Configuration of the α -fetoprotein regulatory domain during development

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The transcription of the mouse α -fetoprotein gene in the fetal liver, gut, and yolk sac is under the control of at least four regulatory sequences, three 5' distal enhancers, and a proximal promoter region. In transgenic mice, the three enhancers exhibited distinct tissue preferences, with all three active in the visceral endoderm of the yolk sac, two in the fetal liver, and one in the fetal gut. To ask whether the enhancers are differentially utilized by the endogenous gene in the three tissues in vivo, we examined their differential sensitivity to the endonuclease DNase I. The experiments indicated that two of the three enhancers exhibited sensitivity to DNase I in all three fetal tissues, as well as in the adult liver, where the gene is transcriptionally repressed. The third enhancer, which exhibited the weakest activity in transgenic mice, was the least sensitive to DNase I in fetal liver and yolk sac and insensitive in the fetal gut. The major changes in the chromatin structure of the gene during postnatal development occurred within the proximal promoter region. The major DNase I cleavage site shifted from a position at 120 nucleotides upstream of the transcriptional start site to the start site itself. Two new sites, at 300 nucleotides upstream of the start site and 1.5 kb within the structural gene, were observed. These results suggest that the distal regulatory regions have the capability to retain biological activity throughout development, and that the primary repression of transcription of the gene proceeds through elements proximal to the promoter of the gene.

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The α -fetoprotein (AFP) gene is expressed in several cell types in the mouse, but at markedly different levels. That is, its transcript is present at very high levels in the visceral endoderm of the yolk sac (20% of total mRNA) and the fetal liver (5% of total mRNA) and at much lower levels in the fetal gastrointestinal tract ($\leq 0.1\%$) (Andrews et al. 1982; Belayew and Tilghman 1982; Tamaoki and Fausto 1984; Tilghman 1985). Shortly after birth, there is a rapid decrease in AFP transcription in the liver, resulting in a 10,000-fold reduction in AFP mRNA by 4 weeks of age (Belayew and Tilghman 1982). In the gut, AFP mRNA is undetectable a few days after birth (R. Compton and S.M. Tilghman, unpubl.).

By utilizing a transient expression assay in a human hepatoma cell line, HepG2, four regulatory regions which affected AFP transcription were identified, including a proximal promoter region, which lies within the first 250 bp upstream of the start of transcription and three distal enhancer elements at -2.5 (EI), -5.0 (EII), and -6.5 (EIII) kb (Godbout et al. 1986, 1988; Widen and Papacostantinou 1986). In HepG2 cells, the three enhancers were equivalent in biological activity and non-

additive. However, when they were tested separately or in combination in vivo by linking them to an AFP minigene and introducing them into the germ line of mice by microinjection, they exhibited distinct tissue preferences (Hammer et al. 1987). High levels of AFP minigene transcription could be obtained in the yolk sac with all three enhancers, together or separately. In contrast, only EI or EII could direct high-level expression in the liver and EI alone was an effective enhancer in the gut. We interpreted these results to suggest that the diversity in the levels of AFP mRNA observed in vivo was achieved, in part, by differential activity of the three enhancers in the three cell types. That is, all three were effective in the yolk sac, which expresses the gene at the highest level, whereas only one enhancer produced high levels of transcription in the gut.

One prediction of such a model might be that the three enhancers would exhibit differential sensitivity to endonucleases, such as DNase I, in the three tissues. DNase I hypersensitive (HS) sites have been found associated with the regulatory regions of transcribed genes (Wu 1980; for review, see Eissenberg et al. 1985) and they are thought to occur as the consequence of regulatory proteins binding to specific DNA sequences (Wu 1984). HS sites have been mapped to the proximal 5' region of numerous genes (for review, see Eissenberg et al. 1985), as well as to enhancer elements. For example the

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immunoglobulin (Chung et al. 1983; Mills et al. 1983) and the HLA-DR (Peterlin et al. 1987) gene enhancers which lie within introns, the mouse albumin enhancer which lies 10 kb upstream of the gene (Pinkert et al. 1987), and the borders of multigene families like the human β -globin gene cluster (Tuan et al. 1985) all exhibit sensitivity to DNase I in chromatin. We have mapped the DNase I HS sites at the 5' end of the AFP gene in the three fetal tissues expressing the gene to ask whether their presence is tissue dependent, in accord with the predictions of the transgenic mice experiments.

Results

DNase I hypersensitivity at the 5' end of the AFP gene in fetal liver

The strategy adopted to map the DNase HS sites in the AFP gene was the indirect end-labeling method of Wu (1980), outlined in the legend to Figure 1. Nuclei were isolated (initially from fetal liver), digested with increasing concentrations of DNase I, and the DNA was purified. The DNA was then digested with *EcoRI*, which

released a 10-kb fragment that spanned from -7.6 to $+2.4$ kb in the AFP gene, and therefore contained all three enhancers and the promoter of the gene, as illustrated by the diagram in Figure 1. This fragment, along with subfragments created by DNase I digestion, was detected with 5' and 3' distal probes A and B, respectively.

Three DNase I HS sites which mapped to EI, EII, and EIII were found in fetal liver chromatin using the 5'-derived probe A (Fig. 1A). These HS sites comigrated with a plasmid marker consisting of the 10-kb *EcoRI* fragment digested with *HindIII*, which cuts within EI and EII and/or *ClaI*, which cuts to the 5' side of EIII (see diagram in Fig. 1; also lane M in Fig. 2). Although it was impossible to map accurately a fourth DNase I HS site because of its high molecular weight, it probably represents the promoter (P) region of the gene. By using a probe from the 3' end of 10-kb fragment, DNase I digestion at EI and EII was observed, along with a 2.5-kb subfragment, which migrated to a position expected for a fragment generated by DNase I cleavage ~ 100 bp upstream of the AFP transcription initiation site (Fig. 1B). The relative intensity of the signals obtained for each enhancer site, taking

Figure 1. DNase I HS sites at the 5' end of the AFP gene in the fetal liver. Fetal liver nuclei were treated with varying concentrations of DNase I. The DNA was purified from each of these samples, digested with *EcoRI*, and electrophoresed on a 1.0% agarose gel. The partial digestion products were detected using (A) a 1.0-kb *EcoRI*–*ClaI* 5' distal probe (probe A) and (B) a 2.1-kb *HhaI*–*EcoRI* 3' distal probe (probe B). Lanes 1 and 2 were loaded with 10 μ g of DNA obtained from nuclei that were not treated with DNase I and kept at 0°C and 25°C, respectively, while lanes 3–8 represent digestion with DNase I for 3 min at 25°C at 1, 2.5, 5.0, 7.5, 10, and 25 μ g/ml, respectively. Reference markers indicating the size of subfragments expected upon cleavage of the DNA at the EI, EII, EIII, and promoter (P) sites are also indicated. The diagram depicts the AFP gene from -7.6 kb to $+2.4$ kb. The enhancers are represented by closed boxes and arrow indicates the transcription initiation site. The lines represent the size of the bands obtained upon cleavage within the promoter and enhancer regions of the gene. Restriction enzyme cleavage sites shown are R (*EcoRI*), C (*ClaI*), and H (*HindIII*).

Figure 2. DNase I HS sites at the 5' end of the AFP gene in the fetal gut and yolk sac. Upstream probe A (see Fig. 1) was utilized to identify the HS sites in fetal gut (A) and yolk sac (B) following the procedures outlined in the legend to Figure 1. Lane 1 represents undigested nuclei (at 0°C), lanes 2–6 indicate nuclei digested with 1, 2.5, 5.0, 10, and 25 µg/ml DNase I, respectively. M is the marker lane and includes *Clal*, complete *HindIII*, and incomplete *HindIII* digestions of a plasmid containing the 10-kb *EcoRI* fragment from –7.6 to +2.4 kb. P (promoter) is used to designate the size of the fragment obtained upon DNase I cleavage within the AFP promoter region. The arrow indicates the presence of an additional HS site mapped between EI and EII in the fetal gut, and the asterisk indicates a contaminant in the gel.

into consideration the proximity of the probe to each HS site and the DNase I concentration required to generate it, would indicate that EIII is the most resistant to DNase I cleavage. This is consistent with our conclusion from analyzing transgenic mice, where EIII had very little activity in fetal liver (Hammer et al. 1987).

Experiments were performed to define further the size of the HS site at EI. These were done by cutting the DNA with restriction enzymes which generated smaller subfragments. These subfragments indicated that DNase I recognizes a chromatin domain of ~100–200 bp of DNA and cuts randomly within this region (data not shown).

DNase I hypersensitivity sites in the yolk sac and the fetal gut

DNase I HS site mapping was carried out in two other fetal tissues expressing the AFP gene, the yolk sac and gastrointestinal tract. As expected, all four DNase I HS sites found in fetal liver were also present in the yolk sac at approximately the same relative intensities (Fig. 2). In the fetal gut, HS sites at EI and EII were clearly visible, however, EIII was not reproducibly detected. It was more difficult to detect HS sites in the gut than in either yolk sac or fetal liver (compare the intensity of completely digested fragments in yolk sac and fetal gut with the intensities of the HS subbands). In addition to the three DNase I HS sites which mapped to defined regulatory elements, an additional faint band (marked with an arrow) was only present in the fetal gut and lies between EI and EII, ~400–500 bp downstream of EII. A high-molecular-weight band (asterisk), seen in both yolk sac and fetal gut DNAs, is probably due to low levels of contamination in this gel as it was not visible in any other experiments.

None of the fetal tissues examined is homogeneous with respect to cell type. The day 19 fetal liver is com-

posed of ~50% hepatocytes, with the remaining cells largely of hematopoietic origin (Silini et al. 1967; Paul et al. 1969) and therefore do not express AFP. Likewise the site of AFP synthesis in the yolk sac is visceral endoderm (Dziadek and Adamson 1978; Andrews et al. 1982) which constitutes ~50% of the cells. Because only 50% of the chromatin should be in the active configuration in both instances, the patterns of digestion can be compared reasonably. As shown in Figure 2, the patterns are very similar, with the promoter, EI and EII exhibiting greater sensitivity than EIII, suggesting no tissue-specific differences.

Little is known about the percentage of cells expressing AFP in the fetal gut, and for this reason it is more difficult to compare its pattern of digestion to that in the other tissues. Nevertheless, the relative intensities of the three enhancers can be compared; from Figure 2 it is clear that EIII is less sensitive to DNase I, relative to the other enhancers, than in the yolk sac or liver.

DNase I hypersensitivity in adult tissues

The rate of transcription of the AFP gene undergoes a 10,000-fold decline shortly after birth (Belayew and Tilghman 1982), and AFP mRNA levels remain low unless the gene is reactivated during liver regeneration or tumorigenesis (Belayew and Tilghman 1982; Tamaoki and Fausto 1984). To ask whether any of the DNase I HS sites identified in fetal liver are lost when AFP transcription is repressed, nuclei from adult liver were isolated. The isolation procedure followed for the fetal tissues did not yield consistent results; therefore, a procedure described by Wu (1988) was also used. As shown in Figure 3, the three DNase I HS sites associated with the enhancers were present in the adult liver, although several consistent differences were noted. First, EIII was barely detectable in all adult nuclei preparations and

Figure 3. Mapping of the DNase I HS sites at the 5' end of the AFP gene in the adult liver. Nuclei were prepared as described using the Durrin and Gorski (1985) protocol for adult liver (A) and fetal liver (B). (C) Nuclei of adult liver were prepared using the procedure of Wu (1988). The DNA was digested with *EcoRI* and analyzed using upstream probe A (see Fig. 1). Lane 1 represents undigested nuclei (0°C) in both A and C. Lanes 2–7 in A indicate samples digested with 1, 2.5, 5.0, 7.5, 10, and 25 µg/ml DNase I, respectively. The DNA shown in B (lanes 3–6) originated from nuclei preparations digested with 2.5, 5.0, 7.5, and 10 µg/ml DNase I. In C, lane 2 contains DNA obtained upon digestion of adult nuclei with 50 µg/ml DNase I. The marker lane (M) as well as the sites marked on the side of this lane are as described in Figure 2.

only at high concentrations of DNase I (see Fig. 3C). Second, the band at the promoter was also reduced in intensity relative to EI and EII. Finally, there was an increase in the sensitivity of EII relative to EI, evident in Figure 3, A and C. However there appeared to be no qualitative changes in the cleavage sites, as indicated by a comparison of panels A and B in Figure 3, where fetal and adult liver DNAs were analyzed together on the same gel.

To test the relevance of the observed HS sites to gene expression, we also examined their presence in nonexpressing tissues at both fetal and adult stages. As illustrated by the analysis of nuclei derived from adult spleen, no AFP HS sites were detected (Fig. 4A). The metallothionein (MT) gene was used as a positive control to ensure that HS sites could be detected in actively transcribed genes in tissues not expressing AFP. MT genes are expressed in virtually every cell type and their regulatory elements have been mapped to the 5' end of the gene (Carter et al. 1984; Stuart et al. 1984). By cutting the DNA with *HindIII*, an HS site at ~1.3 kb should be detected with an intragenic 500 bp *AvaI*–*HindIII* fragment as a probe. A diffuse band of this size (lowest band in Fig. 4) was found in all tissues tested. Similar results were also obtained with fetal heart and brain and with adult brain (data not shown). Interestingly, very light HS bands corresponding to EI and EII were present in the adult kidney (see Fig. 4B). The kidney transcribes the AFP gene, albeit at exceedingly low levels (Nahon 1987), and AFP minigene expression has been observed in the kidneys of some transgenic mice (Hammer et al. 1987). This result shows that even low levels of expression are correlated with the DNase I sensitivity of these two enhancers.

DNase I hypersensitivity in the proximal 5' end of the AFP gene

To examine in greater detail the proximal regulatory region of the AFP gene, purified DNA from fetal and adult tissues was digested with *BamHI* and *EcoRI* to produce a 3.4-kb fragment that spans from –1 to +2.4 kb and ana-

lyzed using probe B from +0.3 to 2.4 kb. Molecular-weight markers were derived from plasmids carrying *Bal31* deletions of the AFP-flanking region, which represent the size of fragments cleaved at –0.6, –0.25, and –0.118 kb.

The DNase I HS site found in fetal liver, fetal gut, and yolk sac corresponded very closely to the –118-bp marker (Fig. 5A,C,E). Based on the width of the band, this represents ~100 bp of DNA with the 3' boundary at the cap site. In adult liver DNA, several striking changes in the pattern of hypersensitivity around the promoter were detected. First, three new adult-specific sites that were not detected in fetal liver were apparent, two strong ones at +1 bp and at +1.5 kb within the gene, and a weak site at –300 bp that is difficult to see in Figure 5B, but was more prominent in other experiments. The band at +1 to +100 bp did not comigrate with the band at –120 bp in fetal liver, as is shown in Figure 5, where the samples have been electrophoresed together. This is most apparent by comparing the distance between the undigested 3.4 kb band and either P1 or P2. Thus, the hypersensitivity at the promoter shifted downstream when transcription was repressed.

A complex pattern emerged upon analyzing the gut at different stages of development. As described above, a single band comigrating with the –118-bp to +2.4-kb marker fragment was seen in the day-17 fetal gut. At day 19, a second band appeared, ~300–400 bp downstream of the first HS site within the gene (P4). The two bands were also present a few hours after birth. However, in the adult gut, only the second band could be detected (Fig. 5D).

Fetal and adult tissues not expressing the AFP gene did not exhibit HS sites in the proximal regulatory region (data not shown), including the adult kidney, where EI and EII HS sites were detected.

Discussion

The discovery of three enhancers at the 5' end of the AFP gene raised the issue of whether all three are required in each of the expressing tissues for AFP transcription. By transient expression assay in a human hep-

atoma cell line, the three enhancers were equivalent in activity (Godbout et al. 1986, 1988). In transgenic mice, however, significant tissue preferences were associated with each enhancer (Hammer et al. 1987). It should be noted that the three enhancers were assayed in an artificial environment, that is, in the absence of the other two enhancers, in a heterologous chromosomal location, and at variable copy number. Thus, it remained possible that the preferences observed did not reflect their true biological activities *in situ*. For this reason, we initiated a series of experiments to ask whether tissue-specific differences in the chromatin structure of the regulatory domains of the AFP gene existed, which might provide ad-

ditional support for their differential utilization *in vivo*.

It is well established that transcriptionally active chromatin is more accessible to endonucleolytic enzymes than the chromatin of nonexpressing genes (Weintraub and Groudine 1976). Furthermore, a more localized hypersensitivity to nucleases has been observed in the regulatory regions of genes in the tissues in which the gene is transcribed (Wu 1980; Eissenberg et al. 1985). It has been proposed that these HS sites are generated when *trans*-acting factors bind to specific regulatory regions and thereby disrupt the normal pattern of histone octamer repeats. The appearance and disappearance of these DNase I hypersensitive sites have been correlated with gene activity, although two different classes of HS sites have been defined. One class is present only when the gene is transcribed and a second class is maintained stably in expressing tissues even in the absence of transcription. For example, in the chicken vitellogenin gene, four HS sites are generated upon initial hormone stimulation, two of which persist upon hormone withdrawal (Burch 1984).

Four DNase I HS sites were found in the fetal liver and yolk sac where the gene is transcribed at a high rate. Three of these mapped to the three enhancers previously identified by the transient expression assay and microinjection into the germ line of mice and the fourth site corresponded to the promoter at the 5' end of the gene (Godbout et al. 1986, 1988; Hammer et al. 1987). The presence of these HS sites correlated with gene expression as they were not detected in tissues not expressing the gene. The comparison of the digestion patterns of nuclei from liver and yolk sac yielded no significant differences; in both cases, HS sites in EI and EII were easily detected, while those in EIII were less evident. Thus, the difference we observed in the transgenic mice, where EIII exhibited only a low level of activity in the fetal liver, cannot be attributed to the inability of this enhancer to assume an active or hypersensitive configuration in fetal liver.

The fetal gut chromatin yielded hypersensitive sites at EI, EII, and the promoter, but EIII was either totally insensitive or much less sensitive to digestion. In transgenic mice high-level expression of the exogenous AFP minigene in gut was achieved only in the presence of EI (Hammer et al. 1987). The equivalent accessibility of EI and EII suggest that both enhancers are capable of assuming an active conformation.

To reconcile these two kinds of observations, we would suggest that the presence of HS sites may be a qualitative rather than a quantitative test of enhancer function in that the assay identifies regions important in transcriptionally active genes but provides no information regarding the relative importance of each individual region. This is perhaps most apparent in the case of the kidney, where transcription of AFP is barely detectable, yet HS sites at both EI and EII are evident. Second, the AFP enhancers have been shown to be complex genetic elements, 200–300 bp in length, and comprise multiple sequence motifs (Godbout et al. 1988). If these enhancers bind both tissue-specific and constitutively ex-

Figure 4. Mapping of the DNase I HS sites in adult spleen and adult kidney. The DNAs were generated and analyzed as described in the legend to Figure 1 using upstream probe A for detecting AFP fragments. Equivalent samples were digested with *Hind*III and the fragments were detected with a metallothionein I gene probe from +755 to 1240 bp. The bottom band is of the size expected for DNase I cleavage at ~100–200 bp upstream of the MT gene transcription initiation site. EI and EII are as described in Figure 1. (A and B) Lanes 1 and 2 represent untreated nuclei at 0°C and 25°C, respectively. Lanes 3–8 represent nuclei digested with 1, 2.5, 5, 7.5, 10, and 25 µg/ml DNase I, respectively.

Figure 5. Precise mapping of the HS sites in the proximal regulatory region of the AFP gene. The nuclei of fetal liver (A), adult liver (B), fetal gut (C), adult gut (D), and yolk sac (E) were treated with DNase I at increasing concentrations and the purified DNA digested with *EcoRI* and *BamHI*. For panels A, C, D, and E the lanes are as follows: (Lane 1) undigested nuclei; (lanes 2–6) nuclei treated with 1, 2.5, 5.0, 7.5, 10, and 25 $\mu\text{g/ml}$ DNase I, respectively. For panel B, lanes 1 and 2 indicate undigested nuclei at 0°C and 25°C, respectively, and lanes 3–8 represent samples treated with 1, 2.5, 5.0, 7.5, 10, and 25 $\mu\text{g/ml}$ DNase I, respectively. Lane M contains *BamHI/EcoRI* digests of plasmids containing variable amounts of 5' flanking DNA to generate marker fragments for DNase I cleavage at –600, –250, and –118 bp upstream of the AFP cap site. The size of the 5 different HS subfragments (P1–P5) obtained are indicated by solid lines. Probe B is represented by the hatched rectangle. The numbers –1 and +2.4 indicate the positions of the *BamHI* (B) and *EcoRI* (R) cleavage sites in kilobases, respectively.

pressed proteins, as is likely based on analogy to the detailed studies on the immunoglobulin μ and κ gene enhancers (Sen and Baltimore 1986; Staudt et al. 1986), it may be that the hypersensitivity of the EII in the gut and EIII in the liver is determined by binding of constitutive proteins which is sufficient to generate a low level of AFP transcription in transgenic mice. Fine mapping of EI showed no significant differences among tissues in the size or sites of sensitivity (data not shown). Due to their positions within repetitive DNAs, similar fine mapping of EII and EIII proved difficult. However, at the level of resolution of the gels in Figures 1–3, no obvious differences were noted.

Nahon et al. (1984) and Turcotte et al. (1986) have examined the chromatin structure of the AFP gene in rat tissues. They mapped a DNase I HS site close to the promoter region and two other sites at –2.5 kb and –3.7 kb 5' of the transcriptional start site in the fetal liver and yolk sac. The absence of HS sites further upstream could reflect a species-related difference in the location and number of distal regulatory domains in the rat and mouse or could simply be due to the method of analysis which failed to resolve HS sites farther upstream in the rat.

The decline in AFP transcription after birth is accompanied by several changes in the accessibility of the AFP regulatory domain to nucleases. The enhancers remain hypersensitive to DNase I, although their relative sensitivities change. EII becomes more sensitive than EI, and EIII is difficult to detect at all. The maintenance of the hypersensitivity of the AFP enhancers in adult liver could represent examples of HS sites stably maintained

in the absence of high-level transcription. In fact, the AFP gene is transcribed at a very low level in the adult. However, their continued presence could also reflect a constitutive role for them in the expression of the 5' distal albumin gene. Experiments using transgenic mice have indicated that under appropriate conditions, these enhancers remain active in adult liver (S. Camper, J. Vacher, and S.M. Tilghman, unpubl.).

The major changes in the chromatin structure of the AFP gene in the adult liver occurred in the region surrounding the start of transcription. The hypersensitive region in the fetal liver is centered around –120 bp, but switches downstream to approximately +1 in the adult liver. This is reminiscent of the changes that occur in the *Drosophila melanogaster* heat shock 70 (hsp 70) gene, where the hypersensitive region shifts from an area around the TATAAA motif upstream to include the heat shock consensus sequence (Wu 1984). It has been argued that the hypersensitivity around the TATAAA box is generated by a protein which binds to this motif, thereby marking the gene for transcription. Upon heat shock induction, the activated heat shock transcription factor binds upstream of the TATAAA motif, and shifts the site of hypersensitivity. By analogy, the hypersensitivity of the AFP promoter in the adult liver is also centered near the TATAAA motif, and may facilitate the rapid inducibility of the gene during liver regeneration (Belayev and Tilghman 1982). Experiments using both transient expression and protein binding assays have identified a crucial regulatory sequence which spans –85 to –125 bp in the AFP gene (M. Feuerman, R. Godbout, and S.M. Tilghman, unpubl.). We propose that hy-

persensitivity of the AFP gene in fetal liver at P1 (Fig. 5) is attributable to protein binding at this site.

Two additional sites appear in adult liver, both of which are absent in fetal liver nuclei, one at -300 bp and one within an intron of the gene itself, at $+1.5$ kb. Both sites are contained within the AFP minigenes which have exhibited appropriate transcriptional repression after birth in transgenic mice (Krumlauf et al. 1985; Hammer et al. 1987), and thus could be important in this process. Neither site has been implicated as a positive regulatory element, and thus functional tests of their role in repression are required.

In conclusion, mapping of the HS sites at the 5' end of the AFP gene suggests that at least two of the three enhancers are in an active conformation in the three tissues expressing this gene. The HS site mapping to EIII is absent in the gut, and weak in the other two tissues, which is consistent with the data derived from transgenic mice, where it exhibited activity only in the visceral endoderm. However, no other significant tissue-specific differences in the sensitivity of the enhancers were observed that could explain their differential activities. The three enhancers maintained their hypersensitivity in the adult liver, although subtle differences in their relative sensitivities were apparent. The major change in the chromatin structure of the AFP gene in the adult was the shift in the site within the promoter of the gene, and the appearance of two new sites, at -300 bp and $+1.5$ kb. These results are consistent with the maintenance of an open chromatin structure in the distal regulatory domains of the AFP gene with finer control in AFP transcription resulting from protein-DNA interactions within the proximal regions of the gene.

Materials and methods

Isolation of nuclei and DNase I digestion

Nuclei were isolated as described by Durrin and Gorski (1985). The tissues analyzed included fetal and neonatal heart, kidney, brain, gut, yolk sac, and liver; and adult liver, kidney, spleen, and brain. In most cases, the tissues were homogenized in cold (4°C) homogenization buffer [10 mM Tris-HCl (pH 7.5), 5 mM MgCl_2 , 0.5 M sucrose, 0.15 mM spermine, 0.5 mM spermidine], layered on a 0.88 M sucrose cushion in homogenization buffer, and spun at $5,000$ rpm for 5 min in an HB-4 rotor. The pellet was resuspended in the homogenization buffer and centrifuged for a second time. The nuclei were resuspended in 10 mM Tris-HCl (pH 7.5), 2.5 mM MgCl_2 , and 0.25 M sucrose (Weintraub and Groudine 1976). DNase I was added to aliquots of nuclei to final concentrations of 0.1 – 50 $\mu\text{g}/\text{ml}$. The nuclei were incubated at 25°C for 5 min for all tissues except brain, which was incubated at 37°C for 5 min in some cases. Digestion was stopped by adding 2 volumes of buffer containing 10 mM Tris-HCl (pH 8.0), 1% SDS, 5 mM EDTA, and 50 $\mu\text{g}/\text{ml}$ proteinase K and incubating at 37°C for 5 – 10 hr. This was followed by chloroform and phenol extractions and precipitation of the DNA in ethanol.

Adult liver nuclei were also isolated as described by Wu (1988). The livers were homogenized in a buffer containing 60 mM KCl, 15 mM NaCl, 5 mM MgCl_2 , 0.1 mM EGTA, 15 mM Tris-HCl (pH 7.4), 0.5 mM DTT, 0.1 mM PMSF, and 0.3 M su-

crose. The homogenate was filtered through gauze and centrifuged in a Sorvall HB-4 rotor for 4 min at $7,000$ rpm. The pellet was resuspended in the same buffer, layered on a 25 ml 1.7 M sucrose cushion in the same buffer, and spun in a HB-4 rotor at $11,000$ rpm for 20 min. The pellet was resuspended in the same buffer containing 5% glycerol and digested as described previously.

Gel electrophoresis and hybridization

The purified DNA was digested with restriction enzymes and electrophoresed on a 1% or a 1.5% agarose gel in 40 mM Tris-acetate and 1 mM EDTA (pH 7.2). The markers utilized included *Hind*III digested λ cl857 DNA and standards prepared by digesting AFP plasmids with restriction enzymes which cut close to the nuclease hypersensitive sites. Ten micrograms of salmon sperm DNA was included along with the trace amounts ($\sim 10^{-5}$ μg) of cloned DNA to avoid electrophoresis artifacts (Burch 1984). The DNA was transferred to nitrocellulose filters (Southern 1975), and the conditions for prehybridization, hybridization, and autoradiography were as described previously (Wahl et al. 1979).

The hybridization probes utilized included a 1 -kb *Eco*RI–*Cl*aI fragment from -6.6 to -7.6 kb of the AFP gene, a 2.1 -kb *Hha*I–*Eco*RI fragment from $+0.3$ to $+2.4$ kb, and an *Ava*I–*Hind*III fragment from the mouse metallothionein I gene ($+755$ to $+1240$; Glanville et al. 1981) obtained from D. Hamer at the National Cancer Institute. Fragments were labeled by nick-translation (Maniatis et al. 1975).

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