6514-6520.

Getzenberg, R.H., Konety, B.R., Oeler, T.A., Quigley, M.M., Hakam, A., Becich, M.J., & Bahnson, R.R. (1996). Bladder cancer-associated nuclear matrix proteins. *Cancer Res.* **56**, 1690-1694.

Guo, B., Odgren, P.R., van Wijnen, A.J., Last, T.J., Nickerson, J., Penman, S., Lian, J.B., Stein, J.L., & Stein, G.S. (1995). The nuclear protein NMP-1 is the transcription factor YY1. *Proc. Natl. Acad. Sci. USA* . **92**, 10526-10530.

Hayes, J., & Scovell, W.M. (1991). *cis*-Diamminedichloroplatinum(II) modified chromatin and nucleosomal core particle. *Biochim. Biophys. Acta.* **1089**, 377-385.

He, D., Nickerson, J.A., & Penman, S. (1990). Core filaments of the nuclear matrix. *J. Cell Biol.* **110**, 569-580.

Hempel, K., & Stratling, W.H. (1996). The chicken lysozyme gene 5' MAR and the *Drosophila* histone SAR are electroelutable from encapsulated and digested nuclei. *J. Cell Sci.* **109**, 1459-1469.

Henderson, B.E., Ross, R., & Bernstein, L. (1988). Estrogens as a cause of human cancer: the Richard and Hinda Rosenthal Foundation Award Lecture. *Cancer Res.* **48**, 246-253.

Hendrix, M.J.C., Seftor, E.A., Chu, Y.W., Seftor, R.E.B., Nagle, R.B., McDaniel, K.M., Leong, S.P.L. & Yohem, K.H. (1992). Coexpression of vimentin and keratins by human melanoma tumor cells: correlation with invasive and metastasis potential. *J. Natl. Cancer Inst.* **84**, 165-174.

Hendrix, M.J.C., Seftor, E.A., Seftor, R.E.B., & Trevor, K.T. (1997). Experimental co-expression of vimentin and keratin intermediate filaments in human breast cancer cells results in phenotypic interconversion and increased invasive behaviour. *Am. J. Pathol.* **150**, 483-495.

Hill, T.L. (1981). Microfilament or microtubule assembly or disassembly against a force. *Proc. Natl. Acad. Sci. USA* **78**, 5613-5617.

Hoffmann, J.S., Hubscher, U., Michot, B., & Villani, G. (1991). Interaction of *cis*-diamminedichloroplatinum(II) with single-stranded DNA in the presence or absence of *Escherichia coli* single-stranded binding protein. *Biochem. Pharmacol.* **42**, 1393-1398.

Holzmann, K., Korosec, T., Gerner, C., Grimm, R., & Sauermann, G. (1997). Identification of human common nuclear-matrix proteins as heterogeneous nuclear ribonucleoproteins H and H' by sequencing and mass spectrophotometry. *Eur. J. Biochem.* **244**, 479-486.

Homberger, H.P. (1989). Bent DNA is a structural feature of scaffold-attached regions in *Drosophila melanogaster* interphase nuclei. *Chromosoma* **98**, 99-104.

Hozak, P., Sasseville, M.J.A., Raymond, Y., & Cook, P.R. (1995). Lamin proteins form an internal nucleoskeleton as well as a peripheral lamina in human cells. *J. Cell Sci.* **108**, 635-644.

Hsieh, T., & Wang, J.C. (1975). Thermodynamic properties of superhelical DNAs. *Biochemistry* **14**, 527-535.

Hsieh, J.T., Zhau, H.E., Wang, X.H., Liew, C.C., & Chung, L.W.K. (1992). Regulation of basal and luminal cell-specific cytokeratin expression in rat accessory sex organs. *J. Biol. Chem.* **267**, 2303-2310.

Ingber, D.E. (1993). Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. *J. Cell Sci.* **104**, 613-627.

Ingber, D.E., Dike, L., Hansen, L., Karp, S., Liley, H., Maniotis, A., McNamee, H., Mooney, D., Plopper, G., Sims, J., & Wang, N. (1994). Cellular tensegrity: exploring how mechanical changes in the cytoskeleton regulate cell growth, migration, and tissue pattern during morphogenesis. *Int. Rev. Cytol.* **150**, 173-224.

Ivanchenko, M., & Avramova, Z. (1992). Interaction of MAR-sequences with nuclear matrix proteins. *J. Cell. Biochem.* **50**, 190-200.

Izaurralde, E., Mirkovitch, J., & Laemmli, U.K. (1988). Interaction of DNA with nuclear scaffolds *in vitro*. *J. Mol. Biol.* **200**, 111-125.

Izaurralde, E., Kas, E., & Laemmli, U.K. (1989). Highly preferential nucleation of histone H1 assembly on scaffold-associated regions. *J. Mol. Biol.* **210**, 573-585.

Jackson, D.A., & Cook, P.R. (1985). Transcription occurs at a nucleoskeleton. *EMBO J.* **4**, 919-925.

Jackson, D.A., Yuan, J., & Cook, P.R. (1988). A gentle method for preparing cyto- and nucelo-skeletons and associated chromatin. *J. Cell Sci.* **90**, 365-378.

Jackson, D.A., Dickinson, P. & Cook, P.R. (1990). The size of chromatin loops in HeLa cells. *Embo J.* **9**, 567-571.

Johnson, N.P., Hoeschele, J.D., & Rahn, R.O. (1980). Kinetic analysis of the *in vitro* binding of radioactive *cis*- and *trans*-dichlorodiammineplatinum(II) to DNA. *Chem. Biol. Interact.* **30**, 151-169.

Kalos, M., & Fournier, R.E.K. (1995). Position-independent transgene expression mediated by boundary elements from the apolipoprotein B chromatin domain. *Mol. Cell. Biol.* **15**, 198-207.

Karin, M., & Hunter, T. (1995). Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Curr. Biol.* **5**, 747-757.

Kas, E., Izaurralde, E., & Laemmli, U.K. (1989). Specific inhibition of DNA binding to nuclear scaffolds and histone H1 by distamycin. The role of oligo(dA)-oligo(dT) tracts. *J. Mol. Biol.* **210**, 587-599.

Kasid, A., Lippman, M.E., Papageorge, A.E., Lowy, D.R., & Gelmann, E.P. (1985). Transfection of v-rasH DNA into MCF-7 human breast cancer cells bypasses dependence on estrogen for tumorigenicity. Science **228**, 725-728.

Kay, V., & Bode, J. (1994). Binding specificity of a nuclear scaffold: supercoiled, single-stranded, and scaffold-attached-region DNA. *Biochemistry* **33**, 367-374.

Keesee, S.K., Meneghini, M.D., Szaro, R.P., & Wu, Y.J. (1994). Nuclear matrix proteins in human colon cancer. *Proc. Natl. Acad. Sci. USA* **91**, 1913-1916.

Kenney, N.J., Saeki, T., Gottardis, M., Kim, N., Garcia-Morales, P., Matrin, M.B., Normanno, N., Ciardiello, F., Day, A., Cutler, M.L., & Salomon, D.S. (1993). Expression of transforming growth factor alpha antisense mRNA inhibits the estrogen-induced proliferation of TGF-alpha and estrogen-induced proliferation of estrogen responsive human breast cancer cells. *J. Cell. Physiol.* **156**, 497-514.

Khan, M.U.A., & Sadler, P.J. (1978). Distribution of a platinum anti-tumor drug in HeLa cells by analytical electron microscopy. *Chem. Biol. Interact.* **21**, 227-232.

Khanuga, P.S., Lehr, J.E., Soule, H.D., Gehani, S.K., Noto, A.C., Choudhury, S, Chen, R., & Pienta, K.J. (1993). Nuclear matrix proteins in normal and breast cancer cells. *Cancer Res.* **53**, 3394-3398.

Kobota, S., Tashiro, K., & Yamada, Y. (1992). Signalling site of laminin with mitogenic activity. *J. Biol. Chem.* **257**, 4285-4288.

Komitowski, D., & Janson, C. (1990). Quantitative features of chromatin structure in the prognosis of breast cancer. *Cancer* **65**, 2725-2730.

Kohwi-Shigematsu, T., & Kohwi, Y. (1990). Torsional stress stabilizes extended base unpairing in suppressor sites flanking immunoglobulin heavy chain enhancer. *Biochemistry* **29**, 9551-9560.

Komitowski, D.D., Hart, M.M., & Janson, C.P. (1993). Chromatin organization and breast cancer: Two-dimensional and three-dimensional image analysis. *Cancer* **72**, 1239-1246.

Kronenberg, M.S., & Clark, J.H. (1985). Changes in keratin expression during the estrogen-mediated differentiation of rat vaginal epithelium. *Endocrinology* **117**, 1480-1489.

Kuller, L.H. (1995). The etiology of breast cancer - from epidemiology to prevention. *Public Health Rev.* **23**, 157-213.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, Lond.* **227**, 680-685.

Landers, J.P., & Spelsberg, T.C. (1992). New concepts in steroid hormone action: Transcription factors, proto-oncogenes, and the cascade model for steroid regulation of gene expression. *Crit. Rev. Euk. Gene Exp.* **2**, 19-63.

LeRoy, A.F., & Thompson, W.C. (1989). Binding kinetics of tetrachloro-1,2-diamminocyclohexaneplatinum(IV) and cis-diamminedichloroplatinum(II) at 37°C with human plasma proteins and with bovine serum albumin. Does aquation precede protein binding? *J. Natl. Cancer Inst.* **81**, 427-436.

Levy-Wilson, B., & Fortier, C. (1989). The limits of DNase I-sensitive domain of the human apolipoprotein B gene coincide with the locations of chromosomal anchorage loops and define the 5' and 3' boundaries of the gene. *J. Biol. Chem.* **264**, 21196-21204.

Li, W., Nagaraja, S., Delcuve, G.P., Hendzel, M.J., & Davie, J.R. (1993). Effects of histone acetylation, ubiquitination and variants on nucleosome stability. *Biochem. J.* **296**, 737-744.

Lilley, D.M.J. (1980). The inverted repeat as a recognizable structural feature in supercoiled DNA molecules. *Proc. Natl. Acad. Sci. USA* **77**, 6468-6472.

Lippard, S.J., & Hoeschele, J.D. (1979). Binding of *cis-* and *trans--* dichlorodiammineplatinum(II) to the nucleosome core. *Proc. Natl. Acad. Sci. USA* **76**, 6091-6095.

Lippard, S.J. (1982). New chemistry of an old molecule: *cis*-$[Pt(NH_3)_2Cl_2]$. *Science* **218**, 1075-1082.

Lippman, M., Bolan, G., Monaco, M., Pinkus, L., & Engels, L. (1976). Model systems for the study of estrogen action in tissue culture. *J. Steroid Biochem.* **7**, 1045-1051.

Lippman, M.E., & Dickson, R.B. (1989). Mechanisms of growth control in normal and malignant breast epithelium. *Rec. Prog. Horm. Res.* **45**, 383-340.

Luderus, M.E.E., de Graaf, A., Mattia, E., den Blaauwen, J.L., Grande, M.A., de Jong, L., & van Driel, R. (1992). Binding of matrix attachment regions to lamin B_1. *Cell* **70**, 949-959.

Luderus, M.E.E., den Blaauwen, J.L., de Smit, O.J.B., Compton, D.A., & van Driel, R. (1994). Binding of matrix-attachment regions to lamin polymers involves single-stranded regions and the minor groove. *Mol. Cell. Biol.* **14**, 6297-6305.

Macoska, J., Hoover, C.N., & Pienta, K.J. (1994). Alterations in cellular gene expression without changes in nuclear matrix protein content. *J. Cell Biochem.* **56**, 502-509.

Mandeville, R., Berrada, F., Ajdukovic, I., Sidrac-Ghali, S., & Zelechowska, M. (1987). An *in vitro model* for cancer invasion: 1. Grading and quantitation of aggressiveness. *Anticancer Res.* **7**, 1133-1142.

Maniotis, A.J., Bojanowski, K., & Inbger, D.E. (1997). Mechanical continuity and reversible chromosome disassembly within intact genomes removed from living cells. *J. Cell. Biochem.* **65**, 114-130.

Mattern, K.A., de Jong, L., & van Driel, R. (1996). Composition and structure of the internal nuclear matrix. In Bird, R.C., Stein, G., Lian, J., & Stein, J. (eds):"Nuclear Structure Gene Expression." Academic Press, San Diego, USA.

Mattern, K. A., van Goethem, R.E.M., de Jong, L., & van Driel, R. (1997). Major internal nuclear matrix proteins are common to different human cell types. *J. Cell. Biochem.* **65**, 42-52.

McCready, S.J., Godwin, J., Mason, D.W., Brazell, I.A. & Cook, P.R. (1980). DNA is replicated at the nuclear cage. *J. Cell Sci.* **46**, 365-386.

McGhee, J.D., Nickol, J.M., Felsenfeld, G, & Rau, D.C. (1983). Higher order structure of chromatin: orientation of nucleosomes within the 30 nm chromatin solenoid is independent of species and spacer length. *Cell* **33**, 831-841.

McKnight, R.A., Shamay, A., Sankaran, L., Wall., R.J., & Hennighausen, L. (1992). Matrix-attachment regions can impart position-independent regulation of a tissue-specific gene in transgenic mice. *Proc. Natl. Acad. Sci. USA* **89**, 6943-6947.

Michelotti, E.F., Michelotti, G.A., Aronsohn, A.I., & Levens, D. (1996). Heterogeneous nuclear ribonucleoprotein K is a transcription factor. *Mol. Cell. Biol.* **16**, 2350-2360.

Mielke, C., Kohwi, Y., Kohwi-Shigematsu, T., & Bode, J. (1990). Hierarchical binding of DNA fragments derived from scaffold-attached regions: correlation of properties *in vitro* and function *in vivo*. *Biochemistry* **29**, 7475-7485.

Millard, M.M., Macquet, J.P., & Theophanides, T. (1975). X-ray photoelectron spectroscopy of DNA-Pt complexes. Evidence of O^6(Gua)•N_7(Gua) chelation of DNA with cis-dichlorodiammineplatinum (II). *Biochim. Biophys. Acta.* **402**, 166-170.

Miller, C.A., & Costa, M. (1989). Analysis of proteins cross-linked to DNA after treatment of cells with formaldehyde, chromate, and *cis*-diamminedichloroplatinum(II). *Mol. Toxicol.* **2**, 11-26.

Miller, C.A., & Costa, M. (1990). Immunodetection of DNA-protein crosslinks by slot blotting. *Mutation Res.* **234**, 97-106.

Mirkovitch, J., Mirault, M.E., & Laemmli, U.K. (1984). Organization of the higher-order chromatin loop: Specific DNA attachment sites on nuclear scaffold. *Cell* **39**, 223-232.

Miyanaga, N., Akaza, H., Ishikawa, S., Ohtani, M., Noguchi, R., Kawai, K., Koiso, K., Kobayashi, M., Koyama, A., & Takahashi, T. (1997). Clinical evaluation of nuclear matrix protein 22 (NMP22) in urine as a novel marker for urothelial cancer. *Eur. Urol.* **31**, 163-168.

Moll, R., Franke, W.W., Geiger, B., Krepler, R., P.L. & Schiller. (1982). The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors and cultured cells. *Cell* **31**, 11-24.

Morris, C.R., & Gale, G.R. (1973). Interactions of an antitumor platinum compound with deoxyribonucleic acid, histones, L-amino acids, poly-L-amino acids, nucleosides and nucleotides. *Chem. Biol. Interact.* **7**, 305-315.

Muller, B.F., Paulsen, D., & Deppert, W. (1996). Specific binding of MAR/SAR DNA-elements by mutant p53. *Oncogene* **12**, 1941-1952.

Murphy, C.S., Pink, J.J., & Jordan, V.C. (1990). Characteristics of a receptor-negative hormone non-responsive clone derived from T-47D human breast cancer cell line kept under oestrogen free conditions. *Cancer Res.* **50**, 7285-7292.

Murphy, L. C., Dotzlaw, H., Hamerton, J., & Schwarz, J. (1993). Investigation of the origin of variant, truncated estrogen receptor-like mRNAs identified in some human breast cancer biopsy samples. *Breast Cancer Res. Treat.* **26**, 149-161.

Nakayasu, H., & Berezney, R. (1991). Nuclear Matrins: Identification of the major nuclear matrix proteins. *Proc. Natl. Acad. Sci.USA* **88**, 10312-10316.

Nickerson, J.A., Krockmalnic, G., Dacheng, H., & Penman, S. (1990). Immunolocalization in three dimensions: immunogold staining of cytoskeletal and nuclear matrix proteins in resinless electron microscopy sections. *Proc. Natl. Acad. Sci. USA* **87**, 2259-2263.

Nickerson, J.A., Krockmalnic, G., Wan, K.M., Turner, C.D., & Penman, S. (1992). A normally masked nuclear matrix antigen that appears at mitosis on cytoskeleton filaments adjoining chromosomes, centrioles, and midbodies. *J. Cell Biol.* **116**, 977-987.

O'Farrell, P.H. (1975). High resolution two-dimension electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007-4021.

Olinski, R., Wedrychowski, A., Schmidt, W.N., Briggs, R.C., & Hnilica, L.S. (1987). *In vivo* DNA-protein cross-linking by *cis*- and *trans*-diamminedichloroplatinum(II). *Cancer Res.* **47**, 201-205.

Osborn, M., & Weber, K. (1982). Intermediate filaments: cell-type-specific markers in differentiation and pathology. *Cell* **31**, 303-306.

Pankov, R., Umezawa, A., Maki, R., Der, C.J., Hauser, C.A., & Oshima, R.G. (1994). Keratin 18 activation by Ha-ras is mediated through ETS and Jun binding sites. *Proc. Natl. Acad. Sci. U.S.A* **91**, 873-877.

Partin, A.W., Getzenberg, R.H., CarMicheal, M.J., Vindivich, D., Yoo, J., Epstein, J.I., & Coffey, D.S. (1993). Nuclear matrix protein patterns in human benign prostatic hyperplasia and prostate cancer. *Cancer Res.* **53**, 744-746.

Pattanaik, A., Bachowski, G., Laib, J., Lemkuil, D., Shaw, F., Petering, D.H., Hitchcock, A., & Saryan, L. (1992). Properties of the reaction of *cis*-dichlorodiammineplatinum(II) with metallothionein. *J. Biol. Chem.* **267**, 16121-16128.

Paulson, J.R., & Laemmli, U.K. (1977). The structure of histone-depleted metaphase chromosomes. *Cell* **12**, 817-828.

Phi-Van, L., & Stratling, W.H. (1988). The matrix attachment regions of the chicken lysozyme gene co-map with the boundaries of the chromatin domain. *EMBO J.* **7**, 655-664.

Pienta, K.J., Partin, A.W., & Coffey, D.S. (1989). Cancer as a disease of DNA organization and dynamic cell structure. *Cancer Res.* **49**, 2525-2532.

Pienta , K.J. & Coffey, D. (1991). Correlation of nuclear morphometry with progression of breast cancer. *Cancer* **68**, 2012-2016.

Pienta, K.J., Getzenberg, R.H., & Coffey, D.S. (1991). Cell structure and DNA organization. *CRC Crit. Rev. Euk. Gene Exp.* **1**, 355-385.

Pienta, K.J, & Coffey, D.S. (1992). Nuclear-cytoskeletal interactions: evidence for physical connections between the nucleus and cell periphery and their alteration by transformation. *J. Cell. Biochem.* **49**, 357-365.

Pinto, A.L., & Lippard, S.J. (1985). Sequence-dependent termination of *in vitro* DNA synthesis by *cis*- and *trans*-diamminedichloroplatinum (II). *Proc. Natl. Acad. Sci. USA* **82**, 4616-4619.

Probst, H., & Herzog, R. (1985). DNA regions associated with the nuclear matrix of Ehrlich ascites cells expose single-stranded sites after deproteinization. *Eur. J. Biochem.* **146**, 167-171.

Quaranta, V., & Jones, J.C.R. (1991). The internal affairs of an integrin. *Trends Cell Biol.* **1**, 2-4.

Ramaekers, F.C.S., Haag, D., Kant, A., Moesker, O., Jap, P.H.K., & Vooijs, G.P. (1983). Coexpression of keratin- and vimentin-type intermediate filaments in human metastatic carcinoma cells. *Proc. Natl. Acad. Sci. USA* **80**, 2618-2622.

Raymond, W.A, & Leong, A.S. (1989a). Vimentin - a new prognostic parameter in breast carcinoma? *J. Pathol.* **158**, 107-114.

Raymod, W.A., & Leong, A.S. (1989b). Co-expression of cytokeratin and vimentin intermediate filament proteins in benign and neoplastic breast epithelium. *J. Pathol.* **157**, 299-306.

Reddel, R.R., Murphy, L.C., & Sutherland, R.L. (1984). Factors affecting the sensitivity of T-47D human breast cancer cells to tamoxifen. *Cancer Res.* **44**, 2398-2405.

Reddel, R.R., Murphy, L.C., Hall, R.E., & Sutherland, R.L. (1985). Differential sensitivity of human breast cancer cell lines to the growth-inhibitory effects of tamoxifen. *Cancer Res.* **45**, 1525-1531.

Renz, A., & Fackelmayer, F.Q. (1996). Purification and molecular cloning of the scaffold attachment factor B (SAF-B), a novel human nuclear protein that specifically binds to S/MAR-DNA. *Nuc. Acids Res.* **24**, 843-849.

Replogle, T.S., & Pienta, K.J. (1996). Role of the nuclear matrix in breast cancer. In R. Dickson and M. Lippman (eds), Mammary Tumour Cell Cycle, Differentiation and Metastasis, Kluwer Academic Publishers, pp.127-140.

Robertson, J.F.R. (1996). Oestrogen receptor: a stable phenotype in breast cancer. *Br. J. Cancer* **73**, 5-12.

Romig,, H., Fackelmayer, F.O., Renz, A., Ramsperger, U., & Richter, A. (1992). Characterization of SAF-A, a novel nuclear DNA binding protein from HeLa cells with high affinity for nuclear matrix/scaffold attachment DNA elements. *EMBO J.* **11**, 3431-3440.

Samuel, S.,K., Minish, T.M., & Davie, J.R. (1997). Altered nuclear matrix protein profiles in oncogene-transformed mouse fibroblasts exhibiting high metastatic potential. *Cancer Res.* **57**, 147-151.

Samuel, S.K., Minish, T.M., & Davie, J.R. (1997). Nuclear matrix proteins in well and poorly differentiated human breast cancer cell lines. *J. Cell. Biochem.* **66**, 9-15.

Sapino, A., Pietribiasi, F., Bussolati, G., & Marchisio, P.C. (1986). Estrogen- and tamoxifen-induced rearrangement of cytoskeletal and adhesion structures in breast cancer MCF-7 cells. *Cancer Res.* **46**, 2526-2531.

Schlake, T., Klehr-Wirth, D., Yoshida, M., Beppu, T., & Bode, J. (1994). Gene expression within a chromatin domain: The role of core histone hyperacetylation. *Biochemistry* **33**, 4197-4206.

Schroder, H.C., Trolltsch, D., Friese, U., Bachmann, M., & Muller, W.E.G.. (1987). Mature mRNA is selectively released from the nuclear matrix by an ATP/dATP-dependent mechanism sensitive to topoisomerase inhibitors. *J. Biol. Chem.* **262**, 8917-8925.

Seely, K.A., & Aggeler, J. (1991). Modulation of milk protein synthesis through alteration of the cytoskeleton in mouse mammary epithelial cells cultured on a reconstituted basement membrane. *J. Cell. Physiol.* **146**, 117-130.

Seibert, K., Shafie, S.M., Triche, T.J., Whang-Peng, J.J., O'Brien, S.J., Toney, J.H., Huff, K.K., & Lippman, M.E. (1983). *Cancer Res.* **43**, 2223-2234.

Seshadri, R., Raymond, W.A, Leong, A.S.Y., Horsfall, D.J., & McCaul, K. (1996). Vimentin expression is not associated with poor prognosis in breast cancer. *Int. J. Cancer* **67**, 353-356.

Simpson, R.T. (1978). Structure of the chromatosome, a chromatin particle containing 160 base pairs and all the histones. *Biochemistry* **17**, 5524-5531.

Sommers, C.L., Walker-Jones, D., Heckford, S.E., Worland, P., Valverius, E., Clark, R., McCormick, F., Stampfer, M., Abularach, S., & Gelmann, E.P. (1989). Vimentin rather than keratin expression in some hormone-independent breast cancer cell lines and in oncogene-transformed mammary epithelial cells. *Cancer Res.* **49**, 4258-4263.

Soule, H.D., Maloney, T.M., Wolman, S.R., Peterson, W.D. Jr., Brenz, R., McGrath, C.M., Russo, J., Pauley, R.J., Jones, R.F., & Brooks, S.C. (1990). Isolation and characterization of a spontaneously immortalized human breast epithelial cell lines, MCF-10^1. *Cancer Res.* **50**, 6075-6086.

Stein, G.S., Lian, J.B., Dworetzky, S.I., Owen, T.A., Bortell, R., Bidwell, J.P., & van Wijnen, A.J. (1991). Regulation of transcription-factor activity during growth and differentiation: involvement of the nuclear matrix in concentration and localization of promoter binding proteins. *J. Cell. Biochem.* **47**, 300-305.

Stuurman, N., Meijne, A.M.L., van der Pol, A.J., De Jong, L., van Driel, R., & van Renswoude, J. (1990). The nuclear matrix from cells of different origin: Evidence for a common set of matrix proteins. *J. Biol. Chem.* **265**, 5460-5465.

Sutherland, R.L., Hall, R.E., & Taylor, I.W. (1983). Cell proliferation kinetics of MCF-7 human mammary carcinoma cells in culture and effects of tamoxifen on exponentially growing and plateau-phase cells. *Cancer Res.* **43**, 3998-4006.

Tait, L., Soule, H.D., & Russo, J. (1990). Ultrastructural and immunocytochemical characterization of an immortalized human breast epithelial cell line, MCF 10. *Cancer Res.* **50**, 6087-6094.

Taylor, Y.C., Zhang, X., Parsian, A.J., & Duncan, P.G. (1991). Image analysis-based measurement of DNA supercoiling changes in transformed and nontransformed human cell lines. *Exp. Cell Res.* **197**, 222-228.

Thompson, E.W., Paik, S., Brunner, N., Sommers, C.L., Zugmaier, G., Clarke, R., Shima, T.B., Torri, J., Donahue, S., Lippman, M.E., Martin, G.R., & Dickson, R.B. (1992). Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. *J. Cell. Physiol.* **150**, 534-544.

Toi, M., Bicknell, R., & Harris, A.L. (1992). Inhibition of colon and breast carcinoma cell growth by interleukin-4. *Cancer Res.* **52**, 275-279.

Trask, D.K., Band, V., Zajchowski, D.A., Yawsen, P., Suh, T., & Sager, R. (1990). Keratins as markers that distinguish normal and tumor-derived mammary epithelial cells. *Proc. Natl. Acad. Sci. USA* **87**, 2319-2323.

Tsutsui, K., Tsutsui, K., & Muller, M.T. (1988). The nuclear scaffold exhibits DNA-binding sites selective for supercoiled DNA. *J. Biol. Chem.* **263**, 7235-7241.

Tsutsui, K., Tsutsui, K., Okada, S., Watarai, S., Seki, S., Yasuda, T., & Shohmori, T. (1993). Identification and characterization of a nuclear scaffold protein that binds the matrix attachment region DNA. *J. Biol. Chem.* **268**, 12886-12894.

Turner, C.E., & Burridge, K. (1991). Transmembrane molecular assemblies in cell-extracellular matrix interactions. *Curr. Opin. Cell. Biol.* **3**, 849-853.

Vic, P., Vignon, F., Derocq, D., & Rochefort, H. (1982). Effect of estradiol on the ultrastructure of the MCF_7 human breast cancer cells in culture. *Cancer Res.* **42**, 667-673.

Villeponteau, B., Lundell, M., & Martinson, H. (1984). Torsional stress promotes the DNase I sensitivity of active genes. *Cell* **39**, 469-478.

Vogelstein, B., Pardoll, D.M., & Coffey, D.S. (1980). Supercoiled loops and eukaryotic DNA replication. *Cell* **22**, 79-85.

von Kries, J.P., Buhrmester, H., & Stratling, W.H. (1991). A matrix/scaffold attachment region binding protein: identification, purification, and mode of binding. *Cell* **64**, 123-135.

Walters, M.C., Fiering, S., Eidemiller, J., Magis, W., Groudine, M., & Martin, D.L.K. (1995). Enhancers increase the probability but not the level of gene expression. *Proc. Natl. Acad. Sci. USA* **92**, 7125-7129.

Wang, D.M., Taylor, S., & Levy-Wilson, B. (1996a). Evaluation of the function of the human apolipoprotein B gene nuclear matrix association regions in transgenic mice. *J. Lipid. Res.* **37**, 2117-2124.

Wang, N., Butler, J.P., & Ingber, D.E. (1993). Mechanotransduction across the cell surface and through the cytoskeleton. *Science* **260**, 1124-1127.

Wang, X., Tolstonog, G., Shoeman, R.L., & Traub, P. (1996b). Selective binding of specific mouse genomic DNA fragments by mouse vimentin filaments *in vitro*. *DNA Cell Bio.* **15**, 209-225.

Ward, W.S, Schmidt, W.N., Schmidt, C.A., & Hnilica, L.S. (1984). Association of cytokeratin p39 with DNA in intact Novikoff hepatoma cells. *Proc. Natl. Acad. Sci. USA* **81**, 419-423.

Watts, C.K.W., Handel, M.L., King, R.J.B., & Sutherland, R.L. (1992). Oestrogen receptor gene structure and function in breast cancer. *J. Steroid Biochem. Molec. Biol.* **41**, 529-536.

Wedrychowski, A., Schmidt, W.N., Ward, W.S., & Hnilica, L.S. (1986). Crosslinking of cytokeratins to DNA *in vivo* by chromium salt and *cis*-diamminedichloroplatinum(II). *Biochemistry* **25**, 1-9.

Wright, C., Nicholson, S., Angus, B., Sainsbury, J.R., Farndon, J., Cairns, J., Harris, A.L., & Horne, C.H. (1992). Relationship between c-erbB-2 protein product expression and response to endocrine therapy in advanced breast cancer. *Br. J. Cancer* **65**, 118-121.

Yanagisawa, J., Ando, J., Nakayama, J., Kohwi, Y., & Kohwi-Shigematsu, T. (1996). A matrix attachment region (MAR)-binding activity due to a p114 kilodalton protein is found only in human breast carcinomas and not in normal and benign breast disease tissues. *Cancer Res.* **56**, 457-462.

Yang, C. H., Lambie, E.J., & Snyder, M. (1992). NuMA: an unusually long coiled coil related protein in the mammalian nucleus. *J. Cell Biol.* **116**, 1303-1317.

Yotsuyanagi, T., Ohta, N., Futo, T., Ito, S., Chen, D., & Ikeda, K. (1991). Multiple and irreversible binding of *cis*-diamminedichloroplatinum(II) to human serum albumin and its effect on warfarin binding. *Chem. Pharm. Bull.* **39**, 3003-3006.

Yu, J., Bock, J.H., Slightom, J.L., & Villeponteau, B. (1994). A 5' β-globin matrix-attachment region and the polyoma enhancer together confer position-independent transcription. *Gene* **139**, 139-145.

Zambetti, G., Ramsey-Ewing, A., Bortell, R., Stein, G., & Stein, J. (1991). Disruption of the cytoskeleton with cytochalasin D induces c-*fos* gene expression. *Exp. Cell Res.* **192**, 93-101.

Zeitlin, S., Parent, A., Silverstein, S., & Efstratiadis, A. (1987). Pre-mRNA splicing and the nuclear matrix. *Mol. Cell. Biol.* **7**, 111-120.

Zeng, C., He, D., & Brinkley, B. (1994). Localization of NuMA protein isoforms in the nuclear matrix of mammalian cells. *Cell. Motil. Cytoskeleton* **29**, 167-176.

Zhao, K., Kas, E., Gonzalez, E., & Laemmli, U.K. (1993). SAR-dependent mobilization of histone H1 by HMG-1/Y *in vitro*: HMG-1/Y is enriched in H1-depleted chromatin. *EMBO J.* **12**, 3237-3247.

Zini, N., Mazzotti, G., Santi, P., Rizzoli, R., Galanzi, A., Rana, R., & Maraldi, N.M. (1989). Cytochemical localization of DNA loop attachment sites to the nuclear lamina and to the inner nuclear matrix. *Histochemistry* **91**, 199-204.