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TRANS-ACTING FACTORS REGULATING S14
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TRANS-ACTING FACTORS REGULATING S14 GENE TRANSCRIPTION

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ABSTRACT

TRANS-ACTING FACTORS REGULATING S14 GENE TRANSCRIPTION

By

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Formation of DNase I hypersensitive site I (Hss-1), located adjacent to the 5' end of the rat liver S14 gene, is regulated by tissue-specific, developmental, nutritional, and hormonal factors. This research tested the hypothesis that sequences within Hss-1 bind transcription factors which regulate S14 gene transcription during these differing physiological states. Characterization of sequences within Hss-1 revealed 4 regions where rat liver nuclear proteins bound S14 promoter DNA. Binding of hepatic proteins to these regions either increased or decreased the basal level of *in vitro* transcription initiation. The effect of these promoter elements on transcription was also observed *in vivo*, both in the absence and presence of a distal enhancer.

Although S14 is transcribed at very low levels in kidney, renal nuclear extracts support *in vitro* transcription from S14 promoter constructs at rates comparable to hepatic nuclear extracts. NF-1 or a related protein in both renal and hepatic nuclear extracts contributes to *in vitro* transcriptional activity from the S14 promoter; however, renal nuclear extracts lack the factors present in liver which increase and decrease this basal level of transcription. Although nuclear factors interacting with S14

promoter elements were regulated in a tissue-specific manner, transcriptional activity of hepatic nuclear factors was not regulated during development or by dietary menhaden oil.

S14 gene transcription is regulated by glucocorticoids and retinoic acid in 3T3-L1 adipocytes. An S14 enhancer located between -1588 and -1381 bp conferred glucocorticoid and adipocyte-specific retinoic acid responsivity to homologous and heterologous promoters in 3T3-L1 cells. The location of this enhancer corresponds to that of rat liver Hss-2. Tissue-specific nuclear proteins bound two sequences within this enhancer designated TSE-1 and TSE-2. TSE-2 overlaps a region sharing high identity with the consensus glucocorticoid response element, and conferred adipocyte-specific glucocorticoid responsivity to the S14 promoter. Hormonal control from this distal enhancer was dependent on the presence of promoter elements.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

bp	basepair
CAT	chloramphenicol acetyltransferase
DEX	dexamethasone
DNase I	deoxyribonuclease I
DTT	dithiothreitol
EDTA	ethylenediamine-tetraacetic acid
FAS	fatty acid synthase
GFC	G-free cassette
GRE	glucocorticoid response element
Hss	hypersensitive site
kb	kilobase
M_r	relative molecular weight
n	number of samples
PMSF	phenylmethylsulfonyl fluoride
PUFA	polyunsaturated fatty acid
RA	retinoic acid
RAR	retinoic acid receptor
RARE	retinoic acid response element
RXR	retinoid X receptor
S.E.M.	standard error of mean
S14	spot 14
TRE	thyroid hormone response element
μCi	microcurie

INTRODUCTION

The ability of cells to metabolize exogenous substrates for energy is the basis for life. Accordingly, vast amounts of research time and money have been directed toward understanding this process. The traditional approach to understanding metabolic processes has been to study enzyme activities in response to various biochemical or physiological manipulations. However, with the advent of protein-specific antibodies, molecular biological techniques, and cloning of many genes involved in metabolism, researchers have been able to extend their analyses to include not only activity of enzymes, but also abundance of enzyme protein, abundance and stability of enzyme mRNA, and rate of gene transcription. Most recently, there has been a large effort to characterize and understand the trans-acting factors and cis-acting elements which regulate initiation of gene transcription. Using these approaches, we may be able to improve human health and lifestyle through understanding and altering our existing metabolism (eg. decreasing obesity). We may also be able to rectify genetic metabolic problems through careful insertion of missing or mutated genetic components under control of promoter/enhancer constructs that allow genes to be expressed and regulated correctly.

This general approach to understanding metabolism has

been applied to the study of S14. Effects of changing physiological state on S14 protein abundance, mRNA abundance and stability, and transcription rates have been carefully documented within the scientific literature. Recently, several laboratories have focused their attention on elucidating cis-acting elements responsible for thyroid hormone, glucocorticoid, retinoic acid (RA), insulin, cAMP, and carbohydrate regulation of transcription of the S14 gene. My dissertation focuses on understanding and characterizing transcriptional initiation and regulation from the S14 promoter and a S14 distal enhancer. It is likely that regulation of S14 gene transcription will be similar to hepatic genes involved in lipogenesis and glycolysis, since they are coordinately regulated. Therefore, information gleaned from research on S14 will have general significance.

CHAPTER 1: LITERATURE REVIEW

The object of this chapter is to review current literature on structure, function, and regulation of S14. I then summarize a portion of the immense amount of research on gene transcription in order to understand how transcription is initiated and regulated in other genes. Hopefully, this allows me to place my research on regulation of S14 gene transcription in context with what has been found in other models.

A. S14 Gene Structure

S14 is a cytosolic protein (17000 M_r, 4.9 pI) initially discovered and studied because of the very rapid response of $mRNA_{S14}$ to thyroid hormone in hypothyroid rat liver (Seelig et al., 1981, 1982). The rat S14 gene has been cloned and is present at 1 copy per haploid genome. The gene is 4.4 kb long and contains 2 exons and 1 intron (Narayan et al., 1984; Liaw and Towle, 1984). Translation is initiated from the ATG found at +23 bp relative to the start site of transcription. Nucleotides surrounding the translational start site correspond to 9 of 13 nucleotides in the GCCGCCA/GCCATGG consensus sequence described by Kozac (1987; Table 1) with no "translation breaking" nucleotides or obvious secondary structure to inhibit initiation of translation. The open reading frame is found

entirely within the 5' exon and codes for a protein of 150 amino acids. Two mRNA species of 1.2 and 1.37 kb are observed in rat due to the presence of 2 polyadenylation signals (AUUAAA and AAUAAA, respectively) in the 3' exon of the S14 gene (Liaw and Towle, 1984). The half-life of the mRNA in both rat liver and mouse 3T3-F442A cells is approximately 90 min (Kinlaw et al., 1987; Lepar and Jump, 1989). In contrast, the half-life in hepatocytes is approximately 5 h (Mariash et al., 1984). In addition to the gene, 11 kb of 5' flanking DNA have been cloned, of which 4.3 kb proximal to the S14 transcription start site have been sequenced. The chromosomal location of the S14 gene has not been reported to date.

Table 1: Comparison of the S14 translational start site with the Kozac consensus sequence.

Position[a]	-9	-8	-7	-6	-5	-4	-3	-2	-1	+1	+2	+3	+4
S14 seq.[b]	**G**	A	A	**G**	**C**	A	**G**	**C**	**C**	**A**	**T**	**G**	C
Freq.(%)[c]	33	19	23	44	39	25	36	49	55	100	100	100	16

[a] Position relative to the ATG start site of translation.
[b] Underlined bolded nucleotides indicate favorable comparison with the consensus sequence identified by Kozac (1987). The guanine in positions -3, -6 and -9 may help ribosomes start translation "in frame" (Trifonov, 1987).
[c] Frequency with which the S14 nucleotide is observed in a large population of actively translated eucaryotic genes. A 25% value would be expected on a random basis.

The chromatin structure of the S14 gene has been analyzed extensively as an aid in localizing prospective cis-

acting regulatory elements. Although no DNase I hypersensitive sites (Hss) are observed within the S14 gene, 6 Hss are observed in the S14 5' flanking region in adult liver. These Hss are located at approximately -65 to -265 bp (Hss-1), -1.5 kb (Hss-2), -2.0 kb (Hss-3'), -2.6 kb (Hss 3), -5.3 kb (Hss-4), and -6.5 kb (Hss-5) relative to the start site for S14 gene transcription (Jump et al., 1987; Jump, 1989a,b). Formation of several of these Hss is regulated by tissue-specific, developmental, nutritional, and hormonal factors. Presence of Hss-1, 2 and 3 is correlated with changes in S14 gene transcription (Jump, 1989a,b; Jump et al., 1988, 1990a). These data have been summarized in Table 2. Correlation between chromatin structure and gene transcription across a broad range of physiological states implicates sequences within these Hss as important targets for regulation of S14 gene expression. My dissertation is focused on defining the role of cis-acting elements within Hss-1 on initiation and regulation of gene transcription, and the role of elements within Hss-2 on tissue-specific hormonal control of gene transcription.

B. Function of S14 Protein

Although the precise function of S14 protein remains unknown, a body of circumstantial data supports a role in some aspect of lipid metabolism. S14 is abundantly expressed only in lipogenic tissues. Hepatic S14 is expressed at very low levels until the rat is weaned onto a chow diet. The hepatic response of S14 to thyroid hormone, insulin,

Table 2: Summary of S14 Chromatin Structure and Transcriptional Activity During Various Physiological States.[a]

Tissue/Physiological Manipulation	Hypersensitive Site 5	4	3	3'	2	1	Transcriptional Activity
Liver:							
a) Before Weaning							
< 15 days of age	+	+			+		Inactive
b) Adult							
Hypothyroid	+	+		+	+	+	Repressed
Euthyroid	+	+	+	+	+	+	Active
Hyperthyroid	+	+	+	+	+	+	Induced
Starved	+	+		+			Repressed
Starved/hypothyroid	+	+					Inactive
+ T3	+	+	+	+	+	+	Repressed
+ sucrose	+	+		+	+	+	Repressed
+ T3+sucrose	+	+	+	+	+	+	Active
Kidney:	+	+			+		Inactive
Spleen/Brain	+	+					Inactive
Lactating mammary gland	+	+	+	+	+	+	Active

[a] Based on a table presented in Jump (1989a).

glucocorticoids, adrenergic agonists and glucagon is very similar to regulation observed with lipogenic genes (e.g. fatty acid synthase (FAS), malic enzyme) and glycolytic genes (e.g. glucokinase, phosphofructokinase, pyruvate kinase) in liver. In addition, the response of S14 to nutritional manipulations such as starvation/refeeding, cafeteria feeding, or feeding high fat, or high carbohydrate/low fat diets closely follows the response of lipogenic enzymes/mRNAs

like FAS. Freake and Oppenheimer (1987) report that in rats, hypothyroidism results in an 80% decrease in $mRNA_{S14}$ in liver and epididymal fat, and that this is correlated with a decrease in lipogenesis. They also have shown that hypothyroidism induces a 3-fold increase in $mRNA_{S14}$ in brown fat which is correlated with an increase in lipogenesis in that tissue. These data provide a strong correlation between S14 and lipogenesis.

In contrast, other studies demonstrate that S14 is not absolutely required for fatty acid and triacylglycerol syntheses. First, S14 is not expressed in liver during fetal development, even though fetal liver is a lipogenic tissue (Perez-Castillo et al., 1987; Little and Hahn, 1990). Second, mouse 3T3-F442A and 3T3-L1 adipocyte-forming cell lines differentiate to the adipocyte phenotype (i.e. synthesize and store triacylglycerol) without S14 being expressed (Lepar and Jump, 1989; Jump et al., 1992). S14 expression in these cell lines depends on the adipocyte phenotype and the presence of hormones. Taken together, these studies suggest that S14 is involved in some aspect of lipid metabolism.

Efforts to identify the function of S14 by comparing DNA or amino acid sequences with proteins of known function have not been successful (Jump and Leykam, unpublished). Nor does the mRNA sequence contain standard motifs for cellular function such as a leader sequence for directing protein synthesis into the endoplasmic reticulum. This approach may

provide insight into the physiological function of S14 as structure and function of more proteins are elucidated.

S14 protein was localized to cytoplasm by radioactively labelling proteins with [^{35}S]-methionine and demonstrating that hepatic cytoplasmic proteins, but not nuclear proteins, contained S14 when purified and analyzed using 2-dimensional gel electrophoresis. Although polyclonal antibodies have been raised against both specific amino acid sequences (Kinlaw et al., 1989) and purified S14-protein A fusion protein (Planells et al., 1991), ultrastructural studies have not been performed to determine the precise cytoplasmic location of S14 protein. Such studies might provide clues as to the function of the S14 protein. Ultimately, a detailed analyses of cellular functions when S14 is overexpressed or its expression is blocked may be required to determine function of S14 protein.

C. Correlation between Regulation of S14 and Hepatic Proteins involved in Metabolism

The expression of hepatic genes involved in metabolizing glucose and synthesizing fatty acids is coordinated during various physiological states. Posttranslational modifications such as phosphorylation/dephosphorylation, and allosteric regulation by end-products or regulatory metabolites, play a very important role in acute minute to minute regulation of several enzyme activities (e.g. 6-phosphofructo-2 kinase/fructose-2,6-bisphosphatase, pyruvate kinase, and phosphofructokinase). However, all of the key metabolic enzymes are regulated chronically, to some extent,

by changing the enzyme abundance through regulation of the amount of protein synthesized (reviewed by Granner and Pilkis, 1990). Fasting, diabetes or glucagon (through cAMP) decrease, while feeding a high carbohydrate diet, or administering insulin, glucocorticoids, or thyroid hormone increase amount of hepatic glycolytic enzymes (e.g. glucokinase, phosphofructokinase, pyruvate kinase (Granner and Pilkis, 1990)); hepatic lipogenic enzymes (ATP-citrate lyase, acetyl CoA carboxylase, FAS, malic enzyme, glucose-6-phosphate dehydrogenase (reviewed by Towle and Mariash, 1986; Goodridge, 1987)), and S14 in a similar fashion. Although the amount, kinetics, and/or mechanism of the response may vary, the direction is invariant. In contrast, key hepatic gluconeogenic enzymes like cytosolic phosphoenolpyruvate carboxykinase or fructose 1,6 bisphosphatase are regulated in the opposite direction by these physiological manipulations, except for glucocorticoids and thyroid hormone (Granner and Pilkis, 1990). In general, regulation of amount of enzyme is at the level of transcription. Cis-acting elements responsible for glucagon/cAMP, carbohydrate, insulin, glucocorticoid, thyroid hormone, and RA have been characterized for many of these genes. Coordinate regulation of these classes of genes is likely because of shared regulatory mechanisms, therefore, information from the relatively well characterized S14 5'-flanking region has broad significance to regulation of genes involved in hepatic metabolism.

D. Regulation of S14 Gene Expression by Tissue Specific Factors

S14 mRNA (Jump, 1989a) and protein (Kinlaw et al., 1989) are expressed at high levels in tissues involved in lipid metabolism. S14 mRNA is present in liver at ca 1200 copies per cell. Other lipogenic tissues such as epididymal fat and lactating mammary gland have 6 and 2 fold higher levels of S14 mRNA expression, respectively (Jump, 1989a). In contrast, nonlipogenic tissues (e.g. testis, kidney and spleen) have S14 mRNA levels of less than 1% of that observed in rat liver. The S14 protein is expressed in a similar pattern to S14 mRNA with detection in liver and epididymal fat, but not in testis, kidney or spleen (Kinlaw et al., 1989). Jump (1989a) has demonstrated that tissue-specific regulation of S14 gene expression is at the transcriptional level, and is correlated with organization of the S14 5' regulatory region into chromatin. For example, Hss-1 is observed in tissues that express S14, (e.g. liver and lactating mammary gland), but not tissues in which S14 is not expressed (e.g. kidney and spleen; Table 2). This correlation between chromatin structure, and gene transcription strongly suggests that sequences within Hss-1 are involved in activation/maintenance of tissue specific gene expression. Wong et al. (1989) have reported that a protein designated P1 binds to a region -288 to -310 bp upstream from the start site of S14 gene transcription. This protein, P1, is present is liver but not other tissues including kidney and spleen. Interestingly, Wong et al.

(1989) did not observe this protein in epididymal fat or brown fat, tissues in which S14 is expressed.

E. Regulation of Hepatic S14 Gene Expression during Development

Hepatic S14 gene expression is regulated during postnatal development in the rat (Jump and Oppenheimer, 1985; Perez-Castillo et al., 1987; Jump et al., 1988; Clarke et al., 1990b). Hepatic S14 mRNA is expressed at very low levels throughout gestation and the postnatal period (<1% of adult hepatic values) until rats are spontaneously weaned at or around day 21. S14 mRNA then rapidly increases to near adult levels by day 22 postpartum. Early weaning of rat pups also stimulates hepatic S14 expression. Jump et al. (1988) have demonstrated that regulation of postnatal expression of S14 mRNA is due to activation of gene transcription, and that this increase in transcription is preceded by induction of Hss-1 (day 18) and Hss-3 (day 20; Table 2).

Induction of S14 mRNA during postnatal development can be influenced by dietary manipulation. For example, weaning pups onto a high carbohydrate diet at day 18 postpartum accelerates induction of S14 mRNA, while weaning pups onto a high fat diet sharply reduces the increase in S14 mRNA (Clarke et al., 1990b). These results suggest that weaning from a high fat milk diet to a chow diet may be the principle activator of S14 gene expression and thus might be responsible for the developmental control observed at weaning. Regulation of FAS during development is very

similar to S14 (Clarke et al., 1990b). Expression is negligible during gestation and suckling stages. At weaning, FAS mRNA increases dramatically to near adult levels at day 30. This process can be inhibited by feeding a high fat diet in a manner similar to S14. Thus, both S14 and FAS show similar control during postnatal development and in response to dietary manipulation.

F. Hormonal Regulation of Hepatic S14 Gene Expression

a) Thyroid hormone

Administration of a receptor-saturating dose of thyroid hormone induces formation of Hss-3 within 5 min (Jump, 1989b; Table 2). This change in chromatin structure is followed by an increase in S14 gene transcription (Jump, 1989b) resulting in increased S14 nuclear mRNA precursor at 10 min (Narayan et al., 1984), a 2-fold increase in mature mRNA by 15 min (Jump et al., 1984; Jump, 1989b) and an increase in S14 protein at 12 h (Kinlaw et al., 1989; Strait et al., 1989). When thyroid hormone is administered to euthyroid animals, a demonstrable increase in S14 protein is observed after 2 h (Kinlaw et al., 1989; Strait et al., 1989). The transition between hypothyroid and euthyroid states appears to influence S14 mRNA levels primarily at the level of transcription while the transition between euthyroid and hyperthyroid states is mainly posttranscriptional. Since binding of glucocorticoid receptors induce Hss formation in the mouse mammary tumor virus promoter (Zaret and Yamamoto, 1984), the rapid and dynamic induction of chromatin structure

in Hss-3 by thyroid hormone suggested that this region was a good candidate for binding of thyroid hormone receptors. The localization of three thyroid hormone response elements to this region was subsequently reported by Zilz et al. (1990) using transient transfection of COS-1 cells. They also reported that thyroid hormone receptors (c-erb Aα and ß) activate S14 gene transcription in cotransfection studies, and that *in vitro* translated c-erb A bind specifically to each of the S14 thyroid hormone response elements. This work was done with COS-1 cells, a monkey kidney cell line, in which S14 is not normally expressed. It may be important to repeat this work using transgenic animals, or at least a more physiologically relevant cell model (eg. primary hepatocytes). This would ensure that this is a general phenomenon, and not due to tissue-specific irregularities within COS-1 cells.

b) Insulin/glucagon

Regulation of the insulin/glucagon ratio influences expression of many genes involved in metabolism (reviewed by Goodridge, 1987) including S14 (Carr et al., 1984). Streptozotocin-induced diabetes represses S14 run-on transcription and mRNA levels to *ca.* 15% of intact levels (Jump et al., 1990b). Readministration of insulin induced S14 run-on activity and mRNA levels 5 and 8 fold by 1 h, respectively. Both transcription and mRNA levels were restored to intact values by 4 h. Administration of fructose to diabetic rats did not restore S14 transcription and mRNA

without administration of exogenous insulin. Since fructose can enter glycolysis in an insulin-independent fashion (through fructokinase), this suggests that most of the carbohydrate regulation of S14 gene transcription is mediated through changes in insulin/glucagon ratio, and not through a mediator induced by carbohydrate metabolism. However, activities of glycolytic enzymes are suppressed in the absence of insulin, and thus, fructose may not be metabolized at a rate sufficient to create physiological concentrations of the putative carbohydrate mediator of gene expression.

Glucagon and other hormones which increase intracellular concentrations of cAMP have proven to be strong negative effecters of S14 gene expression. Administration of glucagon to rats at 2000 h leads to a decrease in hepatic S14 mRNA (Kinlaw et al., 1986) through activation of protein kinase A (Kinlaw et al., 1987). This effect can be reversed by thyroid hormone (Kinlaw et al., 1988). Treatment of 3T3-F442A adipocytes with epinephrine or 8-(4-chlorophenylthio)-cAMP decreases the dexamethasone-induced increase in S14 expression (Lepar and Jump. 1989). In rat liver, the increase in cAMP appears to dominate over the positive effect of insulin since administration of dibutryl cAMP and theophylline with insulin to diabetic rats blocked the increase in S14 transcription and mRNA observed with insulin alone (Jump et al., 1990b). Alterations in insulin/glucagon ratio may well explain the decrease in S14 gene transcription and mRNA observed with 48 h starvation, and the rise

subsequent to a sucrose gavage (Jump et al., 1990a). This is because starvation decreases the insulin/glucagon ratio and sucrose gavage increases the insulin/glucagon ratio.

G. Regulation of Hepatic S14 and FAS Gene Expression by Dietary Fat

Nutrient control of gene expression has recently been recognized as an important regulator of homeostasis. Cholesterol feeds back to inhibit it own synthesis through a trans-sterol regulatory protein interacting with a sterol response element in the HMG-CoA reductase gene (Dawson et al., 1989). Some evidence also exists for a carbohydrate-induced mediator(s) of gene expression (Hamblin et al., 1989) that works through a poorly defined insulin-independent mechanism. Another dietary component that appears to have broad biological effects is dietary fat. Dietary fat influences hepatic lipogenesis by altering expression of apolipoprotein (Williams et al., 1989), LDL receptor (Fox et al., 1987), glucose-6 phosphatase dehydrogenase (Tomlinson et al., 1988), FAS (Wilson et al., 1986), and S14 (Blake and Clarke, 1990; Clarke et al., 1990a,b).

Dietary (n-3) and (n-6) polyunsaturated fatty acids (PUFA) are potent inhibitors of hepatic lipogenic enzymes and lipogenesis (Musch et al., 1974), but have no effect on other metabolic enzymes (e.g. phosphoenolpyruvate carboxykinase) or structural proteins (e.g. actin). Dietary saturated and monounsaturated fatty acids do not suppress hepatic lipogenic enzymes when present at similar dietary levels (Musch et al., 1974). Suppression of hepatic fatty acid synthesis is due to

a reduction in lipogenic enzymes, notably acetyl-CoA carboxylase and FAS (Toussant et al., 1981). The reduction in FAS protein is caused by decreased FAS mRNA (Clarke et al., 1990b) due to inhibition of FAS gene transcription (Blake and Clarke, 1990; Jump and Clarke, unpublished). The mechanism whereby PUFA regulate gene transcription is unknown, but may be mediated through modulation of hormonal pathways.

Hepatic S14 gene expression is regulated by specific dietary fats in weanling and mature rats. As previously mentioned, the increase in S14 mRNA associated with the dietary switch at weaning can be inhibited by weaning onto a high fat diet (68% of energy; Clarke et al., 1990b). However, this same effect can be mimicked by supplementing high carbohydrate diet with only 3% linoleic acid or 3% linolenic acid, but not with 3% palmitic acid or 3% oleic acid (Clarke et al., 1990b). Studies on mature animals have shown that addition of 10% dietary fish oil to a high carbohydrate diet decreases S14 mRNA abundance to ca 30% of that observed when 10% dietary triolein is added (Clarke et al., 1990b). The inhibition of S14 mRNA abundance by (n-6) or (n-3) PUFA is due to inhibition of S14 gene transcription. This is a hepatic-specific event since lipogenic enzyme activities, mRNA levels, and transcription rates are not regulated by PUFAs in other tissues, including white adipose tissue. Defining cis-linked targets for PUFA action on hepatic S14 gene transcription will be important for

understanding the hypolipidaemic effect of dietary fish oils.

H. Regulation of S14 gene expression by tissue-specific factors and hormones in adipocyte cell lines

In vitro models to study S14 gene expression include hepatocytes (Jacoby et al., 1989), COS-1 cells (Zilz et al., 1990), 3T3-L1 adipocytes, and 3T3-F442A adipocytes. Dr. Jump has used 3T3-L1 and 3T3-F442A cells to define cis-linked elements associated with the S14 gene. 3T3-L1 cells are an excellent cell line with which to study the regulation of S14 and other genes expressed in adipose tissue. The 3T3-L1 cell line was originally isolated and characterized by Green and Kehinde (1974). Under appropriate conditions, 3T3-L1 cells differentiate from a fibroblast (preadipocyte) phenotype into cells possessing an adipocyte phenotype. 3T3-L1 cells may truly be adipocyte progenitors since cells transplanted subcutaneously in nude mice in sites normally devoid of fat result in development of tissue indistinguishable from normal white adipose tissue (Green and Kehinde, 1979). Differentiation of 3T3-L1 cells is accompanied by acquisition of many adipocyte characteristics including a massive and dramatic increase in cytoplasmic triacylglycerol due to induction of enzymes involved in fatty acid and triacylglycerol synthesis, and an increase in responsiveness to lipogenic and lipolytic hormones, at least partially due to induction of insulin receptor (Green and Meuth, 1974; Mackall et al., 1976; Mackall and Lane, 1977; Coleman et al., 1978; Reed et al., 1981). Regulation of steady state levels

of mRNAs encoding these adipose-specific proteins is the major mechanism controlling protein synthesis rates during differentiation of 3T3-L1 cells into adipocytes. This increase in mRNA levels is due, almost exclusively, to increased rates of gene transcription (Kaestner et al., 1990; Bernlohr et al., 1985; Christy et al., 1991). Therefore, induction of adipose-specific mRNAs and proteins involved in conferring the adipocyte phenotype appears to be due to the coordinate activation or derepression of lipogenic genes.

S14 mRNA is expressed at < 0.5% of liver levels in both fibroblast and adipocyte phenotypes. However, treatment of 3T3-L1 cells (or a related cell line, 3T3-F442A cells) with either dexamethasone (DEX, a glucocorticoid agonist) or results in a large induction of S14 mRNA in adipocytes, but not in fibroblasts. When 3T3-L1 or 3T3-F442A adipocytes are stimulated by both DEX and RA, S14 mRNA and transcription are induced in a synergistic manner (Lepar and Jump, 1989,1992; Jump et al., 1992). A distal enhancer that confers responsivity to glucocorticoids and RA in the adipocyte phenotype has recently been identified (Jump et al., 1992). The location of this distal enhancer in both cell lines is between -1588 and -1069 bp from the S14 start point. In contrast to the endogenous gene, CAT constructs with S14 5'-flanking regions extending to -1601 bp or beyond show an increase in basal levels of CAT activity as 3T3-L1 cells differentiate into adipocytes. This adipocyte-specific induction is also observed with constructs utilizing only

enhancer sequences -1588 to -1069 bp, suggesting that a tissue-specific transcription factor binds within this enhancer and increases basal levels of transcription in adipocytes. Colocalization of glucocorticoid, RA and tissue-specific control to a distal enhancer suggests that this region may be a hotspot of regulatory activity. In light of the adipocyte-specific control of both glucocorticoid and RA action, and the strong interaction between hormones observed with the endogenous gene, analysis of this region will provide invaluable insight into mechanisms of tissue-specific hormonal control, and synergy between hormonal pathways. I further characterize and delineate tissue-specific hormonal control from this enhancer within my dissertation.

I. Regulation of Gene Expression

The expression of a gene leading to a biologically active protein involves many steps and introduces many potential sites for regulation. Early regulatible events in gene expression include initiation, elongation (Collart et al., 1991), and termination of transcription (Kerppola and Kane, 1991). In general, initiation of transcription is the principle stage at which gene expression is controlled. Intermediate events in gene expression including processing heteronuclear RNA by capping, polyadenylating, splicing, and then transporting the resulting mRNAs out of the nucleus and into cytoplasm. Later events in gene expression involve translation of mRNA into protein, which may fold into its active form, or be synthesized in an inactive form only to be

activated when secreted from cells, or when combined with other proteins. The biological activity of a protein can also be regulated allosterically, or by posttranslational modifications such as phosphorylation, acetylation, or glycosylation. Although all the events leading to expression of biologically active molecules can potentially be regulated, one of the most important sites of regulation for many genes, including S14, is initiation of transcription. Mechanisms for initiating and regulating transcription are complex with many levels of control. This subject will be elaborated on in the following section.

J. Initiation and Regulation of Gene Transcription

a) Formation of Initiation Complexes

Transcription is the process of generating a single stranded RNA molecule complementary to 1 strand of a double stranded DNA template (Lewin, 1990). The process of initiating transcription has been reviewed extensively (Mermelstein et al., 1989; Sawadogo and Sentenac, 1990; Roeder, 1991; Greenblat, 1991a,b). The enzyme which catalyzes the process of transcription for protein-encoding genes is RNA polymerase II. RNA polymerase II is a multi-subunit protein that separates double helical DNA strands and processes in a 5' to 3' direction down the gene incorporating ribonucleotides complementary to DNA bases into a strand of RNA. The initiation of this process involves a group of general initiation factors, including TFIIA, TFIIB, TFIID, TFIIE, TFIIF and RNA polymerase II, which are necessary for

creation of a preinitiation complex, and for correct positioning of the transcriptional start site. Information necessary and sufficient for positioning the start site in many genes is contained within a TATA box element which is usually found about 30 bp upstream from the start site of transcription. TFIID is a multisubunit protein which binds this TATA sequence specifically and stably in a reaction that may involve TFIIA and/or TFIIB (Dynlacht et al., 1991; Matsui et al., 1980; Buratowski et al., 1989). TFIIB may also be involved in promoting the binding of RNA polymerase II (Buratowski et al., 1989), while TFIIE may have ATPase activity (Sawadogo and Sentenac, 1990). TFIIF may have helicase activity (Sopta et al., 1989), and/or recruit binding of RNA polymerase II to preinitiation complexes (Flores et al., 1991). The exact role of these factors in initiation of transcription and clearance of the promoter is unknown. However, as with TFIID (Horikoshi et al., 1989, 1990; Hoey et al., 1990) cloning of the other general transcription factors (Sopta et al., 1989; Ha et al., 1991; Finkelstein et al., 1992), and analysis of structure/function relationships will lead to important information as to their role in this process. What is known is that assembly of this process <u>in vivo</u> is a rate limiting step, and that rate of assembly can be modulated greatly by general or gene-specific transcription factors that bind to proximal or distal control elements.

b) Promoters and Enhancers

The rate of preinitiation complex formation is modulated by ubiquitous, tissue-specific, and/or regulated transcription factors (eg. hormone receptors). Nuclear proteins influence transcription by binding to either modular cis-acting sequences of DNA traditionally defined as either promoter or enhancer elements. Promoter elements are modules of DNA sequences that are in the general vicinity of the transcriptional start site (usually spread out over >100 bp upstream of the start point), and are required for initiation of transcription (Lewin, 1990). Enhancer elements are usually defined as sequences of DNA which enhance transcription, but which are less dependent on orientation and distance from the transcriptional start site than promoter elements (Lewin, 1990). These definitions have become blurred because the same sequence of DNA when located close to the TATA box may act like a promoter (orientation-dependent), but when placed distal to the start site, may act more like an enhancer (orientation-independent; e.g. C/EBP). Because of this, Lewin (1990) stresses that each transcription complex should be assessed in terms of the individual modules and factors involved, rather than trying to distinguish between promoters and enhancers.

Within my dissertation, I refer to cis-acting sequences within Hss-1 as being promoter elements, even though many elements bound by transcription factors within this area are likely to have enhancer properties. I refer to cis-acting

sequences located outside of Hss-1 (and within other Hss) as being located in enhancers. Two S14 gene enhancers are located between -1588 and -1069 bp, and between -2.7 and -2.5 kb. Distal enhancers tend to contain a variety of DNA modules which bind transcription factors, and which work together to influence rate of transcription (Lewin, 1990)

c) **Transcription Factor Structure and Function**

In general, the proteins that regulate transcription by binding to proximal or distal control elements belong to families of structurally distinct transcription factors which can be classified according to common motifs for interacting with proteins or DNA. Transcription factors such as C/EBP, c-fos, and c-jun (Landschultz et al., 1988, Vinson et al., 1989; Curran and Franza, 1988) form dimers through use of a leucine zipper motif, while others, such as myoD, and Id (Murre et al., 1989; Benezra et al., 1990), use a helix-loop-helix structure. A recent class of nuclear factors has been found which contains both leucine zipper and helix-loop-helix domains. These transcription factors may have the ability to form higher order structures. Members of this family include AP-4 (Hu et al., 1990), TFEB (Fisher et al., 1991), c-myc (Blackwell, et al., 1990) and TFE3 (Beckmann et al., 1990). Finally, Forman and Samuels (1990) have proposed that nuclear hormone receptors, such as those for thyroid hormone, vitamin D and RA, utilize a novel mechanism which Forman and Samuels 1990) have termed "the regulatory zipper." Although many transcription factors can be classified into

these families, there appear to be many others which have, at this time, uncharacterized mechanisms for protein-protein interaction.

Several motifs have evolved for binding DNA including: a basic region associated with transcription factors containing leucine zippers or both helix-loop-helix and leucine zippers (Landschultz et al., 1988; Fisher et al., 1991), homeo/POU (e.g. Oct-3; Rosner et al., 1990), helix-turn-helix (reviewed by Brennan and Matthews, 1989), CYS_2-CYS_2 zinc fingers (eg. steroid receptors; reviewed by Forman and Samuels, 1990) and CYS_2-HIS_2 zinc fingers (eg. Gal4). In general, each family of transcription factors contains homologues, which are similar, but not identical (eg. C/EBPα, C/EBPβ, C/EBP gamma, and C/EBPδ; Cao et al., 1991). In a more extreme case, there are at least two families of RA receptors (RAR and RXR). Each family has at least three members (α, β, and gamma), some of which have several mRNA splice products (Giguere et al., 1988; Nohno et al., 1991; Leid et al., 1992; Mangelsdorf et al., 1992). It has recently been demonstrated that RXR family members can facilitate the binding of thyroid hormone receptors to DNA (Leid et al., 1992). However, the functional significance of multiple family members and multiple isoforms is unknown. It may be that specific family members regulate distinct classes of genes, are regulated differently by hormones or second messenger pathways, bind to different accessory factors, and/or share different tissue distributions.

The cloning of many transcription factors, and the genetic dissection of their functional domains has revealed a number of different types of domains involved in conferring transcriptional activation. Some of the functional motifs for trans-activation of transcription include glutamine-rich (SP-1; Courey and Tjian, 1988), proline-rich (NF-1; Mermod et al., 1989), acidic (VP16, TBE3; Beckmann et al., 1990; glucocorticoid receptors), and negatively charged amphipathic α-helical structures. These domains may influence the rate of preinitiation complex formation through direct interaction with factors involved in forming the preinitiation complex, or indirectly through adaptors, coactivators or mediators (reviewed by Martin, 1991). For example, the acidic domain of VP16 can interact directly with TFIIB (Lin et al., 1991) and enhance the binding of TFIIB during formation of the preinitiation complex (Lin and Green, 1991). However, VP16 may be an intermediary for the cellular transcription factor Oct I (Tanaka et al., 1988). Mechanisms for transcriptional activation can easily be visualized when the transcription factors are bound to proximal control elements (e.g. 20 to 100 bp away). However, functional analysis reveals that orientation independent enhancers can be located many thousands of nucleotide basepairs away from transcriptional start sites, and still regulate initiation of transcription.

d) Transcription Factor Binding Sites

Each transcription factor, and to a certain extent, each family of transcription factors, has a particular sequence or

group of gene-associated sequences to which it will bind with high affinity (reviewed by Jones et al., 1988; Wingender, 1988; and Polyanovsky and Stepchenko, 1990). Several to many transcription factors bind to regions associated with each gene, providing each gene with a particular complement of regulatory transcription factors which confer the specific tissue-distribution and regulatory properties observed for that gene. Transcription factors usually bind and regulate transcription from regions flanking the 5' end of the gene, but important regulatory elements have also been localized both within genes and in regions 3' to the gene (Courey et al., 1989; Zajac-Kayne and Levens, 1990; Sap et al., 1990). The location of specific transcription factor binding sites relative to the gene, and even distance between binding sites appear to be important for regulation of some genes, while not others. For example, Benoist and Mathis (1989) report that a B cell specific enhancer located about 2000 bp upstream from the MHC Class II gene functions in a tissue-specific manner when located at least 1000 bp away from the promoter, but not when cloned adjacent to the promoter. Indeed, distance between enhancer and promoter elements is much more conserved between MHC class II genes than is the sequence bound by transcription factors.

Generally, binding of transcription factors to DNA is stable with energy of interactions between 10-15 kcal/mol (corresponding to a K_d of about 10^{-10} M). In comparison, protein-protein interactions are much less stable. DNA-

protein interactions may be even more stable if neighboring regulatory sites are bound by transcription factors due to cooperative binding of transcription factors. Binding of one transcription factor may bend or otherwise alter the conformation of DNA in the vicinity of binding, and thus influence binding of vicinal transcription factors by altering the "fit" between DNA and protein (Kerppola and Curran, 1991). Alternatively, the stability of DNA-protein interactions might be influenced by protein-protein interactions with a neighboring transcription factor. While many sites bound by transcription factors are palindromic, two fold symmetry is not required for binding by all nuclear transcription factors (eg. APF; Cereghini et al., 1988).

The targeting of transcription factors to specific DNA sequences appears to be a daunting problem (reviewed by von Hippel and Berg, 1989), given that each cell contains 3 X 10^9 bp of DNA (therefore about 10^9 possible binding sites), and only on the order of 10^3 (e.g. thyroid hormone receptor; Jump et al., 1992) to 10^5 (e.g. NF-1; Lewin, 1990) molecules of a given regulatory nuclear factor. Rate constants suggest that transcription factors interact with their DNA sequence 1000 times faster than one would expect for a mechanism based on random diffusion. Current proposed mechanisms (von Hippel and Berg, 1989) hypothesize that nuclear transcription factors associate with DNA in a nonspecific fashion, and are then transferred strand to strand in an "intersegment" transfer process. Local DNA sequences are sampled for high

affinity binding sites through a one dimensional diffusional process called "sliding." This process may be quite limited *in vivo* due to blocking by histones or other proteins associated with DNA. Whatever the mechanism, targeting transcription factors to their cognant DNA sequences appears to be a rapid and efficient process.

The dogma that transcription factors bind to a particular sequence that shares identity with a consensus sequence is having to be modified. Work from several laboratories (Park et al., 1990; Christy et al., 1989, 1991; Dikstein et al., 1990; Stewart et al., 1990) has shown that recombinant purified C/EBP can bind to several apparently unrelated sequences in 422(aP2), stearoyl-CoA desaturase 1, alcohol dehydrogenase, and other promoters, and can activate expression of reporter genes from these sites in transient transfection assays (in some cases only if multiple sites or additional sequences are included).

Another recent development is the fact that although binding of transcription factors may be observed *in vitro*, this may not occur *in vivo*. Rigaud et al. (1991) compared *in vitro* and *in vivo* footprinting results in the rat tyrosine aminotransferase gene enhancer. They observed that recombinant C/EBP bound to two separate sites as defined using *in vitro* DNase I footprinting; however, *in vivo* footprinting did not detect footprints at these sites. Furthermore, they demonstrated that although HNF5 bound to the enhancer *in vitro*, production of HNF-5 *in vivo* footprints

was highly dependent on the presence of glucocorticoids. They concluded that glucocorticoids induced a change in chromatin structure that increased accessibility of the HNF5 binding site for binding by HNF5. The results observed in these experiments, that binding *in vitro* may not correspond to binding and function *in vivo*, have been observed in other model systems (Ikuta and Kan, 1991), and point to the fact that *in vitro* observations may be a good place to start an analysis, but that *in vivo* functional or structural analyses using stable transfection, transgenic models, and/or *in vivo* footprinting are critical for repeating and supporting these data.

An additional level of transcriptional control may be introduced by transcription factors sharing similar DNA-binding sites such that different transcription factors compete for the same site. For example, the consensus binding site for AP-1 differs by only one nucleotide from the consensus binding site for ATF/CREB and AP-1 binds ATF/CREB sites, albeit with lower affinity (Angel et al., 1987, 1988; Lee et al., 1987). In another example, Park et al. (1990) demonstrated that recombinant C/EBP bound to a cAMP response element, as well as C/EBP binding sites, while the cAMP response element binding protein bound only to its own element. Therefore, different binding specificities may allow for selective competition. Altering the cellular concentrations of one transcription factor would alter gene expression by two mechanisms: first by increasing it own

effect, and also by decreasing activity from the other transcription factor. In another case, transcription factors may not bind to other DNA elements, but competition is still created because the DNA elements overlap such that only one transcription factor can bind at a time due to steric constraints. These mechanisms allow for an extraordinary number of gene-specific regulatory mechanisms using a finite number of transcription factors.

e) **Interaction between Transcription Factors to form Higher Order Structures**

Transcription factors usually do not bind DNA and function as monomers, instead they interact physically and functionally to form homodimers, heterodimers, or tetramers with other members of their family or class of transcription factors. For example, C/EBPα, ß, and δ have been shown to form homodimers and heterodimers *in vitro* and as far as examined *in vivo* (Cao et al., 1991; Williams et al., 1991). In addition, jun can form homodimers or heterodimers with fos through leucine zipper interaction, and both dimer formations function *in vivo* (Sassone-Corsi et al., 1988; Schuermann et al., 1989). A model for the relative proportion of homodimers and heterodimers as a function of relative abundance of each factor has been proposed by Falvey and Schibler (1991). Higher ordered structures are also possible; for instance, SP-1 has been observed to form tetramers, which in some cases become stacked to form 12-mers (Mastrangelo et al., 1991). This type of formation can dramatically increase concentrations of glutamine-rich

transcriptional activation domain in the proximity of the preinitiation complex, and may partially be responsible for synergistic effects of transcription factors.

Finally, recent work has demonstrated that transcription factors can engage in "crosstalk" between classes of transcription factors. For example, physical and functional interaction has been shown between leucine zippers in fos/jun and glucocorticoid receptors. This interaction appears to be part of a general functional mechanism whereby steroid/thyroid receptors bind to fos/jun (AP-1) in a hormone dependent fashion, and block activation/maintenance of gene transcription by interfering with binding of fos/jun to AP-1 sites (Diamond et al., 1990; Yang-Yen et al., 1990). The converse also appears to be true, in that the constituents of AP-1, c-jun and c-fos, can inhibit the activation of genes by glucocorticoid or thyroid hormone receptors *in vivo*, and block binding of receptors to thyroid hormone response elements *in vitro* (Zhang et al., 1991; Touray et al., 1991).

f) Enhancer Structure and Function

Enhancers can be defined as *cis*-response elements which confer transcriptional control in an orientation independent fashion, and are located distal (>100 bp usually) from the site of initiation of transcription. The mechanism whereby enhancers located far from the start site of transcription can modulate initiation of transcription remains unclear; however, some hints as to possible mechanisms have recently been reported. The mechanism with the most experimental data

to support it is the looping model. Early definitive work was performed in procaryotic models such as the L-arabinose (Martin et al., 1986; Huo et al., 1988; Lee and Schleif, 1989), and lac operons (Griffith et al., 1986) of E. coli. Later work in eukaryotic systems strongly supported a similar looping mechanism. Using conventional and electron microscopy, Mastrangelo et al. (1991) have demonstrated that SP-1 dimers, bound to promoter sequences as well as distal enhancer sequences located 1700 bp downstream of the start site for the herpes simplex virus thymidine kinase gene, interact physically to form tetramers and higher order structures. The SP-1 sites had been implicated earlier as regions where SP-1 bound and synergistically increased transcription (Courey et al., 1989). In further support for the looping model, enhancers from SV40 or cytomegalovirus have been shown to interact with ß-globin promoter elements and activate transcription even when attached to promoter fragments via a protein avidin or streptavidin bridge (Müller et al., 1989), which presumably blocks sliding of proteins from enhancer to promoter sequences. Finally, Farnham and Means, (1990) have shown with an in vitro transcriptional system using a housekeeping gene promoter, dihydrofolate reductase, that enhancer elements can be active even if present in trans. Although it is easy to envision how enhancers located thousands of basepairs away can interact with promoter elements, and regulate the initiation of transcription, it is not easy to create a model in which

multiple enhancer elements located upstream, downstream and/or in the gene apparently interact directly with a single promoter fragment. Functional analyses of many genes reveal that many (10 to 15) proximal and distal control elements can influence rate of preinitiation complex formation, all presumably interacting with components of the preinitiation complex. Future experiments will be required to understand methods for overcoming what would appear to be a steric hindrance problem. Conceivably, one enhancer may function by physically interacting with, and regulating the effects from, another enhancer; however, there is no experimental evidence to support this hypothesis.

g) Complexity in Promoters and Enhancers

What has been surprising has been the incredible complexity of protein-DNA interactions in both promoters and distal enhancers. Approximately 200 DNA-binding transcription factors have been identified with more being reported every week. In contrast to the situation in procaryotes where transcription factors have stringent requirements for the DNA sequence bound, and usually no more than three regulatory transcription factors are found per gene, eucaryotic transcription factors have an imprecise mechanism for recognizing and binding their particular DNA binding sites, and as a result bind to many sites (Beardsley, 1991). Due to the large number of genes in eucaryotic genomes, it makes sense to have a series of transcription factors of various strengths and activities that respond to a

plethora of cellular and extracellular signals, rather than individual regulatory transcription factors for each gene. Presumably, the transcription initiation complex has some mechanism for "adding up" positive and negative signals. The sum total of switches turned on or off results in finely tuned transcriptional control. The system also appears to have a certain level of redundance built in such that the mutation of one DNA binding site, even one as important as the TATA or CAAT box, does not, in some cases, cause a demonstrable alteration in transcriptional regulation (Reach et al., 1991).

As an example of the complexity of promoters, work on the phosphoenolpyruvate carboxykinase promoter has implicated at least 10 regions where proteins bind the promoter (-460 to +73 bp). The identified protein factors are NF-1, HNF-1, RA receptor, thyroid hormone receptor, glucocorticoid receptor, C/EBPα, C/EBPβ, and cAMP response element binding protein as well as many unidentified nuclear factors (accessory factors). Together, these factors confer tissue-specificity, developmental control, and regulation by insulin, thyroid hormone, glucocorticoids, RA, phorbol ester, and cAMP (Ip et. al., 1989,1990; O'Brien et al., 1990, 1991; Liu et al., 1991; Trus et al., 1990; Quinn et al., 1988; Lucas et al., 1991a,b). This gene is also controlled by tissue-specific distal enhancers which bind additional transcription factors including HNF-3 (Ip et al., 1990). In another well studied example, the promoter for the c-fos gene

binds at least 10 proteins with many interacting within a 50 bp hotspot of regulatory DNA sequence called the serum response element to confer responsivity to a multitude of hormone, second messenger, and cell-cycle signals (Lucibello et al., 1991 and references therein).

h) Regulation of Transcription Factors

The formation of transcriptional preinitiation complexes is influenced by binding of transcription factors to neighboring regions of DNA. The amount and/or activity of these transcription factors is under stringent control by various intracellular and extracellular signals (reviewed by Falvey and Schibler, 1991). A common mechanism of tissue-specific and developmental control is regulating synthesis of transcription factors. This can be accomplished by controlling expression of transcription factors at the level of transcription initiation (eg. HNF-1; Frain et al., 1989; Mendal and Crabtree, 1991 and C/EBP isoforms; Cao et al., 1991; Xanthopoulos et al., 1991), or through posttranscriptional mRNA stabilization (eg. HNF-1 is transcribed in spleen, but no mRNA or protein are observed; Xanthopoulos et al., 1991). In addition, regulation of mRNA processing in both developmentally regulated (eg. LAP and LIP; Descombes and Schibler, 1991) and more constitutively expressed (NF-1; Santoro et al., 1988) transcription factors has been described. Finally a posttranslational modification such as phosphorylation has been implicated in nuclear localization of C/EBPß (Metz and Ziff, 1991), in the

activation of Oct-2 (Tanaka and Herr, 1990), and in the DNA-binding activity of E4F (Raychaudhuri et al., 1989) and c-Myb (Lüscher et al., 1990).

As discussed above, formation of transcription factor heterodimers can increase the number of DNA binding sites bound by a given transcription factor. Heterodimer formation also provides an additional level of possible regulation of transcription. For example, Id is a family member of the helix-loop-helix class of transcription factors (Benezra et al., 1990). Id contains a dimerization domain but lacks a DNA binding domain. When Id dimerizes with other transcription factors within the helix-loop-helix class, they lose their ability to bind DNA; therefore, Id acts as an inhibitor. A similar mechanism, through a nuclear protein called CHOP, has recently been described for inhibiting actions of C/EBP family members (Ron and Habener, 1992). Inhibiting transcription factors through this mechanism may be a common method for regulating families of transcription factors.

A further level of control is through sequestration of transcription factors. This has been demonstrated with the glucocorticoid receptor which, in the presence of ligand, binds fos and jun, and abrogates activity from AP-1 binding sites (Diamond et al., 1990; Jonat et al., 1990; Yang-Yen et al., 1990). This effect has also been observed with other members of the steroid/thyroid superfamily of receptors (Zhang et al., 1991).

i) The Role of Chromatin in Initiation and Regulation of Transcription

A further level of complexity is added to regulation of transcription initiation when packaging of DNA into chromatin is considered (reviewed by Kornberg and Lorch, 1991; Felsenfeld, 1992). It has been established that initiation of transcription is prevented *in vitro* by prior assembly of promoter DNA into nucleosomes (Knezetic and Luse, 1986; Lorch et al., 1987; Workman and Roeder, 1987), and that activator proteins may be able to relieve this inhibition (Croston et al., 1991). Inhibition of transcription by histones may also occur *in vivo* since blocking histone synthesis in yeast results in transcription of many genes that were previously inactive (Han and Grunstein, 1988). Accessibility of promoter DNA may be regulated by nucleosomal displacement, as observed experimentally by formation of a DNase I hypersensitive site (reviewed by Elgin, 1988, and Gross and Garrard, 1988). It is likely that nucleosomes must be completely displaced in order to accommodate the preinitiation complex of proteins.

The mechanism of nucleosomal displacement is unknown; however, the process appears to be facilitated by binding of transcription factors with acidic activation domains to promoter elements (Workman et al., 1991). Conceivably, a transcriptional activator may displace all proteins with low affinity (including histones), but allow proteins with high affinity to bind (including proteins involved in preinitiation complex formation; Kornberg and Lorch, 1991),

or an activator protein may contain an enzymatic activity which modifies histones and reduces their affinity for DNA through a posttranslational modification like acetylation. It has been hypothesized that NF-1 has an acetylase activity which plays a role in remodeling of chromatin and the formation of nucleosome-free regions (Oikarinen and Mannermaa, 1990). Finally, Chasman et al. (1990) havepurified a yeast protein, called General Regulatory Factor 2 (GRF2), which binds to GAL upstream activating sequence and creates a nucleosome-free region of about 230 bp. Although several possible mechanisms have been presented for creating DNase I hypersensitive sites based on *in vitro* studies, further experiments will be required in order to understand this process *in vivo*.

In the presence of nucleosomes, binding of activator transcription factors or hormone receptors to promoter or distal elements may be blocked or reduced (Archer et al., 1991). Several factors may limit the binding of transcription factors to nucleosomal DNA (Kornberg and Lorch, 1991). First, DNA sites recognized and bound by the protein may be obscured by wrapping of DNA around histone octomers. Second, even if DNA binding sites are exposed, DNA is bent around histones away from the transcription factor. Usually, proteins bind DNA that is in a linear conformation, or DNA that is bent around the transcription factor. Although most transcriptionally active genes have a DNAse I hypersensitive site (nucleosome-free area) in the promoter region, this is

not always observed. In cases where transcriptionally active genes do not have hypersensitive sites around the start site, it is unclear how transcription factors overcome poor accessibility to DNA caused by nucleosome formation. It may be that these proteins still interact with their partially exposed binding site, but with a much lower affinity, or it may be that positioning of nucleosomes is phased such that binding sites are located in linker regions or on the outward face of nucleosomes. It is also conceivable that DNase I Hss are not observed due to the binding of transcription factors and other proteins to the exposed DNA. While further work will be required to fully delineate mechanisms of protein-DNA interaction in the presence of nucleosomes, it is becoming clear that transcription factors may activate transcription by two mechanisms. Transcription factors may relieve the inhibition of transcription by histones, as well as directly stimulating formation of preinitiation complexes.

j) Mechanisms of RA Action

One of the metabolites which influences S14 gene expression is RA. RA is derived from vitamin A by a series of oxidations. Early research revealed that vitamin A did not have a coenzymatic role typical of many vitamins; instead, there seemed to be a similarity of action between retinol and steroid hormones (Reviewed by Ganguly et al., 1980). With the discovery of a number of nuclear RA receptors with membership in the steroid/thyroid hormone superfamily of receptors, the perception as to the mode of

action of RA was irrevocably altered from that of a vitamin to that of a hormone (reviewed by Wolf, 1990). It has been well established that RA has a profound effect on early development, skeletal growth, and cellular growth and differentiation (Wolf, 1984, 1990; Chytil, 1986; Melton, 1991; Blomhoff et al., 1990). Recently, Jump et al. (1992) proposed that RA also functions in regulation of intermediary metabolism. This is based on RA regulation of genes such as phosphoenolpyruvate carboxykinase, S14, transglutaminase, glycerol-3-phosphate dehydrogenase, alcohol dehydrogenase, and $G_s\alpha$ (Lucas et al., 1991a; Lepar and Jump, 1989; Chiocca et al., 1988; Duester et al., 1991; Chan et al., 1990), and supported by observations that both RA and RA receptors are located in metabolically active tissues such as liver and white adipose tissue (Haq and Chytil, 1991; Jump et al., 1992).

RA receptors appear to be similar to thyroid hormone receptors in that they are always associated with DNA. There are at least 6 different RA receptors, termed RARα, ß, gamma, and RXRα, ß, and gamma (Leid et al., 1992; Mangelsdorf et al., 1992; reviewed by Wolf, 1990). These interact with RA response elements as homodimers or heterodimers. In addition, RXRs can interact as heterodimers with other members of this class (eg. thyroid hormone receptors) to facilitate their binding to DNA (Kliewer et al., 1992; Leid et al., 1992). Binding of ligand activates receptor and relieves receptor-mediated repression of gene transcription

through a domain called τ_i, which is conserved between members of the family of nuclear receptors (Forman and Samuels, 1990). It is not known how τ_i influences rate of preinitiation complex formation. Although effects of RA on gene expression are commonly thought to be mediated through cis-linked RA response elements, expression of transcription factors including AP-2 (Lüscher et al., 1989), and Zif268 (Suva et al., 1991) is regulated by RA, and may result in indirect effects on genes regulated by AP-2 and Zif268. In addition, ligand-bound RA receptors have also been reported to bind fos and jun and thus abrogate transcriptional effects from AP-1 sites. Finally, through a direct effect on the expression of $G_s\alpha$, epidermal growth factor receptor, or interleukin receptor gene expression, an indirect effect on the action of these hormones, or hormones with plasma membrane receptors working through G-protein ($G_{s\alpha}$) might be attained.

k) Mechanisms of glucocorticoid action

Another hormone influencing expression of S14 is glucocorticoid. There are several mechanisms by which glucocorticoids might alter transcription of the S14 gene. The first mechanism involves binding of hormone to glucocorticoid receptor complexed with heat shock protein 90 in the cytoplasm. Binding of ligand induces dissociation of receptor from heat shock protein and receptor is subsequently translocated to the nucleus where it binds to glucocorticoid response elements. The binding of receptor may facilitate

direct activation of transcription through an acidic transcriptional activation domain, or induce an alteration in chromatin such that other transcription factors are able to bind their DNA sites and influence transcription. The second mechanism involves regulation of transcription of other transcription factors such as c-jun, which then influence transcription to become part of the effect of glucocorticoids. The third mechanism involves the ligand-dependent binding of glucocorticoid receptor to AP-1, and blocking c-jun/c-fos action from AP-1 sites. The final mechanism to be discussed is more speculative. Some effects of glucocorticoids and other steroids are so rapid (5 min) that they cannot be explained through effects on transcription. Some investigators have hypothesized that there are plasma membrane steroid hormone receptors which mediate rapid effects in some brain tissues, possibly through regulation of channel or protein kinase activities (reviewed by Touchette, 1990).

1) Summary

Initiation and regulation of gene transcription has been studied in great detail in various model systems. Therefore, there is a good background of scientific literature with which to compare and understand results obtained during analyses of S14 promoter and enhancer regions. Through this review of the scientific literature, it is obvious that mechanisms for initiating and regulating transcription are complex, with involvement of many factors and interactions

between factors. From what is known about S14, the mechanisms for initiating and regulating transcription promise to be equally involved.

K. Rational for current studies

The focus of my dissertation is to increase our understanding of initiation and regulation of S14 gene transcription. Several lines of evidence suggest that the promoter region is important. First, the promoter contains a DNase I Hss that is formed and maintained when the gene becomes transcriptionally active. The fact that Hss formation is regulated during postnatal development, by diet, and in different tissues suggests that sites within this Hss are important for regulation of S14 gene transcription in various physiological states. Second, sequence analysis reveals that S14 promoter sequences share identity with transcription factor consensus sequences. This implicates these transcription factors as potential targets for regulating S14 gene transcription. Third, the position of the promoter at the start site of transcription and the promoters unique role in initiation of transcription suggests that factors interacting within the promoter region will directly influence basal and perhaps regulated transcription.

Analysis of S14 promoter function is a technical challenge since results of transfection studies utilizing primary hepatocytes revealed that S14 promoter sequences -290 to +19 bp had little or no transcriptional activity (Jacoby et al., 1989). In order to gain insight into

transactivational activities of factors binding to promoter sequences, I employ an in vitro transcriptional assay utilizing rat liver nuclear extracts. I support this in vitro data with stable transfection analyses utilizing 3T3-L1 cells and promoter fragments alone or in the context of a S14 distal enhancer. These experimental approaches have allowed me to dissect some of the complex transcriptional regulation within this region.

While the S14 promoter is important for initiating S14 gene transcription, two upstream enhancers play a major role in augmenting and regulating gene transcription (Jump, 1988b; Jacoby et al., 1989; Zilz et al., 1990; Jump et al., 1992). One of these enhancers confers glucocorticoid, RA, and tissue-specific control to the S14 gene. I further delineate cis-acting elements within this enhancer. Finally, studies were initiated to understand the role of promoter elements in mediating glucocorticoid and RA control from a distal enhancer.

The specific aims of this dissertation are to: 1) define S14 promoter elements and determine their effects on initiation of S14 gene transcription; 2) determine if S14 promoter elements are sites for regulation during development, or by dietary or tissue-specific factors; 3) characterize and further define adipocyte-specific glucocorticoid and RA responsive regions in a S14 distal enhancer; 4) determine if there is a functional relationship between S14 enhancer and promoter elements. Although this

dissertation helps fill in one very small part of an exceedingly large story, these analyses will aid in understanding hepatic regulation of genes whose products are involved in metabolism.

CHAPTER 2: CHARACTERIZATION OF THE S14 PROMOTER: DNA-BINDING PROTEINS AND INITIATION OF *IN VITRO* TRANSCRIPTION

INTRODUCTION

The rat liver S14 gene is an excellent model to study multifactorial control of gene expression because S14 is transcriptionally regulated by tissue-specific (Jump, 1989a), developmental (Jump et al., 1988), nutritional (Hamblin et al., 1989; Jump et al., 1990a,b), and hormonal factors (Jump & Oppenheimer, 1984; Lepar & Jump, 1989; Jump, 1989b). S14 gene expression is high in tissues involved in triacylglycerol synthesis such as liver, white adipose tissue, lactating mammary gland and 3T3-F442A adipocytes (Jump et al., 1984; Jump & Oppenheimer, 1985; Jump, 1989a; Lepar and Jump, 1989). The pattern of physiological and tissue-specific control is correlated with that of lipogenic genes, and has led to the hypothesis that S14 protein functions in some aspect of lipid metabolism. Thus, the S14 gene serves as a model to understand regulatory networks involved in lipogenic gene expression.

In previous studies within Dr. Jump's laboratory, analysis of chromatin structure was used to locate prospective cis-acting elements controlling S14 gene transcription (Jump et al., 1987,1988,1990a; Jump, 1989a,b). While 6 DNase I hypersensitive sites (Hss) flank the 5' end of the hepatic S14 gene (Jump et al., 1990a), 2 of these

sites may be particularly important in S14 gene transcription, i.e. Hss-1 located between -65 and -265 bp and Hss-3 located between -2.55 and -2.75 kb. Formation of both sites is regulated by tissue-specific factors, during post-natal development, by dietary manipulation, and by thyroid hormone (Jump et al., 1987,1988,1990a; Jump, 1989a,b). Transfection analysis has shown that Hss-3 contains thyroid hormone response elements which function as upstream enhancers (Zilz et al., 1990). Formation of these Hss correlates with transcriptional activity of the S14 gene.

While highly regulated upstream enhancer elements (-2.55 to -2.75 kb) clearly play a major role in initiation of S14 gene transcription, significance of structural changes and cis-acting elements within Hss-1 remain unclear. Transient transfection of cultured hepatocytes with S14-chloramphenicol acetyl transferase (CAT) fusion genes suggest that constructs utilizing +19 to -290 bp of the 5' end of the S14 gene support very weak promoter activity (Jacoby et al., 1989); however, a detailed analysis of the promoter was not performed. To investigate the role of sequences within Hss-1 in initiating and regulating S14 gene transcription, I use DNase I footprint, gel shift and in vitro transcription initiation assays to identify and assess functions of prospective cis-acting elements. This approach has identified at least 4 prospective hepatic cis-acting elements within the S14 promoter. In addition, my experiments show that some of the transcription factors binding to S14 promoter elements

are expressed in a tissue-specific manner.

MATERIALS AND METHODS

Plasmid Constructions

S14 5' sequences are numbered relative to a transcriptional start site of +1. The pS14T1(.9) plasmid was created by insertion of a Taq I (-464 bp) - Taq I (+480 bp) fragment from the pEMBLS14-13E genomic clone (Jump et al., 1990a) into the Acc I site of pGem-1 (Promega Corporation). The S14 promoter region was isolated by cutting pS14T1(.9) with Sst I and Xba I, treating with exonuclease III and ligating (Henikoff, 1984). The resulting plasmid, pS14T1(.9)7b, contained S14 promoter sequences -464 to -8 bp relative to the S14 transcriptional start site. Restriction of pS14T1(.9)7b with Pst I (cuts at -290 bp and the 5' cloning site) and EcoR I (cuts at the 3' cloning site) released 2 fragments which were subsequently isolated and cloned into pGem-1 to form pS14(-464 to -285) and pS14(-290 to -8). pS14(-290 to -8) was subcloned by isolating the insert, restricting with BstN I (-167, -155, and -152 bp), treating with S1 nuclease, and ligating the resulting fragments into pGem-1 to form pS14(-151 to -8) and pS14(-290 to -168). The insert from pS14(-151 to -8) was isolated, restricted with Hae III (cuts at -88 bp) and ligated into pGem-1 to create pS14(-87 to -8) and pS14(-151 to -88).

Plasmids containing the G-free cassette, $p(C_2AT)_{19}$ (or GFC), and the adenovirus-2 major late promoter (-400 to +10 bp) adjacent to the GFC, $pML(C_2AT)_{19}$, were generously

provided by Dr. R. Roeder (Rockefeller University; Sawadogo & Roeder, 1985). The 3' cloning site in $p(C_2AT)_{19}$ was removed by restricting with BamH I and Hind III, treating with S1 nuclease, and religating to form pGFC(-BH). pS14-GFC1 was prepared by installing EcoR I linkers (Bethesda Research Laboratories) on pS14T1(.9)7b to form pS14T1(.9)7b(EE) and cloning the resulting insert into the EcoR I site of pGFC(-BH). pS14-GFC2 was prepared by installing Sst I linkers on pS14(-290 to -8) and cloning the resulting insert into the Sst I site of pGFC(-BH). pS14-GFC3 and pS14-GFC4 were prepared by ligating inserts from pS14(-151 to -8) and pS14(-87 to -8), respectively, into pGFC(EH). pGFC(EH) was prepared by restricting pGFC(-BH) with Sst I, blunting ends with S1 nuclease, and installing a Hind III linker (Bethesda Research Laboratories). The GFC in $pML(C_2AT)_{19}$ was truncated by restricting with Pst I and BamH I, treating with exonuclease III, and religating. The truncated GFC (pML-GFC2) is about 300 bp long, and transcription products can be resolved on acrylamide sequencing gels or agarose northern gels. Manipulation and cloning of DNA was performed according to Sambrook et al. (1989).

Oligonucleotides

NF-1, SP-1, and AP-3 oligonucleotides were obtained from Stratagene. The NF-1 sequence consists of the adenovirus origin of replication with a single mutated basepair which increases NF-1 binding by four fold (Rosenfeld et al., 1987).

Animal Manipulation

Sprague-Dawley rats (125 to 250 g) were obtained from Charles River (Kalamazoo, MI), and were used in all experiments. Rats were housed at Michigan State University and cared for by University Lab Animal Resources. The animals were kept on a 12 h light: 12 h dark cycle, and were maintained on a stock diet (Teklad) and water ad libitum. All experiments were initiated between 0700 and 0900 h. Animals were anesthetized with ether or CO_2 prior to exsanguination (abdominal aorta) or decapitation, respectively. Liver, kidneys, and/or spleens were rapidly excised and placed in ice cold 0.1 M NaCl. Adipose and connective tissues were removed prior to weighing and further preparation.

Isolation of Nuclei

Hepatic nuclei were isolated essentially as described by Gorski et al. (1986). The procedure was modified to accommodate preparation of nuclear extracts from 6 livers simultaneously. All steps were performed on ice or at +4°C using ice cold buffers, tubes and centrifuges. Each liver (10 to 18 g) was minced, then homogenized in 23 ml of homogenization buffer (10 mM Hepes; pH 7.6, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 10% glycerol, 0.5 mM dithiothreitol (DTT), and protease inhibitors phenylmethylsulfonylfloride (PMSF; 1mM), benzamidine (1mM), leupeptin (0.5 μg/ml), and pepstatin A (1 μg/ml). DTT and protease inhibitors were added just before

homogenization. Homogenate was layered over a 10 ml cushion of the same buffer and spun at 24000 rpm in a SW27 roter for 45 min at -2°C. Nuclear pellets were resuspended in 25 ml of a 9:1 (v/v) mixture of homogenization buffer and glycerol, layered over 10 ml cushions of this new buffer, and centrifuged as above. Pelleted nuclei were resuspended in 6 ml lysis buffer. A 5 μl aliquot was diluted 1:200 in 0.5% SDS, and absorbance measured at 260 nm. The nuclear suspension was diluted to 10 - 20 A260/ml lysis buffer prior to extraction of nuclear proteins. In one gel shift experiment, hepatic nuclei were also isolated by the method of Hewish & Burgoyne (1973) as modified by Jump et al. (1987).

Renal and splenic nuclei were prepared essentially as described by Gorski et al. (1986). To obtain nuclei from which transcriptionally active extracts could be prepared, 15 g kidney or 3 g spleen were minced, homogenized in 2 groups (eg 7.5 g kidney in 23 ml homogenization buffer), and the total volume diluted to 69 ml with homogenization buffer. After mixing, homogenate was layered into 3 tubes, each containing a 10 ml cushion of the same buffer. The rest of the procedure was performed as described for liver except that the 3 nuclei pellets from the first centrifugation were resuspended in 50 ml and combined into 2 tubes for the second centrifugation.

Preparation of Nuclear Extracts

Extracts from hepatic, renal or splenic nuclei were

prepared essentially as described by Parker and Topol (1984) as modified by Gorski et al. (1986). All buffers were ice cold, and unless otherwise stated, all procedures were on ice or at +4°C. Briefly, nuclei were resuspended in lysis buffer (10 mM HEPES; pH 7.6, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 10% glycerol) and diluted to 10 - 20 A260/ml. 0.1 volume of 4 M $(NH_4)_2SO_4$; pH 7.9 was added dropwise to condense chromatin, and the solution was inverted periodically for 30 min. Chromatin was pelleted at 45000 rpm in a Ty65 roter for 90 min. Supernatant was removed and supernatant volume measured. Solid $(NH_4)_2SO_4$ was added to 0.3 g/ml supernatant and allowed to dissolve over 10 - 12 min at room temperature before being placed on ice for 30 min. Nuclear proteins were pelleted at 45000 rpm in a Ty65 roter for 20 min. When smaller volumes were being prepared, centrifugations were performed at 40000 rpm in a SW50.1 rotor. Pelleted nuclear proteins were resuspended at 1 ml/800 A260 units for liver and kidney, and at 1 ml/1600 A260 units for kidney and spleen, and dialyzed for 4 h against a buffer containing 25 mM HEPES; pH 7.6, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol with 1 change of dialysis buffer at 2 h. Samples were spun at 8000 X g for 5 min to pellet denatured precipitated proteins. Supernatant protein concentrations were determined using a Bio-Rad Laboratories protein assay reagent with bovine serum albumin (Sigma) as the standard. Protein concentrations of at least 5 mg/ml were necessary to initiate transcription <u>in vitro</u>. Recovery

of nuclear proteins was about 0.14 mg/g of liver, 0.05 mg/g kidney, and 0.3 mg/g spleen. Ten μl aliquots were stored at -80°C for up to 6 months without appreciable loss of gel shift or *in vitro* transcriptional activity.

DNase I Footprint Analysis

This assay was performed essentially as described by Galas and Schmitz (1978). Genomic DNA fragments to be footprinted were isolated from plasmids by digesting DNA with appropriate restriction enzymes. DNA fragments were purified by electrophoresis and electroelution. Some DNA inserts were further purified using MermaidTM Kit (BIO 101). DNA fragments were end-labelled with the Klenow fragment of DNA polymerase using [α-^{32}P]dCTP or with T_4 polynucleotide kinase using [gamma-^{32}P]ATP. The [^{32}P]-labelled insert was often gel purified on a 6% acrylamide gel to obtain cleaner results. DNA-protein complexes were formed by incubating end-labelled DNA (1 to 3 fmol) for 30 min at 30°C in a reaction mixture containing 0 to 9.0 μg nuclear extract, 25 mM Tris-HCl; pH 7.5, 10% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 100 mM KCl, 0.2 mM PMSF, and 2 mg poly d(I-C) (Boehringer-Mannheim; Johnson et al., 1987). DNase I (Pharmacea) was then added to 6.5 U/ml, and samples incubated on ice for 10 to 90 s. Reactions were terminated by adding 80 μl of termination buffer to make final concentrations of 20 mM EDTA, 1.5% sodium dodecyl sulfate, 1.4 M ammonium acetate, and 500 μg/ml yeast t-RNA. Mixtures were incubated at 85°C for 3 min, extracted with phenol/chloroform (1:1

saturated with TE-8) and chloroform/isoamyl alcohol (24:1), and DNA precipitated with 200 µl ethanol. After incubation at -80°C for 10 min, samples were spun in a centrifuge (Sorvall RC-5B) at 8000 X g for 10 min at 4°C. Pellets were rinsed with 70% ethanol, dried, and resuspended in 10 µl of DNA loading buffer (1X-TBE, 85% deionized formamide, and 0.08% bromophenol blue). Samples were heated to 90°C for 3 min before being cooled on ice and loaded on an 8% acrylamide-8M urea sequencing gel. Gels were dried and autoradiographed at -80°C with intensifying screens. A dimethyl sulfate sequencing reaction was performed Maxam and Gilbert (1980) as outlined by Sambrook et al. (1989), and electrophoresed in parallel with the DNase I footprint. This procedure cleaves [^{32}P]-labelled DNA at guanines and allows DNase I footprints to be localized to specific sequences.

Gel Shift Analysis

This assay was performed essentially as described by Garner and Revzin (1981), and Fried and Crothers (1981). DNA-protein complexes were formed as described above for DNase I footprinting. After reactions were completed, 6 µl reaction buffer containing 0.16% bromophenol blue and 0.16% xylene cyanol was added to DNA-protein complexes just prior to loading on a 6% polyacrylamide gel [acrylamide-bisacrylamide weight ratio, (29:1)] with 90 mM Tris-borate, 2.5 mM EDTA, pH 8.3 (TBE) as buffer. After electrophoresis at 100 V, gels were dried and exposed to X-ray film (Kodak X-OMAT AR) at -80°C with intensifying screens.

In Vitro Transcription Analysis

Transcription reactions were performed essentially as described by Gorski et al. (1986). The reactions (20 µl) contained 25 mM Hepes; pH 7.6, 50 mM KCl, 6 mM $MgCl_2$, 0.6 mM ATP and CTP, 35 µM UTP, 7 µCi [α-^{32}P]UTP (Dupont), 0.1 mM 3'-O-methyl guanosine triphosphate (Sigma), 10% glycerol, 30 units RNAsin (Promega), 1.2 µg of pS14-GFC, 0.1 µg of pML-GFC2, 45 µg nuclear extract, and 15 units T1 RNase. EDTA (35 µM) and DTT (0.35 mM) were contributed by nuclear extract and were held constant by adding nuclear extract dialysis buffer to 7 µl. Reactions were terminated after 30 min by addition of 380 µl of Stop Solution (50 mM Tris-Cl; pH 7.5, 1% SDS, and 5 mM EDTA), and 4 µl of yeast tRNA (10 mg/ml). Solutions were extracted 3 times with chloropane (phenol and chloroform [1:1] saturated with 10 mM sodium acetate; pH 5.0, 0.1 M NaCl, 1 mM EDTA). In the final extraction, 300 µl of supernatant were carefully removed to a clean 1.5 ml microfuge tube and RNA precipitated with 30 µl of 3 M sodium acetate; pH 5.0, and 750 µl ethanol at -80°C for 10 min. After centrifugation (8000 X g for 10 min), pellets were rinsed with 150 µl of 70% ethanol, air dried, and resuspended in 10 µl loading buffer (85% formamide and .08% bromophenol blue in 1X-TBE). Samples were heated to 90°C for 3 min before cooling on ice and loading onto a 6% acrylamide-8 M urea sequencing gel. Gels were electrophoresed at 43 W for 80 min before drying on a slab dryer (BioRad; Model 443) and autoradiography at -80°C with intensifying screens. Bands

were quantified using laser densitometry.

Statistical Analysis

Differences in *in vitro* transcriptional activity of S14 promoter constructs were evaluated using the Bonferroni t-statistic (Gill, 1985).

RESULTS

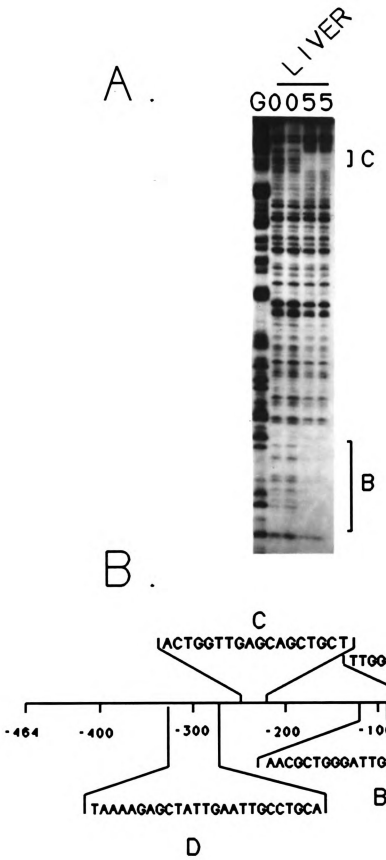
DNase I Footprint Analysis of S14 Promoter Fragments

To localize regions where hepatic nuclear proteins bind S14 promoter sequences, I performed DNase I footprinting analysis on DNA fragments extending from -464 to -8 bp. Fig. 1A illustrates 2 DNase I footprints at -113 to -88 bp (Region B) and -244 to -227 bp (Region C). I also identified footprints at -63 to -48 bp (Region A) and -310 to -286 bp (Region D; not shown). These latter footprints have been reported previously by Wong et al. (1989,1990) as PS-1 and P1, respectively. Core regions of protection for each footprint were confirmed by footprinting the opposite strand. Outer limits of protection for coding and noncoding strands for each footprint are reported in Fig. 1B. In addition, overlapping DNA fragments were analyzed to ensure that binding of a particular liver factor was not disrupted by a cloning site (not shown). Footprinted sequences do not share substantial identity, suggesting that different proteins interact with each of these binding sites. While this analysis locates 4 prominent DNA-protein interactions within 464 bp of the 5' end of the S14 gene, it does not exclude the possibility that other interactions involving low abundance

Figure 1. DNase I Footprint Analysis of the S14 promoter.

A) ^{32}P-labelled noncoding strand of DNA fragment -290 to -60 bp (Pst I and Aha II) digested with DNase I in the absence (0) and presence of 5 μg (5) of hepatic nuclear protein (Materials and Methods). Digested products were electrophoretically separated and autoradiographed. Bracketed Regions B and C identify 2 DNase I footprints. A guanine (G) sequencing ladder was prepared by reacting the ^{32}P-DNA fragment with dimethyl sulfate (Maxam and Gilbert, 1980).

B) Summary of DNase I protection by liver nuclear extracts. Footprints are designated as protected regions A through D. Sequences shown are the outside boundaries obtained when coding and noncoding strands were footprinted. Protected regions A, B, C, and D extend from -63 to -48 bp, -113 to -88 bp, -244 to -227 bp, and -310 to -286 bp, respectively. Footprinted regions are positioned upstream from the S14 transcription start site (→).


A.
LIVER
G0055
C
B
B.
C
ACTGGTTGAGCAGCTGCT
A
TTGGCGTCCTGTCAATJ
S14
-464
-400
-300
-200
-100
AACGCTGGGATTGGCTCAAAACAAGGT
B
TAAAAGAGCTATTGAATTGCCTGCA
D

proteins may exist within this area.

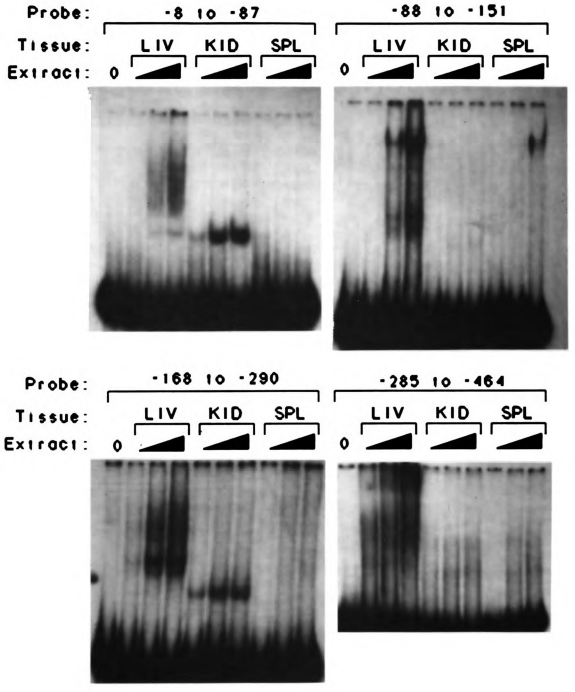
Tissue-Specific Binding of Nuclear Proteins to S14 Promoter Fragments

DNase I footprint analysis revealed 4 different regions recognized by hepatic proteins. To determine whether nuclear proteins from tissues not expressing S14 bind to S14 promoter sequences, I examined DNA-protein interactions using nuclear extracts from spleen and kidney. Four separate DNA fragments, each containing 1 DNase I footprint, were subcloned and used in gel shift assays with nuclear extracts from liver, kidney and spleen (Fig. 2). As expected, hepatic nuclear extracts contained proteins that bound to each of the 4 DNA fragments tested. Gel shift patterns were characterized by both discrete bands and smears. Smeared DNA-protein complexes may represent multiple protein interactions with DNA, dissociation of DNA-protein complexes during electrophoresis, or proteins interacting with DNA-binding proteins. Although DNase I footprint analysis detected only 4 footprints, the more sensitive gel shift analysis suggests protein interaction with S14 promoter sequences may be more complex.

Gel shift patterns using extracts from kidney and spleen were quite different when compared to liver. Kidney extracts promoted shift of -87 to -8 bp and -290 to -168 bp fragments as discrete bands, while they caused little shifting of -151 to -88 bp or -464 to -285 bp fragments. Spleen extracts induced little or no shifting of any of these fragments.

Figure 2. Gel Shift Analysis of S14 Promoter Fragments.

Four probes (-87 to -8 bp, -151 to -88 bp, -290 to -168 bp and -464 to -285 bp) were end labelled with ^{32}P and mixed with nuclear extracts from liver (LIV), kidney (KID) and spleen (SPL). Lane (0) represents the mobility of the DNA fragment without added protein. The amount of protein added to each reaction varied with the DNA fragment used: -87 to -8 bp: 1, 3, 6 μg protein; -151 to -88 bp: 1.5, 3.0, 4.5 μg protein; -290 to -168 bp: 1.5, 3.0, 4.5 μg protein; -464 to -285 bp: 3, 6, 9 μg protein. For each fragment tested, equivalent amounts of nuclear proteins from each tissue were added to the gel shift reaction. Each assay was repeated with 3 to 6 different preparations of liver, kidney and spleen. Representative autoradiographs are presented.


Probe:
-8 to -87
-88 to -151
Tissue:
LIV
KID
SPL
Extract:
0
Probe:
-168 to -290
-285 to -464
Tissue:
LIV
KID
SPL
Extract:
0

This analysis clearly shows that interaction of nuclear proteins with S14 promoter sequences is tissue-specific.

Characterization of DNA-Protein Interaction within the S14 Promoter

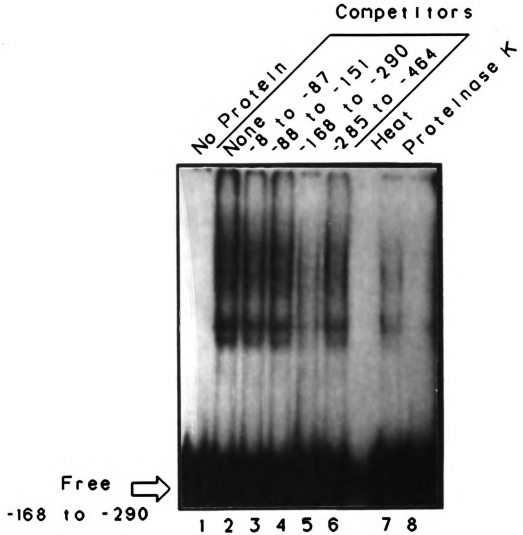
I further characterized interactions between hepatic nuclear factors and S14 promoter fragments by assessing sequence specificity of binding as well as heat and protease sensitivity. While this analysis was performed on each of the 4 DNA fragments used in Fig. 2, results are illustrated in Fig. 3 using only S14 promoter fragment -290 to -168 bp as representative of the others.

Fig. 3 illustrates the pattern of shifting with hepatic nuclear extracts and the -290 to -168 bp region in the absence (lane 2) and presence (lanes 3-6) of unlabelled competing DNAs. While hepatic extracts induced the same pattern as illustrated in Fig. 2, addition of a 500-fold molar excess of unlabelled -290 to -168 bp (lane 5) greatly diminished the fraction of [32P]-labelled -290 to -168 bp fragment shifted. In contrast, addition of 500-fold molar excess unlabelled heterologous DNA (-87 to -8 bp, lane 3; -151 to -88 bp, lane 4; -464 to -285 bp, lane 6) did not influence gel shift profiles. Similar results were observed for the other 3 DNA fragments. Therefore, binding of hepatic nuclear protein(s) to all 4 S14 promoter fragments is sequence specific.

Whereas all shifting of DNA fragments was sensitive to prior proteinase K treatment (Fig. 3; lane 8), proteins shifting the various fragments were differential heat

Figure 3. Characterization of DNA-Protein Interaction within the -290 to -168 bp S14 Promoter Region.

Gel shift analysis was performed using the -290 to -168 bp fragment as described in Fig. 2 and in Materials and Methods. In lane 1, no hepatic protein was added. In lanes 2 - 6, a 500-fold molar excess of competitor DNA was incubated with nuclear extract prior to addition of 1 fmol 32P-labelled DNA fragment -290 to -168 bp: lane 2, no competitor; lane 3, -87 to -8 bp; lane 4, -151 to -88 bp; lane 5, -290 to -168 bp; lane 6, -464 to -290 bp. In lane 7, liver nuclear extract was boiled for 5 min and centrifuged before addition to the reaction mixture. In lane 8, 10 μg proteinase K was added to liver nuclear extract before addition of labelled DNA.


Competitors
No Protein
None
-8 to -87
-88 to -151
-168 to -290
-285 to -464
Heat
Proteinase K
Free
-168 to -290
1 2 3 4 5 6 7 8

sensitive. For example, boiling hepatic extracts for 5 min only partially reduced shifting of the -290 to -168 bp fragment (lane 7). However, shifting of -87 to -8 bp, -151 to -88 bp, and -464 to -285 bp fragments was fully sensitive to heat treatment (not shown). RNase A treatment did not decrease binding to S14 promoter fragments (not shown). Based on these studies, hepatic nuclear proteins which interact with S14 promoter DNA are sequence specific and differentially heat sensitive.

Characterization of the In Vitro Transcriptional Assay

Although DNase I footprint and gel shift analyses indicate that hepatic nuclear proteins bind at 4 loci upstream from the S14 transcription start site, no information was available on the role these proteins play in S14 gene transcription. To assess the function of these elements, I used an *in vitro* transcription initiation assay described by Gorski et al. (1986). S14 promoter elements were fused to the GFC reporter gene and used in assays to examine initiation of transcription. Sequence and structure of promoter templates analyzed is illustrated in Fig. 4.

Characterization of the assay showed that initiation of transcription from S14 promoter deletions was time dependent and linear up to 60 min for all 4 S14 promoter constructs tested (Fig. 5A). Interestingly, *in vitro* transcription from the major late promoter was curvilinear, with a gradual rise in rate of transcription until 30 min, at which time overall rate of transcription increased and was linear until 60 min

Figure 4. Sequence of the S14 Promoter and Structure of DNA templates used in the Transcription Initiation Assay.

A) DNA sequence of the S14 promoter from -480 to +60 relative to the start site of transcription (+1). DNase I footprints are overlined and labelled (Regions A-D). Restriction sites utilized during cloning are underlined and labelled.

B) Structure of the promoter-template constructions used in the in vitro transcription assay. Protected regions D, C, and B were sequentially deleted from the S14-GFC construct by using convenient restriction sites. The S14-GFC template is 400 nucleotides in length. The structure of these templates is illustrated. The G-free cassette (designated by hatching) in pML$(C_2AT)_{19}$ was truncated from the 3' end to about 300 bp to form pML-GFC2.

A.

```
-480 CTTCTGGCCCTGGCTTCGAAATGCTTTGTCCTGTACAGAGCCTGTTCCAGTTCAGGTTAC -421
                    Taq I

-420 TGCTTCCTCTGTTCGTTTCACAGCTAGGTACCCAGGCCGAGAGAGTGCAGCTGTGTGAGT -361

-360 TAGGAGGCAAGGAAGCAGGCAGCAAAACGGTAACTTGACTTCAGGTAACCTAAAAGAGCT -301

      Region D
-300 ATTGAATTGCCTGCAGTCAAGTGTACTGGGTGCCAAGGGGACACAGCTTCCTTCTAACTG -241
                 Pst I
      Region C
-240 GTTGAGCAGCTGCTAAGAAGAGTTGGCCGCCCACTGAGGCAGTCATGCAGACCTGAAGTG -181

-180 ACAAGCAGAAGCCTGGCCAGGTTTGTCCCTGGGTAGATGGATCGCCTGATACGGACACTG -121
               BstN 1          BstN I
                    Region B
-120 GCGACCAAACGCTGGGATTGGCTCAAAACAAGGCCGTGTTGATCCAGTGACTGGGTTTTG -61
                                  Hae III
      Region A
 -60 GCGTCCTGTCAATCTGCTGTCTGCTCAAAAGCCTAGAAATAGTGCGGGGGCAGTTTGCTG -1
     Aha II

  +1 GTCTCTGAGAAAGGAAGCAGCCATGCAAGTGCTAACGAAACGCTACCCCAAGAATTGCCT +60
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B.

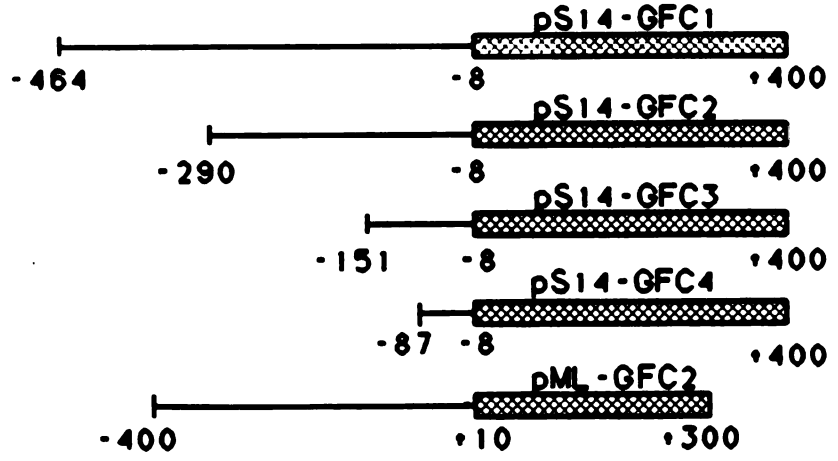
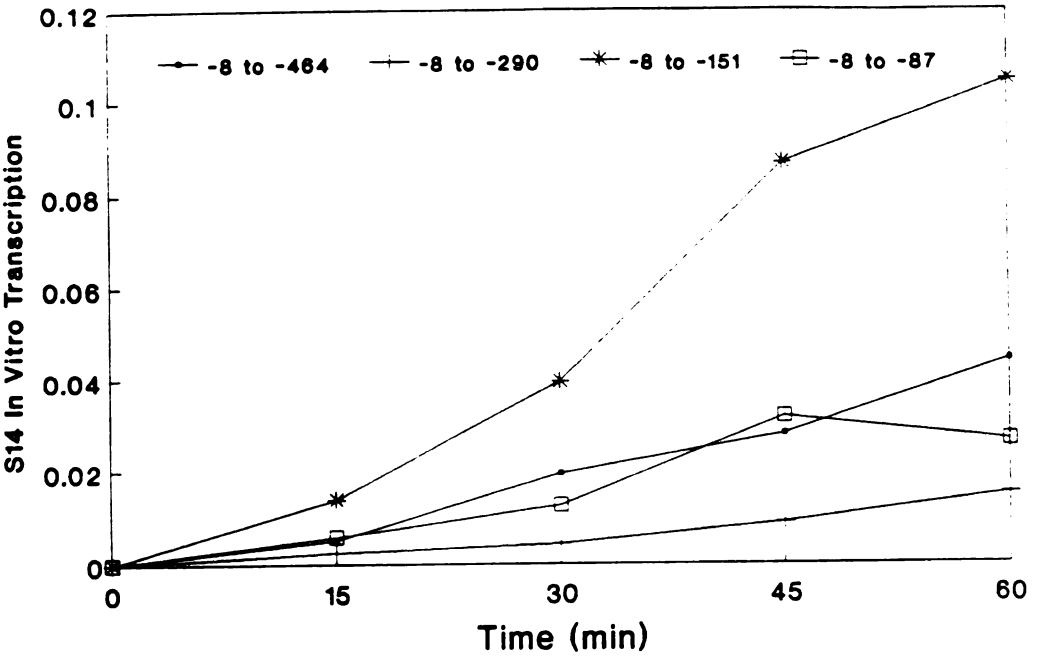


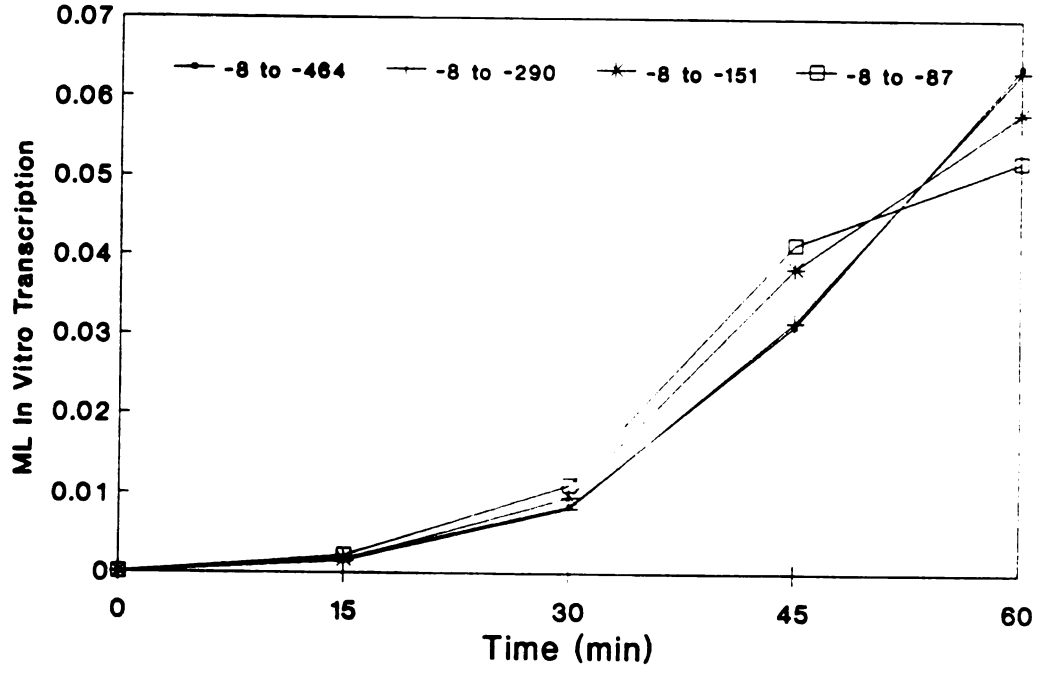
Figure 5. Effect of time on *in vitro* transcription from S14 and major late promoters.

A) Transcription from S14 promoter constructs as indicated. B) Transcription from pML-GFC2 was measured in the presence of S14 promoter constructs as indicated. *In vitro* transcription reactions were initiated as described in Materials and Methods, then stopped at the indicated times. Transcription from S14 and ML promoters was quantified using laser densitometry and is expressed as arbitrary units.

A.


0.12
0.1
0.08
0.06
0.04
0.02
0
S14 In Vitro Transcription
-8 to -464
-8 to -290
-8 to -151
-8 to -87
0
15
30
45
60
Time (min)

B.

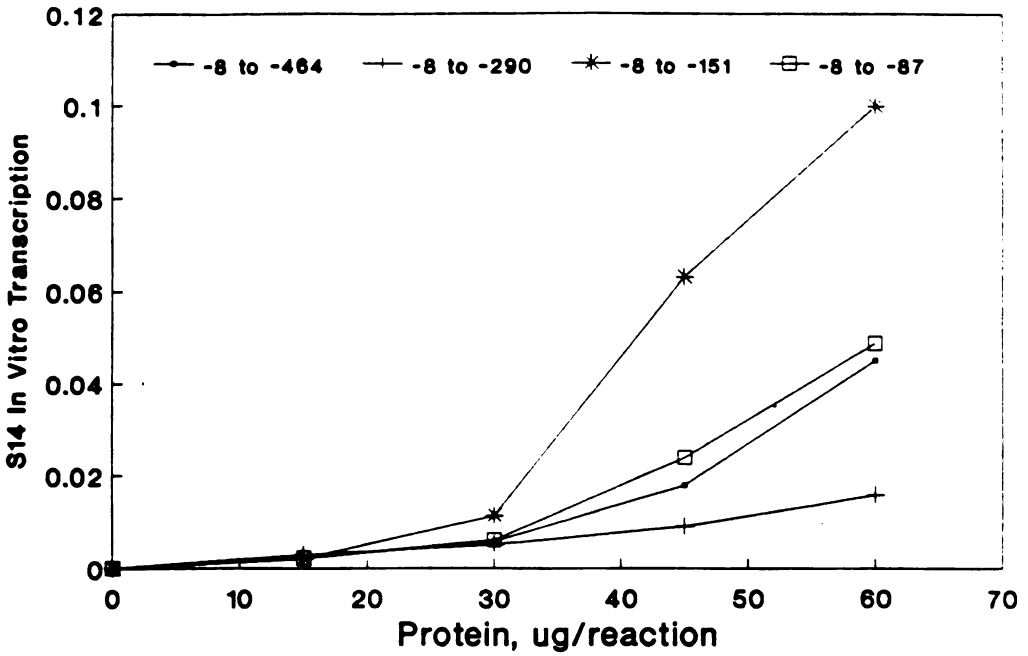

0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
ML In Vitro Transcription
-8 to -464
-8 to -290
-8 to -151
-8 to -87
0
15
30
45
60
Time (min)

(Fig. 5B). Transcription from S14 and major late promoter constructs was dependent on protein concentration and was curvilinear with the linear portion between 30 and 60 μg/20 μl reaction (1.5 and 3.0 mg protein/ml; Fig. 6A and B). Forty five μg of nuclear extract was used in most reactions because: 1) it was within the linear portion of the dose response curve, 2) it provided enough signal to easily detect, and 3) it minimized the quantity of nuclear extracts used. Finally, in vitro transcription from S14 promoters was dependent on concentration of S14 promoter template, and was linear between 0 and 1.6 μg DNA/20 μl reaction (0 to 80 μg DNA/ml; Fig. 7A). Increasing concentration of S14 DNA template did not substantially influence transcription from the major late promoter (Fig. 7B). In these control experiments, addition of different S14 promoter constructs did not influence level of in vitro transcription from the major late promoter. In Figure 7A and B, 1.6 μg of pS14-GFC3 produced a lower rate of S14 and major late in vitro transcription than 1.2 μg of pS14-GFC3. This plasmid consistently produced the highest levels of transcription, therefore, it may be that with very high levels of transcription there is competition for limiting transcription factors which usually would interact synergistically to increase transcription. Alternatively, the observed results may be due to differences in RNA recovery. In any case, 1.2 μg of S14-GFC was used in all experiments since this was in

Figure 6. Effect of concentration of nuclear protein on *in vitro* transcription from S14 and ML promoters.

A) Transcription from S14 promoter constructs as indicated. B) Transcription from pML-GFC2 was measured in the presence of the S14 promoter constructs indicated. *In vitro* transcription reactions were initiated as described in Materials and Methods except the amount of protein was varied from 0 to 60 μg/reaction. Differences in volume contributed by nuclear extract were made up with dialysis buffer. Transcription from S14 and ML promoters was quantified and is expressed as arbitrary units.

A.


-8 to -464
-8 to -290
-8 to -151
-8 to -87
S14 In Vitro Transcription
0.12
0.1
0.08
0.06
0.04
0.02
0
0
10
20
30
40
50
60
70
Protein, ug/reaction

B.

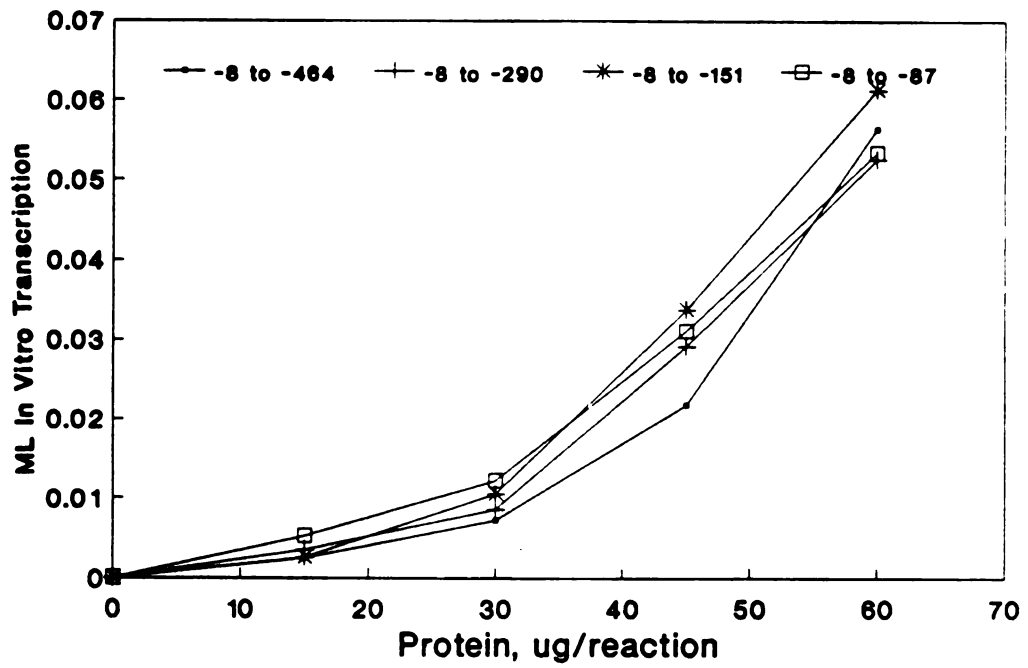
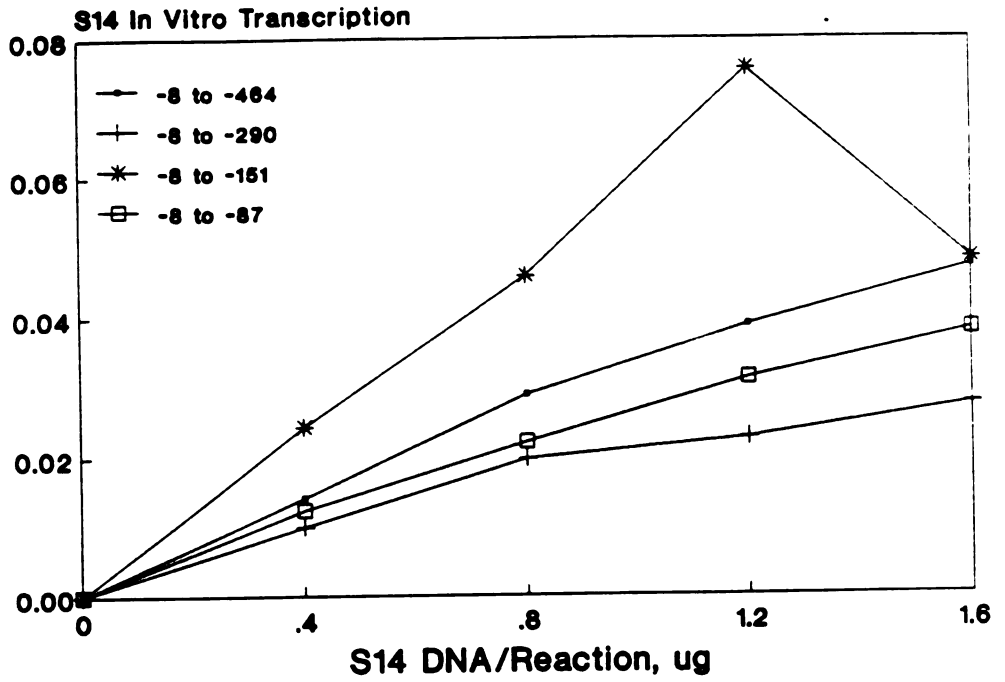

-8 to -464
-8 to -290
-8 to -151
-8 to -87
ML In Vitro Transcription
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
10
20
30
40
50
60
70
Protein, ug/reaction

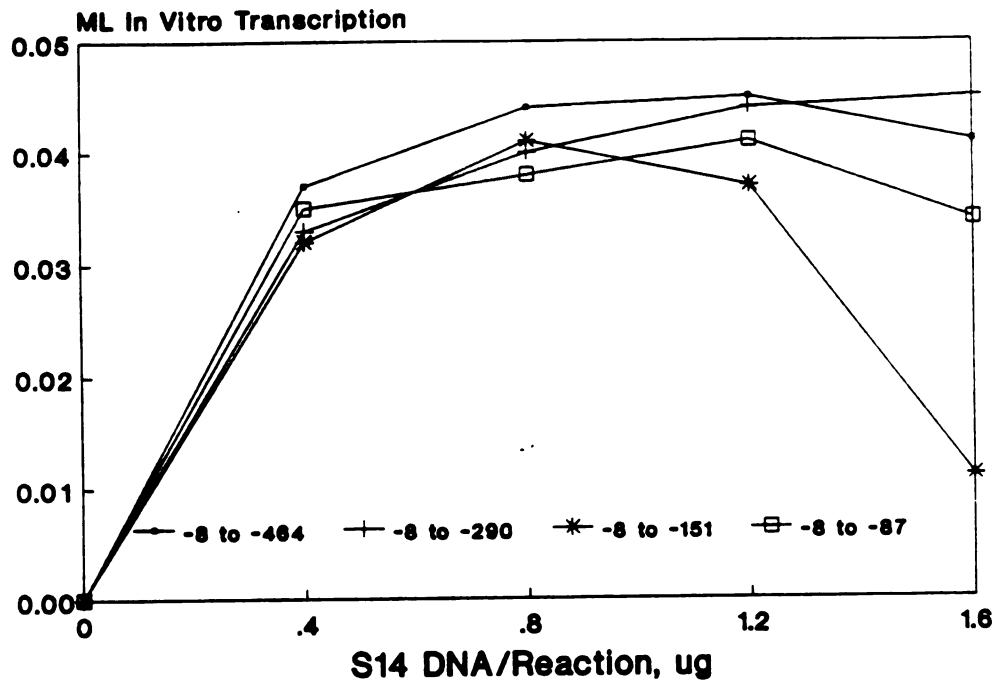
Figure 7. Effect of concentration of S14 promoter template on *in vitro* transcription initiated from S14 promoter constructs and the major late promoter.

A) Transcription from S14 promoter constructs as indicated. B) Transcription from pML-GFC2 was measured in the presence of S14 promoter constructs as indicated. *In vitro* transcription reactions were initiated as described in Materials and Methods except amount of S14 promoter-GFC was varied from 0 to 1.6 μg/reaction. Total amount of template DNA was kept constant by addition of promoterless-GFC. Amount of transcription was quantified using laser densitometry and is expressed as arbitrary units.

A


S14 In Vitro Transcription
0.08
0.06
0.04
0.02
0.00
-8 to -464
-8 to -290
-8 to -151
-8 to -87
0
.4
.8
1.2
1.6
S14 DNA/Reaction, ug

B


ML In Vitro Transcription
0.05
0.04
0.03
0.02
0.01
0.00
-8 to -464
-8 to -290
-8 to -151
-8 to -87
0
.4
.8
1.2
1.6
S14 DNA/Reaction, ug

the linear portion of the DNA dose response and transcription from the major late promoter was not dependent on the S14 promoter construct used.

In addition, transcription was sensitive to addition of 1 μM α-amanitin and 0.4 units RNase A/20 μl reaction, but not to 400 units T1 RNase/20 μl reaction (not shown). These results are summarized in Table 3. Transcription initiated from the S14 promoter *in vitro* satisfies the criteria for an RNA polymerase II catalyzed reaction. Each plasmid was prepared twice, and both preparations yielded essentially identical results. Therefore, differences observed between plasmids are due to deletion of S14 promoter sequences, and not to differences in concentration or preparation of DNA templates. Transcripts from both S14 and ML promoters tended to be of a specific size, with very few shorter products due to premature termination or RNase activity. Wobbe and Struhl (1990) observed that shorter transcripts can be initiated from a weak TATA box within the GFC; however, I did not observe these shorter transcripts in my experiments. In addition, RNA polymerase II pauses at the end of the GFC, therefore, reinitiation of transcription from the same template results in progressively shorter transcription lengths due to the 30 bp covered by RNA polymerase II (Szentirmay and Sawadogo, 1991). The absence of a ladder of transcripts of about 30 bp difference in length may indicate that the reinitiation transcription factor described by Szentirmay and Sawadogo (1991) is not present or active in

Table 3: Summary of control experiments for the *in vitro* transcription assay.

Variable	Effect on In Vitro Transcription	
	S14	ML*
Time	linear (0 to 60 min)	linear (30 to 60 min)
Protein	linear (30 to 60 μg/rxn)	linear (30 to 60 μg/rxn)
S14 DNA	linear (0 to 1.6 μg/rxn)	no effect
α-amanitin	sensitive	sensitive
RNase A	sensitive	sensitive
RNase T_1	resistent	resistent

*ML represents transcription from the adenovirus-2 major late promoter (pML-GFC2).

rat liver nuclear extracts using this extraction protocol.

Transcription from the adenovirus-2 major late promoter (pML-GFC2) was used as an internal standard in all transcription reactions described below. However, transcription from S14 promoters was about 10% of the maximal transcription observed with pML-GFC2. In order to run reactions with comparable rates of transcription from each promoter, concentration of pML-GFC2 was reduced from 40 μg/ml to 5 μg/ml. To ensure that I was measuring rate of transcription and not maximal RNA accumulation, reactions were stopped after 30 to 45 min when all S14 promoters were transcribing RNA in a linear fashion with respect to time (Fig. 5A). Low S14 promoter activity may be due to a weak TATA box. When the TATA box in adenovirus-2 major late promoter, TATAAAA, is mutated to TAGAAAA, it possesses much less ability to initiate transcription *in vitro* (Conaway and Conaway, 1989). Interestingly, this mutant sequence is similar to the putative S14 TATA box sequence, TAGAAAT.

Deletional Analysis of the S14 Promoter

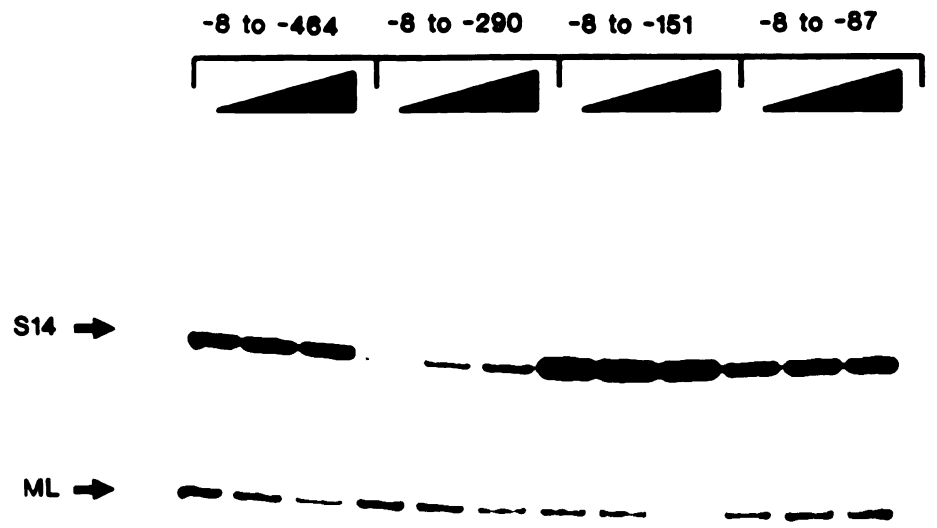
I used various S14 promoter-GFCs constructed by sequential deletion of the 4 footprinted regions (Fig. 4) to determine function of cis-linked elements within these regions. While no transcription was observed when promoterless-GFC was included in transcription reactions (not shown), a "basal" level of transcription was observed when an S14 promoter fragment extending from -87 to -8 bp was ligated upstream from the GFC (Figure 8). The -87 to -8 bp element contains footprinted region A extending from -63 to -48 bp in addition to the prospective TFIID binding site between -27 and -21 bp. Using a promoter element extending from -151 to -8 bp, transcription was increased 1.8 fold ($P < .01$). This region contains two footprinted sequences (Regions A and B, Fig. 1). When a promoter containing S14 promoter region -290 to -8 bp (Regions A, B, and C; Fig. 1) was analyzed, initiation of transcription was suppressed by 88% ($P < 0.01$) when compared to the -151 to -8 bp promoter. Using promoter elements containing all 4 footprinted regions (Regions A, B, C, and D, Fig. 1) and extending to -464 bp, rate of initiation of transcription increased 4-fold ($P < .01$). Selective effects of hepatic nuclear proteins on transcription from S14-GFC constructs suggests that trans-acting factors binding to regions A, B, and D function to increase rate of transcription initiation, while factors binding to region C function to suppress transcription.

Figure 8. Effect of S14 Promoter Deletions on S14 In Vitro Transcription.

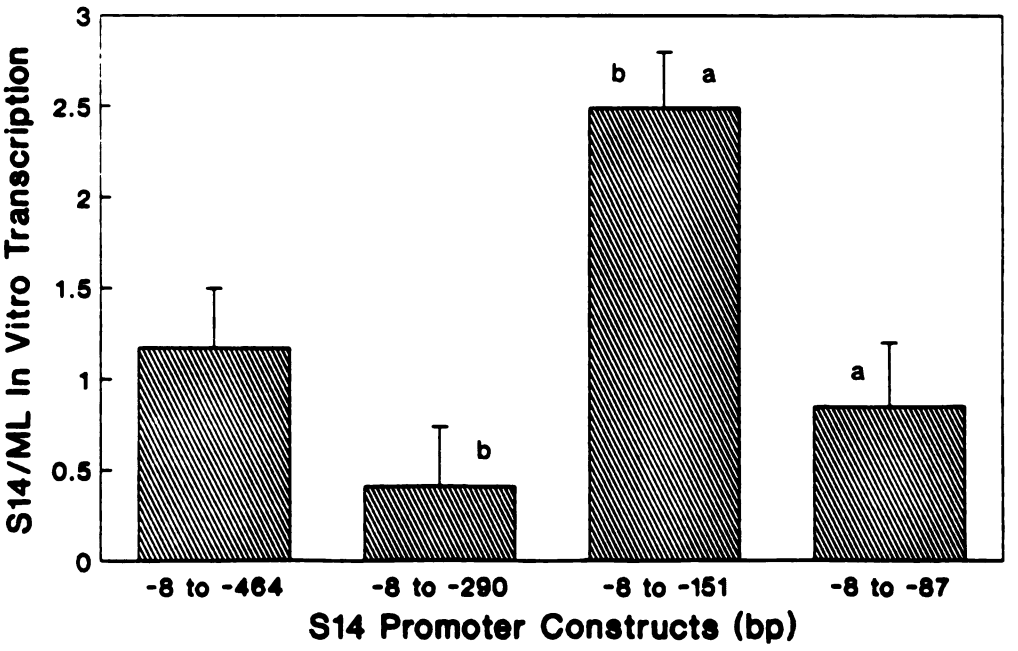
A) Autoradiograph of in vitro transcription from S14 promoter constructs and pML-GFC2. In order to demonstrate that differences between S14 promoter constructs were observed across a range of DNA concentrations, amount of each S14 promoter template added was 0.9, 1.2, and 1.5 μg/reaction. pML-GFC2 was held constant at 0.1 μg/reaction.

B) Transcription from the S14 promoters is expressed relative to transcription from the major late promoter (pML-GFC2). Data from 6 independent preparations of rat liver nuclear extracts were quantified using laser densitometry and are expressed as the mean + pooled S.E.M. Data were statistically analyzed, and -151 to -8 is different from -87 to -8 ($P < .01$), -290 to -8 is different from -151 to -8 ($P < .01$), and -464 to -8 is different from -290 to -8 ($P < 0.01$).

A.


-8 to -464
-8 to -290
-8 to -151
-8 to -87
S14
ML

B.

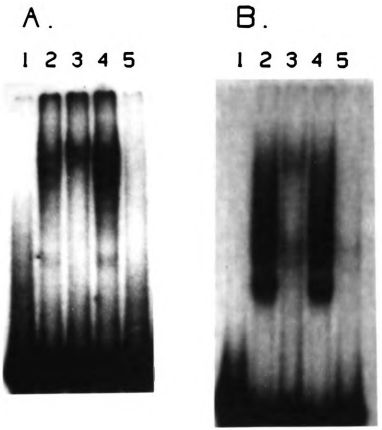

S14/ML In Vitro Transcription
3
2.5
2
1.5
1
0.5
0
b
a
b
a
-8 to -464
-8 to -290
-8 to -151
-8 to -87
S14 Promoter Constructs (bp)

Analysis of NF-1 Interaction with the S14 Promoter

Region A at -63 to -48 bp (TTGGCGTCCTGTCAAT) is similar to the NF-1 consensus sequence (NTTGGCNNNNNGCCAAN; Chodosh et al., 1988) suggesting that hepatic NF-1 may bind to this site and function to increase initiation of S14 gene transcription. To examine this possibility, I used NF-1 oligonucleotide competition in gel shift and in vitro transcription analyses. In gel shift analysis, hepatic nuclei prepared by the method of Gorski et al. (1986; Fig. 9A) and Hewish and Burgoyne (1973; Fig. 9B) were compared. Nuclei prepared by each method were extracted for nuclear proteins using the method of Parker and Topol (1984). Extracts prepared by the "Gorski" method contain DNA-binding and nucleoplasmic proteins capable of initiating gene transcription *in vitro*. Extracts prepared by the "Hewish and Burgoyne" method are depleted of factors required to initiate transcription, but are enriched in DNA-binding proteins. This difference in nuclear extract composition is evident from the shifting pattern of S14 promoter fragment -87 to -8 bp using hepatic extracts prepared by these methods (Fig. 9A and B; lane 2). Addition of a 200-fold molar excess of NF-1 oligonucleotide fully blocked shifting of a single band and smear (Fig. 9A and B; lane 3), but failed to block shifting of a slower-migrating DNA-protein complex. In contrast, addition of a 200-fold molar excess of SP-1 oligonucleotide did not affect the shifting pattern (Fig. 6A and B; lane 4). When hepatic extracts were heat treated (85°C, 5 min) before

Figure 9. Competition by NF-1 for binding to S14 promoter fragment -87 to -8 bp.

Rat liver nuclei were prepared in panel A according to Gorski et al. (1986) and in panel B according to Hewish and Burgoyne (1972). Nuclear proteins were extracted according to Parker and Topol (1984). Otherwise, lane treatments between A and B are identical. Lane 1 contains 1 fmol of labelled -87 to -8 bp alone while in lanes 2-5, DNA was incubated with 7 μg of rat liver nuclear extracts. A 200-fold molar excess of NF-1 oligonucleotide (lane 3) or SP-1 oligonucleotide (lane 4) was added to the reaction mixture. In lane 5, extracts were heated to 85°C for 5 min, centrifuged, and added to the reaction mixture.


A.
1 2 3 4 5
B.
1 2 3 4 5

addition to gel shift reactions, essentially all shifting was abolished suggesting that heat-stable C/EBP (Johnson et al., 1987) is an unlikely candidate for binding to protected region A (Fig. 6A and B; lane 5).

To determine whether NF-1 functions to increase initiation of S14 transcription, NF-1 or SP-1 oligonucleotides were added to *in vitro* transcription reactions. Results are normalized to levels of transcription from pML-GFC2. Addition of 3- or 6-fold molar excess of NF-1 oligonucleotide to transcription reactions decreased S14 transcription by 57 and 67%, respectively, without a concomitant fall in pML-GFC2 transcription (Fig. 10). Addition of SP-1 did not influence *in vitro* transcription from either S14 or ML promoters. In addition, a 6 fold molar excess of NF-1 oligonucleotide, but not AP-3 oligonucleotide, specifically reduced transcription from constructs extending to -290 bp (Figure 11). NF-1 resulted in different levels of inhibition with different promoter constructs. This may be due to the increased contribution from other transcription factors when longer fragments are used. Alternatively, binding of transcription factors to more distal promoter elements may stabilize NF-1 interactions with region A, and thus decrease effects of NF-1 competitive oligonucleotide. Since addition of low levels of NF-1 oligonucleotide selectively inhibits transcription from S14 promoter constructs, NF-1 (or a related protein) may play a functional role in augmenting initiation of S14 gene .ls1

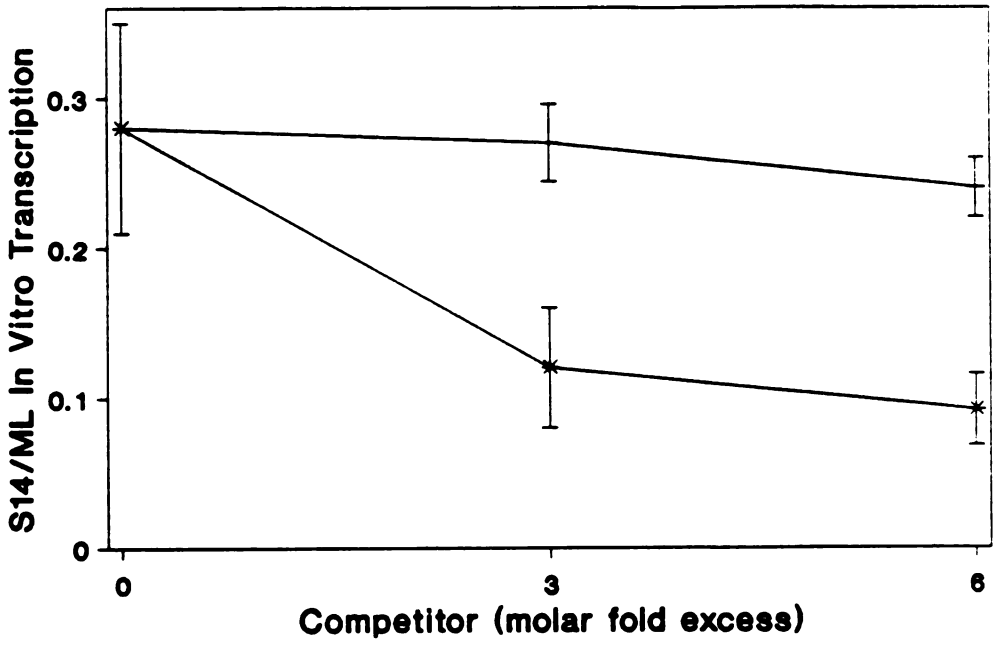


Figure 10. Effect of NF-1 and SP-1 oligonucleotide competition on S14 <u>in</u> <u>vitro</u> transcription.

The <u>in</u> <u>vitro</u> transcription assay was performed as described in Materials and Methods using pS14-GFC4 except that 0, 3-, or 6-fold molar excess of NF-1 (*) or SP-1 (+) oligonucleotide was added to the reaction before transcription was initiated. Data from 3 independent preparations of rat liver nuclear extracts were quantified using laser densitometry and are expressed as means +/- S.D.

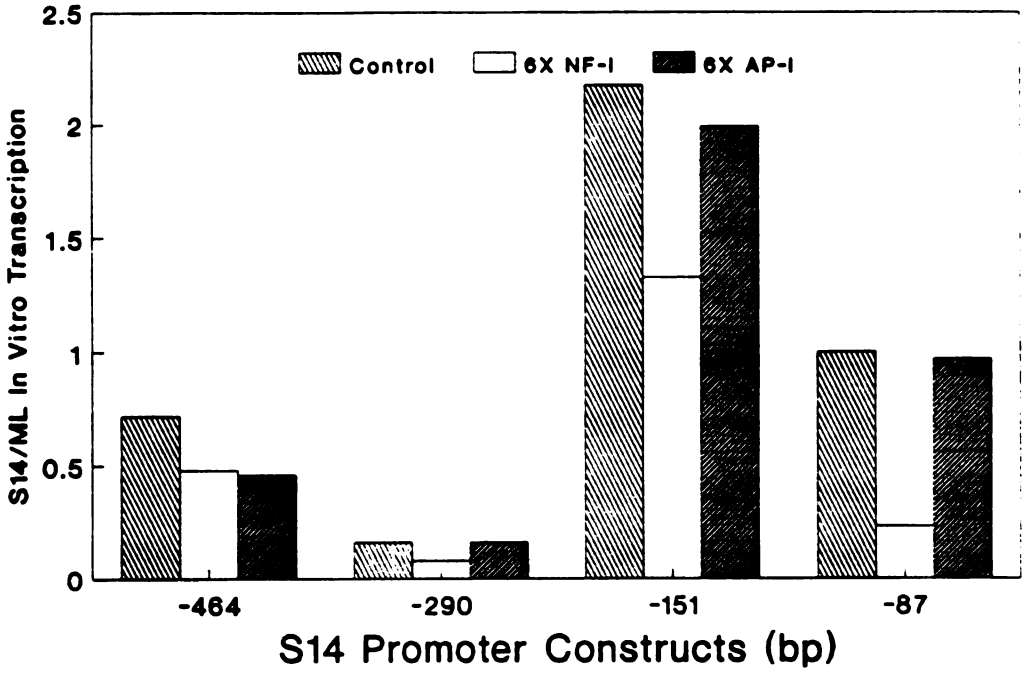


Figure 11. Effect of NF-1 and AP-3 oligonucleotide competition on S14 <u>in vitro</u> transcription.

The <u>in vitro</u> transcription assay was performed as described in Materials and Methods using pS14-GFC1 (-464), pS14-GFC2 (-290), pS14-GFC3 (-151), or pS14-GFC4 (-87) except that 0 or 6-fold molar excess of NF-1 or AP-3 oligonucleotide was added before the reaction was initiated.

transcription in rat liver.

DISCUSSION

In vitro methods were used to identify prospective cis-regulatory elements within rat liver S14 Hss-1. Proximity of Hss-1 to the S14 start site and the regulation of Hss-1 by tissue-specific, developmental, nutritional and hormonal factors suggests that elements within this region function in regulating S14 gene transcription. The *in vitro* approach has allowed me to locate and characterize tissue-specific cis-regulatory elements which affect *in vitro* transcription either positively or negatively. Trans-acting factors which bind these elements may function in the complex multifactorial control of S14 gene transcription.

Using rat liver nuclear extracts, 4 distinct DNase I footprints were identified within 464 bp of the 5'-end of the S14 gene. These footprints are located at -63 to -48 bp (Region A), -113 to -88 bp (Region B), -244 to -227 bp (Region C) and -310 to -286 bp (Region D, Fig. 1). While footprints at Regions A and D were previously reported (Wong et al., 1989; 1990), our studies clearly show that hepatic nuclear proteins bind to additional targets in the 5' flanking end of the S14 gene. Gel shift analysis shows that binding of nuclear proteins to DNA fragments containing footprinted regions are tissue- and sequence-specific. However, gel shift patterns suggest that interactions between hepatic nuclear proteins and each of the four regions is complex. In fact, NF-1 competition studies (Fig. 6 A and B)

show that a fraction of the shifted -87 to -8 bp bands cannot be competed. This fragment contains both a CAAT-like promoter element at -51 bp and a TATA-like element starting at -27 bp (TAGAAAT). While TFIID may bind to the TATA-like element, its binding does not produce a defined footprint under our experimental conditions. DNase I footprint analysis (Figure 1) presumably represent binding of high abundance hepatic proteins to the S14 promoter. For this reason, I cannot exclude the possibility that other proteins interact with S14 promoter sequences.

Interestingly, each of the 4 footprinted regions are similar to hepatic cis-acting elements identified for other genes. S14 "A" footprint shows substantial identity (14 of 15 bp) with the consensus sequence for NF-1 (Chodosh et al., 1988). S14 "B" footprint is similar to a functional sequence (DE III) within the albumin promoter (Herbomel et al., 1989). While sequences within S14 "C" footprint are identical to the AP-4 binding site (CAGCTG; Hu et al., 1990), overlapping regions within this footprint share identity with element IV within the α1-inhibitor III promoter (Abraham et al., 1990) and PKL-I element within the pyruvate kinase promoter (Yamada et al., 1990). S14 "D" footprint shares 8 of 11 bp with DNA sequence recognized by HNF-3 transcription factor (Costa et al., 1989). This analysis suggests that the S14 promoter does not contain novel cis-acting elements, but utilizes an array of cis-acting elements described for other hepatic genes. Regulation of a transcription factor interacting with

multiple genes may represent one mechanism to coordinate hepatic gene expression. In Chapter 3, I investigate the role of postnatal development, nutritional status and tissue-source on transcriptional activity of nuclear proteins binding to S14 promoter elements.

Use of sequentially deleted S14 promoter elements fused to the GFC reporter gene has provided a means to assess the role these four elements play in initiation of gene transcription under in vitro conditions. The minimal component tested, extending from -87 to -8 bp relative to the S14 transcription start site, contained sufficient information to function as a promoter of gene transcription in vitro. The -87 to -8 bp region contains a footprinted sequence similar to the sequence recognized by NF-1. My results suggest that NF-1 or NF-1 like protein may play a functional role in S14 gene transcription. An NF-1 oligonucleotide effectively competed for binding of hepatic nuclear proteins to the S14 promoter and reduced initiation of in vitro transcription (Figures 9, 10, and 11).

In addition, heating hepatic extracts (85°C for 5 mins) essentially obliterated binding of proteins to the -87 to -8 bp region. Heat sensitivity of hepatic nuclear protein binding excludes C/EBP, but not other members of the CCAAT-binding family (for example: CP1, CP2, C/EBP ß, C/EBPδ (Chodosh et al., 1988; Chang et al., 1990; Cao et al., 1991)), as candidates for binding to this region. These studies provide reasonable evidence that NF-1 or a related

protein binds to S14 region A and increases rate of S14 gene transcription.

In vitro functional analysis of the S14 proximal promoter provides evidence that proteins binding to regions B and D may increase rate of S14 gene transcription. Fusing DNA sequences containing footprinted regions A and B upstream from the GFC led to a significant 1.8-fold increase in transcription over region A alone. Cloning DNA sequences containing footprinted regions A through D upstream from the GFC led to a significant 3-fold increase in transcription over regions A through C. Since footprinted region B is similar to a positive cis-acting element found in the albumin promoter, and region D has similarity to the consensus sequence for HNF-3, it may be that trans-acting factors interacting with regions B and D function in tissue-specific control and elevate basal rates of gene transcription. The identity of transcription factors interacting with S14 B and D regions and the mechanism for interacting and influencing preinitiation complex formation remains to be defined.

I was surprised to find that factors which interacted with S14 region C strongly suppressed initiation of gene transcription. This observation supports low CAT activity observed with transfection of S14 promoter sequences extending to -290 bp into cultured hepatocytes or adipocytes (Jacoby et al., 1989; Jump et al., 1992), and suggests that negative transcriptional effects of proteins binding to protected region C may also be dominant *in vivo*. From this

analysis, it is not clear whether transcription factors binding to region C interact by suppressing positive effects from transcription factors associated with regions A or B, or whether they act independently to suppress preinitiation complex formation. However, these studies provide evidence for both positive and negative regulatory elements within the S14 promoter.

Others have reported positive and negative promoter elements including elements within ß-interferon (Goodbourn & Maniatis, 1988) and α1-inhibitor III (Abraham et al., 1990) genes. In addition, positive and negative hepatic elements have been delineated using *in vitro* transcription within mouse albumin (Lichtsteiner et al., 1987), xenopus albumin (Ryffel et al., 1989), xenopus vitellogenin B1 (Corthesy et al., 1989) and vitellogenin II (Vaccaro et al., 1990), aldolase B (Tsutsumi et al., 1989) and α-globin (Kim et al., 1990) gene promoters.

While upstream enhancer elements clearly play a major role in regulating S14 gene transcription (Jacoby et al., 1989, Zilz et al., 1990), identification of proximal elements which have positive and negative effects on transcription and which also are subject to tissue-specific regulation introduces an important second target for control of S14 gene transcription. Although it is not clear whether these proximal elements are involved in formation and/or maintenance of S14 DNase I Hss-I, positive and negative elements clearly add complexity to initiation and potential

regulation of S14 gene transcription. For example, trans-acting factors which interact with the promoter region may regulate initiation of S14 gene transcription as well as facilitate interactions between trans-acting factors bound to upstream enhancer elements and the S14 promoter. I investigate this possibility using a transfection model in Chapter 4.

In summary, I have used *in vitro* methods to identify cis-regulatory elements within the rat liver S14 promoter. This region is assembled into a DNase I Hss *in vivo* that is regulated by developmental, nutritional, tissue-specific, and hormonal factors. The *in vitro* approach described above has allowed me to locate and characterize tissue-specific promoter elements that either enhance or suppress rate of initiation of gene transcription *in vitro*. I speculate that trans-acting factors which bind these elements function in the complex multifactorial control of S14 gene transcription *in vivo*.

CHAPTER 3: REGULATION OF *IN VITRO* TRANSCRIPTION FROM S14 AND FAS PROMOTERS BY POSTNATAL, DIETARY, AND TISSUE-SPECIFIC FACTORS.

INTRODUCTION

S14 and FAS mRNAs are coordinately regulated during postnatal development, and by dietary and tissue-specific factors. S14 and FAS mRNAs are low between birth and weaning (Jump and Oppenheimer, 1985; Perez-Castillo et al., 1987; Clarke et al., 1990a); however, expression of both S14 and FAS increases dramatically to near adult levels 3 days postweaning. Both genes are regulated similarly during dietary manipulations such as fasting/refeeding (or gavage), or feeding diets containing high carbohydrate, high fat, or 10% menhaden oil (Paulauskis and Sul, 1988; Hamblin et al., 1989; Jump et al., 1990; Clarke et al., 1990a,b). Finally, S14 and FAS are expressed at high levels in lipogenic tissues like liver, lactating mammary gland, and white and brown adipose tissues (Jump et al., 1984; Jump and Oppenheimer, 1985; Jump 1989a, Amy et al., 1989; and Schweizer et al., 1989). Tissue-specific regulation of S14 is at the level of transcription (Jump, 1989a). Active transcription of the rat liver S14 gene is correlated with presence of several DNase I hypersensitive sites flanking the 5' end of the gene (Jump et al., 1988, 1989). One of the critical regulatory regions may be Hss-1 which is located within the S14 promoter (-65 to

-265 bp). This site is induced prior to the increase in S14 gene transcription observed during postnatal development and is observed in liver, but not in kidney. This suggests that sequences within this site may be targets for postnatal and tissue-specific regulation (Jump, 1989a; Jump et al., 1987). In this study, I use a cell-free transcription system to determine if hepatic transcription factors initiating transcription from S14 and FAS 5' flanking regions are regulated during postnatal development, by dietary factors, or in different tissues.

MATERIALS AND METHODS

Animal Manipulations

a) Developmental Experiment

Pregnant female rats, 13 to 15 days into gestation, were purchased from Charles River Laboratories (Kalamazoo, MI). These females were housed in individual cages in the Michigan State University Lab Animal Resources Facility in Giltner Hall and allowed to come to term. Twenty seven female pups were sacrificed at day 15 of age by ether anesthesia and decapitation. Livers were rapidly excised, cooled in ice cold 0.1 M NaCl, and weighed. Samples (0.25 g) from 3 livers were stored in tin foil and frozen at -80°C until RNA was extracted. The rest of the livers (18 g) were divided in 2 and homogenized by the standard protocol (Chapter 2) for preparation of nuclei and nuclear extracts. Ten pups were precociously weaned at day 18 of age onto a high carbohydrate diet fed ad libitum between 0800 and 1800

h. At 28 days of age, rats were sacrificed, samples of liver frozen for mRNA analysis, and hepatic nuclear extracts prepared.

b) Dietary Fat Experiment

Pregnant female rats, 13 to 15 days into gestation were purchased from Charles River Laboratories (Kalamazoo, MI). These females were housed in individual cages as before. Thirty female pups were weaned at day 18 onto either a high carbohydrate diet, or a high carbohydrate diet supplemented with 10% triolein or 10% menhaden oil. Dietary composition of high carbohydrate diet is shown in Table 3, while spectrum and amount of fatty acids in menhaden oil is shown in Table 4. Triolein (Sigma Chemical Co.) was approximately 95% pure. All 3 diets were supplemented with 0.1% of the antioxidant butylated hydroxytoluene (Sigma) to prevent oxidation of fatty acids. Oils were stored under nitrogen at +4°C for short periods and at -80°C for long term storage. Diets were prepared fresh each day and were fed between 0800 and 1800 h. Amount of diet consumed was determined by weighing food not eaten during the day. Amount of food offered the next day was pair-fed to the lowest consumption from the previous day. At 27 days of age, 5 rats in each dietary treatment were sacrificed and livers processed for RNA extraction and extraction of nuclear proteins as described previously (Chapter 2). The other half of the animals were processed in an identical fashion at 28 days of age.

Extraction and Analysis of RNA

Total RNA was extracted from rat liver by the guanidinium thiocyanate procedure of Chirgwin et al. (1979). Levels of S14 or FAS mRNA were measured by dot blot hybridization using nick translated inserts from pS14exoPEII-8 or pFAS-17, respectively. pS14exoPEII-8 contains genomic S14 sequences representing +23 to +483 bp and the 5' exon of the S14 gene. pFAS-17 was obtained from S.D. Clarke (Colorado State University) and represents FAS-17 cDNA cloned originally by Nepokroeff et al. (1984). Following hybridization, blots were washed, dried, and exposed to X-ray film. Relative levels of hybridization were quantified using videodensitometry and normalized to an internal hybridization standard from an adult (60 day old) male rat (Jump et al., 1989a). Concentration of $mRNA_{S14}$ has been quantified within this standard, and 1 unit of $mRNA_{S14}$ contains 4.8×10^7 copies of $mRNA_{S14}$ per μg total RNA or 1200 copies of $mRNA_{S14}$ per liver cell.

Table 4: Composition of high carbohydrate diet.

Ingredient	Percentage (w/w)
Sucrose	68.0
Vitamin free casein	18.0
Vegetable oil	8.0
Salt mixture	4.0
Brewers yeast	2.0

Table 5: Composition of menhaden oil.

Ingredient	Specification
Docosahexaenoic acid	12.9%
Eicosapentaenoic acid	18.8%
Total omega 3 fatty acids	36.2%
Total omega 6 fatty acids	4.3%
Omega 3/omega 6 ratio	8.4:1

iii) Tissue-Specific Experiment

All animals and tissues were handled as described in Chapter 2.

Methylation Interference

S14 promoter fragment -87 to -8 bp (25 ng) was end-labelled using [α^{32}P]-dCTP and Klenow fragment, and gel purified from a 6% acrylamide gel. Labelled DNA was partially methylated with dimethylsulfate (Maxam and Gilbert, 1979) and purified exactly as described by Sambrook et al. (1989). Partially methylated DNA was incubated with 90 μg of rat liver nuclear extract under the same buffer conditions as the gel shift assay (total volume of 150 μl) before running on a 6% acrylamide gel at 100 V for 2.5 h. The gel was exposed to film for 1 h for visualization of DNA bands. Bands of interest were excised and electroeluted into dialysis tubing in an electrophoresis buffer of 0.5X TBE. Yeast t-RNA (50 μg) was added before solutions were organic extracted with an equal volume of phenol-chloroform (equilibrated with TE-8), then chloroform-isoamyl alcohol (24:1). Solutions were dehydrated with n-butyl alcohol

before precipitation of DNA with salt and ethanol. DNA was cleaved at methylated guanine nucleotides by addition of piperidine (Maxam and Gilbert, 1979). Purified DNA fragments were electrophoresed on a 6% acrylamide-8 M urea sequencing gel, dried, and autoradiographed.

Plasmid Preparation

The FAS sequences from -1594 (Pst I) to +65 bp (Xho II) were isolated from FASpCAT5 (kindly provided by S.D. Clarke, Colorado State University) and cloned into pGFC(-BH) in correct and reverse orientations to form pFAS-GFC100 and pFAS-GFC101, respectively. Complementary oligonucleotides containing S14 promoter sequences from -38 to -8 bp with a point mutation at -12 bp (G to C) were synthesized by the Macromolecular Facility at Michigan State University and cloned directionally into the BamH 1 and Hind III sites of pGFC(-BH) to form PS14-GFC5. The sequence of these oligonucleotides is shown:

GATCCGCTCAAAAGCCTAGAAATAGTGCGGCGGCAA
GCGAGTTTTCGGATCTTTATCACGCCGCCGTTTCGA

Other Procedures

The preparation of other plasmids and oligonucleotides was described in Chapter 2. Procedures such as preparation of nuclear proteins, and gel shift and *in vitro* transcription analyses were also described in Chapter 2.

Statistical Analyses

Differences in *in vitro* transcriptional activity between pS14-GFC4 and pS14-GFC5 were determined using Students t-test

(Gill, 1985). In the dietary study, all pair-wise combinations of treatment means were examined using Tukey's t-test (Gill, 1985).

RESULTS

Redefinition of the minimal S14 promoter sequences required for initiation of transcription in vitro

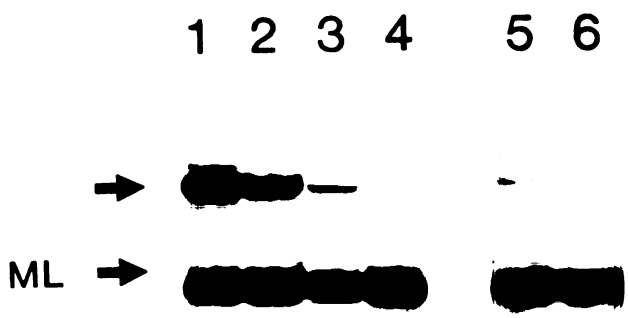
In the previous chapter, I demonstrated that hepatic nuclear extracts support *in vitro* transcription from S14 promoter sequences extending from -87 to -8 bp relative to the 5' end of the gene (MacDougald and Jump, 1991). I was interested in whether deletion of the NF-1 site between -63 and -48 bp would abrogate transcriptional activity from the rest of the promoter. Figure 12 (lane 3) shows that a 30 bp fragment extending from -38 to -8 bp is sufficient to promote transcription of the GFC at 42% ($P < .05$) of that observed with the -87 to -8 bp fragment (lane 2). The -38 to -8 bp fragment contains a sequence resembling a TATA box ($^{-27}$TAGAAAT^{-21}); however, I have not tested whether these sequences are functional. Figure 12 also demonstrates that S14 promoter fragment, -151 to -8 bp, supports 2.5-fold higher transcription than -87 to -8 bp, and that promoterless GFC is not transcribed. These data corroborate results presented in Chapter 2.

FAS 5' flanking sequences function as a promoter

FAS and S14 are regulated in a similar manner during postnatal development and by dietary manipulation, therefore I was interested in determining whether *in vitro*

Figure 12. In vitro transcriptional activity from S14 and FAS promoters using hepatic nuclear extracts.

The lower arrow indicates transcription from pML-GFC2 (ML). The upper arrow indicates transcription from 1.2 μg of pS14-GFC3 (Lane 1), pS14-GFC4 (Lane 2), pS14-GFC5 (Lane 3), and pGFC (Lane 4), or 0.5 μg of pFAS-GFC100 (Lane 5), and pFAS-GFC101 (Lane 6). These results are representative of at least 3 independent experiments.


1 2 3 4 5 6
ML

transcription from FAS and S14 promoters would be coordinately regulated. I obtained the rat FAS promoter from S.D. Clarke (Colorado State University), and subcloned FAS promoter sequences adjacent to a GFC for *in vitro* transcriptional analysis. Hepatic nuclear extracts support *in vitro* transcription when the FAS 5' flanking region (-1594 to +65 bp) is in the correct orientation, but not when in the reverse orientation (Figure 12). This demonstrates that this DNA sequence meets the requirements of a functional promoter. FAS will be included as a control in the following experiments.

Effect of weaning on S14 and FAS *in vitro* transcription

To determine if postnatal induction of hepatic S14 and FAS gene expression is mediated by induction or repression of transcription factors interacting within S14 and FAS promoter regions, I investigated whether hepatic nuclear extracts from rats sacrificed at age 15 (preweaning) supported a reduced level of *in vitro* transcription compared to extracts from rats sacrificed at age 28 (postweaning). Figure 13 demonstrates that S14 and FAS mRNAs were induced during this time period, as previously observed (Jump et al., 1988). Surprisingly, substantial differences in *in vitro* transcriptional activity from S14 and FAS promoters (Figures 14 and 15) were not observed between these different physiological states. These data were supported by my observation that gel shift patterns produced by binding of hepatic factors to S14 promoter fragments were similar

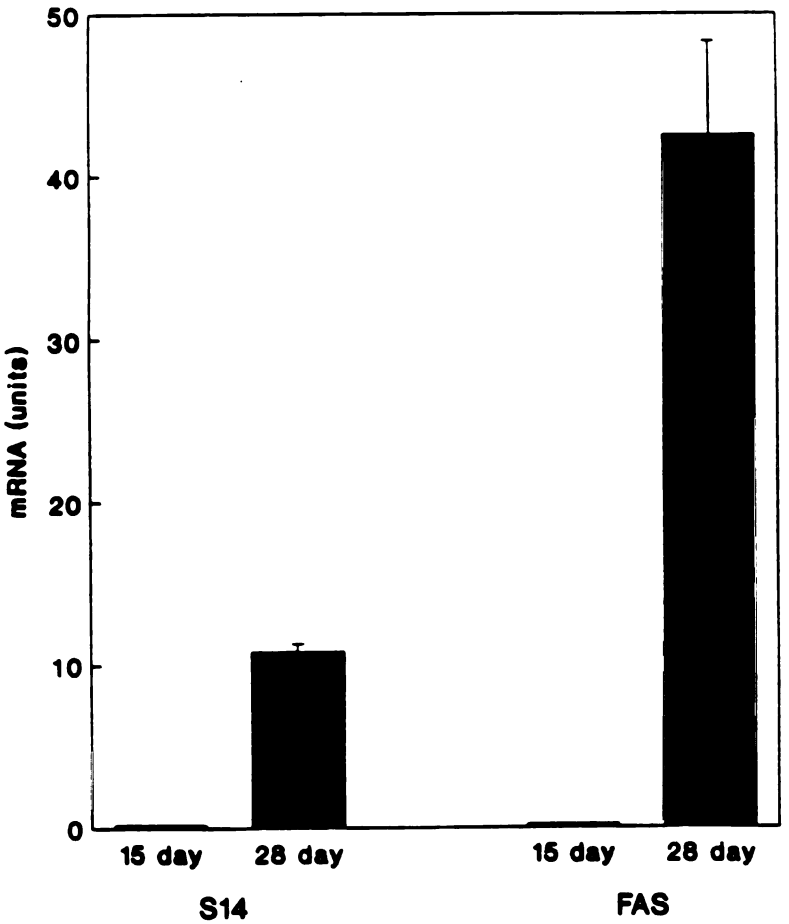


Figure 13. Effect of development on mRNA levels for S14 and FAS.

Rats were sacrificed at day 15 or 28, livers excised, and RNA purified as described in Materials and Methods. S14 and FAS mRNA were analyzed using dot blot analysis, and are expressed as units. Each point represents the mean + S.D. (n = 3).

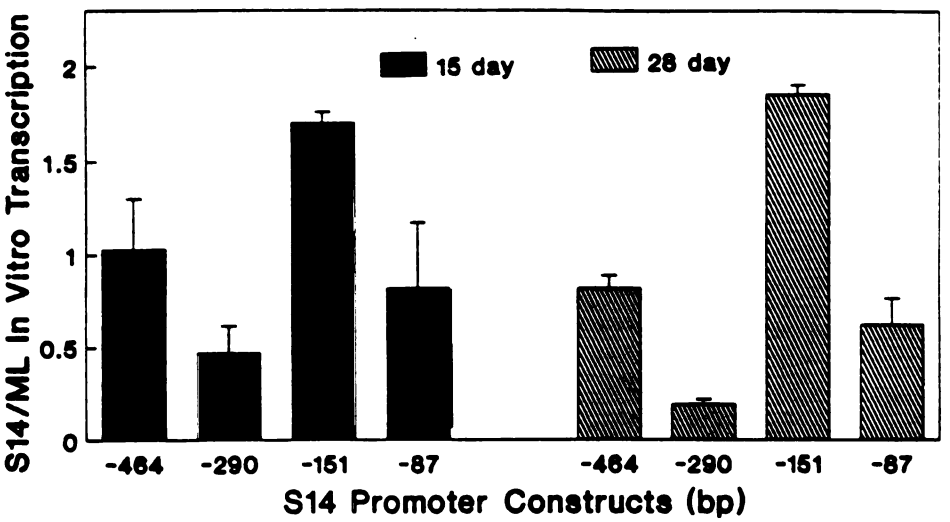


Figure 14. Effect of development on <u>in vitro</u> transcriptional activity from the S14 promoter.

Hepatic nuclear extracts prepared from rats that were 15 or 28 days of age were used to initiate transcription from 4 S14 promoter constructs which extended from a -3 end of -8 bp to the 5' endpoint labelled in the figure. Results are expressed relative to transcription from the major late promoter, and are represented here as mean + range (n = 2).

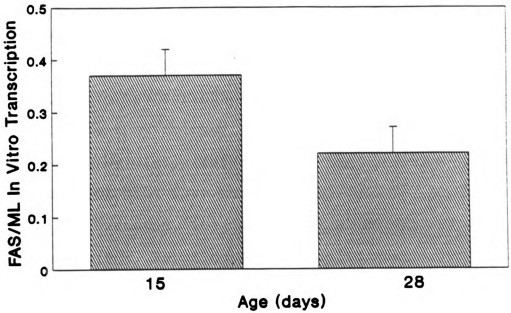


Figure 15. Effect of development on *in vitro* transcriptional activity from the FAS promoter.

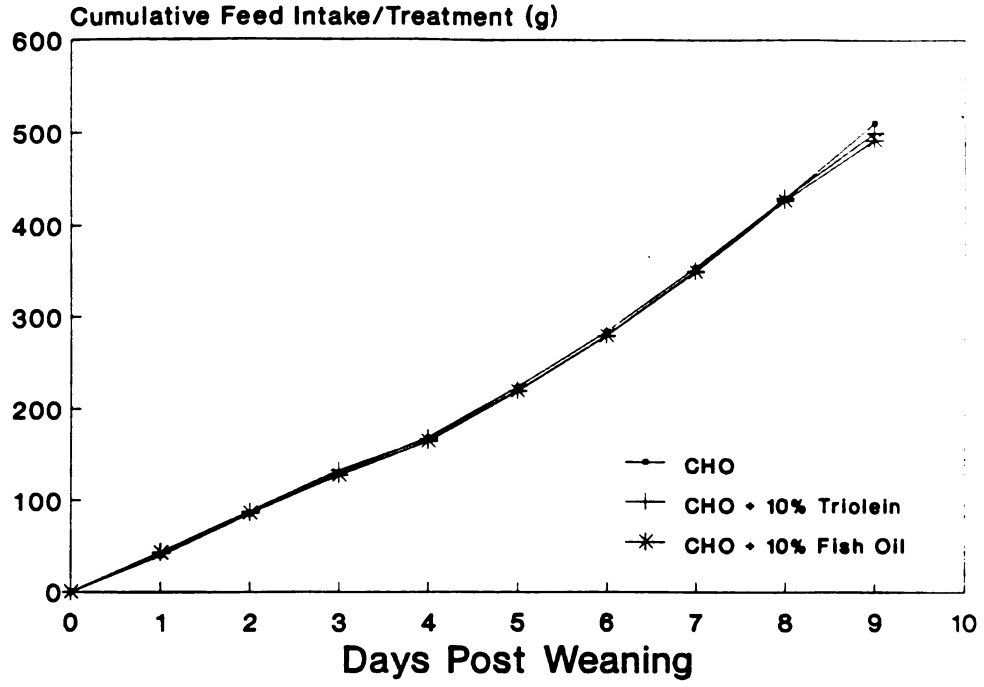
Hepatic nuclear extracts prepared from rats that were 15 or 28 days of age were used to initiate transcription from FAS promoter sequences -1594 to +65 bp. Results are expressed relative to transcription from the major late promoter, and are represented here as mean + range (n = 2).

between extracts from suckling rats and rats weaned onto a high carbohydrate diet (not shown). From these data, it appears that the dramatic increase in S14 and FAS gene transcription at weaning is not due to induction or repression of transcription factors interacting with the S14 promoter region.

Effect of dietary menhaden oil on S14 in vitro transcription

An experiment was conducted to determine if blockade of the postnatal induction in S14 and FAS mRNA by dietary fish oil was reflected in ability of hepatic nuclear extracts to support *in vitro* transcription from these promoters. Figure 16 demonstrates that neither food intake or body weight gain was influenced by diet. This ensures that palatability or digestibility of diet were not influencing the experimental results. As expected, 10% dietary menhaden oil significantly ($P < .05$) blocked the developmental increase in S14 (Figure 17) and FAS (Figure 18) mRNA; however, hepatic nuclear extracts taken from rats in these different physiological states supported similar levels of S14 (Figure 19) and FAS (Figure 20) *in vitro* transcription. This suggests that transcription factors interacting with these promoters are not responsible for induction of gene transcription during post-natal development. This is supported by observations that hepatic nuclear proteins from different dietary treatments produce similar gel shift patterns with S14 promoter fragments (not shown). Therefore, the repression of S14 and FAS gene transcription by dietary

A.



B.

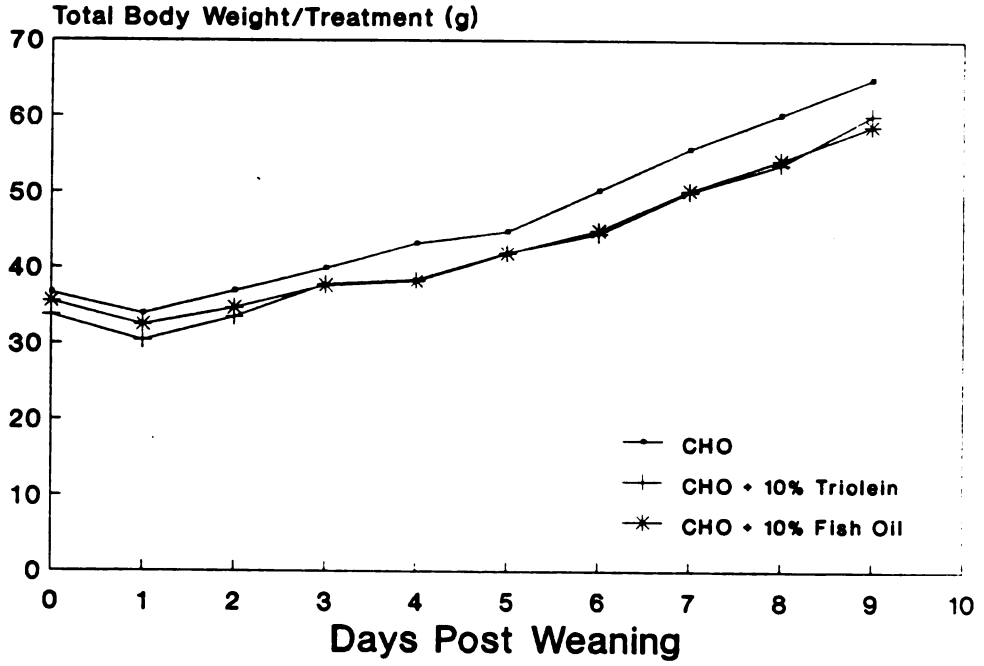


Figure 16. Effect of dietary treatments on food intake and body weight gain.

Rats were fed diet containing either high carbohydrate, high carbohydrate + 10% triolein, or high carbohydrate + 10% fish oil for 10 days. A) Food intake was measured daily and is expressed as the mean of 10 animals. B) Rats were weighed each day and body weights are represented as mean of 10 animals.

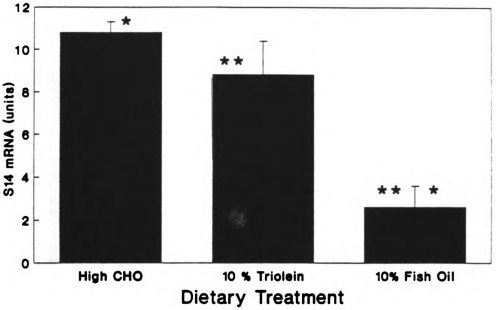


Figure 17. Effect of dietary treatment on mRNA levels for S14.

Rats were weaned onto diets containing either high carbohydrate, high carbohydrate + 10% triolein, or high carbohydrate + 10% fish oil, and were fed these diets for 10 days. Rats were sacrificed, livers excised, and RNA isolated as described in Materials and Methods. S14 mRNA was quantified by dot blot hybridization, and is expressed as units. Data is represented as the mean + S.E.M. (n = 3).

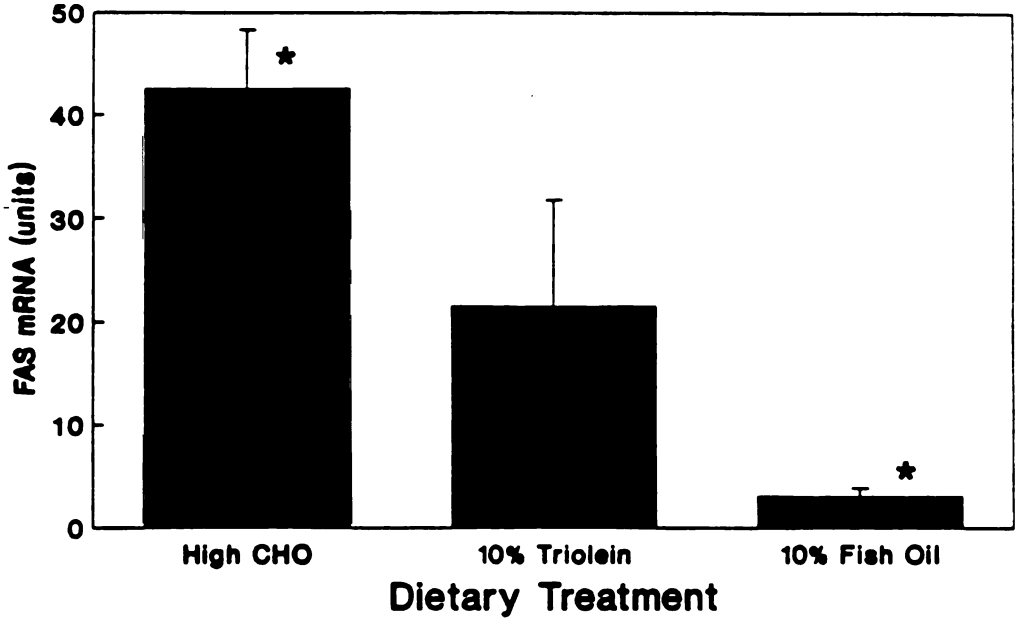


Figure 18. Effect of dietary treatment on mRNA levels for FAS.

Rats were weaned onto diets containing either high carbohydrate, high carbohydrate + 10% triolein, or high carbohydrate + 10% fish oil. Rats were fed these diets for 10 days. Animals were sacrificed, livers excised, and RNA isolated as described in Materials and Methods. FAS mRNA was quantified by dot blot hybridization, and is expressed as units. Data is represented as the mean + S.E.M. (n = 3).

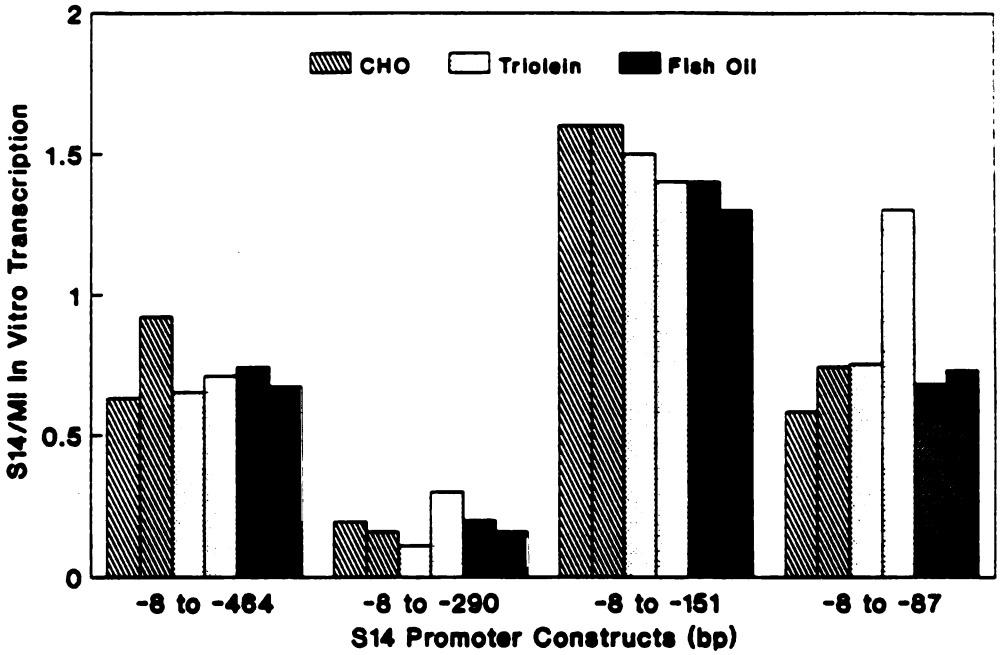


Figure 19. Effect of dietary treatment on <u>in vitro</u> transcriptional activity from the S14 promoter.

Hepatic nuclear extracts were prepared from 2 groups of 5 rats fed diets containing high carbohydrate (CHO), high carbohydrate + 10% triolein (Triolein), or high carbohydrate + 10% fish oil (Fish Oil), and used to initiate transcription from the 4 S14 promoter sequences listed. S14 <u>in vitro</u> transcriptional activity was quantified using laser densiotometry, and is expressed relative to the major late promoter.

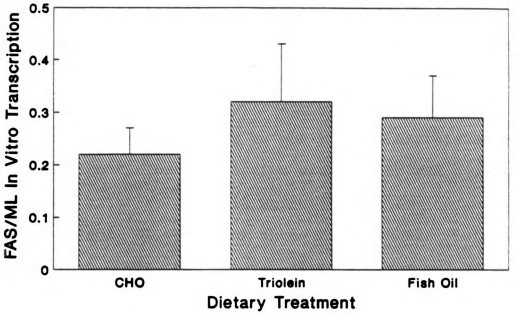


Figure 20. Effect of dietary treatment on in vitro transcriptional activity from the FAS promoter.

Hepatic nuclear extracts were prepared from 2 groups of 5 rats fed diets containing high carbohydrate (CHO), high carbohydrate + 10% triolein (Triolein), or high carbohydrate + 10% fish oil (Fish Oil), and used to initiate transcription from FAS promoter sequences -1594 to +65 bp. FAS in vitro transcriptional activity was quantified using laser densiotometry, normalized to transcription from the major late promoter, and is expressed as mean + range (n = 2).

menhaden oil does not appear to be mediated through abundance of transcription factors interacting within the S14 promoter region.

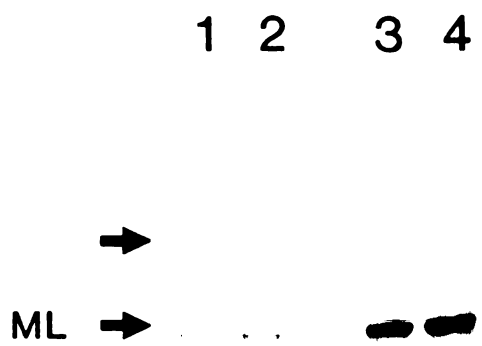
The post-natal development and dietary menhaden oil experiments did not yield data leading to important new mechanisms of transcriptional control. In vitro transcription and gel shift results were correlated and showed little difference in transcriptional activity or DNA-binding activity between treatments, even though treatments greatly altered transcriptional activity of the endogenous S14 gene. I conclude that transcriptional regulation observed between treatments is not through expression of a transcription factor interacting with S14 or FAS promoter elements. Alternatively, it may be that these technologies are not capable of detecting these differences. Future insight into these transcriptional mechanisms may be gained through transgenic or in vivo transfection (eg. retroviral) approaches. In any case, I did not pursue these experiments further.

Tissue-specific in vitro transcription from S14 and FAS promoters

S14 and FAS are expressed in liver at much higher levels than in kidney in the mouse (Paulauskis and Sul, 1988), pig (Mildner and Clarke, 1991), and rat (not shown). Since this is correlated with different DNA-binding proteins interacting with S14 promoter elements between liver and kidney, I wanted to determine whether renal nuclear extracts could initiate in vitro transcription from S14 and FAS promoters (Figure 21).

Figure 21. *In vitro* transcriptional activity from S14 and FAS promoters using renal nuclear extracts.

The lower arrow indicates transcription from pML-GFC2. The upper arrow indicates transcription from 1.2 μg pS14-GFC4 (Lane 1), 1.2 μg pGFC (Lane 2), 1.0 μg pFAS-GFC100 (Lane 3), or 1.0 μg pFAS-GFC101 (Lane 4). These results are representative of at least 3 independent experiments.

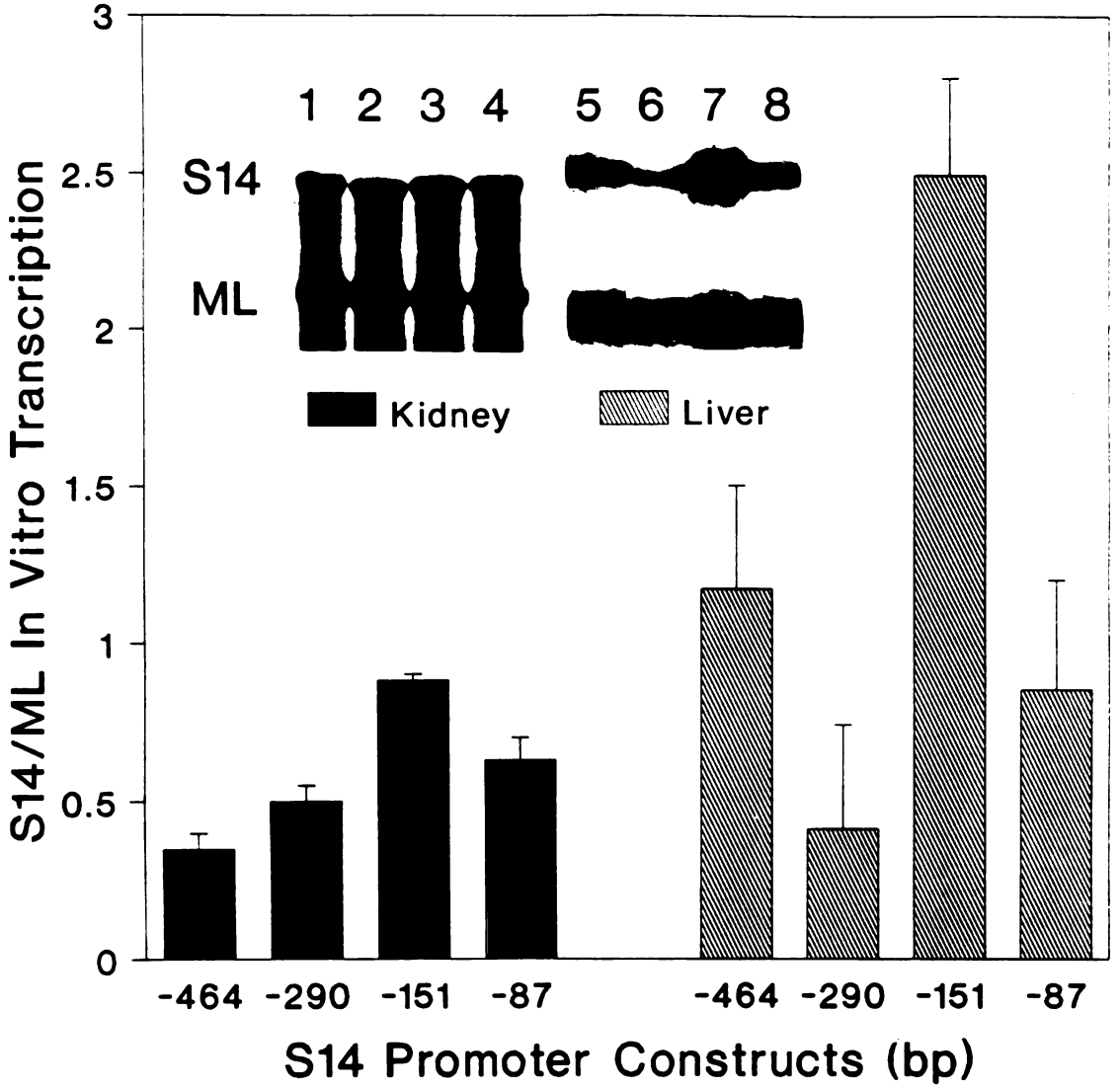

1 2 3 4
ML

Surprisingly, renal nuclear extracts support in vitro transcription from the S14 promoter (-87 to -8 bp) at rates comparable to those observed with hepatic nuclear extracts. In vitro transcription was not observed from the GFC or FAS-GFC constructs, even when the autoradiograph was greatly overexposed (not shown). This suggests that there is discordant regulation of S14 and FAS transcription in liver and kidney. Tissue-specific factors interacting with FAS promoter sequences may function to elevate hepatic gene expression or suppress renal gene expression.

I have observed that strong positive and negative elements reside within the hepatic S14 promoter (Chapter 2, Figure 22). Although slight differences are observed between different S14 promoter constructs, renal nuclear extracts do not contain factors that produce the dramatic differences in in vitro transcriptional activity observed with hepatic nuclear extracts (Figure 22). This is correlated with our previous observation that renal nuclear extracts contain a simplified complement of factors that interact with S14 promoter fragments (Chapter 2). Interestingly, renal nuclear extracts support in vitro transcription from S14 promoter fragment -87 to -8 bp at levels comparable to hepatic nuclear extracts (0.63 +/- 0.05 with kidney versus 0.79 +/- 0.08 with liver), suggesting that similar nuclear factors may be operating within this region of the S14 promoter. In support of this hypothesis, renal nuclear extracts produce the same footprint as hepatic nuclear extracts within this region

Figure 22. In vitro transcriptional activity from S14 promoter constructs using renal and hepatic nuclear extracts.

Inset) ML indicates transcription from pML-GFC2. S14 indicates transcription from 1.2 μg of pS14-GFC1 (lanes 1,5), pS14-GFC2 (lanes 2,6), pS14-GFC3 (lanes 3,7) or pS14-GFC4 (lanes 4,8). Lanes 1-4 and 5-8 represent transcription initiated by renal or hepatic nuclear extracts, respectively. Data were quantified and are graphed as mean + range (n = 2) for kidney, or mean + pooled S.E.M. (n = 4) for liver.


3
2.5
2
1.5
1
0.5
0
S14/ML In Vitro Transcription
1 2 3 4 5 6 7 8
S14
ML
Kidney
Liver
-464 -290 -151 -87 -464 -290 -151 -87
S14 Promoter Constructs (bp)

(Footprint A; -63 to -48 bp), but do not produce a footprint corresponding to Footprint B (not shown).

I have demonstrated that hepatic NF-1 or NF-1-like protein binds to the S14 promoter (-63 to -48 bp) and increases rate of *in vitro* transcription (Chapter 2, MacDougald and Jump, 1991). Sequence identity between S14 and both NF-1 consensus sequence and NF-1 competition oligonucleotide is illustrated in Table 6. I now report that transcriptionally active renal nuclear extracts contain a DNA-binding protein(s) that produces a similar gel shift pattern with S14 promoter fragment -87 to -8 bp (Figure 23A). In addition, this renal DNA-binding protein(s) is effectively competed by an NF-1 oligonucleotide, but not AP-3 oligonucleotide or S14 promoter sequence -38 to -8 bp. Moreover, all of the interactions between renal nuclear factors and this fragment are specific (competed for by S14 promoter fragment -87 to -8 bp, but not -151 to -88 bp) and heat sensitive (85°C for 5 min; not shown). NF-1 oligonucleotide also specifically decreases *in vitro* transcription from the S14 promoter (Figure 23B) as initiated by renal nuclear extracts. In contrast, addition of NF-1 oligonucleotide to hepatic extracts did not suppress *in vitro* transcription from FAS-GFC (not shown) indicating that NF-1 may not function in FAS transcription.

Both hepatic and renal nuclear extracts contain a factor that binds to S14 promoter fragment -87 to -8 bp, but which is not competed for by NF-1 oligonucleotide (Figures 23A and

Figure 23. Competition by NF-1 oligonucleotide for DNA-binding and *in vitro* transcriptional activity of renal nuclear extracts.

A) Gel shift reactions contained 10000 CPM (0.5 ng) of S14 promoter sequence -87 to -8 bp (Lane 1) and 4 μg of renal nuclear extract (Lanes 2-5). A 50 fold molar excess of NF-1 (Lane 3) or AP-3 (Lane 4) oligonucleotide, or S14 promoter sequence -38 to -8 bp (Lane 5) was added as indicated. B) Ability of NF-1 (*) or AP-3 (X) nucleotide to compete for *in vitro* transcription from pS14-GFC4 was examined. A 2 or 4 fold molar excess of NF-1 or AP-3 oligonucleotide was added to the *in vitro* transcriptional reaction before renal nuclear extracts were added. These results are representative of 2 independent experiments.

A
1 2 3 4 5
B
S14/ML In Vitro Transcription
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0X
2X
4X
Molar Fold Excess Competitor

Table 6: Comparison between S14 promoter sequence -63 to -48 bp and NF-1 consensus sequence[a].

S14	**TTGGCGTCCTGTCAAT**
NF-1[b]	**NTTGGCNNNNNGCCAAN**
NF-1 Oligo	ATTTTGGCTTGAAGCCAATATG

[a] Bolded letters indicate sequence identity with S14
[b] NF-1 consensus from Chodosh et al. (1988); "N" indicates any nucleotide

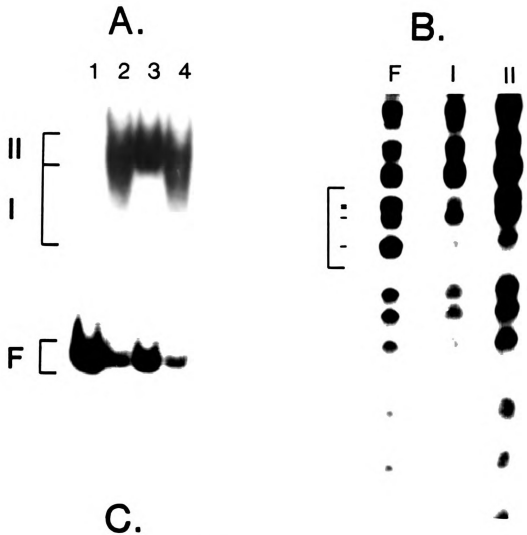
24A). With extracts from both tissues, the binding of this protein(s) actually increased with competition by NF-1 oligonucleotide. This suggests that this nuclear protein may be competing with NF-1 for overlapping binding sites. When I assessed where nuclear proteins from liver and kidney protect DNA from nuclease, I observed footprints in the region from -63 to -48 bp only. To determine where the nuclear factor not competed by NF-1 oligonucleotide was binding, I employed methylation interference analysis to demonstrate that methylation of guanine (at -53 bp) in the 3' half of the footprinted region blocks binding of the factor (Figure 24). Inspection of S14 and NF-1 oligonucleotide sequences reveals that only 5 nucleotides are different, and these tend to be in the region between the sequences bound by NF-1 (Table 6). This suggests that the protein not competed by NF-1 oligonucleotide recognizes and binds to this central region. As expected, bands (smear) corresponding to nuclear factors competed by NF-1 consensus oligonucleotide did not bind DNA if guanines in either of the half-sites bound by NF-1

Figure 24. Methylation interference analysis of nucleotides critical for binding of nuclear proteins to S14 promoter fragment -87 to -8 bp.

A) Labelled -87 to -8 bp was incubated either alone (lane 1) or with 6 μg hepatic nuclear extract (lanes 2-4) before electrophoresis. Lane 3 was also incubated with a 25 fold molar excess of NF-1 oligonucleotide while Lane 4 was incubated with a 25 fold molar excess of -38 to -8 bp oligonucleotide. Three bands or complexes are observed. These correspond to free DNA (F), the complex competed by NF-1 oligonucleotide (I), and the bands not competed by NF-1 oligonucleotide (II).

B) ^{32}P-labelled partially methylated -87 to -8 bp was incubated with hepatic nuclear extracts as in A), electrophoresed, and bands corresponding to F, I, and II purified. DNA was cleaved at methylated guanines, electrophoresed on a sequencing gel, and visualized using autoradiography.

C) Summary of DNA protein interactions in the -63 to -48 bp region. Guanines labelled with an "*" block binding of nuclear protein when methylated while guanines labelled with an "0" do not block binding of nuclear proteins when methylated. Complex I fails to form if guanines at -61, -60, or -53 bp are methylated, while complex II fails to form only if guanine at -53 bp is methylated. Methylation of guanine at -58 bp does not influence binding of either complex.


A.
1 2 3 4
II
I
F
B.
F
I
II
C.
I ----TTGGCGTCCTGTCAAT----
II ----TTGGCGTCCTGTCAAT----

(at -61, -60, and -53 bp) were methylated. In neither case did methylation of guanine between the NF-1 half sites (at -58 bp) disrupt binding of nuclear factors to DNA. These results are summarized in Figure 24C.

DISCUSSION

I demonstrated in Chapter 2 that S14 promoter sequences extending from -87 to -8 bp relative to the start site of transcription supported in vitro transcription of the GFC reporter gene. I now have redefined the minimal promoter element to a promoter sequence from -38 to -8 bp, which contains a modified TATA box, and supports in vitro transcription at about 40% of that observed with -87 to -8 bp (Figure 12).

In addition to what appears to be a weak TATA box ($^{-27}$TAGAAAT^{-21}), the oligonucleotide synthesized also contains a GC rich area ($^{-17}$GCGGCGGC^{-10}). GC-rich elements (consensus GGGGCGGGGC) have been shown to successfully initiate transcription in vitro only if present in multiples (Pugh and Tjian, 1991). Therefore, I speculate that the S14 preinitiation complex is likely formed by binding of the TATA box-binding protein to the S14 TATA-like sequence. A mutation of the TATA box from adenovirus major late promoter to a sequence resembling the S14 sequence, TAGAAAA, results in low but detectable initiation of transcription in vitro (Conaway and Conaway, 1989). Similar results have also been obtained by Wobbe and Struhl (1990).

S14 promoter sequences -38 to -8 bp appear to initiate

transcription at the start of the GFC since mRNA transcript length cannot be distinguished from that transcribed from the major late promoter (when using a 400 bp GFC), the FAS promoter, or the hexokinase II promoter (Thelan and Wilson, unpublished). These three promoter constructs include sequences 3' to the mRNA start site, and therefore have their potential initiator sequences intact. Although S14 promoter sequences extending through the start site of transcription have not been tested, it appears that the S14 gene does not require initiator promoter elements to initiate transcription or correctly position the start site. This is supported by the lack of identity between sequences around the S14 start site and those initiator promoter elements reported to be important by Smale and Baltimore (1989), Means and Farnham (1990), Nakatani et al. (1990) and Roeder (1991).

I have analyzed two physiological states in which S14 and FAS mRNA levels are altered through regulation of gene transcription. In the first experiment, rats were sacrificed at 15 and 28 days of age, during which time S14 and FAS hepatic mRNAs increased >100 fold (Figure 13). In a second experiment, this post-natal increase in S14 and FAS gene expression was significantly suppressed by addition of 10% menhaden oil (Figures 17 and 18). In both studies, the physiological manipulation was successful, as reflected by changes in S14 and FAS mRNA level. Previous work has demonstrated that these changes in mRNA abundance are due to regulation of gene transcription (Jump et al., 1988; Blake

and Clarke, 1991; Jump, unpublished). Surprisingly, hepatic nuclear extracts from rats on these treatments supported similar levels of *in vitro* transcription from both S14 and FAS 5' flanking regions (Figures 14, 15, 19, and 20) and did not show substantial differences in gel shift pattern using S14 promoter fragments and hepatic nuclear extracts.

This lack of regulation during postnatal development or by menhaden oil suggests that sites of regulation may not be through induction or repression of hepatic nuclear transcription factors interacting with S14 or FAS promoters. Instead, regulated transcription factors may interact with genomic sequences not represented in the *in vitro* transcription assay or located too far away from the transcriptional start site to influence rate of transcription *in vitro*. Alternatively, it may be that the regulated protein(s) is a transcription factor not retained with the extraction protocol used, or the posttranslational regulatory signal (e.g. phosphorylation) might have been lost during extraction.

Finally, Hss-I is induced prior to induction of S14 gene transcription during postnatal development (Jump et al., 1988), therefore the regulated step may not be the amount or activity of a transcription factor. Instead, changes in chromatin structure may regulate accessibility of DNA for binding by constitutively expressed transcription factors. Further analysis using *in vitro* transcription will have to be postponed until an alternate approach identifies a putative

transacting factor(s) or cis-acting element(s) through which these postnatal and dietary effects are mediated.

Since renal S14 run-on (*in vivo*) gene transcription is <1% of what is observed in liver (Jump, 1989), it was surprising that renal nuclear extracts initiate *in vitro* transcription from S14 promoter fragment -87 to -8 bp at rates similar to hepatic nuclear extracts (Figure 22). This may be due to tissue-independent expression of a transcription factor(s), since nuclear extracts from kidney and liver produce similar gel shift patterns with promoter fragment -87 to -8 bp. This transcription factor may be NF-1 or a related protein since an oligonucleotide that NF-1 binds to with high affinity (Rosenfeld et al., 1987) competes equally well for DNA-binding proteins and *in vitro* transcriptional activity of hepatic and renal nuclear extracts (Figure 23). In addition, renal nuclear proteins produce a footprint over the same region (-63 to -48 bp) as the hepatic nuclear factor (not shown). Although the ubiquitous expression of NF-1 can explain *in vitro* transcription from the S14 promoter, it also suggests that a mechanism other than tissue-specific expression of transcription factors is responsible for the differences in hepatic and renal transcription rates observed *in vivo*. Once again, renal sequestration of promoter sequences into a nucleosome might block any transactivational effect of NF-1.

NF-1 oligonucleotide competed for most of the binding of renal and hepatic nuclear extracts to S14 promoter fragment

-87 to -8 bp, however, both renal and hepatic extracts contained a nuclear protein not competed by NF-1 oligonucleotide (Figures 23A and 24A). Binding of the non-NF-1 protein was specific and actually increased in intensity in the presence of NF-1 competitive oligonucleotide. This may indicate that this nuclear factor is competing with NF-1 for sequences within -63 to -48 bp that are not represented within the NF-1 oligonucleotide (Table 6), or are not necessary for stable interaction of NF-1 with its DNA element. This nuclear factor produces a defined band upon gel shift analysis; however, footprints were not observed other than between -63 and -48 bp of the promoter fragment. This hypothesis is supported by a methylation interference experiment in which methylation of guanine in the 3' half-site (GTCAA) of the NF-1 consensus element blocked the shifting of the non-NF-1 protein to this region (Figure 24). In contrast, methylation of this guanine and 2 others in the 5' half-site (TTGGC), blocked only shifting competed by NF-1 oligonucleotide. It is unlikely that this non-NF-1 protein is one of the heat sensitive CCAAT binding proteins since binding is not competed by the CAAT portion of the NF-1 oligonucleotide. Therefore, the identity of this factor is unknown. Competition by NF-1 oligonucleotide reduced <u>in vitro</u> transcription to about 40% of control values, which is about the same transcriptional activity observed with the -38 to -8 bp fragment. Therefore, this analysis suggests that the protein not competed by NF-1

oligonucleotide may have no transcriptional activity by itself, but could act as a repressor of transcription by competing with NF-1 for binding to S14 region A. NF-1 and the non-NF-1 protein are not sufficient for induction of Hss-1 since both are present in renal extracts, while Hss-1 is not.

Although renal nuclear extracts successfully initiate in vitro transcription from the S14 promoter, the extracts do not appear to contain the factors present in hepatic nuclear extracts that produce positive and negative effects on rate of transcription in vitro (Figure 22). This is correlated with the simplified number and complexity of renal nuclear factors which bind S14 promoter fragments (Chapter 2). This observation is similar to conclusions made by other investigators. For example, Ryffel et al., (1989) report that a basal promoter construct (-68 to -26 bp) from the Xenopus albumin gene, which contains a TATA box, supports a basal level of in vitro transcription using extracts from L-cells (inactive in vivo), liver (active in vivo), or FTO-2B hepatoma cells (active in vivo). The basal level of in vitro transcription is induced with liver and hepatoma extracts by addition of hepatocyte-specific promoter element (HP1); however, no induction is observed using L-cell extracts. It should be noted that their autoradiograph showed that addition of the HF1 element caused a substantial induction of in vitro transcription in L-cells. An observation has also been made with the hepatic-specific aldolase B promoter and

in vitro transcriptional activity with nuclear extracts from liver and brain (Tsutsumi et al., 1989). Brain extracts supported transcription from the basal promoter element (-92 to -1 bp) at about 5% of the level of hepatic extracts. Addition of promoter sequences to -202 bp increased hepatic transcription 10 fold. Although these authors concluded that these additional sequences did not influence transcription from brain extracts, close examination of the data revealed an induction of at least 4 fold.

The FAS 5' flanking region supported *in vitro* transcription from hepatic extracts but not from renal extracts (Figure 21). Although this could be due to the presence of factors in renal nuclear extracts that repress FAS transcription, it is more likely due to the presence of positive hepatic-specific transcription factors. Monaci et al. (1988) observed that the α1-antitrypsin promoter supported *in vitro* transcription from hepatic extracts but not from splenic extracts. This was due to the binding of two hepatic specific proteins, i.e. LF-B1 and LF-B2. Further studies designed to determine the mechanism of FAS tissue-specific *in vitro* transcription might include a mixing experiment to test whether addition of renal nuclear extracts to hepatic nuclear extracts inhibits hepatic-induced *in vitro* transcription. Finding that renal nuclear extracts do not support detectable levels of transcription from the FAS promoter targets this region for future analyses of elements which function in tissue-specific regulation of FAS promoter

activity.

While hepatic nuclear extracts support *in vitro* transcription from both S14 and FAS promoters, renal nuclear extracts only support *in vitro* transcription from the S14 promoter. This suggests that different mechanisms are operative for tissue-specific expression of S14 and FAS. While tissue-specific expression of transcription factors can explain the FAS observations, it does not account for the S14 data. Hepatic factors might alter chromatin organization in the S14 promoter and possibly in distal enhancers to increase accessibility of DNA for binding by constitutively expressed transcription factors such as NF-1 or related proteins. Alternatively, activity of renal transcription factors might be reduced *in vivo* due to a posttranslational modification (eg. phosphorylation) lost during extraction of nuclear proteins. It is also possible that specific adaptor proteins required by these renal transcription factors are sequestered *in vivo*, and that although the transcription factor is present, it cannot stimulate transcription.

CHAPTER 4: FUNCTION AND INTERACTION OF S14 PROMOTER AND ENHANCER ELEMENTS

INTRODUCTION

In previous chapters, *in vitro* approaches were used to determine the function of proximal promoter elements in initiating and regulating transcription in hepatic nuclear extracts. In order to obtain an *in vivo* correlate, experiments reported within this chapter use stable transfection of 3T3-L1 adipocytes. Expression of S14 mRNA has been examined in 3T3-L1 and 3T3-F442A adipocyte cell lines because of the proposed role of the S14 protein in lipid metabolism (Lepar and Jump, 1989; 1992). 3T3-L1 cells are a well characterized and faithful model for investigation of adipocyte metabolism and differentiation. Differentiation of cells into adipocytes is accompanied by morphological changes, increases in enzymes involved in fatty acid and triacylglycerol synthesis, and an acquisition of responsiveness to lipogenic and lipolytic hormones. 3T3-L1 cells provide an excellent model with which to study function of S14 promoter and enhancer elements *in vivo*.

Although S14 is expressed at very low levels in 3T3-L1 or 3T3-F442A adipocytes, adding DEX or RA to adipocyte medium induces rapid and large increases in S14 mRNA abundance (Lepar and Jump, 1989,1992; Jump et al., 1992). DEX and RA interact synergistically to induce S14 in adipocytes,

resulting in a greater than 150 fold increase in S14 mRNA levels. Efforts to activate S14 gene expression in 3T3-L1 or 3T3-F442A preadipocytes have been unsuccessful. Thus, S14 is an intriguing model for studying transcriptional regulation since both tissue-specific and hormonal factors control S14 gene expression in these adipocyte-forming cell lines.

Stable transfection analyses of 3T3-L1 and 3T3-F442A cells using various deletion/mutation constructs show that the adipocyte-specific DEX and RA regulatory unit is located between -1588 and -1069 bp upstream from the S14 gene (Jump et al., 1992). This enhancer imparts adipocyte-specific hormonal activity since both DEX or RA stimulate CAT activity from this enhancer in adipocytes, but not in preadipocytes, This is similar to regulation of the endogenous S14 gene. Tissue-specific glucocorticoid and RA control is not due to adipocyte-specific expression of hormone receptors, or other factors involved in signal transduction, since glucocorticoids or RA induce CAT activity when initiated from promoters containing multiple glucocorticoid or thyroid hormone response elements, respectively (Jump et al., 1992). This makes the S14 distal enhancer unique and highly interesting in that it contains elements which regulate hormonal control of gene transcription in an adipocyte-specific manner.

The switch from rat liver to mouse adipocyte cell line is justified since previous work (Jump, 1988; Jump et al., 1989; Zilz et al., 1990; MacDougald and Jump, 1991;

MacDougald et al., 1992) has shown that regulation of S14 gene transcription by hormonal, nutritional, and to some extent, tissue-specific factors appears to be mediated through sequences outside the promoter. The function of the promoter may only be to initiate transcription, and to set the basal level of transcription. It may be reasonable to expect that all tissues in which S14 is expressible will have a similar complement of trans-acting regulatory factors since mechanisms of basal expression may not have diverged as they serve a similar function in different tissues and species.

The focus of this chapter is to characterize, within an *in vivo* context, the function of S14 promoter elements defined in previous chapters. Stable transfection of 3T3-L1 fibroblasts and adipocytes with CAT expression plasmids and sequentially deleted proximal promoter elements alone or in the presence of a distal hormonally responsive enhancer is used to further delineate promoter function. In addition, experiments are reported which confirm and extend the definition of DEX and RA responsive regions, as well as tissue-specific control from this multifunctional distal enhancer.

MATERIALS AND METHODS

Plasmid Constructions

a) CAT Vectors

pCAT(An) was obtained from Howard Towle (University of Minnesota). This plasmid contains the bacterial CAT gene and 2 SV40 polyadenylation signals. In order to have an

expression vector into which BamH I/Hind III fragments could easily be inserted, I constructed pCAT(BH) by restricting pCAT(An) with Sal I and Xba I, blunting ends and re-ligating. pS14-2.1-CAT was also obtained from H. Towle; this plasmid contains rat liver S14 genomic sequences extending from -2111 to +19 bp relative to the 5' end of the S14 gene cloned adjacent to the CAT gene. pSV2-NEO and pRSV-CAT plasmids were obtained from S. Conrad (Michigan State University). pTK14Amultimer was obtained from Ron Koenig (University of Michigan) and contains TK-CAT and multiple thyroid hormone response elements (Brent et al., 1987).

b) **Promoter Deletions**

The following series of plasmids were constructed to determine if promoter constructs with a 3' endpoint of -8 bp and 5' endpoints of -464, -290, -151, or -87 bp supported different levels of CAT activity. As a reference for relative promoter activity, pS14(-1601 to +19)CAT was prepared by isolating the Xho I fragment (-1601 to +19 bp) from pS14-2.1-CAT, and ligating this fragment into the Xho I site of pCAT(An). In order to make a compatible -464 to -8 bp insert, pS14T_1(.9)7b(HX) was prepared by restricting pS14T_1(.9)7b with EcoR I, treating with S_1 nuclease, repairing ends with the Klenow fragment and ligating in Xho I linkers. pS14(-464 to -8)CAT was then constructed by ligating the Hind III/Xho I insert from pS14T_1(.9)7b(HX) into pCAT(An). pS14(-290 to -8)CAT was made by purifying the Pst I insert (5' cloning site and -290 bp) away from pS14(-464 to

-8)CAT and re-ligating the plasmid. pS14(-290 to +19)CAT was constructed by restricting pS14(-1601 to +19)CAT with Pst I, treating with S_1 nuclease, Klenow fragment, and re-ligating. pS14(-151 to -8) was constructed by inserting the BamH I/Hind III insert from pS14(-151 to -8) into pCAT(BH). pS14(-87 to -8)(BH) was constructed by replacing the 5' EcoR I site with a BamH I linker. pS14(-87 to -8)CAT was then formed by ligating the BamH I/Hind III insert from pS14(-87 to -8)(BH) into pCAT(BH).

c) **Enhancer/Promoter constructs**

The following set of plasmids were constructed to test whether the presence of promoter elements influences hormonal control from a distally located enhancer. The goal was to create an enhancer construct that could be cloned into the 5' end of the promoter deletions described above. The enhancer construct, pS14(-1601 to -387), was constructed by restricting pS14(-1601 to +19)CAT with Kpn I (-387 bp and 3' to CAT gene), purifying the insert away from the vector and religating the plasmid. The 3' end was modified by replacing an EcoR I site with a BamH I site. The enhancer insert was obtained by restriction with BamH I, followed by electrophoresis, and electroelution of the excised insert. The enhancer, -1601 to -387 bp, was then cloned in the correct orientation into the 5' BamH I sites of pS14(-464 to -8)CAT, pS14(-290 to -8)CAT, pS14(-151 to -8)CAT, pS14(-87 to -8)CAT, and pCAT(BH) to form pS14-CAT103, pS14-CAT102, pS14-CAT101, pS14-CAT100, and pS14-CAT104, respectively.

d) Definition of the Enhancer

The DEX/RA response unit has previously been localized to a region located between -1588 and -1069 bp relative to the transcriptional start site (Jump et al., 1992). This observation was made using a plasmid construction in which this enhancer region was cloned adjacent to pS14(-290 to +19)CAT. In order to confirm and extend this observation, a set of constructs were made in which this region (-1588 to -1069 bp; contained within pS14-Pa(0.5)) was cloned adjacent to S14 promoter sequences -87 to -8 bp. pS14-Pa(0.5) was modified by replacing the Hind III site with a BamH I site. The insert was subsequently purified and subcloned into the BamH I site of pS14(-87 to -8)CAT to form pS14-CAT118 (correct orientation), pS14-CAT119 (reverse orientation), and pS14-CAT120 (2 copies in correct orientation). In order to determine if this enhancer retained hormonal and tissue-specific control when adjacent to a heterologous promoter, the insert containing sequences from -1588 to -1069 bp was also subcloned adjacent to thymidine kinase promoter/CAT gene plasmid (generously provided by Ron Koenig, University of Michigan) to form pTK-CAT208 (correct orientation) and pTK-CAT207 (reverse orientation) and pTK-CAT204 (multiples). I also report CAT activity from plasmids cloned by Kris Knop in which S14 enhancer fragments -1588 to -1381, and -1386 to -1069 bp were inserted into the BamH 1 site of TK-CAT exactly as described above.

The object of the following set of plasmid constructions was to further delineate where DEX and RA cis response elements were located. The region between -1588 and -1069 bp was excised by restriction of pS14-Pa(0.5) with Pst I and the insert isolated. The insert was restricted with Ava I (-1386 bp), and both fragments subcloned into pGEM-1 to form pS14(-1588 to -1381) and pS14(-1386 to -1069). In both plasmids, BamH I linkers were installed to facilitate excision and purification of insert with BamH I. The fragment extending from -1588 to -1381 bp was cloned into the 5' BamH I site of pS14(-87 to -8)CAT to form pS14-CAT121 (correct orientation), pS14-CAT116 (reverse orientation), and pS14-CAT117 (2 copies in correct orientation). The fragment extending from -1386 to -1069 bp was similarly cloned into pS14(-87 to -8)CAT to form pS14-CAT114 (correct orientation) and pS14-CAT115 (reverse orientation). A summary of plasmids cloned for use in this chapter is presented in Table 7.

The region between -1588 and -1381 bp contains a DNase I hypersensitive site in rat liver, and DNase I footprinting of this region showed 2 footprints which changed character during differentiation of 3T3-L1 cells. Accordingly, I had 2 oligonucleotides synthesized by the Michigan State University Macromolecular Facility corresponding to S14 5' flanking sequences -1558 to -1522 bp (TSE-1) and -1482 to 1452 bp (TSE-2) and subsequently cloned them into the 5' BamH I site of pS14(-87 to -8)CAT to form pS14-CAT113 and pS14-CAT111, respectively. In addition, another oligonucleotide

Table 7: Summary of Plasmids used in Transfection Experiments.

Name	S14 5'-Flanking Sequences (bp) Distal	Proximal
Promoter Deletions		
pS14(-464 to -8)CAT	-	-464 to -8
pS14(-290 to -8)CAT	-	-290 to -8
pS14(-290 to +19)CAT	-	-290 to +19
pS14(-151 to -8)CAT	-	-151 to -8
pS14(-87 to -8)CAT	-	-87 to -8
Enhancer/Promoter Constructs		
pS14-CAT100	-1601 to -387	-87 to -8
pS14-CAT101	-1601 to -387	-151 to -8
pS14-CAT102	-1601 to -387	-290 to -8
pS14-CAT103	-1601 to -387	-464 to -8
pS14-CAT104	-1601 to -387	-
Enhancer Definition		
pS14-CAT118	-1588 to -1069	-87 to -8
pS14-CAT119	-1069 to -1588	-87 to -8
pS14-CAT120	2(-1588 to -1069)	-87 to -8
pS14-CAT121	-1588 to -1381	-87 to -8
pS14-CAT116	-1381 to -1588	-87 to -8
pS14-CAT117	2(-1588 to -1381)	-87 to -8
pS14-CAT114	-1386 to -1069	-87 to -8
pS14-CAT115	-1069 to -1386	-87 to -8
pS14-CAT113	-1558 to -1381	-87 to -8
pS14-CAT111	-1482 to -1452	-87 to -8
pS14-CAT112	RARE[a]	-87 to -8
pTK-CAT208	-1588 to -1069	TK[b]
pTK-CAT207	-1069 to -1588	TK
pTK-CAT203	-1381 to -1588	TK
pTK-CAT205	-1069 to -1386	TK

[a] RARE is a synthetic RA response element described by Umesono et al. (1991)
[b] TK: thymidine kinase promoter.

corresponding to a synthetic RARE described by Umesono et al. (1991) was synthesized with BamH 1 ends and cloned into pS14(-87 to -8)CAT to form pS14-CAT112.

Cell Culture

3T3-L1 cells were obtained from H.S. Sul (Harvard

University). Preadipocytes were seeded at ca. 10^5 cells/plate and grown in a maintenance media containing Dulbecco's Modified Eagles Medium (4.5 mg/ml glucose; Gibco Laboratories), 10% calf serum (Armour Pharmaceutical Co.), 100 units penicillin/ml (Sigma Chemical Co.), and 100 units streptomycin/ml (Sigma Chemical Co.). Fibroblasts were maintained at <70% confluence by transferring to new culture dishes until enough plates were obtained to perform the experiment. Cells were differentiated to adipocytes by feeding confluent cells with differentiation media, which contains Dulbecco's Modified Eagles Medium, 10% fetal calf serum (Intergen Co.), 100 units penicillin/ml, 100 units streptomycin/ml, 5 μg bovine insulin/ml (Gibco Laboratories), 0.25 μM DEX (Sigma Chemical Co.), and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma Chemical Co.) added for 72 h. The cells were then maintained on the same media without DEX and 3-isobutyl-1-methylxanthine until harvested. 3T3-L1 cells usually differentiate (>85%) to the adipocyte phenotype under these conditions within 10 days. Media was changed at least every 3 days.

Transfection of 3T3-L1 Cells and CAT Analysis

3T3-L1 fibroblasts were transfected with pSV2-NEO in the presence of promoter/CAT expression plasmids using the lipofection technique (Felgner et al., 1987). Briefly, pSV2-NEO (2μg) and pS14-CAT (20 μg) fusion genes were mixed in 1 ml Opti-MEM (Gibco/BRL) without serum. Two ml of Opti-MEM containing 50 μg of lipofection reagent (BRL) was mixed and

added to the DNA/Opti-MEM solution. This lipofection reagent-DNA mix was added directly to 3T3-L1 fibroblasts that had been washed with phosphate buffered saline. The DNA-lipofection mix was left on the cells for 16 h at 37°C in a 5% CO_2 incubator. Media was then replaced with maintenance media. Within 48 h, cells were trypsinized and transferred to 100 mm plates and fed maintenance media containing 400 μg G418/ml (Geneticin; Gibco/BRL). Within 1 wk, small colonies of G418 resistent cells were evident. Cells were trypsinized, grown to 50-70% confluence on 100 mm petri plates. Cells were then trypsinized and frozen in 10% dimethylsulfoxide at -80°C for 48 h, then in liquid nitrogen until needed. Transfected cells were grown and differentiated as described above except media contained 400 μg G418/ml. Cell cultures were treated with hormones only after preadipocytes were confluent or adipocytes were >85% differentiated. DEX was dissolved in 100% ethanol and administered at 1 μM. RA (all trans; Sigma Chemical Co.) was dissolved in 100% methanol, then diluted with 2 volumes of 100% ethanol (10 mM) before addition to media to make a final concentration of 1 μM. Vehicle was present at only .01%. Cells were treated with hormones for 72 h with 1 change of media after 48 h. CAT activity in fibroblasts represents the mean of duplicate samples. Generally, range between fibroblasts duplicates was <20%. CAT activity in adipocytes represents the mean of triplicate samples. Generally, S.E.M. was <20%.

Following hormone treatment, cell homogenates were prepared for CAT assay (Gorman et al., 1982). Protein concentration of cell extracts were determined using the Biorad Protein Assay (Bradford, 1976) in order to normalize CAT assays for protein concentration. Cell extracts were heated to 70°C for 10 min to inactivate proteases and deacetylases. Reactions (120 µl) contained 125 mM Tris-Cl, pH 7.8, 100 µg protein, 12.5 µg n-butyryl Coenzyme A/ml, and 0.1 µCi of [^{14}C]chloramphenicol (New England Nuclear). Reactions were terminated after 3 h at 37°C by addition of 300 µl mixed xylenes (Aldrich). Tubes were vortexed vigorously for 30 sec, and centrifuged for 1 min in the microcentrifuge. The upper phase (mixed xylenes + butyrylated chloramphenicol) was removed to a new 1.5 ml microfuge tube, and back extracted with 200 µl of Tris-Cl, pH 7.8. The process was repeated as before except 200 µl of the mixed xylene supernatant containing the butyrylated [^{14}C]choramphenicol products were quantitatively removed to a scintillation vial. After addition of 5 ml of scintillation fluid (New England Nuclear), tubes were counted in a liquid scintillation counter (Beckman LS 3150 P) for at least 5 min. Values were compared against a standard curve generated using purified CAT (Pharmacia) and were expressed as CAT units (nmol substrate converted/mg protein/h. All CAT assays were linear with respect to time and protein concentration.

Purification of Nuclei from 3T3-L1 cells

Isolation of nuclei from 3T3-L1 preadipocyte and

adipocyte nuclei was essentially as described by Cook et al. (1985). Briefly, plates were incubated with 3 ml of a solution containing 20 mM Tris-Cl and 2 mM $MgCl_2$ for 5 min before decanting. Cells were scraped into a homogenizing flask in the presence of a buffer containing 10 mM Tris-Cl; pH 7.5, 5 mM $MgCl_2$, 320 mM sucrose, 0.2% Nonidet P-40, 1 mM DTT, and 1 mM PMSF. The homogenate was layered over 10 ml of an identical buffer containing 880 mM sucrose and centrifuged at 3000 X g for 10 min. Pelleted nuclei were washed with 3 ml of an identical buffer containing 250 mM sucrose and without Nonidet P-40. Nuclei were repelleted by centrifuging as before. Nuclei were resuspended in 200 µl of a buffer containing 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 60 µM EDTA, 40% glycerol, 1 mM DTT, and 1 mM PMSF. The absorbance at 260 nm was determined using 5 µl, and the rest was frozen at -80°C until nuclear proteins were extracted. Yield of fibroblast nuclei from 32 100 mm plates was usually about 100 A_{260}/ml and yield of adipocyte nuclei from 24 60 mm plates was usually about 100 A_{260}/ml.

Extraction of Nuclear Proteins

3T3-L1 nuclei were extracted essentially as described by Parker and Topol (1984), as described previously (Chapter 2).

DNase I Footprint and Gel Shift Analysis

DNase I footprint and gel shift assays were performed as described previously (Chapter 2; MacDougald and Jump, 1991) except that 30 µg of nuclear extract was used per DNase I footprint reaction.

Statistical Analyses

Differences between basal CAT activity in the promoter and promoter/enhancer experiments were analyzed using Bonferroni t-statistic (Gill, 1985). Differences in basal CAT activity during differentiation were evaluated using Dunnett's t-test (Gill, 1985). Dunnett's t-test was used to determine which constructs were responsive to DEX. Student's t-test was used to determine whether TSE-1 or TSE-2 were responsive to DEX (Gill, 1985).

RESULTS

Gel shift analysis of promoter element function

To determine if nuclear proteins interacting with S14 promoter elements were conserved between species and expressed in different tissues, I used gel shift analysis to compare DNA-binding nuclear proteins from rat liver, 3T3-L1 fibroblasts, and 3T3-L1 adipocytes (Figure 25). Extracts from all three tissues contained nuclear factors that bound to DNA fragment -87 to -8 bp. The binding of factors from fibroblasts contained a band of the same mobility as a band observed previously in rat liver and kidney (Figure 2). Although this band is observed at low concentrations of hepatic protein, the band shifts up to the smear observed in Figure 25 with addition of more hepatic protein.

Binding of nuclear factors to S14 promoter fragment -151 to -88 bp was qualitatively similar between the three nuclear extracts tested. Shift patterns were very similar between 3T3-L1 fibroblasts and adipocytes. Although many of the same

bands were observed between 3T3-L1 extracts and hepatic extracts, the proportion of DNA shifted into each band was very different. S14 promoter fragment -290 to -168 bp was shifted by all three extracts with rat liver and 3T3-L1 adipocytes containing qualitatively and quantitatively similar DNA-banding activities. Extracts from 3T3-L1 fibroblasts contained at least one unique band, and had neither the number or complexity of bands observed with the other nuclear extracts.

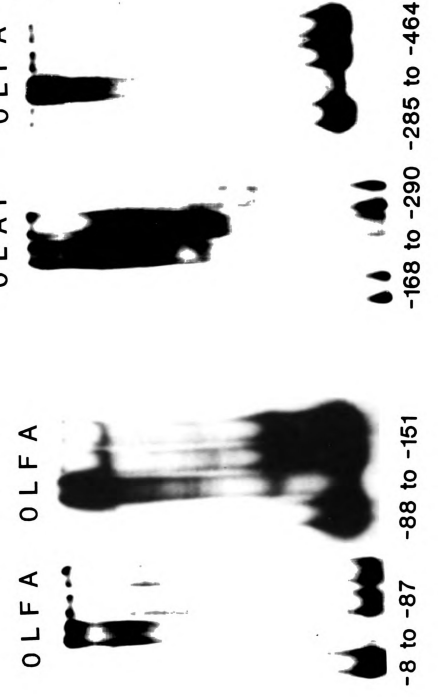
Finally, the presence of nuclear factors that bound strongly to S14 promoter fragment -464 to -285 bp was observed only with rat liver nuclear extracts. This supports a previous observation by Wong et al. (1989) in which they describe a rat hepatic nuclear protein which binds within this region, and is not found in rat adipose tissue. These data suggest that some of the 3T3-L1 nuclear factors binding S14 promoter sequences are under tissue-specific control, and at least some of these DNA binding proteins appear to be similar to factors observed in rat hepatic extracts.

Stable transfection analyses of promoter elements

Activities of transcription factors binding to these regions of S14 5'-flanking DNA were assessed using stable transfection analysis of 3T3-L1 fibroblasts and adipocytes. S14 promoter deletion/mutant constructs were tested that contained 5' endpoints of -464, -290, -151, and -87 bp (Figure 26). S14-CAT constructs containing either -290 to -8 bp or -87 to -8 bp did not support detectable levels of CAT

Figure 25. Gel shift analysis of nuclear extracts from 3T3-L1 fibroblasts and adipocytes with S14 promoter fragments.

Promoter fragments (-87 to -8, -151 to -88, -290 to -168, and -464 to -285 bp) were labelled with ^{32}P as described in Materials and Methods. Labelled DNAs (0.5 ng) were incubated alone (0), or with 5.0 μg nuclear extract from either rat liver (L), 3T3-L1 fibroblast (F), or 3T3-L1 adipocytes (A). The reaction was electrophoresed and the bands visualized by autoradiography. These results are representative of 2 independent preparations of 3T3-L1 nuclear extract.


OLFA
OLFA
OLAF
OLFA
-8 to -87
-88 to -151
-168 to -290
-285 to -464

activity in either 3T3-L1 fibroblasts and adipocytes. This was also observed for the S14-CAT construct containing promoter sequences from -290 to +19 bp (not shown). This indicates that a powerful initiator sequence is not present at the S14 transcriptional start site between -8 and + 19 bp. In contrast, S14 promoter fragment -151 to -8 bp supported CAT activity in both phenotypes. This is remarkably similar to patterns of transcriptional activity observed with *in vitro* transcription from similar S14 promoter deletion constructs as initiated by rat liver nuclear extracts. From these data, it appears that positive transcription factors binding between -151 to -88 bp, and between -464 to -285 bp increase CAT activity, while a repressor of transcription is contained between -290 to -168 bp. Lack of CAT activity in this experiment with S14 sequences extending to -87 bp may be due to insensitivity of the CAT assay. Utilization of a more sensitive reporter gene (eg. luciferase), might improve ability to detect very low levels of transcription.

One of the technical problems associated with the S14 promoter is the extremely low promoter activity observed in Figure 26. In an attempt to overcome this, an enhancer (S14 sequences -1601 to -387 bp) was inserted adjacent to each of the S14 promoter deletion/mutants described above, as well as the promoterless CAT plasmid. The enhancer was cloned 5' to the S14 promoter and in the correct orientation. These plasmids were stably transfected into 3T3-L1 cells, and promoter activity analyzed in both fibroblasts and adipocytes

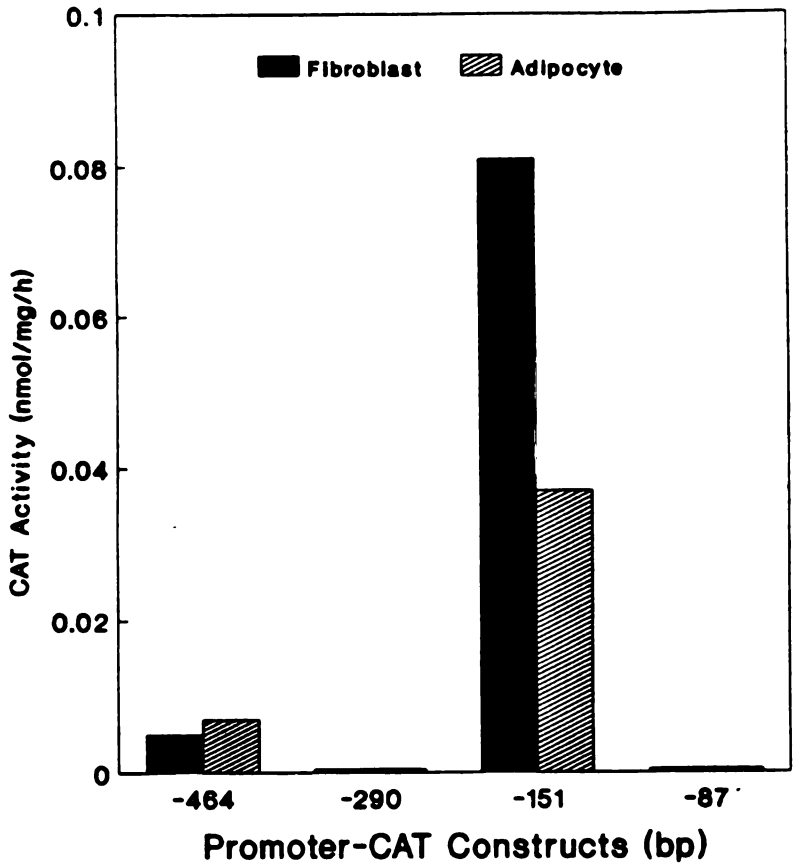


Figure 26. Effect of promoter deletions on CAT activity in 3T3-L1 adipocytes.

Plasmids containing CAT and S14 promoter sequences extending from -8 bp to 5' endpoints of -464, -290, -151, or -87 bp were constructed and stably transfected into 3T3-L1 cells. CAT activity was analyzed in both fibroblast and adipocyte phenotypes and is expressed as nmol/mg/h. Fibroblast and adipocyte data is representative of 2 or 3 replicates, respectively. Statistical analysis revealed that -151 tended to be greater than -290 or -87 ($P < 0.15$). Pooled S.E.M. for adipocyte data is 0.017.

(Figure 27). As expected, addition of the enhancer to promoter-CAT constructs increased basal CAT activity many fold. For example, adding the enhancer to -151 to -8 bp increased basal (without hormones) CAT activity in fibroblasts and adipocytes 3.4 and 4.6 fold, respectively. Interestingly, the pattern of promoter activity observed using enhancer-promoter constructs was similar to results obtained with promoter analyses using stable transfection or *in vitro* transcription.

Various enhancer/promoter deletion constructs were tested for ability to support CAT activity in fibroblasts and adipocytes. Cloning enhancer sequences -1601 to -387 bp adjacent to the CAT gene without a promoter resulted in background levels of CAT activity (Figure 27). However, cloning the enhancer adjacent to -87 to -8 bp induced a detectable level of CAT activity (Figure 27; not shown). This indicates that S14 promoter sequences extending to -87 bp are necessary for basal levels of CAT expression in both 3T3-L1 fibroblasts and adipocytes. Extending the analysis to -151 bp resulted in a dramatic 5 to 6 fold increase in CAT activity in both phenotypes. This high CAT activity was reduced in fibroblasts when sequences extending to -290 bp were analyzed. Finally, sequences extending to -464 bp increased CAT activity in both phenotypes over CAT activity observed promoter sequences extending to -290 bp. Therefore, transcriptional control from the distal enhancer induced basal levels of CAT, but did not influence positive and

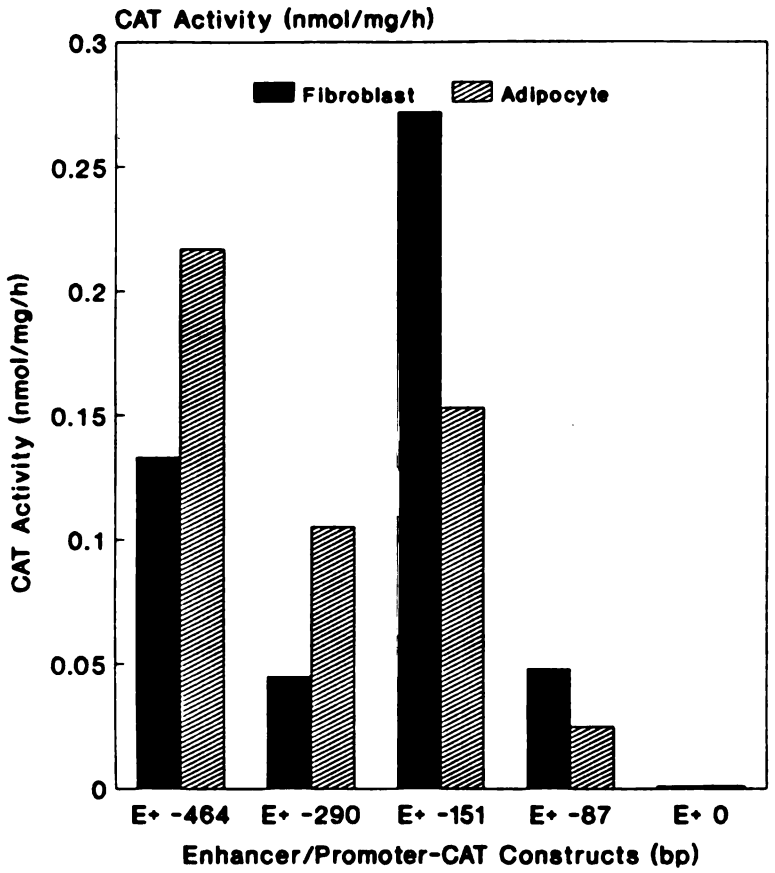


Figure 27. Effect of an enhancer on CAT activity when promoter elements are deleted.

Plasmids containing CAT, S14 enhancer sequences (-1601 to -387 bp), and promoter elements extending from -8 bp to 5' endpoints of -464, -290, -151, -87 or no promoter (0) were constructed and stably transfected into 3T3-L1 cells. CAT activity was analyzed in both fibroblast and adipocyte phenotypes and is expressed as nmol/mg/h. In adipocytes, statistical analysis revealed that -464 is greater than -290 ($P < .01$), and -151 is greater than -87 ($P < .01$). Pooled S.E.M. is 0.023.

negative transcription factors interacting with S14 promoter elements. Although gel shift analysis suggests that at least some of the factors interacting with proximal elements may be regulated during differentiation, effects of these tissue-specific differences do not appear to greatly influence the observed pattern of transcriptional control.

Delineation of the glucocorticoid responsive region

Stable transfection of 3T3-L1 adipocytes was used to test several deletion/mutation constructs for ability to confer glucocorticoid responsivity to the CAT gene (Figure 28). Promoterless CAT constructs are unresponsive to glucocorticoids. Constructs in which enhancer sequences, -1588 to -1069 bp, are cloned adjacent to the -290 bp promoter construct confer glucocorticoid responsivity. This enhancer also works if cloned adjacent to the -87 bp S14 promoter. The -1588 to -1069 bp fragment was split using a convenient Ava I restriction site and both fragments were cloned adjacent and 5' to the -87 bp promoter-CAT construct. The -1588 to -1381 bp fragment induced CAT expression 7.7 fold in response to a 72 h treatment, while the -1386 to -1069 bp fragment did not change CAT expression in response to DEX. Therefore, the glucocorticoid responsive region resides between -1588 and -1381 bp.

To determine whether the S14 glucocorticoid responsive region functions in the context of a heterologous promoter, S14 enhancer fragments (-1588 to -1069, -1386 to -1069, or -1588 to -1381 bp) were ligated adjacent to the thymidine

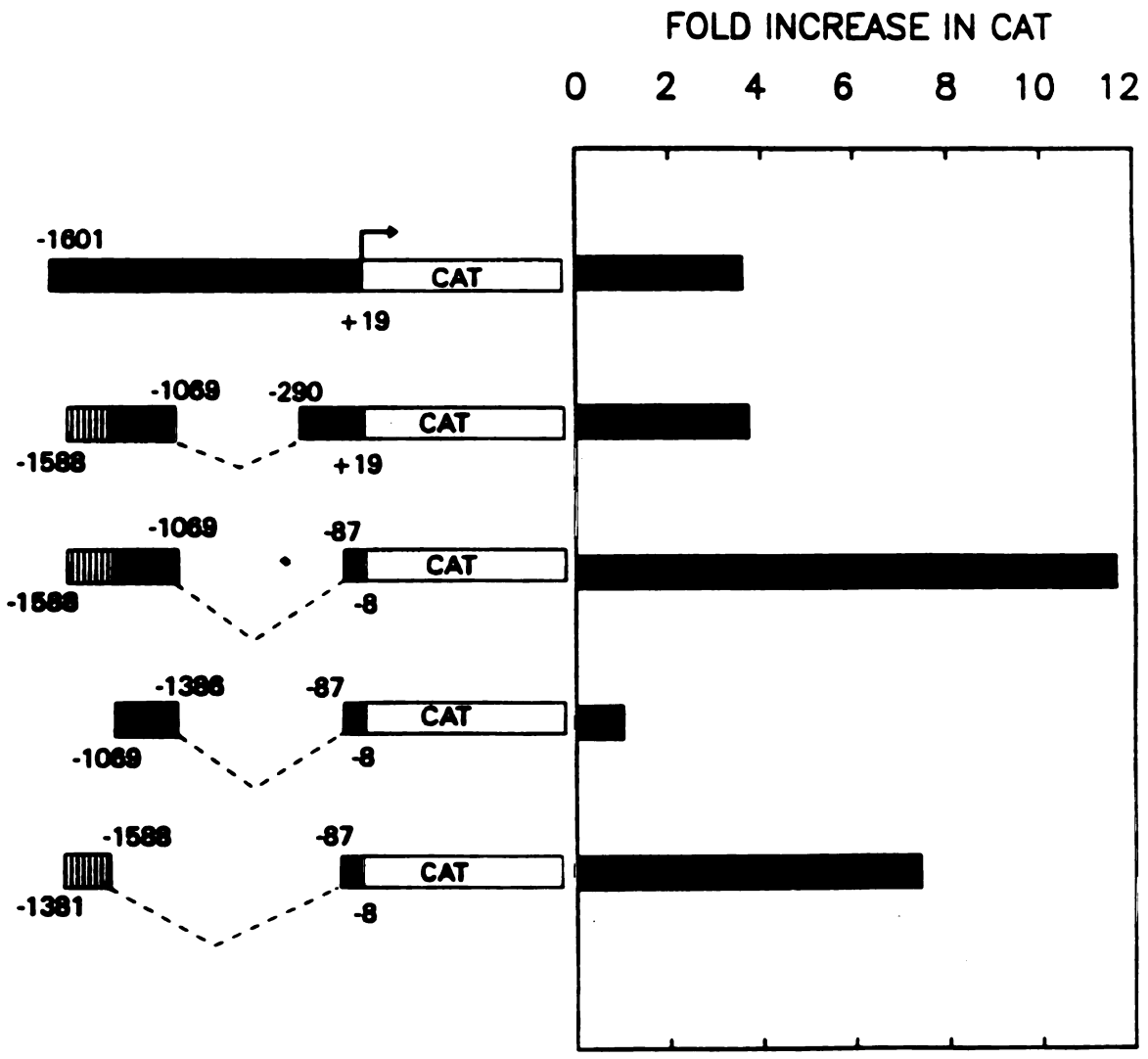


Figure 28. Localization of the S14 glucocorticoid responsive region.

The -1588 to -1069 bp region was inserted into S14-CAT constructs containing S14 sequences -290 to +19 or -87 to -8 bp. The -1588 to -1069 bp fragment was split using Ava I, and the resulting fragments (-1588 to -1381, and -1386 to -1069 bp) were cloned adjacent to the -87 to -8 bp promoter. After plasmids were stably transfected into 3T3-L1 cells, DEX responsiveness was examined in adipocytes following a 72 h hormonal treatment. Data is expressed as fold induction in response to DEX.

kinase promoter (TK-CAT; Figure 29). CAT activity in cells transfected with either TK-CAT or TK-CAT containing thyroid hormone response elements (TRE) did not respond to DEX. However, DEX did induce CAT activity 17 fold and 16 fold in adipocytes transfected with S14TK-CAT(-1588 to -1069) or pS14TK-CAT(-1588 to -1381), respectively (Figure 29). DEX did not induce CAT activity in adipocytes transfected with S14TK-CAT(-1069 to -1386). This analysis confirms that the glucocorticoid responsive region is located between -1588 and -1381 bp, and demonstrates that this enhancer functions as a glucocorticoid responsive region when fused to a homologous or heterologous promoter (Figure 29).

Tissue-specific control from an S14 glucocorticoid responsive region

A consistent increase in basal level of CAT activity was reported when differentiating 3T3-F442A cells transfected with either S14 5' flanking sequences to -1601 bp, or the enhancer region -1588 to -1069 bp (Jump et al., 1992). This increase in basal level of CAT activity has also been observed during differentiation of 3T3-L1 cells. To determine if this tissue-specific enhancer co-localizes with glucocorticoid responsivity, the influence of the glucocorticoid responsive region on basal (unstimulated) CAT activity was examined in 3T3-L1 preadipocytes and adipocytes transfected with various S14TK-CAT fusion genes (Figure 30). Cells transfected with TK-fusion genes containing no enhancer, the thyroid hormone response element, or the -1386 to -1069 bp region showed no change in basal CAT activity as

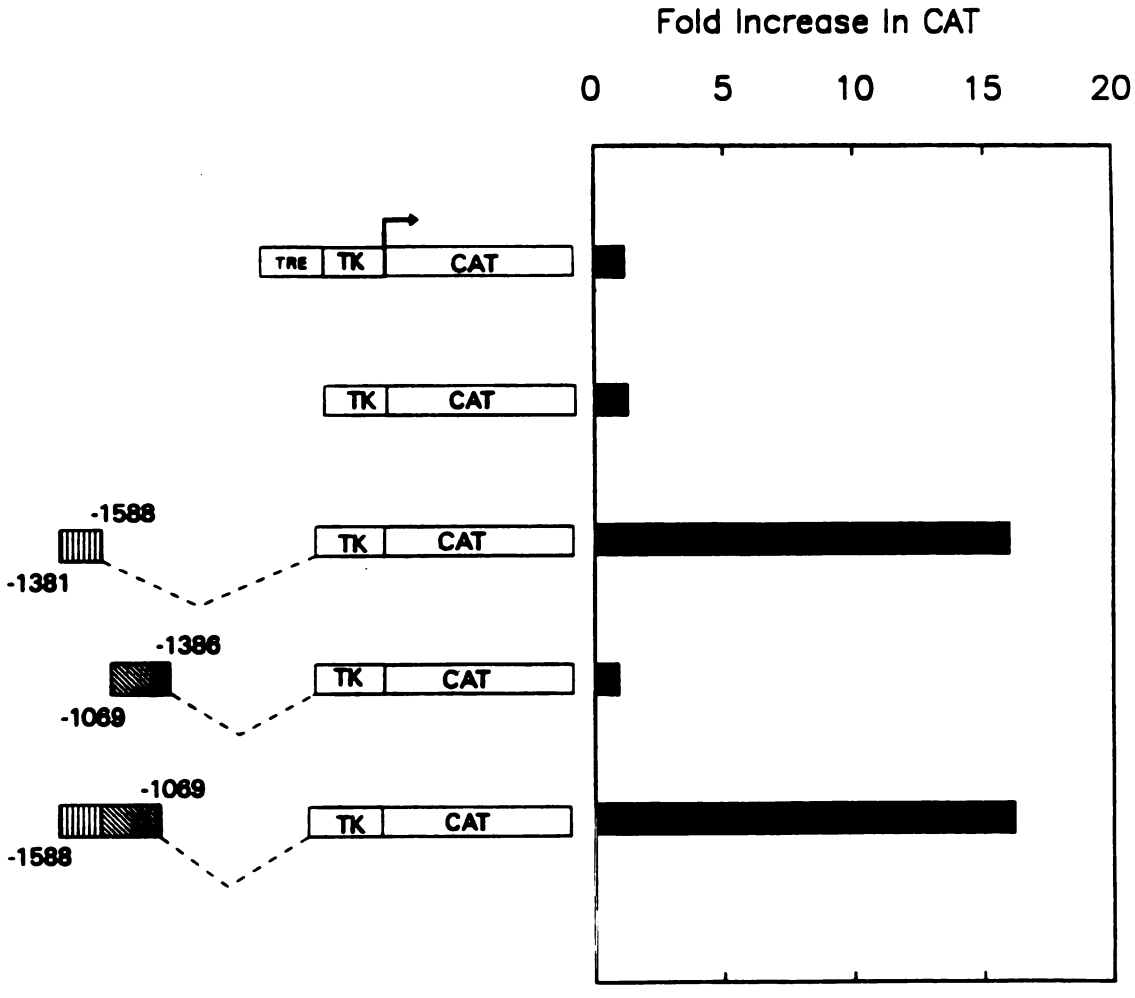


Figure 29. The glucocorticoid responsive region functions on a heterologous TK promoter.

S14 enhancer fragments (-1588 to -1069, -1588 to -1381, and -1386 to -1069 bp) were cloned adjacent to TK promoter sequences in TK-CAT. Control plasmids included enhancerless TK-CAT, and a thyroid hormone response element containing TK-CAT (TRE-TK-CAT). All plasmids were stably transfected into 3T3-L1 cells, and CAT activity analyzed in response to a 72 h treatment with vehicle or 1 μM DEX. Data is expressed as fold induction in response to DEX. Statistical analysis reveals that DEX induced CAT activity from plasmids containing -1588 to -1069 bp or -1588 to -1381 bp ($P < .01$). Pooled S.E.M. is 1.42.

cells differentiated from preadipocytes to adipocytes. However, cells transfected with the -1588 to -1069 bp or -1588 to -1381 bp regions showed an increase in CAT activity of 7 and 4 fold, respectively, as cells differentiated from preadipocytes to adipocytes. Therefore, it appears that the glucocorticoid responsive region (-1588 to -1381 bp) also contains a tissue-specific enhancer.

Transfection studies in 3T3-L1 cells indicate that cis-linked elements involved in both glucocorticoid and tissue-specific control of S14 gene transcription reside between -1588 and -1381 bp. This DNA fragment was analyzed using DNase I footprinting and nuclear extracts from 3T3-L1 fibroblasts and adipocytes (Figure 31). A footprint at Region 1 (TSE-1; -1554 to -1530 bp) was detected using adipocyte nuclear proteins, but was diminished using preadipocyte nuclear extracts. A footprint was also localized at Region 2 (TSE-2; -1477 to -1457 bp) using 3T3-L1 fibroblast nuclear extracts. Addition of nuclear extracts from adipocytes diminished this footprint, although hypersensitive sites induced at both the 5' and 3' ends were maintained. Footprints at both of these regions were observed using nuclear extracts from rat liver (not shown). In addition, rat liver nuclear extracts produced several DNase I footprints not detected using nuclear proteins from 3T3-L1 fibroblasts or adipocytes (not shown). I conclude that 3T3-L1 nuclear proteins interacting with the -1588 to -1381 bp region are under tissue-specific control.

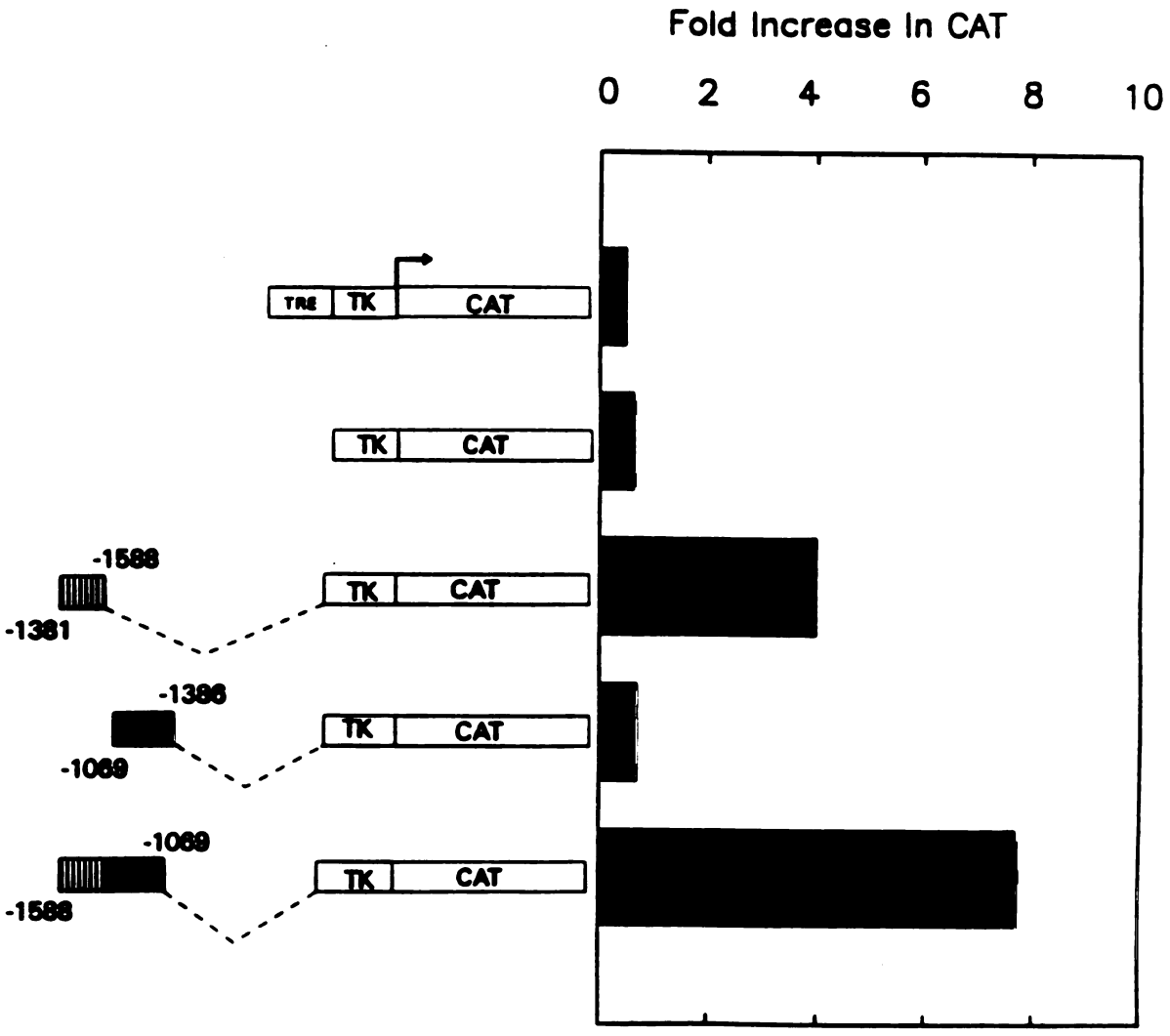


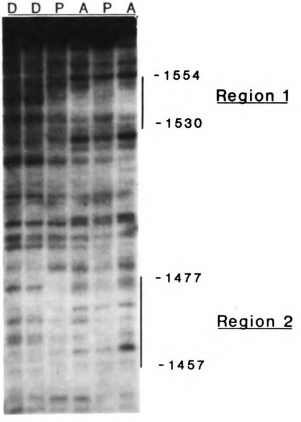
Figure 30. Tissue-specific control from the S14 enhancer.

S14 enhancer fragments (-1588 to -1069, -1588 to -1381, and -1386 to -1069 bp) were cloned adjacent to TK promoter sequences in TK-CAT. Control plasmids included enhancerless TK-CAT, and a thyroid hormone response element containing TK-CAT (TRE-TK-CAT). All plasmids were stably transfected into 3T3-L1 cells, and the basal (unstimulated) level of CAT activity analyzed in fibroblasts and adipocytes. Data is expressed as fold induction during adipocyte conversion. Statistical analysis reveals that CAT activity was induced in plasmids containing -1588 to 1381 or -1588 to -1069 bp ($P < .05$). Pooled S.E.M. is 0.64.

Figure 31. DNase I footprint analysis of the -1588 to -1381 bp region.

The S14 enhancer region (-1588 to -1381 bp) was end-labelled using T_4-polynucleotide kinase, and incubated alone (D) or with 30 μg of nuclear extract from 3T3-L1 fibroblast (P) or 3T3-L1 adipocyte (A). Following digestion with DNase I, ^{32}P-DNA was electrophoretically separated. A DNA sequencing reaction was run in parallel in order to localize sites of DNase I protection (not shown).

DNase I Footprint Analysis of S14 Enhancer Region



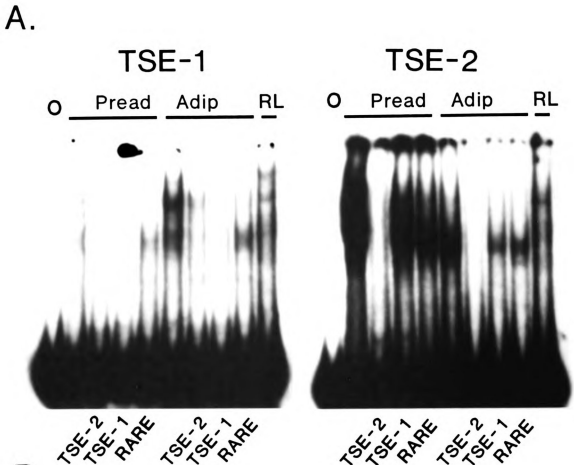
Oligonucleotides corresponding to Region 1 (TSE-1) and Region 2 (TSE-2) were synthesized and used in gel shift analysis (Figure 32A) with nuclear extracts from 3T3-L1 fibroblasts, adipocytes, and rat liver. The oligonucleotide sequences are shown in Figure 32B. Nuclear proteins from adipocyte nuclei induced greater shifting of TSE-1 than nuclear proteins isolated from preadipocyte nuclei. Two bands were observed with adipocyte proteins, while only 1 was observed with fibroblast proteins. Nuclear extracts from preadipocytes contained factors which bound and shifted TSE-2 oligonucleotide much more than nuclear factors from adipocytes. Binding of nuclear proteins to TSE-2 created a gel shift pattern with at least 2 bands. The lower band did not appear to change appreciably during differentiation, while the slower mobility bandshift was reduced in adipocytes. The pattern of binding to both TSE-1 and 2 was qualitatively similar between 3T3-L1 nuclear extracts and rat liver nuclear extracts in that some of the bands appeared to be the same, but both proportions and abundance of banding were quantitatively different. A unique slower migrating band was observed with liver extracts and TSE-1 that was not observed with 3T3-L1 extracts.

Binding of nuclear proteins to TSE-1 and 2 oligonucleotides was specific in that homologous DNA competed for all shifting; however, addition of other oligonucleotides, including a synthetic RARE (Umesono et al., 1991), selectively competed for some binding, probably due to

Figure 32. Gel shift analysis of binding of nuclear extracts from 3T3-L1 preadipocytes and adipocytes to TSE-1 and TSE-2.

A) Oligonucleotides were synthesized to DNase I footprints within the S14 enhancer, labelled with ^{32}P using T_4 polynucleotide kinase, and incubated alone (0) or with 5 μg of nuclear extract from preadipocytes (Pread), adipocytes (Adip) or rat liver (L). Competitor oligonucleotides (TSE-1, TSE-2 or RA response element; RARE) were added to the reaction as labelled. These data are representative of 2 independent experiments.

B) Sequence of oligonucleotides used in A). Sequences sharing identity are either underlined and bolded, or emphasized by bolding and the stippled line.


A.
TSE-1
TSE-2
O
Pread
Adip
RL
O
Pread
Adip
RL
TSE-2
TSE-1
RARE
TSE-2
TSE-1
RARE
TSE-2
TSE-1
RARE
TSE-2
TSE-1
RARE
B.
TSE-1:
GATCCCCACTGGGTCAGAGGCAGCTGTTGCAGGGCTCCAGGG
GGGTGACCCAGTCTCCGTCGACAACGTCCCGAGGTCCCCTAG
TSE-2:
GATCCCTCCCAGCCCTGACCGCAAGGGAACACTCTGG
GGAGGGTCGGGACTGGCGTTCCCTTGTGAGACCCTAG
RARE:
GATCCTCAGGTCACCAGGAGGTCAGAG
GAGTCCAGTGGTCCTCCAGTCTCCTAG

sequences shared between the 3 oligonucleotides (Figure 32B). These data support the DNase I footprint analysis that proteins binding to TSE 1 and 2 are under tissue-specific control. 3T3-L1 nuclear proteins binding to TSE-1 are induced during differentiation, while at least 1 protein binding to TSE-2 is repressed during differentiation.

Glucocorticoid induction of S14-CAT fusion genes containing TSE-1 or TSE-2 oligonucleotides

To determine if TSE-1 or TSE-2 contained sequences sufficient for glucocorticoid responsivity, oligonucleotides containing TSE-1 and 2 were ligated adjacent to the -87 to -8 bp S14-CAT gene and stably transfected into 3T3-L1 preadipocytes. CAT activity was analyzed in both preadipocytes and adipocytes after treatment with vehicle or DEX for 72 h (Figure 33). The S14-CAT construct containing TSE-1 did not confer glucocorticoid responsivity in either phenotype. In contrast, DEX induced a 3.5 fold increase in CAT activity from adipocytes transfected with a TSE-2 containing S14-CAT construct, but did not alter expression of CAT in fibroblasts. This pattern of control closely mimics expression of the endogenous S14 gene. Surprisingly, basal CAT expression from both constructs was not changed during differentiation. From these data, TSE-2 appears to harbor a glucocorticoid responsive element. This is supported by a comparison of sequences from TSE-2 oligonucleotide, S14 sequences overlapping TSE-2, and the consensus GRE sequence (Table 8). The S14 sequence overlapping TSE-2 shares identity with 12 of 15 bp in the consensus GRE (Hard et al.,

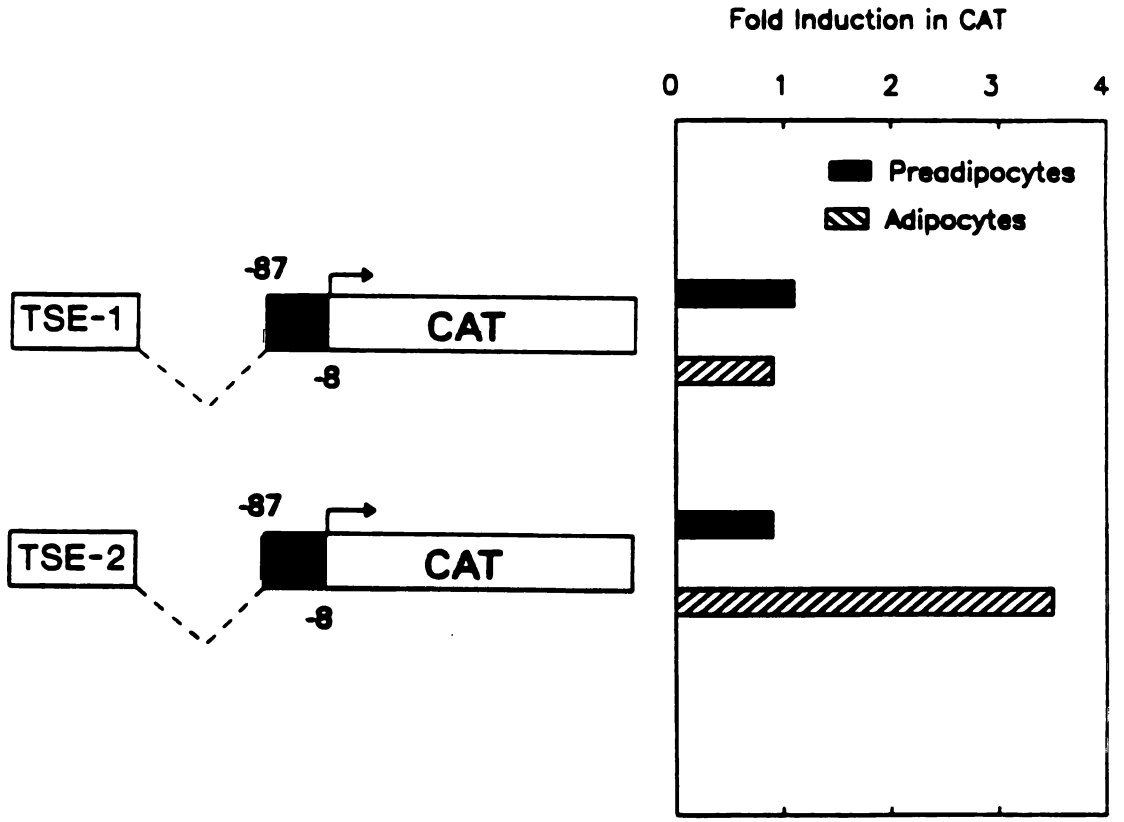


Figure 33. Analysis of glucocorticoid responsivity from TSE-1 and TSE-2.

Oligonucleotides containing sequences from TSE-1 or TSE-2 were cloned adjacent to S14-CAT containing promoters sequences -87 to -8 bp. Plasmids were stably transfected into 3T3-L1 cells and CAT activity was determined in both preadipocytes and adipocytes in response to a 72 h treatment with 1 μM DEX. CAT data is expressed as fold induction in response to DEX. Statistical analysis reveals that DEX induced a significant increase in CAT activity from TSE-2 in adipocytes ($P < .05$).

1990). Part of the GRE 3' half-site was not included in TSE-2 oligonucleotide, since this oligonucleotide was designed to cover only the footprinted sequence. However, the BamH 1 linker replenished a thymine at the 3' end, which may have increased the strength of this GRE. Experiments are ongoing to determine whether glucocorticoid receptors bind this sequence, and whether the putative S14 GRE works with a heterologous promoter.

Table 8: Comparison of the TSE-2 oligonucleotide, GRE consensus, and S14 DNA sequences[a].

TSE-2 Oligo		**GGAACACTCTGGGAT**
GRE Consensus[b]		**GGTACANNNTGTTCT**
S14 Sequence	-1461	**GGAACACTCTGTTTG** -1447

[a] Sequences identical to the consensus GRE sequence are in bold.
[b] Consensus GRE from Hard et al., 1990

Partial definition of a S14 RA responsive region

The -1588 to -1069 bp region also harbors RA responsive cis-acting elements (Jump et al., 1992). Deletion/mutant S14-CAT constructs were analyzed to determine if RA responsivity co-localized with glucocorticoid responsivity (Figure 34). To determine if tissue-specific RA control was due to adipocyte-specific expression of RA receptors or some other part of the RA regulatory network, a synthetic RARE (Umesono et al., 1991) was cloned adjacent to the S14-CAT construct containing S14 promoter sequences -87 to -8 bp. This construct was stably transfected into 3T3-L1 cells, and analyzed for RA responsivity in both fibroblast and adipocyte

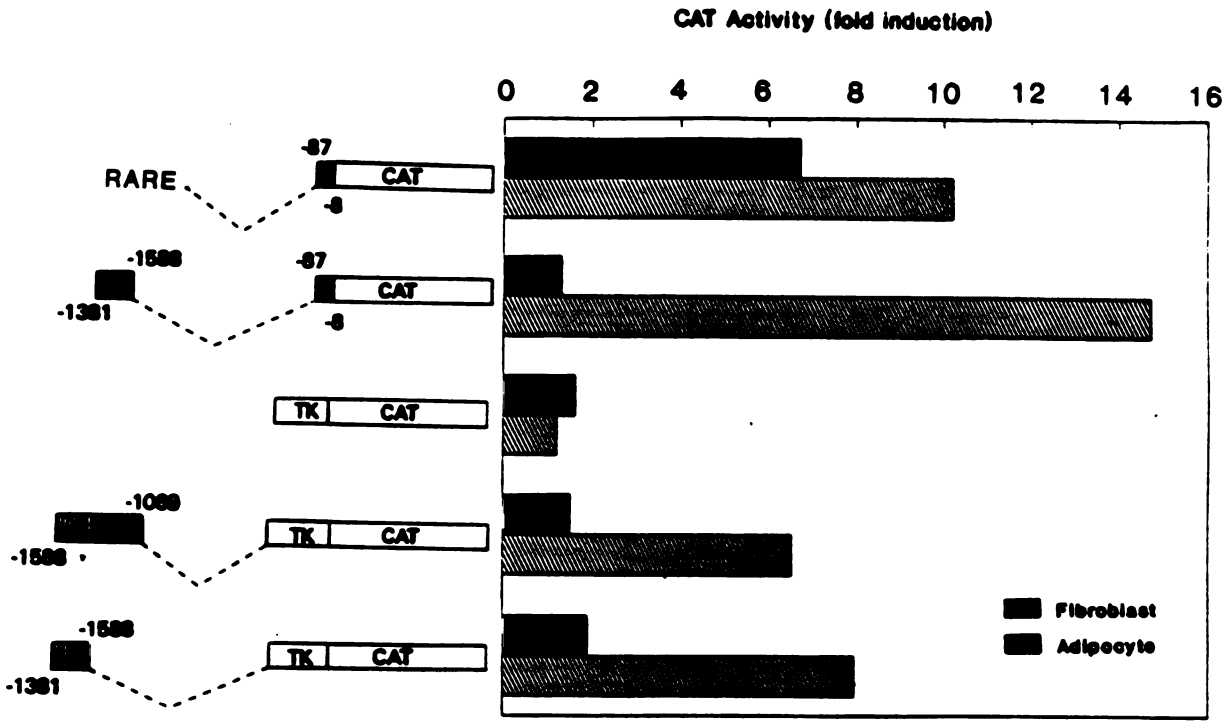


Figure 34. Localization of the S14 RA responsive region.

Several plasmids were analyzed for the ability to confer RA responsivity to a CAT reporter gene. A synthetic RARE as well as S14 enhancer sequences -1588 to -1381 bp were cloned adjacent to S14-CAT (-87 to -8 bp). In addition, S14 enhancer sequences (-1588 to -1069 and -1588 to -1381 bp) were cloned adjacent to the thymidine kinase promoter in TK-CAT. These plasmids and enhancerless TK-CAT were stably transfected into 3T3-L1 cells and analyzed for responsiveness to 1 μM RA in both preadipocyte and adipocyte phenotypes. CAT data is expressed as fold induction over vehicle in both phenotypes.

phenotypes. RA induced CAT activity 6 and 10 fold from this construct in preadipocytes and adipocytes, respectively. In contrast, S14 sequences from -1588 to -1381 bp gave no RA induction in fibroblasts, but conferred a 14 fold induction in adipocytes. Therefore these sequences function to confer not only tissue-specific DEX control, but also adipocyte-specific RA responsivity to a homologous promoter.

To determine if these sequences also confer RA responsivity to a heterologous promoter, experiments were performed with TK-CAT constructs. The TK promoter supported no RA responsivity (Figure 34); however, addition of S14 promoter sequences -1588 to -1069 bp or -1588 to -1381 bp to TK-CAT caused a 6 fold or 8 fold induction in adipocytes, respectively, while no effect was observed in preadipocytes. Therefore, the RA responsive region also bestows tissue-specific RA responsivity to a heterologous TK promoter. S14-CAT constructs containing TSE-1 or TSE-2 did not confer any RA control; therefore, further experiments will be required to delineate the tissue-specific RA regulation from the -1588 to -1381 bp region.

Effect of promoter deletions on hormonal function from a distal enhancer

The S14 promoter contains elements which have positive and negative effects on promoter activity (MacDougald and Jump, 1991). In addition, the DEX and RA responsive region has been localized between -1588 and -1381 bp. In an attempt to determine whether hormonal effects from this distal enhancer are dependent on the presence of specific elements

within the promoter region, a set of promoter deletions were performed while keeping the distal enhancer located at a distance. These plasmid constructs were stably transfected into 3T3-L1 cells, and CAT activity analyzed in fibroblasts and adipocytes (Figure 35). These observations should be regarded as preliminary since they represent results from 1 set of transfection experiments (n = 3). This experiment will have to be replicated numerous times in order to feel confident that results are not due to irregularities in integration site or other problems associated with stable transfection analysis.

As expected, hormonal control was not observed with pCAT alone (not shown). In addition, hormonal control was not observed in either phenotype with an enhancer-CAT construct lacking promoter sequences. However, addition of enhancer region (-1601 to -387 bp) conferred adipocyte-specific DEX and RA control to constructs containing S14 promoter sequences.

The enhancer adjacent to the -464 to -8 bp promoter gave approximately 10 fold and 5 fold induction in response to DEX or RA, respectively. Adding both DEX and RA gave an additive response. When the enhancer was cloned adjacent to -290 to -8 bp, Dex gave a 4 fold induction, while RA had no effect. DEX and RA gave no further increase than DEX alone. Using the enhancer plus -151 to -8 bp promoter construct gave a 6.5 fold induction for DEX and no induction for RA. Once again, DEX and RA did not induce CAT activity more than DEX alone.

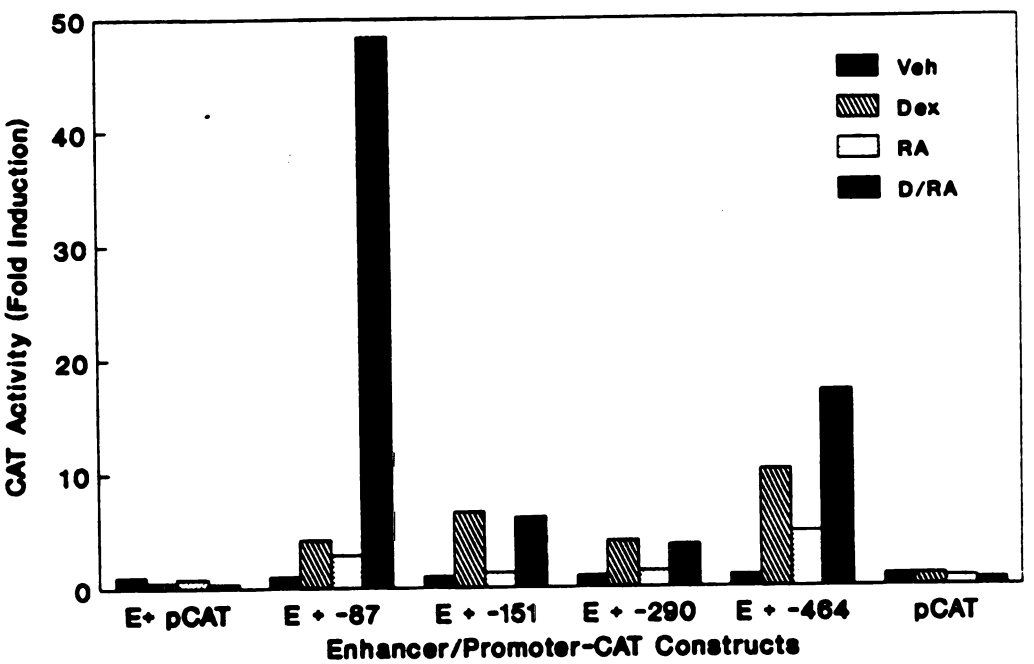


Figure 35. Effect of promoter element deletion on hormonal control from a distal enhancer.

Plasmids containing CAT, S14 enhancer sequences (-1601 to -387 bp), and promoter elements extending from -8 bp to 5' endpoints of -464, -290, -151, -87, or 0 bp were constructed and stably transfected into 3T3-L1 cells. Promoterless pCAT was also transfected as a control. Adipocytes were treated with vehicle, DEX, RA, or DEX and RA for 72 h. Data is expressed as fold induction over vehicle.

Finally, deletion of sequences such that the enhancer was cloned adjacent to -87 to -8 bp gave a 4 fold and 3 fold induction in response to DEX or RA, respectively. Addition of DEX plus RA increased CAT activity in a synergistic manner, increasing CAT activity by 48 fold. From these data, it appears that glucocorticoids can operate from a distal enhancer to induce CAT activity in a manner relatively independent of promoter elements.

In contrast, RA action from the distal enhancer was only observed with promoter elements extending to -464 or -87 bp, implying that sequences between -88 and -151 bp block RA effects from a distal enhancer. However, this inhibitory effect can be overcome by addition of sequences between -290 and -464 bp, or deletion of promoter sequences such that the enhancer is adjacent to -87 to -8 bp. Finally, promoter elements may have a large influence on synergy observed between different hormones. Although RA and DEX effects were additive with a promoter fragment extending to -464 bp, effects of DEX and RA were more than multiplicative when promoter sequences were deleted to -87 bp. This may mean that promoter elements contribute to interaction observed between distal hormone regulatory regions, thus functioning as an additional level of transcriptional control.

DISCUSSION

Gel shift analyses reveal that expression of nuclear proteins interacting with S14 promoter elements is under tissue-specific control. Although rat liver, 3T3-L1

fibroblasts and 3T3-L1 adipocytes express qualitatively similar proteins, the relative expression of each protein is different between tissues (Figure 25). Even though some of the proteins interacting with S14 promoter elements are different between tissue-types, the overall function of S14 promoter regions is similar in rat liver and 3T3-L1 cells. Stable transfection and *in vitro* transcription analyses of promoter deletions show that 3T3-L1 cells and rat liver contain a similar pattern of positive and negative transcription factors (Figure 26; Chapter 2,3). Furthermore, transcription factors binding to S14 promoter elements influence basal level of transcription even when an enhancer is stimulating the overall level of transcription (Figure 27), indicating that these proteins may have an important role in establishing the set point for S14 gene transcription. Tissue-specific differences in DNA-protein interaction may reflect expression of nuclear proteins important for tissue-specific interaction with distal enhancers.

Although I and others (Wong et al., 1989) could not detect substantial binding of nuclear proteins to S14 promoter fragment -464 to -285 bp, addition of this sequence appears to increase baseline level of CAT activity (Figure 25 and 26). It may be that low affinity or low abundance transcription factors bind within this sequence and function to induce S14 gene transcription. It may also be that DNA-binding activities of nuclear factors interacting within this

region are regulated by posttranslational modifications lost during protein extraction.

Baseline levels of CAT activity from S14-CAT constructs (extending to -1601 bp) consistently increase as 3T3-L1 cells differentiate from preadipocytes to adipocytes (Jump et al., 1992). Changes in baseline levels of CAT activity were analyzed from S14TK-CAT constructs stably transfected into 3T3-L1 cells before and after differentiation into adipocytes (Figure 30). The tissue-specific enhancer was located between -1588 to -1381 bp. In an attempt to localize putative tissue-specific element(s) responsible for the induced CAT activity observed in differentiated cells, DNase I footprint analysis was used to show that nuclear proteins from 3T3-L1 cells bind the S14 enhancer in two regions called TSE-1 and TSE-2 (Figure 31). Proteins binding to TSE-1 (-1554 to -1530 bp) are induced during differentiation, while at least some of the proteins binding to TSE-2 (-1477 to -1457 bp) are repressed in the adipocyte phenotype. Footprints for both of these regions are characterized by a complex pattern of DNase I protections and hypersensitivities. The same footprint pattern is observed using hepatic nuclear extracts, perhaps indicating that these proteins are required for regulated expression of S14 in other tissues where S14 is transcribed.

DNase I footprinting analysis indicates that binding of nuclear proteins to the S14 enhancer is tissue-specific. These results are corroborated by gel shift analysis (Figure

32A) using oligonucleotides corresponding to each DNase I footprint and nuclear extract from 3T3-L1 fibroblasts and adipocytes. I observed a very complex pattern of competition with oligonucleotides corresponding to TSE-1, TSE-2, or a RARE (Figure 32A); this may be because sequences within these oligonucleotides share identity (Figure 32B). This complex pattern of nuclear protein binding and competition may indicate that I have scraped the tip of a very complex regulatory iceberg.

I investigated adipocyte-specific glucocorticoid control from a S14 distal enhancer. I demonstrate that in 3T3-L1 adipocytes, glucocorticoid control is mediated by S14 sequences -1588 to -1381 bp (Figure 28), and that much of this control is mediated from a sequence overlapping TSE-2 that has high identity (12/15 bp) to a consensus GRE (Figure 33; Table 5). I also demonstrate that S14 enhancer sequences (-1588 to -1381 bp) confer glucocorticoid control to a heterologous TK promoter. This shows that S14 promoter elements are not required for the action of glucocorticoids from this enhancer (Figure 29).

I also investigated adipocyte-specific RA control from a S14 distal enhancer. I found that RA control co-localizes with the glucocorticoid regulatory region (-1588 to -1381 bp) and works in an adipocyte-specific manner with either homologous or heterologous promoters (Figure 34). Jump et al. (1992) demonstrated that the RA regulatory network was intact in fibroblasts using a RA responsive thyroid hormone

response elements (Brent et al., 1989). I demonstrated that a stably transfected S14-CAT construct containing a single synthetic RARE (Umesono et al., 1991) is induced by RA in both fibroblast and adipocyte phenotypes (Figure 34). This confirms that RA can stimulate gene expression in fibroblasts, and suggests that some other factor must be selectively regulating (blocking) RA control of S14 gene transcription in 3T3-L1 fibroblasts. One possibility is that since the RAREs tested (Brent et al., 1989; Umesono et al., 1991) are trans-activated by RARα, which is found in both 3T3-L1 phenotypes, RA control of S14 gene expression is mediated through another RA receptor family member (eg. RXR) that is expressed in a tissue-specific manner.

Tissue-specific binding of nuclear factors to TSE-1 and TSE-2 may have importance for adipocyte-specific hormonal control from this enhancer. For example, binding of proteins to TSE-2 is repressed in the adipocyte (Figure 32). Since sequences overlapping this element are involved in glucocorticoid control of S14 gene transcription (Figure 33; Table 5), it may be that binding of preadipocyte-specific protein(s) near the S14 GRE block binding of glucocorticoid receptors. Reduced binding of factors to TSE-2 in adipocytes may release the blockade making this site a target for glucocorticoid receptor binding and gene activation. Although I have not localized RA control beyond the -1588 to -1381 bp fragment, it is conceivable that tissue-specific nuclear proteins interacting with TSE-1 or TSE-2 (or other

sites) are involved in a similar manner in regulating binding and/or activity of RA receptors, resulting in adipocyte-specific control by RA. Sequences sharing identity with the RARE described by Umesono et al. (1991) are found in or overlapping both TSE-1 or TSE-2, but no RA control has been ascribed to either of these sequences.

The object of the final set of experiments was to determine if deletion of promoter elements influences hormonal control from a distal enhancer (Figure 35). These data demonstrate that these enhancer/promoter constructs support adipocyte-specific DEX and RA control. They also show that DEX acts relatively independently of promoter elements and distance from the promoter. In contrast, RA induces CAT activity when the enhancer is cloned adjacent to the -464 bp or -87 bp promoter fragments, but provides no induction when cloned adjacent to -290 or -151 bp fragments. This may mean that an inhibitor of RA action is present between -151 and -88 bp, and that elements between -464 and -290 can override this inhibition, or somehow circumvent the negative action by supplying an independent mechanism for RA action.

I also examined interactions between DEX and RA on the induction of CAT activity (Figure 35). These experiments were done with an intact enhancer (-1601 to -387 bp) and various deleted promoter fragments. No interaction was observed between DEX and RA when the enhancer was cloned adjacent to promoter constructs extending to -464, -290, or

-151 bp. The effects of DEX and RA were strictly additive, although there was no RA effect with -290 or -151 bp. This type of additive effect suggests that DEX and RA have independent mechanisms of action.

When the enhancer was cloned adjacent to the -87 to -8 bp construct, DEX plus RA induced CAT activity 48 fold, while DEX alone induced CAT activity 4 fold and RA induced CAT activity by 3 fold. This interaction is much more than multiplicative and implies that RA receptors and glucocorticoid receptors interact while influencing rate of transcription. It should be noted that I have not determined that these hormones act directly through their respective receptors. They could also be working indirectly through regulation of abundance or activity of other transcription factors.

This research demonstrates that S14 promoter sequences -87 to -8 bp are required for initiation of transcription and also for trans-activation of transcription from a distal enhancer. It also shows that the S14 promoter determines the set point from which positive (glucocorticoids, RA) or negative regulators act, and that it plays a role in at least some of the hormonal stimulation and interaction from a distal enhancer. Finally, the S14 promoter contains elements which have either positive or negative effects on gene transcription, and these roles are similar in at least two tissues in which S14 is expressed.

CHAPTER 5: SUMMARY AND CONCLUSIONS

Four research problems are examined within this dissertation. They are to: 1) define S14 promoter elements and determine their effects on initiation of S14 gene transcription; 2) determine if S14 promoter elements are sites for regulation by developmental, dietary or tissue-specific factors; 3) characterize and further define adipocyte-specific glucocorticoid and RA responsive regions in a S14 distal enhancer; 4) determine if there is a functional relationship between the S14 enhancer and promoter.

What we now know about the S14 promoter

Although the S14 promoter has weak activity *in vivo* (Jacoby et al., 1989), it is sufficient and necessary for initiation of S14 gene transcription both *in vitro* and *in vivo*. *In vitro* transcription can be initiated from as little as 30 bp of S14 promoter sequence extending from -38 to -8 bp. Demonstrable reporter CAT gene activity in stably transfected 3T3-L1 cells was observed with promoter sequences -151 to -8 bp. Transcriptional effects from a distal enhancer were dependent on the presence of a promoter. Enhancer sequences functioned with S14 promoter sequences -87 to -8 bp, which was the minimal promoter tested.

The S14 promoter contains elements which bind a number

of hepatic factors having positive and negative effects on initiation of transcription in vitro and in vivo. Binding of factors to these elements is not strictly tissue-specific. Some promoter fragment binding activities are similar between nuclear extracts from 3T3-L1 fibroblasts, 3T3-L1 adipocytes, rat liver, and rat kidney while others are different. Even though some apparent differences were observed in protein/DNA interaction between 3T3-L1 phenotypes, baseline levels of CAT activity were similar between fibroblasts and adipocytes. In addition, the trans-activational activity of each element on transcription appears to be similar between tissues, since rat liver, 3T3-L1 fibroblasts, and 3T3-L1 adipocytes contain the same 3 positive elements and 1 negative element as determined using in vitro transcription and stable transfection analyses.

In spite of exceedingly low rates of renal S14 transcription in vivo, renal nuclear extracts support basal levels of S14 in vitro transcription comparable to hepatic nuclear extracts. This is likely due to the expression of NF-1 in both tissues. Positive and negative control of this basal level is sharply diminished with renal nuclear extracts, probably due to the lack of renal nuclear factors interacting with more distal regulatory regions. In contrast, FAS promoter DNA transcribes the G-free cassette in the presence of hepatic, but not renal nuclear extracts. This is correlated with FAS expression observed in vivo. It is likely that higher level of hepatic FAS mRNA is due to

expression of hepatic-specific transcription factors. Even though gross regulation of S14 and FAS mRNA level is highly correlated across a number of physiological states (Clarke et al., 1990a,b), it appears that tissue-specific regulation from S14 and FAS promoters is through different mechanisms.

Although there is no evidence for regulation of transcription factors binding to S14 promoter elements, it is possible that other regulatory pathways not yet directly evaluated (eg. protein kinase A and C, IP_3, Ca^{2+}, or tyrosine kinase) exert their regulatory effects through these promoter elements. It is clear, however, that one important function of the S14 promoter is to set the basal level of promoter activity. This transcriptional set point can have dramatic effects on absolute levels of gene expression, since irrespective of promoter fragment, glucocorticoids induced CAT activity a relatively constant amount above the baseline expression of CAT. Since presence/absence of S14 promoter elements alters transcription up to 8 fold, this could greatly increase the impact of this hormone.

There are many possible mechanisms whereby S14 promoter elements/transcription factors could influence rate of transcription. Activation domains of transcription factors having a positive effect on transcription may interact directly with factors involved in forming the preinitiation complex, or influence the rate of complex formation indirectly through adaptor proteins. Alternatively, positive transcription factors may sequester adaptors which are

involved in reducing rate of preinitiation complex formation, or directly reduce effects of negative transcription factors by blocking or competing for their interaction with preinitiation complex transcription factors. Effects of negative transcription factors can be modeled in a similar manner, with direct and indirect effects on rates of formation of preinitiation complexes, as well as effects mediated through other DNA-binding transcription factors.

What we now know about the glucocorticoid/RA responsive S14 enhancer

An S14 enhancer involved in conferring tissue-specific hormonal control has been further delineated. Adipocyte-specific DEX and RA control colocalizes between -1588 and -1381 bp upstream of the S14 transcriptional start site, and functions adjacent to homologous or heterologous promoters. This region is also responsible for an induction in CAT activity during differentiation of 3T3-L1 cells into adipocytes. Binding activity of 3T3-L1 nuclear proteins to this enhancer is under tissue-specific control with both induction and repression of transcription factors observed during adipose conversion.

The location of this S14 enhancer corresponds with the location of rat hepatic DNase I Hss-2. Although chromatin organization is likely important in the context of hormonal and/or tissue-specific control in 3T3-L1 cells, hormone-inducible Hss have not been evaluated within these cells. Indeed, adipocyte-specific hormonal control could simply be due to induction of a Hss within this region, thus increasing

the accessibility of hormone receptors and/or ancillary factors to DNA.

Glucocorticoid control appears to be mediated from a site overlapping a binding site for proteins repressed during differentiation of 3T3-L1 cells. Therefore, an alternative mechanism explaining adipocyte-specific glucocorticoid control might be that fibroblastic nuclear factors block interaction of glucocorticoid receptors with the S14 glucocorticoid response element. Once expression or binding activity of these transcription factors is repressed during differentiation of cells into adipocytes, glucocorticoid receptors bind DNA and activate S14 gene transcription. Similar mechanisms could also be operative for adipocyte-specific RA control, with the additional thought that although RARα is expressed in both 3T3-L1 phenotypes, the S14 RA responsive region may be bound by another member of the receptor family (eg. RXR) that is expressed only in adipocytes.

What we now know about functional interactions between enhancer and promoter elements

Interaction between enhancer and specific promoter elements appears to be important for mediating RA control from the S14 distal enhancer, but has little influence on DEX control. Preliminary data is presented which indicates that promoter elements may be important for regulating synergism between hormones. Although the mechanism is unknown, it is possible that enhancer-binding proteins interact directly or

indirectly with transcription factors involved in forming the preinitiation complex, or interact directly or indirectly with proteins binding within the region proximal to the transcription start site.

Importance for Lab and Science

This work has greatly increased my understanding of the S14 promoter by taking what was effectively a DNA sequence and giving it life in terms of its role and importance in initiating and regulating S14 gene transcription. In addition to characterizing DEX and RA response elements, this work has led to hypotheses concerning adipocyte-specific RA and DEX control of S14 gene transcription which are currently being tested. Finally, this research has opened an avenue of study directed towards understanding physical and functional interactions between enhancer and promoter elements. Understanding these interactions has important consequences for understanding hormonal control from distal enhancers, and introduces another possible mechanism for synergy between hormones. Information gained from the S14 gene will likely have general significance, since many hepatic genes involved in metabolism are coordinately regulated.

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