Tissue-specific DNaseI hypersensitive sites in the 5′-flanking sequences of the tryptophan oxygenase and the tyrosine aminotransferase genes

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The genes for tryptophan oxygenase (TO) and tyrosine aminotransferase (TAT) are expressed in a tissue- and development-specific manner and are regulated by glucocorticoids (TO and TAT) and glucagon or its intracellular mediator cAMP (TAT) in rat liver. We have analyzed the chromatin structure of these genes in the vicinity of the 5′ ends with regard to DNaseI hypersensitivity and have found DNaseI hypersensitive sites upstream of each of the promoters. Mapping of this region reveals three closely spaced cleavage sites near the TO promoter and a doublet of sites near the TAT promoter. In both genes additional cleavage sites are found further upstream. All hypersensitive sites of both genes are absent in kidney nuclei and therefore appear to be specific for the tissue expressing the genes. A correlation of expression and modified chromatin structure was also observed in a hepatoma cell line expressing TAT but not TO: hypersensitive sites are present in TAT but not in TO chromatin. Upon glucocorticoid induction an additional hypersensitive site is detected ~2 kb upstream of the TAT promoter in liver and hepatoma cells.

Key words: chromatin structure/DNaseI hypersensitivity/steroid regulation/tryptophan oxygenase gene/tyrosine aminotransferase gene

Introduction

Regulatory processes that lead to differential gene expression are reflected in local changes in chromatin structure. Though correlations have been established between certain features of chromatin structure and gene expression, little is known about their causal relationship (Igo-Kemenes *et al.*, 1982; Weisbrod, 1982). Evidence from studies in several systems suggests that a given domain of chromatin is not transcribed unless its structure is altered as detectable by a modified sensitivity towards digestion by nucleases. For example, DNaseI degrades actively transcribed chromatin much faster than inactive sequences, as first shown by Weintraub and Groudine (1976) for the chick globin genes. These sensitive regions are not uniformly degraded by DNaseI but contain local sites two orders of magnitude more sensitive towards DNaseI cleavage than inactive chromatin and hence are called DNase hypersensitive (HS) sites. Using the indirect end-labeling technique as first applied by Wu (1980) and Nedospasov and Georgiev (1980) such sites have been localized in the chromatin of many genes. Hypersensitive sites frequently map near the 5′ ends of actively transcribed genes and their presence has been correlated in many systems with their state of expression (Elgin, 1981).

To understand the nature and the regulatory significance of these chromatin structures, it is necessary to study which DNA sequences and proteins are required to define a DNaseI hypersensitive site in the control region of the active genes and how this structure is related to transcriptional activation. We believe that analysis of gene control of the tryptophan oxygenase (TO) and the tyrosine aminotransferase (TAT) genes are well suited for this approach. Both genes are expressed in a tissue-specific fashion and are developmentally regulated (Greengard, 1970). The activity of both genes is controlled by glucocorticoids (Danesch *et al.*, 1983; Granner and Hargrove, 1983 for review) and the TAT gene is also regulated by glucagon, insulin and cAMP (Holten and Kenney, 1967). Furthermore, a control region located on chromosome 7 in the vicinity of the albino locus appears to be required for expression of TAT as suggested from genetic and biochemical analysis of lethal albino mutants (W.Schmid, G.Schütz, S.Gluecksohn-Waelsch, unpublished data; Gluecksohn-Waelsch, 1979).

In the present set of experiments we characterize DNaseI hypersensitive sites around the 5′ end of the TO and the TAT genes. We have limited the analysis to these parts of the genes since transfection studies with hybrid genes containing 2 kb of 5′-flanking TO and TAT sequences appear to be sufficient for expression (U.Danesch, M.Jantzen, R.Renkawitz and G.Schütz, unpublished results). The DNaseI hypersensitivity of these two genes has been analyzed in rat liver and kidney nuclei with respect to hormone dependence and tissue specificity. In addition these chromatin structural changes have been examined in hepatoma cells, in which the TAT but not the TO gene is expressed.

Results

DNaseI cleaves at multiple sites near the 5′ end of the TO gene

To visualize DNaseI HS sites, liver nuclei were prepared and treated with increasing DNaseI concentrations. After purification the DNA was digested with *Eco*RI or *Pst*I (Figure 1B) and fragments carrying 5′ TO sequences were visualized by Southern blotting employing the indirect end-labeling method developed by Wu (1980) and Nedospasov and Georgiev (1980). When DNaseI-treated DNA is cut with *Eco*RI and probe 'b' (Figure 1B) is used for Southern hybridization the region which can be scored for HS sites extends to the next *Eco*RI site 450 bp upstream of the TO cap site. Figure 1A shows an autoradiogram resulting from such an experiment. *Eco*RI cleavage without prior incubation of nuclei with DNaseI generates a prominent 3.6-kb band. With increasing concentrations of DNaseI a significantly shorter fragment appears in increasing intensity (thick arrow, Figure 1A and B), which corresponds to a HS region near the cap site of the TO promoter. In addition a number of bands are visible which represent DNaseI cuts within the transcribed region of the gene (Figure 1A and B). Using DNA size markers and the 3.6-kb *Eco*RI fragment as an internal standard, a hypersensitive region was localized in close proximity

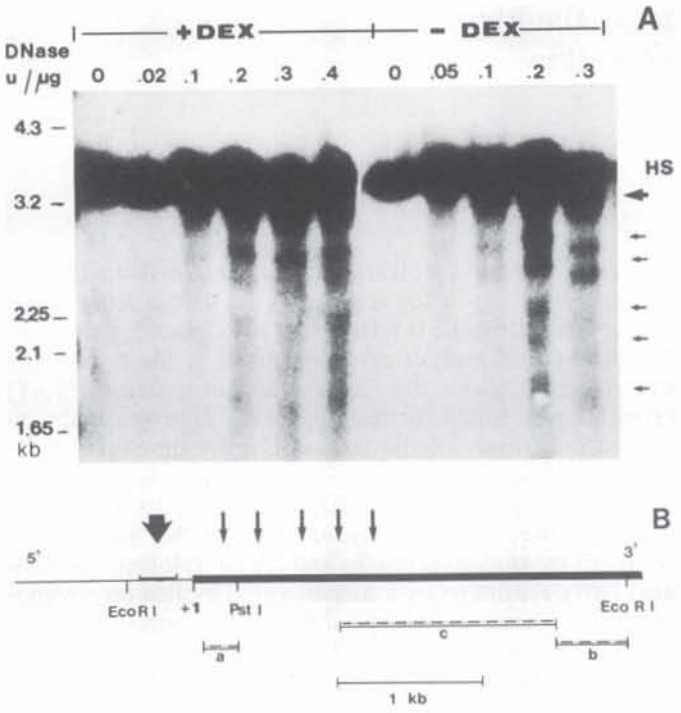

Fig. 1. DNaseI HS sites in TO chromatin. **A**: Nuclei from glucocorticoid-induced (+Dex) and uninduced (−Dex) rat livers were digested with increasing amounts of DNaseI. After purification the DNAs were cut with *Eco*RI and 50 µg of each sample was separated on a 1.0% vertical agarose gel which was blotted as described. As a probe for the resulting Southern filter probe 'b' (see **B**) was used. **B**: A map of the DNase HS cuts near the 5′ end of the TO gene. The thick line represents the transcribed sequence. The fragments 'a'−'c' show the probes used for the various blots (see Materials and methods). '+1' indicates the cap site for the TO major transcript. The HS sites are marked with arrows.

to the TO cap site. The internal cuts are regularly spaced and the approximate positions were determined to be: +220, +470, +780, +1020 and +1250 (numbers in base pairs from the transcription start at '+1').

To demonstrate that the hypersensitive sites exist as a consequence of chromatin structure, high mol. wt. protein-free DNA from rat liver was digested with DNaseI and analyzed as above. The blot (Figure 2) shows that the broad HS region 5′ of the cap site has no counterpart in protein-free DNA and hypersensitivity therefore must be a feature of chromatin. The cleavage sites within the TO gene, however, are also visible on a longer exposure in protein-free DNA, but the bands are much less intense.

The pattern of fragments resulting from a limited DNaseI digest of the TO gene is not different when comparing nuclei from uninduced or hormone-induced animals and moreover appears at approximately the same extent of DNaseI digestion (Figure 1A).

To allow a more detailed analysis of the nuclease-sensitive region at the 5′ end of the TO gene, the DNaseI cleavage sites were mapped from the *Pst*I site at about +300 using a small fragment (probe 'a') abutting with the *Pst*I cleavage site (Figures 1B and 3). *Pst*I cleavage alone yields a 3.8-kb fragment hybridizing to probe 'a', thus allowing the scoring of DNaseI HS sites in 3.5 kb of TO flanking DNA. Figure 3 shows a Southern blot that was obtained with probe 'a' after DNaseI digestion of nuclei and subsequent *Pst*I cleavage of the purified DNA. The HS region resolves into three distinct bands. The positions of the DNaseI cuts were mapped using

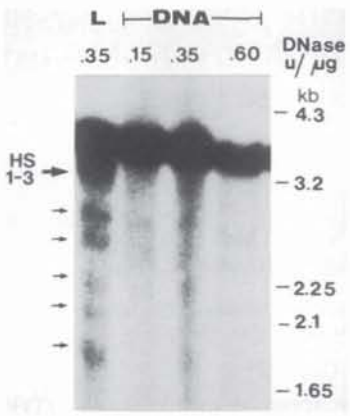

Fig. 2. HS sites in TO chromatin and protein-free DNA. Protein-free high mol. wt. rat liver DNA was digested with DNaseI to the same extent as the chromatin samples. The DNAs were treated as described in the legend to Figure 1A, 70 µg of DNA from the protein-free samples was loaded per slot on the gel; 'L' represents 50 µg DNA from liver chromatin treated as described in Figure 1. The filter was hybridized with probe 'b' (Figure 1B).

calibration curves from three different blots as follows: HS 1: −130 to −180 (±20); HS 2: −210 to −250 (±20); HS 3: −420 to −470 (±40); (mean values ± standard deviation). The cap site is not part of the hypersensitive region. This has also been observed in the chromatin of the chicken β-globin gene (McGhee *et al.*, 1981). At this level of fine structure mapping no significant difference in digestion patterns from nuclei of hormone-treated and uninduced animals is visible. Further upstream of HS 3 (between 0.45 and 3.8 kb upstream of the TO promoter) no additional prominent band is evident.

DNaseI HS sites near the 5′ end of the TAT gene: one upstream site is induced by glucocorticoids

To screen the 5′ region of the rat TAT gene in liver for preferential cleavage sites for DNaseI, DNA from DNaseI-treated nuclei of glucocorticoid induced and uninduced livers was digested with *Hind*III. To demonstrate DNaseI hypersensitive sites in the 4.4-kb *Hind*III fragment containing the cap site and 2.9-kb 5′-flanking sequences, the *Eco*RI/*Hind*III fragment (Figure 4B) was used for indirect end-labeling. An autoradiogram from a Southern blot analysis is presented in Figure 4A. With increasing amounts of DNaseI smaller bands appear below the *Hind*III fragment. DNaseI cleavage within a broad region produces hybridizing fragments of ~1.7 kb. in length in both glucocorticoid-induced and uninduced nuclei. On other gels (e.g., Figure 6) this broad band is clearly resolved into a doublet of distinct bands (HS 1, HS 2). Another site (HS 3) is common to both induced and uninduced nuclei and appears at similar DNaseI concentrations, whereas HS 4, visualized as a band directly below the *Hind*III fragment, is present only in hormone-induced nuclei. The HS sites were mapped using calibration curves from three different gels: HS 1 and HS 2 cover a region of ~200 bp in close proximity to the cap site. HS 3 and the hormone-induced sites HS 4 were mapped to positions −750 and −2000, respectively. On a shorter run of the gel an additional HS site becomes visible which maps to a position +700 within the transcribed region (not shown). A DNaseI digest of protein-free genomic DNA to control for possible preferential cleavage sites within protein-free genomic DNA to control for possible preferential cleavage sites within protein-free DNA is shown in Figure 6. On a longer exposure (not shown) discrete bands become

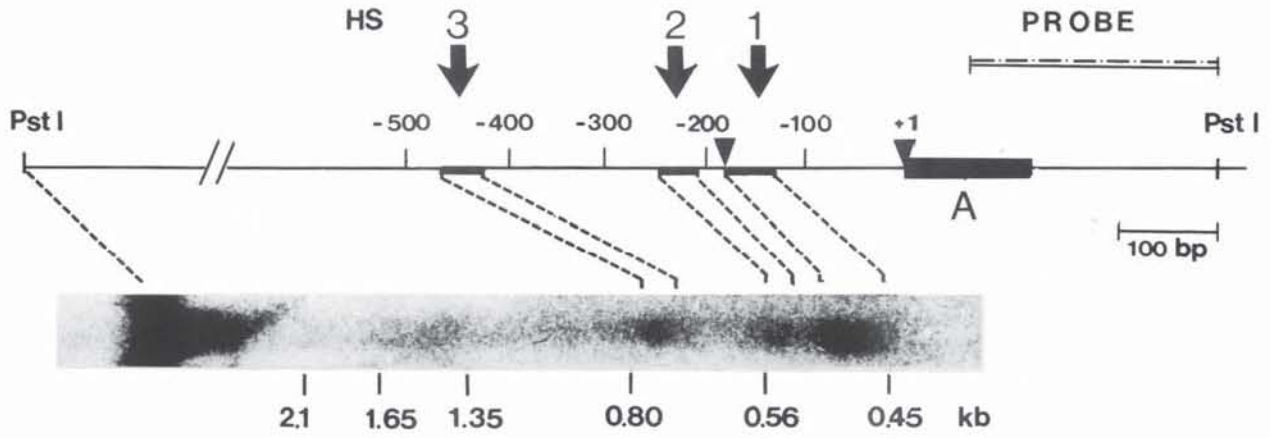

Fig. 3. Fine mapping of the 5′-flanking HS sites of the TO gene. Nuclei from hormone-induced rat livers were digested with 0.3 U DNaseI per μg DNA. After purification the DNA was cut with *PstI* and 50 μg/slot were separated on a 2.0% agarose gel and blotted for 2 h only in order to retain smaller fragments. The filter bound DNA was hybridized with probe 'a' (Figure 1B). The genomic *PstI* fragment shown in the map is ~3.8 kb long. The triangles point to the positions of the major transcriptional start of the gene at +1 and the minor upper start at −180. Numbers indicate distance in base pairs from the cap site. The first exon ('A') is shown as a thick bar. HS sites 1–3 are indicated by arrows.

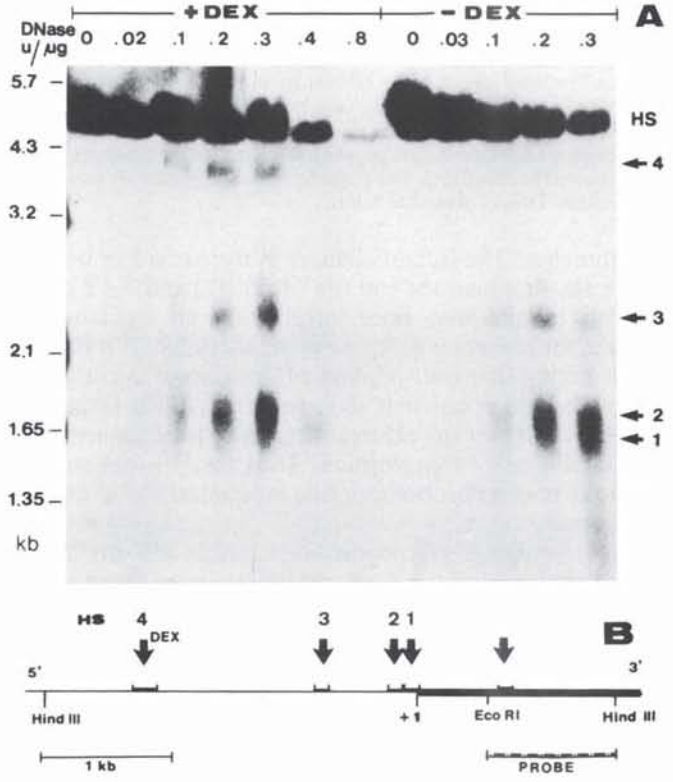

Figure. 4. DNaseI HS sites in TAT chromatin. **A**: Nuclei from hormone-induced (+Dex) and uninduced (−Dex) rat livers were treated with increasing amounts of DNaseI. After purification the DNAs were cut with *Hind*III and 50 μg/slot were separated on a 1.0% agarose gel. The DNA was blotted and hybridized to the *Eco*RI/*Hind*III fragment shown in **B**. **B**: A map of the 5′ end of the TAT gene is presented. The thick line symbolizes the transcribed region, the cap site is marked +1. The 5′ HS sites 1–4 are indicated with arrows. An additional site within the gene found with shorter runs of the gel (data not shown) is also indicated. The hormone-inducible site (HS 4) is labeled 'DEX'.

faintly visible in the protein-free samples, but they clearly do not correspond to those found in chromatin.

All HS sites are correlated with expression of the TAT and TO gene

TO and TAT are exclusively expressed in liver (Knox, 1955; Hargrove and Mackin, 1984). To determine whether the DNaseI HS sites found in chromatin are limited to tissues expressing the TO and TAT genes, nuclei were prepared from kidneys of hormone-induced rats, and analyzed for HS sites as described. Figure 5B shows that chromatin prepared from kidney and liver can be digested to a comparable degree using similar amounts of DNaseI. Autoradiograms resulting from an analysis of the TO (Figure 5A) and the TAT chromatin (Figure 6) show that there are no hypersensitive sites in the non-expressing tissue suggesting that all sites in both genes are tissue specific.

Hepatoma cells have widely been used in the analysis of hormonal control (Tomkins *et al.*, 1972) and cell differentiation (Weiss *et al.*, 1981), since controlled expression of liver-specific proteins continues *in vitro*. In most rat hepatoma cell lines the TAT gene is transcribed and regulated by steroids and cAMP, whereas the TO gene is not expressed (Ramanarayanan-Murthy *et al.*, 1976). To relate this difference in the activity of the two genes in hepatoma cells to the characteristic features of chromatin structure observed in liver cells, the presence and pattern of HS sites in hepatoma chromatin was examined. Hepatoma cells were kept with and without glucocorticoids and the chromatin in the TO and TAT 5′ region was analyzed for DNaseI HS sites. As shown in Figure 7, the same cleavage sites are observed following DNaseI digestion of hepatoma nuclei as are found in the TAT chromatin of liver. The HS site 4 upon dexamethasone induction is present in hepatoma cells as well. In contrast, no HS sites are visible in the TO chromatin of hepatoma cells, independent of hormone treatment. In this respect, TO chromatin resembles the DNaseI-resistant pattern found in kidney, suggesting that hypersensitivity within these genes correlates with their transcriptional activity.

Discussion

We have identified regions in the chromatin of the TAT and TO genes characterized by structural differences as evidenced by preferential cutting by DNaseI. Both genes show a prominent HS region at or near the site of transcription initiation. The TAT gene displays two additional HS sites further upstream, the distal one of which appears to be glucocorticoid inducible. The broad HS regions near the cap site appear

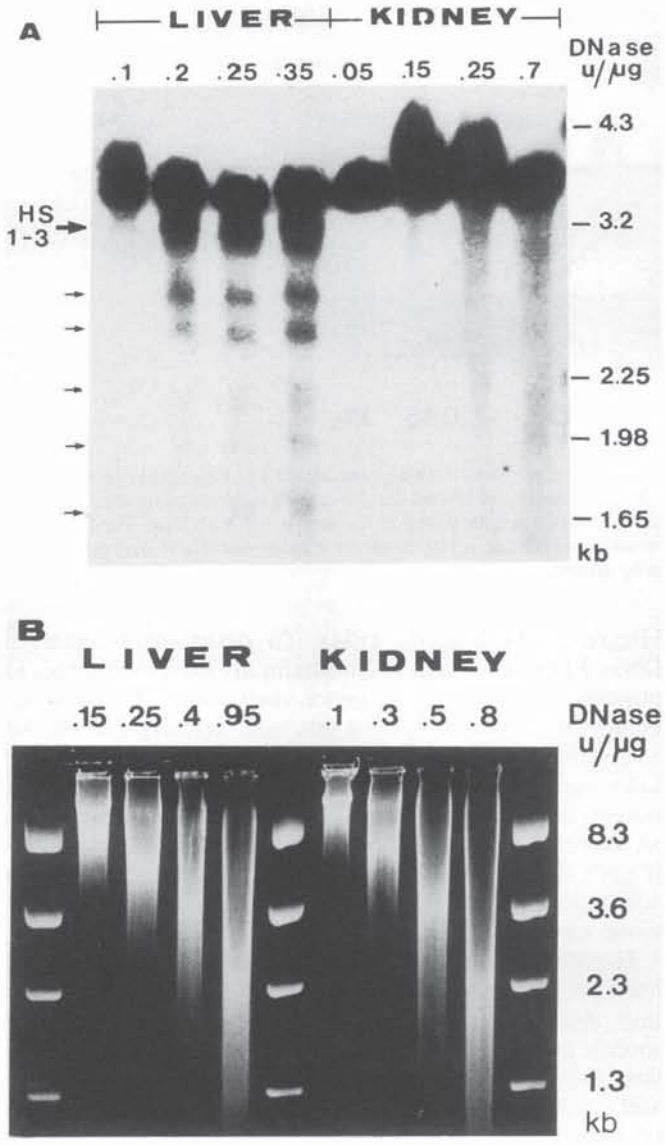

Fig. 5. Tissue specificity of HS sites in TO chromatin. Nuclei from kidney and liver of hormone-induced rats were digested with DNaseI to similar extents. Purified DNAs were cut with *Eco*RI, separated on 1% agarose gels and blotted as described **(A)**. To achieve a strong hybridization signal probe 'c' (Figure 1B) was used. To assess the extent of DNaseI digestion the DNA obtained from comparative DNase digestions of nuclei was run on an analytical gel **(B)**.

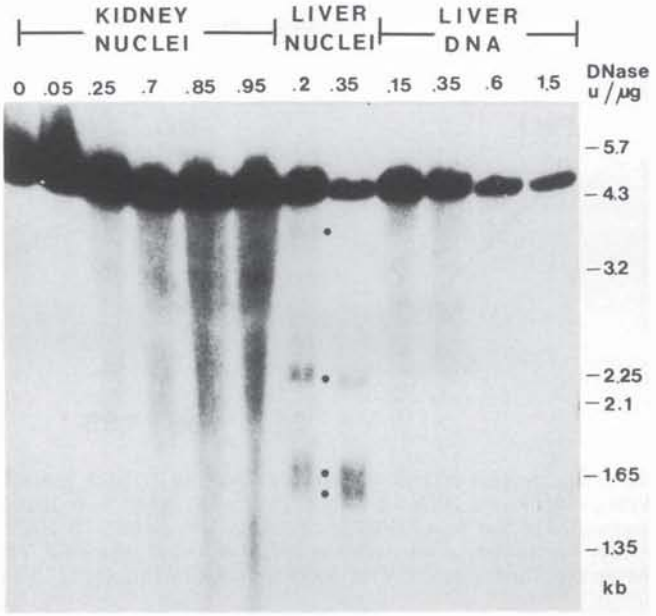

Fig. 6. Tissue specificity of HS sites in TAT chromatin and comparison with protein-free DNA. Liver and kidney nuclei and protein-free DNA from rat liver were digested with DNaseI to similar degrees. All DNA samples were treated as described in the legend to Figure 4A, except that 70 μg of DNA of the protein-free samples and 50 μg for the chromatin samples were loaded on the gel per slot. As probe for the Southern hybridization the *HindIII/EcoRI* fragment shown in Figure 4B was used. Dots indicate DNaseI-generated bands.

not to be homogeneously hypersensitive to DNaseI digestion, but structured. In the TO gene a triplet of bands is found upstream of the TATA box whereas the TAT chromatin displays a doublet of bands. A relatively protected stretch within a hypersensitive area could result from the binding of a factor yet to be defined. Emerson and Felsenfeld (1984) recently obtained evidence suggesting a causal relationship between the binding of a protein factor to the DNA in expressing tissue and the appearance of a HS site in reconstituted β-globin chromatin at about this position.

Sequences which confer steroid responsiveness and receptor binding have been identified near the cap site in several steroid-inducible genes (Hynes *et al.*, 1983; Dean *et al.*, 1983; Scheidereit *et al.*, 1983; Payvar *et al.*, 1983; Karin *et al.*, 1984; Renkawitz *et al.*, 1984). Thus far no studies are reported to relate the binding of the steroid receptor to chromatin structural changes such as the appearance of DNaseI hypersensitive sites. The lack of changes in the pattern or intensity of the HS sites near the cap site of the TO and TAT gene is difficult to interpret, since information on regulatory sequences for these two genes is not yet available. It is however worth noting that transcription of both genes occurs in the absence of glucocorticoids (Scherer *et al.*, 1982; Danesch *et al.*, 1983) and that steroid treatment leads to enhancement of this basal level of transcription. Thus the HS sites near the promoter may reflect transcription independent of glucocorticoid action.

In this study a glucocorticoid-inducible HS site (HS 4) ~2 kb upstream of the TAT cap site has been detected. The large distance from the glucocorticoid-induced HS site to the 5′ end of the TAT gene and the possible regulatory significance of this site should be interpreted cautiously since it is not known whether an as yet unidentified gene is located in that area. It remains to be clarified whether the influence of steroid hormones on gene expression involves structural changes in the chromatin at positions rather distant from the promoter. An estradiol-dependent HS site ~700 bp upstream of the cap site has been found in the chicken vitellogenin gene (Burch and Weintraub, 1983).

All HS sites in both the TAT and the TO genes appear to be specific for the tissues in which the genes are transcribed. Since the expression of TAT is limited to hepatocytes (Granner and Hargrove, 1983) we assume that the observed chromatin changes are specific for this portion of the liver. We do not find any HS site in the TO chromatin of kidney and hepatoma cells in which the gene is not expressed, suggesting a correlation of the presence of HS sites with the transcriptional activity of the gene. Tissue-specific formation of DNase hypersensitivity so far has been found in all genes that are known to be differentially expressed in various tissues (Igo-Kemenes *et al.*, 1982). However, a causal relationship

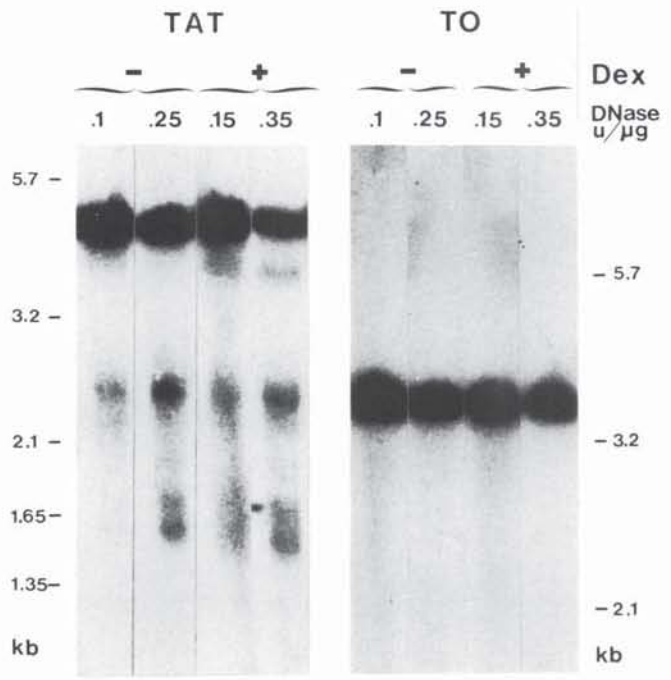

Fig. 7. HS sites in TAT and TO chromatin in hepatoma cells. Nuclei from hormone-induced (+Dex) and uninduced (−Dex) hepatoma cells were digested with DNaseI as indicated. After purification of the DNAs, 50 µg samples were cut with *Hind*III or *Eco*RI for analysis of the HS sites in TAT and TO chromatin, respectively, separated on a 1% agarose gel and blotted. The same DNA was analyzed with respect to both genes. As probes for Southern hybridization probe 'b' (Figure 1B) was used in the case of the TO gene and the *Eco*RI/*Hind*III fragment (Figure 4B) in the case of the TAT gene.

between DNaseI hypersensitivity and gene expression still remains to be established. Studies are in progress to identify sequences in the TO and TAT gene which are important for transcription and its control by steroids and cAMP, and which confer steroid receptor binding. These studies may reveal, whether the DNaseI hypersensitive sites observed here have a regulatory function in the expression of the TO and TAT genes.

Materials and methods

Animals and hormone treatment

For all experiments male Wistar rats of ~200 g body weight were used. The animals were adrenalectomized 5–7 days prior to killing. Hormone induction was carried out by injecting 10 µg dexamethasone (Sigma)/100 g body weight in 1 ml saline i.p., 1 h prior to removal of the liver.

Cell culture and dexamethasone induction

Hepatoma cells (H4-IIE-C3, provided by M.Weiss) were grown to ~75% confluence in Dulbecco's modified Eagle medium (Seromed) including 10% fetal calf serum (Gibco) and antibiotics. Cells were kept 10–12 h in serum-free medium prior to induction with 10^{-6} M dexamethasone for 1 h. Control cells were kept 12 h serum free prior to preparation of nuclei.

Isolation of nuclei

Liver and kidney. Nuclei were prepared from fresh tissue; all steps were carried out at temperatures between 0 and 4°C. Liver or kidney tissue was homogenized in 60 mM KCl, 15 mM NaCl, 15 mM Tris-Cl pH 7.5, 0.5 mM spermidine, 0.15 mM spermine (buffer A) containing 0.3 M sucrose, 2 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) (freshly added from 100 mM solution in isopropanol) using a loose fitting motor-driven potter homogenizer. The homogenate was filtered through four layers of gauze and pelleted by centrifugation at 3000 r.p.m. for 5 min. The nuclear pellet was resuspended in homogenization buffer containing 0.1% Triton X-100 and collected nuclei were washed twice in homogenization buffer without Triton. Finally the nuclear suspension was filtered through a 44 µm polyester-filter (Tripette et Renaud, Strassbourg) and resuspended in buffer A + 1 mM PMSF, 0.2 mM EGTA. Yields of nuclei were estimated by counting aliquots under the microscope.

Hepatoma cells. Cells from 10 plates (15 cm diameter) were pooled for preparation of nuclei. Plates were washed twice with PBS (120 mMNaCl, 28 mM Na_2PO_4 pH 7.3, 2.5 mm KH_2PO_4) and the cells scraped into PBS and collected by centrifugation for 5 min at 3000 r.p.m. The pellet was resuspended in 20 ml of cold homogenization buffer, then combined with another 20 ml of homogenization buffer containing 1% NP40, mixed and left on ice for 5 min. Nuclei were pelleted and washed twice with homogenization buffer without detergent (all steps at 0–4°C). Finally the pellet was resuspended in buffer A containing 1 mM PMSF, 0.2 mM EGTA for DNaseI digest.

DNaseI digest

The DNaseI digestion of nuclei and protein-free DNA was essentially carried out as described by Fritton *et al.* (1983) except that the DNaseI digestion of protein-free DNA was performed at 0°C with higher amounts of DNaseI. DNA of digested nuclei was recovered by three successive ethanol precipitations. Purified DNA was digested with 2–3 units of restriction enzyme per µg DNA overnight according to the suppliers' recommendations.

Plasmids and fragments used as probes for Southern analysis

All probes used for the analysis of the two genes were pUC8-subclones derived from the genomic clones λTO1 (Schmid *et al.*, 1982) or λrTAT1 (Shinomiya *et al.*, 1984). In some experiments isolated fragments were used. For detection of TAT gene sequences a 0.95-kb *Eco*RI/*Hind*III fragment including the exons B and C (Figure 4) was subcloned into pUC8 and the resulting plasmid used as probe. The TO probes are depicted in Figure 1B. Probe 'a' is a *Hga*I/*Pst*I fragment of 250 bp in length that was isolated from the corresponding subclone and concatemerized prior to nick translation. Probe 'b' is a 500-bp *Kpn*I/*Eco*RI fragment from the third intron of the gene which was isolated from the corresponding subclone and concatemerized prior to nick translation. For probe 'c' a 1.5-kb *Pst*I/*Kpn*I fragment adjacent to fragment 'b' and thus including the third exon and parts of the flanking introns was subcloned into pUC8 and the resulting plasmid used as such. Gel electrophoresis of the cleaved DNAs was carried out on 4 mm thick vertical agarose gels (0.8–2.0%) in a Tris acetate buffer. Routinely 50 µg of DNA was loaded per slot. End-labeled fragments of *Hind*III cut λlys30 DNA was employed as size marker. Transfer of DNA to nitrocellulose (Schleicher und Schüll), nick translation of DNA and hybridization were according to Maniatis *et al.* (1982) and Wahl *et al.* (1979), respectively. Final wash of the Southern filters after hybridization was in 0.1 x SSC, 0.1% SDS at 65°C. The air dried filters were exposed to Kodak XAR5 film in cassettes with Kyokko HS intensifying screens at −70°C for 2–4 days. All DNaseI HS sites were visible after an overnight exposure.

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