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 glucocorticoid hormones (TO and TAT) and growth factor or 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 5' end of rat liver DNA sequences and protein is 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 is 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 (Danesh et al., 1983; Granner and Hargrove, 1983 for review) and the TAT gene is also regulated by glucagon, insulin and cAMP (Holten and Kennedy, 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as suggested from genetic and biochemical analysis of lethal albino mutants (W.Schmid, G. Schütz, S.Gluecksohn-Waelsch, unpublished data; Gluecksohn-Waelsch, 1979).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' ends 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TO and TAT sequences appear to be sufficient for expression (M. Danesh, M. Jantzen, R. Renkawitz and G. Schütz, unpublished results).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.

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 (Iigo-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 nuclease. 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 DNaseI 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 is 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 (Danesh et al., 1983; Granner and Hargrove, 1983 for review) and the TAT gene is also regulated by glucagon, insulin and cAMP (Holten and Kennedy, 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).

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 EcoRI or PstI (Figure 1B) and fragments carrying 5' TO sequences were visualized by Southern blotting employing the indirect end-labeling method developed by Wu (1980) and Geogiev (1980). When DNaseI-treated DNA is cut with EcoRI and probe 'b' (Figure 1B) is used for Southern hybridization the region which can be scored for HS sites extends to the next EcoRI site 450 bp upstream of the TO cap site. Figure 1A shows an autoradiogram resulting from such an experiment. EcoRI 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 EcoRI fragment as an internal standard, a hypersensitive region was localized in close proximity...
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 DNase I 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 DNase I 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 DNase I digestion (Figure 1A).

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

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

To demonstrate DNase I hypersensitive sites in the 4.4-kb HindIII fragment containing the cap site and 2.9-kb 5′-flanking sequences, the EcoRI/HindIII 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 DNase I smaller bands appear below the HindIII fragment. DNase I 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 DNase I concentrations, whereas HS 4, visualized as a band directly below the HindIII 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 DNase I 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
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 DNasel 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 DNasel. 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 DNasel HS sites. As shown in Figure 7, the same cleavage sites are observed following DNasel 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 DNasel-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 DNasel. 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
not to be homogeneously hypersensitive to DNase 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; Scheiderer 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 DNase 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 (Graner 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

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Fig. 5. Tissue specificity of HS sites in TO chromatin. Nuclei from kidney and liver of hormone-induced rats were digested with DNase to similar extents. Purified DNAs were cut with EcoRI, 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 DNase 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 DNase 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 DNase-generated bands.
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 (H4-11E-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 10−4 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) (frequently 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 (Triplet et Renault, Strasbourg) 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 mM NaCl, 28 mM NaH2PO4, pH 7.3, 2.5 mM KH2PO4) 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 steps for was resuspended in buffer A containing 1 mM PMSF, 0.2 mM EGTA for DNaseI digestion.

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 TO1 (Schmid et al., 1982) or κTAT (Shinomya et al., 1984). In some experiments isolated fragments were used. For detection of TAT gene sequences a 0.95-kb EcoRI/HindIII 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 Hpal/PstI fragment of 250 bp in length that was isolated from the corresponding subclone and concatenated prior to nick translation. Probe ‘b’ is a 500-bp KpnI/EcoRI fragment from the third intron of the gene which was isolated from the corresponding subclone and concatenated prior to nick translation. For probe ‘c’ a 1.5-kb PstI/KpnI 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 HindIII cut λlys30 DNA was employed as size marker. Transfer of DNA to nitrocellulose (Schleicher und Schuell), nick translation of DNA and hybridization were according to Maniatis et al. (1982) and Wahl et al. (1979). 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 cassette 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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