DNase I hypersensitivity and methylation of the 5'-flanking region of the α₁-fetoprotein gene during developmental and glucocorticoid-induced repression of its activity in rat liver

Bernard Turcotte+, Michel Guertin, Mario Chevrette§, Hélène LaRue and Luc Bélanger*

Centre de Recherche en Cancérologie de l'Université Laval, L'Hôtel-Dieu de Québec, Québec G1R 2J6, Canada

Received 15 August 1986; Revised and Accepted 11 November 1986

ABSTRACT

Three major regions of DNase I hypersensitivity (DH) were found in α₁-fetoprotein (AFP) chromatin of rat liver. DH site I is located at the transcription initiation site and associated with ongoing AFP transcription. DH site II is located 2.5 kb upstream from the cap site; it is developmental stage-dependent but dissociable from ongoing AFP transcription. DH site III, 3.7 kb upstream from the cap site, behaves as hepatocyte-constitutive. DH sites are present in similar regions of liver albumin chromatin. Dexamethasone-induced AFP gene repression is accompanied by the selective loss of AFP DH site I, a likely result of glucocorticoid receptors binding to a DNA recognition sequence located 5'-adjacent to DH site I. Si nuclease-hypersensitive sites were found on naked superhelical AFP and albumin DNA, but do not appear to contribute DH sites in liver chromatin. The extent of hypomethylation of HpaII sites at the 5'-end of the AFP gene correlates positively with the level of potential and actual expression of the gene. We conclude that developmental and hormonal regulation of the AFP gene is confined within ~4 kb of 5'-flanking DNA, and we discuss possible hierarchical interactions among DH sites, in relation to DNA methylation and replication.

INTRODUCTION

Albumin and α₁-fetoprotein (AFP) are homologous proteins encoded by structurally closely related genes, derived by duplication of a common ancestral gene, and organized in tandem (5' albumin, AFP 3'), 14 kb apart, with the same polarity of transcription, in the mouse (1), human (2) and rat (3) genomes. The AFP and albumin genes operate under some common controls, as their similar pattern of tissue-specific expression indicates, but they respond differentially to developmental signals. At early stages of development, hepatocytes express both genes. Around the time of birth, the AFP gene is silenced. In mature liver, the AFP gene can be reactivated by stimuli, generally carcinogenic, that alter the hepatic differentiation state. The AFP/albumin gene system thus provides a model to analyse developmental gene controls superimposed on tissue-specific controls in normal cells and defective in cells undergoing neoplastic changes.

© IRL Press Limited, Oxford, England.
The developmental cessation of liver AFP gene activity has been related to the decline in liver DNA synthesis (4), the appearance of liver histone H1o (5), and the triggering of extra-hepatic endocrine functions (6). The effect of adrenal hormones is of particular interest. Exogenous glucocorticosteroids, administered to immature rats, abruptly interrupt liver AFP gene transcription, at the level of polymerase initiation, independent of protein synthesis, and with kinetics parallel to the accumulation of glucocorticoid-receptor complexes in liver nuclei (7). This is consistent with a direct down-regulating interaction of the glucocorticoid receptor with the AFP gene. Thus, a well-characterized differentiation factor is available with which to probe the developmental control of AFP gene expression. The system also constitutes a rare model amenable to molecular analysis of negative gene regulation by steroid hormones. Here, we sought further insights into the mechanisms by which the AFP and albumin genes are differentially regulated in rat liver during normal development, and under the action of glucocorticoid hormones.

Active or potentially active chromatin is more sensitive to nucleasea than "bulk" chromatin, suggesting a more relaxed structure, accessible to diffusible gene regulators (8-9). This is the case for AFP. AFP chromatin of adult kidney (10-11), fibroblasts (10), brain (12), embryonal carcinoma cells (12), or Friend erythroleukemic cells (13), in which the AFP gene is not expressed, is either no more sensitive to DNase I than bulk chromatin, or less sensitive to DNase I than AFP chromatin of fetal liver (11-12), visceral endoderm (11-12), or Morris 7777 hepatoma cells (10), in which the AFP gene is expressed. DNase I (10-12) or micrococcal nuclease (14) also cleave preferentially AFP chromatin of adult liver, which is AFP-inactive but AFP-permissive. These data thus conform to the rule that active, once-expressed or inducible genes reside in "open" chromatin domains (8-9), and point to subtler chromatin alterations determining the state of AFP gene activity.

Active or potentially active chromatin also contains sites hyperreactive to DNA cleavage agents, particularly DNase I, superimposed on its general nuclease sensitivity (15-17). These DNase I-hypersensitive (DH) sites are related to gene regulatory functions and are believed to be nucleosome-free regions, specified by DNA sequences and/or proteins to provide access for diffusible gene regulators. We have mapped such DH sites in AFP and albumin chromatin; in parallel we tested nuclease sensitivity of isolated AFP and albumin genes.
Changes in the activity of genes are also correlated with methylation changes in their regulatory regions (18). The current view is that methylation changes can modulate specific or nonspecific interaction between DNA and proteins, altering gene activity either directly, or as a permissive event, or as a secondary mechanism that imprints the functional state, and/or that hypomethylation footprints DNA-binding proteins that exclude DNA-methylase (18-19). Cause or effect, methylation patterns, like DH sites, pinpoint regions of interest in gene function [and indeed, hypomethylated sites and DH sites at times are correlated (20-21)].

Methylation analyses have been conducted on the structural portion of the AFP gene. In the mouse, a positive correlation was established between at least partial hypomethylation and gene activity in AFP-producing cells (fetal liver, hepatoma, yolk sac or its endoderm) as compared to AFP-nonproducing cells (adult liver, hepatoma, yolk sac mesoderm) (22). However, active as well as inactive genes are hypomethylated in mouse yolk sac endoderm (23). In the rat, the AFP gene was found to be hypomethylated in AFP-producing and AFP-nonproducing hepatoma cells (24), and to be extensively methylated in fetal or adult liver (25), fetal or adult hepatocytes from normal liver (26), and oval cells (AFP-producing) or hepatocytes (AFP-nonproducing) from preneoplastic livers (27). Thus, at least in the rat, methylation sites potentially relevant to AFP gene regulation seem not to be found in the structural portion of the AFP gene. Here, we extend the methylation analysis to the 5'-regulatory region of the gene.

MATERIALS AND METHODS

Tissues, cells and nuclei

Liver, kidney and brain were taken from Sprague-Dawley rats. Partial (70%) hepatectomy was conducted on male 30-day old rats. Glucocorticoid-treated livers were from rats injected from 4 days of age with dexamethasone (DEX) 5 μg/g intraperitoneally twice a day. Hepatocytes were isolated from post-natal livers by collagenase perfusion in vivo (28), and from pre-natal livers by collagenase digestion in vitro followed by cell attachment to petri dishes (3 h at 37°C in Dulbecco's modified Eagle medium): isolated hepatocytes were >95%-pure, based on differential immunostaining of cytokeratin A and vimentin (28). Morris 7777 hepatoma cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal calf serum, and harvested in exponential growth. Nuclei were purified by the procedure of Marshall and Burgoyne (29), except for initial centrifugation of isolated cells in 1.2 M sucrose.
Nucleic Acids Research

Genes

A Sprague-Dawley rat genomic library was constructed in lambda EMBL4 (3). EcoRI fragments of the AFP and albumin genes were subcloned in the EcoRI site of plasmid pSP64. Figure 1 presents restriction maps of the 5' regions of the AFP and albumin genes, established from subclones spanning 7 and 7.4 kb upstream from the transcription initiation sites. The albumin restriction sites match those predicted from the genomic sequence reported by Sargent (30) and the first-exon AFP sites are in agreement with our cDNA sequence analysis (7). Gene fragments contained in recombinant plasmids pHQ861 and pHQ8.4B (Fig. 1) served to map nuclease-sensitive sites on naked AFP and albumin DNA. Restriction fragments I to V (Fig. 1), purified and 32P-labeled by nick translation to 0.2-1 x 10^9 cpm/µg, served as hybridization probes.

Nuclease-sensitivity assays

DNase I-hypersensitive sites in AFP and albumin chromatin were analysed by the indirect end-labeling technique (15,31). Nuclei isolated from whole liver, kidney and brain, and from purified hepatocytes, were incubated at 500 µg DNA per ml in 15 mM Hepes, pH 7.4, 0.35 M sucrose, 60 mM KCl, 15 mM NaCl, 5 mM MgCl2, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM spermidine, 0.3 mM spermine, 0.5 mM phenylmethylsulfonylfluoride, in the presence of 25 to 800 U/ml of DNase I (Boehringer Mannheim), 30 min at 10°C. Reaction mixtures were then adjusted to 10 mM EDTA, diluted with three volumes of water, brought to 0.5% SDS, and digested overnight at 37°C with 50 µg/ml of proteinase K. DNA was then purified (phenol/chloroform extraction, ethanol precipitation), treated 4 h at 37°C with RNase A (0.4 µg/µg DNA) and with restriction endonucleases (2 U/µg DNA), reextracted, and analysed on Southern blots. EcoRI digests were analysed with probes I and V, BglI digests with probe III and XmnI digests with probe IV (Fig. 1). In these and other Southern analyses, DNA was electrophoresed in 5-10 µg aliquots in 0.8 - 1% agarose gels, then transferred to nitrocellulose filters and hybridized with 32P-labeled probes, at 68°C without formamide (32).

DNase I sensitivity of naked AFP and albumin genes was tested on total genomic DNA (isolated from adult rat kidney by phenol extraction and ethanol precipitation), and on DNA from recombinant plasmids pHQ861 and pHQ8.4B (purified by banding in cesium chloride, cut with EcoRI and mixed in a 1:30 ratio with salmon sperm DNA). DNA preparations were incubated at 30 µg/ml in the conditions described above for nuclei, in the presence of 80 U/ml of DNase I. Total genomic DNA was then cut with EcoRI, and genomic and plasmid DNAs were analysed on Southern blots using hybridization probes I and V.
Fig. 1 Restriction maps and cloned fragments of rat AFP and albumin genes. 0 on scale is cap site. Maps: E, EcoRI; H, HpaII; X, XmnI; B, BglII; S, ScaI; A, AvaI; Hd, HindII. H1 and B1 are polymorphic sites. Boxes are exons. The location of the first three exons on the albumin gene is from Sargent (30). B2 probably falls in the third AFP exon, by homology with the mouse AFP gene. Gene fragments. Restriction fragments I to V are hybridization probes (arrow heads indicate the direction of DH site mapping); pHDQ 861 and pHDQ 8.4B fragments are inserts contained in pSP64 [the 5' end of the pHDQ 861 insert extends to the EcoRI site 9 bp in the polylinker of the genomic cloning vector EMBL4 (3)].

S1 nuclease-hypersensitive sites on supercoiled AFP and albumin DNA were mapped on plasmids pHDQ 861 and pHDQ 8.4B. Supercoiled plasmid DNA (purified by banding in cesium chloride) was incubated at 200 μg/ml in 30 mM sodium acetate, pH 4.6, with 50 U/ml of S1 nuclease (BRL), 1 to 10 min at 37°C, according to Ruiz-Carrillo (33). DNA was then digested with EcoRI, mixed in a 1:100 ratio with salmon sperm DNA, and analysed on Southern blots with probes I and V.

Sensitivity of the AFP gene to the restriction endonucleases MspI and HpaII was tested on high molecular weight DNA isolated by the method of Blin and Stafford (34) from brain, kidney, purified hepatocytes and hepatoma cells. DNA was digested with EcoRI/MspI or EcoRI/HpaII, and analysed on Southern blots with probe II.

RESULTS AND DISCUSSION

DH sites

In the indirect end-labeling procedure, native chromatin in isolated nuclei is subjected to mild DNase I treatment, and the DNA is then purified, cleaved with a restriction enzyme and analysed on Southern blots with a DNA
Fig. 2 Mapping of DH sites in the 5' regions of AFP and albumin chromatin. Nuclei were prepared from adult liver and brain, and from purified hepatocytes of 4 day old rats before and 6 h after one injection (5 μg/g) of DEX. Nuclei were digested with increasing (left to right lanes) amounts of DNaseI; DNA was then purified, cut with EcoRI, and analysed on Southern blots with probes I or V (Fig. 1). M, lambda markers; C, cap site markers consisting of restriction fragment HpaII (+3 bp)/EcoRI (+1 kb) from pHDQ861, and restriction fragment HindII (+10 bp)/EcoRI (+1 kb) from pHDQ.4B (Fig. 1), mixed with salmon sperm DNA (AFP) or rat genomic DNA (albumin); O, nuclei incubated without exogenous DNase I.

probe that abuts on the restriction site: this makes it possible to locate regions that were preferentially digested by DNase I within the restriction fragment. We conducted our analyses with EcoRI, which generates AFP and albumin gene fragments spanning +1 kb to -7/-7.4 kb relative to transcription initiation sites, with BglI and XmnI, which generate fragments reaching +1.9 kb into the AFP gene and 2.9 kb into the albumin gene, and with their corresponding probes I, III, IV, V (Fig. 1). For both genes, major DH sites were found in liver chromatin in the vicinity of transcription initiation sites, and between 2 and 4 kb upstream from cap sites (Figs. 2 and 5). Bulk chromatin of brain was digested by DNase I to a similar as bulk chromatin of liver, judging from DNA electrophoretic pattern prior to restriction cleavage, but showed no DH sites (Fig. 2). Thus, DH sites are tissue-specific in reference to brain, and most likely also in reference to other AFP- and/or
albumin-nonexpressing tissues, in view of the general insensitivity of AFP chromatin to nucleases in nonpermissive tissues (10-13).

Chromatin active in AFP gene transcription contained three major regions of DNase I hypersensitivity, located -125 to +35 bp (site I), -2300 to -2600 bp (site II), and -3550 to -3800 bp (site III) relative to the AFP cap site (Figs. 2 and 5). Additional minor cleavage sites were also apparent, particularly between -1 kb and site I: these may be genuine DH sites or perhaps reflect a greater sensitivity of linkers between nucleosomes specifically positioned adjacent to major DH sites (15). Because end-labeling tends to underestimate probe-distal relative to probe-proximal DH sites, one can infer from their relative intensity that sites II and III are most sensitive to nuclease [and indeed, they occasionally showed up in newborn liver nuclei in the absence of exogenous nuclease (Fig. 2)]. DH sites around the AFP and albumin transcription initiation sites contained segments resistant to DNase I digestion: these segments are presumably protected by transcriptional factors bound to the promoter. AFP site II also frequently appeared as a doublet, resembling the doublet site in the same region of the albumin locus, which again points to protein binding within site II. DNase I-cleavage sites in AFP and albumin chromatins did not coincide with preferential cleavage sites in genomic or plasmid AFP and albumin DNA (Fig. 3); thus they appear structure-dependent rather than sequence-specific.

The similarity of the location of DH sites in the AFP and albumin loci suggests common structural features of their chromatin, which is in line with the structural and functional homologies of the genes. However, DH sites in AFP chromatin have a distinct operational significance. Site I is associated with ongoing transcription; its intensity, relative to the corresponding albumin site, fits the relative transcriptional rates of the genes (7). Site II is absent from normal adult liver but persists in newborn liver after the AFP gene is silenced by dexamethasone; site II is thus developmental stage-dependent but dissociable from ongoing AFP transcription. The farther upstream site III was detected in all liver cell-derived chromatin, and thus it appears to be hepatocyte-constitutive.

Transcriptional and developmental correlations indicate that AFP sites I/II are a functional part of the AFP chromatin unit. It seems unlikely that site III belongs to another transcriptional unit inserted in the AFP/albumin intergenic region. Site III is absent from brain, it is part of the overall pattern of AFP/albumin DH similarities, it resides within a DNA domain the methylation of which correlates with AFP gene activity (below), and it falls
Fig. 3 Nuclease sensitivities of naked AFP and albumin genes, tested with recombinant plasmids pHQ861 and pHQ8.4B. Left two autoradiograms: SI nuclease sensitivity of supercoiled DNA. Plasmid DNA was digested with SI nuclease, then with EcoRI, and analysed on Southern blots with probes I or V. Lanes 1: DNA untreated with SI; lanes 2,3,4: DNA treated 1, 5 and 10 min with SI; lanes 5: DNA linearized with EcoRI, then treated 10 min with SI; lane 6: longer exposure of lane 3. Right autoradiogram: DNase I sensitivity of linear DNA. Plasmid DNA was digested with EcoRI, then with DNase I, and analysed on Southern blots with probes I or V. Arrows mark cap sites.

within a region, the homolog of which in the mouse has AFP-enhancer properties shared by further upstream sequences (35) and contains an element reiterated in the structural AFP and albumin genes (36). Also, our analysis provides no indication for DH sites beyond site III, and with intergenic probes we detected only reiterated transcripts, more abundant in adult than newborn liver whereas site III is stronger in the newborn (unpublished results). Taken together, this makes a strong argument that all three DH sites are operationally connected with AFP gene transcription.

It is noteworthy that DH sites fall into two regions the homologs of which in the mouse have striking effects on AFP promoter functions tested by transient expression of genes reconstructed with 5'-flanking segments of the AFP gene: DH site I fits a proximal (-118/-52 bp) segment needed for AFP gene expression, and DH sites II/III fall within a distal (-1/-3.8 kb) segment
which enhances AFP gene expression 20-fold (35). However, it is not clear that AFP-activating events in these transient transfection assays reflect the AFP-activating events in which DH sites may be involved in vivo. In particular, the AFP proximal cis-activating segment (35) is dispensable for transient expression when a heterologous enhancer is used (37), the AFP enhancer segment is not strongly tissue-specific (35) while DH sites probably are (and furthermore site II is developmental stage-dependent), and AFP gene sequences further upstream (between -3.8 and -7.6 kb) also have strong enhancer capabilities (35), with no evidence for DH sites in the corresponding regions of native chromatin (although our analysis is admittedly less resolutive in far upstream regions). Thus, while transient expression assays identified AFP-flanking DNA sequences with a propensity to activate transcription, their exact relevance to AFP gene modulations in vivo, as reflected by DH sites formation, remains to be clarified.

Repression of liver AFP gene transcription by glucocorticoid hormones suppressed selectively DH site I (as tested 6 h and 48 h through DEX treatment of newborn rats). DH site I is flanked by the glucocorticoid receptor-binding consensus sequence TGTGA (38), conserved between -159 and -166 in the rat, mouse and human AFP loci (3,37,39); our transfection analyses also indicate that 360 bp 5'-adjacent to the AFP cap site are sufficient to confer glucocorticoid responsiveness upon reconstructed genes (unpublished results). Thus, it is reasonable to think that DH site I is eliminated by the action of DEX receptors binding to their recognition sequence flanking DH site I. Whether the loss of DH site I might then be the cause or the effect of AFP transcription arrest induced by DEX is open to more detailed kinetic analysis. Our preliminary results indicate that after one hour of DEX treatment a suppressive effect on DH site I is detectable but is not proportional to AFP gene repression, which is near-complete (7): this suggests that site I repression is at least partly the consequence of AFP transcription arrest. The fact that DEX does not affect DH site II, which is developmentally regulated, indicates that glucocorticoid hormones, if involved at all, are not the only factor determining the developmental extinction of the hepatic AFP function. DEX did not alter albumin DH sites in liver chromatin of newborn rats, consistent with its lack of an effect on albumin gene transcription in neonatal liver (7).

DNase I hypersensitivity of AFP chromatin was essentially the same in 30 day old rat liver before or 40 h after partial hepatectomy, which induces massive hepatocyte division but minimal AFP transcription [at 40 h, we
detected no transcription signal in nuclear run-on assays (7)]. Thus, DNA replication may perhaps contribute (17), but per se is not sufficient to establish AFP DH sites. Also, DH site III persists in nonproliferating adult liver, and DH sites other than site I are not modified up to 48 h after DEX treatment of newborn rat liver, at which time liver DNA synthesis is ≈ 90% suppressed (40), which further indicates that DNA replication per se is not required for the long-term maintenance of site III and the short-term maintenance of site II.

**S1 sensitivity**

Upon torsional stress certain double-stranded DNA sequences can adopt secondary structures sensitive to the "single strand-specific" nuclease S1 (41). Such sequences may be privileged locations for the formation of DH sites (17), and indeed for some genes (41-42), but not for all (43), DH regions in chromatin correspond to S1-sensitive regions in superhelical DNA.

S1 cleavage of supercoiled plasmids pHQ861 and pHQ8.4B yielded a cluster of four strong bands in 5'-flanking AFP DNA, and one major and three minor cleavage sites dispersed in 5'-flanking albumin DNA (Figs. 3 and 5) (an additional band in albumin DNA did not correspond to a structure under torsional stress since it was detected in linearized plasmid; Fig 3, lane 5). Preferential cleavage of AFP and albumin DNA by DNase I in genomic or plasmid DNA (Fig. 3) did not coincide with S1 sites, except for a fragment that fell within the region of AFP S1 sites and which may indicate sequence specificity at one S1 cleavage site.

S1 sites on supercoiled AFP and albumin DNA did not correspond to DH sites in native chromatin (note, however, that our S1 analysis does not reach all AFP DH regions). Thus, except perhaps for the major albumin S1 site bordering a DH site (Fig. 5), the data provide little indication that particular DNA sequences with a propensity to undergo structural changes under torsional stress are involved in the conformational changes that preside over DH site formation and AFP/albumin activation in native chromatin. Perhaps such sequences play a role in enhancer effects of transfected episomic gene fragments, noted above.

**Methylation**

Five CCpGG sites were mapped in the 5' region of the AFP locus, at +3 bp, -747 bp, -2.5 kb, -3.0 kb, and -4.1 kb relative to the AFP cap site (Figs. 4-5). The sites at +3 bp and -2.5 kb were extensively demethylated in AFP-producing cells, i.e. fetal hepatocytes, newborn hepatocytes, and 7777 hepatoma cells; in adult liver (AFP-permissive, nonproducing), the +3 bp and
Fig. 4  Restriction analysis of CCpCGG methylation in the 5' region of the AFP gene. DNA from isolated hepatocytes of 19-day fetal liver (lanes 2/12), isolated hepatocytes of newborn liver, 0 h (3/13), 1 h (4/14), 6 h (5/15) and 48 h (6/16) through DEX treatment (5 μg/g twice a day, started at 4 days of age), isolated hepatocytes of adult liver (7/17), Morris 7777 hepatoma cells (8/18), adult brain (9/19), and adult kidney (10/20), was digested with EcoRI/HpaII, or EcoRI/MspI, and analysed on Southern blots with probe II (Fig. 1). Lanes 1/11: lambda markers. The faint bands at 0.75, 1 and 2.5 kb in lane 15 showed up clearly on other blots.

-3.0 kb sites were strongly methylated, and the -2.5 kb and -4.1 kb = 50% methylated; all sites were extensively methylated in adult brain (nonpermissive); in adult kidney, a tissue in which substantial amounts of AFP transcripts are detectable in the neonatal period, methylation was intermediate between adult liver and brain. Thus, a positive correlation clearly emerges between the degree of hypomethylation in the 5' region of the rat AFP gene and the potential or actual activity of the gene; this contrasts with the lack of such a correlation in the structural gene (24-27).

The usual question arises as to whether methylation state would change or reflect AFP functional state. The -747 site is polymorphic and always heavily methylated, and thus it presumably has no bearing on AFP expression. Methylation at any of the four other sites does not strictly correlate with expression; for instance, the +3 bp site is strongly demethylated in adult
Fig. 5 Diagram summarizing the positions of DNase I-hypersensitive sites in chromatin (boxes), SI nuclease-hypersensitive sites on naked supercoiled DNA (triangles), and potentially methylated (HpaII/MspI) sites (dots) in the 5' region of rat AFP and albumin genes (0, cap site; ← direction of transcription).

Kidney and the -2.5 kb site is ~50% demethylated in adult liver, which do not express AFP. But, of course, only a fraction of methylated sites were located here, and methylation changes at any single of these or other sites might modulate the action of AFP gene regulators, possibly in a cell- or developmental stage-dependent manner; or alternatively, general patterns of methylation may confer regional particularities to chromatin structure, favoring protein actions. However, extensive treatment of embryonal carcinoma cells (23) and neonatal or partially hepatectomized rat liver (our unpublished results) with 5-azacytidine and other DNA-hypomethylating agents, does not result in AFP gene activation. And in fact, there is no CpG from the TATA box to -360 bp in the rat AFP gene (3), yet this region confers expression and hormonal modulation of fusion genes in transfection assays (our unpublished results). Thus the role of methylation, if any, in rat AFP gene function, may be secondary or permissive, and it has to be exerted in promoter-distal regions.

DEX did not change AFP gene methylation in newborn rat liver, indicating that its action on the gene is not mediated by methylation changes, as anticipated from its fast kinetics, and the argument above.

CONCLUSIONS

We have identified three major regions of nuclease hypersensitivity at the 5' end of the AFP gene in liver chromatin, apparently all necessary for
AFP gene expression, but with distinct operational specificities. A promoter-proximal site is linked with ongoing transcription: it may provide access to transcriptional factors and it may be formed, at least in part, as a consequence of AFP gene activity. DH site II, at -2.5 kb, and additional minor sites within -1 kb, are developmental stage-dependent and thus emerge as regions of prime interest in the mechanisms of AFP gene modulations. The further upstream DH site III behaves as a hepatocytic lineage marker, and may represent a developmentally stable chromatin structure, also found upstream of other developmentally regulated loci [e.g. globin gene (44)], marking determination events that imprint the potential for gene activation. Thus, one may speculate on a functional model of cooperation among DH sites (45), in which hierarchical controls, generating and/or utilizing DH sites, would proceed downward from an upstream cell lineage-permissive site, through a developmental stage-dependent site exerting coarse-control over gene activity, to promoter-proximal sites involved in fine regulation of gene transcription. This pattern would follow that of other developmentally regulated genes (46).

Because upstream sites were found to be not essential for AFP expression in our reconstituted systems, they might serve conformational functions rather than as entry sites for molecules with "looping" effects or scanning the DNA towards the promoter. In any event, the location of DH sites, and the correlations established here between AFP gene methylation and function, with other evidence from gene transfer studies (35,47), indicate that AFP regulatory elements exert their functions within a region of 4 kb adjacent to the transcription initiation site.

The available data on AFP gene repression by glucocorticoid hormones are consistent with a mechanism whereby activated glucocorticoid receptors bind to a DNA recognition sequence around -160 bp from the AFP transcription initiation site, which results in closing off the adjacent "open" chromatin structure and blocking polymerase initiation on the AFP promoter. DEX receptors perhaps dislodge an AFP gene activator from a promoter-proximal region: the structural homology between the glucocorticoid receptor and the ErbA oncogene product (48), which impinges on cell differentiation, seems particularly intriguing in this regard. However, AFP repression is partly irreversible upon DEX withdrawal, which indicates that secondary mechanisms operate in DEX-induced AFP repression, plausibly connected with the concomitant arrest of liver cell growth, a signal to which the AFP gene is highly sensitive.
ACKNOWLEDGEMENTS

We thank Adolfo Ruiz-Carrillo, Markus Affolter, Jean Renaud, Normand Marceau, Hélène Baribault and Alan Anderson for advice, Diane Hamel and Michel Lambert for technical assistance, and Elisabeth Lemay for secretarial work. B.T. was supported by a doctoral studentship from "Le Fonds de la Recherche en Santé du Québec" and M.G. by a scholarship from the Quebec Ministry of Science and Technology. This work is supported by a grant from the Medical Research Council of Canada.

* Present address: Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Institut de Chimie Biologique, 11 rue Humann, 67085 Strasbourg, France

§ Present address: Comprehensive Cancer Center, University of Southern California, Los Angeles, CA 90033, U.S.A.

* To whom correspondence should be addressed.

REFERENCES

1. Ingram, R.S., Scott, R.W. and Tilghman, S.M. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 4694-4698.
2. Urano, Y., Sakai, M., Watanabe, K. and Tamaoki, T. (1984) Gene 32, 255-261.
3. Chevrette, M., Guertin, M., Turcotte, B. and Bélanger, L. Nucl. Acids Res., in press.
4. Sell, S., Nichols, M., Becker, F.F. and Leffert, H.L. (1974) Cancer Res. 34, 865-871.
5. Roche, J., Gorka, C., Goeltz, P. and Lawrence, J.J. (1985) Nature 314, 197-198.
6. Bélanger, L., Hamel, D., Lachance, L., Dufour, D., Tremblay, M. and Gagnon, P.M. (1975) Nature 256, 657-659.
7. Turcotte, B., Guertin, M., Chevrette, M. and Bélanger, L. (1985) Nucl. Acids Res. 13, 2387-2398.
8. Weintraub, H. and Groudine, M. (1976) Science 193, 848-856.
9. Stalder, J., Groudine, M., Dodgson, J.B., Engel, J.D. and Weintraub, H. (1980) Cell 19, 973-980.
10. Nahon, J.L., Gal, A., Erdos, T. and Sala-Trepat, J.M. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 5031-5035.
11. Kunnath, L. and Locker, J. (1985) Nucl. Acids Res. 13, 115-129.
12. Latchman, D.S., Brzeski, H., Lovell-Badge, R. and Evans, M.S. (1984) Biochim. Biophys. Acta 783, 130-136.
13. Balcarek, J.M. and McMorris, F.A. (1983) J. Biol. Chem. 258, 10622-10628.
14. Koropatnik, J., Andrews, G. and Duerksen, J.D. (1983) Nucl. Acids Res. 11, 3255-3267.
15. Wu, C. (1980) Nature 286, 854-860.
16. Stalder, J., Larsen, A., Engel, J.D., Dolan, M., Groudine, M. and Weintraub, H. (1980) Cell 20, 451-460.
17. Eisenberg, J.C., Cartwright, I.L., Thomas, G.H. and Elgin, S.C.R. (1985) Ann. Rev. Genet. 19, 485-536.
18. Doerfler, W. (1983) Ann. Rev. Biochem. 52, 93-124.
19. Bird, A.P. (1986) Nature 321, 209-213.
20. Keeshet, I., Lieman-Hurwitz, J. and Cedar, M. (1986) Cell 44, 535-543.
21. Groudine, M. and Conkin, K.F. (1985) Science 228, 1061-1068.
22. Andrews, G.K., Dziadek, M. and Tamaoki, T. (1982) J. Biol. Chem. 257, 5148-5153.
23. Young, P.R. and Tilghman, S.M. (1984) Mol. Cell. Biol. 4, 898-907.
24. Kuo, M.T., Iyer, B., Wu, J.R., Lapeyre, J.-N. and Becker, F.F. (1984) Cancer Res. 44, 1642-1647.
25. Kuninath, L. and Locker, J. (1983) EMBO J. 2, 317-324.
26. Vedel, M., Gomez-Garcia, M., Sala, M. and Sala-Trepat, J.M. (1983) Nucl. Acids Res. 11, 4335-4354.
27. Pertopoulos, C.J., Yaswen, P., Panzica, M. and Fausto, N. (1985) Nucl. Acids Res. 13, 8105-8118.
28. Baribault, H., Leroux-Nicollet, I. and Marceau, N. (1985) J. Cell. Physiol. 122, 105-112.
29. Marshall, A.J. and Burgoyne, L.A. (1976) Nucl. Acids Res. 3, 1103-1110.
30. Sargent, T.D. (1981) Ph.D. Thesis, California Institute of Technology.
31. Fritton, H.P., Sippel, A.E. and Igo-Kemenes, T. (1983) Nucl. Acids Res. 11, 3467-3485.
32. Wahl, G.M., Stern, M. and Stark, G.R. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3683-3687.
33. Ruiz-Carrillo, A. (1984) Nucl. Acids Res. 12, 6473-6492.
34. Blin, N. and Stafford, D.W. (1976) Nucl. Acids Res. 3, 2303-2308.
35. Godbout, R., Ingram, R. and Tilghman, S.M. (1986) Mol. Cell. Biol. 6, 477-487.
36. Kioussis, D., Eiferman, F., van de Rijn, P., Gorin, M.B., Ingram, R.S. and Tilghman, S.M. (1981) J. Biol. Chem. 256, 1960-1967.
37. Scott, R.W. and Tilghman, S.M. (1983) Mol. Cell. Biol. 3, 1295-1309.
38. Yamamoto, K.R. (1985) Ann. Rev. Genet. 19, 209-252.
39. Sakai, M., Morinaga, T., Urano, Y., Watanabe, K., Wegmann, T.G. and Tamaoki, T. (1985) J. Biol. Chem. 260, 5055-5060.
40. LaRue, H., Bissonnette, E. and Bélanger, L. (1983) Can. J. Biochem. Cell Biol. 61, 1197-1200.
41. Larsen, A. and Weintraub, H. (1982) Cell 29, 609-622.
42. Shimada, T. and Nienhuis, A.W. (1985) J. Biol. Chem. 260, 2468-2474.
43. Renaud, J. and Ruiz-Carrillo, A. (1986) J. Mol. Biol. 189, 217-226.
44. Groudine, M. and Weintraub, H. (1982) Cell 30, 131-139.
45. Shermoen, A.W. and Beckendorf, S.K. (1982) Cell 29, 601-607.
46. Burch, J.B.E. and Weintraub, H. (1983) Cell 33, 65-76.
47. Krumlauf, R., Hammer, R.E., Tilghman, S.M. and Brinster, R.L. (1985) Mol. Cell. Biol. 5, 1639-1648.
48. Weinberger, C., Hollenberg, S.M., Rosenfeld, M.G. and Evans, R.M. (1985) Nature 318, 670-672.