Biochem. J. (1991) 280, 761–767 (Printed in Great Britain)

Identification of functional *cis*-acting elements within the rat liver S14 promoter

Ormond A. MACDOUGALD and Donald B. JUMP*
Cell and Molecular Biology Program, Department of Physiology, Michigan State University, East Lansing, MI 48824-1101, U.S.A.

The structure of DNAase I hypersensitive site 1 (Hss-1), located adjacent to the 5′ end of the rat liver S14 gene, is regulated by tissue-specific factors, and its formation correlates with the transcriptional activation of the S14 gene. We propose that tissue-specific *trans*-acting factors interacting with key *cis*-linked elements within this site function in the initiation of S14 gene transcription. To examine this hypothesis we used DNAase I footprint, gel shift and *in vitro* transcriptional analyses to identify *cis*-linked elements that function in the control of S14 gene transcription. Binding of rat liver nuclear proteins to the S14 promoter (from −8 to −464 bp) produced four DNAase I footprints (designated A–D). Gel shift studies showed that DNA–protein binding was tissue- and sequence-specific, differentially heat-sensitive, and abolished by proteinase K. The function of the four *cis*-acting elements was assessed by using an *in vitro* transcription initiation assay in which the S14 promoter was fused to a reporter gene (G-free cassette). Deletion studies showed that nuclear factors binding to regions A (−48 to −63 bp), B (−88 to −113 bp) and D (−286 to −310 bp) enhanced the rate of initiation of transcription, while proteins binding to region C (−227 to −244 bp) suppressed the rate of initiation of transcription. Based on oligonucleotide competition studies, we suggest that hepatic NF-1 (or a related protein) binding to the A region enhances the rate of initiation of S14 gene transcription. Since *trans*-acting factors interacting with regions B and D are found in liver but not in spleen or kidney, we suggest that the proteins interacting with these regions may be involved in the tissue-specific augmentation of S14 gene transcription.

INTRODUCTION

The rat liver S14 gene is an excellent model with which to study the multifactorial control of gene expression, because the gene is regulated at the transcriptional level by tissue-specific (Jump, 1989*a*), developmental (Jump *et al.*, 1988), nutritional (Hamblin *et al.*, 1989; Jump *et al.*, 1990*a*,*b*) and hormonal (Jump & Oppenheimer, 1985; Lepar & Jump, 1989; Jump 1989*b*) factors. The S14 gene is expressed at high levels 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, 1989*a*; Lepar & Jump, 1989). The pattern of physiological and tissue-specific control has led to the suggestion that the S14 protein functions in lipid metabolism. Thus the S14 gene serves as a model for understanding the interaction of regulatory networks involved in lipogenic gene expression.

In previous studies, we used chromatin structure analysis to locate prospective *cis*-acting elements controlling S14 gene transcription (Jump *et al.*, 1987, 1988, 1990*a*; Jump, 1989*a*,*b*). While six DNAase I hypersensitive sites (Hss) flank the 5′ end of the hepatic S14 gene (Jump *et al.*, 1990*a*), two of these sites may be particularly important in S14 gene transcription, i.e. Hss-1, located at −65 to −265 bp, and Hss-3, located between −2.55 and −2.75 kb. The 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, 1990*a*; Jump, 1989*a*,*b*). The dynamic regulation of these sites suggests that key *trans*-acting factors bind to these sites to regulate chromatin structure and gene transcription. This view has recently been supported by the finding that Hss-3 contains thyroid hormone response elements which function as upstream enhancers (Zilz *et al.*, 1990). Although the sequences within Hss-3 clearly play a role in S14 gene transcription, the significance of structural changes within Hss-1 remain unclear. Transfection of cultured hepatocytes with S14–chloramphenicol acetyltransferase (CAT) fusion genes suggested that the region extending to −290 bp relative to the 5′ end of the S14 gene supports only weak promoter activity (Jacoby *et al.*, 1989); however, a detailed analysis of the promoter was not performed.

We have investigated the role that the Hss-1 region plays in regulating initiation of S14 gene transcription by using DNAase I footprint, gel shift and *in vitro* transcription initiation analyses. This approach has identified four prospective *cis*-acting elements within the S14 promoter. Each element functions in the initiation of S14 gene transcription *in vitro*. In addition, our studies show that expression of proteins which bind to these elements is subject to tissue-specific control.

MATERIALS AND METHODS

Plasmid constructions

The S14 promoter is numbered relative to a transcriptional start site of +1. The pS14T1(.9) plasmid was created by insertion of a *Taq*I (−464)–*Taq*I (+480) fragment from the pEMBLS14-13E genomic clone (Jump *et al.*, 1990*a*) 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 re-ligating (Henikoff, 1984). The resulting plasmid, pS14T1(.9)7b, contained the S14 promoter sequence −8 to −464 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 *Eco*RI (cuts at the 3′ cloning site) released two fragments which were subsequently isolated and cloned into

Abbreviations used: Hss, hypersensitive site; CAT, chloramphenicol acetyltransferase; GFC, G-free cassette; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; TBE, Tris/borate/EDTA.

* To whom correspondence should be addressed.

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X33553.

pGem-1 to form pS14(−285 to −464) and pS14(−8 to −290). pS14(−8 to −290) was subcloned by isolating the insert, restricting with *Bst*NI (at −152, −155 and −167 bp), treating with S1 nuclease, and ligating the resulting fragments into pGem-1 to form pS14(−8 to −151) and pS14(−168 to −290). The insert from pS14(−8 to −151) was isolated, restricted with *Hae*III (cuts at −88 bp) and ligated into pGem-1 to create pS14(−8 to −87) and pS14(−88 to −151).

Plasmids containing the G-free cassette, $p(C_2AT)_{19}$ (or GFC), and the adenovirus-2 major late promoter adjacent to the G-free cassette, $pML(C_2AT)_{19}$, were generously provided by Dr. R. G. Roeder (Rockefeller University New York, NY, U.S.A.; Sawadogo & Roeder, 1985). The 3′ cloning site in $p(C_2AT)_{19}$ was removed by restricting with *Bam*HI and *Hind*III, treating with S1 nuclease and re-ligating to form pGFC(-BH). pS14-GFC1 was prepared by installing *Eco*RI linkers (Bethesda Research Laboratories) on pS14T1(.9)7b to form pS14T1(.9)7b(EE) and cloning the resulting insert into the *Eco*RI site of pGFC(-BH). pS14-GFC2 was prepared by installing *Sst*I linkers on pS14(−8 to −290) 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(−8 to −151) and pS14(−8 to −87) respectively into pGFC(EH). pGFC(EH) was prepared by restricting pGFC(−BH) with *Sst*I, blunting ends with S1 nuclease and installing a *Hin*dIII linker (Bethesda Research Laboratories). The G-free cassette in $pML(C_2AT)_{19}$ was truncated by restricting with *Pst*I and *Bam*HI, treating with exonuclease III and re-ligating. The truncated G-free cassette (pML-GFC2) is approx. 300 bp long, and transcription products can be resolved on acrylamide sequencing gels or agarose northern gels. All manipulations and cloning were performed according to Maniatis *et al.* (1982).

Oligonucleotides

NF-1, SP-1, AP-1 and AP-3 oligonucleotides were obtained from Stratagene. The NF-1 sequence consists of the adenovirus origin of replication with a single mutated base pair which increases NF-1 binding by 4-fold (Rosenfeld *et al.*, 1987).

Isolation of nuclei and preparation of nuclear extracts

Male Sprague–Dawley rats were used in all studies. Hepatic, renal and splenic nuclei were isolated as described by Gorski *et al.* (1986), except that the protease inhibitors phenylmethanesulphonyl fluoride (PMSF; 1 mM), benzamidine (1 mM), leupeptin (0.5 μg/ml) and pepstatin A (1 μg/ml) were included in the homogenization buffers. In one gel shift experiment, hepatic nuclei were isolated by the method of Hewish & Burgoyne (1973), as modified by Jump *et al.* (1987). Nuclear extracts from liver, kidney or spleen were prepared as described by Parker & Topol (1984), as modified by Gorski *et al.* (1986). Following $NH_4(SO_4)_2$ precipitation, the pelleted nuclear proteins were resuspended at 1 ml/400 A_{260} units for liver or kidney, and at 1 ml/800 A_{260} units for spleen. Protein concentrations were determined using the Bio-Rad Laboratories protein assay reagent. Recovery of nuclear proteins ranged from 0.12 to 0.16 mg/g of liver. Aliquots of 10 μl were stored at −80 °C for up to 6 months without appreciable loss of gel shift or transcriptional activity.

DNAase I footprinting analysis

This assay was performed essentially as described by Galas & Schmitz (1978). Genomic DNA fragments to be footprinted were isolated from plasmids by digesting DNA with the appropriate restriction enzymes. The DNA fragments were purified by electrophoresis and electroelution. Some DNA inserts were further purified using a Mermaid 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 [γ-^{32}P]ATP. DNA–protein complexes were formed by incubating end-labelled DNA (1–3 fmol) for 30 min at 30 °C in a reaction mixture containing 0–9.0 mg of nuclear extract, 25 mM-Tris/HCl (pH 7.5), 10% glycerol, 5 mM-$MgCl_2$, 0.1 mM-EDTA, 1 mM-dithiothreitol (DTT), 0.1 M-KCl, 0.2 mM-PMSF and 2 μg of poly[d(I-C)] (Boehringer-Mannheim; Johnson *et al.*, 1987). DNAase I (Pharmacia) was then added to 6.5 units/ml, and samples were incubated at 0 °C for 10–90 s. The reaction was terminated by adding 80 μl of termination buffer to give final concentrations of 20 mM-EDTA, 1.5% SDS, 400 mM-ammonium acetate, and 500 μg of yeast tRNA/ml. The mixture was incubated at 85 °C for 3 min, extracted with phenol/chloroform (1:1, v/v) and chloroform/3-methylbutan-1-ol (24:1, v/v), and precipitated with ethanol. Pellets were rinsed with 70% ethanol before loading on to an 8% acrylamide/8 M-urea sequencing gel. Gels were dried and autoradiographed at −80 °C with intensifying screens. A dimethyl sulphate sequencing reaction was performed as outlined by Maxam & Gilbert (1980), and electrophoresed in parallel with the DNAase I footprint.

Gel shift assay

This assay was performed essentially as described by Garner & Revzin (1981) and Fried & Crothers (1981). DNA–protein complexes were formed as described above for DNAase I footprinting. After the reaction, 6 μl of reaction buffer containing 0.16% Bromophenol Blue and 0.16% xylene cyanol was added to the DNA–protein complex just prior to loading on to a 6% polyacrylamide gel (acrylamide/bisacrylamide, 29:1, w/w) with 90 mM-Tris/borate/2.5 mM-EDTA, pH 8.3 (TBE), as buffer. After electrophoresis, the gels were dried and exposed to X-ray film at −80 °C with intensifying screens.

In vitro transcription assay

Transcription reactions were performed essentially as described by Gorski *et al.* (1986). The reactions (20 μl) contained 1.2 μg of pS14-GFC, 0.1 μg of pML-GFC2, 2.25 mg of nuclear extract/ml and 15 units of T_1 RNAase. EDTA (35 μM) and DTT (0.35 mM) were contributed by the nuclear extract and were held constant by adding dialysis buffer to 7 μl. The reaction was terminated after 30 min, and then extracted with chloropane before precipitation of RNA with sodium acetate and ethanol. After centrifugation (8000 *g*, 10 min) pellets were rinsed with 70% ethanol before resuspension in 10 μl of loading buffer (85% formamide/0.07% Bromophenol Blue in 1 × TBE) and loading on to a 6% acrylamide/8 M-urea sequencing gel. Gels were dried and autoradiographed at −80 °C with intensifying screens. Bands were quantified using laser densitometry. Differences between means were evaluated using the Bonferroni *t* statistic (Gill, 1985).

RESULTS

DNAase I footprint analysis of the S14 proximal promoter

To localize regions where hepatic nuclear proteins bind the S14 proximal promoter, we performed DNAase I footprinting analysis on DNA fragments extending from −8 to −464 bp. Fig. 1(*a*) illustrates two DNAase I footprints at −88 to −113 bp (region B) and −227 to −244 bp (region C). We also identified footprints at −48 to −63 bp (region A) and −286 to −310 bp (region D; results not shown). These 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 strands. The outer limits

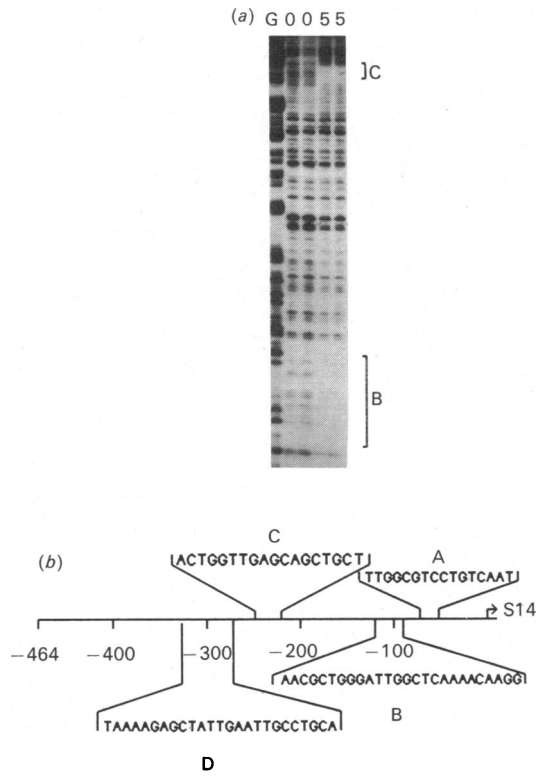

Fig. 1. DNAase I footprint analysis of the S14 promoter

(*a*) ^{32}P-labelled non-coding strand of DNA fragment −60 to −290 (*Aha*II and *Pst*I) digested with DNAase I in the absence (0) and presence of 5 mg (5) of hepatic nuclear protein (see the Materials and methods section). Digested products were electrophoretically separated and autoradiographed. Regions B and C identify two DNAase I footprints. A sequencing ladder was prepared by reacting the [^{32}P]DNA fragment with dimethyl sulphate (Maxam & Gilbert, 1980) which marked the location of guanine (G) residues. (*b*) Summary of DNAase I protection by liver nuclear extracts. Footprints are designated as protected regions A–D. Sequences shown are the outside boundaries obtained when coding and non-coding strands were footprinted. Protected regions A, B, C and D extend from −48 to −63 bp, −88 to −113 bp, −227 to −244 bp and −286 to −310 bp respectively. The footprinted regions are positioned upstream from the 5′ end of the S14 transcription start site (↱).

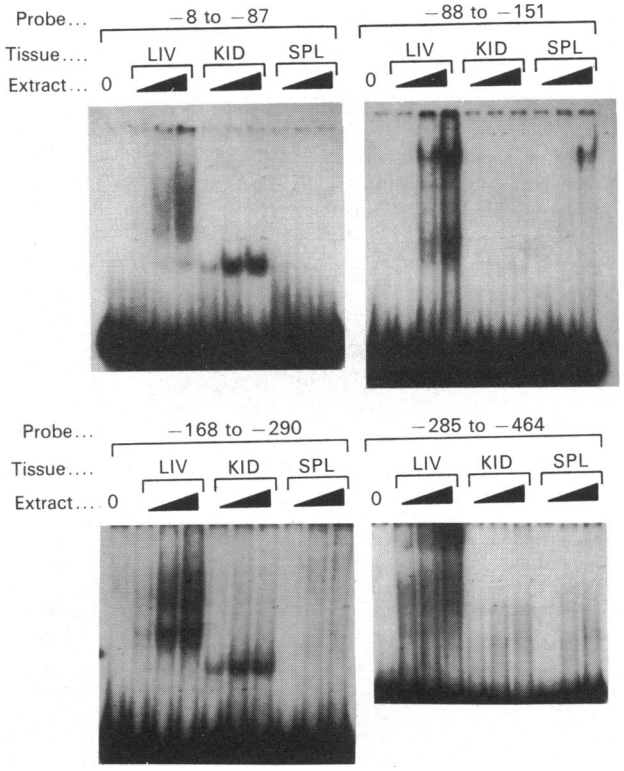

Fig. 2. Gel shift analysis of the S14 promoter

Four probes (−8 to −87 bp, −88 to −151 bp, −168 to −290 bp and −285 to −464 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: −8 to −87 bp: 1, 3 and 6 mg of protein; −88 to −151 bp: 1.5, 3.0 and 4.5 mg of protein; −168 to −290 bp: 1.5, 3.0 and 4.5 mg of protein; −285 to −464 bp: 3, 6 and 9 mg of 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 three to six different preparations of liver, kidney and spleen. Representative autoradiographs are presented.

of protection of the coding and non-coding strands of each footprint are reported in Fig. 1(*b*). In addition, overlapping DNA fragments were analysed to ensure that binding of a particular liver factor was not disrupted by a cloning site (results not shown). Although this analysis locates four prominent DNA–protein interactions within 464 bp of the 5′ end of the S14 gene, we do not exclude the possibility that other interactions involving low-abundance proteins may exist within this area.

Tissue-specific binding of nuclear proteins to the S14 proximal promoter

The DNAase I footprint analysis described four different regions recognized by hepatic proteins. To determine whether nuclear proteins present in tissues which do not express S14 bind to DNA sequences within the S14 proximal promoter, we examined DNA–protein interactions using nuclear extracts from spleen and kidney. Four separate DNA fragments, each containing one DNAase 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 four DNA fragments tested. The pattern of shifted DNAs was characterized by both discrete bands and smears. The smeared DNA–protein complexes may represent multiple protein interactions with DNA or dissociation of DNA–protein complexes during electrophoresis. Although the DNA footprint analysis indicated that only four footprints were detected, gel shift analysis suggests that protein interaction within these sites may be complex.

The pattern of gel shift analysis using extracts from kidney and spleen was quite different when compared with that of liver. Kidney extracts promoted shift of the −8 to −87 bp and −168 to −290 bp fragments as discrete bands, while inducing little shifting of the −88 to −151 bp and −285 to −464 bp fragments. Spleen extracts induced little or no shifting of the S14 promoter fragments. In order to confirm that the observed differences were not due to differences in the activity of the extracts, we demonstrated that each of the extracts induced a similar amount of shifting of AP-1 and AP-3 oligonucleotides (results not shown). This analysis clearly shows that interaction of nuclear proteins with the S14 promoter is tissue-specific.

Characterization of DNA–protein interaction within the S14 promoter

We characterized further the interaction of hepatic nuclear factors with the S14 promoter by assessing the sequence specificity

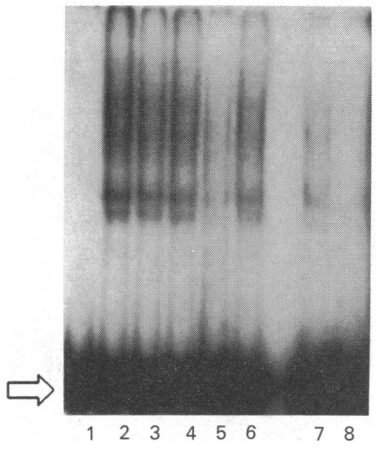

Fig. 3. Characterization of DNA–protein interaction within the −168 to −290 bp S14 promoter region

Gel shift analysis was performed using the −168 to −290 bp fragment as described in Fig. 2 and in the Materials and methods section. 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 the addition of 1 fmol of ^{32}P-labelled DNA fragment −168 to −290: lane 2, no competitor; lane 3, −8 to −87 bp; lane 4, −88 to −151 bp; lane 5, −168 to −290 bp; lane 6, −285 to −464 bp. In lane 7, liver nuclear extract was boiled for 5 min and centrifuged before addition to the reaction mixture. In lane 8, 10 mg of proteinase K was added to liver nuclear extract before addition of labelled DNA. The arrow indicates free −168 to −290 bp.

of binding as well as heat- and protease-sensitivity. Although this analysis was performed on each of the four DNA fragments used in Fig. 2, we illustrate our findings in Fig. 3 using only the −168 to −290 bp region as representative of the others.

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

Whereas all shifting of DNA fragments was sensitive to prior proteinase K treatment (Fig. 3, lane 8), we observed differential heat-sensitivities of protein shifting of the various fragments. For example, boiling hepatic extracts for 5 min only partially decreased the extent of shifting of the −168 to −290 bp fragment (Fig. 3, lane 7). However, shifting of the −8 to −87 bp, −88 to −151 bp and −285 to −464 bp fragments was fully sensitive to heat treatment (results not shown). RNAase A treatment did not decrease binding to the four fragments (results not shown). Based on these studies, it can be concluded that hepatic nuclear proteins which interact with the S14 promoter are sequence-specific and differentially heat-sensitive.

Characterization of the *in vitro* transcription assay

Although the DNAase I footprint and gel shift analyses indicated that hepatic nuclear proteins bind at four loci upstream from the S14 5′ transcription start site, we had no information on the role these proteins played in S14 gene transcription. To assess the function of these elements, we used the *in vitro* transcription

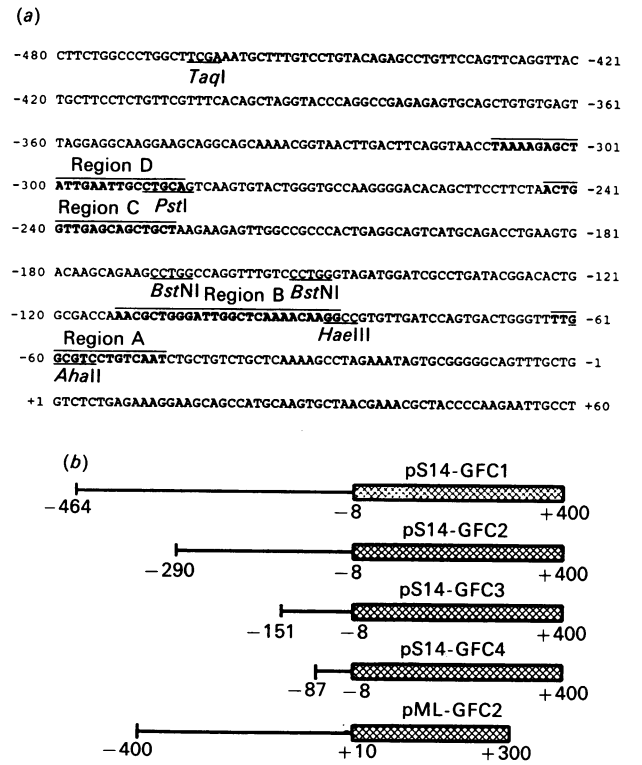

Fig. 4. Sequence of the S14 promoter and structure of DNA templates used in the transcription initiation assay

(*a*) DNA sequence of the S14 promoter from −480 to +60 relative to the start site of transcription (+1). DNAase 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 approx. 300 bp to form pML-GFC2.

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. The sequence and structure of the promoter templates analysed is illustrated in Fig. 4.

Characterization of the assay showed that initiation of transcription from the S14 promoter was time-dependent and linear up to 60 min for all four S14 promoter constructs tested. Transcription was dependent on protein concentration and was linear between 1.5 and 3.0 mg of protein/ml. Transcription was dependent on concentration of DNA template and was linear between 0 and 80 μg of DNA/ml. Transcription was sensitive to α-amanitin and RNAase A, but not to T_1 RNAase. These results suggest that transcription of the GFC driven from the S14 promoter satisfies the criteria for an RNA polymerase II-catalysed reaction.

Similar results were obtained using the adenovirus major late promoter fused to the GFC (pML-GFC2). The pML-GFC2 was used as an internal standard in all transcription reactions described below. However, transcription from S14 promoters was approx. 10% of the maximal transcription observed with pML-GFC2. In order to run the reactions with comparable rates of transcription from each promoter, the concentration of pML-GFC2 was decreased from 40 μg/ml to 5 μg/ml. To ensure that we were measuring the rate of transcription and not maximal RNA accumulation, reactions were stopped after 30 min, when

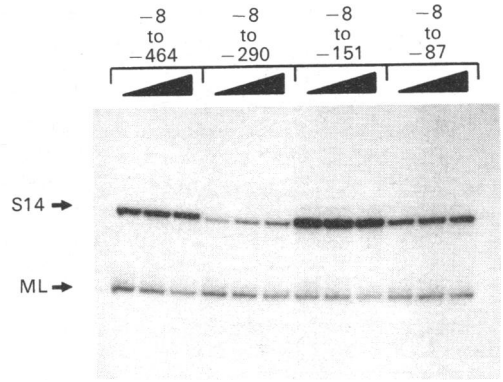

Fig. 5. Effect of S14 promoter deletions on S14 *in vitro* transcription

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, the amount of each S14 promoter template added was 0.9, 1.2 or 1.5 μg/reaction. pML-GFC2 was held constant at 0.1 μg/reaction. Transcription from the four S14 promoters, expressed relative to transcription from the major late promoter (pML-GFC2), was as follows: −8 to −464 bp, 0.72±0.08; −8 to −290, 0.18±0.08; −8 to −151, 1.46±0.08; −8 to −87, 0.79±0.08. Data from six independent preparations of rat liver nuclear extracts were quantified using laser densitometry and are expressed as the means±pooled S.E.M. The data were statistically analysed, and −8 to −151 is different from −8 to −87 ($P < 0.01$), −8 to −290 is different from −8 to −151 ($P < 0.01$) and −8 to −464 is different from −8 to −290 ($P < 0.01$).

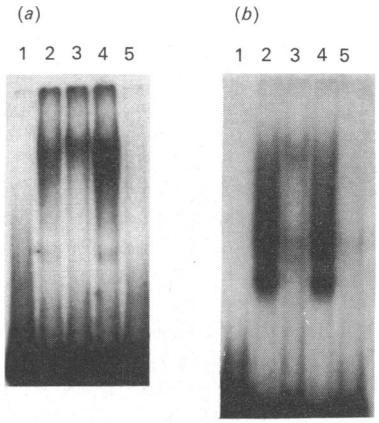

Fig. 6. Competition by NF-1 for binding to the S14 −8 to −87 bp fragment

Rat liver nuclei and extracts were prepared according to Gorski *et al.* (1986) (*a*) or Hewish & Burgoyne (1973) (*b*). Nuclear proteins were extracted according to Parker & Topol (1984). Otherwise, lane treatments in (*a*) and (*b*) were identical. Lane 1 contains 1 fmol of labelled −8 to −87 fragment alone, while in lanes 2–5 DNA was incubated with 7 mg 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.

all S14 promoters were transcribing RNA in a linear fashion with respect to time (results not shown).

Deletion analysis of the S14 promoter

We used various S14 promoter–GFCs constructed by sequential deletion of the four footprinted regions (Fig. 4) to determine the function of *cis*-linked elements within these regions. While no transcription was observed when the promoterless GFC was included in the transcriptional assay (results not shown), a basal level of transcription was observed when an S14 promoter fragment extending from −8 to −87 bp was ligated upstream from the GFC (Fig. 5). This element contains the footprinted region A extending from −48 to −63 bp in addition to the prospective TFIID binding site at −27 bp.

Using a promoter extending from −8 to −151 bp, the rate of initiation of transcription was increased 1.8-fold ($P < 0.01$) when compared with the level of transcription from the −8 to −87 bp promoter. The −8 to −151 bp promoter contains two footprinted regions (regions A and B, Fig. 1). When a promoter containing regions A, B and C (−8 to −290 bp, Fig. 1) was analysed, the rate of initiation of transcription was suppressed by 88% ($P < 0.01$) when compared with the −8 to −151 bp promoter. When using a promoter containing all four footprinted regions (regions A, B, C and D, Fig. 1) and extending to −464 bp, the rate of initiation of transcription increased 4-fold ($P < 0.01$). In Fig. 5 it appears that the dose–response of S14/ML transcription to increasing amounts of S14 template is due to decreased transcription of pML-GFC2; however, we generally observed an increase in S14 transcription with little or no change in pML-GFC2. The observed results may be due to differences in RNA recovery. Alternatively, in some nuclear extracts higher concentrations of S14 template may decrease pML-GFC2 transcription by competing for transcription factors. 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, not differences in concentration or preparation of DNA templates. The selective effect of hepatic nuclear proteins on the transcription of different S14-GFCs suggests the *trans*-acting factors binding to regions A, B and D function to enhance the rate of initiation of transcription from the S14 promoter, while factors binding to region C function to suppress transcription.

Analysis of the interaction of NF-1 with the S14 promoter

The footprint at −48 to −63 bp (TTGGCGTCCTGTCAAT) is similar to the NF-1 consensus sequence (NTTGGCNNNNNGCCAAN; Chodosh *et al.*, 1988), suggesting that an NF-1 hepatic protein may bind to this site and function in the initiation of S14 gene transcription. To examine this possibility we used NF-1 oligonucleotide competition in both the gel shift and *in vitro* transcription analysis. In gel shift analysis, hepatic nuclei prepared by the methods of Gorski *et al.* (1986) (Fig. 6*a*) and Hewish & Burgoyne (1973) (Fig. 6*b*) were compared. Nuclei prepared by each method were extracted for nuclear proteins using the method of Parker & Topol (1984). While the 'Gorski' method yields DNA-binding and nucleoplasmic proteins capable of initiation of gene transcription *in vitro*, the 'Hewish and Burgoyne' method yields extracts depleted of the factors required to initiate transcription but enriched in DNA-binding proteins. This difference in nuclear extract composition is evident when comparing the shift patterns of the −8 to −87 bp fragment using liver extracts prepared by the two methods (Figs. 6*a* and 6*b*, lane 2). Addition of a 200-fold molar excess of the NF-1 oligonucleotide fully blocked the shifting of a single band and smear (Figs. 6*a* and 6*b*, lane 3), but failed to block the 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 (Figs. 6*a* and 6*b*, lane 4). When hepatic extracts were heat-treated (85 °C, 5 min) before addition to the gel shift reactions, all shifting was abolished, suggesting that the heat-stable C/EBP (Johnson *et al.*, 1987) is an unlikely candidate for binding to the S14 promoter (Figs. 6*a* and 6*b*, lane 5).

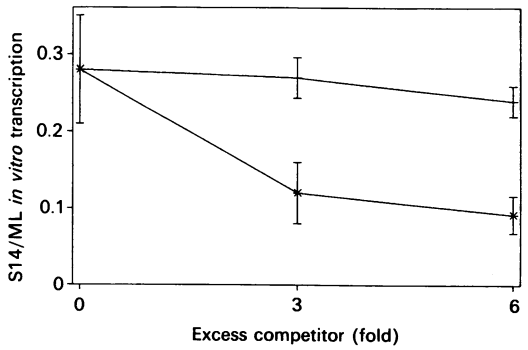

Fig. 7. Effect of NF-1 and SP-1 oligonucleotide competition on S14 *in vitro* transcription

The *in vitro* transcription assay was performed as described in the Materials and methods section, except that a 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 three independent preparations of rat liver nuclear extracts were quantified using laser densitometry and are expressed as means ± S.D.

To determine whether NF-1 functions in the initiation of S14 transcription, the NF-1 oligonucleotide was added to the *in vitro* transcription assay. The results are normalized to the level of transcription for pML-GFC2. Addition of 3- and 6-fold molar excesses of NF-1 to the transcription reaction decreased S14 transcription by 57 and 67% respectively (Fig. 7), while addition of SP-1 had no effect. In addition, a 6-fold molar excess of NF-1 oligonucleotide, but not of AP-3 oligonucleotide, specifically decreased transcription from constructs extending to −290 bp (results not shown). Since addition of low levels of NF-1 oligonucleotide selectively inhibited transcription from the S14 promoter, NF-1 (or a related protein) may play a functional role in augmenting the initiation of S14 gene transcription in rat liver.

DISCUSSION

Rat liver nuclear extracts produced four distinct DNAase I footprints within the S14 promoter. Interestingly, each of the four footprinted regions is similar to hepatic *cis*-acting elements identified for other hepatic genes. The A footprint showed substantial identity (14 of 15 bp) with the consensus sequence for NF-1 (Chodosh *et al.*, 1988). The S14 B footprint is similar to a functional sequence (DE III) within the albumin promoter (Herbomel *et al.*, 1989). While sequences within the S14 C footprint are identical to the AP-4 transcription factor binding site (CAGCTG; Hu *et al.*, 1990), overlapping regions within this footprint show identity to element IV within the α_1-inhibitor III promoter (Abraham *et al.*, 1990) and the PKL-I element within the pyruvate kinase promoter (Yamada *et al.*, 1990). The S14 D footprint shares 8 of 11 bp with the DNA sequence recognized by the 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 that function in the transcription of other hepatic genes.

The 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 the initiation of gene transcription under *in vitro* conditions. The minimal component tested, extending from −8 to −87 bp relative to the S14 transcription start site, contained sufficient information to function as a promoter of gene transcription. The −8 to −87 bp region contains a footprinted sequence similar to the sequence recognized by NF-1. An NF-1 oligonucleotide effectively competed for both hepatic nuclear protein binding to the S14 promoter and initiation of *in vitro* transcription (Figs. 6*a*, 6*b* and 7). In addition, heating hepatic extracts (85 °C for 5 min) essentially obliterated all protein binding to the −8 to −87 bp region. The heat-sensitivity of hepatic nuclear protein binding excludes C/EBP, but not other members of the CCAAT-binding family [e.g. CP1, CP2 (Chodosh *et al.*, 1988) or AGP/EBP (Chang *et al.*, 1990)], as candidates for binding to this region. Interestingly, the single shifted band induced with kidney extracts (Fig. 2) was effectively competed with the addition of the NF-1 oligonucleotide (results not shown). Thus our studies provide reasonable evidence for a role of NF-1 or a closely related protein in binding to the S14 promoter and functioning in the initiation of S14 gene transcription.

The *in vitro* functional analysis of the S14 proximal promoter provides evidence that proteins binding to regions B and D increase the rate of initiation 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. Addition of protected region D caused a 4-fold increase in transcription compared with a fragment containing regions A, B and C. Since the footprinted region B is similar to a positive *cis*-acting element found in the albumin promoter, and region D is similar to the consensus binding site for the HNF-3 transcription factor, it would appear that the *trans*-acting factors which bind to regions B and D may function in the tissue-specific control of S14 gene transcription. The identity of the *trans*-acting factors interacting with the B and D regions and their interaction with the transcription initiation apparatus, however, remains to be defined.

We were surprised to find that factors which interacted with the C region strongly suppressed the initiation of gene transcription. This observation is consistent with the low CAT activity observed during transient transfection of hepatocytes with S14–CAT fusion genes extending to −290 bp (Jacoby *et al.*, 1989). The presence of positive and negative elements within the promoters of the β-interferon (Goodbourn & Maniatis, 1988), α_1-inhibitor III (Abraham *et al.*, 1990) and vitellogenin II (Vaccaro *et al.*, 1990) genes has been previously reported.

While the upstream enhancer elements play a major role in hormonal regulation of S14 gene transcription (Jacoby *et al.*, 1989; Zilz *et al.*, 1990), the identification of tissue-specific promoter elements introduces an important second target for control of S14 gene transcription. Although it is not clear whether these elements are involved in the formation and/or maintenance of Hss-I, the positive and negative elements clearly add complexity to the initiation and potential regulation of S14 gene transcription. For example, *trans*-acting factors which interact with the proximal promoter region may regulate initiation of S14 gene transcription as well as facilitating the interaction between *trans*-acting factors bound to upstream enhancer elements and the S14 promoter. A transfection approach may determine whether these promoter elements modulate the activity of upstream enhancer function.

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

This work was supported by grants from the American Diabetes Association (Michigan Affiliate), the UpJohn Company, Sigma Xi and

the Michigan State University Biotechnology Center. O.A.M. was supported by the Barnett Rosenberg Fellowship. We thank Zack Burton and Steve Clarke for critical review of this manuscript.

REFERENCES

Abraham, L. J., Bradshaw, A. D., Shiels, B. R., Northemann, W., Hudson, G. & Fey, G. H. (1990) Mol. Cell. Biol. **10**, 3483–3491
Chang, C.-J., Chen, T.-T., Lei, H.-Y., Chen, D.-S. & Lee, S.-C. (1990) Mol. Cell. Biol. **10**, 6642–6653
Chodosh, L. A., Baldwin, A. S., Carthew, R. W. & Sharp, P. A. (1988) Cell **53**, 11–24
Costa, R. H., Grayson, D. R. & Darnell, J. E., Jr. (1989) Mol. Cell. Biol. **9**, 1415–1425
Fried, M. & Crothers, D. M. (1981) Nucleic Acids Res. **9**, 6505–6525
Galas, D. J. & Schmitz, A. (1978) Nucleic Acids Res. **5**, 3157–3170
Garner, M. M. & Revzin, A. (1981) Nucleic Acids Res. **9**, 3047–3060
Gill, J. L. (1985) Design and Analysis of Experiments in the Animal and Medical Sciences, vol. 1, The Iowa State University Press, Ames, Iowa
Goodbourn, S. & Maniatis, T. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 1447–1451
Gorski, K., Carneiro, M. & Schibler, U. (1986) Cell **47**, 767–776
Hamblin, P. S., Ozawa, Y., Jefferds, A. & Mariash, C. N. (1989) J. Biol. Chem. **264**, 21646–21651
Henikoff, S. (1984) Gene **28**, 351–359
Herbomel, P., Rollier, A., Tronche, F., Ott, M.-O., Yaniv, M. & Weiss, M. C. (1989) Mol. Cell. Biol. **9**, 4750–4758
Hewish, D. R. & Burgoyne, L. A. (1973) Biochem. Biophys. Res. Commun. **52**, 504–510
Hu, Y.-F., Luscher, B., Admon, A., Mermod, N. & Tjian, R. (1990) Genes Dev. **4**, 1741–1752
Jacoby, D. B., Zilz, N. D. & Towle, H. C. (1989) J. Biol. Chem. **264**, 17623–17626
Johnson, P. F., Landschulz, W. H., Graves, B. J. & McKnight, S. L. (1987) Genes Dev. **1**, 133–146
Jump, D. B. (1989*a*) in Gene Regulation by Steroid Hormones IV: Thyroid Hormone Regulation of Rat Liver S14 Gene Expression (Roy, A. K. & Clark, J. H., eds.), pp. 144–160, Springer-Verlag, New York
Jump, D. B. (1989*b*) J. Biol. Chem. **264**, 4698–4703
Jump, D. B. & Oppenheimer, J. H. (1985) Endocrinology (Baltimore) **117**, 2259–2266
Jump, D. B., Narayan, P., Towle, H. & Oppenheimer, J. H. (1984) J. Biol. Chem. **259**, 2789–2797
Jump, D. B., Wong, N. C. W. & Oppenheimer, J. H. (1987) J. Biol. Chem. **262**, 778–784
Jump, D. B., Veit, A., Santiago, V., Lepar, G. & Herberholz, L. (1988) J. Biol. Chem. **263**, 7254–7260
Jump, D. B., Bell, A. & Santiago, V. (1990*a*) J. Biol. Chem. **265**, 3474–3478
Jump, D. B., Bell, A., Lepar, G. & Hu, D. (1990*b*) Mol. Endocrinol. **4**, 1655–1660
Lepar, G. J. & Jump, D. B. (1989) Mol. Endocrinol. **3**, 1207–1214
Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor
Maxam, A. M. & Gilbert, W. (1980) Methods Enzymol. **65**, 499–560
Parker, C. S. & Topol, J. (1984) Cell **36**, 357–369
Rosenfeld, P. J., O'Neill, E. A., Wides, R. J. & Kelly, T. J. (1987) Mol. Cell. Biol. **7**, 875–886
Sawadogo, M. & Roeder, R. G. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 4394–4398
Vaccaro, M., Pawlak, A. & Jost, J.-P. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 3047–3051
Wong, N. C. W., Perez-Castillo, A. M., Sanders, M. M., Schwartz, H. L. & Oppenheimer, J. H. (1989) J. Biol. Chem. **264**, 4466–4470
Wong, N. C. W., Deschamps, B. J., Yeomans, Z. A. & Cannon, P. D. (1990) J. Biol. Chem. **265**, 8775–8781
Yamada, K., Noguchi, T., Matsuda, T., Takenaka, M., Monaci, P., Nicosia, A. & Tanaka, T. (1990) J. Biol. Chem. **265**, 19885–19891
Zilz, N. D., Murray, M. B. & Towle, H. C. (1990) J. Biol. Chem. **265**, 8136–8143

Received 21 May 1991/29 July 1991; accepted 6 August 1991