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ISOLATION AND CHARACTERIZATION OF THE RAT PLACENTAL LACTOGEN-II GENE

BY

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF THE RAT PLACENTAL LACTOGEN-II GENE(rPL-II). Paresh C. Shah, Department of Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, Canada.

Growth Hormone (GH), Prolactin (PRL) and Placental Lactogen (PL) comprise a family of polypeptide hormones that appear to have arisen from a common ancestor by gene duplication. In our laboratory, a cDNA clone to rPL-II has been isolated and sequenced, revealing that in the rat, unlike the human, placental lactogen is more closely related to the PRL's than to the GH's. We have now isolated and characterized the gene for rPL-II. A 714 bp cDNA clone (c52-A) to rPL-II was used to screen a rat genomic DNA library constructed in the lambda vector EMBL3. Two overlapping genomic clones, designated GC-I (18.5 Kilobases) and GC-II (9.4 Kilobases) were isolated. The GC-I clone appears to contain the entire rPL-II gene on 5 Eco RI fragments, while GC-II contains 3' coding and flanking regions. A detailed restriction map shows that like the PRL and GH genes the rPL-II gene is made up of at least five exons and four introns. The gene is about 5.4 Kilobases, making it smaller than rPRL (10 Kilobases) and larger than rGH (2.1 Kilobases). Like the rPRL gene, the rPL-II gene appears to contain a repetitive element within the fourth exon. In summary, it appears that the rPL-II gene shares a number of structural similarities with rPRL and rGH genes.

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LIST OF ABBREVIATIONS

HORMONES

GH	Growth Hormone
PRL	Prolactin
PL	Placental Lactogen

HORMONE PREFIXES

b	bovine
h	human
m	mouse
o	ovine
r	rat

UNITS OF MEASURE

pg	picogram
ng	nanogram
ug	microgram
mg	milligram
g	gram
kg	kilogram
cm	centimeter
mm	millimeter
uM	micromolar
mM	millimolar
M	molar
ul	microliter
ml	millilter
l	liter

LIST OF ABBREVIATIONS (cont.)

MISCELLANEOUS

^{o}C	degrees centigrade
bp	basepairs
Kb	kilobases
V	volts
mA	milliamps
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
cDNA	complementary DNA
mRNA	messenger RNA
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
$dCTP^{32}$	[$-^{32}P$]-deoxycytidine 5'-triphosphate
ATP	adenosine triphosphate
DNase	deoxyribonuclease
RNase	ribonuclease
BSA	bovine serum albumin
DTT	dithiothreitol
$CHCl_3$	chloroform
CsCl	cesium chloride
LMP	low melting point
EDTA	ethylenediaminetetraacetic acid
Tris	Trizma base
TE	Tris-EDTA

LIST OF ABBREVIATIONS (cont.)

SDS	Sodium dodecyl sulphate
$O.D._{550}$	Optical density determined by spectrophotometry at wavelength of 550nm.

INTRODUCTION

A. THE PLACENTA

The placenta plays an integral role in mammalian reproduction. Its importance to the developing fetus is not surprising, as it is the tissue that provides the anatomical and nutritional link between mother and fetus. The placenta is derived from embryonic tissue, and as it develops, it forms a barrier between the fetal and maternal circulations. It has been known for years that this organ allows for exchange of nutrients, waste products, and gases between the two circulations, so as to provide a favorable environment for fetal maturation. Along with the fetus, it acts as a multi-organ system, serving different physiologic roles for the fetus throughout its development. It is not a passive filter, but is rather active and selective in the type of substances with which it allows the fetus to interact. The rate of glucose transfer between maternal and fetal circulations via the placenta is much greater than normal, indicating that its diffusion must be facilitated by a transport system (Newsholme,1977). In addition, the amino acid concentrations are higher in the fetal circulation compared to the maternal side, indicating that transfer to the fetus is against a concentration gradient (Crumpler et al.,1950) In this respect, the placenta not only helps sustain fetal growth, but also provides the fetus with a form of protection.

Being a temporary organ, the placenta must also create an immunologic barrier, since it is essentially an

allograft that is foreign to the mother's body. In addition, the placenta produces many hormones with which it is able to regulate specific maternal body functions. In a matter of weeks or months, depending on the species, the placenta differentiates into a mature state. During this period, it performs a series of tasks which continuously change throughout pregnancy so as to adapt to the needs of the developing fetus.

The autonomy of the placenta is best exemplified by the observation that it continues to survive and produce hormones in the uterus after fectectomy (Petropoulos,1973). The placenta utilizes approximately one-third of the oxygen and glucose that is provided from the maternal side for the fetus (Newsholme,1976). The role of the placenta in exchanging substances between the fetus and mother has been well studied, but its endocrine functions are not as well understood.

Why does the placenta have an endocrine role? Is it not redundant to produce hormones that are the same or similar to those produced by the maternal system? Do these hormones affect the fetal and maternal systems independently or in synergy with the maternal hormones? These hormones may also serve a paracrine function by aiding the growth and maintenance of the placenta. The endocrine role of the placenta varies from species to species. There are some features that have been conserved, and this warrants further investigation to try and

elucidate the significance of these pregnancy-specific hormones.

B. THE PLACENTA AS AN ENDOCRINE GLAND

Since the early 1900's, the placenta has been implicated as a source of pregnancy-specific proteins. The first evidence of an endocrine function for the placenta was demonstrated when a sow placental extract referred to as "chorinine", was shown to stimulate lactation in women (Bouchacourt,1902). It was later demonstrated that a gonadotropin-like substance in the human placenta was capable of maintaining corpus luteum function during the early weeks of pregnancy (Halban,1905; Fellner,1913). The corpus luteum produces progesterone, a hormone involved in the maintenance of pregnancy. For this reason, it is essential that the corpus luteum is sustained, so that pregnancy may be maintained. Ascheim and Zondek (1928) later identified a gonadotropin in the urine of pregnant women and gave it the name "prolan". This, the first placental hormone to be characterized, is now referred to as human chorionic gonadotropin (hCG). Human CG is a glycoprotein composed of α and β-subunits; the latter has structural homology to the β-subunits of three pituitary derived hormones: human luteinizing hormone (hLH), human follicle stimulating hormone (hFSH) and human thyroid stimulating hormone (hTSH), (Closset,1973). These comprise a family of hormones which may have evolved from an ancestral protein by gene duplication and mutation

(Archer,1976).

Further evidence in support of an endocrine role for the placenta came from studies with rats. Pencharz and Long (1931) found that hypophysectomy in the first half of pregnancy in the rat terminated pregnancy. However, if hypophysectomy was performed after day ten or eleven of pregnancy, the fetuses survived. In addition, Greep (1938) demonstrated that after hypophysectomy, if these corpora lutea were maintained and transferred to non-pregnant animals, they were non-functional. This showed that although the pituitary was important for maintaining early pregnancy, other extra-pituitary factors were essential during late pregnancy to allow it to continue. Astwood and Greep (1938) subsequently demonstrated that the placenta produced a substance capable of maintaining corpus luteum function in the pseudopregnant rat. This was demonstrated by injections of rat placental extracts which prolonged corpus luteum function. They concluded that during normal pregnancy, a hormone with luteotropic activity was secreted by the placenta. More recently, Jayatilak et al. (1985) have identified a prolactin-like hormone produced by rat decidual tissue. They describe this hormone as possessing luteotopic activity as it has a marked effect on luteal cell function. Some of these effects include an increase in ovarian steroidogenesis <u>in vivo</u>, as well as stimulation of luteal cell production of progesterone and luteinizing hormone stimulated steroidogenesis <u>in vitro</u>.

The first evidence that the rat placenta could elicit

a mammotropic effect was shown by Lyons (1943) when mammary gland development and lactation in hypophysectomized, ovariectomized virgin rats were stimulated on injection with a combination of estrogen and rat placental extract. This was the same response observed when estrone, progesterone and pituitary prolactin were injected into the rats. Lyons (1952) also demonstrated a simultaneous luteotropic and mammotropic effect in hypophysectomized rats that were only treated with estrone and rat placental extract. He suggested that rat placenta induced progesterone secretion from rat ovary and that a synergistic effect between estrone and rat placental extract caused mammary gland growth. These findings that the rat placenta was capable of carrying out functions previously thought to be exclusive to the pituitary led investigators to look for further similarities between placental and pituitary hormones. Confirmation of Astwood and Greep's work came when it was demonstrated that a rat placental substance, "cyonin", stimulated progestin secretion by the corpus luteum (Averill et al.,1950). In 1954, Ray et al. demonstrated that the rat placenta produced a substance or substances with luteotropic, mammotropic, lactogenic and crop-sac stimulating activities comparable to pituitary prolactin.

Prolactin-like activity in the placenta is not exclusive to the rat. It had previously been demonstrated in the mouse (Newton,1939; Gardner,1942) and the rhesus

monkey (Agate,1952). The earliest report of prolactin-like activity in human placenta was that of Ehrhardt (1936). This substance was later isolated by Ito and Higashi (1961). The following year, Josimovich and Maclaren (1962) isolated a substance from human placenta that exhibited lactogenic activity and was immunologically similar to pituitary growth hormone. They called this substance human placental lactogen (hPL). This finding was confirmed by Kaplan and Grumbach (1964) who localized its production to the syncytiotrophoblast cells. Although they coined the term human chorionic growth hormone-prolactin for this substance, it is now referred to as chorionic somatomammotropin (CS) or more commonly, placental lactogen (PL).

C. PLACENTAL LACTOGENS

Although placental lactogens are thought to be involved in many functions, they have not been clearly identified as being essential for any particular physiological event. It has been suggested that placental lactogens may be involved in the preparation of the mammary gland for postpartum lactation (Blank,1977; Forsyth,1974), in the stimulation of ovarian and placental steroidogenesis (Simpson and MacDonald,1981), and in the promotion and maintenance of fetal growth (Forsyth,1974). The possibility that placental lactogens act in synergy with other placental or maternal hormones to carry out specific functions cannot be excluded.

(i) Human Placental Lactogen

Human placental lactogen is a single-chain polypeptide hormone of molecular weight 21,000 daltons and very similar in structure to hGH and hPRL (Niall et al.,1973). Although it is chemically similar to pituitary hGH, its somatotropic activity is approximately 0.1-1% of that which hGH possesses (Chard,1982). In the rat, hPL displays some luteotropic activity as defined by its ability to influence corpus luteum progesterone secretion in hypophysectomized rats (Josimovich,1980) and to enhance placental progesterone secretion (Josimovich and Archer,1977). Human PL has no effect on human corpus luteum function, but it may be involved in progesterone regulation in the placenta and fetus (Josimovich,1974). It possesses lactogenic activity and may be involved in mammary gland development during pregnancy (Josimovich and Maclaren,1962; Forsyth,1974). In addition, hPL has been implicated in promoting fetal growth by affecting maternal metabolism. This is accomplished by mobilizing amino acids and glucose from maternal stores so that they may be transferred to the fetus (Munro and Chatterjee,1977). This action is thought to be mediated by an anti-insulin type role that hPL is thought to possess (Simpson and MacDonald,1981).

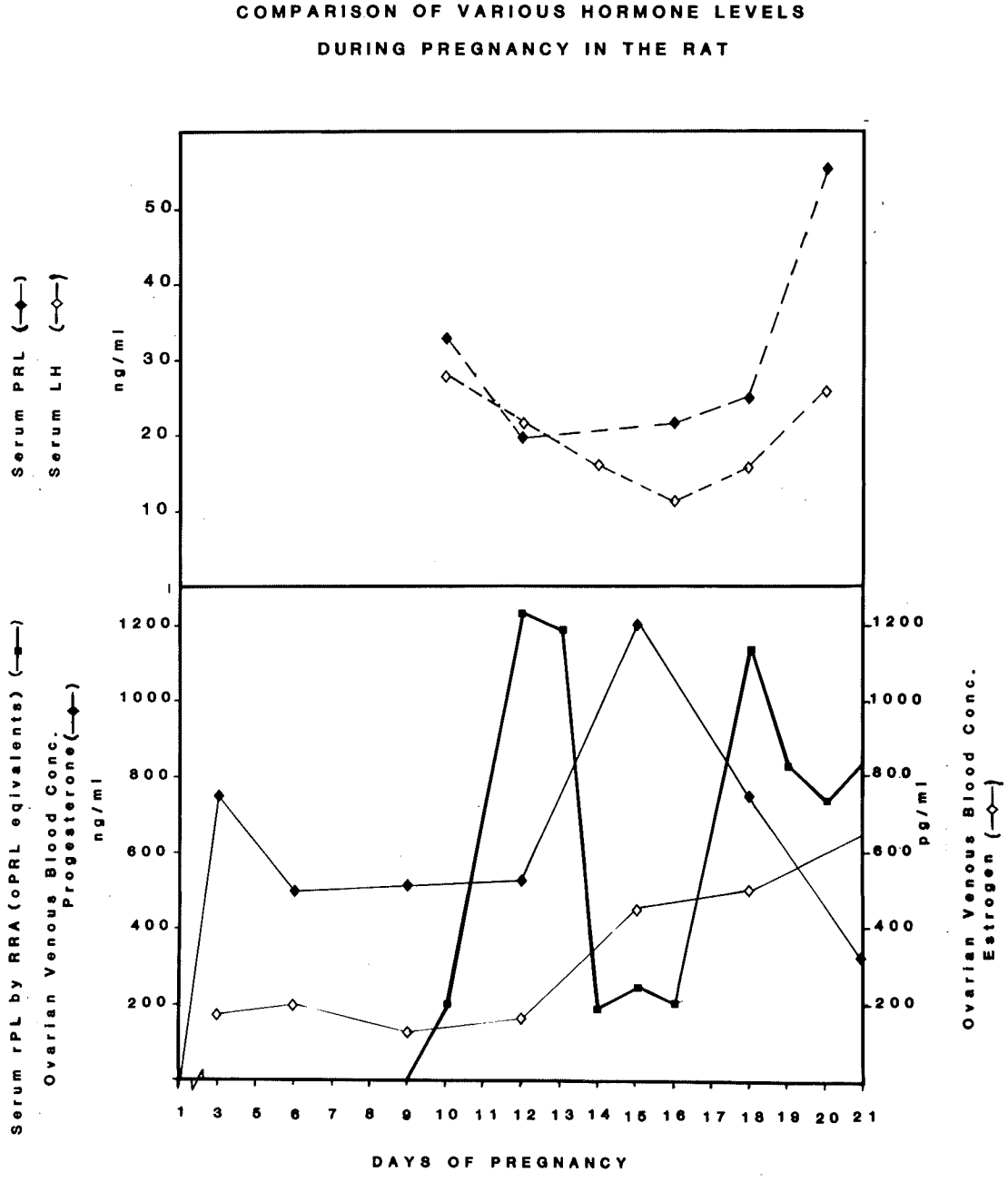
During the third trimester of pregnancy, the rate of production of hPL is an astonishing 1-2 grams/day. This is much greater than the production of any other protein hormone during a normal state (Chard,1982). For this reason alone, it seems peculiar that no definitive role has

been assigned to this hormone. Adding to the uncertainty are reported cases of normal pregnancies from mothers with no detectable levels of hPL in maternal serum or placental tissue (Nielsen et al.,1979; Sideri et al.,1983). In both cases, the babies were normal and in good health. Thus, another shadow has been cast over the physiologic role of hPL. It is therefore not surprising that research on placental lactogens has led to investigation of PL's in other species in hopes of gaining a better understanding as to their role during pregnancy and parturition. Placental lactogen production has been documented in a variety of species and many have been purified and biochemically characterized (Kelly et al.,1976; Blank et al.,1977; Talamantes,1980). Since the gestation period of a rat is only 21 days this species appears to be a suitable candidate in which to further our studies on placental lactogens. Figure A shows the levels of various hormones produced in the rat throughout gestation (Shiu and Friesen,1975 and Blank et al.,1977).

(ii) Rat Placental Lactogen

The development of a radioreceptor assay (RRA) for lactogenic hormones (Shiu et al.,1973) allowed Kelly et al. (1975) to identify two peaks of activity in maternal serum for rat placental lactogen (rPL). They found that the first peak, between days 11-13, was a 40-50,000 dalton species while the second peak, between days 17-21, was about 20,000 daltons. The latter was immunoreactive in an

FIGURE A


COMPARISON OF VARIOUS HORMONE LEVELS
DURING PREGNANCY IN THE RAT
Serum PRL (—◆—)
Serum LH (—◇—)
ng/ml
50
40
30
20
10
Serum rPL by RRA (oPRL eqivalents) (—■—)
Ovarian Venous Blood Conc.
Progesterone (—◆—)
ng/ml
1200
1000
800
600
400
200
Ovarian Venous Blood Conc.
Estrogen (—◇—)
pg/ml
1200
1000
800
600
400
200
1 3 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
DAYS OF PREGNANCY

RIA developed against a lactogenic hormone present in late pregnant rat placenta (Robertson and Friesen,1981). Both the early and late forms, designated rPL-I and rPL-II respectively, have since been characterized by Robertson et al. (1982).

As is the case with hPL, no specific biological function has been assigned to the rPLs. The involvement of rPL in mammary gland development was suggested by the observations that rats hypophysectomized in mid-pregnancy still showed mammary gland development and transient lactation at parturition (Talamantes,1980). Bussman et al.(1983) concluded that in the absence of prolactin and progesterone, rPL could still induce lactose synthesis during late pregnancy. In addition, it has been shown that the progressive removal of fetoplacental units on day 12 or 16 of pregnancy reduced the weight of mammary glands to nonpregnant control levels by day 21 (Forsyth,1974). The importance of the rat placenta in mammary gland development has been investigated and direct evidence exists documenting that rPL possesses lactogenic activity, as determined by radioreceptor assay and bioassay (Blank et al.,1977).

Glaser et al.(1984) have investigated the actions of both rPL-I and rPL-II. They report that rPL-I, present in the maternal circulation during mid-pregnancy (days 11-13) has luteotropic properties and designate it as rat placental luteotopin. This fits well with the earlier finding that the primary site of the luteotropic activity

shifts from the pituitary (pituitary prolactin) to the placenta, at around day 11 (Blank et al.,1977). Furthermore, Morishige and Rothchild (1973) had suggested that a two phased control mechanism exists to enhance sustained progesterone secretion throughout gestation. They stated that from days 1-7, PRL, along with either estrogen, FSH, LH or a combination was responsible for maintaining progesterone secretion, while during days 8-12, a placental luteotropin-LH complex was responsible. This luteotropin may be rPL-I, as a search for a rat equivalent to hCG has proved to be unsuccessful (Tabarelli et al.,1982; Wurzel et al.,1983). Rat PL-II possesses no luteotropic activity but is lactogenic and may possibly be a luteolytic agent (Glaser et al.,1984).

Rat PL is secreted in an episodic fashion which does not seem to correlate well with the ultradian patterns exhibited by rGH and rPRL (Klindt et al.,1981). The episodic secretion of these hormones is of a pulsatile nature (Klindt et al.,1982). It has been proposed that rPL is an inhibitor of pituitary PRL surges. The biphasic diurnal and nocturnal PRL surges which begin after mating, terminate after days 8 and 10 respectively (Smith and Neill,1976). An inverse relationship with respect to rPL and rPRL has led to the suggestion that an increase in rPL secretion at mid-pregnancy has a inhibitory influence on rPRL release (Tonkowicz and Voogt,1983,1984; Voogt,1984).

The difficulty in defining a biological role for rPL

is reinforced by the difficulty in defining physiological control mechanisms for its synthesis and release. No inhibiting or releasing factors have been clearly defined as has been the case with GH and PRL. Therefore, an investigation of rPL at the level of the gene may help answer some questions about the structure, function and regulation of placental lactogens.

D. GH - PRL GENE FAMILY

Growth hormone, prolactin, and placental lactogen comprise a family of polypeptide hormones that appear to have arisen from a common ancestor by gene duplication (Niall et.al,1971). Evidence has accumulated to show that these hormones are related functionally, immunochemically, and structurally. Growth hormone and PRL are produced in the anterior pituitary, while in the human, PL is synthesized in the syncytiotrophoblast of the placenta. All three mature proteins are of similar size, ranging from 190 to 199 amino acids. Each has two homologous disulfide bonds and four internal regions of homology. These four internal regions are also homologous among the other members of the family (Niall,1971; Miller and Eberhardt,1983). Human GH and hPL are more closely related to one another than to hPRL. Although these hormones are produced in different tissues, each possesses lactogenic and growth-promoting properties (Miller and Eberhardt,1983). Human GH and hPL share 85% homology at the amino acid level while hGH shares only about 35%

homology to hPRL (Bewley,1972; Barsh,1983). In addition, hGH and hPL share greater than 90% sequence homology at the nucleotide level (Fiddes,1979) but, hGH and hPRL have only 42% homology to each other (Cooke,1981). It is not that surprising therefore, to find that hGH and hPL are located in close proximity on the long arm of chromosome 17 and consist of a cluster of several genes (Owerbach,1980; Moore,1982). Human PRL is found as a single copy on chromosome 6 (Owerbach,1981; Truong,1984).

Until recently, hPL remained the only PL to be cloned and sequenced. In our laboratory, a cDNA clone to rPL-II has recently been sequenced and analyzed (Duckworth et al.,1986a). The results reveal that rPL-II is more closely related to rPRL than to rGH. This finding coincides with reports about other mammalian species where it has been suggested that bovine PL and ovine PL (Hurley,1977) and also rabbit PL (Bolander,1976) may all be more closely related to PRL rather than GH. This thesis describes the isolation and characterization of clones derived for the gene that codes for rPL-II. To put this information in context with other members of the PRL-GH gene family, further details pertaining to the structure of the rGH and rPRL genes shall be discussed.

E. rPRL - rGH GENE STRUCTURE

The cDNA and genomic DNA clones for rPRL have been isolated, sequenced and analyzed (Cooke,1980,1982; Gubbins,1980). Similar information has also been published

in several reports for rGH cDNA and genomic DNA clones (Seeburg,1977; Barta,1981; Page,1981). The coding regions of the genes for rPRL and rGH are present in five exons which are interrupted by four introns. The rPRL gene spans approximately 10 Kb (Gubbins,1980) while rGH is only about 2.1 Kb long (Barta,1981). The difference in length between the two genes is due largely to the size of the introns, as the coding regions are approximately the same size. It is interesting to note that when the coding regions of rGH and rPRL are aligned to maximize homology, the splice junctions of all four introns occur at analogous positions in both genes (Cooke,1982). Thus although there is only 25% amino acid homology and 39% nucleotide homology between rGH and rPRL, the conservation of splice junctions supports the hypothesis that both genes evolved from a common ancestral gene.

Cooke et al. (1982) postulated that although the structures of rPRL and rGH suggest a common precursor, the 5' ends of both genes may have originated independently. This was suggested because the exon 1 regions of these genes are of different lengths and lack homology to one another. Secondly, exon 1 in both genes is flanked by a direct repeat sequence that is 80% homologous in the two genes. The presence of these direct repeats suggests that they may have been involved in the insertion of different exon 1 regions into the genes at this location. In addition, the sequences flanking the 5' repeat are greater than 75% homologous between rGH and rPRL. The repeat at

the 3' end of the exon 1 is part of the first intron of both genes.

Repetitive DNA sequences were initially detected within the rPRL gene by Gubbins (1980) and have now been extensively mapped by Weber et al. (1984). Repeated sequences were found throughout most of the 3' region flanking the last exon and in a couple of regions upstream of the first exon. These major repeated sequences near the rPRL gene hybridize to a known family of repetitive DNA characterized by a 1.3 Kbp EcoRI fragment. Also, there is an 820 bp repeat sequence within the fourth intron which has limited homology to human Alu elements. In addition, a 508 bp repeated sequence has been identified in the second intron of the rGH gene (Barta,1981). Homology between the rPRL repeats that are members of the 1.3 Kb family of rat repetitive DNA has been established within this 508 bp rGH repeated sequence (Weber,1984).

The 5' flanking regions of numerous genes have been studied extensively and are known to contain structures which are vital for efficient transcription of the gene. They may also possess regions that allow the gene to be regulated by different hormone-receptor complexes and can contain sequences which may be responsible for tissue specific expression of a gene (Miller and Eberhardt,1983). The promoter region of a gene is involved in directing proper initiation of transcription. The structure TATAAA is associated with the promoter region of a gene. It is

usually found about 30 bp upstream from the site of transcription (Goldberg,1979). Some other eukaryotic promoters possess a structure similar to GC(CT)CAATCT situated approximately 40 bp upstream from the TATAAA sequence (Benoist,1980). This sequence may or may not have an important influence on transcription.

Analysis of the rPRL and rGH genes has shown they contain the probable promoter element, TATAAA, in the 5' flanking region. This structure is situated within the two direct repeats that flank exon 1 of both genes. Neither gene has a CAAT sequence 40 bp upstream of the TATAAA sequence, but they both possess what may be a non-functional promoter sequence upstream from the 5' direct repeat. This sequence is AATAAA and in the rPRL gene is preceded by a CAAAT sequence 42 bp upstream. Thus, Cooke et al. (1982) have postulated that an insertion of non-homologous first exons along with regulatory signals 5' to the genes, may be the reason for the divergence of function and regulation of rGH and rPRL.

Other important structural features of the rPRL and rGH genes include the finding that sequences in the immediate 5' flanking region of both genes are responsible for conferring sensitivity to hormonal regulation (Leff et al.,1986). Further to this, Nelson et al. (1986) have reported the identification of 5' flanking nucleotide sequences that are involved in transferring cell-specific expression to heterologous genes. This was determined by examining the expression of fusion genes containing these

"enhancer" sequences from the PRL and GH genes in cell lines specific for the production of either GH or PRL. On the other hand, Birnbaum and Baxter (1986) have shown that the 5' flanking region may not be exclusively important for the efficient expression of the rGH gene. They detected almost full length transcripts after deleting the 5' flanking sequences of rGH. Also, after making the deletions, the gene still possessed sequences that were sensitive to glucocorticoid regulation. These sequences may be within the gene or in the 3' flanking region.

It would be interesting to see if rPL-II gene has similar structural features to rPRL and/or rGH. As mentioned, we do know that rPL-II is more related to rPRL than rGH at the nucleotide level. Duckworth et al. (1986) have shown that rPL-II has 52% amino acid homology to rPRL and only 34% homology to rGH. When comparing nucleotide sequences , rPL-II has 54% homology to rPRL and only 36% to rGH. These results are the opposite of what is seen in humans. A more detailed look at the gene itself may answer more pressing questions as to its regulation both _in vivo_ and _in vitro_. It would also be important to see how the rPL-II gene compares to its family members with respect to its size, intron/exon structure, and regulatory elements. As mentioned earlier, the work in this thesis has involved isolating clones of the rPL-II gene from a library of rat genomic DNA sequences and subsequent characterization of the size, restriction map and intron/exon structure of the

gene.

F. NEW PLACENTAL CLONES IN THE GH-PRL GENE FAMILY

Besides rPL-II, several other major proteins are produced in late-term rat placenta. Work in our laboratory has led to the discovery of several day 18 placental cDNA clones which exhibit varying degrees of homology to rPRL and hPRL, as well as to one another. These clones show different restriction enzyme maps and exhibit varied mRNA induction patterns through gestation (Duckworth et al.,1986b and unpublished observations). One of these clones, rat prolactin-like protein A (rPLP-A) has been characterized in detail (Duckworth et al.,1986b). Sequence analysis shows that it possesses greater than 40% amino acid homology to rPRL, hPRL and rPL-II, but only 29% homology to rGH. Similar analysis on the other clones is currently being done.

Recently, Linzer and Nathans (1983, 1984) reported the isolation and characterization of a novel member of the GH-PRL gene family, They identified the production of several growth related mRNAs during the proliferative response of mouse 3T3 cells to serum. One of these mRNAs coded for a protein which they termed proliferin. Subsequent studies have shown the presence of proliferin mRNA in the mouse exclusively in the placenta (Linzer et al.,1985). Mouse proliferin has structural similarities to the prolactins and is distinct from mPL. In comparison to bovine PRL, it shows 55% nucleotide sequence homology. Linzer and Nathans

(1985) have also identified another PRL-like protein present in mouse placenta. They called it proliferin related protein (PRP) as it has a very similar sequence to proliferin.

The identification of these new members to the GH-PRL gene family from both mouse and rat placenta poses interesting questions as to their role in pregnancy. Because of mPLF's association with cell proliferation, there is speculation that it may serve a role as a growth factor. Further studies may lead to a better understanding as to their biological significance as well as their evolutionary patterns.

G. CONSTRUCTING A LAMBDA GENOMIC DNA LIBRARY

A DNA genomic library is essentially a cloned collection of DNA fragments representing the entire genome of a particular species. The first libraries had to enrich for the DNA sequences of interest before they could be cloned into the bacteriophage vectors. The enrichment was done by separating DNA fragments on preparative agarose gels (Tilghman et al.,1977; Tonegawa et al.,1977), reverse-phase chromatography (Hardies and Wells,1976; Landy et al.,1976) or both. The fractions containing the sequences of interest were detected by hybridization to cDNA probes before cloning into bacteriophage vectors. The subsequent development of efficient *in vitro* packaging systems (Hohn and Murray,1977; Sternberg et al.,1977) and *in situ* hybridization techniques by Benton and Davis (1977)

have eliminated the need for this extensive enrichment of sequences of interest. At present, complete libraries are constructed from eukaryotic genomic DNA and then the sequences of interest are identified by hybridization to the recombinant phage that contain DNA sequences of interest.

A genomic library is prepared by partially digesting total, unsheared genomic DNA with one or two restriction endonucleases that cut DNA at frequent intervals. The DNA is then size fractionated either on a preparative agarose gel or a sucrose density gradient. This allows for the isolation of fragments of the proper size to be cloned in the appropriate lambda cloning vector. In the case of EMBL3, the fragments to be cloned are between 10 and 20 Kb in length. The DNA fragments are ligated to the vector DNA following which they are placed in an <u>in vitro</u> packaging system along with bacteriophage proteins, so that together, viable phage particles may be formed. The packaging system consists of the necessary bacteriophage proteins which together, may be assembled into a viable bacteriophage that contains some of the foreign DNA. The recombinant phage which now have target DNA incorporated, are used to infect a host bacterial strain over the surface on an agar plate. The phage particles are allowed to infect the bacterial cells and form visible plaques on the surface of the agar, representing regions of lysed bacterial cells. The phage DNA from these plaques is transferred and bound to nitrocellulose membrane filters. These filters are then

screened for the DNA sequence of interest by hybridizing them to a radioactively labelled cDNA probe (Benton and Davis,1977).

Construction of a genomic DNA library using a plasmid cloning vector would be impractical as one would be limited to much smaller insert sizes. Although it is possible to clone fairly large pieces of DNA in a plasmid, an insert of 8-10 Kb or larger would result in a low efficiency of transformation, thereby reducing the yield of recombinants obtained. For this reason, it would be necessary to screen many more recombinants to obtain a representative genomic library because one would be forced to clone smaller pieces of DNA. Also, a very large quantity of bacterial culture would be needed to perform the transformations, making the procedure quite laborious.

The advent of lambda cloning vectors has made producing and screening a genomic DNA library many times more efficient. The lambda bacteriophage is a double-stranded DNA virus with a genomic size of about 50 Kbp. The DNA is in a linear duplex form with small single-stranded "cohesive" ends (12 nucleotides in length) that are complementary to one another, These cohesive ends allow the DNA to circularize after the phage infects its host. The phage may then enter either a lytic or lysogenic replicative pathway. During lysogeny, the phage DNA itself is incorporated into the host genome and is replicated and passed down to the progeny as the bacteria divide. During

lytic growth, a large number of phage particles are formed and the cell eventually lyses when these new phage mature into infectious virus particles. The lytic growth cycle is the basis by which a genomic DNA library may be grown and amplified (Maniatis,1982).

The primary advantage of lambda vectors is their capacity to accept large pieces of foreign DNA for cloning purposes. The middle one-third of the lambda phage genome may be replaced with another piece of DNA without affecting its ability to grow lytically (Blattner,1977). However, the insert should be no longer than 105% or shorter than 78% of the wild type genome so as not to decrease the viability of the phage (Maniatis,1982). In most cases, these vectors have a cloning capacity of 9 to 23 Kb (Karn,1980; Frischauf,1983). By taking full advantage of this cloning capacity, a library of 10^6 to 10^7 DNA fragments of approximately 20 Kb each would adequately represent a mammalian genome which is usually about 1-2 x 10^9 bp. By using a plasmid vector, it would necessitate screening 4 to 5 times more recombinants to represent this genome.

Performing a limited digestion of the genomic DNA rather than a complete digestion, increases the number of different fragments that are created. In addition, if a total digest was carried out, the entire gene might not get cloned, depending on the frequency of the sites for the enzyme being used. This in turn enables one to generate a series of overlapping clones so that it is possible to use

one clone as a probe to "walk" along the genome and identify other overlapping clones. A single complete digest would leave the possibility that certain sequences would not be represented because the fragments were either too large or too small to be cloned (Maniatis,1978). Partial digestion allows for larger fragments to be cloned and therefore decreases the number of recombinants to be screened. There is also no need to worry about the distribution of restriction enzyme sites within and around the sequences of interest (Maniatis,1982).

There is no assurance that the recombinants in a library represent 100% of the sequences in the genome. Although it is theoretically possible, it is more likely that approximately 5% of the sequences may be under-represented or not present at all in the library. This is due to the distribution of the the restriction enzyme sites within and around the sequence of interest. If the sites are too frequent or not frequent enough, the piece may not be of clonable size. Thus, the use of two frequent cutting enzymes can help offset this problem.

MATERIALS AND METHODS

ENZYMES

Restriction endonucleases were obtained from either Bethesda Research Laboratories (BRL), Boehringer Mannheim Corporation (BMC), or New England Biolabs (NEBL). Alkaline Phosphatase, Deoxyribonuclease I, Lysozyme, Proteinase K, and T4 DNA Ligase were all from BMC. Ribonuclease H was supplied by BRL.

AGAROSE

Agarose-DNA grade (Electrophoresis Reagent) from Bio-Rad was used for analytical gel electrophoresis. Specially purified Agarose-NA from Pharmacia (PL) was used in preparative gels for isolating fragments to be subcloned. Ultra pure low melting point agarose from BRL was also used to isolate insert DNA. Ethidium bromide for staining DNA gels was from Sigma.

POLYACRYLAMIDE

Acrylamide, N,N'-methylene bis acrylamide (Electrophoresis Purity Reagents), Ammonium Persulfate and N,N,N,'N'-tetraethylethylenediamine (TEMED) were all purchased from Bio-Rad.

BACTERIAL STRAINS

The following two strains were used as hosts:

E.coli WA803 (rk-,mk-,supF+ was used for propagating the rat genomic libraries.

E.coli RRI (F-,recA+,rk-,mk-) was used for

transforming the following recombinant plasmid vectors: pRP52-A, pRP52, and pRP52-3.

RAT GENOMIC LIBRARIES

The Eco RI partial cleavage library was prepared from adult male Sprague-Dawley rat liver DNA. The library was cloned in Charon 4A, a lambda vector derivative. It was obtained from Dr. Tom Sargent at the National Institutes of Health, Bethesda Maryland. The EMBL3 library was generously supplied by Dr. Michael Crerar at York University, Toronto, Canada. This was created by a partial Sau3A/MboI double digest and cloned into the Bam HI site of the lambda vector derivative. The DNA was also isolated from adult male Sprague-Dawley rat liver DNA.

CULTURE MEDIA AND ANTIBIOTICS

Bacto-tryptone, Bacto-agar, Bacto-yeast extract were purchased from Difco. Agarose (Electrophoresis Grade) was from Bio-Rad. Tetracycline, ampicillin, and chloramphenicol were obtained from Sigma.

E.coli RRI was grown in L Broth prepared as follows: 10 g/liter Bacto-tryptone, 5 g/l Bacto-yeast extract, and 8 g/l NaCl. The pH was adjusted to 7.5, following which the volume was brought up to 1 liter with distilled water. The solution was then autoclaved. When antibiotic selection was necessary, ampicillin was added to a final concentration of 50-100 ug/ml and tetracycline to a final concentration of 12.5-15.0 ug/ml. E.coli 803 was used to

amplify the genomic DNA libraries and this was also grown in L Broth, but with the addition of 0.2% maltose and 10 mM $MgSO_4$ (final concentrations). Bacteriophage stocks were stored at 4°C in sterile SM Buffer consisting of: 10 mM Tris-Cl (7.5), 10 mM $MgCl_2$, 100 mM NaCl, 0.05% gelatin, and 0.3% chloroform.

The DNA genomic library and plasmid preparations were routinely plated out on LB Agar plates with slight modifications for each one. For plasmid preparations, one liter of LB Agar consisted of L Broth and 15 grams of Bacto-agar. Any necessary antibiotics were added after autoclaving, to their appropriate concentrations. One liter of LB Agar for phage preparations consisted of L Broth, 15 grams of Bacto-agar, 0.2% maltose and 10 mM $MgSO_4$. Top Agarose for plaque lifts was composed of the following: 7 g/liter agarose, 10 g/l Bacto-tryptone, and 2.5 g/l NaCl. The volume was brought up to 1 liter with distilled water and the solution was sterilized by autoclaving.

CLONING VECTOR

The plasmid vector pAT153 was used for subcloning all cDNA and genomic DNA inserts. pAT153 is a high copy variant of pBR322 and possesses selectable markers for ampicillin and tetracycline resistance (Twigg and Sherratt,1980).

CENTRIFUGES AND ROTORS

The following is a list of centrifuges that were used and the types of rotors that were used in each:

Beckman LS-70M Ultracentrifuge: Ti-60 and Ti-75.
Beckman LS-65 Ultracentrifuge: Ti-60 and Ti-75.
Beckman J2-21 centrifuge: JA-10, JA-20, JS-13 and JS-7.5.

TRANSFORMATION OF PLASMID DNA

This procedure of Mandel and Higa (1970) allows bacteria to be transformed with plasmid DNA by a calcium dependent method. A single bacterial colony of E.coli RRI was inoculated in 5 ml of L Broth. The culture was allowed to grow overnight at 37°C in a shaking incubator. The following day, 1 ml of the overnight culture was added to 100 ml of L Broth in a 500 ml sterile flask. The cells were grown with vigorous shaking at 37°C for approximately 1-1.5 hours (density of 5×10^7 cells/ml or O.D.550 = 0.2). The culture was chilled on ice for 10 minutes and then divided into two 50 ml sterile Corning polystyrene centrifuge tubes. The cells were centrifuged in a PR-6000 centrifuge at 2000 rpm for 5 minutes at 4°C. The supernatant was gently decanted so as not to disrupt the small cell pellet. Each pellet was then resuspended in 20 ml of cold 50 mM calcium chloride ($CaCl_2$) and left on ice for 20 minutes. The suspension was once again centrifuged at 2000 rpm for 5 minutes at 4°C. The supernatant was poured off and the cells were resuspended in 4.5 ml of cold 50 mM $CaCl_2$. These competent cells were kept on ice and if they were not immediately required, were stored on ice at

0°C for up to 48 hours.

The plasmid DNA to be transformed was dissolved in 1.0 mM Tris/ 0.1 mM EDTA (pH 8.0), at a concentration of 10 ng/ul. In an Eppendorf centrifuge tube, 4 ul of DNA was then mixed with 200 ul of competent cells and the reaction was incubated on ice for 40 minutes. The tube was transferred to a 42°C waterbath for 3 minutes. Following the heat shock treatment, 200 ul of fresh L Broth was added to the tube and incubated at 37°C with shaking for 30-60 minutes to allow the cells to express the drug resistance. No antibiotics were added to the L Broth. After this period, the entire contents of the tube was spread onto an LB agar plate with antibiotics. The plates were left at room temperature in a laminar flow hood until the liquid had been absorbed. They were then incubated at 37°C for 12-16 hours in an inverted position. Plates were stored at 4°C for upto 4-6 weeks before being replated.

PLASMID DNA ISOLATION - LARGE SCALE

The following plasmid DNA preparation is a modification of the procedure described by Birnboim and Doly (1979). A single bacterial colony containing the plasmid DNA of interest was picked off a plate and inoculated into 5 ml of L Broth along with the appropriate antibiotic. The culture was grown overnight at 37°C with vigorous shaking (300 rpm). The following morning, 5 ml of the overnight was diluted 1:100 in 500 ml of L Broth (containing the appropriate antibiotic) in a sterile 2

liter culture flask. The cells were allowed to grow with vigorous agitation at 37°C until the culture reached an optical density (O.D.550) of 0.6-0.7. This usually took anywhere from 2-4 hours, at which point 2.5 ml of chloramphenicol was added per 500 ml, to a final concentration of 170 ug/ml. The cells were left to shake for approximately 16-18 hours at 37°C. Chloramphenicol is a bacteriostatic agent which prevents the bacteria from dividing by inhibiting the peptidyl transferase activity of the 50S ribosomal subunit, but allows the plasmid to continue replicating inside the bacteria.

The cells were harvested by centrifugation in a JA-10 rotor at 8000 rpm for 10-15 minutes at 4°C. The bacterial pellets were resuspended in 9.5 ml of lysis buffer (25 mM Tris-Cl,pH 8.0; 10 mM EDTA; 50 mM glucose) and transferred to sterile 50 ml Oak Ridge PC tubes (Nalgene). Following this, 0.5 ml of lysis buffer containing 20 mg/ml lysozyme was added to the cells and the tube was left on ice for 15 minutes with occasional gentle swirling. The lysozyme solution was prepared immediately prior to use. After the lysozyme treatment, 10 ml of a NaOH/SDS solution (0.2 N NaOH; 0.2% SDS) was added to the cells and incubated on ice for 10 minutes with occasional swirling. Finally, 10 ml of 3 M sodium acetate pH 4.8, was added and the mixture was left on ice for 30 minutes with intermittent swirling. The cells were centrifuged in a JA-20 rotor at 18,000 rpm for 30 minutes at 4°C. The supernatant was transferred to a 50

ml Corning tube and extracted once with an equal volume of phenol/chloroform (buffered with 50 mM NaAc; 5 mM EDTA; 10 mM NaCl). The extracted supernatant was transferred to a 125 ml siliconized Corex bottle and precipitated with 0.6 volumes of isopropanol at room temperature. The solution was mixed well and let stand at room temperature for 15 minutes. Doing this at room temperature prevents the SDS from coming out of solution. DNA was then recovered by centrifugation in a JS-7.5 rotor at 7,000 rpm for 20-30 minutes at room temperature. The supernatant was discarded and the pellet was dried in a vacuum desiccator for approximately 5-10 minutes. The DNA was dissolved in 6.0 ml of TE (7.5) and transferred to a 50 ml Corning tube. To the dissolved DNA, 0.5 ml of 10 mg/ml ethidium bromide was added, followed by the 6.6 grams of cesium chloride (CsCl). The CsCl was dissolved and the mixture was centrifuged in a PR-6000 centrifuge for 5 minutes. This removes insoluble complexes between the ethidium bromide and RNA present in the mixture which would otherwise interfere with the gradient formation. The supernatant was transfered by syringe and needle into a Beckman 16 mm x 76 mm Quick Seal tube and then overlaid with mineral oil. All tubes to be centrifuged were balanced to within 50 mg and then sealed by heat. The tube was placed in a Beckman Ti-75 rotor, covered with a titanium cap, and centrifuged at 55,000 rpm for at least 16-18 hours at 20^{o}C.

After centrifugation, the tubes were removed from the rotor very carefully so as not to disrupt the bands within

the CsCl gradient. The tube was attached to a clamp on a ring stand and the bands were viewed by illuminating with long-wave UV light. Two bands of DNA were visible, the upper containing linear bacterial DNA, and nicked circular plasmid DNA, both of which are not wanted. The lower band, composed of closed circular plasmid DNA, contained the DNA of interest. A 16-gauge needle was used to puncture a hole in the top of the tube to release some of the pressure. Then, a 3 ml syringe and 21-gauge needle were used to draw out the lower band. The needle was inserted into the tube approximately 5 mm below the band and about 1-2 ml of the band was drawn out, being careful not to take up any of the top band. The band was transferred to a 15 ml polypropylene tube (Falcon) wrapped in foil so as to minimize the amount of exposure to visible light. Since ethidium bromide is light sensitive, the DNA may become disrupted with prolonged exposure to light. The ethidium bromide was extracted out with three times the volume of iso amyl alcohol. The extraction was repeated 4-5 times. Following the final extraction, the contents were transferred to a 15 ml Corex tube and three times the volume of water was added. The DNA was then precipitated by the addition of twice the volume of 95% ethanol and 3 M sodium acetate to a final concentration of 0.2 M. The contents were mixed and precipitated at -70^{o}C for 2 hours or at -20^{o}C overnight. After precipitating, the tube was centrifuged in a JS-13 rotor at 12,000 rpm for 30 minutes.

The ethanol was decanted out of the tube and the DNA pellet was washed with 70% ethanol and recentrifuged. The 70% ethanol was poured off and the pellet dried and redissolved in 100 ul of TE (7.5) in an Eppendorf tube. Quantitation of the DNA was determined by measuring a dilution in a spectrophotometer (1 O.D.260 = 50 ug/ml), or by running a small quantity on a gel and comparing it to the intensity of a standard nucleic acid digest (i.e. lambda DNA digested with Hind III).

PLASMID DNA ISOLATION - MINI PREPS

This rapid, small scale isolation of plasmid DNA is that described by Maniatis et al. (1982) and is a modification of a method by Birnboim and Doly (1979). The protocol allows for a number of samples to be prepared simultaneously. A single bacterial colony was used to inoculate 5 ml of L Broth with the appropriate antibiotic. The culture was grown overnight with vigorous shaking at 37^{o}C. Approximately 1.2 ml of the overnight culture was transferred to an Eppendorf tube and centrifuged for 1 minute in an Eppendorf centrifuge. The remainder of the culture was stored at 4^{o}C. The supernatant was removed by aspiration so as to leave the pellet as dry as possible. The pellet was resuspended by vortexing in an ice-cold solution of 50 mM glucose; 10 mM EDTA; 25 mM Tris-Cl (8.0) and 4 mg/ml lysozyme. The mixture was allowed to stand for 5 minutes at room temperature. Then, 200 ul of freshly prepared 0.2 N NaOH/1% SDS was added and the contents mixed

by inverting the tube 2 or 3 times very rapidly. The tube was stored on ice for 5 minutes. After this, 150 ul of an ice-cold solution of 3 M sodium acetate (4.8) was added and each tube was vortexed in an inverted position for 5-10 seconds. The tubes were left on ice for 5 minutes. Samples were then spun in an Eppendorf centrifuge at 4°C. The supernatant was transferred to a fresh tube by a Gilson pipettor, following which a phenol/chloroform extraction was performed. The tubes were spun for 2 minutes and the top layer was transferred to a fresh tube. Two volumes of 95% ethanol were added at room temperature and vortexed. After 1 minute, the samples were centrifuged for 5 minutes in an Eppendorf centrifuge and the supernatant removed and replaced with 1 ml of 70% ethanol. Tubes were vortexed and recentrifuged. All of the supernatant was removed an the pellet dried in a vacuum desiccator. Approximately 30-50 ul of TE (7.5) was used to resuspend the pellet and in addition, 20 ug/ml of RNase was added as well. About 5-10 ul of the DNA is sufficient for restriction enzyme analysis.

AGAROSE GEL ELECTROPHORESIS

Agarose gels are commonly used to analyze or purify large DNA and RNA fragments. The location of DNA within the gel may be readily determined by staining the gel with ethidium bromide. This allows for nanogram quantities of DNA or RNA to be detected by direct viewing under ultraviolet light (Sharp,1973). Furthermore, the

electrophoretic mobility of DNA through agarose gels depends on four parameters, including: molecular size, agarose concentration, the conformation of the DNA, and the applied voltage. Since DNA fragments of a particular size migrate at different rates through gels containing different agarose concentrations, it is possible to separate a wide range of DNA fragment sizes by varying the concentration of agarose (Maniatis et al.,1982). Both preparative and analytical gels were run in horizontal tanks after being poured on a removable glass or plastic plate. The gels were completely submerged under the surface of the electrophoresis buffer.

The two most commonly used electrophoresis buffers were Tris-Acetate-EDTA (TAE) or Tris-Borate-EDTA (TBE), the latter of which has a higher buffering capacity of the two (Maniatis et al.,1982). These buffers were routinely made up as concentrated stocks and stored at room temperature. The working concentration of TAE buffer was 0.04 M Tris-Acetate; 0.02 M EDTA, while that of TBE buffer was 0.089 M Tris-Acetate; 0.089 M boric acid; 0.02 M EDTA.

Most of the agarose gels had a 200 ml volume and were cast and run on glass plates (14 x 18 cm) with 1 mm thick wells. The appropriate amount of powdered agarose was added to a measured quantity of electrophoresis buffer and the volume was brought up with deionized, distilled water. The mixture was heated to a boil on a hot plate until the agarose was completely dissolved. In the meantime, a clean, dry, glass plate was sealed with tape around all

four edges so as to form a mold in which a gel could be cast. The dissolved agarose was cooled to approximately 50°C and ethidium bromide was added to a final concentration of 0.5 ug/ml. The edges of the mold were sealed with a small amount of agarose solution and allowed to set for a few minutes, following which the remainder of the gel solution was poured. The appropriate sized comb was then immediately placed into the gel to allow the formation of sample wells. Usually 0.5-1.0 mm was left between the bottom of the teeth and the base of the gel. Once the gel had completely set (30-45 minutes), the comb and surrounding tape were removed. The gel was then carefully transferred to the electrophoresis tank while still on the plate. Electrophoresis buffer was then added to the tank so as to submerge the entire gel completely.

The DNA samples and markers to be run were routinely mixed with loading buffer composed of 0.25% bromphenol blue; 0.25% xylene cyanol and 30% glycerol in water. The samples were then loaded into the submerged wells by a Gilson micropipettor. Gels were usually run at 100-150 volts in TBE for 2-3 hours depending on the fragment sizes to be separated. Following electrophoresis, the gel was rinsed in water for 15-20 minutes to remove some of the ethidium bromide dye that had not intercalated with the DNA. This reduced some of the background staining to yield better pictures. The gel was then photographed using ultraviolet light and Polaroid type-57 film.

POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis (PAGE) is similar to agarose gel electrophoresis except that the gel pores are smaller and therefore allow for the separation of smaller DNA and RNA fragments (usually less than 1 Kb in length). These gels may also be cast in various concentrations depending on the fragment size of interest. Polyacrylamide gels are normally poured between two glass plates that are held apart by thin spacers. Since exposure to oxygen inhibits polymerization, this limits the amount of exposure of the gel to the air. The length of these gels may vary from 10 cm to 100 cm and are usually run in a vertical position (Maniatis et al.,1982).

Before preparing the gel solution, two glass plates were washed with detergent and rinsed in deionized water. They were then rinsed in ethanol and dried thoroughly. The two plates were placed on top of one another, separated by two spacers running the length of both sides. The plates were then clamped tightly and fixed onto the gel casting apparatus.

The acrylamide was routinely made up as a 30% stock solution containing 29 grams of acrylamide and 1 gram of bis-acrylamide in 100 ml of water. The effective range of separation from a 3.5% acrylamide gel is 100-1000 nucleotides (Maniatis et al.,1982). This was prepared in a 100 ml volume consisting of 11.6 ml of 30% acrylamide stock solution, 76.3 ml water, 10 ml of 10 x TBE buffer and 2.1

ml of 3% ammonium persulfate. This volume allows for two gels to be poured. If a different percentage was required, the amounts of 30% acrylamide and water were adjusted accordingly, with the remaining ingredients staying the same. To begin with, the acrylamide, TBE and water were added to a vacuum flask and deaerated by vacuum suction. Following this, 2.1 ml of freshly prepared 3% ammonium persulfate and 30 ul of TEMED were added. The TEMED serves as a catalyst for the polymerization reaction to begin and so it is necessary to pour the gel immediately following its addition to the gel mixture.

Once the gel was poured into the small opening between the plates created by the spacers, an appropriate sized comb was inserted into the gel and the acrylamide was allowed to polymerize for at least one hour at room temperature. After polymerization, the comb was removed and the wells rinsed out with 1 x TBE buffer using a needle and syringe. The gel was then attached to the electrophoresis tank and the reservoirs filled with 1 x TBE. A pasteur pipette was used to remove any air bubbles trapped at the bottom of the gel. The DNA samples were mixed with the same dye used for agarose gel elctrophoresis. Samples were loaded with a Hamilton microsyringe. For one gel, the current was set at 15 mAmps for 2-3 hours. The current was doubled for two gels. Following the run, the gel was placed in a glass dish and submerged with a staining solution of 1 x TBE containing 0.5 ug/ml ethidium bromide. After 20-25 minutes of

staining, the gel was viewed by UV light and photographed.

ISOLATION OF DNA FROM LOW-MELTING POINT AGAROSE

Ultra pure low melting point (LMP) agarose is a specific grade of agarose which has hydroxymethyl groups incorporated into the molecule. This modification allows the agarose to gel at 30°C and to melt at 65°C, a temperature below the melting temperature for most DNAs. By employing these special properties, a simple technique has been developed for recovering DNA from LMP gels (Maniatis et al.,1982).

Powdered LMP agarose was dissolved in electrophoresis buffer by heating to a temperature greater than 70°C. After dissolving the agarose, the gel mixture was cooled to 35-40°C and ethidium bromide dye was added to a final concentration of 0.5 ug/ml. The gel was poured at 4°C and allowed to solidify for at least one hour. Samples were prepared and electrophoresed at 4°C to ensure that the gel did not melt during the run. Following the run, the desired fragment was cut out of the gel, placed in an Eppendorf tube, and placed in a 65°C waterbath for 5 minutes to melt. The volume was then diluted two times or to a volume of 0.7 ml, with 20 mM TE (pH 8.0). The mixture was extracted with an equal volume of phenol and the aqueous phase was then re-extracted with phenol/chloroform and then chloroform. Following the chloroform treatment, the volume was reduced to 200 ul by extracting with 1-butanol. The DNA was then recovered by ethanol

precipitation in the presence of 0.2 M sodium acetate at -20°C overnight. The precipitated DNA was washed with 70% ethanol, dried, and dissolved in TE (pH 7.5).

ISOLATION OF DNA BY ELECTROELUTION

Electroelution of specific DNA fragments from agarose gels is a quick and simple way to isolate a large quantity of specific DNA. The DNA sample of interest was electrophoresed on a preparative agarose gel containing ethidium bromide at a concentration of 0.5 ug/ml. The concentration of the gel was adjusted according to the size of the DNA fragment to be purified. If a large quantity of DNA was to be loaded, several wells were taped together to form one large well, rather than overloading a smaller well. Once the fragment of interest had run far enough into the gel to separate it from any other DNA fragments that might be present, the gel was removed from the tank. The gel was placed over a UV transilluminator and the fragment of interest was carefully cut out using a scalpel blade. This space was then enlarged on all four sides to create a well about twice the size of the gel piece which had been removed. The entire gel was placed back into the reservoir and buffer was added so that only the ends of the gel were in contact with the solution, allowing for a current to flow.

A piece of dialysis tubing larger than the well was wetted in the buffer and placed in the well. All four edges of the tubing were propped up in the air by small

bits of agarose so that they did not come in contact with the gel surface. The gel slice containing the insert DNA was then placed back into the enlarged trough on top of the tubing. Approximately 200-500 ul of buffer from the tank was placed in the trough containing the gel slice. The power was turned on to 100 volts and the DNA was allowed to elute out of the gel slice and into the buffer in the trough. The dialysis tubing that lined the well prevented the DNA from passing through it. The movement of the ethidium bromide stained insert was monitored by a hand-held UV lamp. The rate at which the insert elutes out of the gel will vary depending on the size and mass of the DNA. As the DNA eluted into the trough, the buffer was transferred to a polypropylene tube and replaced with fresh buffer. This was repeated until all of the DNA had flowed out of the gel, at which point the current was reversed for 10 seconds to allow any DNA attached to the dialysis tubing to be electrophoresed back into the buffer. Once all of the buffer aliquots were pooled together, the ethidium bromide was immediately removed by several extractions with iso amyl alcohol (3x volume). Following this, the DNA was precipitated with twice the volume of 95% ethanol and 0.2 M sodium acetate at 20^{o}C overnight, or at -70^{o}C for 2-3 hours. DNA was dissolved in TE (pH 7.5) and quantified by absorbance at A260.

SOUTHERN TRANSFER OF DNA TO NITROCELLULOSE FILTERS

The Southern transfer technique involves the transfer and immobilization of size separated DNA fragments from an agarose gel to a nitrocellulose filter (Southern,1975). The relative positions of the DNA fragments in the gel are preserved through the transfer to the filter. The filter may then be hybridized to a radioactively labeled cDNA probe to identify any bands that have sequences complementary to the radioactive probe. This technique allows for the identification of specific sequences in cloned DNA or in digests of total eukaryotic DNA (Jeffreys and Flavell,1977). A limitation of this procedure is that fragments smaller than 200-300 bp bind poorly to nitrocellulose and so their transfer may not be detected (Meinkoth and Wahl,1984).

The following procedure is one developed by Southern (1975), with slight modifications. Following electrophoresis of DNA restriction enzyme digests, the gel was photographed with a ruler along its length so that the distances of each band could be measured directly from the photograph. The gel was then trimmed with a scalpel blade to remove any unused areas and placed in a baking dish. The DNA was denatured by soaking the gel in several volumes of 1.5 M NaCl and 0.5 M NaOH for one hour at room temperature with constant gentle shaking. Following this, the gel was rinsed in distilled water and placed in several volumes of a neutralizing solution consisting of 3 M NaCl and 0.5 M Tris-Cl (pH 7.5). The gel was soaked for 45

minutes with gentle shaking at room temperature, and then transferred to fresh neutralizing solution for another 45 minutes. The gel was ready to be blotted at this point.

During the denaturing and neutralizing steps, apparatus for the transfer was prepared. Fifteen pieces of Whatman 3MM paper and one piece of nitrocellulose (Schleicher and Schuell, BA85), were cut to the size of the gel. In addition, a piece of 3MM paper larger than the gel was also cut so that it could be used as a wick to set up a flow of buffer for the transfer. The nitrocellulose was wetted in a container of boiling water for 15-30 seconds to extract any detergents. It was then placed in a solution of 20 x SSC (transfer buffer) along with one piece of 3MM paper. A glass dish was filled with 20 x SSC and a clean Plexiglass plate was supported upon this dish. The large piece of 3MM paper was wetted in the 20 x SSC and then draped over the Plexiglass plate with both ends submerged in the SSC transfer solution. The gel was then carefully placed on top of the wet wick. Excess liquid was removed from the gel surface, a sheet of Saran Wrap was placed over the entire dish and a hole was cut in the middle so that only the gel was exposed. The soaked nitrocellulose was then laid on top of the gel taking care not to trap any air bubbles beneath it. The presence of air bubbles would prevent transfer of DNA in that particular region. The 3MM paper that was wetted in 20 x SSC was then carefully placed on top of the nitrocellulose, followed by the remaining dry

pieces of 3MM paper. A 5-8 cm stack of absorbant paper towels was placed on top of the exposed 3MM papers, on which were placed another plate and a weight of about 500 grams. The wick in contact with the 20 x SSC allows for a flow of liquid to be started from the reservoir, through the gel and the nitrocellulose, so that the DNA fragments are eluted onto the filter. The DNA binds to the nitrocellulose in high salt concentrations.

The transfer was usually left for 14-24 hours, following which the paper towels and 3MM filters werre removed. The nitrocellulose was labeled with a waterproof marker in order to mark the position and orientation of the lanes. The filter was then lifted off the dried gel and transferred to a solution of 2 X SSC. The nitrocellulose was left at room temperature for 5-10 minutes in 2 x SSC. It was then blotted dry and placed between fresh pieces of 3MM paper, following which it was baked for 2 hours at 80^{o}C under vacuum. The filter was left at room temperature until it was ready to be used for hybridization.

NICK TRANSLATION OF DNA FRAGMENTS

Nick translation is a procedure developed by Rigby et al. (1977), which enables one to label double-stranded DNA radioactively with deoxynucleotide 5'-[^{32}P]triphosphates to specific activities greater than 10^{8} cpm/ug. This method employs the use of DNAse I to generate random nicks in the DNA duplex while DNA Polymerase I sequentially adds nucleotide residues to fill the gaps that were created.

Some of the nucleotides that are being incorporated by DNA Polymerase I are radioactive and therefore allow the DNA to be radioactively labeled (Meinkoth and Wahl,1984). A nick translation kit (Amersham Corp.) was used to prepare radioactively labeled DNA probes. The kit includes radiolabeled α-dCTP^{32}, a nucleotide solution (solution-1) and an enzyme solution (solution-2). Solution-1 contains 100 uM concentrations of dATP, dGTP and dTTP in a buffer. Solution-2 contains both DNA Polymerase I and DNAse I in a buffered solution as well. A 20 ul reaction was routinely set up in an Eppendorf tube. The reaction mix included: 7 ul dCTP^{32}; 4 ul solution-1; 2 ul solution-2; DNA and water to 20 ul. The enzymes were always added last and then the mix was spun down and incubated at 15^{o}C for 90 minutes.

During the incubation period, a column to fractionate the reaction was prepared using Sephadex G-100 (3 grams in 200 ml of sterile water). This allowed for the separation of the labeled probe from unincorporated nucleotides. A 5 ml Fisher polystyrene pipette was cut at the 1 ml mark with a sharp blade so as to leave a 4 ml pipette in which to make the column bed. A small amount of siliconized glass wool was pushed down to the tip of the pipette to form a porous plug and then a 3-way stopcock was attached to the tip, secured by Parafilm. With the pipette clamped onto a stand in a vertical position, the Sephadex was poured into the pipette to form a column. The Sephadex G-100 was removed from 4^{o}C and allowed to warm up at 65^{o}C for 30 minutes prior to use, in order to reduce bubbles. Once the

Sephadex had packed down to form a 3-4 ml bed, the column was equilibrated with an equal volume of 10 mM Tris-Cl (pH 7.5)/0.1 mM EDTA (elution buffer).

Ninety minutes was enough time for the DNA to be labeled to a specific activity of approximately 10^8 cpm/ug. The reaction was stopped by the addition of an equal volume of stopping buffer (20 mM EDTA; 0.2% SDS; 2 mg/ml denatured salmnon sperm DNA) and incubated at 65°C for 10-15 minutes. Prior to applying the reaction to the column, 4 ul of yeast tRNA was added to serve as a carrier for the labeled DNA. The reaction was applied to the column by a Gilson pipettor and eluted with TE buffer. The fractions were collected in Eppendorf tubes. The drops were monitored for radioactivity by a Geiger counter. The void fraction was collected in one tube until radioactivity was detected, at which point the drops were collected in fresh tubes at a frequency of two drops/tube. Two peaks of radioactivity were observed: the first peak included the radioactive probe while the second peak contained free nucleotides.

The amount of radioactivity incorporated was determined by adding 1 ul of probe to a liquid scintillation vial filled with Aquasol scintillation fluid. The vial was counted in an LKB Rack Beta counter and the amount of radioactivity incorporated in the 1 ul sample was determined by the counter in counts per minute (cpm). This value was then used to determine the specific activity of the probe by multiplying the total volume collected along

with the total counts and the amount of DNA being labeled. The probe was stored at -20°C in a lead container until it was required for hybridization. Usually it was within 2-3 days.

HYBRIDIZATION OF DNA/RNA BOUND TO NITROCELLULOSE

With the development of methods for immobilizing DNA on nitrocellulose paper (Nygaard and Hall,1963) and for detecting the fixed nucleic acid with a radioactive probe (Denhardt,1966), it is now possible for DNA analysis to become a more routine procedure. Nucleic acid hybridizations are sensitive enough to detect less than 1 pg of complementary sequence, fast enough to be performed within 24 hours, and convenient enough to require simple materials (Meinkoth and Wahl,1984). There are several methods of hybridization available. All of them differ slightly in conditions with respect to the solvents, temperature, duration, probe concentration, stringency of washes, and use of components such as dextran sulfate or formamide (Maniatis,1982).

All hybridizations were performed under aqueous conditions in 6 x SSC at 65-68°C. Initially, the baked filter was wetted evenly in 6 x SSC for 5 minutes, following which it was prehybridized for at least one hour in a prehybridization solution. Prehybridization has been suggested as an effective means of reducing background hybridization with substances designed to bind nonspecific nucleic acid binding sites on the nitrocellulose filters

(Denhardt,1966). The prehybridization solution was essentially the same as the hybridization solution, but lacked the labeled probe. It was composed of 6 x SSC; 1 x Denhardt's solution; 0.1% SDS; 10 ug/ml Poly A; and 100 ug/ml denatured salmon sperm DNA. Denhardt's solution was stored at -20^{o}C as a 4 times concentrated stock;it consists of 8 grams/liter of Ficoll, PVP-360 (polyvinylpyrrolidone), BSA (Bovine serum albumin) and water to one liter. The salmon sperm DNA was denatured by sonicating and then boiling for five minutes. It was stored at -20^{o}C at a concentration of 5 mg/ml.

During the prehybridization, sites on the nitrocellulose that bind DNA non-specifically, are saturated by either the salmon sperm DNA, SDS, or components of the Denhardt's solution. Poly A was included to prevent the labeled cDNA probe from binding to T-rich sequences that are common in eukaryotic DNA (Maniatis et al.,1982). Routinely, 25-50 ml of solution was used for prehybridizing 1 or 2 12 cm x 18 cm filters. Volumes were scaled up or down depending on the size and number of filters being probed. If there were a large number of filters such as during a library screening, a plastic box was used with a tightly sealed lid to prevent evaporation of any liquid.

Prior to the start of hybridization, the nick translated ^{32}P-labelled DNA probe was denatured by boiling for 5 minutes. The specific activity of the probe was in the range of 1-2 x 10^{8} cpm/ug for most homologous DNA

fragments being probed. During the screening of genomic library filters, the minimum concentration of probe DNA used was 5ng/ml to ensure an adequate mass in the hybridization mixture.

Although the prehybridization may be performed in a bag or a plastic box, the hybridizations were always carried out in a Sears Seal-a-Meal bag. Typically, enough solution was added to the bag so that the liquid covered the entire filter or filters. The hybridization was carried out by shaking at 65°C (18-24 hours).

Following the hybridization, the filters were transferred to a washing solution of 2 x SSC/0.1% SDS in a plastic box. These washes were done at room temperature and repeated four times for 5-10 minutes each. The filters were then washed more stringently in 1 x SSC/0.1% SDS for 60-90 minutes at 65°C. This wash was performed two times in order to remove any background hybridization. If the background was still high, the washes were made more stringent by reducing the salt concentrations down to 0.1-0.2 x SSC/0.1% SDS (65°C) for 15-30 minutes. Filters were then wrapped in Saran Wrap and a piece of tape was placed on top and labeled with radioactive ink for orientation purposes. The filters were then placed in a film cassette and exposed to X-ray film (Kodak XAR) using Dupont Lightning Plus intensifying screens at -70°C.

DEXTRAN SULFATE HYBRIDIZATION

Hybridization in the presence of dextran sulfate accelerates the rate of association of nucleic acids. This is accomplished by excluding the nucleic acids from the volume that is occupied by the dextran sulfate polymer, thereby increasing their effective concentration (Maniatis et.al,1982). It has been reported that the rate of association may be increased 10-fold in the presence of 10% dextran sulfate (Wahl,1979). Dextran sulfate is useful when the rate of hybridization would be limited due to a low abundance of the sequences of interest. This type of hybridization procedure was used solely when probing Southern blots of genomic DNA digests.

The hybridization was performed at 65^{o}C under aqueous conditions, but in the presence of 6.6 x SCP (1 x SCP: 2 M NaCl; 0.6 M Na_2HPO_4; 0.2 M EDTA; pH 6.2). The prehybridization solution was made up in a 150 ml volume and comprised of the following: 50 ml 20 x SCP; 4.3 ml 35% N-Lauryl sarcosine; 3 ml 5 mg/ml denatured salmon sperm DNA; 37.5 ml 4 x DH; water to 150 ml. The solution was degassed and 100 ml was used for prehybridizing the filter at 65^{o}C for at least one hour. The remainder of the solution was saved for the hybridization. For the hybridization, 2 grams of dextran sulfate was dissolved by heating in 20 ml of the hybridization solution. The mixture was degassed thoroughly, and the boiled cDNA probe was then added. Following prehybridization, the solution was drained from the bag and replaced with the labeled

probe solution. The bag was heat sealed and placed in a 65°C water bath for hybridization overnight.

The washes were also performed in SCP solutions. Two washes of 15 minutes duration were performed at 65°C in 6.6 x SCP/1% SDS. Then, three washes were done at 65°C in 1 x SCP/1% SDS. The first two were for 90 minutes and the last one for 15 minutes. The filter was then wrapped in Saran Wrap and exposed to Kodak XAR film at -70°C using a Dupont Lightning Plus intensifying screen for 2-5 days. Usually, faint bands were seen overnight with 5ug of DNA, but a better exposure was seen after 4 or 5 days.

RESTRICTION ENZYME DIGESTION OF DNA CLONES

Restriction enzyme maps of the various clones were constructed using a number of different restriction endonucleases. By using a combination of single and double digests with both frequent and infrequent cutters, maps were generated for all DNA clones. Restriction enzyme buffers were the same for all enzymes except that their salt requirements were as recommended by Maniatis et al. (1982). All buffers consisted of a final concentration of 10 mM Tris-Cl (7.5); 10 mM $MgCl_2$ and 1 mM Dithiothreitol (DTT). The concentration of NaCl varied from 0 mM to 100 mM depending on the requirement of each enzyme. All reactions were performed in a volume of 20 ul and contained a concentration of DNA ranging from 0.2-1.0 micrograms. Tris-Cl and $MgCl_2$ were stored as 100 mM stocks while NaCl was at a concentration of 1 M. Dithiothreitol was stored

in small aliquots of 10 mM solutions at -20°C and a fresh tube was thawed each time a set of digestions were carried out.

A typical 20 ul reaction was always set up in a 1.5 ml Eppendorf tube, placed in a rack on ice. Two different enzymes were added simultaneously only if they were able to work with the same buffer conditions. If the requirements were different, the enzyme in the buffer with the lower concentration was used first, and then the salt and second enzyme was added later on. Enzyme volumes were kept to no more than 1/10 the reaction volume so that the glycerol in the enzyme solution would not inhibit the enzyme activity (Maniatis et al.,1982). The tube was then spun down in a Beckman microfuge and placed in a 37°C water bath overnight. The reaction was always stopped by placing the tube in a 65°C waterbath for 10-15 minutes to inactivate the endonuclease. The sample was then run immediately on a gel for analysis or stored at 4°C until required.

LAMBDA GENOMIC LIBRARY SCREENING

To screen a mammalian genomic DNA library, approximately 5×10^5 to 1×10^6 recombinant phage must be examined in order to represent the genome adequately. The EMBL3 library was made up of clones ranging from 10-18 Kb and so at least 1×10^6 recombinants were screened in order to find the rPL-II gene. Benton and Davis (1977) have developed an efficient method for screening recombinant phage.

The initial screening of the library was plated out on 150 mm Petri dishes. It has been reported that the maximum number of plaques that can be plated on a dish of this size is 50,000 (Maniatis et al.,1982). The EMBL3 library was separated into 25 different aliquots, and each was plated out to confluence on a separate 150mm dish. To a sterile 15 ml metal-cap glass tube (Fisher-borosilicate) a portion of a bacteriophage stock was added that represented 50,000 bacteriophage particles. Usually the volume was below 100 ul. To this, 300 ul of plating bacteria (E.coli 803) was mixed in and the tube was incubated in a 37°C water bath for 20 minutes, allowing the phage to adsorb to the bacterial cells. The cells had been grown overnight from a single bacterial colony inoculated into 5ml of L Broth supplemented with 10 mM $MgSO_4$ and 0.2% maltose. After the twenty minutes, the tubes were moved into a laminar flow hood where approximately 7 ml of lambda top agarose (0.7%) was added into each tube. The top agarose was melted and left at 65°C and brought out to cool down to about 50°C 5-10 minutes prior to use. The tubes were quickly vortexed and the contents were poured over the surface of an LB agar plate and spread around evenly by swirling the plate by hand. Once all twelve tubes were plated, the plates were left for 5 minutes and then incubated in an inverted position at 37°C overnight or until the surface was almost confluent.

Once the plaques had grown adequately, the plates were

removed and placed at 4°C for at least one hour to allow the top agarose to harden. In the meantime, twelve large 150 mm nitrocellulose filters were being labeled with a waterproof, heat stable marker. After the plates had cooled, the nitrocellulose filter was carefully placed onto the surface of the plate so that it was in direct contact with the plaques. Care was be taken to avoid trapping air bubbles underneath the filter. A small needle was then dipped in drawing ink and poked through the filter and the agar surface in three asymmetric locations to create a unique orientation pattern for each plate and filter. After 60 seconds, the filter was removed by Millipore forceps and immersed in a denaturing solution of 1.5 M NaCl/0.5 M NaOH for 60 seconds. The filters were always placed phage-side up so as not to wash any phage DNA off the surface. The filter was then transferred to a neutralizing solution of 0.2M Tris-Cl (7.5) for 60 seconds followed by another 0.2M Tris-Cl treatment. The last treatment was in a solution of 2xSSC for 60 seconds. Once all the filters were treated, they were air dried and baked at 80°C under vacuum for 2 hours. Filters were then ready to be hybridized to a labeled cDNA for screening.

Following the hybridization to the cDNA probe, the filters were washed and wrapped in Saran Wrap. On top of the Saran Wrap, tape was placed around each filter, upon which an orientation mark was made with radioactive ink. The filters were then exposed to x-ray film. The autoradiograms were matched to their corresponding filters

by using the ink marks. If any filters showed signals on the film, the corresponding plate was aligned on top of the autoradiogram, and the region that hybridized was picked by using the large end of a sterile Pasteur pipette. During the first two screens, the plates were so crowded that it was not feasible to distinquish individual plaques, and so the entire region around the signal had to be picked. The plug of agarose from a positive signal was placed in an Eppendorf tube containing 1 ml of fresh SM buffer. The tube was vortexed and left at room temperature for two hours to allow the phage particles to diffuse out of the agarose and into the medium. The stocks were then stored over a few drops of chloroform at 4°C until rescreened.

The plaques corresponding to the positive signals from the first screen were plated out on 85 mm Petri dishes for the second screen. The phage for this second screen were also plated out at a density close to confluence. The screening was performed as before and the positive signals that appeared were picked from the large end of a Pasteur pipette to increase the chance of the signal being carried over to the next screen. The next few successive screenings were plated out at low density so that single plaques could be localized to a given signal on the autoradiogram. Once a plate was screened such that all the plaques showed a positive hybridization to the cDNA probe, this was an indication that a pure genomic clone had been isolated. The DNA was then isolated from phage stocks of

the clone and characterized further.

LIQUID CULTURE PHAGE DNA PREPARATION

In this technique it is important to take note that in the initial infection, the ratio of bacteriophage to bacterial cells (multiplicity of infection - m.o.i.) should be one. Small changes in this ratio can greatly affect the final yield of bacteriophage. Therefore, it was necessary to have a properly titered phage stock and to monitor the growth of the bacteria before infection to ensure that an appropriate ratio would be attained. Although the following protocol describes a 100 ml preparation, all of the values may be scaled up or down depending on the quantity of phage DNA that is required.

A single colony of E.coli 803 was inoculated into 5 ml of L Broth supplemented with 10 mM Mg^{++} and 0.2% maltose, and grown overnight at 37°C with shaking. The entire 5 ml overnight culture was used to inoculate 100 ml of fresh media in a sterile 1 liter culture flask. It is important to keep at least a 1:10 ratio and to shake vigorously at 37°C so as to provide sufficient aeration for growth. The culture was grown to an O.D.650 of 0.4 - 0.48, which represents about 2-3 x 10^8 cells/ml. At this point, the appropriate phage stock was added to the cells to ensure that the multiplicity of infection equaled 1.

The infection was monitored closely by measuring the O.D.650 in a spectrophotometer. The O.D. rose as high as 1.8 and then began to drop as the cells started to lyse.

The amount of time for the lysis to begin varied from 2 to 6 hours depending on the viability of the cells, phage stock, and the m.o.i. The presence of bacterial debris in the culture was a good indication that lysis was taking place. In addition, clearing of the culture becomes noticeable.

The O.D.650 was monitored until it reached a minimum, indicating that the lysis was complete (the O.D. may drop to 0.6 or even lower). At this point, 250 ul of $CHCl_3$ (1 ml/500 ml) was added and vigorous shaking was continued for 10 minutes. After this, 4 grams of NaCl was added with 100 ul of 1 mg/ml DNase and 100 ul of 10 mg/ml Rnase. The flask was incubated at 37°C for 15 minutes with gentle shaking. The culture was then divided among four 30 ml Corex tubes and centrifuged at 10,000 rpm for 20-30 minutes to remove the bacterial cells. The supernatants were then transferred to four Ti60 ultracentrifuge tubes and spun at 20,000 rpm for 2 hours at 20°C. The pellets were usually small and brown or yellow in color due to bacterial debris. The supernatants were decanted carefully so as not to disrupt these pellets. The tubes were left overnight on an angle at 4°C, with 1 ml of SM dilution buffer covering the pellets. If the pellets were not fully resuspended the following day, they were carefully resuspended by using a 10 ml pipette. It is critical not to disrupt the integrity of the phage at this point, because if they are broken, the subsequent DNAse treatment would destroy the phage DNA.

The resuspended phage were transferred to Eppendorf

centrifuge tubes and treated with 20 ul of 1 mg/ml DNAse and 40 ul of 10 mg/ml RNAse at 37°C for 30 minutes. Next, 100 ul of 10 mg/ml Proteinase K was added and allowed to digest for 30 minutes at 65°C. After 30 minutes, 20 ul of 0.5 M EDTA was added. The tubes are incubated for 60 minutes at 65°C. All samples were extracted three times successively with phenol, phenol-chloroform and chloroform. DNA was precipitated in ethanol at -20°C overnight or at -70°C for 2 hours. The DNA was were spun down in an Eppendorf centrifuge for 15 minutes and and the pellets were rinsed with 70% ethanol and spun again for 15 minutes. The pellets were then dried down in a vacuum desiccator and resuspended in 50 ul of Tris/EDTA (pH 7.5) following which the quantity of DNA was determined by measuring its O.D.260 absorbance reading.

PLATE LYSATE PHAGE DNA PREPS

The appropriate dilution of a phage stock was used to infect a culture of E.coli 803. The mixture was plated out on an LB Agar plate and incubated at 37°C until confluence. Five milliliters of SM dilution buffer was added to the plate and it was then left at room temperature for two hours with shaking. The SM was harvested into a 15 ml polystyrene tube and this served as a stock from which phage DNA was isolated.

To the harvested stock, 10 ul of 1 mg/ml DNase and 10 ul of 500 units/ml RNase was added and left at 37°C for one hour. The tube was then centrifuged at 3000 rpm for 30

minutes at 0-4°C to remove bacterial debris. The supernatant was transferred to a fresh tube using a sterile Pasteur pipette following which 5 ml of 2 M NaCl-20% Polyethylene Glycol (PEG) in SM buffer, was added. The tube was kept on ice for at least 2-3 hours, although overnight gave the best results. Precipitation with PEG allows for the concentration of phage lysates. Following the precipitation, the tube was centrifuged at 3000 rpm for 30 minutes at 0-4°C. The supernatant was decanted and the pellet resuspended in 500 ul of fresh SM buffer. Then, Proteinase K was added at a final concentration of 50 ug/ml and incubated at 65°C for 30 minutes. At this point, 5 ul of 10% SDS and 5 ul of 0.5 M EDTA were added to the reaction and incubated for a further 30 minutes at 65°C. The solution was extracted sequentially with phenol, phenol-chloroform and then chloroform. The supernatant was then ethanol precipitated at -20°C overnight. The DNA was spun down, rinsed with cold 70% ethanol, dried down and resuspended in 1 mM Tris/0.1 mM EDTA (pH 7.5).

SUBCLONING DNA FRAGMENTS INTO PLASMID VECTORS

DNA fragments from the genomic DNA clones were isolated by electroelution on agarose gels and subcloned into the plasmid vector pAT153 for fine structure mapping. Five micrograms of pAT153 DNA were digested with Eco RI in a 50 ul reaction for 2-3 hours with the appropriate buffer. Following the digestion, the DNA was treated with 1 ul of Calf Intestinal Phosphatase and incubated at 37°C for an

additional 30 minutes. The removal of the terminal 5' phosphate groups from the linear plasmid DNA prevents the DNA from recircularizing. However, the foreign DNA to be inserted may still be ligated to the dephosphorylated plasmid DNA because its 5'-terminal phosphates can form a bond with the plasmid's 3'-hydroxyl group (Maniatis et al.,1982). The phosphatase treatment was stopped by heat inactivation at 65°C for 15 minutes. The mixture was then extracted successively with phenol, phenol/chloroform and chloroform, at which point it was ethanol precipitated. The DNA was then resuspended in 20 ul to yield a final concentration of 250 ng/ul. The ligation reaction was carried out in a 20 ul volume. The reaction included the following: 2 ul pAT153 (cut/dephosphorylated); 500 ng insert DNA; 2 ul ligation buffer; 2 ul T4 DNA Ligase; water to 20 ul. The 10 x ligation buffer consisted of 0.66 M Tris-Cl (7.6);10 mM ATP; 10 mM spermidine; 0.1 M $MgCl_2$; 150 mM DTT; and 2 mg/ml BSA. The contents were incubated at 15°C overnight in a 500 ul Eppendorf tube.

It was important to carry out control reactions. The two controls used included a sample containing no ligase and a sample containing ligase and plasmid DNA. The former should yield no colonies as it shows how well the vector was cut, while the latter should show how well the vector was dephosphorylated.

Freshly prepared competent cells (E.coli RRI) were transformed in the usual manner and plated onto LB agar

plates containing the antibiotic ampicillin. The plates were incubated at 37°C in an inverted position removed for 14-16 hours. The colonies were picked and mini plasmid preps were performed to determine if the inserts had been subcloned.

ISOLATION OF HIGH MOLECULAR WEIGHT EUKARYOTIC DNA

Frozen rat liver or kidney tissue was weighed and then pulverized with a mortar and pestle. The tissue powder was resuspended in 25 volumes/gram of 10 mM Tris-Cl (pH 8.0)/1% SDS/10 mM EDTA and digested overnight at 37°C with 100 ug/ml Proteinase K with gentle shaking for. The homogenate was extracted twice with water saturated phenol followed by a single extraction with chloroform:isoamyl (24:1). The aqueous phase was dialyzed in 4 liters of 10 mM Tris-Cl (pH 8.0)/1 mM EDTA/10 mM NaCl at 4°C. The solution was changed 3 times and the dialysis was carried out for 2 days. The solution was then incubated with 100 ug/ml DNAse-free pancreatic RNAse for 3 hours at 37°C. Sodium dodecyl sulfate was added to a concentration of 1%, EDTA to 10 mM, and Proteinase K to 50 ug/ml and incubation was continued for 1 hour at 37°C. The solution was extracted once with phenol and then with chloroform:isoamyl. The aqueous phase was then dialysed once again with 10 mM Tris-Cl (pH 8.0)/1 mM EDTA/10 mM NaCl as before, with 3 changes. All of the phenol must be eliminated so that the restriction enzymes are not inhibited. The DNA was resuspended in TE (pH 7.5) by gentle mixing on a rotary wheel at 4°C for 1-2 days, and

the concentration was determined by absorbance reading at A260.

ISOLATION OF RNA

RNA was isolated by the Guanidinium thiocyanate/Cesium Chloride method (Chirgwin et al.,1979). The tissue was homogenized in 10 volumes of guanidinium solution per gram of tissue. The solution was made up of the following ingredients: 473 g guanidinium isothiocyanate (4 M); 5 g N-lauryl sarcosine (0.5%); 7 ml b-mercaptoethanol; 7.4 g sodium citrate and water to 1 liter. The solution was adjusted to pH 7.0 and filtered through a Whatmann #1 filter by gravity or suction. Following filtration, 4 ml/liter of anti-foam A was added. Frozen tissue was placed in guanidinium solution in a 50 ml Corning tube. Homogenization was carried out in a polytron at room temperature. The polytron was turned on at full speed and the tissue was treated with four 30 second bursts. The tube was then centrifuged in the benchtop IEC for 15 minutes at full speed.

A Cesium chloride (CsCl) solution of 5.7 M CsCl/0.001 M EDTA (pH 7.4) was prepared and filtered as above. Ten ml of this CsCl solution was placed into a Beckman Ti-60 Quickseal tube by syringe. Then, 20-25 ml of the cell homogenate was added to the tube. The tubes were balanced with fresh guanidinium solution. The tubes were sealed and centrifuged at 27,000 rpm for 16-19 hours at room temperature. It was imperative to remove the pellet from

the tubes immediately following the spin so as not to lose the pellet.

Using a 20 ml syringe, the top layer of the gradient was removed and discarded. The top of the tube was then carefully cut off. The remaining guanidinium solution was removed by syringe and the CsCl phase was poured off. With a Kimwipe, the bottom of the tube was cleaned of any excess CsCl. The pellet was then dissolved in 1 ml of 65°C sterile distilled water or 10 mM Tris (pH 7.0)/1 mM EDTA. The solubilized RNA was then transferred to a sterile 15 ml polypropylene tube and extracted with phenol/chloroform. The RNA was precipitated in ethanol at -20°C The RNA was spun down in a JS-13 rotor, collected, and stored in water.

ELECTROPHORESIS AND NORTHERN TRANSFER OF RNA GELS

Electrophoresis of RNA was carried out in denaturing gels containing 2.2 M formaldehyde. Electrophoresis was carried out as described by Maniatis et al.(1982). The running buffer was made as a 10 x stock composed of 0.2 M MOPS (pH 7.0); 50 mM sodium acetate 10 mM EDTA (8.0). The buffer was then diluted to 1 x in sterile water prior to use. Formaldehyde was obtained as a 37% stock solution in water (12.3 M), with a pH greater than 4.0. The gel was prepared by melting agarose in water, cooling to 60°C, and adding 10 x buffer and formaldehyde to yield a 1 x and 2.2 M final concentration of each respectively. A 1.3% agarose concentration was used for the gels. Samples were prepared by mixing the following in a sterile Eppendorf tube: 1 ul

10 x buffer; 3.5 ul Formaldehyde; 10 ul Formamide (deionized); RNA (up to 20 ug); and water to 20 ul. The mixture was heated at 55°C for 15 minutes following which 2 ul of sterile loading buffer was added. The buffer was composed of: 50% glycerol; 1 mM EDTA; 0.4% bromphenol blue: 0.4% xylene cyanol.

Prior to running the gel, all the apparatus was thoroughly washed in detergent to minimize RNAse contamination, followed by a rinse in distilled water. The gel was run in 1 x running buffer and the DNA markers were loaded after treating with formaldehyde and formamide. The gel was run at 30 volts overnight. The gel was ethidium bromide stained, and photographed on an ultraviolet transilluminator that had been cleaned to minimize RNAse contamination. For blotting the gel, the only pretreatment was a 30 minute wash in sterile 20 x SSC. After this treatment, the blot was treated in the same manner as a Southern blot, except that sterile 20 x SSC was used. The nitrocellulose was soaked briefly in 5 x SSC to remove any formaldehyde before it was dried and baked at 80°C in a vacuum oven.

RESULTS

I. GENOMIC SOUTHERN BLOT ANALYSIS

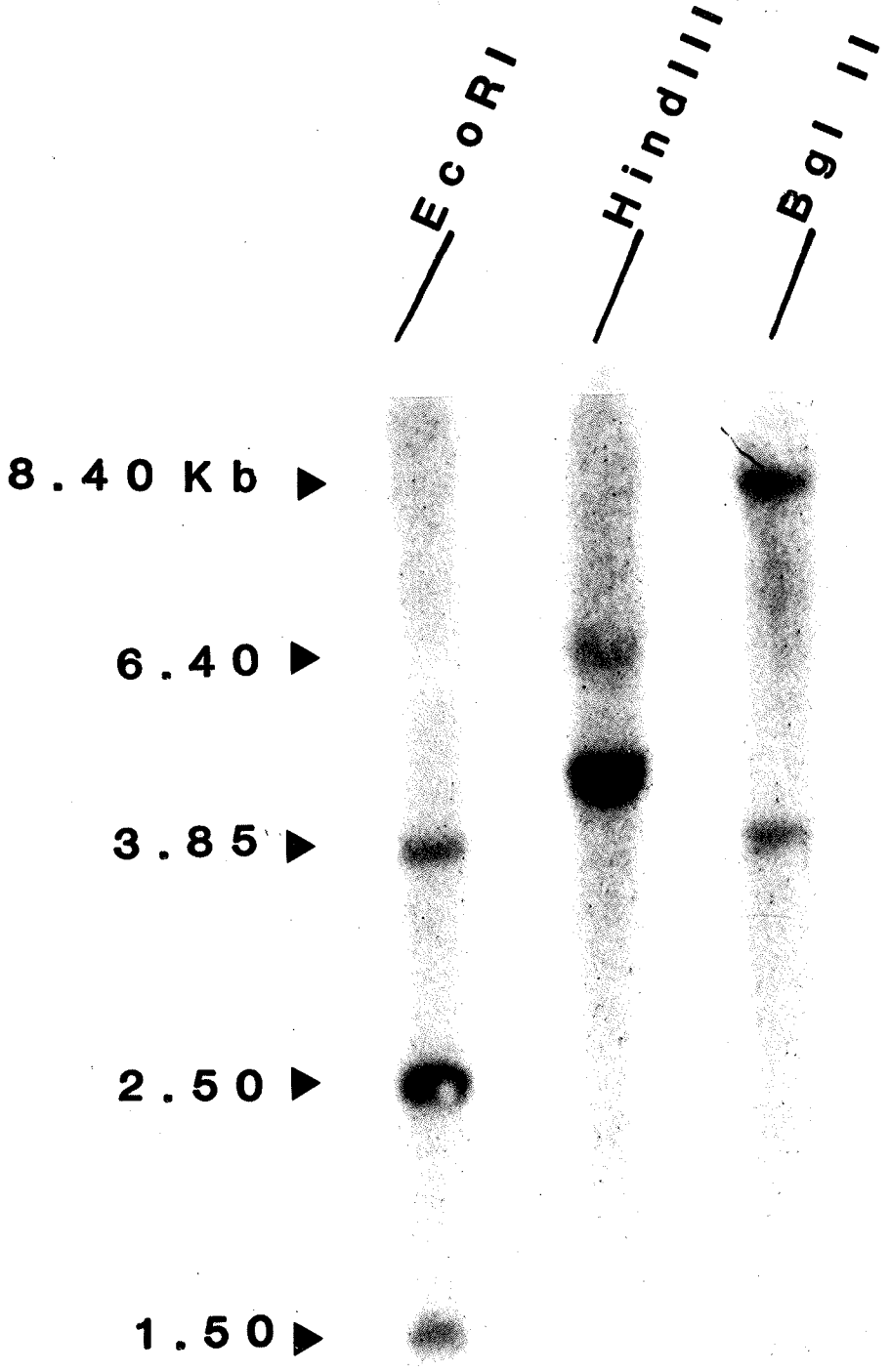
Five micrograms of rat liver DNA was digested with a series of restriction enzymes, size fractionated, and the Southern blot was hybridized to c52-A, a 714 bp cDNA representing most of the coding region of rPL-II (Duckworth et al.,1986). The resulting autoradiogram gave an indication as to the particular sites within and around the rPL-II gene as well as an approximation of the gene size (Figure I). Examination of the Eco RI digest indicates that the rPL-II gene may include at least three Eco RI fragments of sizes 3.85, 2.5, and 1.5 Kb, totaling 7.85 Kb. Since the cDNA has only one EcoRI site within its sequence, the other three sites are in introns or flanking sequences. Furthermore, since the cDNA is not of full length, it is possible that there may be more EcoRI fragments that were not detected on the genomic Southern.

II. LAMBDA GENOMIC LIBRARY SCREENING

Two rat genomic DNA libraries were screened for the structural gene coding for rPL-II. The probe used for both libraries was clone 52-A (c52-A). This clone along with the other cDNA clones to rPL-II are shown in figure V. The EcoRI partial digest lambda Charon 4A library was screened first, and after examining approximately 1×10^6 recombinant phage plaques, no genomic clones were isolated. Subsequently, a Sau 3A/Mbo I partial digest lambda EMBL3 library was examined in the same manner. On the initial

FIGURE I. GENOMIC SOUTHERN BLOT. Autoradiogram of Southern blot representing rat liver genomic DNA restriction digests, hybridized to c52-A. All samples contain 5 µg of DNA per lane, and were run on a 0.6% agarose gel.

FIGURE I



screening, nine putative positive signals were observed on the autoradiograms. Following five rounds of plaque purification, two genomic clones to rPL-II were isolated and purified to homogeneity. These clones were designated GC-I and GC-II.

III. ANALYSIS OF rPL-II GENOMIC CLONES

The DNA was prepared from phage stocks of GC-I and GC-II (as described in Materials and Methods). The fragments generated by the Sau 3A/Mbo I digests were capable of being cloned into the Bam HI site of the EMBL3 lambda cloning vector. The Bam HI cloning sites are situated within Sal I sites, and quite often the Bam HI sites are not regenerated after the foreign DNA is cloned into the vector. Therefore, by digesting GC-I and GC-II with Sal I, it was possible to determine the size of the genomic insert fragments. It was apparent from the Sal I digests that there were no Sal I sites within either insert. In the GC-I digest (Figure IIA), three bands were present, representing the 19.4 Kb large arm, the 9.4 Kb small arm of lambda and an insert of 18.5 Kb, indicating that it was cut out as one intact piece. On hybridization to c52-A, only the 18.5 Kb insert hybridized (Figure IIB).

For a Sal I digest of GC-II (Figure III), only two bands appear on the ethidium bromide stained gel - the 19.4 Kb large arm and the 9.4 Kb small arm. However, the increased intensity of ethidium bromide stain present in the 9.4 Kb band suggests that the insert may be of the same

FIGURE IIA. RESSTRICTION ENZYME DIGESTS OF GC-I. Arrows designate bands which hybridize to c52-A. M = Markers (Øx174/Hae III and λ/Hind III); Eco = Eco RI, Sal = Sal I. Samples were run on a 1.0% agarose gel.

FIGURE IIA

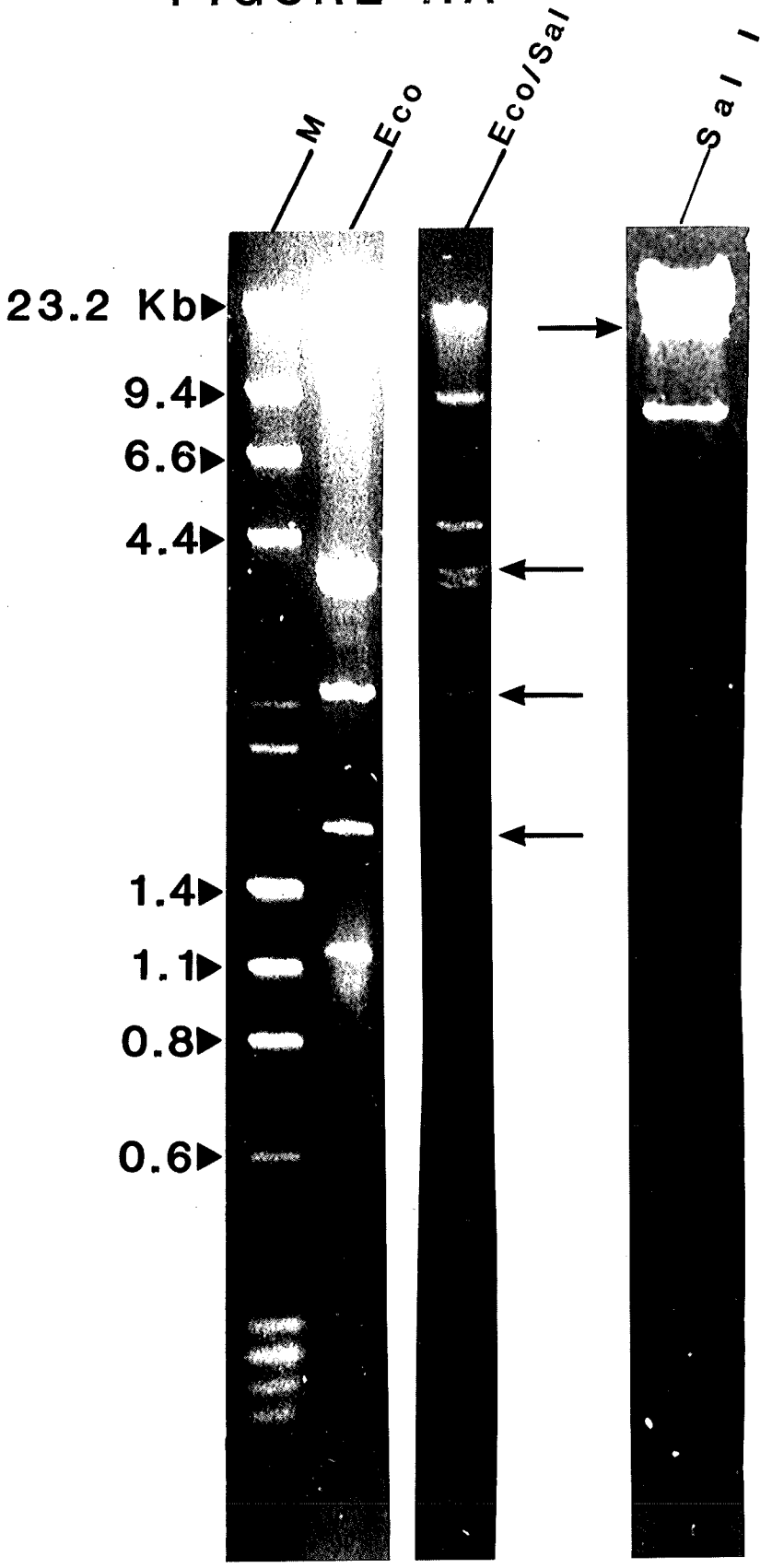


FIGURE IIB. SOUTHERN BLOT OF GC-I. Autoradiogram of GC-I digests, hybridized to c52-A. Eco = Eco RI and Sal = Sal I.

FIGURE IIB

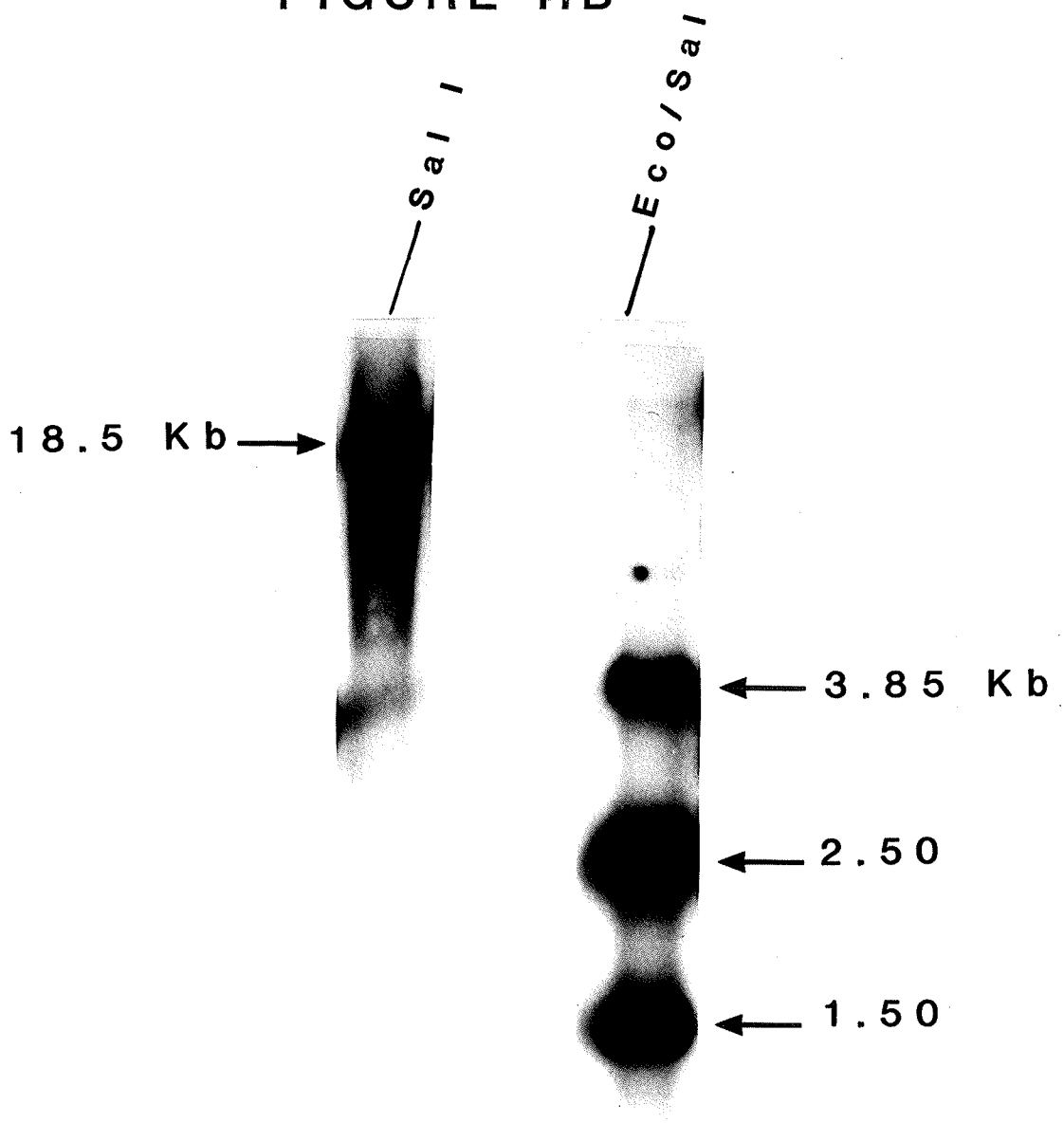
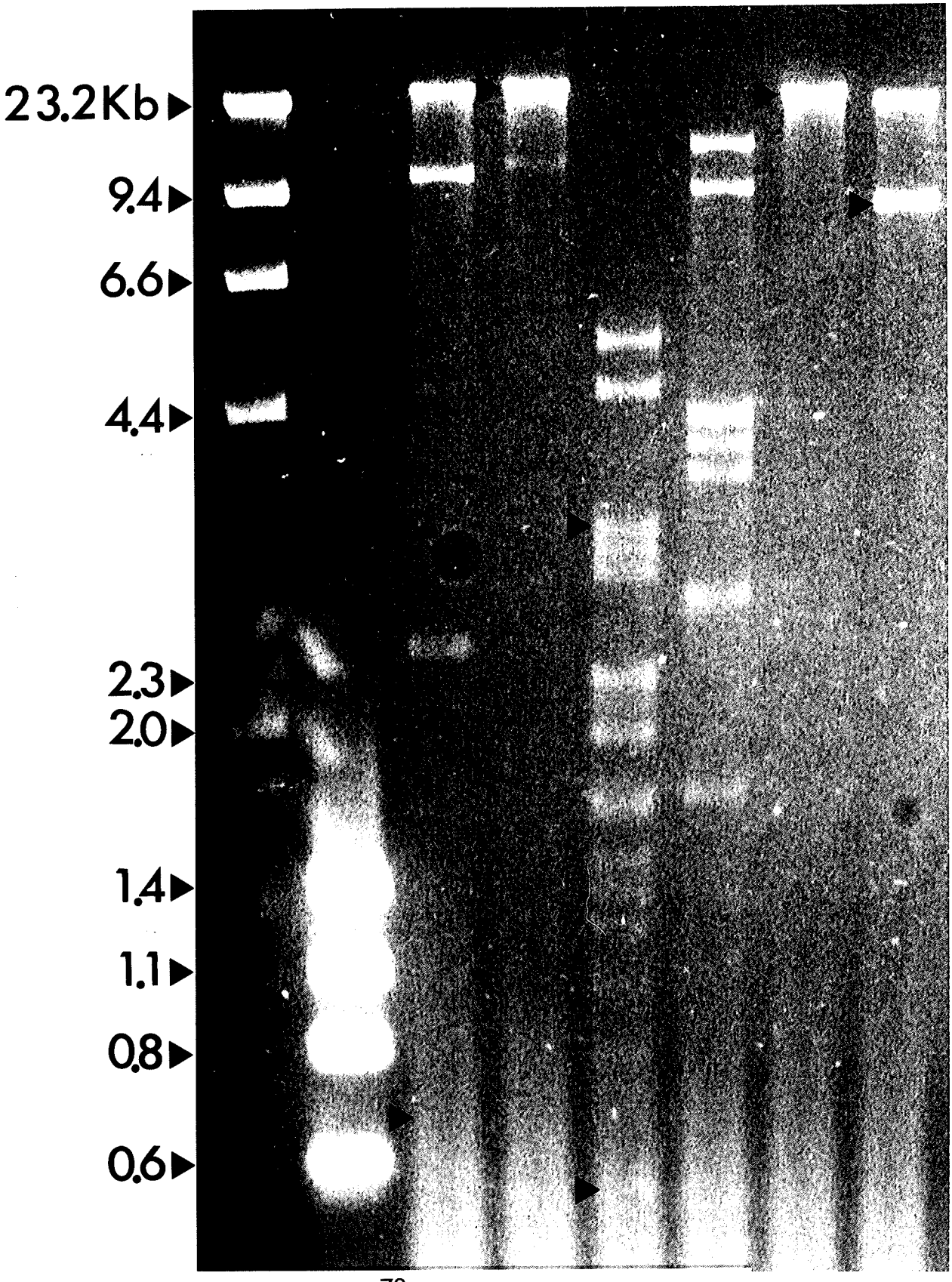


FIGURE III. RESTRICTION ENZYME DIGESTS OF GC-II. Black arrows designate bands which hybridize to c52-A. M = markers (ϕx174/Hae III and λ/Hind III); E = Eco RI, H = Hinc II, P = Pvu II, X = Xba I, B = Bam HI, and S = Sal I. Samples were run on a 1.0% agarose gel.

FIGURE III



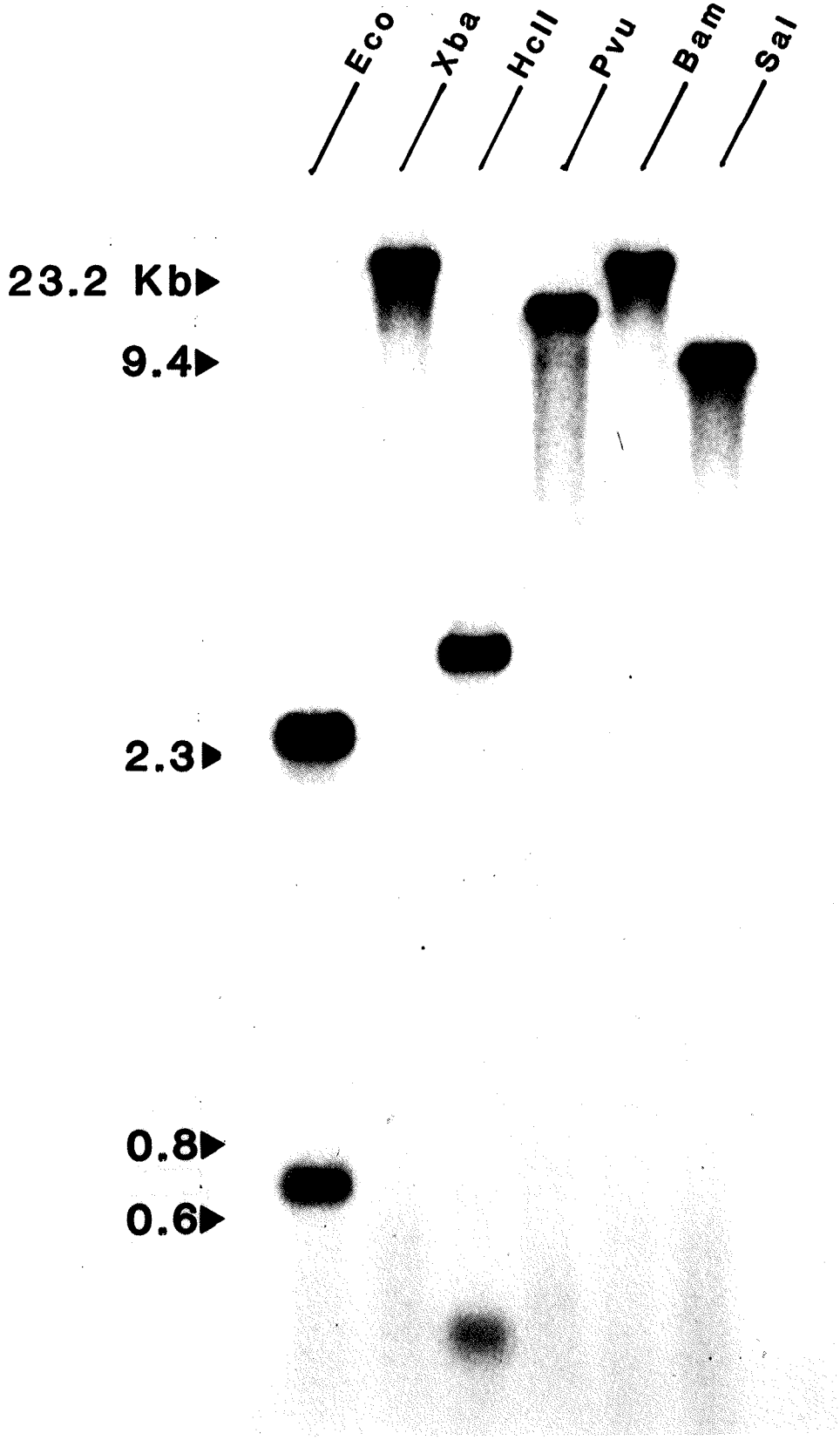
size as the small arm of EMBL3. On hybridization to c52-A, only the 9.4 Kb fragment hybridizes, indicating that the insert in GC-II is indeed the same size as the vector's small arm (Figure IV).

An Eco RI digest of GC-I (Figure IIA) generates seven fragments, three of which hybridize to c52-A (Figure IIB). The 3.85, 2.5, and 1.5 Kb fragments that hybridize are the same size as those which hybridized to c52-A on the rat liver genomic DNA blot. There are two other Eco RI fragments of 3.55 and 1.1 Kb that do not hybridize to c52-A and therefore represent either flanking regions of the gene or intervening sequences within the gene. The two large bands represent the arms of EMBL3, with a portion of the genomic insert attached to each. These two pieces are released from the arms by an Eco RI/Sal I double digest. In this digest, two extra bands are present, representing the portions of the insert that were attached to each arm. The 4.85 Kb fragment is attached to the small arm, while the 1.05 Kb fragment is attached to the large arm. This is evident because the small and large arms in the Eco RI digest are reduced in size respectively by 4.85 and 1.05 Kb in the Sal I/Eco RI digest.

An Eco RI digest of GC-II (Figure III) produces four bands, of which two hybridize to c52-A. The smallest band which hybridizes is about 650 bp and although it is not readily seen on the ethidium stained gel, its appearance is reproducible and the fragment has been isolated and subcloned. This 650 bp fragment overlaps with a small

FIGURE IV. SOUTHERN BLOT OF GC-II. Autoradiogram of GC-II digest (derived from Figure III), hybridized to c52-A. Eco = Eco RI, Xba = Xba I, HcII = Hinc II, Pvu = Pvu II, Bam = Bam HI, and Sal = Sal I.

FIGURE IV



portion of the 1.50 Kb hybridizing Eco RI fragment of GC-I. It contains a Pvu II site 400 bp from its 3' Eco RI site. This Pvu II site is also located in the same position in the 1.50 Kb Eco RI fragment of GC-I. This suggests that an Eco RI site may have been created in GC-II by mutation, resulting in a 650 bp piece, which would explain why this piece was not detected in any GC-I digests. Sequence analysis would confirm this possibility. The other band which hybridizes is a 2.5 Kb fragment, the same size as one of the hybridizing Eco RI fragments in GC-I. The other two pieces include the vector arms with portions of the insert attached to each. An Eco RI/Sal I digest reveals that a 1.2 Kb piece of the insert is attached to the large arm, while a 5.1 Kb piece is attached to the small arm of the vector.

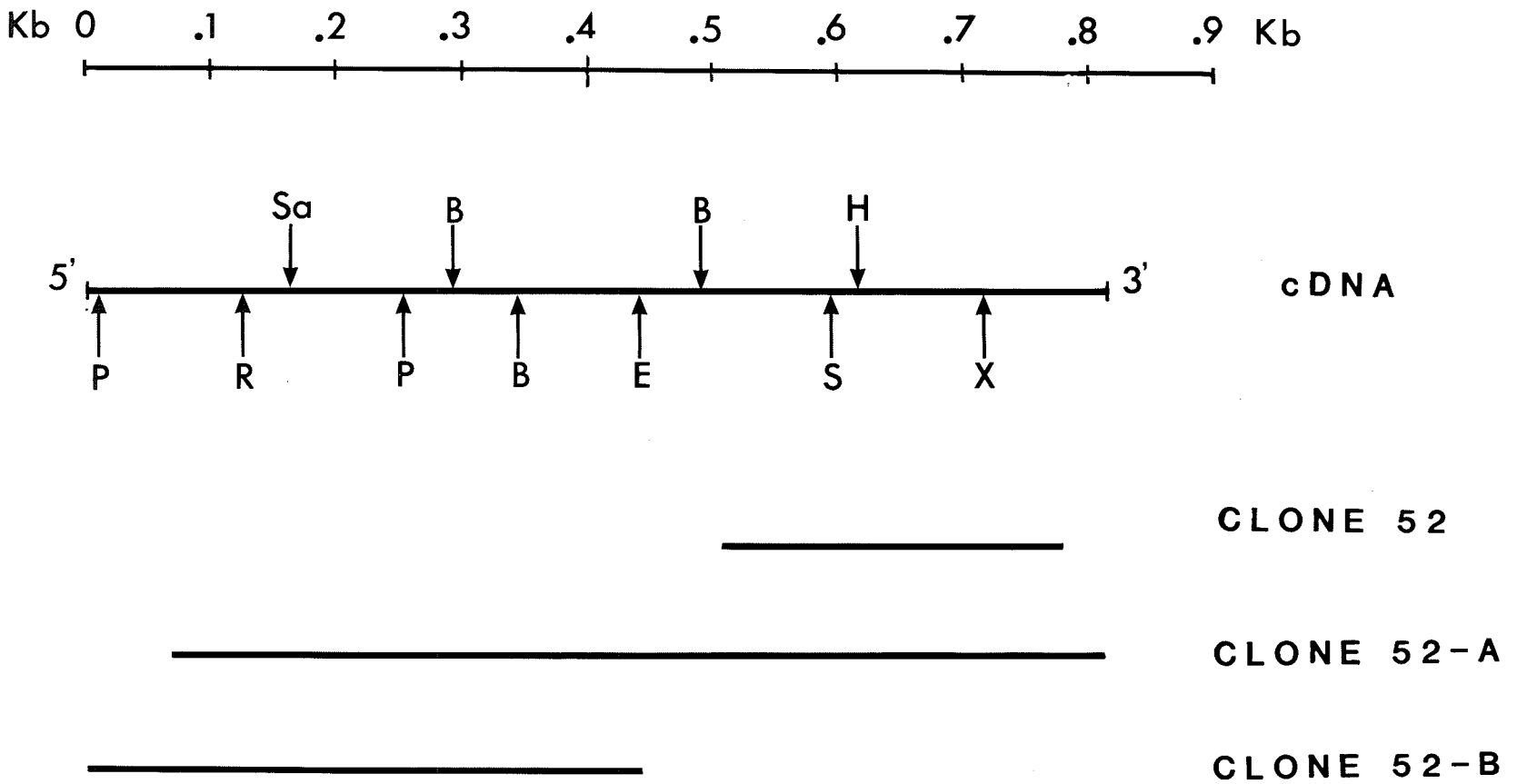
Three different cDNA clones to rPL-II were used as hybridization probes throughout the analysis of the genomic clones (Figure V). Clone 52 represents the 3' portion of the rPL-II message, while clone 52-B represents the 5' portion. Clone 52-A extends further 3' than c52, but does not extend as far as c52-B in the 5' direction. Together, the three cDNAs cover all of the rPL-II coding sequence except for a few nucleotides in the most 5' region of the cDNA (Duckworth et al., 1986).

When the GC-I Eco RI digest was hybridized to c52-B, only the 3.85 and 1.5 Kb fragments hybridized. On the other hand, c52 hybridized to the 2.5 Kb fragment. These results suggest that the 3.85 and 1.5 Kb fragments

FIGURE V. CDNA RESTRICTION MAPS. Restriction enzyme map of the rPL-II cDNA clones used in the analysis of GC-I. The cDNA represents the entire cDNA sequence of 814 bp, and the restriction sites of importance. c52-3 represents a 3' cDNA clone of 327 bp, c52-B represents a 5' cDNA clone of 435 bp, and c52-A covers the 3' and part of the 5' region of the rPL-II message and is 740 bp in length.

FIGURE V

rPL-II cDNA CLONES



represent the 5' portion of the gene while the 2.5 Kb piece is part of the 3' portion. The same analysis with the GC-II Eco RI digest showed that the 650 bp fragment hybridized to c52-B while the 2.5 Kb piece hybridized only to c52. This implies that the two genomic clones share an overlapping region. Both 2.5 Kb Eco RI fragments were subsequently isolated and a fine structure restriction map was created for both fragments. The maps derived were identical for both fragments, confirming that GC-I and GC-II are actually overlapping clones.

To determine if the 3' end of the gene had been isolated, the most 3' Xba I/Pst I fragment (107 bp) from c52-A was purified by electroelution, nick translated, and used as a probe. This piece hybridized to the 2.5 Kb Eco RI fragments from both GC-I and GC-II, indicating the 3' end of the rPL-II had been isolated. Further to this, the most 5' Eco RI/Rsa I fragment (124 bp) of c52-B was isolated and nick translated. On hybridization to the GC-I and GC-II Eco RI digests, only the 3.85 Kb fragment in GC-I hybridized to this piece. This indicates that the 5' end of the gene may be represented in GC-I, but not in GC-II. Thus, the data suggests that GC-I not only encompasses the coding sequences found within GC-II, but it may in fact contain the entire rPL-II gene and its flanking sequences.

IV. RESTRICTION MAPPING OF GC-I

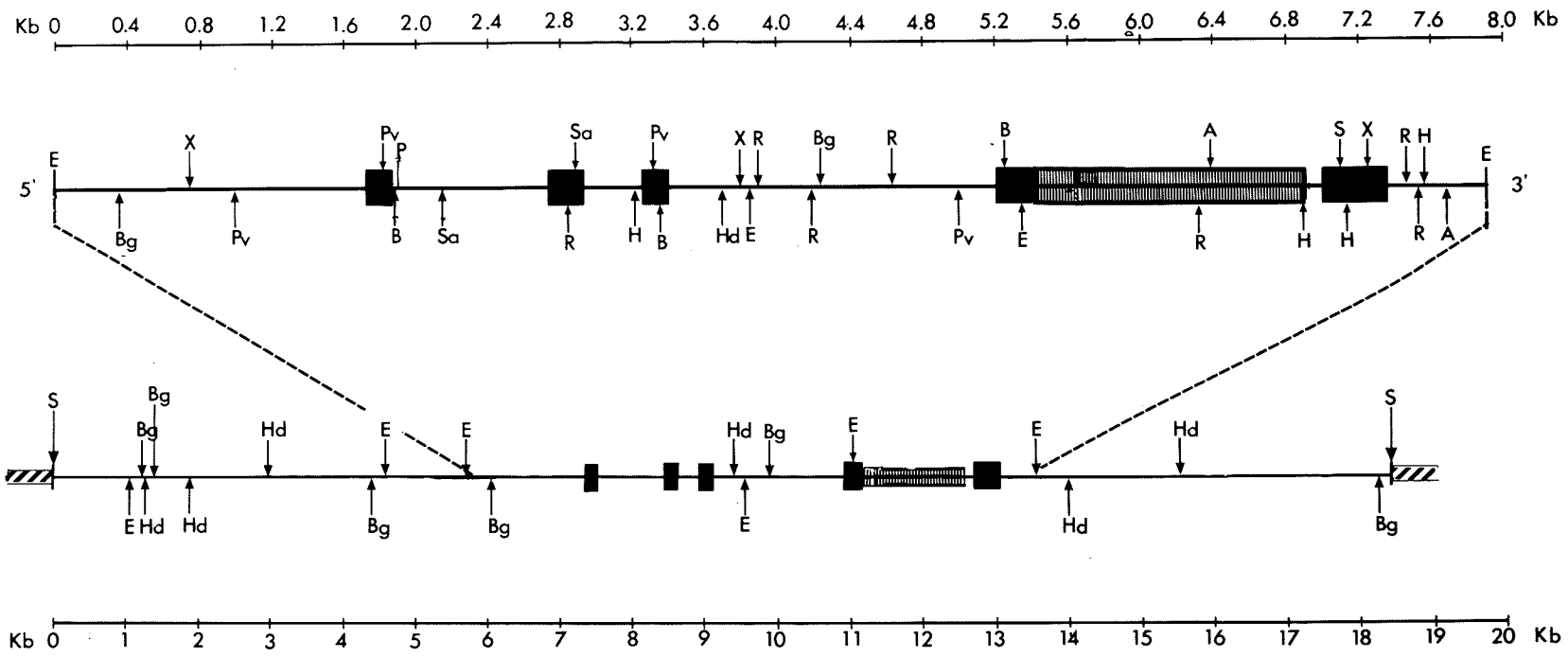
Numerous enzymes were used to create a restriction map of GC-I, but in most cases, the enzymes had so many sites

within the vector or the clone that the large number of bands made it very difficult to orient all of the fragments. However, Eco RI, Hind III and Bgl II produced a manageable number of fragments with which to work, and so they were used in mapping GC-I. The map that was determined for GC-I is shown in Figures VI, VIIA and VIIB, and the data is shown in Figures VIII and IX.

As mentioned, an Eco RI/Sal I digest of GC-I results in seven bands of the following sizes: 1.05, 3.55, 1.10, 3.85, 1.50, 2.50, and 4.85 Kb. The 1.05 and 4.85 Kb fragments are attached to the large and small arms of the vector respectively. The 3.85, 2.5 and 1.5 Kb Eco RI pieces all hybridize to c52-A, while the 3.55 and 1.1 Kb pieces are nonhybridizing fragments. Before subcloning these fragments for fine structure mapping, they were oriented with respect to one an other within GC-I, by creating single and double digests with Hind III, Bgl II and Eco RI.

The Hind III digest results in nine bands due to eight sites within the GC-I insert and one site in the small arm of EMBL3. When Sal I and Hind III are used together, eleven bands are created, three of which represent the arms of EMBL3 (Figure VII). The size of these bands are as follows: 18.5, 6.40, 4.80, 4.60, 4.40, 2.85, 1.70, 1.45, 1.10, 1.10, and 0.69 Kb. In this digest, the short arm is split into two pieces of 4.80 and 4.40 Kb, while the large arm remains intact. The 6.40 and 4.60 Kb bands are the only two fragments that hybridize to c52-A

FIGURE VI. RESTRICTION ENZYME MAP OF GC-I. The three EcoRI subclones that hybridized to c52-A are shown in the enlarged map with more detail. Solid boxes represent exons (▬■▬). Shaded rectangular box represents a repetitive DNA sequence (▥). Cross-hatched regions at the end of GC-I represent the arms of EMBL3 (▨). E = Eco RI, A = Acc I, B = BstNI, Bg = Bgl II, P = Pst I, Pv = Pvu II, X = Xba I, S = Sph I, Sa = Sau 3A, H = Hinc II, Hd = Hind III, R = Rsa I, and S = Sal I.



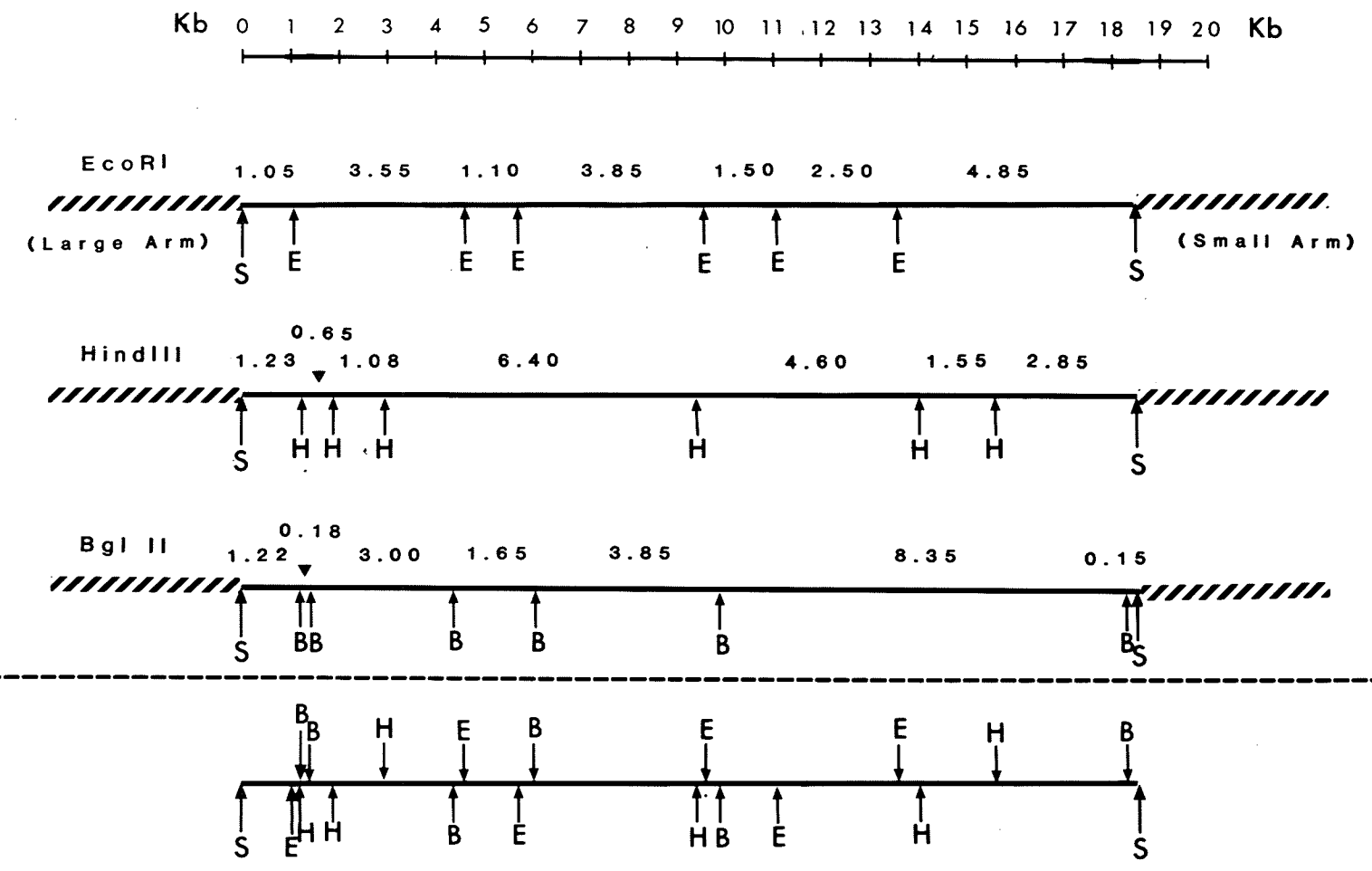
RAT PLACENTAL LACTOGEN-II GENOMIC CLONE

FIGURE VI

FIGURE VIIA. GC-I RESTRICTION MAPS. Individual restriction enzyme maps of GC-I for Eco RI, Hind III and Bgl II. Fragment sizes are shown above each fragment, in kilobases. A composite map of GC-I showing all three enzymes is at the bottom of the figure. Cross-hatched region represents the arms of EMBL3 (////). E = Eco RI, H = Hind III, B = Bgl II, and S = Sal I.

FIGURE VIIA

RESTRICTION SITES AND FRAGMENT LENGTHS OF GC-I



MAP OF GC-I

FIGURE VIIB. GC-I RESTRICTION FRAGMENTS. Various restriction fragments of the genomic clone, used in mapping GC-I. The restriction enzyme representing each fragment is shown in parentheses and fragment lengths are above each piece, in kilobases. The cross-hatched region represents the arms of EMBL3. E = Eco RI, H = Hind III, and S = Sal I.

FIGURE VIIB

RESTRICTION FRAGMENTS OF GC-I

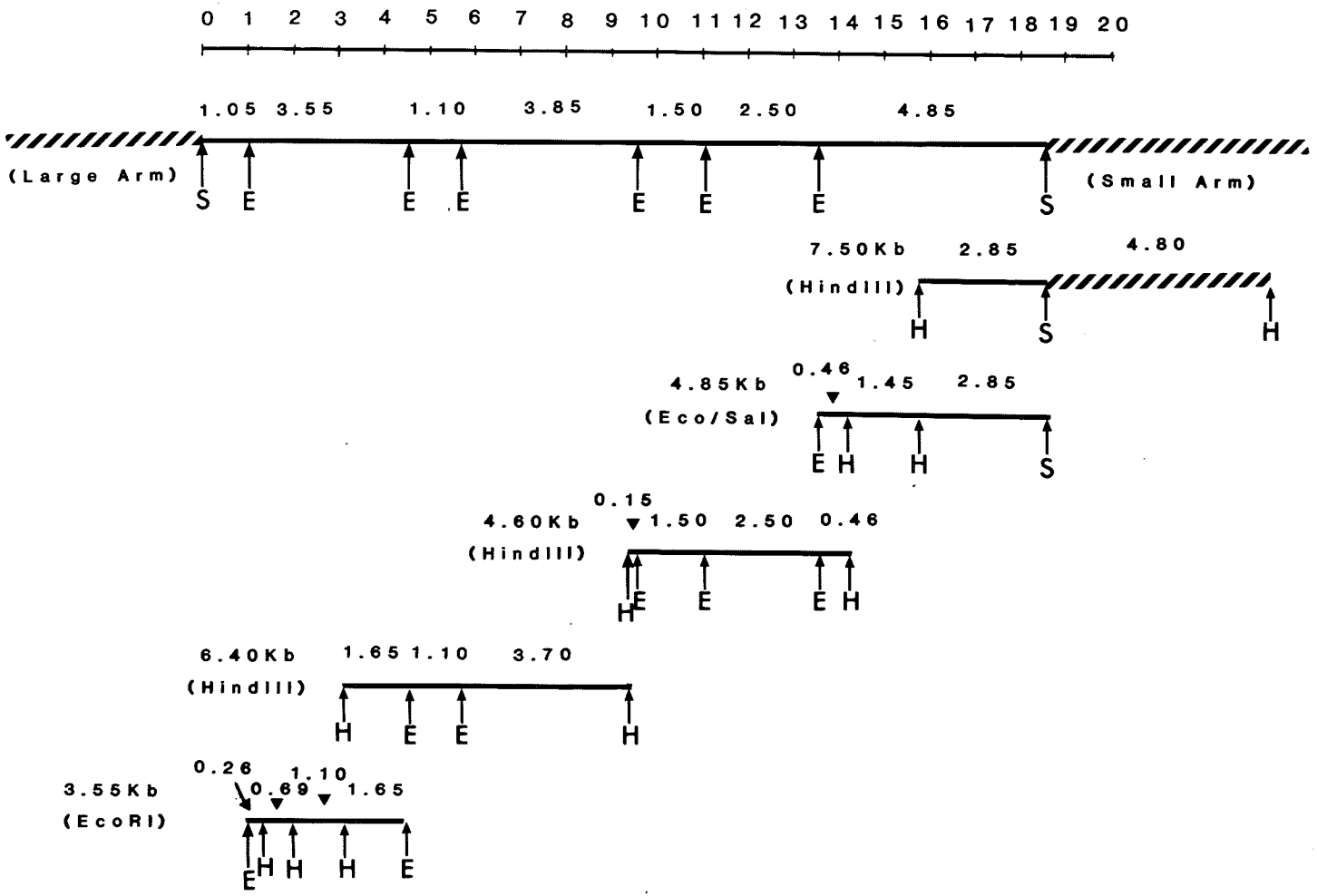


FIGURE VIII. RESTRICTION ENZYME DIGESTS OF GC-I. White arrows represnt bands which hybridize to c52-A. M = markers (øx174/HaeIII and λ/Hind III); E = Eco RI, X = Xba I, B = Bgl II and H = Hind III. Samples were run on a 0.6% agarose gel.

FIGURE VIII

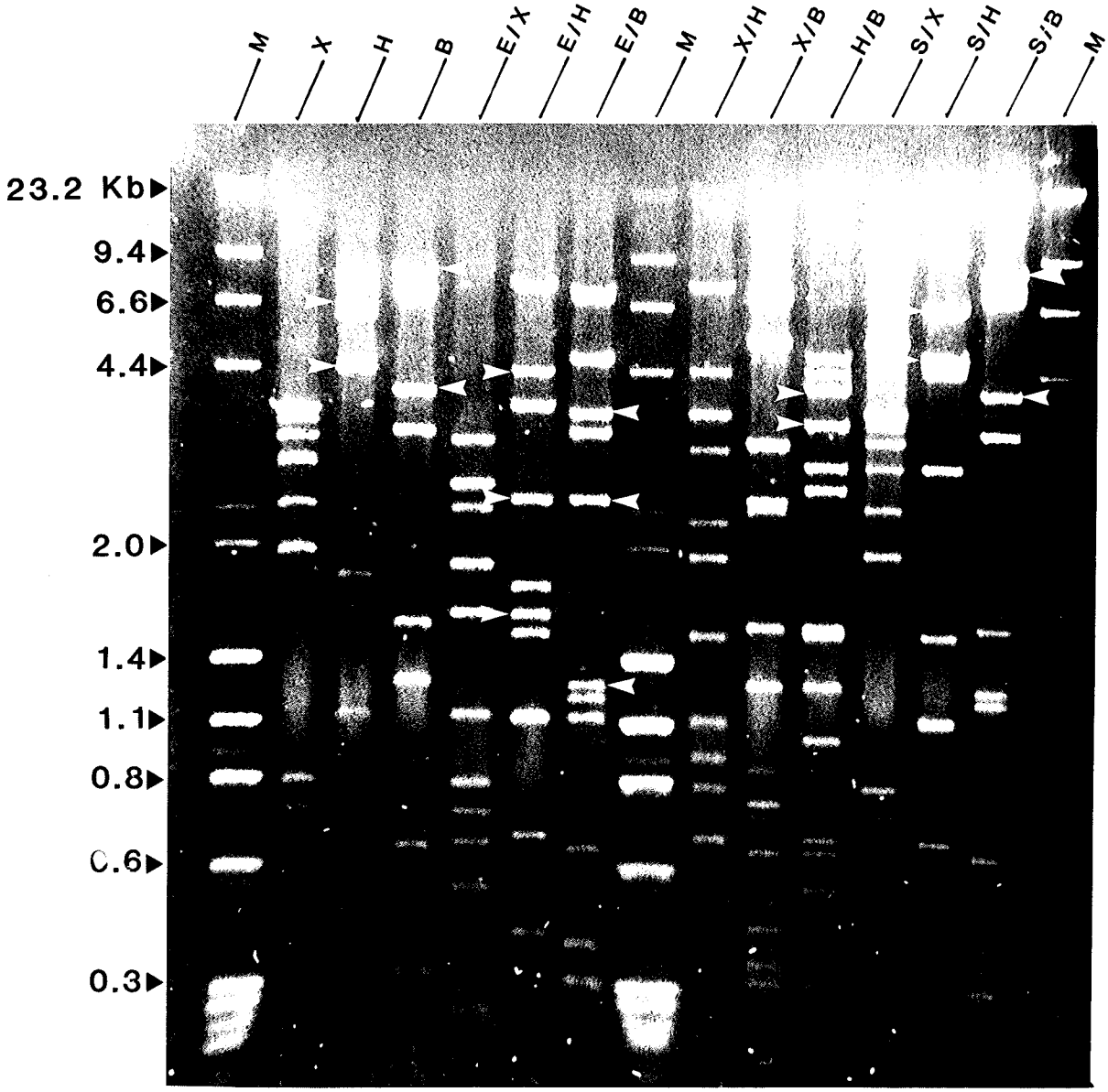
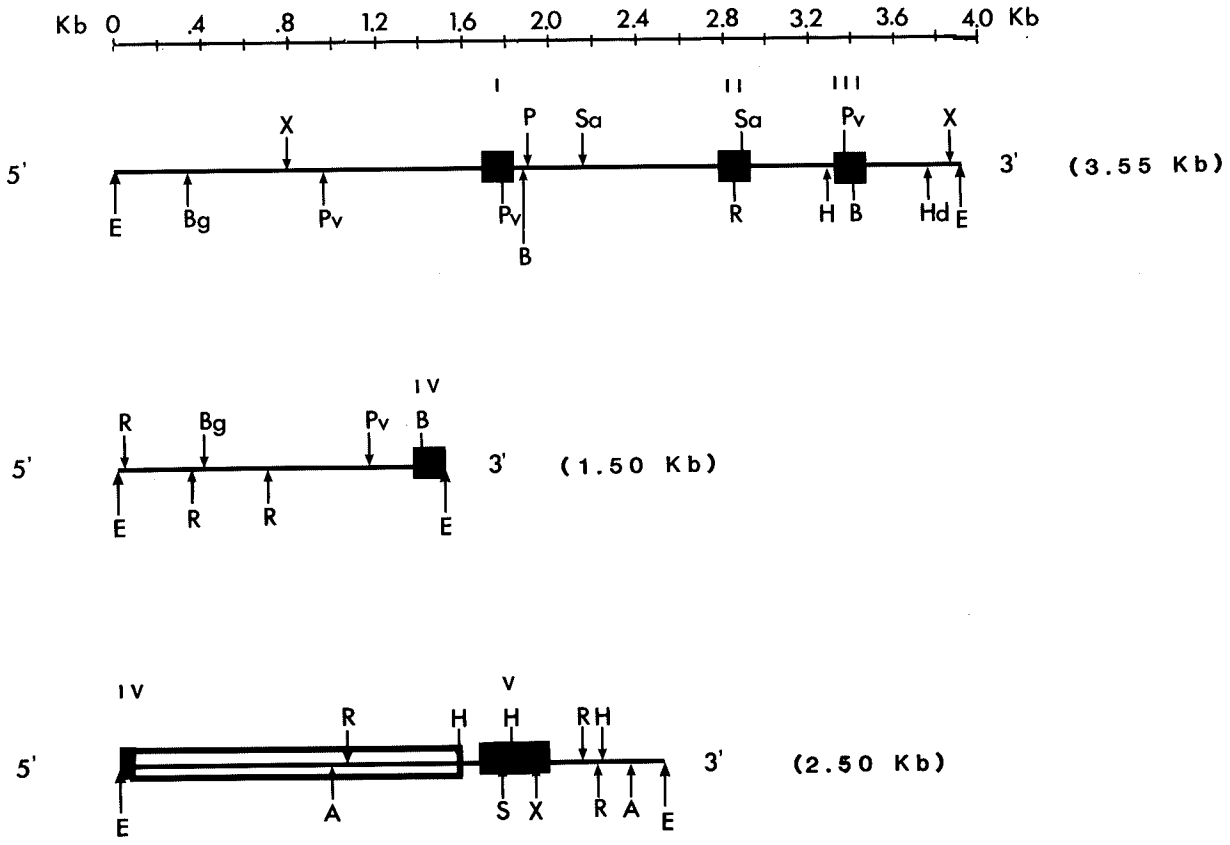


FIGURE IX. RESTRICTION MAPS OF THE HYBRIDIZING GC-I SUBCLONES. Restriction enzyme maps of the 3.55, 1.50 and 2.50 Kb Eco RI genomic subclones which hybridize to c52-A. Solid boxes represent exons (—■—) and the open rectangular box represents the presence of a repetitive DNA sequence. Exons are numbered with Roman numerals. Exon IV is split by Eco RI and is represented on two of the subclones. A = Acc I, B = Bst NI, Bg =Bgl II, E = Eco RI, H = Hinc II, Hd = Hind III, P = Pst I, Pv = Pvu II, R = Rsa I, S = Sph I, Sa = Sau3A, and X = Xba I.

FIGURE IX


Kb 0 .4 .8 1.2 1.6 2.0 2.4 2.8 3.2 3.6 4.0 Kb
I
II
III
5'
X
P
Sa
Sa
Pv
X
3'
(3.55 Kb)
E
Bg
Pv
Pv
B
R
H
B
Hd
E
IV
5'
R
Bg
Pv
B
3'
(1.50 Kb)
E
R
R
E
IV
V
5'
R
H
H
RH
3'
(2.50 Kb)
E
A
S
X
R
A
E

(Figure VIII). Not surprisingly, these are the same sized fragments that hybridized to c52-A in the rat liver genomic DNA blot (Figure I). The 2.85 Kb fragment is attached next to the small arm. This was determined because the 7.5 Kb piece in the Hind III digest represents the 4.8 Kb of the small arm plus an additional 2.85 Kb of the GC-I insert. The 2.85 Kb piece is released from the arm by the Sal I/Hind III digest. The 1.1 Kb fragment in the double digest has more intense ethidium bromide staining suggesting that this band represents two fragments of the same size. One of the 1.10 Kb pieces is released from the large arm by the Sal I/Hind III digest.

In order to orient the Eco RI fragments, a double digest with Eco RI and Hind III was carried out. This combination creates the following fragments: 20.0, 7.50, 4.40, 3.70, 2,50, 1.65, 1.50, 1.45, 1.10, 1.10, 0.69, 0.46, and 0.29 Kb. The 20.0 Kb band represents the large arm plus 1.05 Kb of the GC-I insert. The 7.50 Kb fragment represents the 4.80 Kb piece of the small arm and the 2.85 Kb Hind III/Sal I fragment. The 4.85 Kb Eco RI/Sal I piece which is next to the small arm is split by Hind III into at least a 2.85 Kb piece (as mentioned) and should therefore leave a 2.00 Kb fragment. This is not the case, and so there must be another Hind III site within this region. The 4.85 Kb Eco RI/Sal I fragment is actually split into three pieces of 2.85, 1.45 and 0.46 Kb by Hind III.

When used as nick translated probes, the 1.50 and 2.50 Kb Eco RI fragments hybridize to the 4.60 Kb Hind III

fragment. This suggests that the 2.50 and 1.50 Kb Eco RI fragments are contained within this 4.60 Kb Hind III piece. Furthermore, when using the 1.10 Kb Eco RI fragment as a probe, it hybridized to the 6.40 Kb Hind III fragment and the 1.10 Kb Eco RI/Hind III fragment. Therefore, this piece must be from the 6.40 Kb Hind III fragment. The 3.55 Kb Eco RI fragment used as a probe revealed hybridization to the 6.40 Kb Hind III piece as well as to the large arm, indicating that this fragment is adjacent to the large arm and is flanked by the 1.10 Kb Eco RI fragment. The 3.85 Kb Eco RI fragment must therefore be adjacent to the 1.10 Kb Eco RI fragment, in the 3' direction. Since the 1.50 Kb Eco RI fragment hybridizes to c52-B (5'), while the 2.50 Kb Eco RI fragment hybridizes to c52 (3'), the 1.50 Kb piece must lie between the 3.85 and 2.5 Kb Eco RI fragments. The 4.85 Kb Eco RI/Sal I fragment, therefore, is situated next to the 2.50 Kb Eco RI fragment.

The 6.40 Kb Hind III fragment is split into three fragments of 3.70, 1.10 and 1.65 Kb by Eco RI. The 3.70 Kb piece hybridizes to c52-A and is derived from the Hind III site in the 3.85 Kb Eco RI fragment and the 3' Eco RI site of the same piece. The 1.10 Kb piece represents the 1.10 Kb Eco RI fragment, while the 1.65 Kb piece arises from the 3' Eco RI site in the 3.55 Kb Eco RI fragment to the most 3' Hind III site in the same fragment. The other 1.10 Kb piece and the 0.69 Kb piece are derived from internal Hind III sites within the 3.55 Kb Eco RI fragment. The sizes of

the fragments from Hind III/Eco RI digests of the 3.55 Kb Eco RI band were confirmed from digests with the subcloned fragment.

It therefore appears that the Sal I/Eco RI fragments of GC-I are oriented in the following sequence from a 5' to 3' direction: 1.05, 4.00, 1.10, 3.85, 1.50, 2.50 and 4.85 Kb. The 1.05 and 3.55 Kb fragments, which do not hybridize to the cDNA, must include 5' flanking information while the 4.85 Kb piece contains 3' flanking information.

The orientation of the Eco RI fragments that was set up using the Hind III and Eco RI digests mentioned above, were confirmed in the same manner with digests involving Bgl II and Eco RI/Bgl II. The sites for Bgl II are shown in the map of GC-I (Figure VIII). The major point to be made from these digests is the fact that Bgl II sites within the 3.85 and 1.5 Kb Eco RI fragments gave rise to a 3.85 Kb Bgl II piece that hybridizes to c52-A. This supports the conclusion that the 3.85 and 1.5 Kb Eco RI fragments are contiguous. In addition, the Bgl II digest in the rat liver DNA blot (Figure I) showed two bands, one of which was about 3.9 Kb. The other hybridizing Bgl II fragment in GC-I is about 8.4 Kb and extends in the 3' direction from the Bgl II site in the 1.5 Kb fragment all the way to a Bgl II site approximately 200 bp from the small arm.

V. FINE STRUCTURE MAPPING OF GC-I

Since the Eco RI digest produced the least number of

restriction fragments, each of the five GC-I Eco RI bands was isolated and subcloned into the plasmid vector pAT153. This allowed for easier restriction mapping to be carried out since smaller pieces of DNA could be analyzed. The five GC-I Eco RI fragments that were subcloned were of the following sizes: 3.55, 1.10, 3.85, 1.50, and 2.50 Kb. The last three hybridized to the cDNA probes. All of the subclones were mapped in a similar manner and hybridized to various subfragments of the three cDNA clones to rPL-II (Figure V). The method used to construct a restriction map for the 1.50 Kb Eco RI fragment which hybridized, will be given in detail as an example to indicate how mapping was accomplished. The map constructed for this 1.50 Kb subfragment is shown in Figures IX and X, and the data are shown in Figures XI, XII, XIII, and XIV.

Numerous restriction enzymes were used to find restriction sites within clone 1.5, but the only sites found were with Bgl II, Pvu II, Rsa I and Bst NI. Figure IX represents a series of single enzyme digests of clone 1.5 and clone 2.5. Only the lanes representing digests of clone 1.5 will be discussed. Bgl II has no sites within pAT153, and so the single band in this digest represents the only Bgl II site in this clone. Rsa I has two sites within pAT153 and the four bands in this digest indicate that it has two sites within clone 1.5. PvuII has no sites in pAT153, and so the single band indicates that there is one site within the insert. If there were no sites in either the vector or the clone, then the DNA would be

FIGURE X. RESTRICTION ENZYME MAPS OF THE 1.50 Kb HYBRIDIZING Eco RI FRAGMENT OF GC-I. Sizes of the fragments cut by the various enzymes are indicated in kilobases. The position of the Rsa I sites in pAT153 are indicated above the respective sites (in basepairs). Regions of hybridization to the various cDNA probes are noted for each set of digests (Hybn. or No Hybn.). The cross-hatched region represents the vector pAT153 (////). B = BstNI, Bg = Bgl II, E = Eco RI, P = Pvu II, and R = Rsa I.

FIGURE X

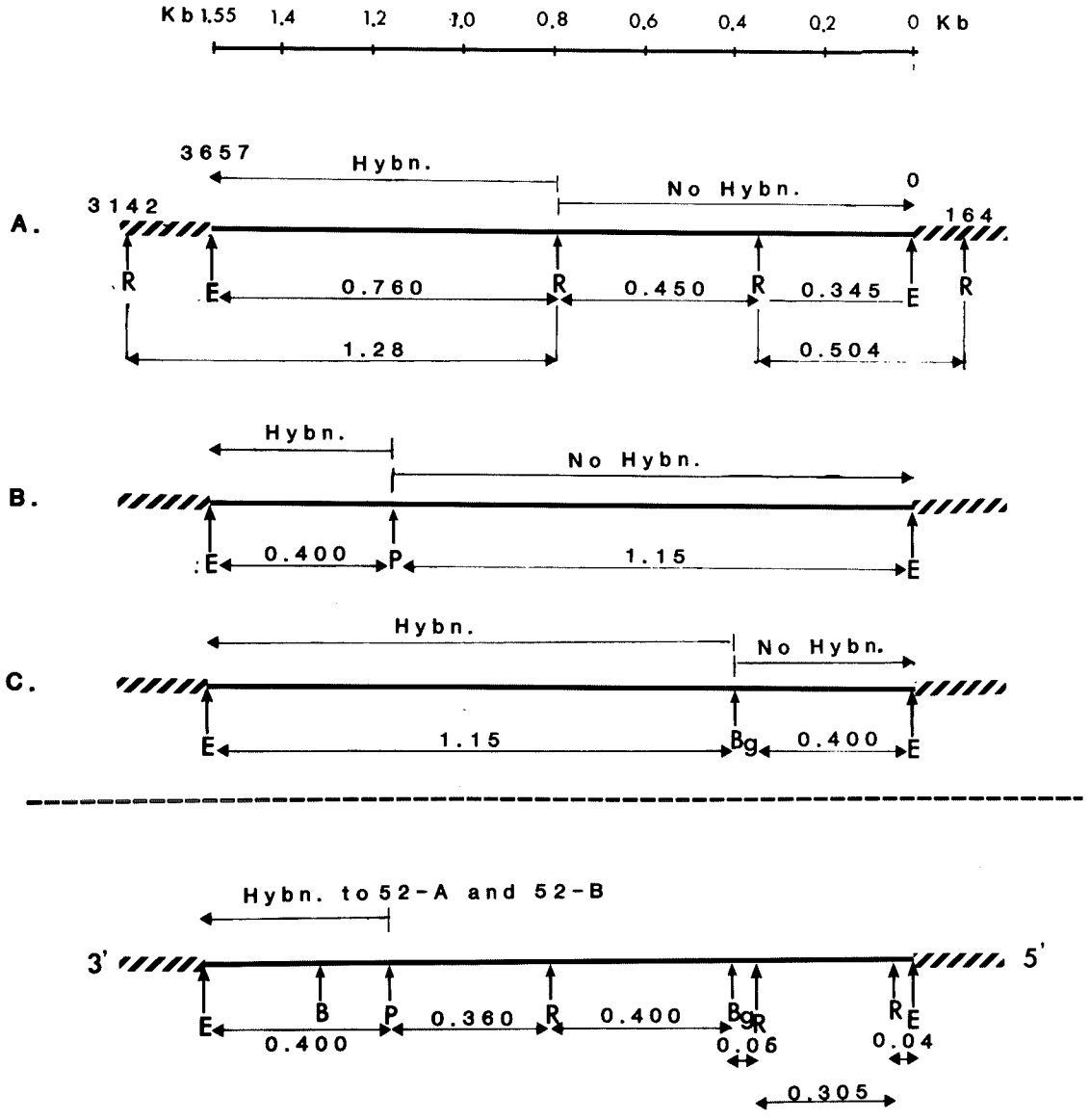

1.50 Kb EcoRI SUBCLONE
Kb 1.55 1.4 1.2 1.0 0.8 0.6 0.4 0.2 0 Kb
3657
Hybn.
No Hybn.
0
3142
164
A.
R
E
0.760
R
0.450
R
0.345
E
R
1.28
0.504
Hybn.
No Hybn.
B.
E
0.400
P
1.15
E
Hybn.
No Hybn.
C.
E
1.15
Bg
0.400
E
Hybn. to 52-A and 52-B
3'
5'
E
B
P
0.360
R
0.400
Bg
R
R
E
0.400
0.06
0.04
0.305

FIGURE XI. RESTRICTION ENZYME DIGESTS OF THE 1.50 and 2.50 Kb HYBRIDIZING Eco RI FRAGMENTS OF GC-I. The white arrows designate the fragments of the 1.50 Kb subclone which hybridize to c52-A. M = Markers (ØX174/Hae III and λ/Hind III); E = Eco RI, X = Xba I, H = Hinc II, P = Pvu II, B = Bgl II, R = Rsa I and A = Acc I. Samples were run on a 1.0% agarsose gel.

FIGURE XI

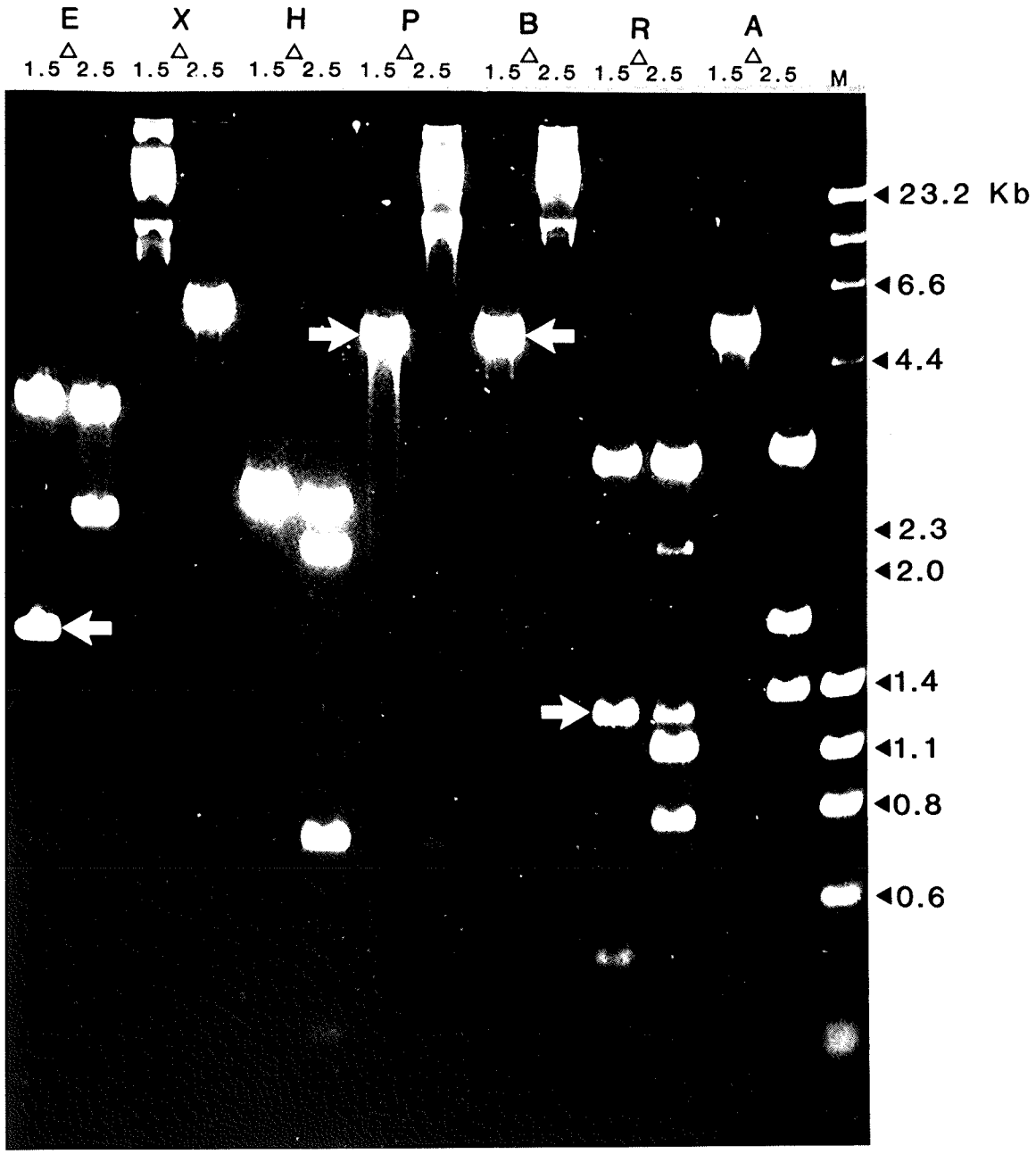

E
X
H
P
B
R
A
1.5 2.5 1.5 2.5 1.5 2.5 1.5 2.5 1.5 2.5 1.5 2.5 1.5 2.5
M
23.2 Kb
6.6
4.4
2.3
2.0
1.4
1.1
0.8
0.6

FIGURE XII. RESTRICTION ENZYME DIGESTS OF THE 1.50 Kb HYBRIDIZING Eco RI FRAGMENT OF GC-I. The white arrows designate the fragments which hybridize to c52-A. M = Markers (øx174/Hae III and λ/Hind III); E = Eco RI, P = Pvu II, B = Bgl II, R = Rsa I, and H = Hind III. Samples were run on a 1.0% agarose gel.

FIGURE XII

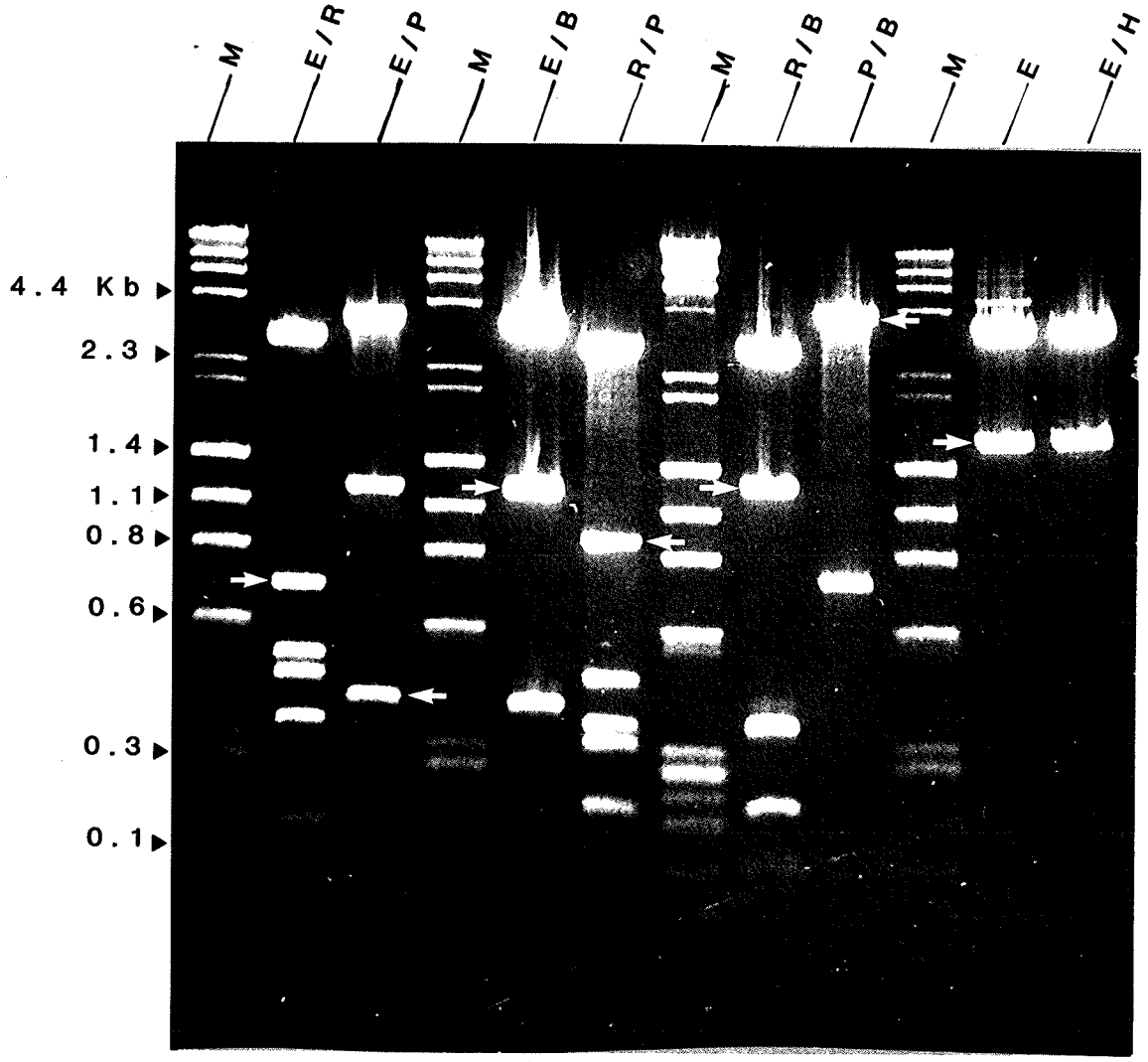


FIGURE XIII. SOUTHERN BLOT OF 1.50 Kb Eco RI FRAGMENT. Autoradiogram of the 1.50 Kb Eco RI subclone restriction digests (see Figure XII). Hybridization to c52-A. E = Eco RI, P = Pvu II, B = Bgl II, R = Rsa I, and H = Hind III.

FIGURE XIII

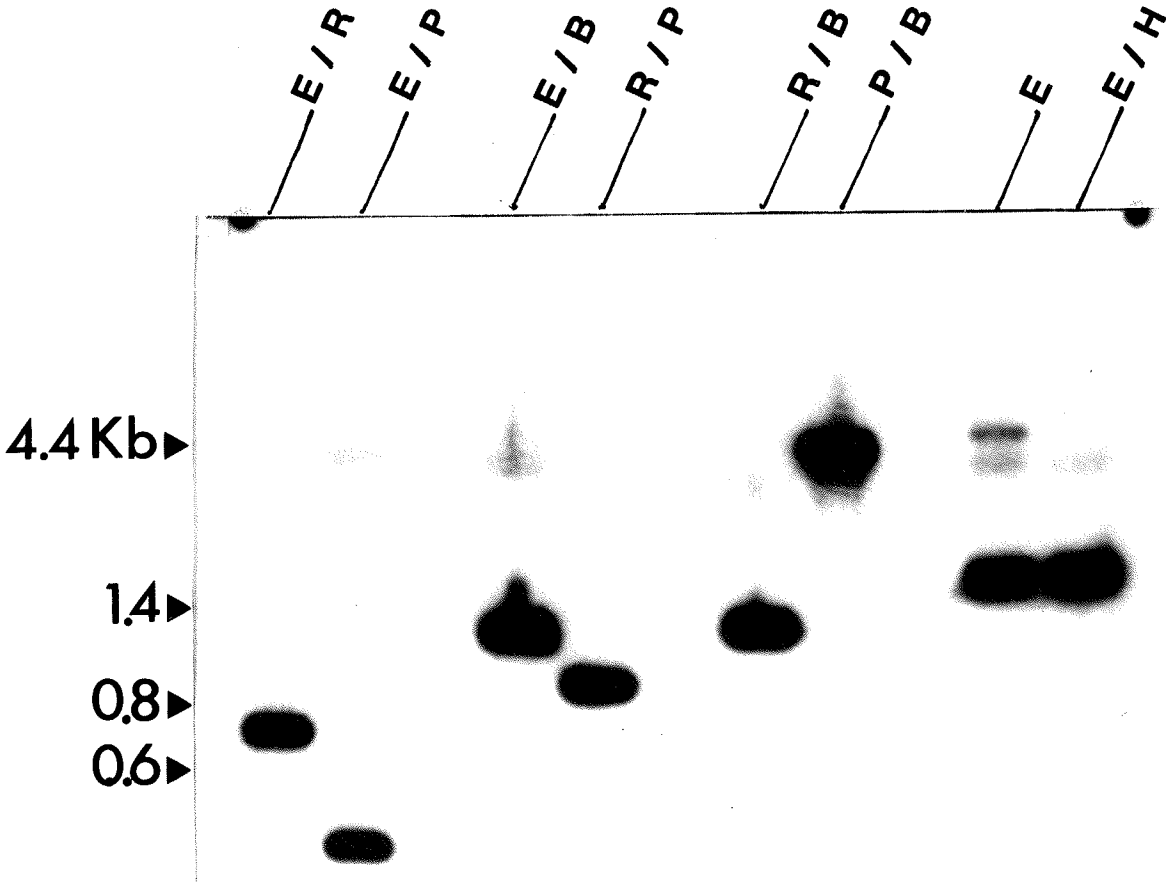
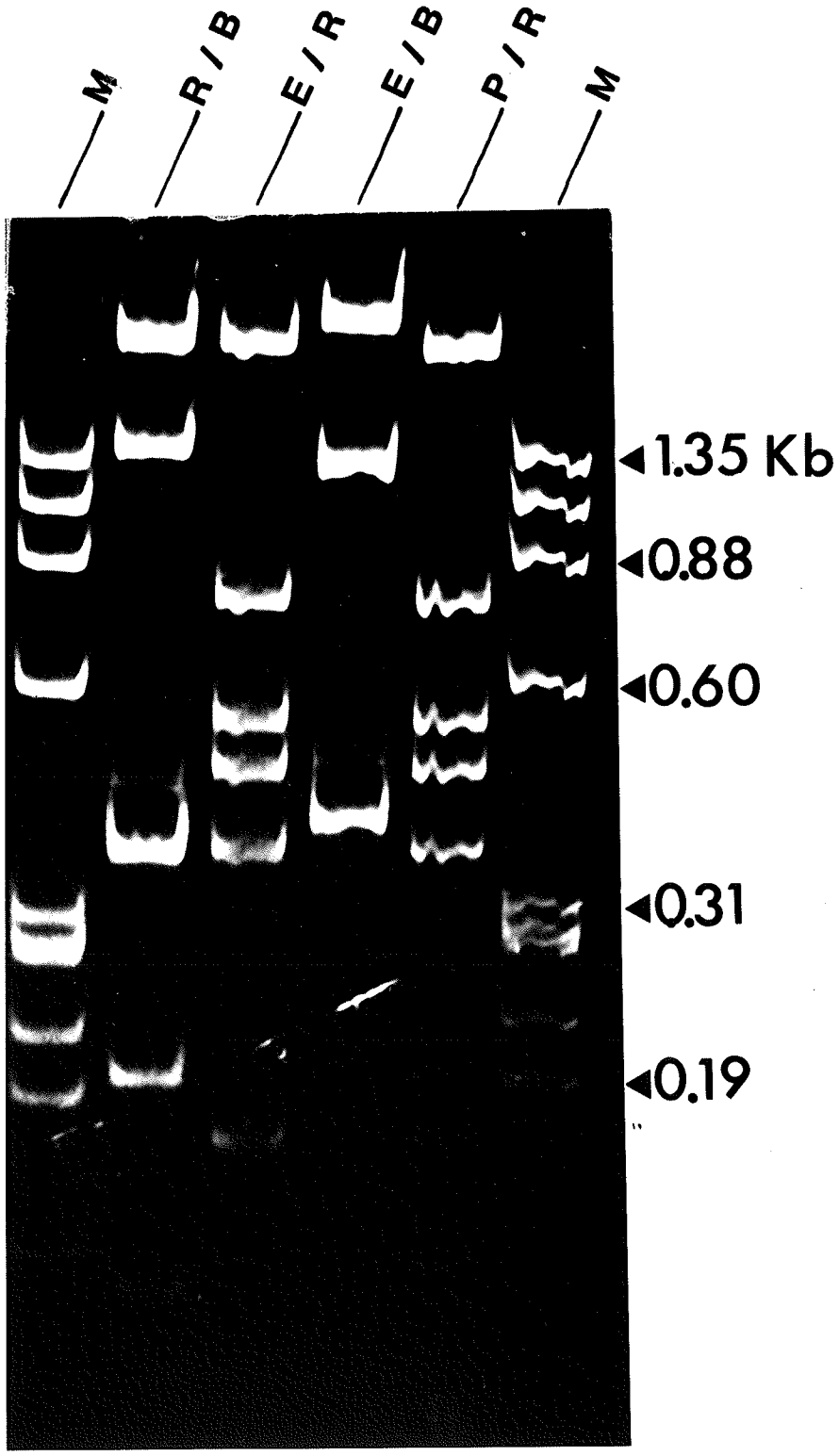


FIGURE XIV. RESTRICTION ENZYME DIGESTS OF THE 1.50 Kb HYBRIDIZING Eco RI FRAGMENT OF GC-I. M = Marker (ØxI74/Hae III); R = Rsa I, B = Bgl II, E = Eco RI, and P = Pvu II. Samples were run on a 3.5% polyacrylamide gel.

FIGURE XIV

1.50 Kb EcoRI



represented as several large high molecular weight bands in the gel, rather than one discrete band (nicked, circular, and supercoiled DNA bands). This is exemplified with the Xba I digest. A Bst NI digest (not shown) reveals a single site within the clone. Of all of these enzymes, only Bst NI is found within the coding sequence of rPL-II (Figure V).

Since Rsa I has sites within pAT153, these sites were used to orient the internal insert fragments created by its digestion. Rsa I cuts pAT153 at positions 164 and 3142 with respect to the Eco RI cloning site. The Eco RI cloning site by convention, starts at position zero and ends at position 3657. In the Rsa I digest, four bands are present (Figure XII). The 1.28 and 0.504 Kb fragments include a portion of the insert and vector together. The other two fragments represent an internal portion of the insert (0.45 Kb) and a large piece of pAT153 cut out by the Rsa I sites within the vector (3.14 Kb). A double digest with Eco RI and Rsa I (Figures XII and XIV) results in two extra bands created by the Eco RI sites that are within the two fragments containing the vector and insert pieces together. The 1.28 Kb fragment is split into two pieces of 760 and 515 bp. The former is part of the vector while the 760 bp band represents the piece of insert attached to the 515 bp piece of pAT153 and flanks one side of the 450 bp insert fragment (Figure X). The 515 bp band is a fragment of pAT153 that extends from position 2142 to 3657. The 0.504 Kb Rsa I band is split into two pieces of 340 bp and

164 bp by an Eco RI/Rsa I digestion. The 164 bp fragment is a part of pAT153 while the 340 bp band represents the third piece of the insert which flanks the other side of the 450 bp insert fragment. When comparing the Rsa I digest with the Eco RI/Rsa I digest, two bands are of the same size in both digestions. The 3.0 Kb bands represent the large vector fragment, while the 450 bp band does not change, implying that this fragment must be in between the two Rsa I sites in the insert. On hybridization to c52-A, the only piece that hybridizes is the 760 bp Eco RI/Rsa I fragment (Figure XIII), indicating that this fragment contains some coding sequence for rPL-II.

A digest of clone 1.5 with Pvu II (Figures X and XII) results in one intact fragment since there are no sites for Pvu II within the vector. An Eco RI/Pvu II double digest gives rise to three bands on the gel, the largest of which represents pAT153 (Figures XII and XIV). The sizes of the two smaller bands are 1.15 and 0.4 Kb, which together make up the 1.5 Kb subclone (Figure X). Only the smallest piece hybridizes to c52-A suggesting that the Pvu II site is contained within the 760 bp Rsa I/Eco RI fragment that also hybridizes to c52-A. This is confirmed by a double digest with Pvu II and RsaI where a 915 bp fragment hybridizes to c52-A (Figure XIII). This fragment is made up of the 400 bp insert and 515 bp of pAT153 from the Rsa I site at position 3142 to the Eco RI site at position 3657.

The Bgl II/Eco RI double digest produces two fragments

of 1.15 Kb and 0.4 Kb in length (Figure XII and XIV). They are the same sizes as the Pvu II fragments, but in this case, the large piece hybridizes to c52-A rather than the small piece (Figure XIII). Therefore, the Bgl II site must fall within the 460 bp Rsa I fragment in the subclone (Figure X). This is confirmed in a Bgl II/ Rsa I digest in which the 460 bp Rsa I fragment has been cut into two smaller pieces of 400 and 60 bp (Figure XIV). The only hybridizing fragment in this case is the same 1.27 Kb piece that hybridizes in the single Rsa I digests.

Before digesting the clone with Bst NI, the insert was cut out by Eco RI digestion and isolated by electroelution to free it from the vector, because there are six sites for Bst NI in pAT153. Digestion of the insert with Bst NI produced two bands of 1.4 and 0.1 Kb, both of which hybridize to c52-A. This indicates that the Bst NI site is probably situated in an exon located within the 400 bp Pvu II/Eco RI fragment of clone 1.5. When looking at the cDNA sequence of c52-A, a Bst NI site is situated 95 bp in the 5' direction from the Eco RI site. Since this is the same size as the small Bst NI/Eco RI hybridizing fragment, it supports the fact that the Bst NI site in clone 1.5 is the same as that found in the cDNA sequence. From these results, a restriction map of genomic subclone 1.5 was constructed (Figure IX).

In the restriction map of clone 1.5, the solid box represents the presence of an exon within the subclone, but its 5' limit is estimated to within a 50 bp range from the

Bst NI site. This can be concluded because the cDNA sequence has another Bst NI site 50 bp 5' to the Bst NI site shown in this clone. If this second Bst NI site were present in clone 1.5, another fragment should be seen in a Bst NI digest. As this is not the case, it appears that the remainder of clone 1.5 upstream from the solid box represents an intron. Restriction maps of the other four GC-I genomic subclones were constructed in the same manner as described for clone 1.5 (Figure IX). The 3.85 Kb clone has been mapped and is known to possess the 5' end of the gene. It contains three exons, three introns, and over 1.5 Kb of 5' flanking sequence. The limits of the exons as represented by the solid boxes in the diagrams are once again estimated boundaries. Their precise locations may be determined by DNA sequence analysis. These exon boundaries were estimated by matching known restriction fragments created by specific sites within the rPL-II cDNA sequence to the same sites and fragments within the genomic subclones. In the case of all the three hybridizing subclones, the exons were placed on the maps when those particular regions hybridized to their homologous regions within the cDNA sequence. Also, when two or more sites within the cDNA sequence were found in a subclone, the predicted fragment lengths within the subclone were confirmed to be the same as that predicted by the cDNA sequence before an exon boundary was outlined on the map. Fragments adjacent to exon boundaries indicated on the maps

were shown not to hybridize to any cDNA sequence.

The 124 bp 5' Rsa I/Eco RI fragment of c52-B hybridizes to exons A and B of clone 3.85. Exon B contains the RsaI site while exon A contains the Pvu II site, both found in the cDNA sequence. However, c52-A which does not extend far enough to include the 5' Pvu II site, only hybridizes to exon B and exon C, but not to exon A. Therefore, these findings seem to support this map for the subclone.

The open rectangular box in clone 2.5 represents a region in the clone that possesses a eukaryotic repetitive DNA sequence. Its presence was detected when clone 2.5 was nick translated and used as a probe to hybridize against a rat liver genomic DNA Southern blot (Figure XV). The hybridization pattern was characteristic of a repeat sequence as the entire lane of each sample hybridizes to the probe. The repetitive sequence was located to a more specific region within the subclone by hybridizing nick translated rat liver DNA to a Southern blot containing various restriction fragments of clone 2.5 (Figures XVI and XVII). The hybridization was limited to the 5' Eco RI/Hinc II fragment within the region indicated by the open rectangular box in Figure VI.

As mentioned earlier, the 3' Xba I/Eco RI fragment from c52-A hybridizes to clone 2.5. Further characterization of the restriction map for clone 2.5 has revealed that the 3' end of the cDNA sequence is represented within this genomic subclone. The sites within

FIGURE XV. GENOMIC SOUTHERN BLOT HYBRIDIZED TO GC-I SUBCLONES. Autoradiogram of a rat liver genomic DNA Southern blot hybridized to $dCTP^{32}$ labeled 2.50 Kb Eco RI fragment of GC-I. The autoradiogram shows the presence of a repetitive DNA sequence within the genomic subclone. E = Eco RI, X = Xba I, Hd = Hind III, Pv = Pvu II, P = Pst I, B = Bam I, S = Sal I, Bg = Bgl II, H = Hinc II and R = Rsa I. The 5 ug samples were run on a 1.0% agarose gel.

FIGURE XV

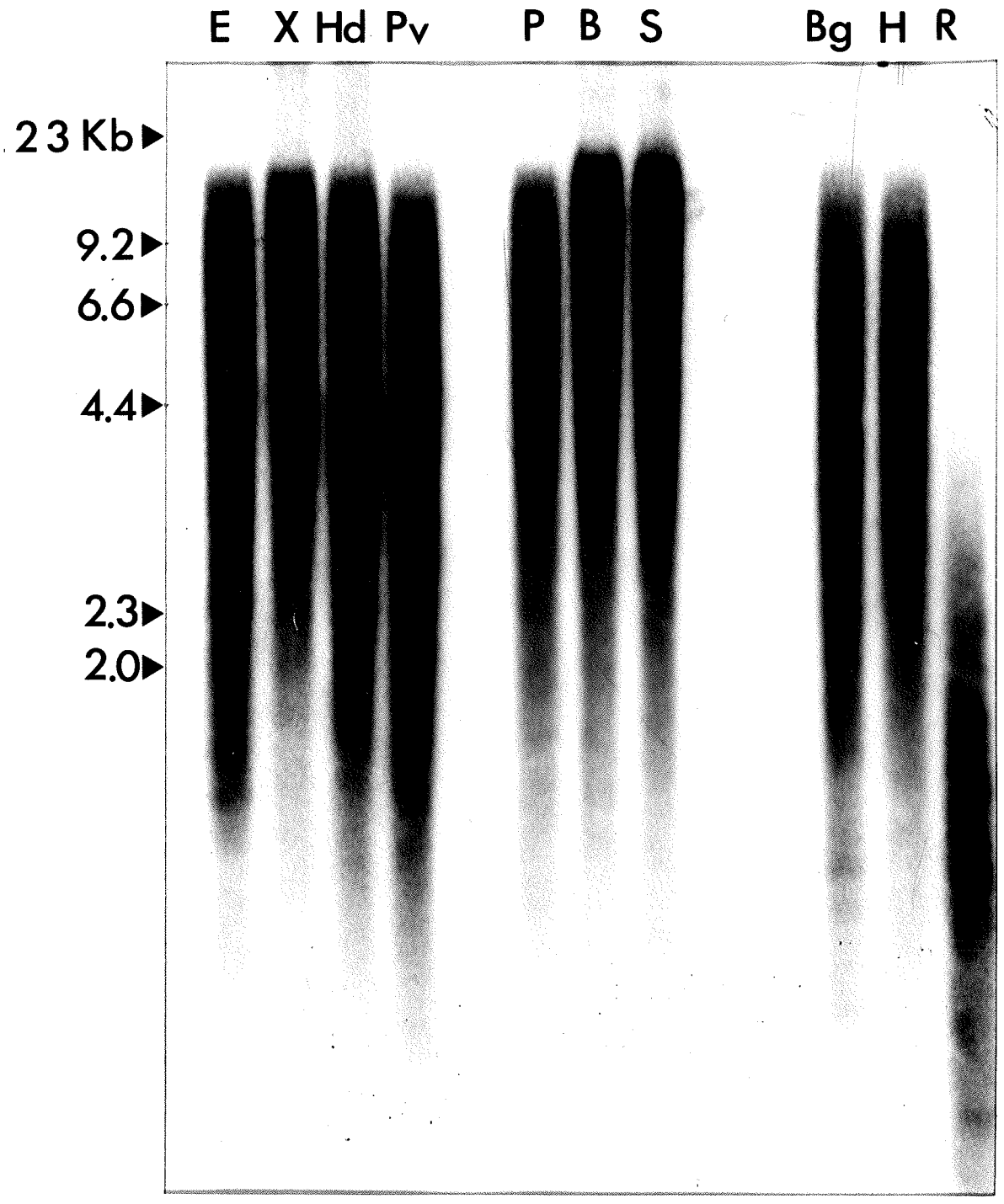


FIGURE XVI. RESTRICTION ENZYME DIGESTS OF THE 2.50 Kb Eco RI HYBRIDIZING FRAGMENT OF GC-I. This is the genomic subclone which possesses the repetitive DNA sequence (see Figure XV). Arrows represent the fragments which hybridize to $dCTP^{32}$ labeled rat liver genomic DNA (see Figure XVII). M = Marker (ØX174/Hae III and λHind III); E = Eco RI, R = Rsa I, A = Acc I, H = Hind III, X = Xba I, and S = Sph I.

FIGURE XVI

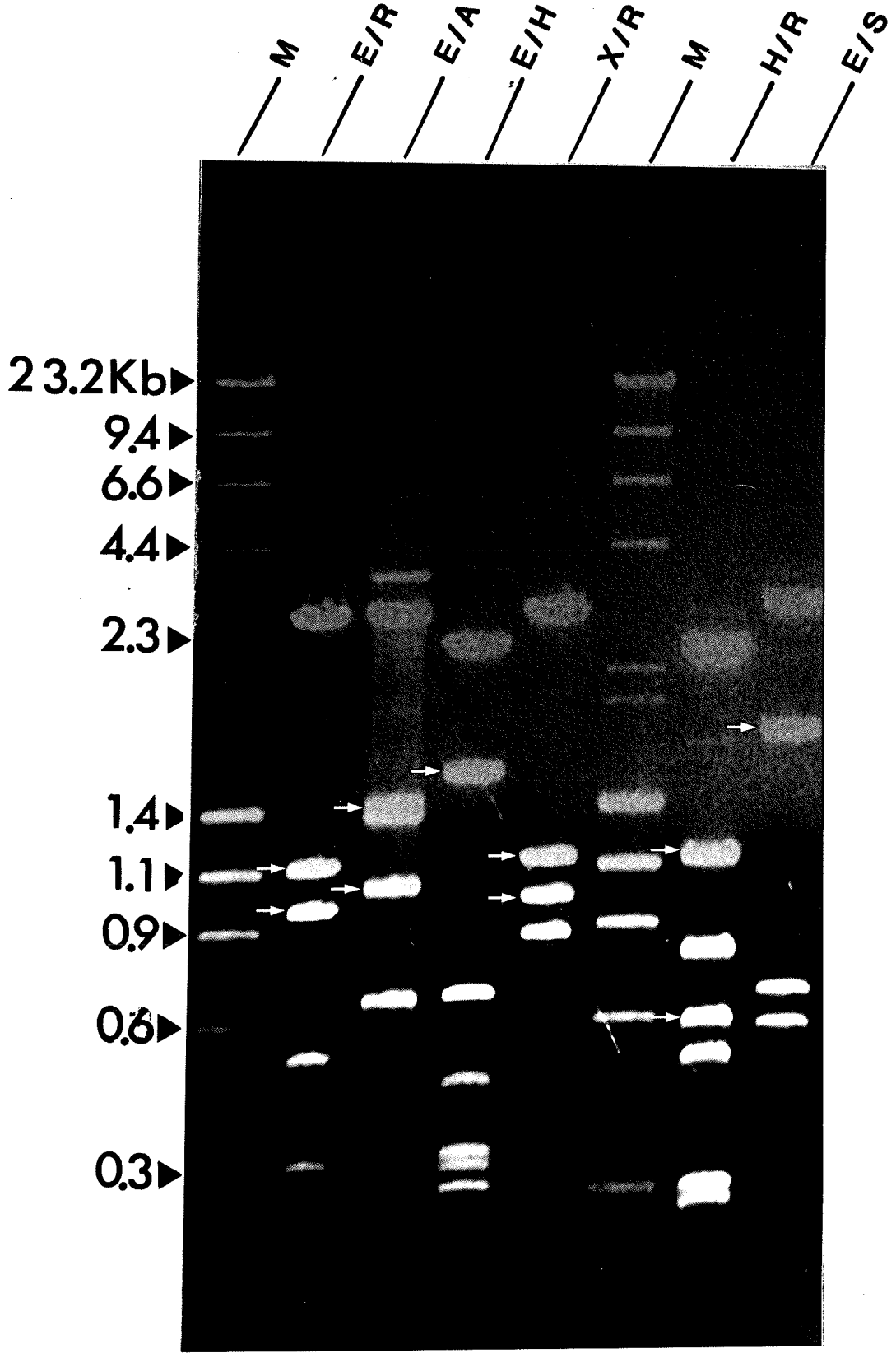
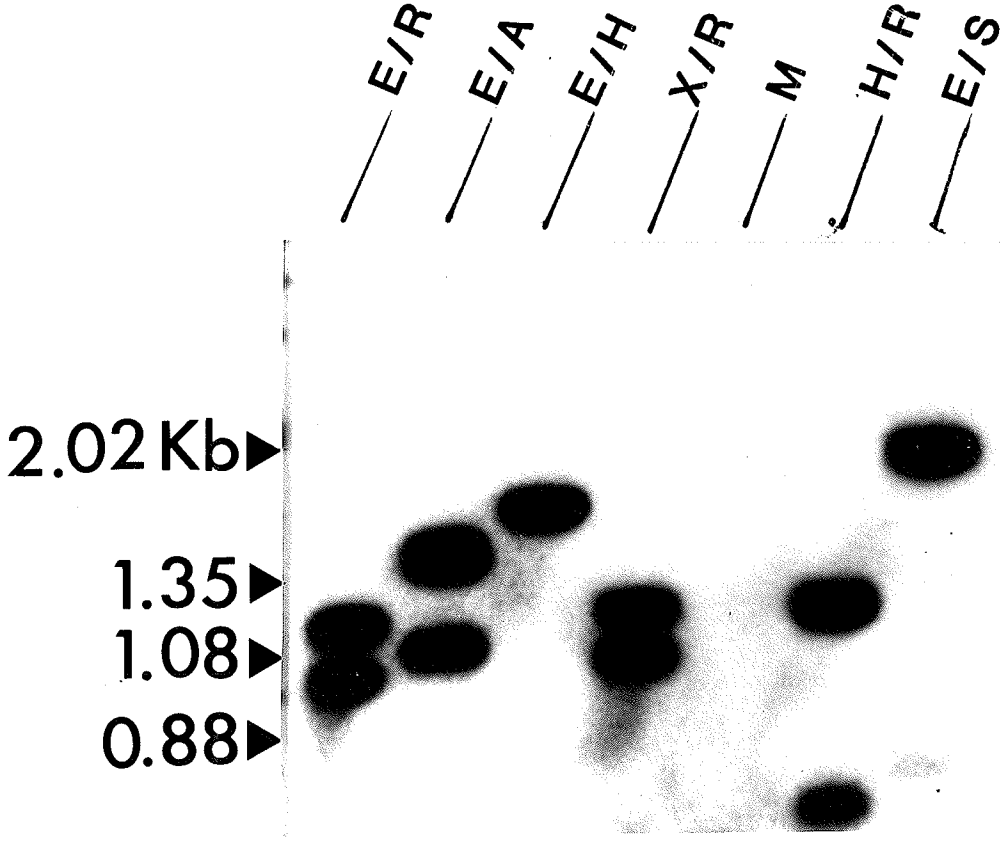


FIGURE XVII. SOUTHERN BLOT OF 2.50 Kb Eco RI FRAGMENT. Autoradiogram of the 2.50 Kb Eco RI genomic subclone restriction digests (see Figure XVI). Hybridization to $dCTP^{32}$ labeled rat liver genomic DNA. The autoradiogram shows localizes the repetitive DNA sequences to specific fragments of the 2.50 Kb Eco RI fragment. M = Marker (Øx174/ Hae III); E = Eco RI, R = Rsa I, A = Acc I, X = Xba I, H = Hind III and S = Sph I.

FIGURE XVII



exon E show the same restriction enzyme map as that seen in c52-A. In addition, clone 2.5 also contains about 300-400 bp of 3' flanking sequence.

A series of single and double digests were carried out on both the 3.55 and 1.10 Kb subclones with Hind III, EcoRI, and Bgl II. This was done in order to obtain better size estimates of the fragments. No sites were found in the 1.10 Kb clone, but there were three sites for both Bgl II and Hind III in clone 3.55. Individual maps are not shown for these clones, but their sites are represented in the map of GC-I (Figure VI).

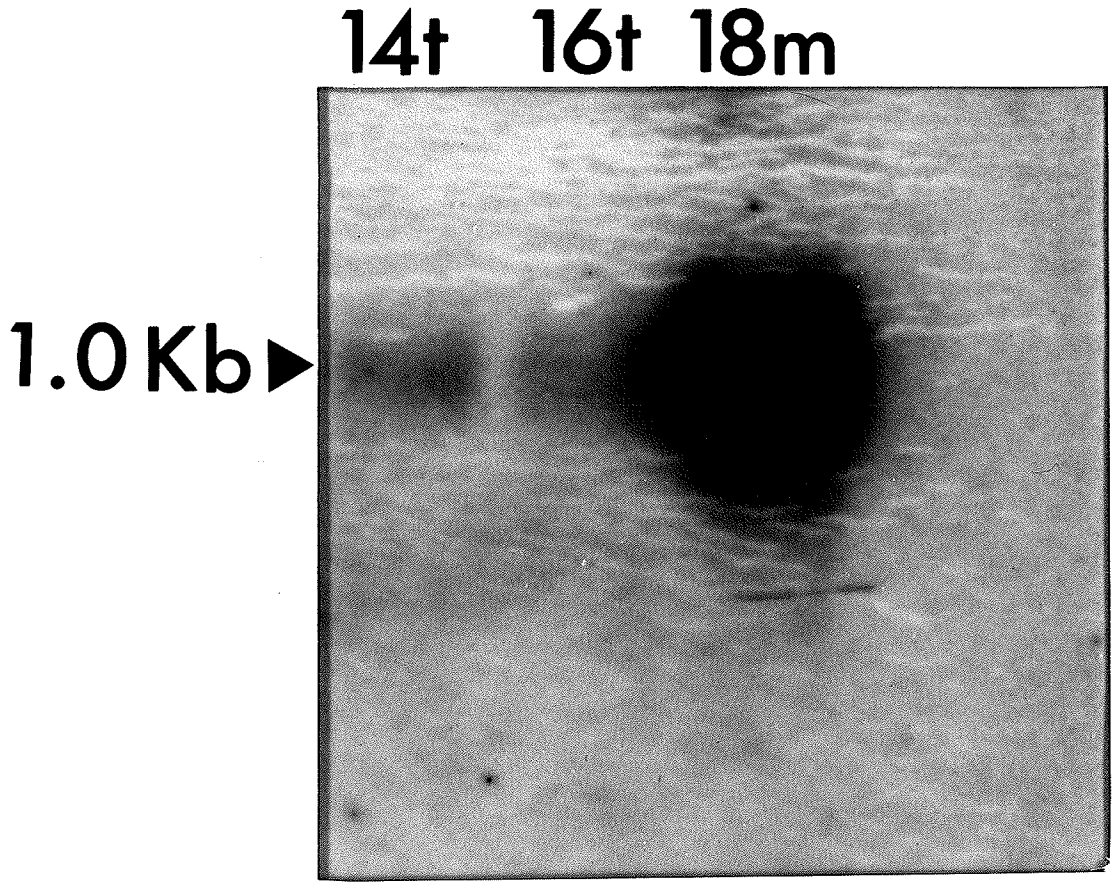
The deduced restriction enzyme map of GC-I is shown in Figure VI. The rPL-II gene is composed of at least five exons interrupted by four introns. If a very small exon of less than 30 bp was present, it could be possible to miss detecting it by hybridization. Therefore, sequence analysis would be necessary to confirm this possibility. The size of the gene is about 5.5 Kb, including untranslated regions. There is a repetitive DNA sequence within the fourth intron of the gene. In addition, 5' and 3' flanking sequences are also represented. It is certain that GC-I does not contain any portion of the rat prolactin gene as this clone does not hybridize to the rPRL cDNA at 65^{o}C.

To confirm that the rPL-II genomic clone actually represents the rPL-II gene, each subclone was nick translated and hybridized to a rat placental RNA Northern blot (Figure XVIII). Each clone recognized the same 1.0 Kb

transcript in total RNA or mRNA preparations.

FIGURE XVIII. NORTHERN BLOT. Autoradiogram of a Northern blot hybridized to a $dCTP^{32}$ labeled 1.50 Kb Eco RI fragment of GC-I. The 1.50 Kb fragment detects a transcript of 1.0 Kb, the ame size of the rPL-II message. 14t = Day 14 total placental RNA, 16t = Day 16 total placental RNA, and 18m = Day 18 messenger RNA.

FIGURE XVIII



DISCUSSION

A rat genomic DNA library was screened for the rPL-II gene by using a 714 bp cDNA clone. Two overlapping genomic clones, GC-I and GC-II, were isolated. Restriction enzyme mapping and hybridization analysis has shown that the entire rPL-II gene exists within GC-I. These results have shown that the rPL-II gene is approximately 5.4 Kb in length. This places it between the rPRL gene which is approximately 10 Kb and the rGH gene which is approximately 2.1 Kb in length. The gene consists of five exons and four introns, the same arrangement as that found within the rPRL and rGH genes. The major reason for the difference in size between these genes is due to the varied lengths of their intervening sequences. Without DNA sequence analysis, the size of the introns and exons are limited to the best estimates available through restriction enzyme mapping. Nevertheless, these estimates are still fairly precise and are presented in Table I for comparison with the lengths of the exons and introns of the rGH and rPRL genes. From these data it is apparent that although the analogous structural gene segments are within 200 bp in size with respect to one another, the sizes among the introns differ by as much as 9.0 Kb.

Three classes of intron splice junctions are possible- class 0, class 1 and class 2. Class 0 introns interrupt the reading frame between codons, class 1 introns interrupt codons between the first and second nucleotide, and class 2

TABLE - I

EXON	INTRON	APPROXIMATE LENGTH IN KILOBASES rPL-II	rPRL	rGH
1		??	.027	.010
	A	1.10	1.53	0.19
2		0.17	0.17	0.16
	B	0.30	1.54	0.72
3		0.10	0.18	0.12
	C	1.70	2.75	0.16
4		0.18	0.18	0.16
	D	1.50	3.15	0.21
5		0.33	0.19	0.37
TOTAL INTRON SIZE		4.60	8.97	1.29
TOTAL EXON SIZE		0.81	0.83	0.82
TOTAL GENE SIZE		5.4 Kb	10.0 Kb	2.1 Kb

Reference for rPRL and rGH figures:

1. Cooke and Baxter, 1982.
2. Gubbins et al., 1980.

introns interrupt codons between the second and third nucleotides (Sharp,1981). When the coding sequences of rGH and rPRL are aligned to maximize homology, the splice junctions of introns A, B, C, and D of both genes are not only of the same class, but occur at analogous positions (Cooke et al.,1982). This is in spite of the fact that rGH and rPRL share only 25% amino acid homology and the rPRL gene is almost five times larger than that for rGH. This feature is seen frequently among individual members of particular gene families (Efstratiatis et al., 1980). It would be interesting to see if this same relationship exists between rPL-II and rPRL and rGH. If the splice junctions were conserved, it might not be surprising since rPL-II shares 34% amino acid homology to rGH and 52% homology to rPRL. In the case of rGH and rPRL the intron sizes do not seem to affect the splice junction locations, and so this could hold true for the splice sites in rPL-II. If these splice junctions were actually conserved in rPL-II, then by aligning the coding sequence of rPRL with rPL-II so as to maximize homology, it is possible to predict where they occur within the cDNA sequence. This type of analogy with rPRL was used to estimate where the introns might occur within rPL-II. By doing this, and also knowing that the restriction sites within the rPL-II exons are the same as in the cDNA sequence, the intron/exon boundaries were estimated in the restriction map of rPL-II. However, DNA sequence analysis would still be required to pinpoint the precise locations and determine the class of splice

junctions that occur within rPL-II.

A repetitive DNA sequence was found within intron D of the rPL-II gene. Since repeat sequences have been found within the upstream and downstream flanking regions of rPRL as well, it would be interesting to see if the same regions in rPL-II also possess similar repetitive elements. The repeat sequence in intron D of rPRL has limited homology to human Alu elements. In addition, the 508 bp repeat sequence in intron B of rGH has homology to some of the repeats in the flanking region of rPRL, although not to the repeat in intron D. This again suggests that the rPL-II gene may be more closely related to rPRL than to rGH since it also has a repeat sequence in intron D. However, it would still be worthwhile investigating the possibility of a similarity between the repeats of rGH and rPL-II as well. The fact that the intron/exon structures of rGH and rPRL have been conserved since the genes diverged and yet a different intron in each gene contains related repeat sequences, suggests that the repeats came into the introns after divergence (Weber et al.,1984). Further characterization of the downstream repeats of both rGH and rPRL may reveal more as to their evolution. Furthermore, similar analysis of rPL-II repeat sequences may help explain the evolution of this gene as well.

Although the functions of repetitive DNA are unknown, it has been proposed that dispersed repetitive sequences may serve as "hot spots" for genetic recombination

(Gilbert, 1978), and that their presence in introns may be reponsible for exon shuffling and gene rearrangement (Darnell, 1978). Another group has suggested that some of the dispersed repetitive elements may have a nuclear role in selecting which mRNA molecules enter the cell cytoplasm (Davidson and Britten, 1979).

Although the general structure of the rPL-II gene has been deduced, this serves only as the foundation on which more information may be compiled. Many more questions need to be addressed pertaining to rPL-II gene structure. Before sequence analysis can be performed, it is important to obtain a proper restriction enzyme map of the gene. This enables one to know which particular fragments of the gene are being sequenced and in what orientation they are with respect to the other portions of the gene. Since a map has now been constructed, sequencing can readily be accomplished. Furthermore, S1-nuclease mapping may be used to localize the definitive site in the DNA where transcription originates (Berk and Sharp, 1977).

Sequence analysis will also be helpful in examining the genes' 5' flanking region for possible promoter elements as well as the location of each of the intron/exon boundaries. The promoter comprises specific nucleotide segments involved in the control of efficient transcription. Both rGH and rPRL possess the probable promoter element TATAAA (Goldberg-Hogness Box), but lack the CAAT sequence which is usually found 40 bp upstream from this sequence. The TATAAA sequence appears to be

important in specifying the transcription initiation site for RNA Polymerase II, while the function of the CAAT sequence is not known, although it is present in most genes. If rPL-II is similar, it too may have the same features and sequence in the 5' flanking region.

As mentioned in the introduction, Cooke et al. (1982) have postulated that the 5' ends of the rPRL and rGH genes may have had separate origins due to their lack of homology and the different length of exon 1 in the two genes. Evidence of a direct repeat flanking exon 1 in both rPRL and rGH suggests that the repeat may have been involved in the insertion of two different first exons into the genes at this location. Therefore, it would be interesting to see if sequence analysis of rPL-II also reveals a different 5' end due to the presence of a direct repeat. The theory that the insertion of homologous first exons and 5' regulatory signals may have been responsible for the divergence of function between rGH and rPRL should be further investigated with rPL-II. Since rPL-II is more closely related by coding sequence to rPRL, this could possibly mean its 5' regulatory signals may be more conserved in relation to rPRL than to rGH. Any striking differences or similarities may shed some light as to the types of sequences that may be involved in the regulation of these genes.

The most important aspect in the study of rPL-II is an attempt to elucidate a specific role for the hormone. One

way to look at this is to examine what regulates rPL-II gene expression. Once the nucleotide sequence of rPL-II and its flanking regions has been determined, it will be possible to locate specific sequences, other than the promoter elements, that may be important in affecting the regulation of gene expression. This can be accomplished by producing deletion mutants of the gene in the 5' flanking region of the gene where certain structures which regulate gene expression may exist. For example, the 5' flanking region of the rPRL gene contains certain elements which allow the transfer of hormonal regulation to heterologous transcriptional units (Supowitz et al., 1984). Furthermore, detailed analysis has shown that a 5' flanking sequence of less than 60 bp is responsible for transferring this type of regulation to fusion genes in a cloned rat pituitary cell line (GH cells). In addition, similar sequences responsive to hormonal regulation have been found in the rGH 5' flanking region.

All of these findings make it even more worthwhile to investigate whether rPL-II possesses similar features. Does rPL-II contain hormone responsive sequences? Are rPL-II transcription rates affected in the same way that rPRL rates respond to thyrotropin releasing hormone (TRH) or epidermal growth factor (EGF)? Is rPL-II affected by any other factor? These questions need to be answered and can be studied by employing the use of cell culture and expression vector systems. An example of this type of study is that which was done to examine a potential

glucocorticoid regulatory element (GRE) in the hGH gene by DNA-binding and gene transfer experiments (Slater et.al, 1985). They first assayed for sequences which bind to glucocorticoid-receptor complexes by nitrocellulose filter binding. Then, fragments of the gene which possessed this binding capacity were assayed for biological activity by fusing them to the human metallothionein-IIA gene promoter depleted of its GRE. The gene was also linked to the thymidine kinase gene. The hybrid was transfected into a cell line and the gene expression in response to glucocorticoids was measured. Similar experiments may be done to localize different regulatory sequences for rPL-II.

It will be important to see if there are any internal sequences within the rPL-II gene that may affect its expression. This may be investigated by examining the production of RNA transcripts in response to hormones or cAMP, after deleting the 5' flanking region of rPL-II the same way that Birnbaum and Baxter (1986) have done with the rGH gene in response to glucocorticoids. They found that almost full length transcripts were still produced when all of the rGH 5' flanking sequences were deleted, suggesting that sequences may exist within the gene that are sensitive to hormonal regulation.

A very important question to be addressed pertains to the type of structure or structures within the rPL-II gene that allow it to be expressed specifically in the placenta, though it is homologous to pituitary rPRL and rGH. The

development of a rat placental giant cell cell line capable of producing only rPL-II may help in resolving this question. Similar 5' sequences to those localized by Nelson et al. (1986) in rPRL, that are reported to confer cell-specific expression to heterologous genes, may be found within rPL-II. If similar sequences are found within the 5' flanking region of rPL-II, the differences between them may help us to understand how particular genes within a gene family are regulated and why they have evolved in a specific manner.

Enhancer elements which influence gene expression in a positive or negative manner would be interesting to look for as well. Enhancer elements are short DNA segments that activate gene transcription, in either orientation and over distances of many kilobases (Banerji et al.,1981). Enhancers may be identified by using an SV40 "Enhancer Trap" which is essentially a vector that possesses linear SV40 DNA lacking the 72 bp enhancer region (Weber et al., 1984). Lack of the enhancer causes low levels of SV40 gene expression. By inserting different fragments of the rPL-II gene into this expression vector system, DNA fragments which possess enhancer activity could be detected by their effect on the expression of the SV40 gene after transfection into a cell line (monkey CV-I cells).

Finally, in humans, GH and PL are so closely related to one another that they consist of a cluster of genes on chromosome 17, while rPRL is found on chromosome 6. Rat PRL, and rGH have recently been localized to chromosomes 17

and 10 respectively (Cooke et al., 1986). It would not be surprising to find rPRL and rPL-II together, in the light of their degree of homology. This is a question that should be looked at to see if any answers can be drawn with respect to their evolution.

Thus, although we now know that the rPL-II gene is similar in its basic structure to rPRL and rGH, much more is still not known about the gene. Once the DNA sequence of the gene has been elucidated and the origin of transcription and intron/exon boundaries have been localized by S1-nuclease mapping and sequencing, further questions may be addressed. These would include questions pertaining to the regulation of gene expression and and function of rPL-II. Thus, the construction of a restriction map for the rPL-II gene, although only a beginning, is an essential first step for further study of structural features within the gene.

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