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Chromatin Structure and Methylation State of a Thyroid Hormone-responsive Gene in Rat Liver*

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The gene for S14 in the rat codes for an mRNA which in lipogenic tissues (liver, fat, mammary gland) responds both to L-triiodothyronine and a high-carbohydrate, fat-free diet. In an effort to understand the molecular basis for the tissue-specific regulation of mRNA-S14 expression, we have examined the organization of this gene in chromatin. Specifically, we examined the distribution of DNase I-hypersensitive sites and DNA methylation sites associated with a 25-kilobase chromosomal domain containing the S14 gene. Our results show that DNase I preferentially digests four regions of the DNA flanking the 5′ end of the hepatic S14 gene which have characteristics of DNase I-hypersensitive sites. The sites, identified as HS-1, HS-2, HS-3, and HS-4, are located at or adjacent to the site of transcription initiation and at 1.2, 7, and 8 kilobases upstream from this site, respectively. In lactating mammary gland where the S14 gene is also highly expressed and regulated by L-triiodothyronine, sites HS-1, HS-2, and HS-4 are present, but HS-3 is absent. No hypersensitive sites were detected either within the gene or flanking the 3′ end of the gene. In brain, kidney, and spleen, tissues in which mRNA-S14 is expressed at levels ≤10% of that found in euthyroid liver, sites HS-1 and HS-3 were absent. Despite the marked effect of thyroid state on the abundance of hepatic mRNA-S14, no significant alterations were observed in the DNase I sensitivity of hepatic chromatin containing the S14 gene. Analysis of the DNA methylation pattern at *Hpa*II and *Hha*I sites showed a positive correlation of hypomethylation of the gene and the contiguous flanking regions with S14 gene expression. All *Hpa*II/*Msp*I sites and most *Hha*I sites either within or flanking the S14 gene were undermethylated in liver and lactating mammary gland. Although thyroid status had no generalized effect on the site-specific DNA methylation state of the hepatic S14 chromosomal domain, one site (H3) situated in the second exon close to the 3′ terminus of the S14 gene appeared to undergo demethylation in the transition from hypo- to euthyroidism. In essence, our results show that the 5′ DNA flanking region of the S14 gene contains a tissue-specific DNase I-hypersensitive site which, although not influenced by thyroid status, appears essential for the expression of S14 and its regulation by L-triiodothyronine. On the other hand, thyroid hormone administration results in the demethylation of a specific DNA cytosine in the 3′ region of the gene.

The recent description of the hepatic mRNA sequence coding for the protein "Spot 14" (S14) (M_r 17,010; pI 4.9) has provided a promising model for the study of L-triiodothyronine (T_3)[1] action at the cellular level (1–6). We have shown that hepatic mRNA-S14 expression is regulated by hormonal, tissue-specific, developmental, nutritional, and circadian factors. Although the function of the S14 is unknown, the tissue distribution and regulation of S14 gene expression by T_3 and diet suggest a role in some aspect of fatty acid synthesis, storage, or metabolism (4, 5). Since administration of T_3 to hypothyroid rats leads to an increase in hepatic nuclear pre-mRNA-S14 within 10 min (7), it appears likely that the effect of T_3 is not mediated by an antecedent translational product stimulated by this hormone. The rise in the precursor mRNA precedes the increase in the mature mRNA-S14 (4, 7) which is first detected at 20 min. Additional studies suggest that T_3 mediates accumulation of hepatic mRNA-S14 by augmenting gene transcription and enhancing mRNA-S14 stability (8).

In an effort to understand the molecular basis for the tissue-specific and hormonal regulation of mRNA-S14 expression, we have examined the organization of the S14 gene in chromatin. Several features distinguish transcriptionally active from inactive chromatin (9, 10). In addition to a general preferential sensitivity of active genes to DNase I digestion (11), active chromatin is punctuated by local discontinuities in the nucleosomal array to form sites that are hypersensitive to DNase I (12–15). Often, but not always, these sites flank the 5′ ends of active genes and are present prior to initiation of gene transcription. These structures appear to be important but not sufficient for gene activation (14). The high endonuclease sensitivity of these structures is considered to represent a local opening in the nucleosomal packaging of chromatin to permit interaction of specific DNA sequences with regulatory proteins and enzymes involved in specific gene expression.

In addition to the increased DNase I sensitivity, active genes are in general hypomethylated at cytosine residues in the sequence CpG (10, 16–20). DNA methylation has been implicated as an important factor in gene expression (16). Whereas the majority of cytosines in the sequence CpG are methylated within the mammalian genome, many active genes display undermethylation at critical sites (18–20).

In this report, we compare the characteristic DNase I-hypersensitive sites and the DNA methylation profile of the S14 gene in liver with that found in other rat tissues. In

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[1] The abbreviations used are: T_3, L-triiodothyronine; SDS, sodium dodecyl sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; bp, base pair; kb, kilobase.

lactating mammary gland, mRNA-S14 is expressed at levels comparable to liver and is regulated by T_3 (4, 5); whereas in brain, kidney, and spleen, mRNA-S14 is expressed at ≤10% of the hepatic value, and T_3 regulation is absent (4). Using this comparison, we have found one DNase I-hypersensitive site flanking the 5′ end of the gene that may be important for tissue-specific and hormonal regulation of the S14 gene.

MATERIALS AND METHODS

Animals—Euthyroid male Sprague-Dawley rats (2 months old, approximately 220–250 g) and lactating female rats (330–350 g plus litters of rats 10–15 days postpartum) were obtained from Biolabs (St. Paul, MN). Animals were fed standard lab chow (Purina). Animals were rendered hypothyroid either by thyroidectomy combined with ^{131}I radioablative treatment (2) or by maintaining animals for at least 3 weeks on methimazole (0.025%) in drinking water (21). In each case, animals were used when they ceased to gain weight. Euthyroid animals were made hyperthyroid by daily injections of a receptor-saturating dose of T_3 (200 μg/100 g of body weight) for 7 days (22).

Isolation of Nuclei—Nuclei were isolated from all tissues by a modification of the procedure described by Hewish and Burgoyne (23). All steps were carried out at 4 °C, and all buffers were adjusted to 1 mM phenylmethylsulfonyl fluoride just before use. Briefly, tissue (2 g) was homogenized in 20 ml of Buffer A (15 mM Tris, pH 7.5, 60 mM KCl, 15 mM NaCl, 0.15 mM spermidine, 0.5 mM dithiothreitol, 2.0 mM EDTA, 0.5 mM EGTA) containing 0.34 M sucrose in a glass-Teflon homogenizer. The homogenate was filtered through one layer of gauze, centrifuged at 600 × *g* for 10 min, and resuspended in 10 ml of Buffer B (Buffer A with 1.0 M sucrose). The suspension was adjusted to 0.5% Triton X-100 and centrifuged at 2500 × *g* for 10 min. The pellet was resuspended in 20 ml of Buffer C (Buffer A containing 0.25 M sucrose, 0.1 mM EDTA, 0.1 mM EGTA), adjusted to 0.5% Triton X-100, and centrifuged at 600 × *g* for 10 min. This step was repeated once using Buffer C containing no Triton. Nuclei were then resuspended in 5.0 ml of Buffer C and stored at 4 °C for use the same day. The DNA content was determined by a diphenylamine assay (24) or more routinely by absorbance of 260 nm (20 $A_{260\ nm}$ units/mg of DNA).

Nuclease Digestion and DNA Extraction—Nuclei were resuspended in Buffer D (Buffer C without spermine, spermidine, EDTA, or EGTA and containing 3 mM $MgCl_2$ and 0.05 mM $CaCl_2$) at a concentration of 20 A_{260nm} units/ml and prewarmed to 30 °C for 2 min. Aliquots of nuclei were digested for 5 min at 30 °C with increasing concentrations of pancreatic DNase I (Worthington). DNase I concentrations ranged from 0.5 to 10.0 units/20 A_{260nm} units. The control digest represents nuclei resuspended in Buffer D and incubated for 5 min at 30 °C in the absence of DNase I. Digestions were stopped by the addition of EDTA and EGTA to a final concentration of 20 mM each. DNA was extracted by adding 3 volumes of a solution containing 0.15% SDS, 100 μg/ml proteinase K in TEN buffer (10 mM Tris, pH 7.5, 0.1 M NaCl, and 1.0 mM EDTA) to the nuclear suspension and incubation at 37 °C overnight. The suspension was then extracted with phenol/chloroform/isoamyl alcohol (50:49:1) and then with ether. Extracts precipitated with ethanol at −20 °C were centrifuged, and the DNA pellets were resuspended in TEN buffer, treated with DNase-free RNase A (10 μg/ml for 20 min at 37 °C), and re-extracted as above. Ethanol-precipitated pellets were resuspended in TE buffer (10 mM Tris, pH 7.5, 1.0 mM EDTA) and stored at 4 °C. DNA concentration was determined by absorbance at 260.

Cloning of cDNA and Genomic DNA of S14 Gene—The cloning of two cDNAs containing sequences homologous to the mRNA-S14 has been described previously (7). Excision of a cloned insert from the pS14-C1 plasmid with *Pst*I yields a 420-base pair DNA fragment containing sequences homologous to the 3′ exon of S14 gene. Digestion of pS14-C2 plasmid with *Pst*I yields two cDNA fragments (610 and 310 base pairs). The 610-base pair fragment contains sequences homologous to the 5′ and 3′ exons of the S14 gene. The inserts from C1 (470 bp) and C2 (610 bp) were used to localize the position of hypersensitive sites (Fig. 1). All DNAs were labeled to a specific activity of 1–2 × 10^8 cpm/μg DNA with [^{32}P]dCTP by nick translation as previously described (4).

Restriction Enzyme Digestion, Southern Blotting, and Hybridization—DNA isolated from control or DNase I-digested nuclei was exhaustively digested with restriction enzymes under conditions recommended by the suppliers. The hypersensitive site studies used *Eco*RI (Bethesda Research Laboratories), whereas the methylation studies used the isochizomeric enzyme pairs *Hpa*II/*Msp*I (recognizing CCGG) and *Hha*I/*Cfo*I (recognizing GCGC). Digestions were carried out at 3 units of enzyme/μg of DNA at room temperature overnight. Following restriction enzyme digestion, DNA was electrophoresed at 50 V for 14 h in 0.7% agarose gel with 89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA, pH 8.3, as buffer. DNA size standards included a mixture of λ phage cut with *Eco*RI (21.7, 7.8, 5.9, 5.5, 4.9, and 3.4 kb) and *Pvu*I (14.6, 12.8, 11.9, and 9.7 kb) and φX174 replicative form cut with *Hae*III (1.35, 1.03, 0.72, 0.61, and 0.31 kb). Following electrophoresis, gels were stained with ethidium bromide and photographed. DNA in the gels was depurinated, denatured, neutralized, and then transferred to nitrocellulose (Schleicher & Schuell, BA83) or Zetabind (AMF) as described by Southern (25). Zetabind was used in our studies on hypersensitive sites, and nitrocellulose was used for the DNA methylation studies. After DNA transfer, blots were baked at 80 °C for 2 h *in vacuo* and stored *in vacuo* until hybridization.

Nitrocellulose blots were prehybridized overnight in buffer containing 50% formamide (deionized), 5 × SSC; 5 × Denhardt's, 0.5% SDS, 50 mM $NaPO_4$, pH 6.5, 250 μg/ml salmon testis DNA at 68 °C. Blots were hybridized with nick-translated [^{32}P]cDNA at 2.0 × 10^6 cpm/ml in the same buffer for 20 h at 42 °C. Following hybridization, blots were washed twice in 2 × SSC, 0.1% SDS at room temperature 15 min each and three times in 0.1 × SSC, 0.5% SDS at 68 °C 1 h each. Zetabind blots were hybridized and washed according to the procedures outlined by the manufacturer. Blots were then air-dried, wrapped in plastic film, and exposed to XAR-5 x-ray film for 5–10 days at −80 °C using Du Pont Cronex intensifying screens.

RESULTS

Localizing Hypersensitive Sites Associated with Spot 14 Gene—The S14 gene is a single-copy gene measuring 4.4 kb in length containing one intervening sequence flanked by two exons. DNA sequence data indicate that all protein coding information is found within the 5′ exon (26). Fig. 1 shows an *Eco*RI restriction map for the Spot 14 gene and the sequence homology between the gene and the two cDNA probes. The 470-bp insert excised from the pS14-C1 plasmid hybridizes to the 4.9-kb *Eco*RI genomic fragment (Fig. 1, *map*). This fragment contains the 3′ exon and the contiguous 3′-flanking DNA. The 610-bp insert excised from the pS14-C2 probe hybridizes to two *Eco*RI genomic fragments. The 12-kb fragment contains the 5′ exon plus contiguous 5′-flanking DNA, and the 4.9-kb fragment is the same fragment recognized by the C1 cDNA.

Fig. 1 shows the pattern of digestion of DNA from euthyroid rat liver nuclei digested to varying extents with DNase I. In order to localize the hypersensitive sites associated with the S14 gene, Southern blots of *Eco*RI-digested genomic DNA from control and DNase I-treated nuclei were probed with either the ^{32}P-labeled C1 insert probe (Fig. 1*A*) or the ^{32}P-labeled C2 insert probe (Fig. 1*B*). In Fig. 1*A*, decreasing levels of hybridization of the ^{32}P-labeled C1 probe reflect increasing levels of DNase I digestion of this fragment. However, no subfragments are generated, indicating the absence of DNase I-hypersensitive sites at the 3′ end of the gene. The subfragment detected at 2.9 kb which fails to change in intensity with increasing DNase I digestion does not exhibit the characteristics of a hypersensitive site and may represent microheterogeneities of the sequence within the gene.

Hybridizing the same DNA samples with the ^{32}P-labeled C2 probe revealed the two prominent bands at 12 and 4.9 kb and four subfragments. These four subfragments are labeled HS-1, HS-2, HS-3, and HS-4 and are 1.85 ± 0.1, 3.0 ± 0.2, 8.3 ± 0.3, and 9.0 ± 0.3 kb in length, respectively. Since the four subfragments hybridize only with the C2 probe (5′ probe) but not with the C1 probe (3′ probe), we conclude that all four bands are generated as a result of the combined action of DNase I (in nuclei) and *Eco*RI (on isolated DNA) on the 12-kb fragment. These same fragments are generated by lim-

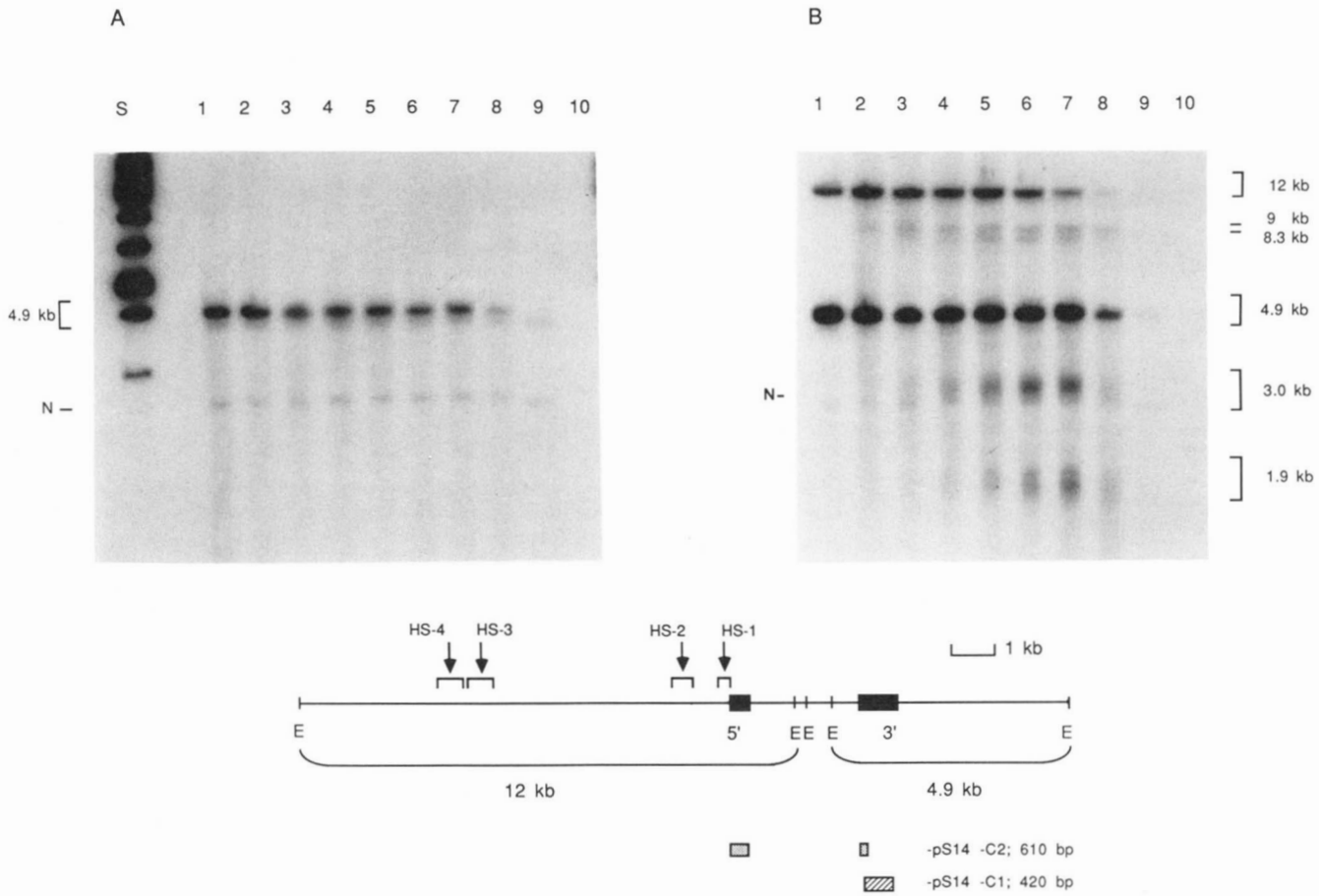

FIG. 1. **Localization of endonuclease-hypersensitive sites associated with rat liver S14 gene.** Euthyroid rat liver nuclei were digested with increasing concentrations of DNase I (*lanes 1–9*: 0.0, 0.05, 0.1, 0.25, 0.5, 1.0, 2.0, 4.0, and 10.0 units of DNase I/20 A_{260nm} units). DNA isolated from undigested (*lane 1*) and DNase I-digested (*lanes 2–9*) nuclei was exhaustively digested with *Eco*RI, electrophoresed, and transferred to Zetabind as described under "Materials and Methods." DNA standards (see "Materials and Methods") were electrophoresed in adjacent lanes (*lane S*). Blots were hybridized with either the C1 (*A*) or C2 (*B*) nicked-translated probes. *A*, blot of *Eco*RI-digested DNA from nuclei treated with increasing concentrations of DNase I (*lanes 2–9*) and from undigested nuclei (*lane 1*). Blot is hybridized with the nicked-translated ^{32}P-labeled C1 probe. A single restriction fragment (4.9 kb) corresponding to the 3′ end of the gene and contiguous flanking DNA is detected (see Fig. 1). *B*, blot of *Eco*RI-digested DNA from nuclei treated with increasing concentrations of DNase I (*lanes 2–9*) and from undigested nuclei (*lane 1*). Blot was hybridized with the nicked ^{32}P-labeled C2 probe. Two restriction fragments resolved in the control DNA (*lane 1*) are 12 and 4.9 kb in length. The DNA samples obtained from nuclei treated with increasing concentrations of DNase I and subsequently digested with *Eco*RI show four additional fragments designated HS-1, 1.85 ± 0.1 kb; HS-2, 3.0 ± 0.2 kb; HS-3, 8.3 ± 0.3 kb; and HS-4, 9.0 ± 0.3 kb. The size of each band is the mean ± S.D. of five separate blots. In both blots, a band migrating just distal to HS-3 (3.0 kb) has been labeled *N* because we are uncertain of its origin at this time; clearly, it does not seem to be present in all digestions, as evident in Fig. 3. *Map*, restriction map of the S14 gene, homology of the cDNA probes, and location of DNase I-hypersensitive sites associated with the S14 gene. The S14 gene is a single copy gene containing two exons (*closed boxes*) flanking a single intervening sequence. The gene is 4.4. kb in length; the first exon, which contains all the protein coding sequences, is 494 bp, the intervening sequence is 3.1 kb, and the second exon is 820 bp. The location of homology between the inserts derived from the two cDNA clones designated pS14-C1 (insert 420 bp) and pS14-C2 (insert 610 bp) is indicated. *Eco*RI (*E*) digestion of genomic DNA cleaves the gene and flanking DNA at five sites; three are located within the intervening sequence and two are located in the DNA flanking the gene. The C1 probe will hybridize to a 4.9-kb fragment containing the 3′ end of the gene and contiguous flanking DNA. The C2 probe will hybridize to 12- and 4.9-kb fragments. The 12-kb fragment contains the 5′ end of the gene and contiguous flanking DNA. Neither cDNA probe will hybridize to the small *Eco*RI fragments generated from the intron DNA sequences. The positions of the four hypersensitive sites are positioned relative to the designated *Eco*RI digestion site in the intervening sequence. The positions of the four hypersensitive sites are designated by the *arrows* and *brackets*. The *brackets* designate the range of cleavage sites.

ited micrococcal nuclease digestion, indicating that both strands of DNA within the hypersensitive site are accessible to nuclease (results not shown).

No subfragments were detected in control DNA, *i.e.* those samples not previously exposed to DNase I (Fig. 1, *lanes 1*), nor were they found in samples treated with DNase I (Fig. 2*A*) or micrococcal nuclease (results not shown) but without subsequent *Eco*RI digestion. Naked DNA (Fig. 2*C*) treated with DNase I followed by *Eco*RI also did not result in the formation of subfragments labeled HS-1 to HS-4. This indicates that tissue-specific proteins associated with chromatin are required for the generation of DNase I-hypersensitive sites. The intron *Eco*RI site, located nearest the 5′ exon (see Fig. 1, *map*), serves as the reference point to allow us to

position each hypersensitive site relative to the 5′ end of the S14 gene. Accordingly, the four sites are positioned along the gene as illustrated in Fig. 1 (*map*). HS-1 is located at or adjacent to the 5′ cap site; whereas HS-2, HS-3, and HS-4 are located 1.2, 7, and 8 kb upstream from the cap site, respectively. Because bands HS-1 and HS-2 are broad, precise positioning of the sites is not possible. These broad bands probably represent heterogeneity of the DNase I cleavage site owing to a very open portion of the chromatin.

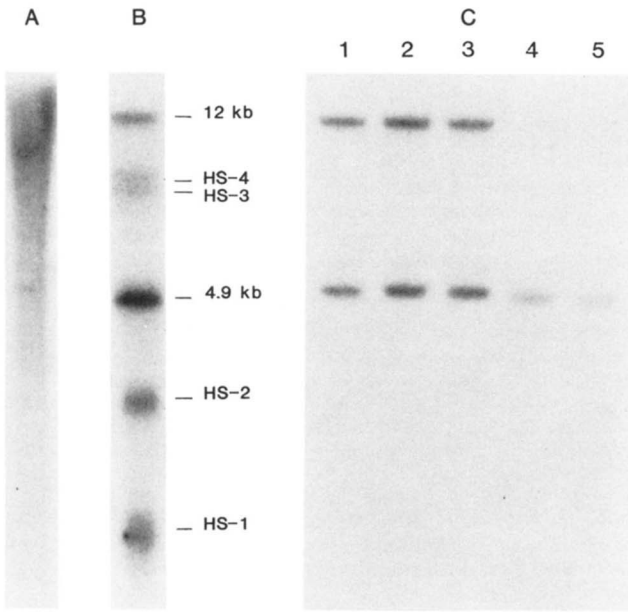

FIG. 2. **Southern gel analysis of euthyroid rat liver chromatin or naked DNA with DNase I.** *A*, euthyroid rat liver nuclei digested with 0.5 unit of DNase I/20 $A_{260\ nm}$ units. The digested DNA was not treated with *Eco*RI. The Southern blot is hybridized with the ^{32}P-labeled C2 probe. *B*, an aliquot of the DNase I-treated DNA sample in *A* was digested to completion with *Eco*RI, and the resultant products were separated by electrophoresis. The blot was hybridized with the C2 probe. *C*, naked euthryoid rat liver DNA dissolved in Buffer D was digested with DNase I at 5×10^{-5}, 5×10^{-4}, 2.5×10^{-3}, 5×10^{-3}, and 1×10^{-2} units/20 $A_{260\ nm}$ units in *lanes 1–5*, respectively. The blot was hybridized with the C2 probe.

Packaging of Spot 14 Gene in Chromatin Is Subject to Tissue-specific Factors—In liver and lactating mammary gland, mRNA-S14 is expressed at high levels and is regulated by T_3, whereas in brain, kidney, and spleen, mRNA-S14 is expressed at levels ≤10% of the values measured in liver, and T_3 regulation of this sequence is absent (4, 5). To determine whether the varying levels of mRNA-S14 expression could be attributed to organization of the gene in chromatin, we compared the pattern of DNase I-hypersensitive sites associated with the S14 gene in lactating mammary gland, kidney, spleen, and brain nuclei with that found in liver. Nuclei from these euthyroid tissues were digested with DNase I, and isolated DNA was digested with *Eco*RI, electrophoresed, blotted, and hybridized to the ^{32}P-labeled C2 probe as described in the legend to Fig. 1. Fig. 3 illustrates the pattern of hypersensitive sites in the various tissues. The pattern of hypersensitive sites found in lactating mammary gland differed from that found in liver. In the mammary gland, HS-1, HS-2, and HS-4 are present, but HS-3 is absent. Moreover, at least two additional sites were detected as indicated by the ladder-type effect (bands labeled X and Y, measuring 7.2 and 6.3 kb, respectively) above the 4.9-kb fragment. The size of these fragments suggests that these hypersensitive sites are also located at the 5′ end of the S14 gene.

The organization of the S14 gene in brain, spleen, and kidney nuclei also differs from that found in liver. Whereas HS-4 is present in these tissues, sites HS-1 and HS-3 are absent. Although HS-2 is also apparent in kidney, the level of DNase I required to detect this fragment is much greater than that found in liver and mammary gland. HS-1 was the only site found in all those tissues in which mRNA-S14 is expressed at high levels and is regulated by T_3.

Although not shown here, we have also examined the DNase I sensitivity of hepatic chromatin which contains the S14 gene in hypothyroid and hyperthyroid animals. As previously shown by Jump *et al.* (4), there is a 20-fold increase in the level of mRNA-S14 during the transition from hypothyroidism to hyperthyroidism. However, despite this, we failed to perceive perceptible differences in the DNase I digestibility of S14-containing chromatin. Furthermore, the pattern of digestion and the intensity of the resultant bands corresponding to HS-1 to HS-4 were not different among the three thyroidal states (hypo/eu/hyper).

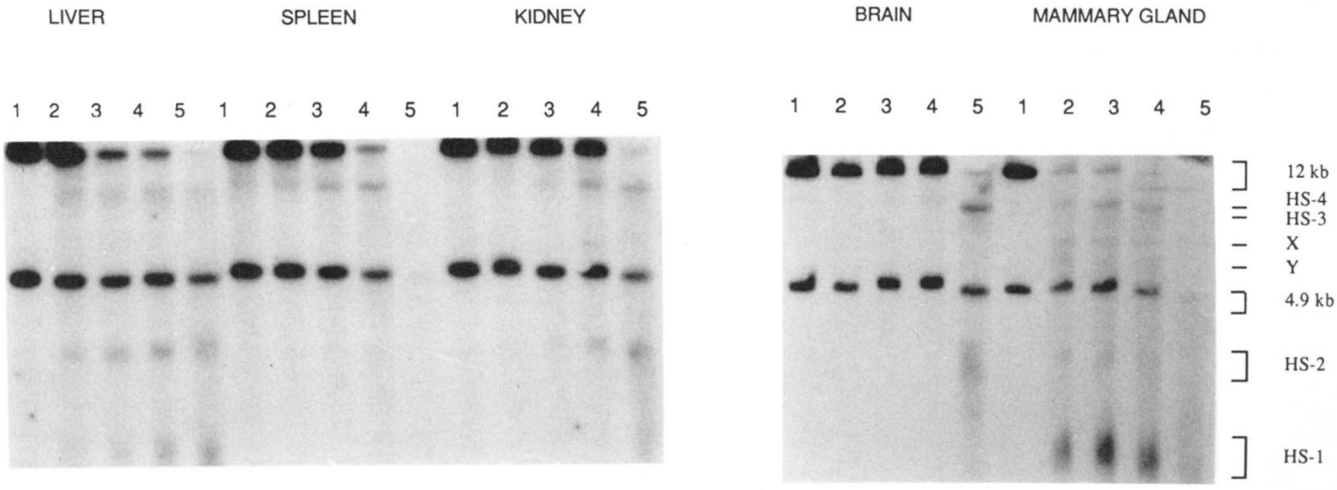

FIG. 3. **Tissue-specific DNase I-hypersensitive sites associated with S14 gene.** Nuclei from several rat tissues were digested with increasing concentrations of DNase I. The tissues include euthyroid liver, spleen, kidney, brain, and lactating mammary gland. Isolated nuclei were digested with DNase I at 0.0, 0.1, 0.25, 0.5, and 1.0 unit of DNase I/20 $A_{260\ nm}$ units. Isolated DNA was digested with *Eco*RI, electrophoresed, blotted, and hybridized with the C2 probe as described in the legend to Fig. 2. The positions of the hypersensitive sites (HS-1, HS-2, HS-3, X, Y, and HS-4) are indicated.

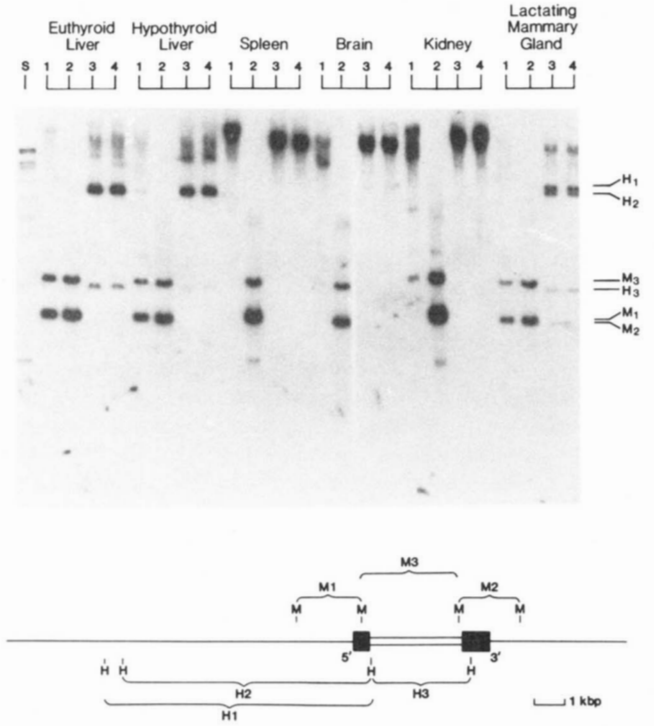

FIG. 4. **Tissue-specific methylation of S14 gene.** *Blot*, genomic DNA from euthyroid and hypothyroid liver, euthyroid brain, kidney, spleen, and mammary gland was digested with *Hpa*II (*lane 1*), *Msp*I (*lane 2*), *Hha*I (*lane 3*), and *Cfo*I (*lane 4*) and hybridized with the ^{32}P-labeled C2 probe. DNA standards were run in an adjacent lane (*S*). The various fragments generated by *Msp*I (M1, M2, and M3) and *Hha*I/*Cfo*I (H1, H2, and H3) are indicated. *Map*, *Hpa*II and *Msp*I (*M*) cut the S14 gene at four sites. The fragments generated and detectable using the ^{32}P-labeled C2 probe are indicated by M1, M2, and M3. Digestion of the palindromes CCGG by *Hpa*II is blocked by cytosine methylation, whereas *Msp*I digestion of this sequence is not blocked. *Hha*I and *Cfo*I (*H*) cuts the S14 gene at four sites. The fragments generated by *Hha*I/*Cfo*I are H1, H2, and H3. Digestion of the GCGC palindrome by either *Hha*I or *Cfo*I is blocked by cytosine methylation (26, 27).

Tissue-specific Methylation of Spot 14 Gene—We next examined the methylation state of the S14 gene. Our analysis is restricted only to those *Hpa*II/*Msp*I- and *Hha*I/*Cfo*I-generated fragments containing sequences homologous to the C2 probe. The *Hpa*II/*Msp*I isochizomeric enzyme pair has the identical recognition sequence except for the fact that *Hpa*II will only cut at the site if the cytosine residue is unmethylated. Consistent with the finding of others, we also noted that *Hha*I/*Cfo*I would not cut at the GCGC palindromic sequence when the cytosines were methylated (27, 28). Consequently, we were not able to estimate the degree of methylation of this palindrome as we have for the CCGG palindrome cut by the *Hpa*II/*Msp*I combination. Genomic DNA from liver, lactating mammary gland, kidney, brain, and spleen was digested to completion with each of the four restriction enzymes. The blots probed with the ^{32}P-labeled C2 probe are shown in Fig. 4. Fig. 4 also illustrates a restriction map and the identity of the fragments generated by these restriction enzymes. Cleavage by *Msp*I generated three fragments labeled M1, M2, and M3. Similarly, *Hha*I (*Cfo*I) digestion results in three fragments labeled H1, H2, and H3. Comparisons of the fragments excised by *Hpa*II and *Msp*I suggest that in both euthyroid (Fig. 4, *lanes 1* and *2*) and hypothyroid (*lanes 1* and *2*) rat liver, a major portion of *Hpa*II sites are unmethylated. In lactating mammary gland, as in liver, most *Hpa*II sites are undermethylated. In general, the degree of hypomethylation

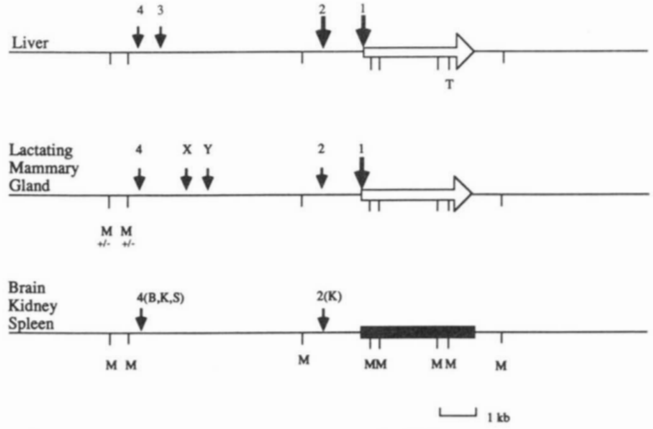

FIG. 5. **Summary of tissue-specific DNase I-hypersensitive sites and methylation sites.** The *open horizontal arrows* represent the transcriptionally active S14 in liver and lactating mammary gland. The direction of transcription is from left to right. The *closed horizontal box* represents the inactive S14 gene in brain, kidney, and spleen. The *thin horizontal line* represents untranscribed DNA flanking the S14 gene. The locations of the four DNase I-hypersensitive sites (HS-1, HS-2, HS-3, and HS-4) are designated by *vertical arrows* and are labeled *1–4*, respectively. The *thickness* of the *arrows* reflects the relative intensity of the various fragments shown in the autoradiograms (Figs. 2 and 3). The locations of the two additional hypersensitive sites flanking the S14 gene in lactating mammary gland nuclei are noted. The *letters* in *parentheses* adjacent to the *arrows* indicate which hypersensitive sites were found in: *B*, brain; *K*, kidney; and *S*, spleen. The eight DNA methylation sites are designated by *vertical lines* extending below the *horizontal lines* (see *map* in Fig. 4). Three states of methylation are noted at each site: fully methylated (*M*), partially methylated (*M* +/−), and unmethylated. The location of the *Hha*I site which is influenced by thyroid status is indicated in the liver map (*T*).

of CCGG correlated with high levels of mRNA-S14 expression.

Although the majority of *Hpa*II sites (Fig. 4, *lane 1* in all tissue) in DNA isolated from spleen, brain, and kidney are methylated, some tissue variation is evident. In spleen, the M3 site is fully methylated; whereas in kidney and lactating mammary gland, one of the sites bounding the M3 site is partially methylated. The *Hpa*II sites flanking the S14 gene which contribute to the generation of the M1 and M2 fragments are fully methylated in spleen, brain, and kidney. A minor fragment found in only spleen and kidney, migrating faster than M2, was not found in liver or mammary gland, nor was it found in digests of S14 genomic clones. The identity of this fragment is unknown.

Since digestion of the GCGC sequence by both *Hha*I and *Cfo*I is inhibited by methylation, we were not able to assess the degree of methylation at the various *Hha*I sites. However, we are able to draw the same correlation between methylation states at most *Hha*I sites and levels of mRNA-S14 expression as we found for the *Hpa*II sites. In our analysis, we did detect a significant and reproducible difference in the intensity of the H3 fragment excised from euthyroid and hypothyroid liver DNA. The two sites which define the H3 fragment are within the coding region of the gene. This finding suggests that thyroid status may influence the methylation state of the S14 gene. In lactating mammary gland, the degree of methylation at sites H1 and H2 which are more than 6 kb upstream from the 5′ end of the gene was found to vary with different preparations. These results may reflect heterogeneity of cell types characteristic of mammary gland. In contrast to the results in liver and mammary gland, all the *Hha*I sites are fully methylated in brain, kidney, and spleen. These results are summarized in Fig. 5.

DISCUSSION

In this report, we examined the organization of a 25-kb chromosomal domain containing the S14 gene. This is a single copy gene, and the cDNA probes employed in these studies hybridize only to the S14 gene under the stringency conditions used. Our results on the methylation pattern and the location of DNase I-hypersensitive sites associated with the S14 gene domain in several rat tissues are summarized in Fig. 5. In liver, four hypersensitive sites flank the 5′ end of the S14 gene. Eight potential DNA methylation sites either flank the gene or are located within it. No hypersensitive sites were detected within or flanking the 3′ end of the gene. In general, the presence of DNase I-hypersensitive sites at the 5′ end of the S14 gene and the hypomethylation of most *Hpa*II and *Hha*I sites correlate with high levels of mRNA-S14 expression in liver and lactating mammary gland. Conversely, the absence of the two DNase I-hypersensitive sites (*i.e.* HS-1 and HS-3) and the hypermethylation of the S14 genomic DNA correlate with low levels of mRNA-S14 in brain, spleen, and kidney. This pattern of DNase I hypersensitivity and DNA methylation is typical for many genes expressed in a tissue-specific manner (15–20).

The spread of the HS-1 band suggests that multiple sites are exposed to DNase I digestion within a 200–300-base pair region near the 5′ end of the gene. This area includes the TATA box (TAGAAT) which is located 27 bases upstream from the site of mRNA-S14 transcription initiation (26). Thus, the openness of this region in liver and the virtual absence of this site in nuclei of brain, kidney, and spleen suggest that this structural modification of chromatin may be required for the tissue-specific regulation of S14 gene expression. This contention is supported by the finding that HS-1 is detected in lactating mammary gland, a tissue in which mRNA-S14 is also expressed at high levels and regulated by T_3.

We anticipated a pattern of S14 chromatin digestion in mammary gland similar to that in liver based on the finding of high levels of mRNA-S14 expression in both tissues. Although sites HS-1, HS-2, and HS-4 were detected, HS-3 was absent. Moreover, at least two additional sites were detected 7.2 and 6.3 kb (labeled X and Y in Fig. 3) from the S14 cap site. We cannot ascribe these results to any one cell type since lactating mammary gland has a more complex cellular composition than does liver (29). Our results could reflect the fact that only a small fraction of the cells in the gland express mRNA-S14. In addition to the sites close to the S14 gene, we detected two sites (HS-3 and HS-4) located 7 and 8 kb upstream from the 5′ end of the hepatic S14 gene. Others (30) have reported that hypersensitive sites are not always associated with the expressed genes. Using a subcloned portion of genomic DNA derived from a genomic library (26), we found that no DNA sequences are expressed as mRNA in the region from −4500 bp to the 5′ cap site for S14 (results not shown). If HS-3 and HS-4 are not associated with the S14 gene, then the lack of expression of the sequences between the −4500 bp to the cap site of mRNA-S14 suggests that these hypersensitive sites may not be associated with an actively expressed gene. The data do not, however, exclude the possibility that these two sites may indeed be associated with the S14 gene, despite the distance separating them from the site of S14 gene transcription. Alternatively, these sites may mark the 5′ boundary of a gene that is transcribed in a direction opposite to S14. The diverse pattern of hypersensitive sites and methylation state found in the 12-kb fragment indicate that the chromatin structure within this region is significantly influenced by tissue-specific factors. Clearly, the significance of these structural changes on S14 gene expression will require additional study.

Several recent studies have shown that changes in chromatin structure at hypersensitive sites are associated with gene activation. Burch and Weintraub (31) previously reported that estrogen induced additional sites at the 5′ end of the vitellogenin gene in chicken liver and suggested the estradiol receptor may interact with chromatin at this site. Recently, rapid changes in DNase I hypersensitivity were detected following glucocorticoid administration to cells containing a portion of the murine mammary tumor virus long terminal repeat (32), a region previously found to bind glucocorticoid receptors *in vitro* (33). In the case of the hepatic S14-containing chromatin, T_3 does not appear to alter the DNase I sensitivity pattern despite a marked increase in the expression of this gene. This stands in sharp contrast to the recent findings of Nyborg and Spindler (34) which showed that T_3 leads to an increase in the DNase I sensitivity of the growth hormone gene.

The methylation studies in this report have examined the DNA within the S14 gene as well as the 5′- and 3′-flanking regions. In general, most methylation sites with the sequence CCGG or GCGC are hypomethylated in liver and lactating mammary gland and hypermethylated in brain, kidney, and spleen within this region. In other studies, methylation of sites (*Hha*I) which extend 15 kb downstream from the 3′ end of the S14 gene shows a similar pattern of methylation. This pattern of methylation for the S14 gene domain is more typical of the pattern found for tissue-specific gene expression than is the pattern for the expression of "housekeeping" genes (35). Housekeeping genes display a 5′ hypomethylation and 3′ hypermethylation, whereas tissue-specific genes are typically hypomethylated in the 5′- and 3′-flanking regions (19, 35, 36).

The finding that identical patterns of cytosine methylation were seen at all *Hpa*II/*Msp*I sites associated with the S14 gene in both hypo- and euthyroid liver suggested that these sites were not affected by thyroid status. In contrast, the generation of the H3 fragment by *Hha*I/*Cfo*I is influenced by thyroid status (Fig. 4, *lanes 3* and *4*) for euthyroid and hypothyroid liver). According to the *Hha*I restriction map, the GCGC site which defines the 3′ end of the H1/H2 fragment is the same one which gives rise to the 5′ end of the H3 fragment. Since there does not appear to be a difference in the relative amount of the H1/H2 fragment in either hypo- or euthyroid animals, the degree of methylation at these sites is probably not different in the various thyroid states. This implies that the GCGC site at the 3′ end of the H3 fragment is the site which changes with thyroid status. This site is designated by *T* in Fig. 5. It is generally assumed that for genes to undergo demethylation, ongoing DNA synthesis is required (18, 37). The demethylation of the H3 site in the transition from hypothyroidism to euthyroidism may therefore reflect stimulation of DNA synthesis by T_3. Studies are presently underway to determine whether T_3 can induce the demethylation of this site in the absence of DNA synthesis. We are also interested in defining the relationship of this modification in DNA structure to the hormonal induction of S14 gene expression.

Finally, our studies have demonstrated differences in the structure of S14 chromatin from various rat tissues. These differences likely play a role in the tissue-specific expression of the S14 gene. In addition, the DNA methylation pattern of this gene indicates that cytosine demethylation at sites examined is associated with increased gene expression.

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