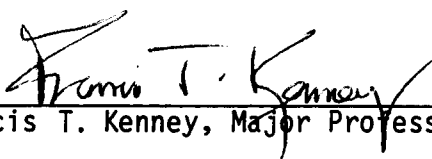
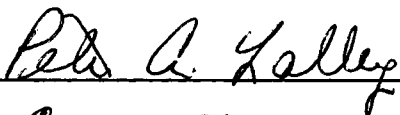


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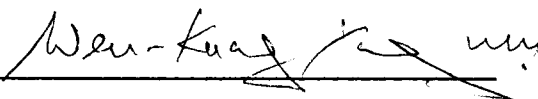

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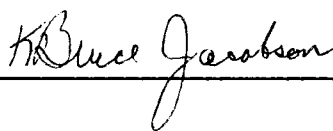
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TISSUE SPECIFIC EXPRESSION OF RAT GENES
SUBJECT TO MULTIHORMONAL REGULATION

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Lisa Ann Francine Balogh
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TO MOM AND DAD, WHO MADE
IT ALL POSSIBLE

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ABSTRACT

Two genes, tyrosine aminotransferase (TAT) and another as of yet unidentified gene referred to as gene 33, expressed in rat liver and subject there to control by several hormones and to regulation of expression during differentiation of the liver have been of particular interest due to the similarities in the control of their expression in rat liver. Extensive research has been focused on the expression of these two genes in rat liver but the tissue specificity of their expression has not been explored. Immunochemical analyses of partially purified protein from nonhepatic tissues utilizing TAT specific antibodies and analyses of RNA in nonhepatic tissues using a cDNA clone for TAT revealed that TAT is expressed only in the liver. In contrast to TAT, hybridization analyses of gene 33 RNA in nonhepatic tissues using a cDNA clone demonstrated that this gene is expressed at varying levels in kidney, brain, heart, lung, and testis. Uniform size mRNA, 3.4kb, was detected in each of these tissues and the mRNA was present in the cytoplasm where it has the potential to be translated into a protein product. Several hormonal agents known to regulate expression of gene 33 in rat liver, cAMP, insulin, and hydrocortisone, were investigated for their effect on expression in these tissues. Both cAMP and insulin increased the levels of gene 33 mRNA in kidney and liver. On the other hand, hydrocortisone effectively elevated the gene 33 mRNA in each of the tissues. In vitro transcription runoff assays in hydrocortisone-induced kidney

nuclei determined that the mechanism by which hydrocortisone exerts its action in kidney is via increased transcription, as was previously shown for its action on liver. In summary, two rat genes subject to similar multihormonal and developmental expression in rat liver were found to be differentially expressed in nonhepatic tissues.

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CHAPTER I

INTRODUCTION

Tissue Specific Expression

The mechanisms regulating the expression of specific genes in mammalian cells are being studied intensively in a number of laboratories, especially since the advent of recombinant DNA techniques and other recently developed techniques that permit identification and analysis of RNA gene products and of the genes themselves. Among the aspects of gene regulation that are of special interest is the phenomenon of tissue specific expression. While it has been recognized for many years that the complement of proteins in various organs or tissues is widely variable, the mechanisms responsible for this differential phenotypic expression of a constant genotype were until recently poorly understood. However, using currently available techniques investigators have made impressive inroads into this problem. Considerable progress has been made towards defining elements which confer tissue specific expression. These factors include specific DNA sequences, differential usage of specific DNA sequences, modification of DNA, and specific proteins which interact with DNA sequences. In addition, alternative RNA splicing events and differential post-translational processing of protein are mechanisms which can also dictate tissue specific expression.

Many investigators have introduced plasmids containing parts of cloned genes, attached to an assayable test gene, into appropriate cell lines to define DNA sequences that are important for gene expression. Utilizing this approach several DNA sequences important for transcription of genes by RNA polymerase II were identified: the TATA box, which helps determine the correct transcriptional start site (Breatnach and Chambon, 1981) and other sequences upstream from the TATA box, including the CAAT box (Benoist and Chambon, 1981; McKnight and Kingsbury, 1982). Finally, another set of sequences, referred to as enhancers, have been identified in variable locations with respect to their associated genes.

Enhancers were first identified in mammalian viruses and were found to increase the transcription of an associated gene from a variable distance away from the gene and regardless of its orientation with respect to the gene (Benoist and Chambon, 1981; Gruss et al., 1981; Everett et al., 1983). Enhancer elements have been found associated with DNA viruses such as SV40 (Benoist and Chambon, 1981; Gruss et al., 1981), polyoma virus (de Villiers and Schaffner, 1981; Tyndall et al., 1981; Herbomel et al., 1985; Veldman et al., 1985), human papovavirus (Rosenthal et al., 1983), bovine papilloma virus (Lusky et al., 1983) as well as RNA viruses (Laimins et al., 1982; Jolly et al., 1983; Kriegler and Botchan, 1983; Luciw et al., 1983; Laimins et al., 1984a; Laimins et al., 1984b). In addition to enhancing transcription these elements also confer cell or tissue specificity

to the virus (de Villiers et al., 1982; Laimins et al., 1982) and also may determine the host range of the virus (Laimins et al., 1982; Byrne et al., 1983; Chatis et al., 1983; Even et al., 1983). Enhancers have been identified in specific regions of several cellular genes: 5' to the insulin, chymotrypsin (Walker et al., 1983) and major histocompatibility genes (Gilles et al., 1984), and within immunoglobulin (Banerji et al., 1983; Gilles et al., 1983; Queen and Baltimore, 1983; Bergman et al., 1984) and β -globin genes (Charnay et al., 1984). Enhancer elements have been proposed to be the tissue specific modulators of transcription. However, in some cases other elements in addition to the enhancer are required for tissue specific expression (Bergman et al., 1984; Gopal et al., 1985; Grosschedl and Baltimore, 1985). These include other DNA sequences, i.e. promoters, or regulatory proteins.

In several cases multiple promoters associated with a gene are used to express mRNA of different sizes in a tissue specific manner. The mouse α -amylase gene synthesizes 2 different size mRNAs with the same coding region but different 5' termini in expressing tissues, parotid gland and liver, by use of 2 different promoters. In nonexpressing tissues, kidney, spleen, and brain, an additional promoter upstream from the parotid gland and liver promoters is used to produce a precursor mRNA that is apparently processed incorrectly since no cytoplasmic α -amylase RNA is detected (Schibler et al., 1983). Therefore differential use of the promoters can lead to tissue specific expression.

Analysis of methylated cytosine content, 5-methylcytosine (m^5C), in DNA showed that tissue specific differences exist (Gama-Sosa et al., 1983). The presence of m^5C , predominantly in the sequence CpG, is also suggested to be a factor determining the transcriptional state of the gene. Extensive research towards analyzing the tissue specific pattern of DNA methylation suggests a causal relationship between undermethylated sites and active transcription from a gene: genes active in transcription contain specific unmethylated sites, whereas those not active in transcription are usually, but not always, methylated (Waalwijk and Flavell, 1978; Mandel and Chambon, 1979; van der Ploeg and Flavell, 1980; Neveh-Many and Cedar, 1981). Tissue specific variations in undermethylation of particular cytosines that correlates with active transcription of a gene has been reported for globin (Christman et al., 1977; Waalwijk and Flavell, 1978; van der Ploeg and Flavell, 1980), phosphoenolpyruvate carboxykinase (Benvenisty et al., 1985), and ovalbumin (Mandel and Chambon, 1979). However, undermethylation may be necessary but not sufficient for allowing transcription of a gene (Ott et al., 1982; Grainger et al., 1983). Huang et al. (1984) have identified a human placental protein which preferentially binds to m^5C -rich DNA and implied that such a protein may be involved in repressing transcription from specific genes after binding to these regions. In a more general context, histone H1 has also been shown to be associated with m^5C -containing DNA (Ball et al., 1983; Weintraub, 1984) whereas nonhistone high mobility group

proteins 14 and 17 are associated with undermethylated DNA (McCarthy et al., 1982). Therefore, interaction of histone and nonhistone with m^5C -containing DNA may determine the transcriptional state of a gene and confer tissue specificity to that gene.

Recently interaction of nuclear proteins with specific DNA sequences have been described in a number of systems (Dylan and Tjian, 1983; Borgmeyer et al., 1984; Sienbenlist et al., 1984; Dylan et al., 1985; Gronostajski et al., 1985). Interaction of proteins with specific DNA sequences may be important in determining if a gene will be transcribed and thus may confer tissue specificity to a gene. Initiation of transcription in soluble extracts has shown that formation of initiation complexes on a cloned β -globin DNA requires factors from an expressing tissue (Triadon et al., 1983). Thus there may be tissue specific transcription factors.

Genes active in transcription have regions within their chromatin which display an unusually high sensitivity to nucleases (Weisbrod, 1982). Emerson and Felsenfeld (1984) have demonstrated that several proteins from partially purified erythrocyte nuclear extracts interact with cloned DNA containing the region of a DNase I hypersensitivity site in β -globin chromatin. Nucleoprotein complexes reconstituted with the cloned DNA, nuclear extracts, and histones exhibit DNase I sensitivity in the same region as in intact chromatin. In these in vitro experiments proteins were found that increase the nuclease sensitivity. In an additional series of experiments Jackson and

Felsenfeld (1985) located protein-DNA interactions in vivo by demonstrating protection of specific DNA sequences from nuclease attack. Two regions were protected in adult erythrocyte nuclei but little protection of these same areas was exhibited in nonexpressing tissues. Recently tissue specific chromatin proteins were also described (Vanderbilt and Anderson, 1983). Therefore, in addition to the tissue specific proteins described above that may be involved in initiation of transcription from a specific gene and there may also be other proteins that work in a more general way to control tissue specific transcription.

Tissue specific expression can be manifested by alternative processing of RNA transcripts or differential posttranslational processing of protein products. Alternative processing of an RNA transcript made from the calcitonin gene produces two distinct mRNAs. One transcript produced in thyroid encodes for calcitonin and a second transcript produced in hypothalamus encodes for a calcitonin gene-related peptide (Amara et al., 1982; Rosenfeld et al., 1983; Jonas et al., 1985). Differential posttranslational processing of a precursor protein occurs in tissues expressing somatostatins. Somatostatins are peptide hormones produced as a non-biologically active pre-prosomatostatin precursor of 92 amino acids. In hypothalamus neurons, rat retina, pancreatic islets and gastric endocrine cells this precursor is processed into an active peptide somatostatin-14, which contains 14 amino acids, whereas in intestinal mucosa and bovine retina the precursor is processed into another active form, somatostatin-28, which

contains 28 amino acids. Several larger peptides are produced from this same precursor in brain. Studies indicate that the proteases which process this precursor are not specific to the tissues normally expressing somatostatin so that other factors are required for differential processing of this protein (for review see Low et al., 1985). Therefore differential processing at the transcriptional or translational levels can result in tissue specific expression of different products produced from the same gene.

In summary, interplay of many factors, including DNA sequences, utilization of DNA sequences, modification of DNA sequences, proteins as well as differential processing of RNA or protein gene products may dictate tissue specific expression.

Two genes expressed in rat liver and subject there to control by several hormones and to regulation of expression during differentiation of the liver have been of particular interest to this laboratory, but as yet the tissue specificity of their expression has not been adequately defined. In this dissertation I have studied this problem, analyzed their gene products in a variety of rat tissues, and determined whether the hormones known to regulate their expression in the liver are also capable of doing so in other tissues expressing the mRNA products of these genes. Our current knowledge of these genes, their mRNA and protein products, and their developmental and hormonal regulation will here be reviewed.

Tyrosine Aminotransferase

Expression of rat liver tyrosine aminotransferase (TAT; L-tyrosine:2-oxoglutarate aminotransferase; E.C.2.6.1.5) is subject to a complex set of hormonal controls. TAT mRNA and protein are induced following treatment with hydrocortisone (Lin and Knox, 1958; Kenney, 1962a; Kenney et al., 1962b; Rowekamp et al., 1976; Nickol et al., 1978) or the pancreatic hormones, insulin and glucagon (Holten and Kenney, 1967; Ernest and Feigelson, 1978; Noguchi et al., 1978; Hill et al., 1981), the latter acting via the intracellular mediator cyclic adenosine 3',5'-monophosphate (cAMP; Wicks et al., 1969). In isolated nuclei each hormonal agent increases the amount of mRNA by increasing the rate of TAT mRNA transcription (Becker et al., 1984; Hashimoto et al., 1984; Lee et al., 1985a). In contrast to the bulk of liver proteins and mRNAs, the rates of TAT protein and mRNA degradation are rapid (Kenney, 1967; Stiles et al., 1976; Nickol et al., 1978). TAT protein and message turnover are blocked by cycloheximide (Rowekamp et al., 1976; Stiles et al., 1976; Ernest, 1982). Induction of TAT by both insulin and cAMP is particularly interesting since these agents generally incite opposing effects on metabolic processes, including the synthesis of liver proteins (Kenney, 1970).

TAT expression is subject not only to multihormonal regulation but also developmental regulation. TAT mRNA and protein are virtually absent in fetal liver. Concomitant with birth both mRNA and protein

attain adult levels (Perry et al., 1983; Rothrock, 1983). In the rat fetus on days 20 and 21 of gestation (birth is on day 22) TAT is refractory to glucocorticoid and insulin induction, but cAMP can elicit a response similar to that observed in the adult (Perry et al., 1983; Johnson, 1985). In utero treatment of 20 day fetuses with 5-azacytidine can cause precocious expression of TAT (Rothrock et al., 1983). Thus, it seems that certain requirements necessary for glucocorticoid- and insulin-mediated induction of TAT are not yet fulfilled during fetal life, although those for cAMP-mediated induction of this gene have been. Treatment with 5-azacytidine appears to accelerate a normal process that occurs during development, perhaps demethylation of particular DNA sequences, that is required for expression of the TAT gene. Whether any of the inducing hormones are also required for developmental activation of TAT expression has not been determined unequivocally, but some data suggest that they are not (Perry et al., 1983).

Biochemical and genetic experiments suggest that regions controlling TAT expression are located on mouse chromosomes 7 and 11 (Gleucksohn-Waelsch, 1979; Killary and Fournier, 1984). X-ray induced deletions of a region containing the albino (c) locus on mouse chromosome 7 cause a lethal mutation which results in early postnatal death associated with impaired liver function, including reduced levels of proteins such as glucose-6-phosphatase, serine dehydratase, and TAT. Cells derived from these mutant mice do not express TAT. However

after fusion of these cells with rat hepatoma cells, mouse-specific TAT is re-expressed (Cori et al., 1981). These data suggest that the TAT structural gene is intact in these mouse cells since it can be expressed again after fusion with rat cells, and a product of the deleted region containing the c locus may be required for expression of TAT and other liver specific genes. Another series of experiments suggest that there is a regulatory element on mouse chromosome 11 (Killary and Fournier, 1984). In rat hepatoma-mouse fibroblast microcell hybrids retaining the entire complement of rat chromosomes but only mouse chromosome 11 TAT expression normally occurring in the hepatoma cell is "extinguished." However, if the fused cells are allowed to divide and lose chromosome 11 rat TAT is re-expressed. Thus these experiments suggest that a product of a gene on mouse chromosome 11 represses expression of TAT. A product of the region containing the c locus on mouse chromosome 7 may be the factor that allows expression of TAT in liver, while the product of a specific gene on chromosome 11 can act to repress TAT expression elsewhere.

Recently cDNA and genomic clones for rat TAT have been isolated (Scherer et al., 1982; Shinomiya et al., 1984). A 2.7 kb mRNA is produced by this gene. The structural gene extends over 11 kbp in the rat genome, is single copy, and is interrupted by 11 exons. Features common to other eukaryotic structural genes have been found at the 5'-end of the gene: a TATA-box located between -31 and -25 of the transcription start site, a CAAT-box sequence further upstream,

and 6 areas of sequence homologous to putative glucocorticoid receptor binding sites. No DNA sequence has been identified that renders the TAT gene responsive to insulin or cAMP.

TAT is a soluble protein consisting of 2 identical subunits with molecular mass of 53 kDa on denaturing gels (Lee et al., 1979). Multiple forms of the dimeric enzyme are formed by proteolysis of the subunit (Iwasaki et al., 1973; Johnson et al., 1973; Hargrove et al., 1980; Hargrove et al., 1982). Gohda and Pitot (1980, 1981) have identified and characterized a protease, cathepsin T, that cleaves a peptide from the carboxy terminus converting the 53 kDa subunit to 50 kDa, which results in the generation of multiple forms of the enzyme.

TAT has been regarded as a liver specific protein, although transaminase activity utilizing tyrosine as an amino donor is ubiquitous in mammalian tissues (Canellakis and Cohen, 1956; Lin and Knox, 1958; Wurtman and Larin, 1968; Fellman et al., 1969; Fuller, 1970). Whether the activity in nonhepatic tissues is TAT or another transaminase has been the subject of discussion for many years. Investigators have approached this question by utilizing enzymatic assays and attempting to purify the activity from nonhepatic tissues. Interpretation of enzymatic assay data is difficult since transaminases other than TAT, such as aspartate aminotransferase, can utilize tyrosine as an amino donor (Ohisalo and Pispa, 1976; Hargrove and Mackin, 1984). This further complicated interpretation of purification data which relied on use of enzymatic assays to monitor the purification.

Becker et al (1984) have shown that DNase I hypersensitivity sites occur in chromatin at the 5' end of the TAT gene in liver nuclei but not in kidney nuclei. In many systems DNase I hypersensitivity sites within chromatin have been associated with active transcription of a gene or those that have the potential to be transcribed (Weintraub and Groudine, 1976; Elgin, 1981; Weisbrod, 1982). Although not directly shown it was suggested that the TAT gene is not transcribed in kidney since the DNase I hypersensitivity sites were not present.

Thus, due to difficulties in interpretation of studies involving enzymatic assay and in the purification of TAT from nonhepatic tissues, and lack of alternative methods by which to approach the question, the true tissue specificity of TAT expression has not been fully defined.

Gene 33

Gene 33 is an unidentified gene with properties much like TAT. A cDNA clone for this gene was isolated by differential screening of a library prepared from hydrocortisone-induced rat liver poly(A) mRNA (Lee et al., 1985b). Gene 33 mRNA, a 3.4 kb message, is induced by hydrocortisone, as well as by insulin and cAMP. The kinetics of these inductions parallel those of TAT. Insulin and cAMP cause a rapid 5- to 6-fold increase in gene 33 mRNA which peaks in 1 hr; the induction of gene 33 mRNA by glucocorticoids is much slower, mRNA levels increasing 6 fold with peak induction occurring at 3 hr. Gene 33 mRNA undergoes turnover at a rate similar to TAT mRNA

($t_{1/2} = 1$ hr). As may be common for mRNAs with rapid turnover rates (Rowekamp et al., 1976; Stiles et al., 1976; Ernest, 1982), gene 33 mRNA is stabilized by cycloheximide.

In addition to multihormonal regulation, gene 33 is also developmentally regulated. Gene 33 mRNA is low, but detectable, in fetal rat liver throughout gestation with levels reaching that of adults just prior to birth (Rothrock, 1983; Johnson, 1985). Gene 33 mRNA can be increased to adult levels by treatment of 20- or 21-day fetuses in utero with hydrocortisone or cAMP (Johnson, 1985). Thus, elements conferring glucocorticoid as well as cAMP-mediated responses to this gene are mature before birth. Gene 33 mRNA, like TAT, can be prematurely elevated to adult levels by in utero treatment of 20-day rat fetuses with 5-azacytidine (Rothrock, 1983). This suggests that demethylation of a particular DNA sequence is important in the activation of gene 33 at parturition.

Recently, a genomic clone of gene 33 was isolated and characterized (Tindal, 1985). This single copy gene covers 13.1 kbp in the haploid rat genome. Regulatory elements common to other eukaryotic structural genes have also been identified: a TATA box flanked by GC-rich regions occurs at -31, an inverted CAAT box at -81, and 4 areas homologous to putative glucocorticoid receptor binding sites occur 5' to the cap site at +1.

Little is known about the protein product of gene 33, except that in vitro translation of gene 33 hybrid-selected mRNA yields

a 53 kDa species on a denaturing protein gel and a protein with a pI of 6.65, as determined by isoelectric focusing. This protein is probably intracellular since the mRNA is associated with nonmembrane-bound polysomes (Lee et al., 1985b). The function of the protein product of gene 33 is not known.

Extensive research has focused on expression of gene 33 in rat liver but the tissue specificity of its expression has not been explored.

CHAPTER II

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (300-500 g, from the Charles River Breeding Laboratory) were used in all experiments. When involved in hormone induction studies rats were bilaterally adrenalectomized, maintained on 1% NaCl and food ad libitum for 3 to 5 days, and fasted over night prior to the experiment. All hormonal agents were dissolved or suspended in 0.15 M NaCl.

Tyrosine Aminotransferase Purification and

Protein Assays

For the analyses of expression of the TAT protein it was necessary to partially purify the enzyme. Purification of TAT from kidney, liver, and testis was done using modifications of established methods (Lee et al., 1979; Hargrove et al., 1982). Rats were intraperitoneally injected with 5 mg of hydrocortisone (Sigma) per 100 g body weight, and killed by decapitation 5 hr later.

Tissues were excised, rinsed in cold (4°C) extraction buffer, and held on ice in cold extraction buffer until all tissues had been removed. All subsequent steps were carried out at 4°C unless otherwise noted. Tissues were homogenized with 8 strokes of a glass homogenizer fitted with a tight teflon pestle using 4 volumes of cold extraction buffer per g tissue. Extraction buffer contains 50 mM Hepes

(N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid), pH 7.6, 0.1 M KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 2.5 mM 2-ketoglutarate, 0.1 mM pyridoxal-5'-phosphate, and 0.1 mM phenylmethylsulfonyl fluoride. Postmitochondrial supernatants were prepared by centrifugation in a Beckman JA-17 rotor using 270 x g for 10 min, 12,100 x g for 10 min, and 27,000 x g for 20 min. After lipid was removed, the supernatant was decanted and recentrifuged in a Beckman 30 Ti rotor at 100,000 x g for 2 hr. The high speed supernatant was decanted into an Erlenmeyer flask which was then placed in an 85°C H₂O bath, and stirred constantly until the temperature reached 62°C. After heating at 62°C for 5 min the flask was cooled in an ethanol-ice bath and denatured protein was removed by centrifugation (JA-17 rotor 27,000 x g for 10 min).

Supernatants from the heat step were applied to a DEAE-Sephadex A-50 column, previously equilibrated with 50 mM K₂PO₄, pH 7.6, 5 mM 2-ketoglutarate, 1 mM EDTA, and 1 mM 2-mercaptoethanol. After washing the column with 6 to 8 column volumes of equilibration buffer containing 30 µg/ml pyridoxal-5'-phosphate and 100 mM KCl, protein was eluted with equilibration buffer supplemented with 30 µg/ml pyridoxal-5'-phosphate and 400 mM KCl. A trial purification of TAT from liver allowed monitoring of elution of TAT activity from the column. The fractions which normally contain TAT activity were precipitated by adding solid ammonium sulfate to 70% and stirring overnight. Precipitated protein was recovered by centrifugation as described above,

and salt was removed by dialyzing against 3 changes of 50 mM NaH_2PO_4 , pH 6.8, 2.5 mM 2-ketoglutarate, 3 $\mu\text{g/ml}$ pyridoxal phosphate, 1 mM EDTA, and 1 mM DTT.

TAT activity was determined by the method of Kenney (1959) or Diamondstone (1966); a unit of activity is defined as the amount of enzyme catalyzing the formation of 1 nmole of p-hydroxyphenylpyruvate per min at 37°C. The assay time was extended from 10 min to 1 hr when activities were less than 1 U/mg protein. Protein concentration was determined by the method of Bradford (1976) utilizing bovine serum albumin, fraction V, (Sigma) as a standard.

Western Immunoblotting

Protein was subjected to SDS-polyacrylamide electrophoresis according to the method of Laemmli (1970). Briefly, proteins were denatured in similar volumes by adding 0.5 volume of sample buffer (30% glycerol, 15% 2-mercaptoethanol, 9% SDS, 0.2 M Tris-HCl, pH 6.8, 0.15% bromophenol blue) and heating in a boiling H_2O bath for 2 min. Protein was electrophoresed through 1.5 mm thick gels consisting of a 1 to 2 cm stacking gel (3% acrylamide, 0.13 M Tris-HCl, pH 6.8, 0.1% SDS) and 9 to 10 cm resolving gels (10% acrylamide, 0.38 M Tris-HCl, pH 8.8, 0.1% SDS). Electrophoresis was carried out using 30 mA (constant current) for 1 hr followed by 100 volts (constant voltage) for 5 to 6 hr.

Protein immunoblots, which will hence be referred to as western blots or western immunoblots, was carried out according to Burnette

(1981). Proteins were electroblotted from acrylamide gels onto nitrocellulose (Schleicher and Schuell BA(85), pore size 0.45 μ m) using a Hoefer Scientific transfer apparatus, Model TE series, equipped with a TE 50 power supply (0-100 volts, 0-2 amperes). Transfer was achieved using 250 mA/60 volts (constant current) for 24 hr at 4°C in 6 l transfer buffer (150 mM glycine, 20 mM tris, 0.1% SDS, 20% methanol).

After the transfer nitrocellulose filters were placed in a glass tray containing stop buffer (3% bovine serum albumin, fraction V; Sigma) in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4) for 1 hr at room temperature to occupy all the free protein binding sites on the nitrocellulose.

Steps to follow involve using several buffers made by adding components to one basic buffer, 50 mM Tris, pH 7.4, 5 mM EDTA, pH 7.4, and 0.25% gelatin (bacterial gelatin from BBL Becton-Dickinson and Co.). Basic buffer No. 1 is 150 mM NaCl, 0.05% NP-40, basic buffer No. 2 is 1 M NaCl, 0.4% sodium N-lauroyl sarcosine, and basic buffer No. 3 is 150 mM NaCl, 0.5% triton X-100, and 0.1% SDS.

After transfer blots were rinsed briefly in H_2O , placed in a Seal-N-Save bag, and incubated in 5 ml of antibody solution for 2 hr at room temperature. Antibodies prepared against purified TAT subunit or holoenzyme (provided by Dr. K.-L. Lee) were purified from serum with two-33% ammonium sulfate precipitations and batch-wise elution from DEAE-cellulose. Antibodies were diluted in basic buffer

No. 1 (TAT subunit antibody used 1:51 dilution of stock; TAT native enzyme antibody used 102,000 units/5.0 ml). Blots were washed 2 times for 15 min with basic buffer No. 1. Blots were incubated with 5 ml ^{125}I -Protein A solution (10^6 cpm) for 0.5-1 hr at room temperature to detect antibody bound to antigen. Protein A was iodinated using the Bolten-Hunter reagent (Bolten and Hunter, 1973; Feitelson et al., 1981). Blots were washed 3 times (minimum of 30 min each time) with basic buffer No. 2. The final wash was carried out in a glass tray for 30 min with basic buffer No. 3. Blots were rinsed in H_2O , allowed to air dry, and then wrapped in Saran Wrap. Nitrocellulose filters were then exposed to XAR-5 Kodak X-Ray film using 2 Cronex Lightning Plus intensifying screens at -70°C for 2 to 3 days.

Total Cellular RNA Isolation

Total cellular RNA was isolated from various rat tissues according to the methods of Chirgwin et al. (1979) and Glisin et al. (1974). Tissues were removed from decapitated rats using sterile instruments, rinsed in cold PBS, and immediately homogenized using 1 g tissue per 15 ml G buffer (5 M guanidine thiocyanate, 0.5% sodium-N-lauroyl sarcosine, 25 mM sodium citrate, pH 7.0, 0.1 M 2-mercaptoethanol, 0.1% antifoam A). Routinely, 8 strokes of a 40 ml glass homogenizer fitted with a tight teflon pestle (rheostat on 25 of a Lourdes motor driven homogenizer) were required to disrupt tissue. Homogenates were centrifuged at $12,100 \times g$ for 10 min at 10°C to remove any cell debris. Seven ml of homogenate were layered over a 4 ml cushion

of 5.8 M CsCl, 0.1 M EDTA, pH 7.4, in an SW 41 polyallomer tube, and centrifuged at 35,000 rpm for 24 hr at 21°C. Under these conditions DNA will band at the interface between the CsCl and G buffer, and the RNA will pellet. RNA pellets were then resuspended in 1 mM EDTA, pH 7.4, heated briefly at 65°C, and precipitated by addition of 3 M sodium acetate, pH 5.2, to 0.2 M and ethanol to 70%. RNA was precipitated overnight at -20°C, recovered by centrifugation, and washed twice with 3 M sodium acetate, pH 5.2, to remove any contaminating DNA. RNA was ethanol precipitated at least 2 to 3 times before use in any analyses. RNA concentration was determined by reading $A_{260\text{ nm}}$ of a diluted sample utilizing 1 $A_{260\text{ nm}}$ per ml of RNA as 40 ug RNA per ml (Maniatis et al., 1982). All solutions were prepared in autoclaved H_2O and reautoclaved when possible.

Cytoplasmic and Nuclear RNA Isolation

Several protocols were combined to isolate cytoplasmic and nuclear RNA (Cox, 1968; Nickol et al., 1978; Derman et al., 1981; Lamers et al., 1981; Schibler et al., 1983). Buffers used in the following section include buffer H (0.3 M sucrose in buffer A (60 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM 2-mercaptoethanol, 15 mM HEPES, pH 7.5, 0.5 mM EGTA, and 2 mM EDTA; pH 7.5 at room temperature)), guanidine-HCl buffer (7.5 M guanidine-HCl, 25 mM sodium citrate, 5 mM DTT, and 3 mM EDTA), buffer B (buffer B is same as buffer A except that EGTA and EDTA are at a concentration of 0.1 mM and 0.1 mM,

respectively), and nuclei storage buffer (40% glycerol, 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.5 mM DTT).

Tissues were homogenized in ice cold buffer H and homogenates were filtered through 4 layers of sterile cheesecloth. A crude nuclear pellet was obtained by centrifugation at 1000 x g for 10 min at 0°C in a Beckman JS-13 rotor. The supernatant was decanted and used for isolation of cytoplasmic RNA. Ten ml of the cytoplasmic supernatant was added to 12 g guanidine-HCl crystals (Schwartz-Mann) which had previously been preweighed out in a 40 ml Oak Ridge tube. The crystals were dissolved by gentle inversion, then 2-mercaptoethanol was added to 1%. RNA was precipitated by addition of 0.5 vol of absolute ethanol and incubation at -20°C for 1 hr. RNA was recovered by centrifugation and twice precipitated from a guanidine-HCl buffer followed by several washes with 3 M sodium acetate, pH 5.2.

Nuclear RNA was isolated from the crude pellet obtained from the low speed centrifugation step. Nuclei were resuspended in 2 ml buffer H by gentle swirling. To the nuclear suspension 4.5 ml of guanidine thiocyanate buffer was added (same buffer as used above for isolation of total cellular RNA) and the solution was made homogeneous by homogenizing with a Dounce homogenizer (30 strokes in a 40 ml Dounce with a tight pestle). CsCl was added at 0.6 g per ml of sample. This was layered over a 4 ml cushion of 5.8 M CsCl, 0.1 M EDTA, pH 7.4, in an SW41 polyallomer tube. Centrifugation and subsequent steps are as described for the total RNA isolation above.

Nuclei were isolated for transcription runoff experiments starting from the crude nuclear pellet obtained above. The crude nuclear pellet was resuspended in buffer H and recentrifuged. The nuclear pellet was recovered and resuspended in H buffer containing 0.15% Triton X-100 and was layered over a cushion of 30% sucrose in buffer A. After centrifugation at 4000 x g for 10 min in a JS-13 rotor nuclei were recovered and purified by high speed centrifugation. Nuclei from the last step were resuspended in 4 ml 30% sucrose in buffer B and 4 ml of 2.0 M sucrose in buffer B was added after nuclei were resuspended. This was then layered over a 2.0 M sucrose cushion in an SW41 tube and centrifuged at 36,000 rpm at 2°C for 80 min. The nuclear pellet was resuspended in 100 μ l of nuclei storage buffer per g starting weight and stored at -70°C. Transcription runoff experiments were as described by Lee et al. (1985b).

Dot Hybridization Analysis

Dot hybridization analysis is as described by White and Bancroft (1982) and Thomas (1980, 1983). SSC is 0.15 M NaCl, 0.015 M tetrasodium citrate. Master mix is 3 parts 20XSSC and 2 parts 37% w/w formaldehyde (Fisher). To one volume of RNA (starting vol was never larger than 100 μ l) one volume of a master mix was added and RNA was denatured by heating at 50°C for 15 min and quick cooling in an ethanol-ice bath. Denatured RNA was serially diluted (1:2 dilutions) into 20XSSC, and applied, by use of a BRL hybridot manifold, onto nitrocellulose filters, which had previously been equilibrated in 20XSSC.

Hybridization of nitrocellulose filters is described below. After autoradiography x-ray film was scanned with an LKB Bromma ultro-scan interfaced with an Apple II computer. The linear range of the intensity of hybridization versus ug RNA was determined and the best fit line was determined by linear regression. When possible intensities at a fixed amount of RNA within the linear range of the response was used for comparison of samples.

Northern Blot Analysis

Routinely, 70 ug total cellular RNA was aliquotted into 1.5 ml sterile Eppendorf tubes and ethanol precipitated. For RNA gel analysis, commonly referred to as northern blotting, the RNA was recovered by centrifugation of samples in a Beckman Eppendorf Micro-fuge, 5413, for 10 min (6500 x g). Ethanol was poured off and the RNA pellet was washed with 80% ethanol (-20°C). Samples were lyophilized briefly and resolubilized in sterile H₂O. RNA was denatured in 2.4 M formaldehyde, 54% deionized formamide, 10.9 mM NaH₂PO₄, pH 6.5, by heating at 50°C for 15 min. Gel loading buffer (10X stock containing 25% ficoll, 0.25% each bromophenol blue and xylene cyanol) was added and A_{260nm} readings were retaken. The appropriate amount of RNA (50 ug for analysis of total cellular RNA) was then loaded onto a 300 ml gel containing 1.2% agarose, 2.2 M formaldehyde, and 10 mM NaH₂PO₄, pH 6.5 (Lehrach et al., 1977). Electrophoresis was carried out at 100 volts (constant voltage) for 6 to 9 hr using 10 mM NaH₂PO₄, pH 6.5, running buffer. The integrity of RNA was verified

by observing rRNA bands in total cellular RNA on a portion of the gel which had been stained with acridine orange (Carmichael and MacMaster, 1980). The distance that the rRNA migrated on the stained portions of the gel were noted and were used as molecular weight markers for the corresponding gel that was transferred. After electrophoresis the portion of the gel to be transferred was soaked in 15XSSC for 15 minutes and transferred onto nitrocellulose filters (Schleicher and Schuell, BA85) in 15XSSC. Transfer was allowed to proceed over night.

Preparation of Hybridization Probes

Plasmid DNA was isolated by a modification of the method of Clewell (1972) as described by Tindal (1985). Plasmid DNA was cut with an appropriate restriction enzyme to release the entire insert DNA in one fragment using conditions suggested by Bethesda Research Laboratories. Restriction digests were phenol- CHCl_3 extracted and ethanol precipitated. Verticle 1.5 mm thick 4.8% acrylamide, 0.2% bis-acrylamide gels were prepared in 89 mM Tris, 89 mM boric acid, 2 mM EDTA buffer and were preelectrophoresed at 200 volts (constant voltage) for 30 min. Restriction digests were electrophoresed through the gel at 200 volts (constant voltage) for 1 hr. The gel was stained in ethidium bromide, the insert was identified and a gel slice containing the insert was excised from the gel with a razor blade. DNA was extracted from the gel slice by incubating the slice in 0.5 M $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$, 10 mM $\text{MgC}_2\text{H}_3\text{O}_2$, 1 mM EDTA, and 0.1% SDS at 37°C several times (Maxam and Gilbert, 1980).

DNA was radiolabeled with [α - ^{32}P]-dCTP (specific activity of $> 3200 \text{ Ci/mmol}$ from ICN). Nick translation was carried out with a nick translation kit from either Amersham or New England Nuclear. Following nick translation the labeled probe was separated from unincorporated nucleotide by gel filtration using a Biogel A 0.5 m column (Biorad; mesh 200-400) built in a 10 ml disposable pipet and previously equilibrated with 80 mM Na Cl, 10 mM Tris-HCl, pH 8.0, and 0.2 mM EDTA. Peak fractions were pooled and ethanol precipitated. Carrier tRNA was added to aid in recovery of probe. Specific activities ranging from $7 \times 10^7 \text{ cpm}$ to $1 \times 10^8 \text{ cpm}$ per μg DNA were used in hybridizations.

DNA inserts were cloned into an SP6 system from Promega Biotec and RNA probes were made by in vitro transcription utilizing [^{32}P]-UTP as described by the manufacturer. Unincorporated label was removed by repeated precipitation of the RNA (addition of $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$ to 2.5 M and 2.0 volume absolute ethanol).

Hybridization of Dot and Northern Blots

Following transfer or dotting of RNAs, the nitrocellulose filters were baked in vacuo at 80°C for 3 hr. If blots were not to be hybridized immediately then they were stored at room temperature under vacuum (Maniatis et al., 1982).

Before prehybridization blots were floated on 6XSSC, then submerged to wet the nitrocellulose thoroughly. Blots were prehybridized at 42°C for at least 4 hr in 10 ml of 1X prehybridization

solution (50% formamide, 5XSSC, 100 mM NaH_2PO_4 , pH 6.5, 250 ug/ml sheared, denatured, calf thymus DNA, 0.02% each of ficoll 400, polyvinylpyrrolidone, bovine serum albumin, fraction V, and 10 ug/ml poly(A) (Thomas, 1980; Thomas, 1983). Radiolabeled probe, [^{32}P]-labeled as described above, was denatured in a solution containing 80% deionized formamide by heating in a boiling H_2O bath for 5 min. The denatured probe was quick cooled on ice and an equal volume of 2X hybridization stock (same as 2X prehybridization stock except it contains 20% dextran sulfate) was added. A 1X hybridization stock was made fresh and an additional amount was added along with the denatured probe. Hybridization was carried out at 42°C for 18 to 24 hr using 3 to 5 x 10⁵ cpm labeled probe per ml of hybridization solution. Following hybridization blots were then washed with 2XSSC, 0.1% SDS at 65°C until the radioactivity in the wash reached background. This wash was followed by another wash using 0.2XSSC, 0.1% SDS at 65°C. Nitrocellulose filters were then blotted dry, wrapped in Saran Wrap, and exposed to Kodak XAR-5 X-ray film using 2 Cronex Lightning Plus intensifying screens at -70°C.

Hybridizations were essentially the same when cRNA probes were used, except that it was not necessary to denature the probe. Hybridizations were carried out at 65°C, and washed at 70°C. The last wash of the blots was in 0.1XSSC, and 0.1% SDS at 70°C.

Rehybridization of Nitrocellulose Filters

Hybridized probe was removed from blots by incubating filters in 0.1XSSPE (0.18 M NaCl, 10 mM NaH_2PO_4 , pH 7.4, and 1 mM EDTA)

containing 0.1% SDS which had been heated to 95°C. After the solution had cooled to room temperature the process was repeated. Nitrocellulose filters were exposed to Kodak XAR-5 X-ray film using 2 Cronex Lightning Plus intensifying screens at -70°C for at least 24 hr to determine if hybridized probe had been completely removed (Gatti et al., 1984).

CHAPTER III

RESULTS

Tyrosine Aminotransferase in Kidney, Liver, and Testis

A transaminase activity using tyrosine as an amino donor is present in many nonhepatic tissues, including brain, kidney, heart, spleen, thyroid, and skeletal muscle (Canellakis and Cohen, 1956; Lin and Knox, 1958; Wurtman and Larin, 1968; Fellman et al., 1969; Fuller, 1970). Furthermore, it is reported that the transaminase activity, presumably TAT, in brain and kidney is increased by glucocorticoids, although not to the extent observed in liver (Elam and Barnett, 1981; Snape and Badaway, 1981). For many years investigators have sought to determine if the transaminase activity in nonhepatic tissues was TAT or some other transaminase. However several problems were encountered 1) transaminases other than TAT, i.e. aspartate aminotransferase (Ohisalo and Pispä, 1976), can transaminate tyrosine and 2) levels of activity in these tissues are much lower than the basal level observed in liver and difficult to assay. Since the activity is low the background caused by other transaminases complicates interpretation of enzymatic assay data. Initial analyses involved use of a functional assay of tyrosine aminotransferase. This does not determine if TAT protein, possibly altered so that it is no longer catalytically active, is present in these tissues.

To determine if TAT protein was present in nonhepatic tissues western immunoblotting techniques were employed. This method involves electrophoresis of proteins on SDS-polyacrylamide gels, electroblotting of protein to nitrocellulose filters, and detection of the protein utilizing specific antibodies to the protein. In the following analyses two polyclonal antibodies were used. The use of polyclonal antibodies increases the chance of detecting TAT protein, even if it is altered, due to recognition of multiple antigenic determinants by these antibodies. Furthermore, the use of two different polyclonal antibodies, one prepared against TAT dimer and the other prepared against TAT subunit (Donohue et al., 1982), increases the chance of detecting TAT protein even more since each antibody preparation likely recognizes different sets of determinants. Preliminary experiments involving analysis of liver TAT by these methods showed that it would be necessary to partially purify and concentrate this protein to allow detection. This meant that transaminase assays would still be requisite to this analysis. It must also be assumed that if altered TAT were present in nonhepatic tissues it would still retain similar purification characteristics as liver TAT.

Two tissues, in addition to liver, were chosen for partial protein purification and western immunoblot analyses: kidney, since previous studies had reported the presence of transaminase activity that was also inducible by glucocorticoids in this tissue (Snape and Badaway, 1981), and testis since it was a likely nonexpressing tissue.

Before purification of transaminase from these tissues assays were performed on post mitochondrial supernatants to establish the tyrosine transaminase levels on which to follow further purification steps (see Table 1). Our results for kidney and liver were in accord with published results (Lin and Knox, 1958). Activity, although less than 1.0% of that observed for liver, was also detected in testis samples.

Several steps in a typical TAT purification scheme were performed. High speed supernatants ($100,000 \times g$) from rats given hydrocortisone to elevate the TAT levels were heated to 62°C and denatured protein was removed by centrifugation (heat step). Supernatants were then applied to DEAE-Sephadex columns, washed with buffer containing 0.1 M KCl, and eluted with buffer containing 0.4 M KCl (see Table 2). After the heat step no activity remains in the testis sample, and less than 1% of the activity remained in the kidney sample after elution from the DEAE-Sephadex column. Neither testis or kidney activities purified in a manner similar to the liver TAT, as had been suggested previously by other investigators (Hargrove and Mackin, 1984).

Protein from the heat step and DEAE-Sephadex columns was concentrated by ammonium sulfate precipitation, dialyzed, subjected to SDS-polyacrylamide electrophoresis, and electroblotted to nitrocellulose filters. Blots were subsequently probed with TAT-specific polyclonal antibodies. Autoradiograms of blots probed with TAT subunit

TABLE 1
Transaminase Activity in Kidney, Liver, and Testis^a

Tissue	Specific Activity ^b	% Liver Activity
	U/mg Protein $\pm$ Standard Error	
Liver	15.0 $\pm$ 6.0 (5)	100.0
Kidney	0.5 $\pm$ 0.1 (5)	3.3
Testis	< 0.2 (5)	< 1.0

^a27,000 x g supernatants were assayed for transaminase activity as described in Materials and Methods.

^bNumbers in parentheses indicate the number of determinations.

TABLE 2

Transaminase Purification from Kidney, Liver, and Testis[†]

Tissue	Step ^a	Total Units	Units per mg Protein	Recovery ^b (%)
Liver	A	1.48x10 ⁵	560	100
	B	1.33x10 ⁵	1100	90
	C ^c	2.17x10 ⁴	1.17x10 ⁴	31
	D	0	0	0
Kidney	A	760	0.9	100
	B	639.0	1.7	85
	C	6.2	1.0	0.8
	D	670	2.4	90
Testis ^d	A	0	0	0

[†]Hydrocortisone was intraperitoneally administered to fasted rats (5 mg per 100 g body weight). After 5 hours animals were killed by decapitation. Purification of transaminase and assays were as described in Materials and Methods.

^aSteps refer to A, high speed supernatant; B, heat step; C, DEAE-Sephadex column eluate; D, DEAE-Sephadex column run through.

^bRecovery is based on the number of units recovered from the step divided by the number of units in step A.

^cOnly 48% of the heat step supernatant was applied to the DEAE-Sephadex column.

^dNo activity was detectable in this large volume of supernatant. After ammonium sulfate precipitation low amounts of activity were detected.

and dimer antibodies are shown in Figures 1 and 2. Two major species, 53 and 50 kDa as determined from a standard curve (Figure 3) were detected in liver samples. These correspond to the intact subunit and the degraded form of the subunit which is generated by proteolysis during isolation (Godha and Pitot, 1980). Several other immunoreactive bands were also detected in liver samples. These may be due to antibodies present as minor components of the antibody preparation reaction with their corresponding antigens. To test this hypothesis blots could be probed with antibody prepared from preimmune serum. Several of the immunoreactive bands may also represent turnover products of TAT (Fan et al., 1977). A series of serially diluted liver protein samples were run on gels to determine the limits of detection with this assay (Figure 1, lanes 5-10; Figure 2, lanes 4-10) and to use as a relative measure of TAT protein on which to compare levels of TAT protein if it was detected in nonhepatic tissues. However, no immunoreactive bands of the correct molecular mass were observed for protein fractions purified from testis or kidney.

Through partial purification of transaminase from kidney and testis it was demonstrated that the activity did not purify as authentic TAT. Analysis of the purified protein by western immunoblotting did not reveal any immunoreactive species in either testis or kidney. By these means it has been shown that the protein product of the TAT gene is not present in kidney or testis at detectable levels.

Figure 1. Western blots of kidney, liver, and testis heat step protein fractions.

Heat step fractions from hydrocortisone induced rats were subjected to SDS-polyacrylamide electrophoresis, electroblotted to nitrocellulose filters, and probed with TAT antibodies.

Lanes: 1, ^{125}I -protein standards; 2, 69 ug testis protein; 3, 69 ug kidney protein; 4-10, liver protein serially diluted 1:2, beginning with 12.5 units of TAT activity in lane 4. For kidney and testis 69 ug represents 0.10 and 0.004 units of TAT activity, respectively. A, blot probed with TAT subunit antibody; B, blot probed with TAT dimer antibody.

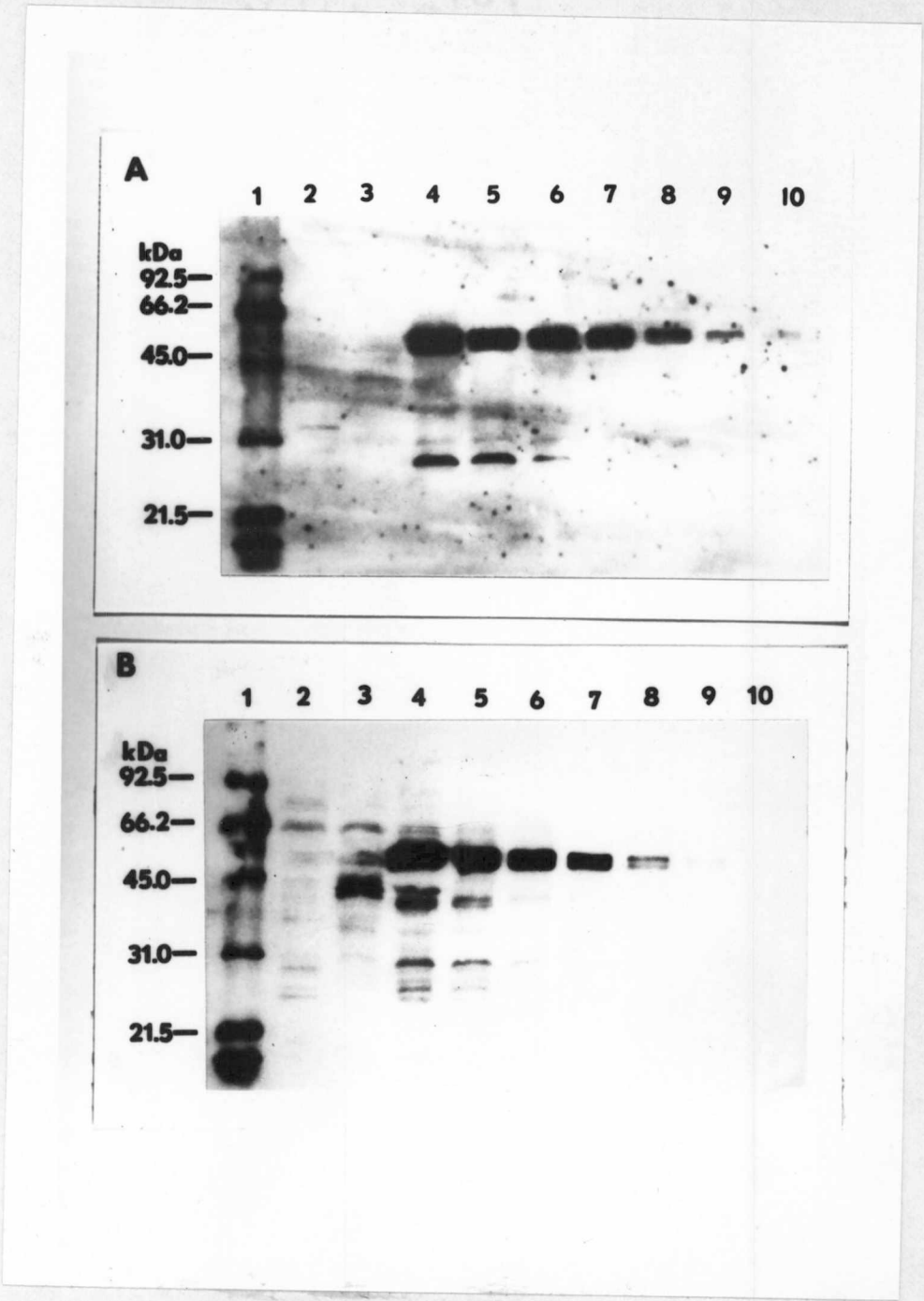


Figure 1

Figure 2. Western blots of kidney, liver, and testis DEAE-Sephadex purified protein fractions.

Lanes: 1, [^{125} I]-standard proteins; 2, testis; 3, kidney; 4, liver; 5-10, 1:2 dilutions of liver TAT beginning with 12.5 units in lane 5. In lanes 2-4 100 ug of protein were loaded which represent 0.15, 0.10, and 1170 units of testis, kidney, and liver activity, respectively. A, blot probed with TAT subunit antibody; B, blot probed with TAT dimer antibody.

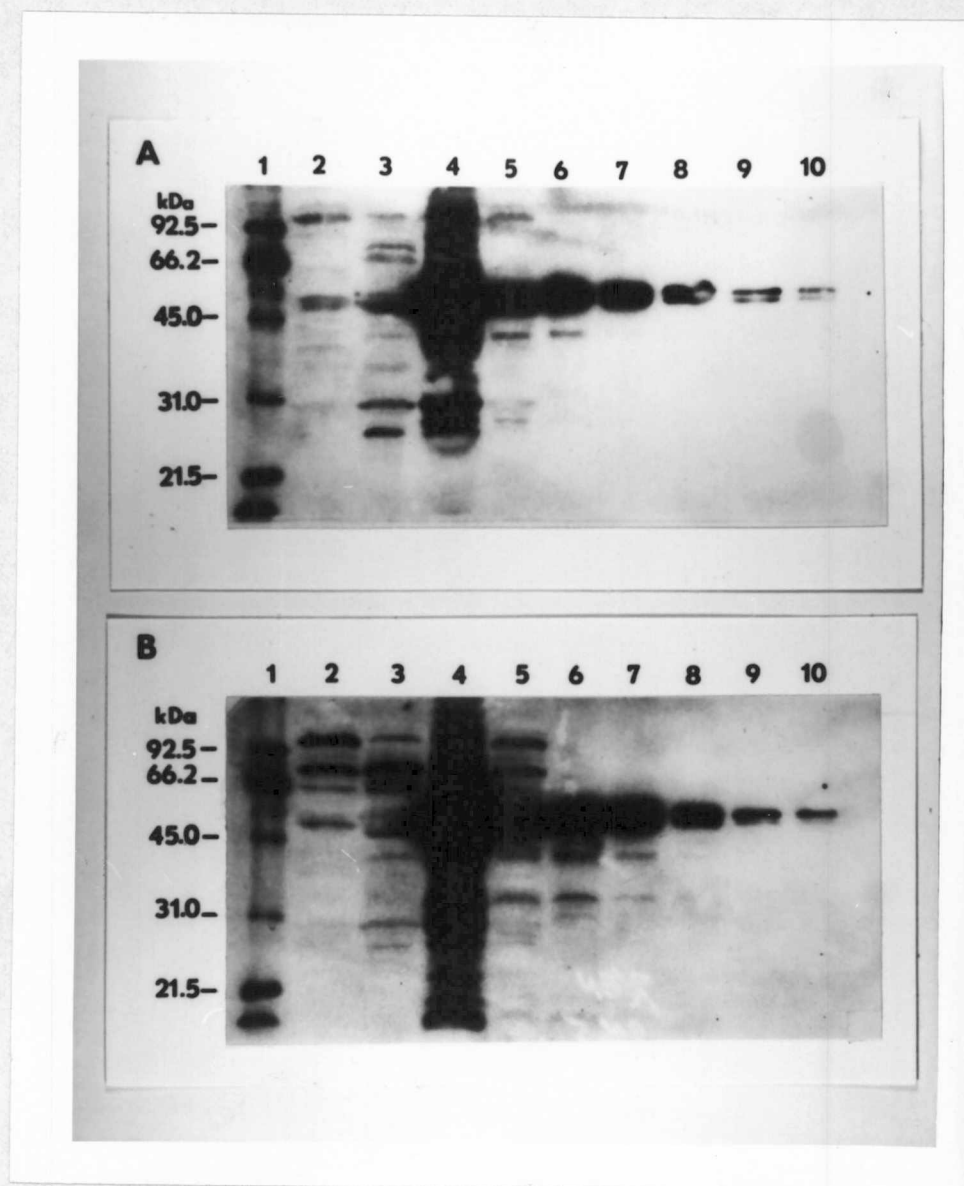


Figure 2

Figure 3. Standard curve for Western blot analysis.

^{125}I -labeled standard proteins were electrophoresed along with other samples on gels and blotted as described in materials and methods. The relative mobility is the distance the protein traveled relative to the dye front. (-●-●-) standard proteins; (◆) bands detected on western blots of liver protein.

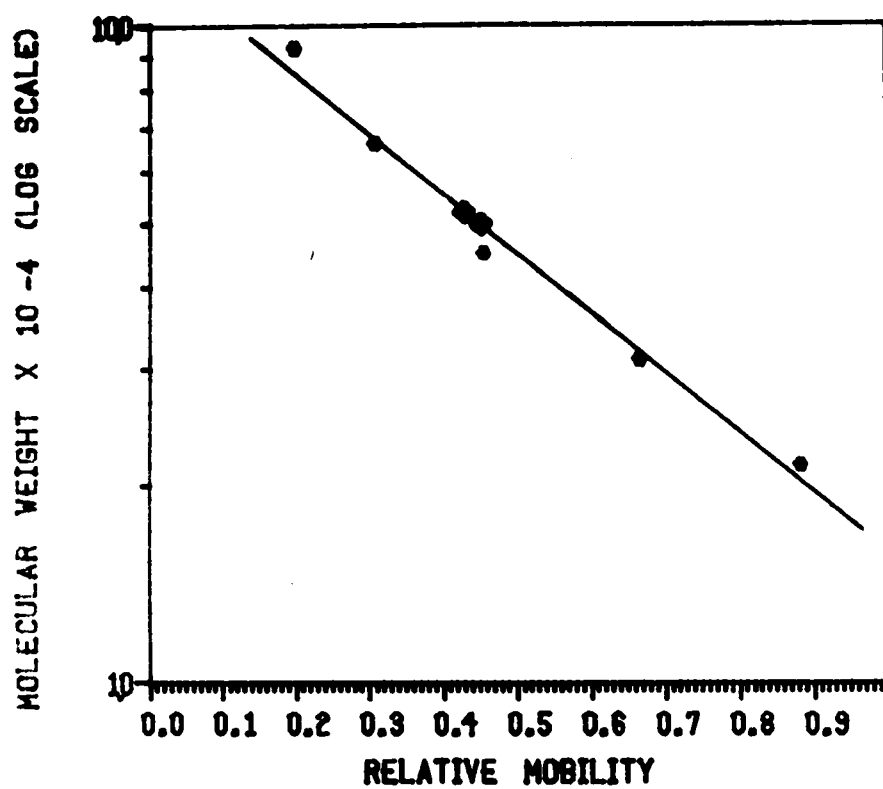


Figure 3

Expression of TAT and Gene 33 mRNAs

Assays of tyrosine aminotransferase activity from nonhepatic tissues suggested that TAT may not be liver specific. As was shown in the previous section this activity did not purify as authentic TAT and was not detected on western blots by polyclonal antibodies to TAT. These data suggest that TAT protein is not present in kidney or testis and probably not in other tissues in which similar transaminase activities have been noted. Alternative methods for analysis are necessary before a final conclusion about the expression of TAT in nonhepatic tissues can be drawn.

The cloning of the rat TAT cDNA (Scherer et al., 1982) provides more direct and highly sensitive means for determination of TAT tissue specificity. This cloned DNA can be used to determine whether TAT mRNA is synthesized in tissues other than liver. Towards this end, total cellular RNA was isolated from a variety of rat tissues by a combination of the methods of Chirgwin (1979) and Glisin (1974). This protocol proved to be highly suitable for isolation of intact RNA from a variety of tissues due to direct homogenization of samples in guanidinium thiocyanate, a potent protein denaturant. RNA was purified from several tissues suggested to exhibit transaminase activity, notably kidney, brain, and heart, and others that were not expected to express TAT, testis, and lung. Purified RNA was then denatured in formaldehyde and either subjected to electrophoresis on denaturing gels and transferred to nitrocellulose filters to determine the size

of TAT mRNA in expressing tissues or applied directly to nitrocellulose using the dot blotting technique to determine the level of TAT mRNA in expressing tissues relative to the level of expression in liver (Thomas, 1980; Thomas, 1983). Blots were subsequently probed with the [^{32}P]-labeled cDNA clone, pcTAT-3 (Scherer et al., 1982). Figures 4 and 5 contain the results of such as experiment. By both dot hybridization and northern analyses this cDNA probe detects TAT mRNA only in liver. The size of the mRNA on the northern blot is as expected, 2.7 kb. These data confirm that TAT expression is liver specific.

The similarities in regulation of gene 33 and TAT in rat liver are numerous (see Introduction). To determine if gene 33 is also similar to TAT in the tissue specificity of its expression, dot hybridization and northern blot analyses were completed using a [^{32}P]-labeled cDNA cloned probe to gene 33. As shown in Figure 6 by dot hybridization analysis, gene 33 mRNA is present in all tissues examined. Levels of gene 33 mRNA relative to liver were determined by laser scanning of dot hybridization autoradiograms and comparison of spot intensity within the linear range of the scan (Table 3). Gene 33 mRNA is most abundant in liver, with moderate levels in lung and kidney, and much lower levels in brain, testis, and heart. Determination of relative differences by this analysis are difficult when the difference is 10 fold or greater. Thus, this analysis probably underestimates the difference.

In the preceding section northern blot analysis of total cellular RNAs reveals that the major species hybridizing to a gene 33 cDNA probe

Figure 4. Dot hybridization analysis of TAT mRNA in total cellular RNA from a variety of rat tissues.

RNA was formaldehyde treated, serially diluted, and blotted onto nitrocellulose filters in high salt. The blot was probed with a [^{32}P]-labeled TAT cDNA cloned probe, washed, and exposed to X-ray film for 5 days.

Row: a, kidney; b, liver; c, testis; d, brain; e, heart; f, lung.

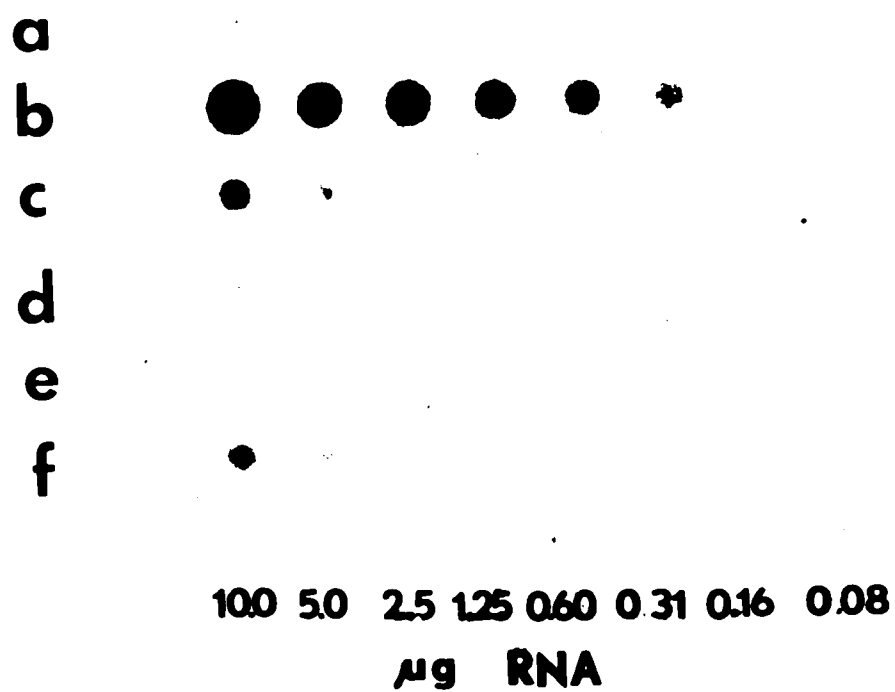


Figure 4

Figure 5. Northern blot analysis of TAT mRNA in total cellular RNA from a variety of rat tissues.

Total cellular RNA (50 ug) was subjected to electrophoresis on a denaturing gel, transferred to nitrocellulose, and probed with a [³²P]-labeled TAT cDNA cloned probe. Blots were washed and exposed to X-ray film for 5 days. E. coli rRNA was run in an extra lane and stained with acridine orange to provide a standard.

Lanes: 1, brain; 2, heart; 3, lung; 4, kidney; 5, liver; 6, testis.

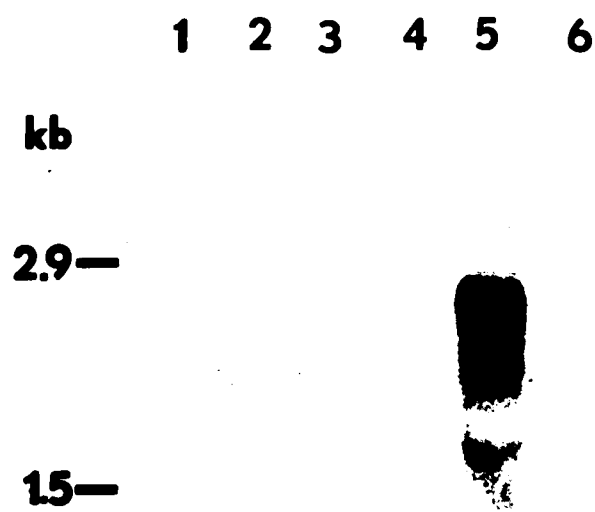


Figure 5

Figure 6. Dot hybridization analysis of gene 33 mRNA in total cellular RNA from a variety of rat tissues.

RNA was formaldehyde treated, serially diluted, and blotted onto a nitrocellulose filter in high salt. The blot was probed with a [^{32}P]-labeled gene 33 cDNA, washed, and exposed to X-ray film for 5 days.

Row: a, kidney; b, liver; c, testis; d, brain; e, heart; f, lung.

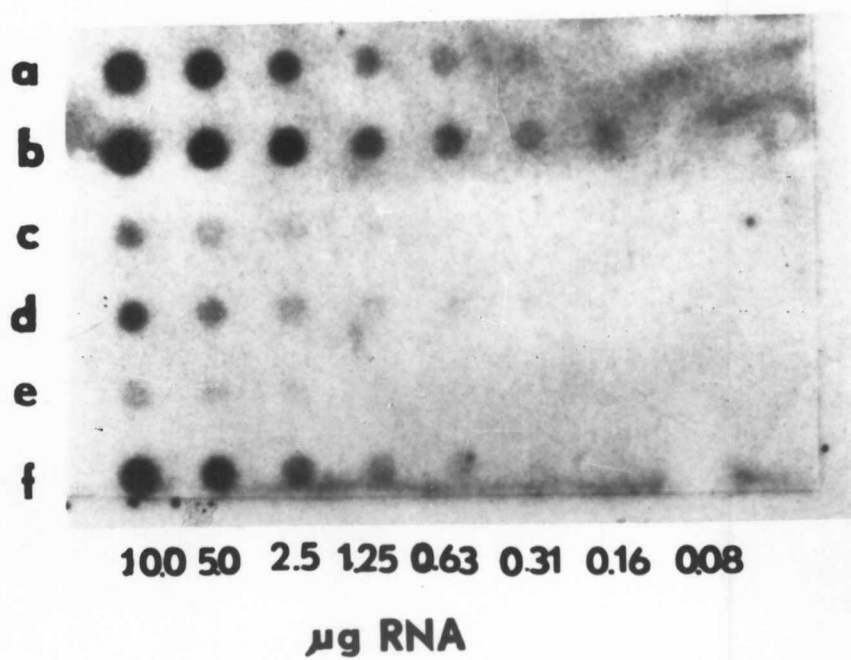


Figure 6

TABLE 3

Relative Levels of Gene 33 mRNA in Total Cellular
RNA from Rat Tissues^a

Tissue	Gene 33 mRNA Level % of Liver Value ^b			Avg. $\pm$ Std. Dev. ^c
	I	II	III	
Liver	100.0	100.0	100.0	100.0
Kidney	56.3	53.9	74.2	55.1 $\pm$ 1.7
Testis	29.6	5.2	4.4	17.4 $\pm$ 17.2
Brain	10.7	15.6	15.6	13.2 $\pm$ 3.5
Heart	< 2	6.4	6.7	< 4.2
Lung	25.4	26.7	20.7	26.1 $\pm$ 0.9

^aAn autoradiogram of a dot hybridization was scanned with a laser densitometer and relative intensities of dots were compared to liver within the linear portion of the response.

^bRelative intensity at a fixed amount of RNA for a given sample is divided by the intensity of the liver sample at that same amount of RNA, and then multiplied by 100.

^cThe average and standard deviation were determined for set I and II. Set III RNA was isolated from adrenalectomized, fasted rats, and therefore was not included in the calculation.

is of a uniform size in each of the tissues (Figure 7). The differences in level of gene 33 mRNA as demonstrated by dot hybridization analysis are also reflected by this method. Routinely 50 ug of RNA were run per well for these analyses to illustrate the difference in level of expression between the tissues. Electrophoresis of this large amount of RNA causes broad bands to be detected on the blots and also affects the migration of the RNA through the gel, perhaps masking subtle differences in the size of gene 33 mRNA in tissues other than liver.

These data show that although TAT and gene 33 are subject to similar multihormonal and developmental control in rat liver, these two genes are differentially expressed in rat tissues; TAT is liver specific and gene 33 is not.

Analysis of Cytoplasmic Gene 33 mRNA

Lee et al. (1985b) have demonstrated that gene 33 mRNA in liver is associated with polysomes, and therefore is probably translated into a protein product. In the preceding section northern analysis of total cellular RNA demonstrated that gene 33 mRNA of a uniform size occurs in all tissues examined. This is an important finding but does not unequivocally prove that a gene 33 protein product could be produced in these tissues. Therefore, it was of interest to determine if gene 33 transcripts in tissues other than liver are present in the cytoplasm, where they have the potential to be translated.

Figure 7. Northern blot analysis of gene 33 mRNA in total cellular RNA from a variety of rat tissues.

The blot shown in Figure 5, page 44, was deprobed as described in Materials and Methods and rehybridized with a [^{32}P]-labeled cDNA cloned probe to gene 33. The blot was washed and exposed to X-ray film for 7 days. *E. coli* rRNA was run in an extra lane and stained with acridine orange to provide a standard. The distance that the rRNA migrated on the gel is indicated on the blot.

Lanes: 1, brain; 2, heart; 3, lung; 4, kidney; 5, liver; 6, testis.

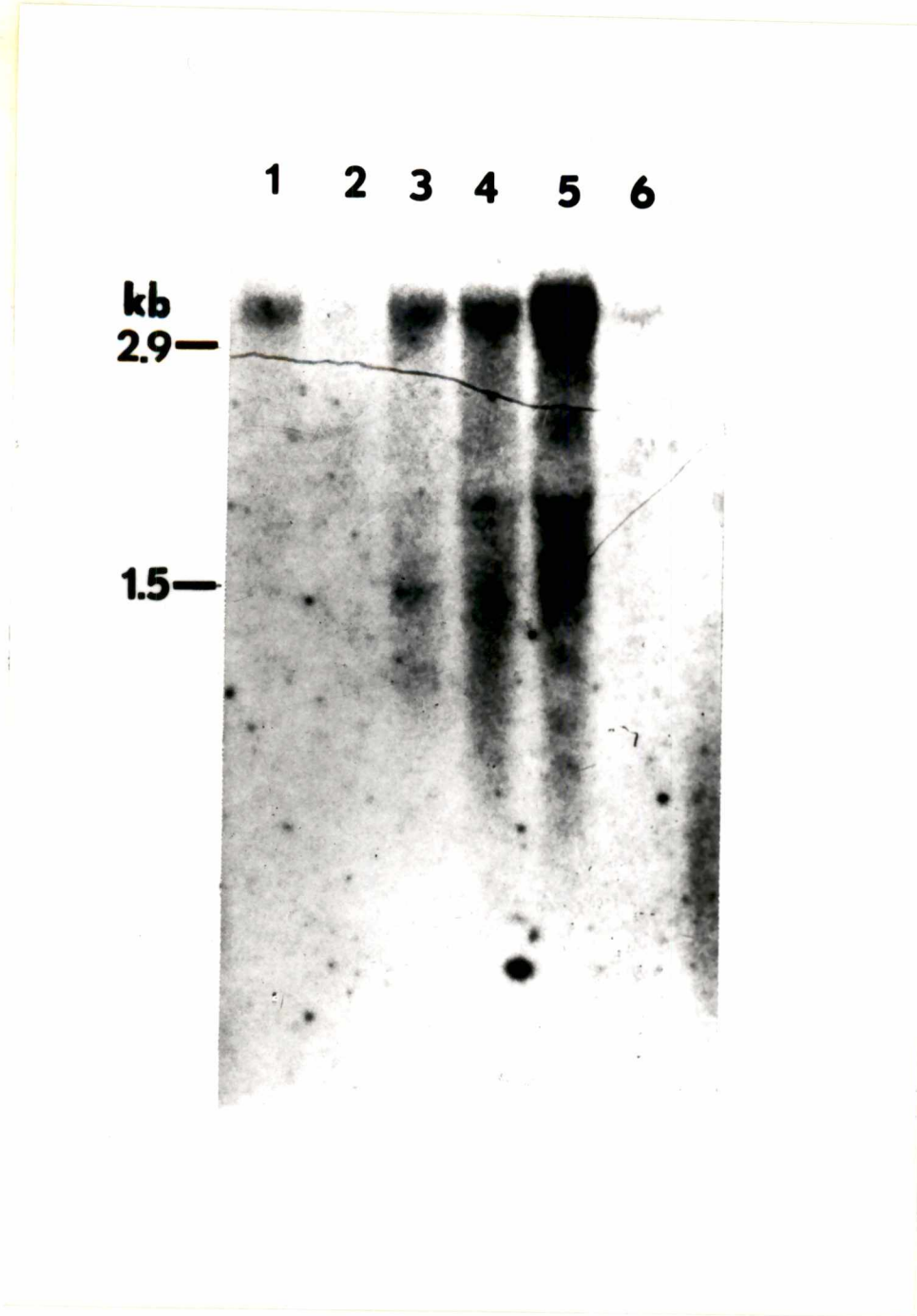


Figure 7

Cytoplasmic RNA was consequently isolated and analyzed by dot hybridization analysis (Figure 8 and Table 4). Cytoplasmic gene 33 mRNA was detected in each tissue. However, greater relative differences in the levels of expression with respect to liver were found for each tissue than were found in analysis of total cellular RNA, although the basic pattern reflected in analysis of total cellular RNA was retained, i.e. mRNA is most abundant in liver followed by kidney, lung, brain, testis, and heart. Variability in levels of mRNA between individual determinations were also noted for kidney and brain samples. These differences, in the relative levels with respect to liver and the variability between replicate samples, probably have to do with the method of isolation. In the isolation of total cellular RNA tissues were homogenized directly in guanidinium thiocyanate buffer, thus denaturing any RNases immediately. When cytoplasmic RNAs were isolated, tissues were homogenized in a sucrose-spermidine buffer as suggested by Schibler et al. (1983), followed by a low speed centrifugation, then guanidine-HCl was added. Thus in this isolation RNases are not denatured until several steps after the homogenization of tissues so that there is time for them to begin to degrade the RNA. This analysis shows that gene 33 mRNA is present in the cytoplasm of kidney, liver, testis, brain, heart, and lung, and thus, could potentially be translated.

Hormonal Regulation of Gene 33 mRNA Expression

In rat liver gene 33 mRNA expression is subject to regulation by glucocorticoids, insulin, and cAMP. In the preceding section it

Figure 8. Dot hybridization analysis of gene 33 sequences in cytoplasmic RNA from a variety of rat tissues.

RNA was formaldehyde treated, serially diluted, and blotted onto a nitrocellulose filter in high salt. The blot was probed with [³²P]-labeled cDNA cloned probe to gene 33, washed, and exposed to X-ray film for 8 days.

Row: a, kidney; b, liver; c, testis; d, brain; e, heart; f, lung.

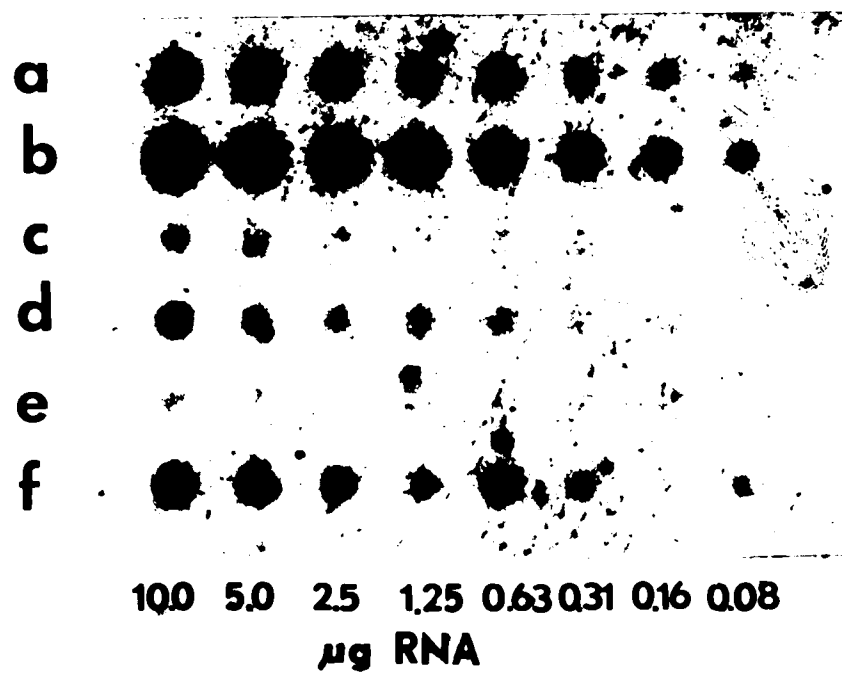


Figure 8

TABLE 4
Relative Levels of Gene 33 Cytoplasmic
mRNA in Rat Tissues^a

Tissue	Gene 33 mRNA Level % of Liver Value			Avg. $\pm$ Std. Dev.
	I	II	III	
Liver	100.0	100.0	100.0	100.0
Kidney	3.7	29.1	19.2	17.3 $\pm$ 12.8
Testis	4.7	4.7	6.3	5.2 $\pm$ 0.9
Brain	9.5	< 2 ^b	16.1	9.2 $\pm$ 7.0
Heart	< 2	< 2	< 2	< 2
Lung	7.5	6.2	8.3	7.3 $\pm$ 1.1

^aAn autoradiogram of a dot hybridization was scanned with a laser densitometer. Relative intensities of dots were compared to liver within the linear portion of the scan.

^bUsed 2% in calculation of the average and standard deviation.

was shown that gene 33 mRNA is detected in a variety of rat tissues. Therefore, it was of interest to test whether gene 33 mRNA expression is modulated by these hormonal agents in these tissues.

It was realized that the route of injection would be an important consideration when many different tissues were challenged with these agents. If a tissue has the potential to respond to these agents, to do so it must also receive a dose sufficient to elicit a response. Previous studies have demonstrated that each of the tissues in question has glucocorticoid receptors (Munick and Leung, 1977). Also, specific physiological responses occur in many of these tissues when insulin or cAMP levels in the body change (Krahl, 1961; Fritz, 1972; Butcher et al., 1972). There is evidence, however, that insulin cannot pass through the blood-brain barrier (Haugaard et al., 1954). Assuming that most of the tissues have the potential to respond to these hormonal agents this effort was begun by intra-peritoneally administering hydrocortisone, insulin, or cAMP to adrenalectomized and fasted rats utilizing doses and induction times, that were known to increase gene 33 mRNA in liver (all expressed as amount per 100 g body weight; 2.5 mg hydrocortisone for 3 hr; 1 U insulin for 1 hr; 5 mg 8-bromo cAMP for 1 hr). Saline injected animals were used as the controls. Total cellular RNA was isolated as before since this method proved to give intact RNA from each tissue and analyzed by dot hybridization and northern blots. Figure 9 is an insulin induction experiment that illustrates the gradient in

Figure 9. Dot hybridization analysis of gene 33 mRNA in total cellular RNA from various rat tissues following insulin treatment.

Insulin (1 U per 100 g body weight) was administered to rats by intraperitoneal injection. Rats were killed after 1 hr and total cellular RNA was extracted from each tissue as described in Materials and Methods.

Rows: 1-2, kidney; 3-4, liver; 5-6, testis; 7-8, brain; 9-10, heart; 11-12, lung. Odd and even numbered rows correspond to control RNA (saline injected) and RNA isolated from insulin treated rats, respectively.

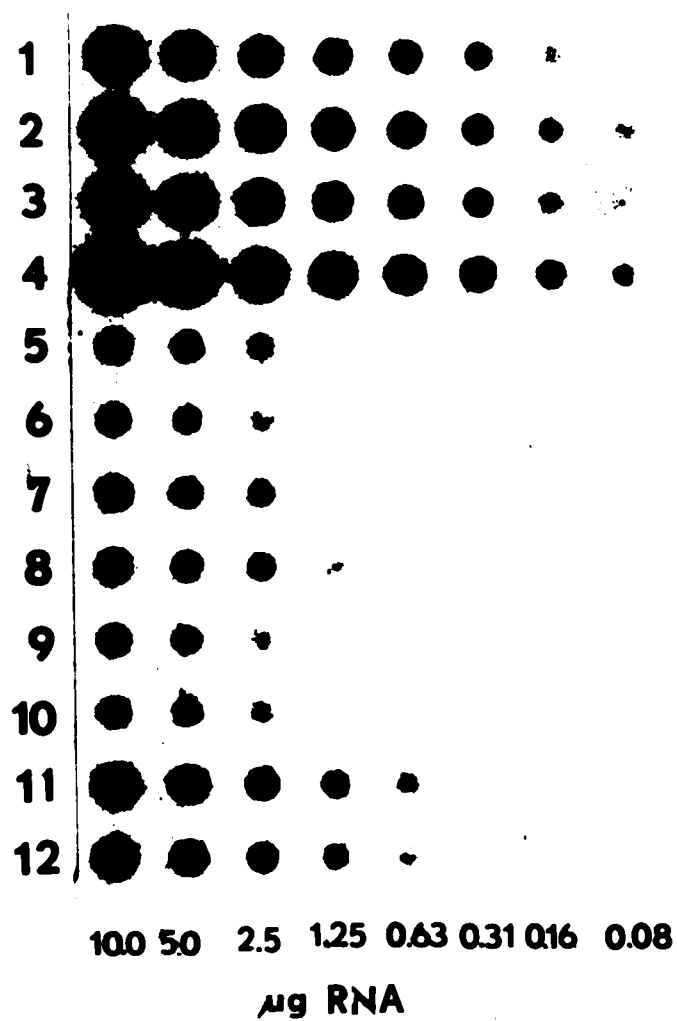


Figure 9

expression of gene 33 between the different tissues. It also shows that gene 33 mRNA is induced 2- to 4-fold in kidney and liver by insulin under these conditions. No apparent induction by insulin was detected for the other tissues. Northern blot analysis reveals that a 3.4 kb band is increased in kidney and liver following insulin treatment (Figure 10). A similar result was obtained for cAMP, gene 33 mRNA was elevated 2- to 5-fold in kidney and 2- and 3-fold in liver but the gene 33 mRNA remained the same in the other tissues (see Figures 11 and 12). However, hydrocortisone administration caused a dramatic increase in the level of gene 33 mRNA in each of the tissues (see Figure 13, Table 5, and Figure 14 on pages 66, 68 and 69, respectively). Therefore, gene 33 in kidney responds to all of these hormonal agents in a manner similar to liver. In brain, heart, lung, and testis gene 33 mRNA levels are increased by hydrocortisone but not by cAMP or insulin under the conditions used. The induction by hydrocortisone is most dramatic in the lung. Perhaps these tissues do not receive an adequate dose of these agents to elicit the response. This may be true for insulin since this agent has a half life of 3 to 4 minutes in the blood stream (Lehninger, 1975).

Analysis of Kidney Cytoplasmic and Nuclear mRNAs

Following Hydrocortisone Treatment

By using a number of cloned cDNAs to rat liver genes it has been shown that transcriptional control is the major basis for liver specific mRNA production (Derman et al., 1981; Clayton and Darnell,

Figure 10. Northern analysis of gene 33 mRNA in total cellular RNA from various rat tissues following insulin treatment.

Rats were treated with 1 U insulin per 100 g body weight by intraperitoneal injection and were killed after 1 hour. Northern gel analysis is as described in Materials and Methods. Total cellular RNA was run in an extra lane and was stained to use rRNA as standards. Controls were saline injected.

Lanes: A, 1-2, kidney; 3-4, liver; 5-6 testis, 7-8, brain; 9-10, heart; 11-12, lung (10 day exposure); B, 2 day exposure of lanes 1-4. Odd and even numbered lanes correspond to control RNA and RNA isolated from insulin treated animals, respectively.

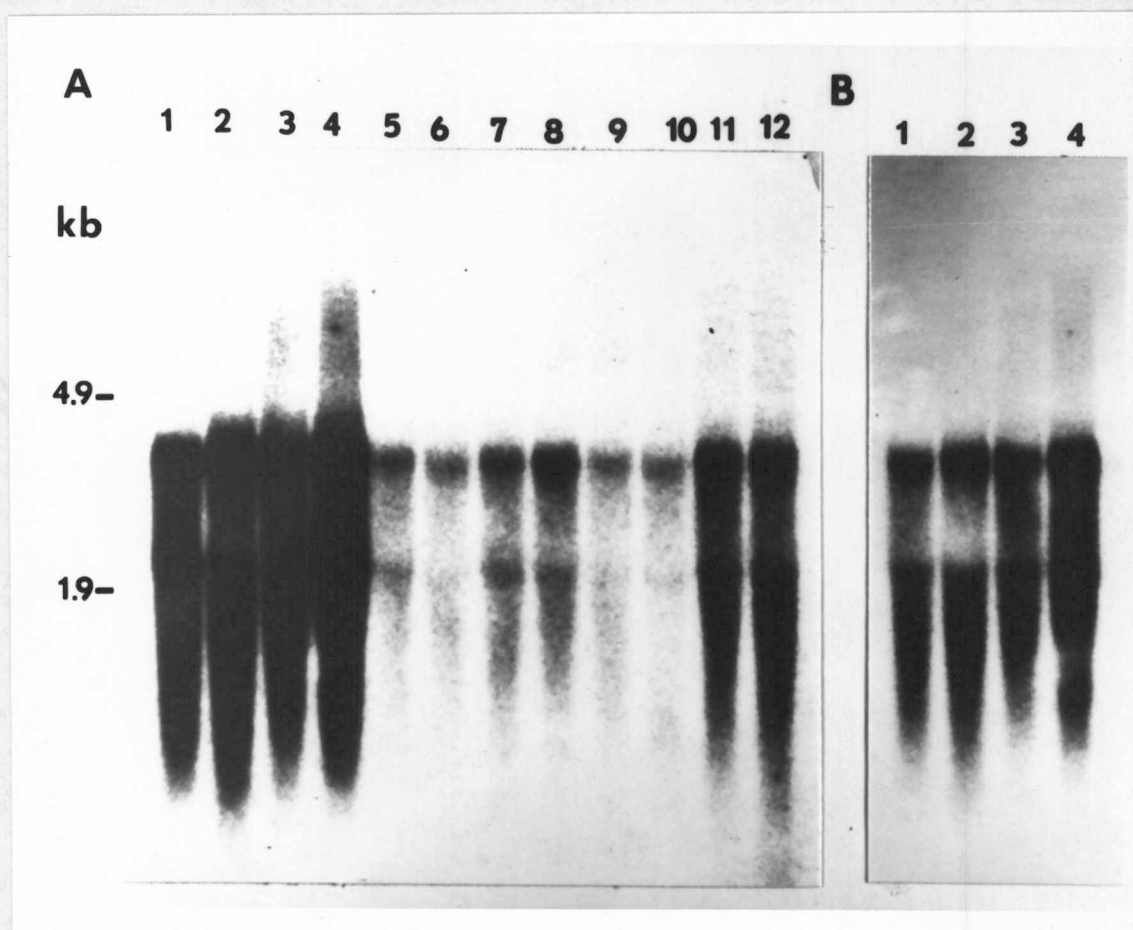


Figure 10

Figure 11. Dot hybridization analysis of gene 33 mRNA in total cellular RNA from various rat tissues following cAMP treatment.

Rats were given 5 mg 8-bromo cAMP per 100 g body weight intraperitoneally and were killed after 1 hr. Total cellular RNA was isolated as described in Materials and Methods. Controls were saline injected.

Rows: 1-2, kidney; 3-4, liver; 5-6, testis, 7-8, brain; 9-10, heart; 11-12, lung. Odd numbered rows are control RNA and even numbered rows are the cAMP treated sample.

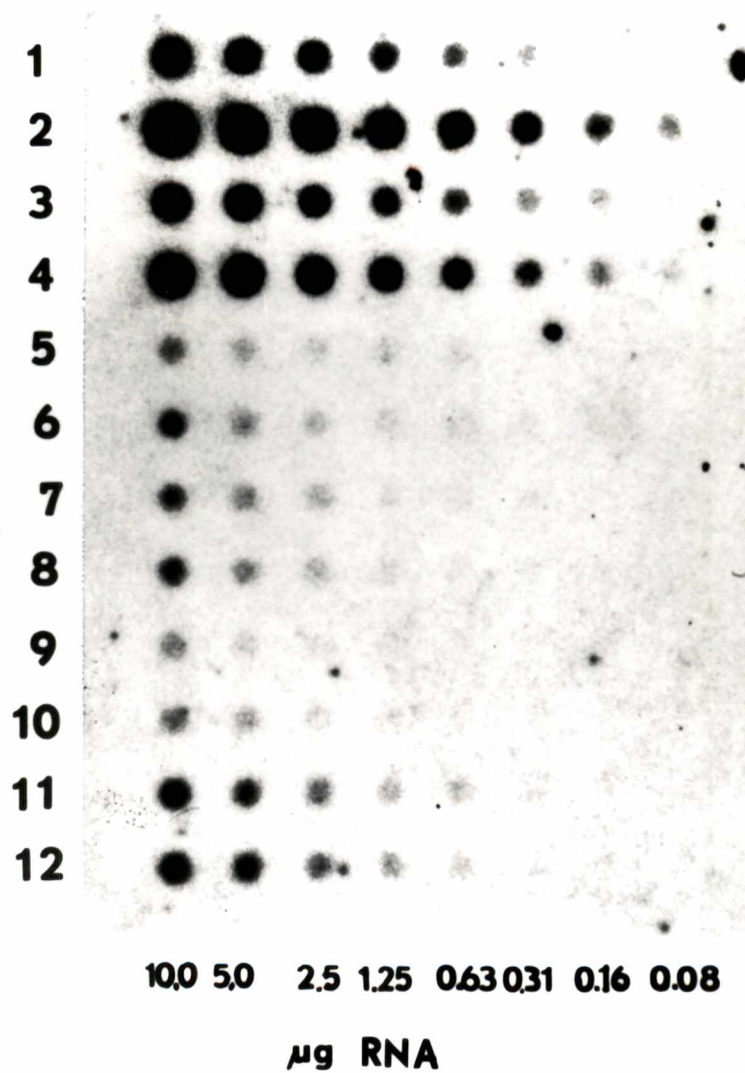


Figure 11

Figure 12. Northern analysis of gene 33 mRNA in total cellular RNA from various rat tissues following cAMP treatment.

Rats were given 5 mg 8-bromo cAMP per 100 g body weight intra-peritoneally and were killed after 1 hr. Total cellular RNA was isolated as described in Materials and Methods. Controls were saline injected. Total cellular RNA was run in an extra lane and was stained with acridine orange following electrophoresis to use rRNA as standards.

Lanes: A, 1-2, kidney; 3-4, liver; 5-6, testis; 7-8, brain; 9-10, heart; 11-12, lung (10 day exposure); B, 2 day exposure of lanes 1-4. The first number represents the lane in which the control was run and the second number represents the lane in which RNA isolated from cAMP treated rats was run.

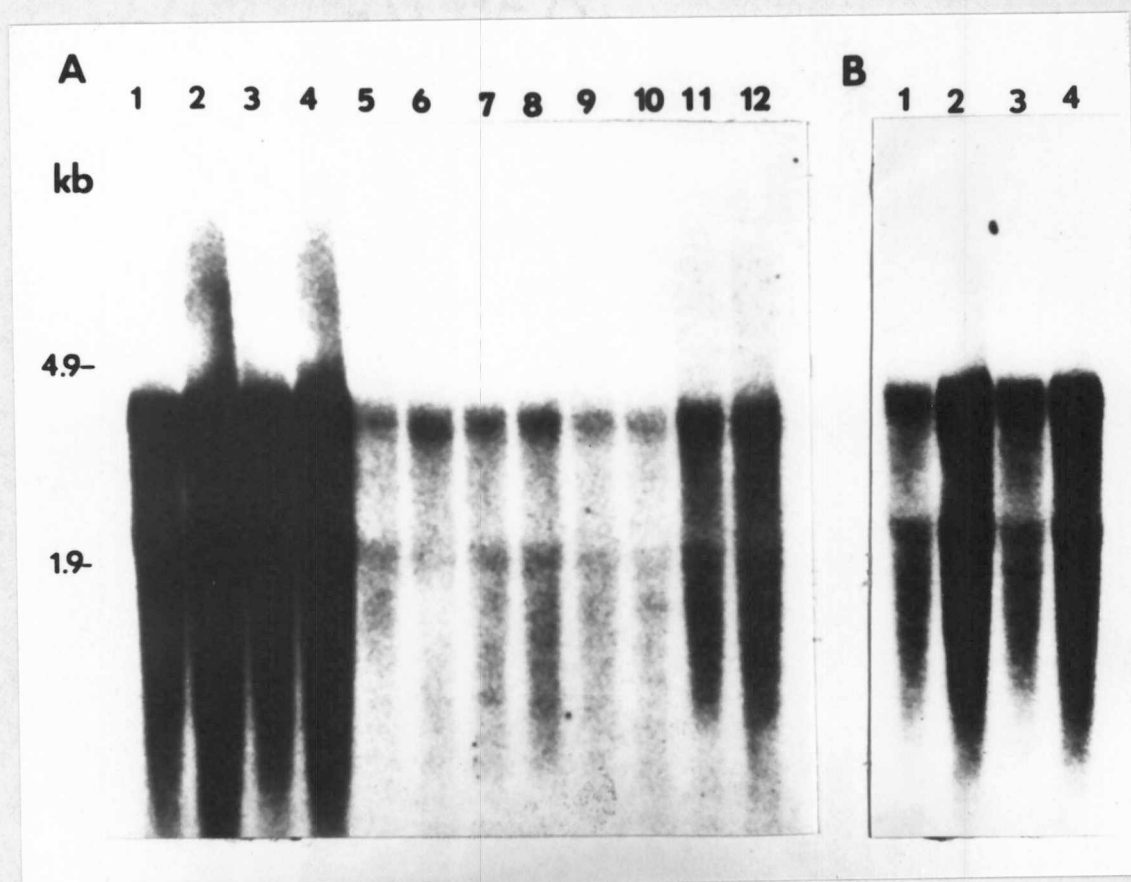


Figure 12

Figure 13. Dot hybridization analysis of gene 33 mRNA in total cellular RNA from various rat tissues following hydrocortisone treatment.

Rats were given 2.5 mg hydrocortisone per 100 g body weight and killed after 3 hr. Total cellular RNA was isolated as described in Materials and Methods.

Rows: 1,7 kidney; 2,8 liver; 3,9 testis; 4,10 brain; 5,11 heart; 6,12 lung. The first number is row containing the control RNA and the second number is the row containing the RNA isolated from hydrocortisone treated rats sample.

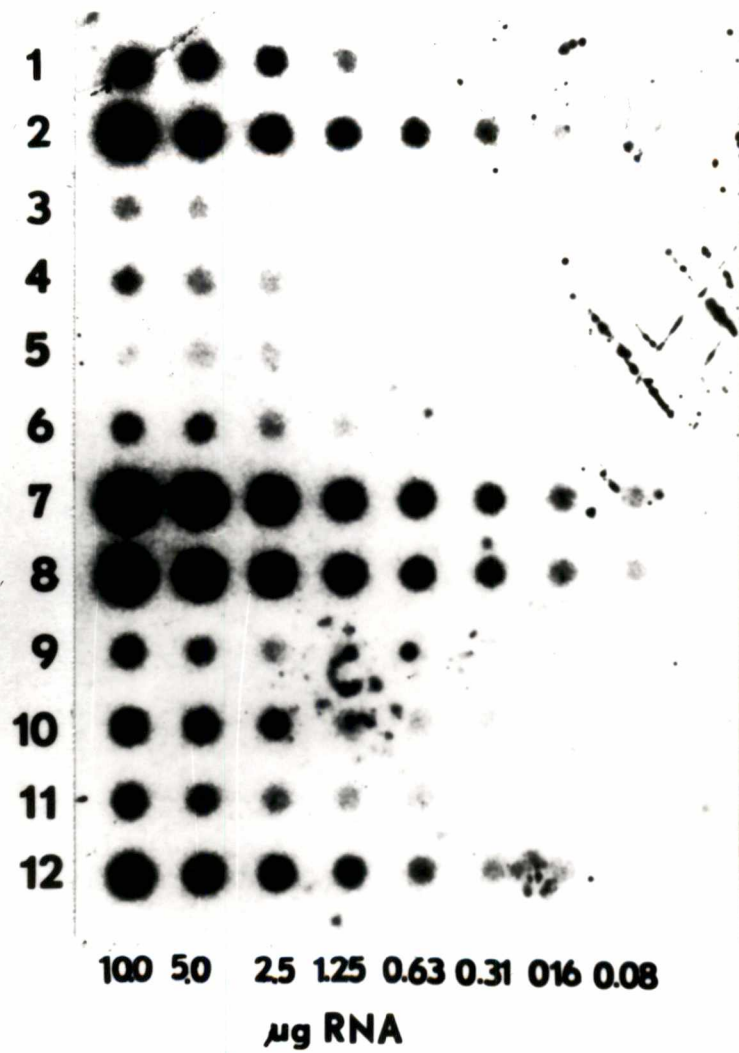


Figure 13

TABLE 5

Levels of Gene 33 mRNA in Total Cellular RNA
from Hydrocortisone Treated Rats^a

Tissue	Gene 33 mRNA Level Treated/Control x 100			Avg. $\pm$ Std. Dev.
	I	II	III	
Liver	2.1	2.1	3.5	2.6 $\pm$ 0.8
Kidney	4.6	1.6	2.0	2.7 $\pm$ 1.6
Testis	3.9	1.9	1.8	2.5 $\pm$ 1.2
Brain	4.7	2.9	2.0	3.2 $\pm$ 1.3
Heart	6.9	4.5	4.0	5.1 $\pm$ 1.5
Lung	8.5	7.5	6.7	7.6 $\pm$ 0.9

^aRats were treated with 2.5 mg hydrocortisone per 100 g body weight and killed 3 hr later. Total cellular RNA was isolated and was subject to dot hybridization analysis. Saline injected animals served as the control. Autoradiograms were scanned with a laser densitomer and relative intensities of dots were compared to liver in the linear portion of the scan.

Figure 14. Northern analysis of gene 33 mRNA in total cellular RNA from various rat tissues following hydrocortisone treatment.

Rats were given 2.5 mg hydrocortisone per 100 g body weight and killed after 3 hr. Total cellular RNA was isolated as described in Materials and Methods. Total cellular RNA was run in an extra lane and was stained with acridine orange following electrophoresis in order to use rRNA as a standard.

Lanes: 1-2, kidney; 3-4, liver; 5-6, testis; 7-8, brain; 9-10, heart; 11-12, lung. The first number is the control RNA (saline injected) and the second number is RNA isolated from hydrocortisone treated rats.

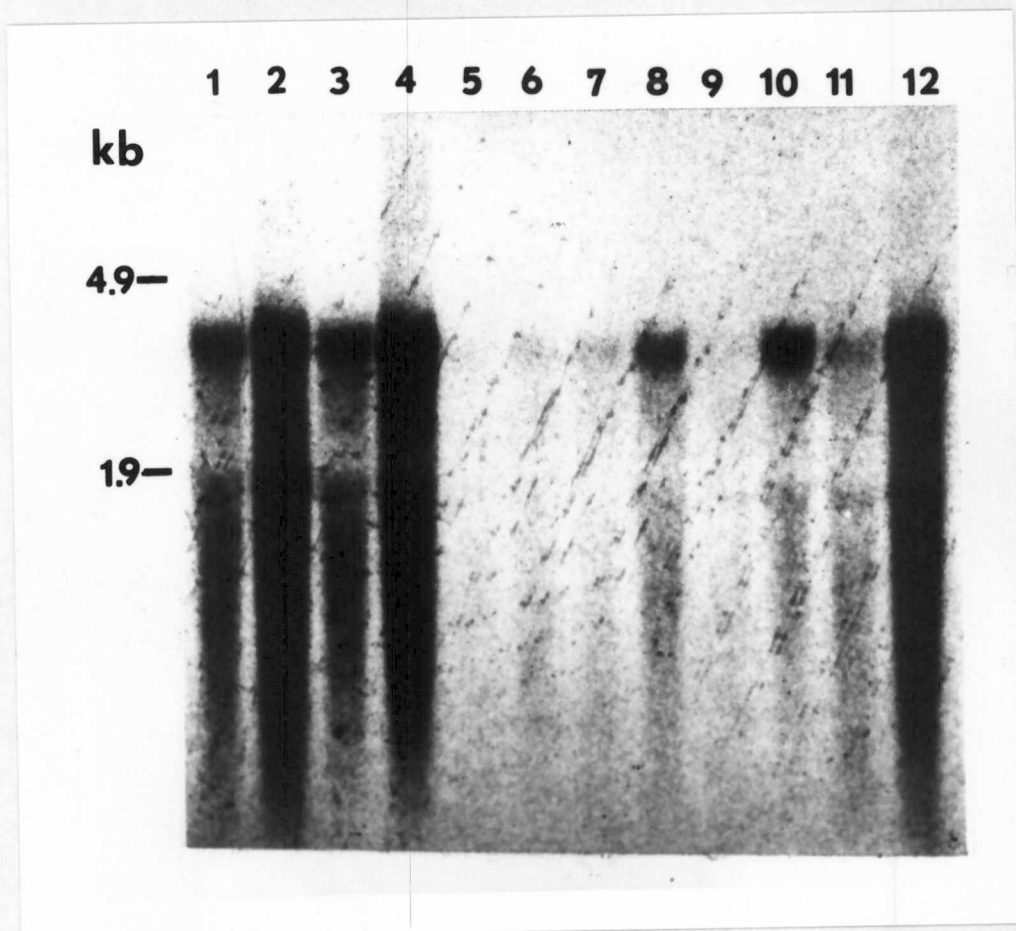


Figure 14

1873; Clayton et al., 1985a; Clayton et al., 1985b). For both TAT and gene 33 the increase in mRNA levels following treatment with cAMP, insulin, or hydrocortisone can be accounted for by a corresponding increase in the transcription rate from these genes (Hashimoto et al., 1984; Lee et al., 1985a; Lee et al., 1985b). However, changes in the level of tissue mRNA may also be a consequence of altered stability of precursor RNA, processing intermediates, or the mature mRNA (Darnell, 1982). Estrogen, for example, stabilizes cytoplasmic vitellogenin mRNA against degradation (Brock and Shapiro, 1983), and thyroid hormone stabilizes the mRNA precursor to a specific rat liver mRNA, designated spot 14 (Narayan and Towle, 1985).

As a means of determining whether synthesis or turnover of the mRNA is the basis for the increase in gene 33 mRNA in the kidney following hydrocortisone treatment, both cytoplasmic and nuclear RNAs were isolated from kidneys of hydrocortisone-treated rats and analyzed by dot hybridizations (Figure 15 and Table 6). Levels of gene 33 mRNA increase to the same extent in the nucleus and in the cytoplasm, a result consistent with the conclusion that glucocorticoids stimulate transcription in this organ or stabilize the nuclear precursor of gene 33. To differentiate between an increase in the transcription rate and stabilization of the RNA by this agent direct measurement of transcription rate was made by a "transcriptional runoff assay in isolated nuclei, wherein gene transcripts initiated in vivo are completed and labeled in vitro, then assayed for specific

Figure 15. Dot hybridization analysis of gene 33 sequences in kidney cytoplasmic and nuclear RNA after hydrocortisone treatment.

Rats were given 2.5 mg hydrocortisone per 100 g body weight and were killed after 3 hr. RNA was isolated as described in Materials and Methods.

Row: a,b nuclear RNA; c,d cytoplasmic RNA. Rows are stated as RNA from hydrocortisone treated rats (a,c) and RNA from control rats (saline injected; b,d).

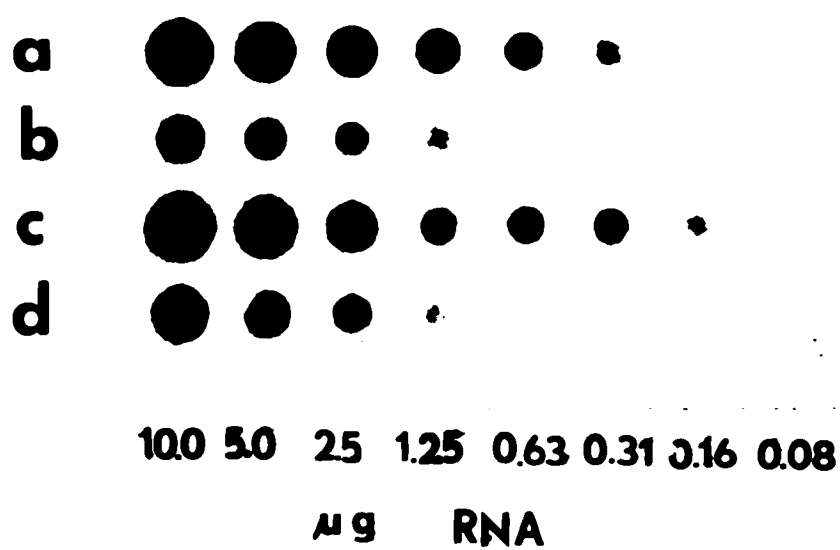


Figure 15

TABLE 6

Hydrocortisone Increases Kidney Gene 33
Cytoplasmic and Nuclear RNA^a

RNA	Treated/Control x 100 Avg. $\pm$ Std. Dev. ^b
Nuclear	2.8 $\pm$ 0.3 (2)
Cytoplasmic	3.6 $\pm$ 0.5 (3)

^aAutoradiograms of dot blots were scanned with a laser densitometer and the relative intensities of induced and control RNA samples were compared in the linear portion of the scan.

^bNumber in parentheses is number of individual determinations.

gene 33 transcripts by hybridization. The assay, in effect, is a measure of the relative number of RNA polymerase molecules engaged in transcribing a particular gene at the time the tissue was taken and nuclei prepared. Using the methods of Lamers et al. (1982) and Derman et al. (1981) the rate of transcription of gene 33 was compared in nuclei from kidneys of control and hydrocortisone-treated rats. The data from this experiment (Table 7) confirm that transcription is enhanced about 3-fold in kidney nuclei. While absolute confirmation will require repetition of this analysis, it seems reasonable to conclude that the same mechanism is involved in induction of gene 33 mRNA in both kidney and liver.

TABLE 7

Effect of Hydrocortisone on Transcription
of Gene 33 in Kidney Nuclei^a

Treatment	Input RNA cpm x 10 ⁶	Hybridized pBR322	cpm p33	Relative Rate of transcription, ^b ppm
Saline	8.34	62	102	36
Hydrocortisone	6.90	67	152	101

^aNuclei were isolated from kidneys of adrenalectomized rats that were treated with saline or 2.5 mg hydrocortisone per 100 g body weight for 3 hr. In vitro transcription protocol is as described in Lee et al. (1985b).

^bThe relative rate of transcription is calculated according to Lamers et al. (1982).

CHAPTER IV

DISCUSSION

Gene 33 and TAT expression in rat liver are subject to control by several hormones and to regulation during differentiation. Until the identification of gene 33 it was thought characteristics of TAT gene expression were unique to TAT, the increase in gene products following cAMP, insulin, and hydrocortisone as well as the developmental control of the gene. In this dissertation immunochemical analyses of TAT protein in nonhepatic tissues and RNA analyses confirm that TAT is liver specific. Most hormonally or developmentally regulated genes, e.g. phosphoenolpyruvate carboxykinase (PEPCK; Benvenisty et al., 1985), mouse major urinary protein (Knopf et al., 1983), α_{2u} -globulin (Kulkarni et al., 1985), are expressed in a tissue specific manner. In contrast, data from northern and dot blot analyses of total and cytoplasmic RNAs have shown that gene 33, although subject to the same elaborate multihormonal and developmental control as TAT in liver, is expressed in a variety of tissues, including kidney, lung, brain, testis, and heart. These results suggest that there may be a common mechanism whereby TAT and gene 33 are regulated in the liver but by other mechanisms unique to TAT or gene 33 which control their tissue specificity.

Biochemical and genetic experiments suggest that a regulatory element for TAT expression is contained on mouse chromosome 7 including

the c locus. Mice carrying deletions within this region die shortly following birth, and have reduced levels of TAT activity (Gluecksohn-Waelsch, 1979) and mRNA (Schmid et al., 1985). Furthermore, by Southern analysis it was shown that the TAT gene is intact, and neither glucocorticoids nor cAMP could elicit a response from this gene. These experiments suggest that a protein product of this region is required for function and inducibility of the TAT gene. Isham et al. (1985) have shown that gene 33 levels are not diminished in these mutants, but the mRNA level of PEPCCK, another gene subject to multihormonal, developmental, and tissue specific control, is also reduced. Sala-Trepat et al. (1985) also found that the mRNA of aldolase B, another liver specific enzyme, is decreased in these mutants. A gene product made from the region containing the c locus might be suggested to be the factor which confers expression of TAT, aldolase B, and PEPCCK in the liver. Perhaps this is a general factor that acts on many genes that are subject to tissue specific control. Since it is possible that a common factor regulates the expression of TAT, aldolase B, and PEPCCK in liver it is also possible that this factor interacts with a common DNA sequence, perhaps an enhancer sequence like the tissue specific sequence associated with the immunoglobulin light chain (Grosschedl and Baltimore, 1985) to exert its effect.

Gene 33 steady state mRNA levels were analyzed by applying serially diluted RNA onto nitrocellulose filters, hybridization of

filters with a nick translated gene 33 cloned probe, and comparing relative intensities of hybridization signals of various tissues to liver on autoradiograms of hybridized filters. Analysis of mRNA levels in a variety of tissues revealed that gene 33 is expressed to different extents in each of the tissues examined (see Table 3, page 48). In the liver both gene 33 and TAT gene products undergo rapid turnover, a quality generally associated with highly specialized functions. However, gene 33 is expressed in several tissues which is more consistent with genes that are constitutively expressed. Genes that are constitutively expressed are generally not inducible. However, gene 33 mRNA is increased by glucocorticoids (see Table 5, page 68) in all tissues examined, and by all three effectors in kidney and liver (see Figures 9, 10, 11, 12, pages 57, 60, 62 and 64, respectively). Although gene 33 exhibits many properties of a highly specialized function, it also exhibits qualities of a nonspecialized function.

In rat liver gene 33 mRNA levels are increased by cAMP, insulin as well as hydrocortisone by increasing transcription from the gene (Lee et al., 1985b). As a means to determine whether differential rates of synthesis or turnover of mRNA was the basis for the increase in gene 33 mRNA in kidney following hydrocortisone treatment cytoplasmic and nuclear mRNA levels and in vitro transcription run off assays were carried out. Levels of gene 33 mRNA increased approximately 3- or 4-fold in both nuclear and cytoplasmic RNA following hydrocortisone treatment (see Figure 15, page 72), a result that is consistent with the conclusion that glucocorticoids either stimulate

transcription in this organ or stabilize the nuclear precursor which may also be reflected in a corresponding increase in cytoplasmic gene 33 mRNA. Transcription runoff assays in isolated kidney nuclei indicated that transcription is enhanced 3-fold after hydrocortisone treatment (see Table 7). The same mechanism, an increase in transcription, is employed in kidney and liver to increase the level of gene 33 mRNA by hydrocortisone. The same mechanism may also be used to elevate gene 33 mRNA levels in brain, heart, lung, and testis following hydrocortisone treatment (see Figures 13 and 14, pages 66 and 69, respectively).

In Table 5 (page 68) the steady state levels of gene 33 mRNA in several rat tissues following hydrocortisone treatment were summarized. If hydrocortisone action is mediated by an enhancer element as has been previously suggested by Zaret and Yamamoto (1984) the change in the mRNA levels in each tissue would be expected to occur to the same extent. This was not the case. Gene 33 mRNA was increased approximately 8- and 5-fold in lung and heart, respectively, whereas only 2- to 3-fold in liver, kidney, testis, and brain. The role of an enhancer in this process should not be disregarded since it must also be considered that each tissue may not have received equivalent doses of hydrocortisone by intraperitoneal administration of the agent.

In northern blot analyses the size of gene 33 mRNA in various rat tissues was examined. Equal amounts of total cellular RNA, 50 ug,

were electrophoresed on denaturing gels, transferred to nitrocellulose filters, and hybridized to a nick translated gene 33 cDNA clone. A broad band of hybridization was detected in brain, heart, lung, kidney, and liver (see Figure 7, page 50). The mRNAs were of uniform size in each of the tissues, approximately 3.4 kb. Since such a large amount of RNA was electrophoresed a diffuse pattern of hybridization was detected on northern blots. This may mask subtle differences in mRNA size or hinder detection of multiple species of mRNA with subtle size differences. Identification of uniform size of mRNA in all of the tissues suggests that the RNA may be processed similarly in each tissue, although differential processing may also result in the same size of mRNA. The smear of hybridizing material below the major 3.4 kb band may reflect the rapid turnover of this mRNA.

Several mechanisms can be proposed for control of gene 33 expression. First, the variation in the levels of gene 33 expression may be due to different rates of transcription or altered stability of the mRNA in the different tissues which may be the result of interaction with nuclear or cytoplasmic factors therein. Second, the level of gene 33 mRNA may also be regulated by methylation of DNA at particular sites. Interaction of regulatory proteins with DNA may be determined by the extent of methylation at specific sites within gene 33, and the interaction with the proteins dictates the amount of RNA made. This is an attractive mechanism since the 5' end of gene 33 is 70% GC, and the sequence CpG is reiterated many times within this GC rich area (Tindal, 1985). I have obtained

evidence that there is at least one site methylated in this region in heart DNA that is not methylated in liver DNA (data not shown). This hypothesis can be easily approached by determining sites of methylation within the 5' end of gene 33 DNA from these tissues using isoschizomers, restriction enzyme pairs which recognize the same DNA recognition site but one will not cut within the sequence if particular cytosines are methylated. The level of expression may not only be due to the presence of these methylated sites but also might be consequence of regulatory factors having to interact at the sites of methylation, i.e. the protein may recognize a methylated site, bind, and allow limited access of RNA polymerase, or protein may bind and allow increased access to RNA polymerase. In vivo protein-DNA interactions can be investigated and possibly correlated with specific DNA methylations using techniques described by Jackson and Felsenfeld (1985).

In summary, two rat genes with similar multihormonal and developmental expression in rat liver were found to be differentially expressed in nonhepatic tissues. The complex mechanisms responsible for the differential phenotypic expression of a constant genotype will remain subject to extensive research for many years.

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APPENDIXES

APPENDIX A

CHROMOSOMAL LOCALIZATION OF TAT AND GENE 33

BY IN SITU HYBRIDIZATION

Introduction

It was of interest to determine the chromosomal location of gene 33 and TAT due to the similarities and the differences in expression of these genes.

Materials and Methods

Human Lymphocyte Cultures

Blood (10 to 20 ml) was collected from a human male donor using a heparinized syringe. Buffy coats were isolated by centrifugation in an IEC clinical centrifuge (centrifugation at top speed 8 min using a 221 rotorhead). Buffy coat (containing mostly lymphocytes) recovered from 3 ml of blood was added to a medicine type culture bottle containing 9.7 ml of RPMI 1640 media, 15% heat inactivated fetal calf serum to which 300 ul of phytohemagglutinin (PHA; Wellcome; 5 ml freeze dried stock reconstituted with 5 ml sterile H₂O) had previously been added. PHA is a mitogenic agent which will stimulate the G₀ lymphocytes to divide. Cultures were incubated at 37°C in 5% CO₂ for 72 hr. Cells were arrested in metaphase of the second cell cycle by addition of colchicine (Sigma) to a final

concentration of 10^{-5} M and incubating the cultures at 37°C for an additional 2 hr.

Preparation of Metaphase Chromosome Spreads

Cells from cultures in the preceeding section were collected by centrifugation, washed with Hank's balanced salt solution, and incubated in hypotonic solution (0.075 M KCl) at 37°C for 15 min. Cells were then fixed in 3:1 methanol, glacial acetic acid (fixative). Preparations were washed several times with fixative then resuspended in 0.3 to 0.5 ml of fixative per starting 10 ml culture. Using a Pasteur pipet one drop of cell suspension was dropped onto a wetted glass slide (Clay Adams precleaned Rite-On microscope slides) from several feet above the slide. Slides were stored at room temperature for 1 week prior to beginning the next steps of the experiment.

Prebanding of Metaphase Chromosomes

The major problem with carrying out in situ hybridization was identifying chromosomes after the hybridization. Previous attempts at G-banding (Yunis et al., 1978) or R-banding (Donlon, 1985) following hybridizations were unsuccessful. Therefore, it was necessary to identify chromosomes before hybridization.

Air dried slides which had been aged for 1 week at room temperature were G-banded (Yunis et al., 1978) by incubating slides for 20 min in Wright's stain (Sigma; 0.3% w/v in methanol diluted to 0.25% with H₂O and filtered through a 0.45 um Millipore filter)

diluted 1:3 in phosphate buffer, pH 6.8 (1:1 0.06 M NaH_2PO_4 and 0.06 M NaHPO_4). Slides were rinsed in phosphate buffer and were temporarily mounted with phosphate buffer and a coverglass (1 mm thick, 22 x 50 mm, Fisher brand). Well banded metaphase spreads containing complete karyotypes were identified under oil immersion using a Zeiss photomicroscope. Metaphase coordinates were recorded and were photographed using Kodak technical pan 2415 35 mm film. Slides were destained in 95% ethanol for 2.5 min and 100% methanol for an additional 2.5 min.

Preparation of Radiolabeled Probe

Radiolabeling of plasmid DNA (pcTAT-3 or p33, cDNA to gene 33) was carried out with a nick translation kit from Amersham. A typical reaction was carried out in 100 μl consisting of 1.0 μg plasmid (twice purified over CsCl gradients), 20 μM [^3H]-dNTPs (dATP, 27 Ci/mmol; dTTP, 64 Ci/mmol; dCTP, 30 Ci/mmol; all from ICN), 20 μM dGTP, 5.5 units DNA polymerase I, 110 pg DNase I, in 50 mM Tris-HCl, pH 7.8, 5 mM MgCl_2 , and 10 mM 2-mercaptoethanol. The reaction mixture was incubated at 15°C for 5 hr. The labeled DNA was separated from unincorporated nucleotide by chromatography on BRL Nacs-52 columns. The nick translation mixture was applied to the column in 0.5 M NaCl, TE (20 mM Tris-HCl, pH 7.2, 1 mM EDTA), washed with 15 ml of the same buffer, and eluted with 300 μl 2.0 M NaCl, TE. Carrier tRNA was added to the eluted fraction to 50 μg per ml and the fraction was ethanol precipitated. After recovering the labeled DNA by

centrifugation, it was resuspended in a small volume of H₂O and formamide was added to a final concentration of > 98%. DNA was denatured by heating in an H₂O bath for 7 min followed by quick cooling in an ethanol-ice bath. An equal volume of 2X hybridization buffer (4XSSC, 0.08 M NaPO₄, pH 6.5, 0.04% bovine serum albumin, 0.04% ficoll, 0.04% polyvinylpyrrolidone, and 20% dextran sulfate) was added to the denatured DNA. Probe specific activities ranged from 2 to 5 x 10⁷ cpm per ug DNA. The final concentration of probe in the hybridization mix was 0.2 ug DNA per ml.

In Situ Hybridization

Human metaphase spreads which had been prebanded and destained were hybridized by the method of Harper and Saunders (1981). Slide preparations were treated with 100 ug/ml RNase A (bovine pancreatic, Millipore) in 2XSSC at 37°C for 1 hr. Slides were then rinsed in 2XSSC, dehydrated in 95% ethanol, and air dried. Slides were immersed in 70% formamide, 2XSSC at 70°C for 2 min to denature the chromosomal DNA. Then, 30 ul of denatured ³H-probe (6 ng DNA) was applied to each slide and coverslips were mounted with rubber cement. Hybridization was carried out on a slide warmer at 37°C for 19 hr. Slides were rinsed four times with 250 ml of 50% formamide, 2XSSC and three times with 250 ml of 2XSSC, each at 39°C. Slides were dehydrated in 95% ethanol and air dried.

Autoradiography and Chromosome Identification

Hybridized slides were coated with Kodak NTB₂ emulsion, placed in a Clay Adams slide box with dessicant, and stored at -70°C for

1 week to 2 months. Slides were processed at 15°C as follows: developed in Kodak Dektol for 3 min, rinsed in distilled H₂O, fixed in Kodak general fixer for 5 min, and rinsed in distilled H₂O. Slides were stained as described above and mounted in euparal mounting medium with a 20 x 50 mm coverglass. Metaphases which had previously been photographed were relocated and photographed again. Negatives of pre-banded metaphases and their corresponding hybridized spread were developed for 6 min at 68°F in HC110 developer (diluted 1:10 with distilled H₂O) and printed on Kodak F3 or F4 kodabromide paper. G-banded human chromosomes were identified according to Yunis et al. (1978). On hybridized metaphases a grain was considered to be associated with a chromosome if it was within less than a width of a chromatid away from a chromosome.

Results and Discussion

Figure 16 represents a metaphase spread that was G-banded before hybridization. Chromosomes from this cell were identified (Figure 17) and the grains associated with the chromosomes were recorded. Figure 18 shows the same metaphase as in Figure 16 after it was hybridized to pcTAT-3 plasmid. In 12 human metaphases hybridized to pcTAT-3, 127 grains were associated with chromosomes. The distribution of grains over the chromosomes is shown in Figure 19. This figure displays the number of grains associated with the p arm (short arm) and the q arm (long arm) of the chromosome. Young et al.

Figure 16. G-banded human metaphase chromosomes.

Slide preparations were G-banded with Wright's stain as described in Materials and Methods.



Figure 16

Figure 17. Identification of G-banded human chromosomes.

Chromosomes were identified according to Yunis et al. (1978).

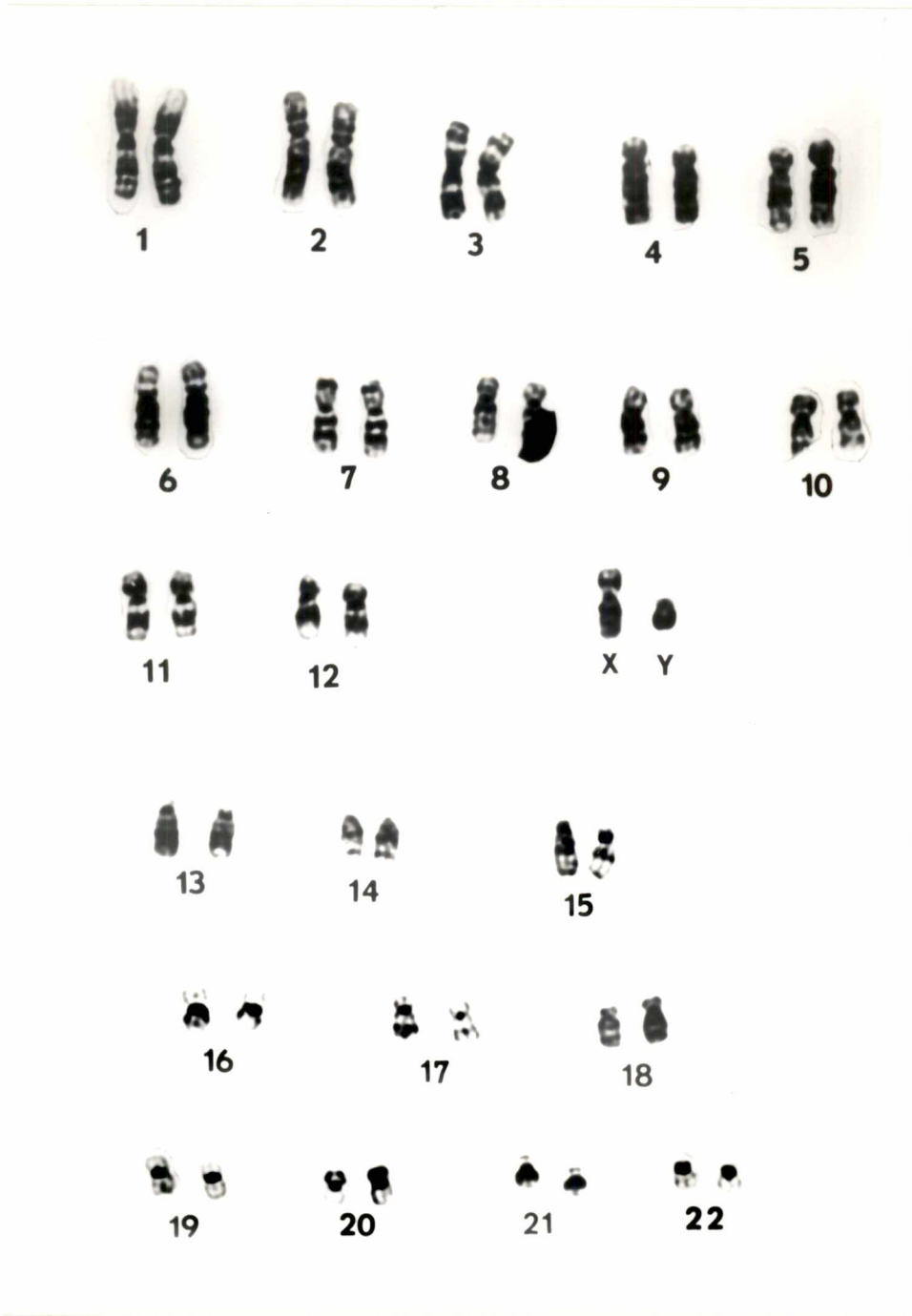


Figure 17

Figure 18. Human metaphase chromosomes hybridized with pcTAT-3 plasmid DNA.

Slide preparations were hybridized as described in Materials and Methods. The G-banded counterpart to this metaphase spread is shown in Figure 17. Circles represent grains associated with chromosomes.



Figure 18

Figure 19. Distribution of grains over human metaphase chromosomes hybridized to pcTAT3 plasmid.

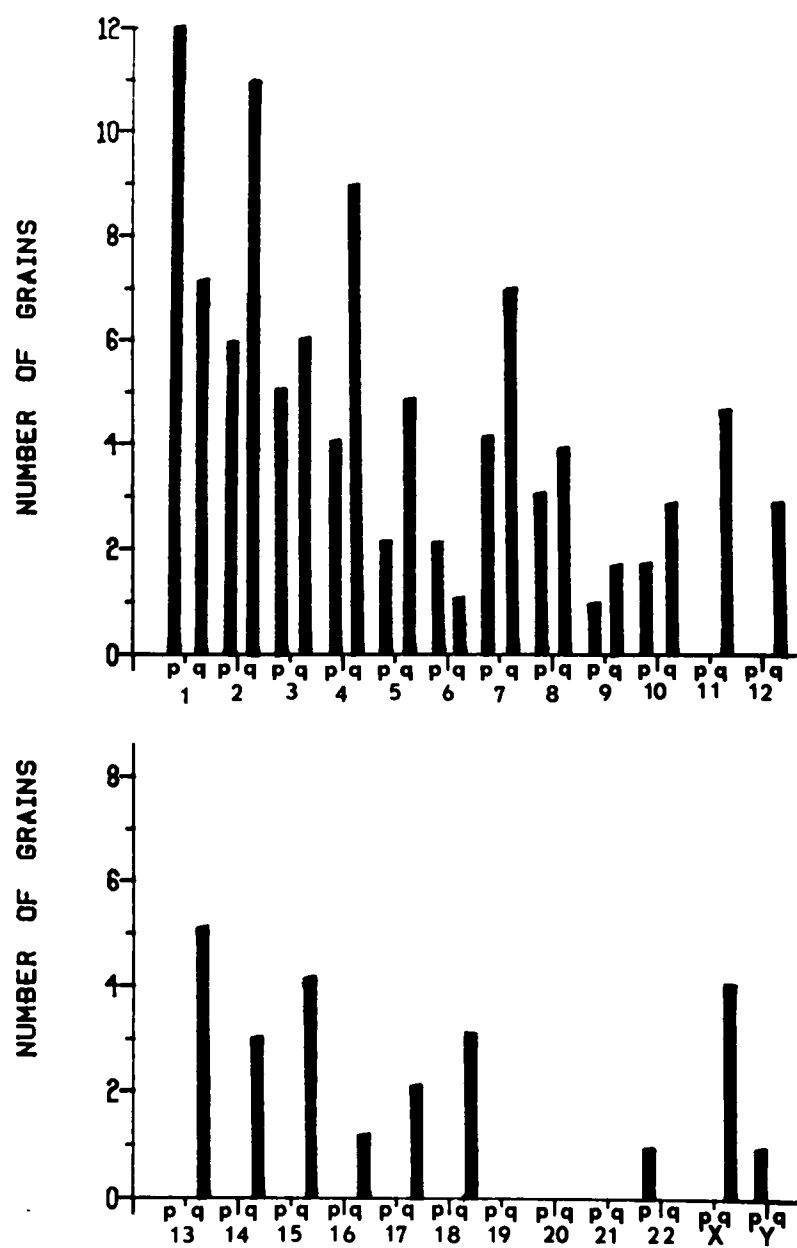


Figure 19

(1983) have demonstrated that the number of grains randomly associated with chromosomes is dependent on the length of the chromosome arms, e.g. chromosome 1 would have more nonspecifically associated grains than chromosome 21 since the chromosome arms are longer (see Figure 17 for the size of each chromosome). Specific hybridization is well above that expected for random distribution over the chromosome length so that the chromosome the probe specifically hybridizes to is easily distinguished from the background hybridization. Distributions of grains on chromosomes after hybridization with pcTAT3 appears to be random. In this experiment 5 to 22 grains were associated with each metaphase. Several investigators report finding as few as 2 to 5 grains per metaphase (Chandler and Yunis, 1978; Harper and Saunders, 1981; Young et al., 1983). Therefore it was concluded that background was too high in this particular experiment. High background may have been caused by incomplete destaining of chromosomes before the hybridization. A component of Wright's stain, methylene blue, can reduce the silver in the emulsion (Donlon, 1985) and cause an increase in background. Therefore it appears that high background was obtained with the protocol used. Also, after hybridization many chromosomes did not retain their morphology and could not be used in the analyses.

To alleviate problems with high background and loss of chromosome morphology several measures were taken in more recent experiments. First, chromosomes were prebanded before hybridization but were also

destained using more extensive conditions: 2 min in 3:1 methanol, glacial acetic acid, rinse in methanol, rinse in xylene, rinse in 95% ethanol, and a rinse in 70% ethanol. Second, to preserve chromosome morphology during hybridization, slide preparations were pre-treated prior to denaturation with 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% ficoll, in 2XSSC for 1 hr at 25°C and dipped in 0.05% chrome alum, 0.5% gelatin (Rasch, 1985). Several slides were examined after hybridization with pcTAT-3 and p33 after using this updated protocol. Chromosomes retained their morphology and the number of grains observed in several metaphases was 3 to 5. Chromosome identification and grain distributions remain to be completed.

APPENDIX B

CHROMOSOMAL ASSIGNMENT OF GENE 33 USING SOMATIC CELL HYBRIDS

Introduction

A panel of mouse-hamster somatic cell hybrids containing varying complements of mouse chromosomes were generously provided by Dr. P. A. Lalley and used as another means to determine the chromosomal location of gene 33. Peterson et al. (1985) have recently assigned the TAT structural gene to mouse chromosome 8.

Materials and Methods

Isolation of Genomic DNA

Genomic DNA was isolated according to the method of Lacy et al. (1983). Cells were removed from culture plates by trypsinization. After washing the cells several times in serum free medium the cells were frozen as a pellet at -70°C until DNA was to be isolated. Frozen cells, equivalent to amount grown on one-100 mm plate, were added to 4 ml cold ET buffer (100 mM EDTA, 50 mM Tris-HCl, pH 8.0) in a 50 ml Falcon tube and allowed to thaw on ice. To this 0.7 ml 5 M NaCl, 0.12 ml 30% sodium-N-Lauroyl sarcosine, and 0.7 ml 2.5 mg/ml proteinase K (type XI, Tritirachium album; Sigma) was added. This mixture was adjusted to 7 ml with ET buffer, incubated in an H₂O bath at 50°C for 3 to 5 hr, and 0.81 g CsCl was added per g of sample

(the final density of the sample was 1.55 g/ml). The refractive index of each sample was read (it should be 1.3856) and, if necessary, more CsCl was added. After addition of 30 μ l 10 mg/ml ethidium bromide, the mixture was transferred to a 50 Ti polyallomer tube and underlayered with 2 ml 6.0 M CsCl in ET buffer. Tubes were capped and filled to capacity with light mineral oil. Centrifugation was carried out in a 50 Ti rotor at 35,000 rpm at 21°C for 48 hr. Banded genomic DNA was visualized by brief UV illumination and removed from the tube with a sterile 16 guage needle attached to a disposable 5 ml syringe. DNA was extracted with isopropanol (preequilibrated with CsCl-saturated H₂O) and dialyzed extensively against 10 mM Tris-HCl, and 1 mM EDTA, pH 8.0.

Restriction Enzyme Digests and Southern Blots

DNA was digested with 3 to 5 units of restriction endonuclease per μ g DNA over night at 37°C using buffer conditions specified by Bethesda Research Laboratories. Spermidine-HCl, pH 7.0, was added to a final concentration of 1 mM to aid in digestion of DNA (Bouche, 1981). Digests were phenol-CHCl₃ extracted, ethanol precipitated, and resolubilized in sterile H₂O. Gel loading buffer was added to resolubilized DNA (1/9 volume of 10X stock: 25% ficoll, 0.25% bromophenol blue-xylene cyanol) and samples were loaded onto a 300 ml gel containing 0.6% agarose, 89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH 8.3. DNA was electrophoresed for 1000 volt-hr using constant voltage. Following electrophoresis gels were stained in ethidium bromide and DNA was visualized by UV illumination.

DNA gels were transferred and processed according to the method of Southern (1975). After staining, gels were incubated at room temperature two times each in 250 ml 0.25 N HCl, 250 ml 0.5 N NaOH, 1.5 M NaCl, and 250 ml 0.5 M Tris-HCl, pH 7.0, 3.0 M NaCl for 15 min each time. DNA was transferred to nitrocellulose filters (Scheicher and Schuell, BA85) in 20XSSC for at least 12 hr. After transfer filters were baked and hybridized as described for northern transfers (see page 25). After hybridization filters were washed in 2XSSC, 0.1% SDS at 50°C until radioactivity in the washes reached background. Occasionally blots were also incubated in prehybridization solution at 42°C as a final wash. Autoradiography is as described for northern transfers (see page 25).

Results

Preliminary experiments involved digestion of DNA from the somatic cell hybrid parent cells, E36 hamster cells and RAG mouse cells, with a series of restriction enzymes followed by Southern blot analysis. Nitrocellulose filters were hybridized with a gene 33 cDNA clone to determine hybridization patterns for the hamster and mouse DNA. Several different hybridization patterns were observed using gene 33 plasmid, p33, as a probe (Figure 20). Hind III, Pst I or Sst I each generate a different size restriction fragment which hybridizes to p33 for hamster DNA as compared to mouse DNA. A panel

Figure 20. Hybridization of gene 33 plasmid to a Southern blot of E36 (hamster) and balb/c mouse liver DNA.

Lanes: E (#36, hamster DNA); M (balb/c mouse liver DNA). Restriction enzymes are indicated above the corresponding lane. Standards, Hind III digested DNA and Hae III X174 DNA, were visualized on the ethidium bromide stained gel. The migration distances were noted and used as standards on the transfer. In this experiment mouse liver DNA was used instead of RAG cells DNA. These DNAs are equivalent.

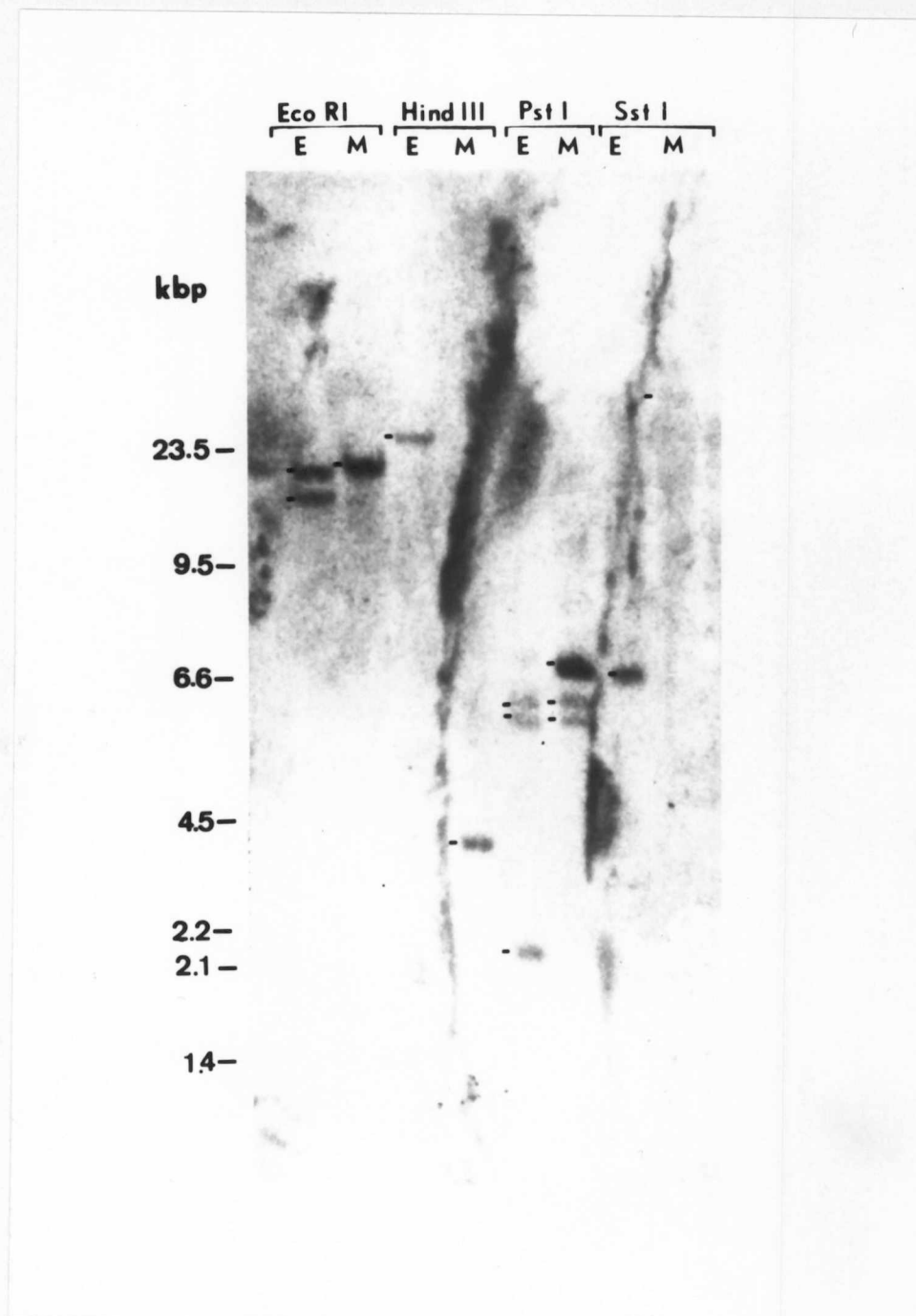


Figure 20

of 13 well-characterized mouse-hamster somatic cell hybrids in which hamster chromosomes are retained but many mouse chromosomes are lost will be used to determine the chromosomal location of gene 33 in the mouse genome. Southern blots will be used to screen the panel for presence of the unique size mouse DNA restriction fragment that was determined by the analyses in Figure 19. Knowing the complement of mouse chromosomes in each hybrid and in which hybrid(s) the unique size mouse DNA restriction fragment is detected by the gene 33 probe the location of gene 33 in the mouse genome will be deduced.

VITA

Lisa Ann Francine Balogh was born on October 11, 1958 in Norristown, Pennsylvania. She attended the Phoenixville Area High School in Phoenixville, Pennsylvania from which she was graduated in June of 1976. She majored in Biochemistry at the Pennsylvania State University in State College, Pennsylvania and was graduated with a Bachelor of Science degree in June 1980. In September 1980 she began graduate study at the Oak Ridge Graduate School of Biomedical Sciences located at the Biology Division of the Oak Ridge National Laboratory. She received a Ph.D. in Biomedical Sciences in October of 1985 for research carried out under the direction of Dr. Francis T. Kenney. After receiving her Ph.D. she began research in the laboratory of Dr. Jerard Hurwitz at the Memorial Sloan-Kettering Cancer Center in New York, New York.