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The Mitochondrial Uncoupling Protein Gene in Brown Fat: Correlation between DNase I Hypersensitivity and Expression in Transgenic Mice

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The mitochondrial uncoupling protein gene is rapidly induced in mouse brown fat following cold exposure. To identify *cis*-regulatory elements, approximately 50 kb of chromatin surrounding the uncoupling protein gene was examined for its hypersensitivity to DNase I. Seven DNase I-hypersensitive sites were identified in the 5′-flanking DNA, and one site was identified in the 3′-flanking DNA. Transgenic mice with an uncoupling protein minigene were generated by microinjection of fertilized eggs with a transgene containing 3 kb of 5′-flanking DNA and 0.3 kb of 3′-flanking DNA. Expression of the transgene is restricted to brown fat and is cold inducible. Four additional transgenic lines were generated with a second transgene containing a 1.8-kb deletion in the 5′-flanking DNA, and expression of this minigene is absent in all tissues analyzed. A DNase I-hypersensitive site located in the 1.8-kb deletion contains a cyclic AMP response element that binds a brown fat tumor enriched nuclear factor. On the basis of these observations, we propose that a *cis*-acting regulatory sequence between −3 and −1.2 kb of the 5′-flanking region, possibly at a DNase I-hypersensitive site, is required for controlling uncoupling protein expression in vivo.

Brown fat, through a mechanism called nonshivering thermogenesis, is an important site for heat production during birth, cold acclimation, and arousal from hibernation in mammals. This process is initiated from signals originating in the hypothalamus and propagated through the sympathetic nervous system, resulting in the release of norepinephrine from the sympathetic nerve terminals at the surface of the adipocyte. Norepinephrine released from the sympathetic nerve terminals binds to adrenergic receptors on the brown fat cell. Adrenergic stimulation leads to an increase in intracellular cyclic AMP concentration, brown fat cell lipolysis, increased intracellular free fatty acid concentration, and the rapid induction of the mitochondrial uncoupling protein (3, 18, 27, 34, 40).

The uncoupling protein (UCP) is a 32,000-M_r inner mitochondrial membrane protein that functions as a proton channel to uncouple mitochondrial respiration from ATP synthesis and produce heat (34). *Ucp* is a nuclear gene on mouse chromosome 8 containing six exons, each encoding a transmembrane domain (28, 29). The promoter region has a TATA box and sequence resembling a cyclic AMP response element.

We are pursuing an analysis of two problems associated with *Ucp* expression. One problem is to explain how the expression of *Ucp* is limited to brown fat, since it has been suggested that brown fat and white fat have similar developmental origins (2, 12, 48). The other problem is to determine how gene expression is controlled by β-adrenergic stimulation. The role of the β-adrenergic system in the control of metabolism and physiology is well known (31); however, its action on gene expression is just beginning to be investigated (22, 26).

DNase I-hypersensitive (HS) sites represent gaps in the distribution of nucleosomes along the chromatin fiber that reveal the location of regulatory motifs (13). *trans*-acting factors may interact with regulatory DNA sequence elements through the developmental positioning of HS sites along the chromatin fiber (13, 20). Clusters of DNase I-HS sites are often found at the 5′ end of tissue-specific and inducible genes and are associated with transcriptional start sites (5) as well as enhancers and promoters (13, 20). We have compared the pattern of DNase I-HS sites flanking the *Ucp* gene in several tissues to identify potential regions of open chromatin that may interact with *trans*-acting factors. Transgenic mice were generated to define the *Ucp* regulatory domain in vivo and to localize potential *cis*-acting regulatory regions within the flanking DNA.

MATERIALS AND METHODS

Animals. Inbred strains of mice were obtained from the Animal Resources of The Jackson Laboratory. (The Jackson Laboratory is fully accredited by the American Association of Accreditation of Laboratory Animal Care.) Mice were housed at an ambient temperature of 21°C on a 12-h light-dark cycle. For cold-induction experiments, mice were maintained at 27°C in a thermostatically controlled chamber for 3 days and then transferred to 5°C in a walk-in cold room for 18 h.

***Ucp* minigene constructs.** Gene regions used in subcloning the *Ucp* minigenes were derived from overlapping genomic clones described previously (29). The *Ucp*-1 minigene was constructed by isolating a 6.2-kb *Eco*RI-*Sac*I fragment which contained 3.7 kb of 5′-flanking DNA and the first two exons of *Ucp* (see Fig. 5). *Not*I linkers were blunt end ligated onto the free ends, the fragments were digested with *Not*I, and the products were ligated into the *Not*I site of pBluescript SK+ (Stratagene). Secondly, a 1.9-kb *Sac*I-*Hin*dIII fragment containing 300 bp of 3′-flanking DNA and exon 6 were cut out of a *Hin*dIII genomic clone, *Hin*dIII linkers were blunt end ligated onto the *Sac*I end, and the fragment was inserted into the *Hin*dIII site of pBluescript SK+. The final *Ucp*-1 minigene was then cut out with *Sph*I and *Xho*I

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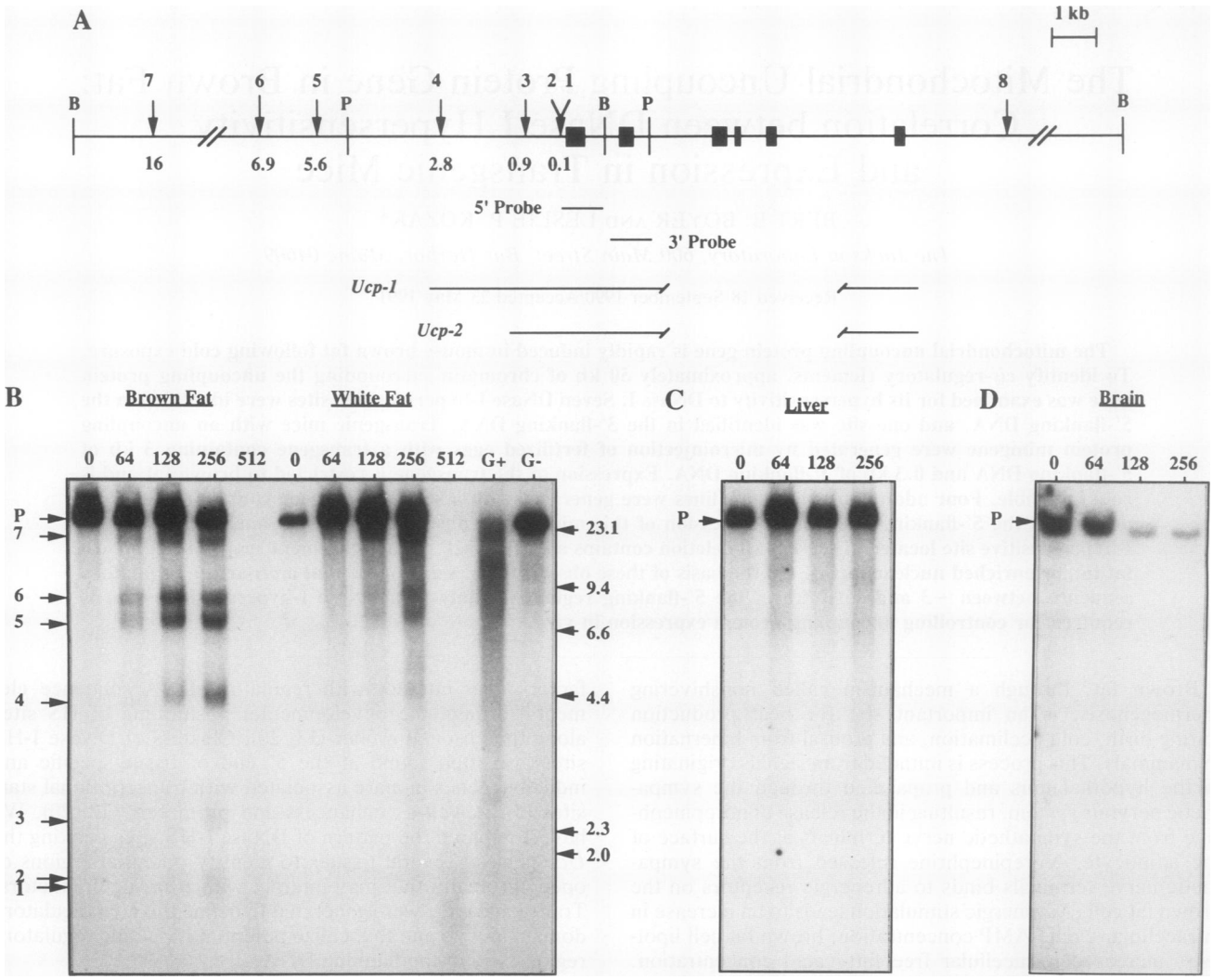

FIG. 1. (A) DNase I-HS site map. Numbered arrows denote the locations of the eight HS sites. The number under each DNase I-HS site indicates the distance (in kilobases) of the site from the first exon. Restriction enzyme sites (B, *Bam*HI; P, *Pvu*II) are indicated. The gels show the pattern of DNase I-HS sites upstream of the *Ucp* gene in brown and white fat (B), liver (C), and brain (D). Following DNase I digestion and DNA purification, 20 μg of DNA was digested with a 10-fold excess of *Bam*HI, electrophoresed in 1% agarose gels, transferred to Zetabind, and hybridized to a *Kpn*I-*Pst*I restriction fragment probe (5′ probe) as described in Materials and Methods. DNase I concentrations (in units per milliliter) are indicated above each lane of the autoradiograph. Control incubations with protein free genomic spleen DNA in the presence (G+) or absence (G−) of 2 U of DNase I per ml are also indicated. The 23-kb upstream parent band (P) represents the size of the *Bam*HI restriction fragment.

and contained 3 kb of 5′-flanking DNA and 300 bp of 3′-flanking DNA, as well as 49 bp of the multiple cloning site of pBluescript SK+ (see Fig. 5). *Ucp*-2 was identical to *Ucp*-1 except that the 3-kb 5′-flanking region was reduced to 1.2 kb by cutting the 5′ end of the minigene in pBluescript with *Sna*BI instead of *Sph*I.

Transgenic animals. The minigene constructs were purified by preparative electrophoresis through agarose gels followed by either extraction using the Geneclean DNA purification system (Bio 101, Inc., La Jolla, Calif.) or electroelution. The isolation of zygotes from C57BL/6J females, microinjection of the minigene constructs into male pronuclei, and transfer of the morulae to pseudopregnant (C57BL/6J × SJL/J)F_1 hybrid females were carried out by the Jackson Laboratory transgenic mouse facility using methods described previously (47). Founder animals were screened for the presence of the transgene by Southern blot analysis after digestion with *Bam*HI or *Pst*I (23, 43). Hybridization was to a gel-purified, random primer-labeled (Boehringer) 1.4-kb *Pst*I-*Hin*dIII fragment which contained the first and second exons of the mouse *Ucp* gene. Filters were washed with 0.1× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (final stringency) at 60°C. Total RNA was isolated (7), and Northern RNA blots were performed as described by Derman et al. (9) by using a 320-bp *Bgl*II restriction fragment derived from the first exon of the *Ucp* gene and radiolabeled with random primers (14). Northern blots demonstrating tissue-specific expression of the *Ucp* gene were reprobed with a *Bam*HI-*Eco*RI mouse 18S rRNA restriction fragment. Transgene copy number was estimated by densitometric analysis and radioanalytic imaging (Ambis Systems, San Diego, Calif.).

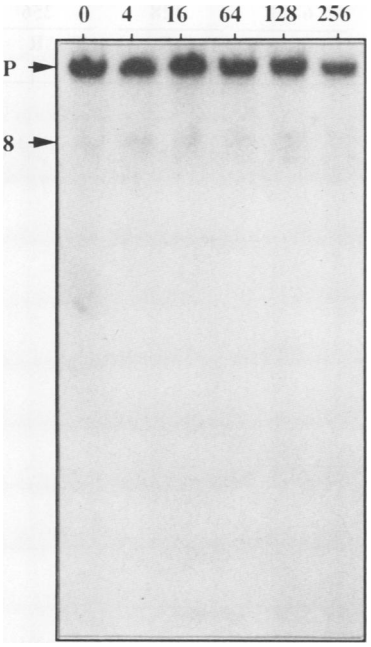

FIG. 2. Pattern of DNase I-HS sites downstream of the *Ucp* gene in brown fat. Conditions are identical to those described in the legend to Fig. 1 except that the DNA was hybridized to a *Bam*HI-*Hin*dIII restriction fragment 3′ of the *Bam*HI restriction enzyme site (3′ probe). The 30-kb downstream parent band representing the *Bam*HI restriction fragment is indicated (P).

Nucleus isolation. Nuclei were isolated from 6-week-old male BALB/cByJ mice housed at room temperature. Brown fat was isolated from the interscapular fat pad, and white fat was isolated from the epididymal fat pad. Tissues were removed and placed into ice-cold isolation buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM 2-mercaptoethanol, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2 mM EDTA, and 0.5% Nonidet P-40 (Sigma). Tissues were minced with scissors and homogenized with a polytron for 10 s at 10% maximum power. The resulting slurry was homogenized further with three strokes in a Dounce homogenizer (B pestle). Following homogenization, the Nonidet P-40 concentration was increased to 1% final concentration and the nuclei were pelleted and washed as described previously (11).

DNase I digestion. Nuclease digestions were performed on ice for 10 min as described by Fritton et al. (17). Following digestion with DNase I (Worthington Biochemical, Freehold, N.J.), nuclei were pelleted for 1 min at 2,000 × *g*. The supernatant was discarded, and the pellet was washed two times with DNase I wash buffer (0.15 mM spermine, 0.5 mM spermidine, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 15 mM Tris-HCl [pH 7.4], 0.5 M sucrose, and 1 mM phenylmethylsulfonyl fluoride). Resuspended nuclei were incubated in 200 μl of proteinase K buffer (1% sodium dodecyl sulfate, 20 mM Tris-HCl [pH 7.4], and 10 mM EDTA), containing proteinase K (Boehringer) at 100 μg/ml at 55°C for 15 h. Proteinase K was removed by phenol-chloroform extraction and ethanol precipitation. The DNA was resuspended in 10 mM Tris-HCl (pH 7.4)–1 mM EDTA. Restriction enzyme reactions were carried out under the reaction conditions suggested by the manufacturer (New England Biolabs, Beverly, Mass.).

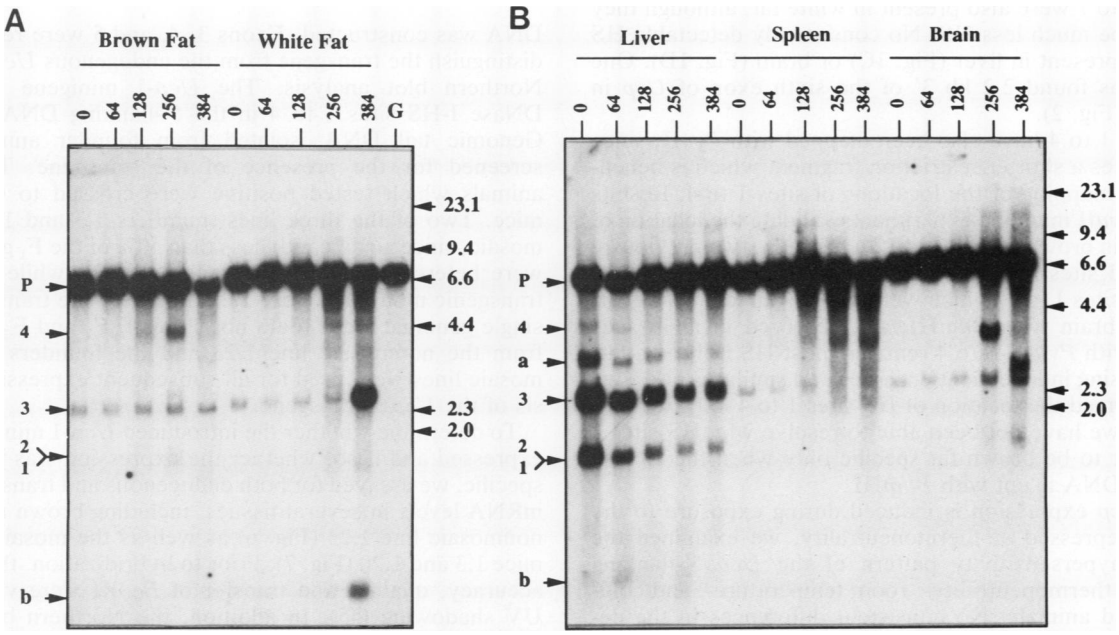

FIG. 3. Pattern of DNase I-HS sites 1 to 4 upstream of the *Ucp* gene in brown and white fat (A) and liver, spleen, and brain (B). Conditions for DNase I digestion and DNA purification are identical to those described in the legend to Fig. 1, except that the DNA was digested with a 10-fold excess of *Pvu*II. The Southern-blotted DNA was hybridized to the same *Kpn*I-*Pst*I restriction fragment probe as in Fig. 1. DNase I concentrations (in units per milliliter) are indicated above each lane of the autoradiograph. The 6.8-kb upstream parent band (P) represents the size of the *Pvu*II restriction fragment.

Nuclear extracts and gel shift assays. Nuclei were pelleted as described for the DNase I digestion, except phenylmethylsulfonyl fluoride was included to a final concentration of 0.5 mM. Following the initial pelleting of nuclei in the 500 × *g* spin, nuclear extract was prepared exactly as described elsewhere (19). Small aliquots of extract were frozen in liquid nitrogen for future use. Gel shift assays were performed as described by Carthew et al. (6) with slight modifications. Nuclear extract was incubated with approximately 40,000 cpm (0.1 to 0.5 ng) of an end-labeled single-stranded 30-bp oligonucleotide DNA fragment in the presence of 0.5 μg of poly(dI-dC) in a final volume of 20 μl. Incubations were carried out for 1 h on ice in 12 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.9)–60 mM KCl–5 mM $MgCl_2$–4 mM Tris-HCl (pH 8.0)–0.6 mM EDTA–0.6 mM dithiothreitol. At the end of the incubation period, 2 μl of loading buffer (25% Ficoll, 0.05% bromophenol blue, and 0.05% xylene cyanol) was added to each sample. Samples were layered onto low-ionic-strength 6% nondenaturing polyacrylamide gels and electrophoresed as previously described (6).

RESULTS

DNase I-HS sites. To identify the locations of potential *cis*-acting regulatory elements, we surveyed the *Ucp* chromatin domain in brown fat, white fat, liver, and brain for DNase I-HS sites unique to brown fat. Nucleic were digested with low concentrations of DNase I so that a selected region of DNA bounded by a particular restriction enzyme site would be cut less than one time on the average. All nuclease digestions were performed on ice to reduce endogenous nuclease activity. *Bam*HI was initially used to cut the DNase I-digested DNA because approximately 50 kb of chromatin surrounding the *Ucp* gene could be surveyed for HS sites. Seven DNase I-HS sites were found 5′ to the first exon of the *Ucp* gene in brown fat (Fig. 1A and B). DNase I HS sites 4 to 7 were also present in white fat, although they appear to be much less HS. No consistently detectable HS sites were present in liver (Fig. 1C) or brain (Fig. 1D). One HS site was found 2.2 kb 3′ of the sixth exon of *Ucp* in brown fat (Fig. 2).

HS sites 1 to 4 have also been mapped with *Pvu*II, since this produces a shorter restriction fragment which is beneficial in fine mapping of the locations of sites 1 to 4. Results from the *Pvu*II mapping experiments validate the location of sites 1 to 4 in brown fat (Fig. 3A). In addition, two previously unidentified sites (a and b) are present in several tissues. However, sites 1 to 3, which were undetectable in white fat, liver, and brain with *Bam*HI, are observed in all tissues analyzed with *Pvu*II. Site 4 remains most HS in brown fat. Thus, by using independent enzymes and similar probes, we have confirmed the location of HS sites 1 to 4 in brown fat. However, we have not been able to resolve why HS sites 1 to 3 appear to be brown fat specific only when the DNase I-digested DNA is cut with *Bam*HI.

Since *Ucp* expression is induced during exposure to the cold and repressed at thermoneutrality, we examined the DNase I hypersensitivity pattern of the *Ucp* 5′-flanking DNA from thermoneutrality-, room temperature-, and cold-acclimatized animals. No consistent differences in the degree of hypersensitivity or pattern of HS sites were observed in the DNA obtained from animals acclimated to thermoneutrality, room temperature, or the cold (Fig. 4).

***Ucp*-1 transgenic animals.** A *Ucp*-1 minigene (Fig. 5) containing 3 kb of 5′-flanking DNA and 300 bp of 3′-flanking

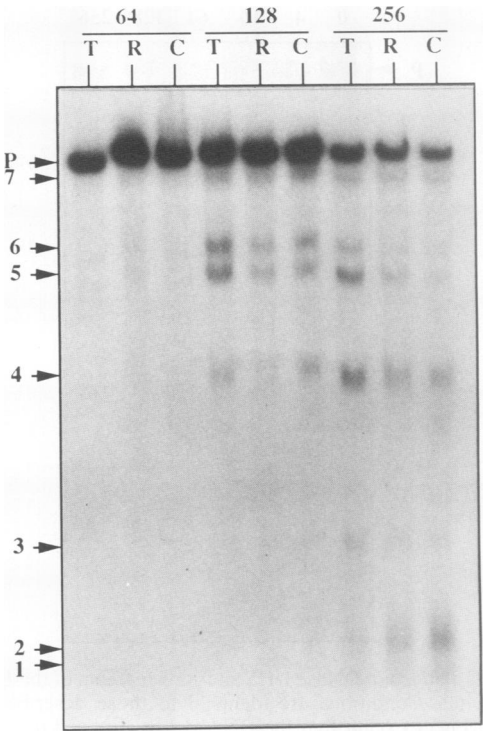

FIG. 4. Effect of temperature on DNase I hypersensitivity upstream of the *Ucp* gene. Nuclei were isolated from animals housed at a thermoneutral temperature of 29°C for 3 days (T) or at room temperature (R) or from animals housed at thermoneutrality for 3 days and then placed in the cold at 5°C for 12 h (C). Conditions for the DNase I digestion, DNA purification, restriction enzyme digestion, and DNA hybridization are identical to those for Fig. 1; DNase I concentrations are indicated above the lanes in units per milliliter.

DNA was constructed. Exons 3, 4, and 5 were removed to distinguish the transgene from the endogenous *Ucp* gene by Northern blot analysis. The *Ucp*-1 minigene contained DNase I-HS sites 1 to 4 in the 5′-flanking DNA (Fig. 1). Genomic tail DNA isolated from founder animals was screened for the presence of the transgene. The three animals which tested positive were crossed to C57BL/6J mice. Two of the three lines (numbers 1.3 and 1.20) were mosaic, since significantly less than 50% of the F_1 generation were heterozygous for the transgene (32), while the third transgenic mouse (number 1.23) inherited the transgene at a single genomic locus (data not shown). F_1 and F_2 offspring from the nonmosaic line 1.23 and the founders from the mosaic lines were used for all subsequent expression analysis of the *Ucp*-1 transgene.

To determine whether the introduced *Ucp*-1 minigene was expressed and if so, whether the expression was brown fat specific, we assayed for both endogenous and transgene *Ucp* mRNA levels in several tissues, including brown fat, of the nonmosaic line 1.23 (Fig. 6) as well as the mosaic founder mice 1.3 and 1.20 (Fig. 7). Prior to hybridization, the loading accuracy, quality, and transfer of *Eco*RI were verified by UV shadowing (45). In addition, the Northern blots were reprobed with a mouse 18S rRNA probe (*Bam*HI-*Eco*RI fragment) to document any variations in loading accuracy. The major and minor mRNA species detected for the endogenous *Ucp* gene result from the use of two poly(A) signal sequences in vivo (29). A minor mRNA species for the

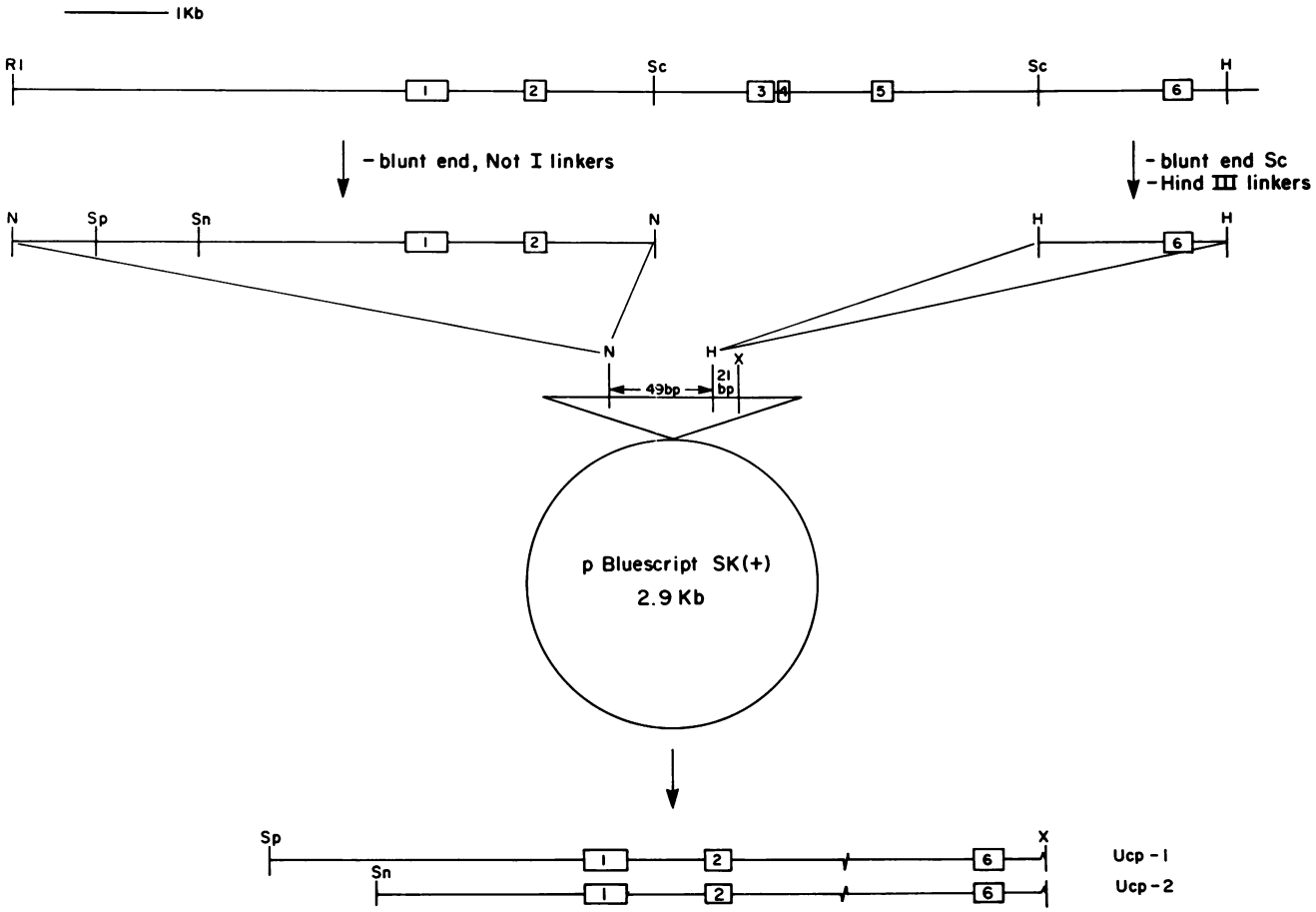

FIG. 5. Subcloning and structure of the *Ucp*-1 and *Ucp*-2 minigenes. Shown are the *Ucp* gene with 3.7 kb of 5′-flanking sequence and 300 bp of 3′-flanking DNA. Forty-nine base pairs of pBluescript SK(+) multiple cloning site was incorporated into both *Ucp*-1 and *Ucp*-2 between exons two and six and contains a *Bam*HI and *Pst*I restriction enzyme site used for screening transgenic animals. The *Xho*I site located at the 3′ end of both minigenes is also derived from 21 bp of the multiple cloning site of pBluescript. Restriction enzyme sites: RI, *Eco*RI; Sc, *Sac*I; H, *Hin*dIII; N, *Not*I; Sp, *Sph*I; X, *Xho*I; Sn, *Sna*BI.

minigene may have been obscured by the endogenous gene transcript. Expression of the transgene was specific to brown fat in line 1.23 (nonmosaic; Fig. 6) and one of the mosaic founders (line 1.20; Fig. 7). We were unable to detect expression of the *Ucp*-1 minigene in any tissue in line 1.3 by this analysis. Having determined that there were approximately six copies of the *Ucp*-1 minigene in line 1.23 (data not shown), the actual quantitative level of transgene expression appears to be less than that of the endogenous gene.

To determine whether the *Ucp*-1 minigene was cold inducible, both positive and negative transgenic animals from line 1.23 were cold and thermoneutrality acclimatized and mRNA was isolated from brown fat for Northern analysis. In the thermoneutrality-acclimatized animals, both the *Ucp*-1 minigene and the endogenous *Ucp* gene mRNA are repressed to extremely low levels, while both the *Ucp*-1 minigene and the endogenous *Ucp* gene in cold-acclimatized animals are highly induced (Fig. 8). These data indicate that the regulatory elements for both tissue-specific and cold-inducible expression of *Ucp* are present in the *Ucp*-1 minigene.

***Ucp*-2 transgenic animals.** To further define the *Ucp* regulatory regions in vivo, we deleted 1.8 kb from the 5′ end of *Ucp*-1 and microinjected this minigene deletion construct (*Ucp*-2, Fig. 5) into fertilized mouse eggs. The *Ucp*-2 minigene contained DNase I-HS sites 1 to 3, while *Ucp*-1 contained sites 1 to 4. Four different transgenic lines tested positive for the presence of the *Ucp*-2 minigene. The tissue specificity and cold inducibility of *Ucp*-2 mRNA were examined by cold acclimatizing positive F_1 transgenic offspring as described previously for *Ucp*-1 screening. Expression of the *Ucp*-2 transgene deletion construct could not be detected in cold-acclimatized transgenic mouse brown fat in any of the four lines (Fig. 9) or in any of the other normally analyzed tissues (data not shown), suggesting that a regulatory element was deleted from this transgene. The copy numbers for lines 2.3, 2.5, 2.14, and 2.28 were approximately 7, 30, 5, and 5 copies, respectively.

DNase I-HS site 4. Since the deletion of HS site 4 from the *Ucp*-2 transgene resulted in a loss of expression, we thought that there may be a brown-fat-specific *trans*-acting factor interacting at this site. We sequenced the region surrounding HS site 4 and discovered a partial consensus sequence to the cyclic AMP response element (CRE). To determine whether brown fat nuclear proteins bound to this CRE, a 30-bp oligonucleotide containing the CRE was synthesized, labeled, and used in a gel shift assay (Fig. 10, oligo 323). A single complex was formed (Fig. 11) in extract obtained from

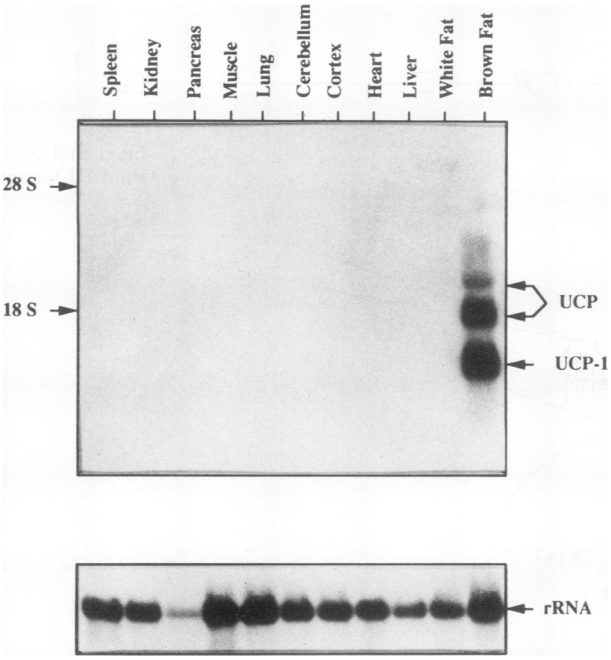

FIG. 6. Northern blot analysis of mouse *Ucp*-1.23 minigene mRNA expression in homozygous cold-adapted transgenic animals. Ten micrograms of total RNA was resolved by electrophoresis in 1.25% formaldehyde-agarose gels. Hybridization was performed as described in Materials and Methods.

liver and a brown fat tumor known to be responsive to norepinephrine (30). Because of the difficulty of obtaining nuclear extract from normal brown fat, nuclear extracts were obtained from brown fat tumor cells having a high level of *Ucp* mRNA (30) and DNase I hypersensitivity pattern identical to that of brown fat (4). The sequence specificity of the binding reaction is demonstrated by the ability of nonradioactive oligonucleotide to compete for binding (lanes 3 to 5 and 7 to 9), while poly(dI-dC), a nonspecific competitor, does not reduce the binding (compare lanes 9 and 10). Although the relative amount of binding protein in the brown fat tumor appears to be much greater than in liver, it is not clear whether this nuclear binding protein is as abundant in brown fat as in the tumor.

To demonstrate that the nuclear binding protein interacts with the CRE, an additional gel shift assay was performed with the intact CRE as the probe (Fig. 10, oligo 323) and a 15-bp oligonucleotide that contained the first 4 bp of the CRE (Fig. 10, oligo 356) as the nonradioactive competitor. The single complex formed with the liver and brown fat tumor nuclear extract is reduced, although much less efficiently by the 15-bp oligonucleotide 356, indicating sequence specificity for binding of the nuclear protein to the CRE (Fig. 12).

DISCUSSION

DNase I-HS sites of the *Ucp* gene. HS sites have helped to define transcriptional regulatory elements for several genes, including the β-globin (16, 46), immunoglobulin κ (8, 36), and phosphoenolpyruvate carboxykinase (24, 25) genes and the proto-oncogene c-*fos* (10, 39). Although the presence of cell type-specific HS sites does not always correlate with *cis*-regulatory elements, a *cis* element that affects a chromosomal process will be associated with an HS site (13, 20, 35). In fact, it has been proposed that promoters and enhancers come into contact through interactions between *trans*-acting factors bound at HS sites by looping out the intervening chromatin (37). Accordingly, we analyzed approximately 50 kb of chromatin flanking the mouse *Ucp* gene in brown fat, white fat, liver, spleen, and brain for its hypersensitivity to DNase I.

The four most distal 5′ HS sites (DNase I-HS sites 4 to 7) have been detected in brown fat and white fat but not in liver or brain. Three HS sites closest to the *Ucp* transcription start site (sites 1 to 3) have been mapped in four tissues with two restriction enzymes and appear to be present in all tissues. Sites 1 and 2 are probably associated with proteins that bind to the CAAT box and TATA box, as DNase I-HS sites are frequently associated with these elements (5, 17). Site 3 is HS in all tissues when the DNA is cut with *Pvu*II, while site 4 remains strongly HS only in brown fat regardless of the restriction enzyme used to cut the DNA.

Although DNase I-HS sites have been shown to be specific to genes being actively transcribed (44), DNase I-HS sites can be found in nonexpressing, DNase I-resistant regions of chromatin (15). Therefore, the presence of DNase I-HS sites 4 to 7 when mapped with *Bam*HI and sites 1 to 4 when mapped with *Pvu*II in white fat chromatin may not be so surprising, even though no *Ucp* mRNA can be detected in RNA isolated from white fat by Northern blot analysis (Fig. 6). In fact, the presence of these HS sites in white fat chromatin may indicate that a common cell lineage exists for these two cell types or that the white fat cell was able to transcribe the *Ucp* gene at an earlier developmental stage.

DNase I-HS sites 1 and 2 span a region of chromatin-containing sites homologous to thyroid, glucocorticoid, and cyclic AMP-regulatory elements as well as TATA box- and CAAT box-binding protein sites (3, 29). These HS sites were predicted, since most *cis*-acting elements this close to the start site bind nuclear factors which participate in transcriptional complex stabilization (1). The only DNase I-HS site detected in 25 kb of 3′-flanking DNA was located 2.2 kb 3′ of the sixth exon.

Eight DNase I-HS sites have been identified in the flanking DNA surrounding the *Ucp* gene in brown fat nuclei. Two restriction enzymes which generate large and small parental fragments have been utilized in this analysis to map the brown fat HS sites. Although we are unable to resolve the discrepancy in HS site tissue specificity using these two restriction enzymes, the combined results corroborate the location of the brown fat HS sites. Furthermore, we have used these mapping results to begin a systematic analysis for potential *trans*-acting factors that may interact with *cis* elements at each of the HS sites.

Experiments were also carried out to determine whether any changes in the degree of hypersensitivity or pattern of hypersensitivity occur during the induction or repression of *Ucp* expression. The lack of any significant changes in hypersensitivity suggests that these DNase I-HS sites may be necessary for gene activation and function as windows at which inducible *trans*-acting factors can interact with *Ucp*.

Expression of the *Ucp*-1 transgene in brown fat. Having identified a structural chromatin domain surrounding the *Ucp* gene in mouse brown fat by its hypersensitivity to DNase I, a functional assay was necessary to elucidate potential regulatory elements required for in vivo expression of *Ucp*. As there are no continuous brown fat cell culture systems, transgenic mice were used to delineate the *cis*-acting requirements for expression of the *Ucp* gene in the

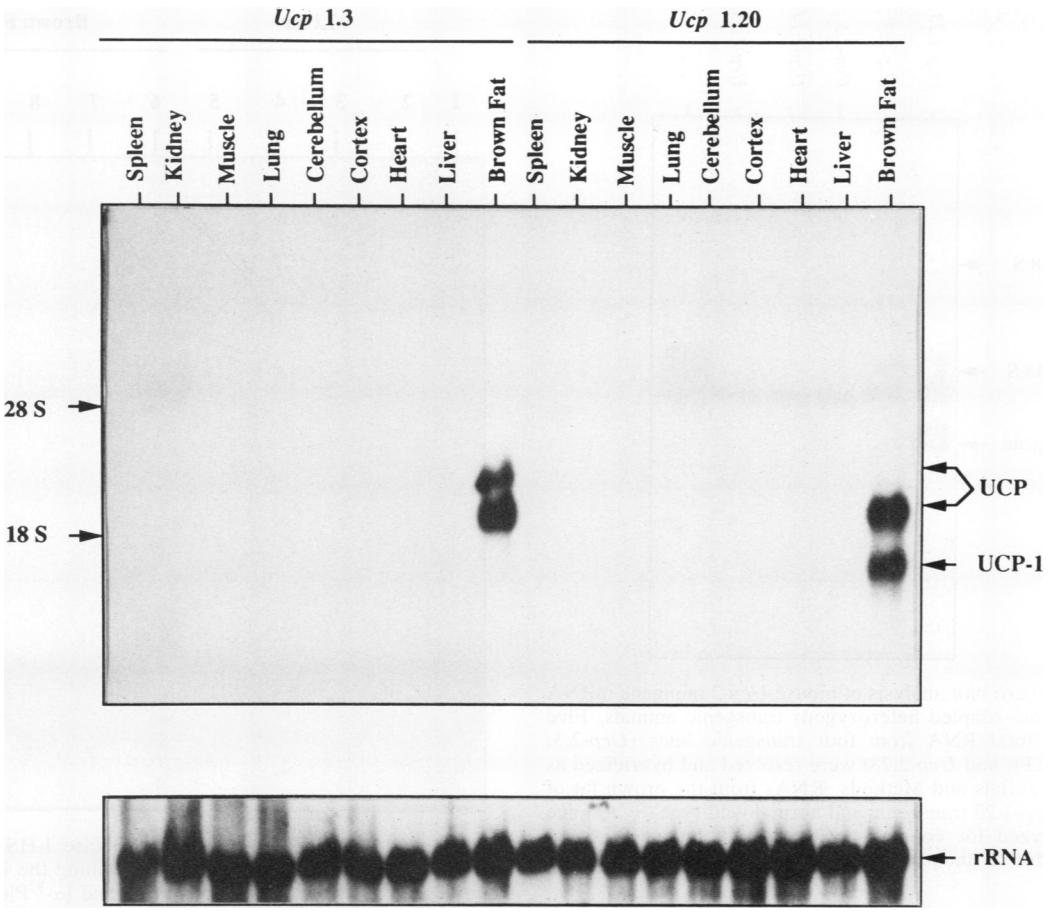

FIG. 7. Northern blot analysis of mouse *Ucp*-1.3 and -1.20 minigene mRNA expression in cold-adapted founder transgenic animals. Ten micrograms of total RNA was resolved by electrophoresis in 1.25% formaldehyde-agarose gels. Hybridization was performed as described in Materials and Methods.

mouse. Transgenic mice are an ideal genetic tool for determining the temporal and spatial control of inducible mammalian gene expression, since the tissue-specific expression of transgenes is often independent of the integration site.

We show that sequences within the *Ucp*-1 minigene are sufficient to direct brown fat-specific expression and cold induction of *Ucp* in vivo. Quantitatively, expression of the *Ucp*-1 minigene appears less than that of the endogenous *Ucp* gene. Similarly, Grosveld et al. (21) demonstrated that high levels of human β-globin transgene expression are obtained only when erythroid-specific super-HS sites located 50 kb 5′ and 20 kb 3′ of the β-globin gene are included. Additional experiments demonstrated that a single 5′ super-HS site located far upstream of the β-globin gene was responsible for the enhanced β-globin transgene expression (41), while two of these 5′ super-HS sites could stimulate high levels of human α-globin gene expression in erythroid cells of transgenic mice (42). Since DNase I-HS sites 5 to 7 were not present in the *Ucp*-1 minigene where both tissue-specific and cold-inducible expression are maintained, it is conceivable that full quantitative expression of a *Ucp* transgene will require additional upstream sequence associated with these DNase I-HS sites.

Loss of a *Ucp* regulatory element in the *Ucp*-2 transgene. Our results indicate that a *cis*-acting regulatory region within the *Ucp* gene 5′-flanking sequence between −3,000 and

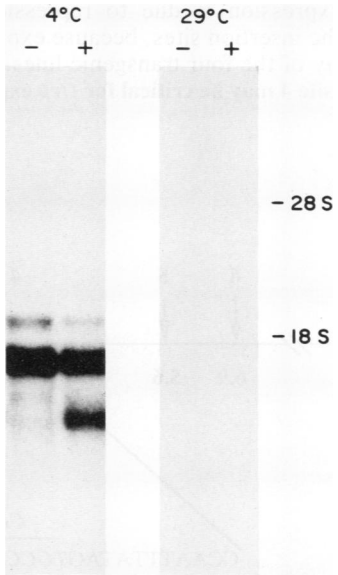

FIG. 8. Northern blot analysis of cold-adapted versus thermoneutrality-adapted brown fat mRNA expression from *Ucp*-1.23 transgenic animals. Temperature acclimation was as described in Materials and Methods. Positive F_1 transgenic animals (+) and negative wild-type F_1 animals (−) are indicated. Two micrograms of total RNA was loaded per lane and hybridized as described in Materials and Methods.

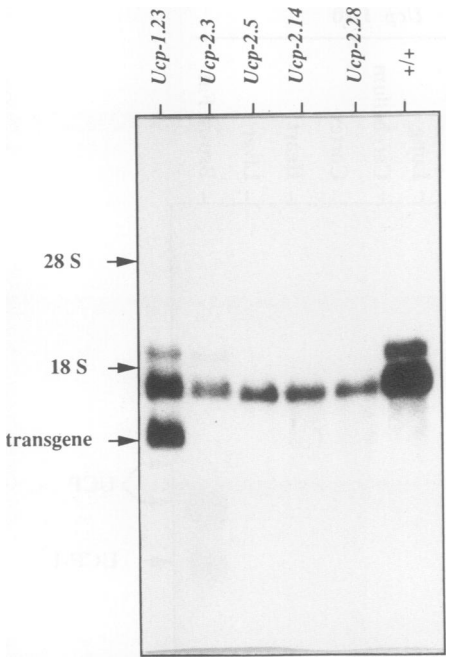

FIG. 9. Northern blot analysis of mouse *Ucp*-2 minigene mRNA expression in cold-adapted heterozygous transgenic animals. Five micrograms of total RNA from four transgenic lines (*Ucp*-2.3, *Ucp*-2.5, *Ucp*-2.14, and *Ucp*-2.28) were resolved and hybridized as described in Materials and Methods. RNAs from the brown fat of cold-adapted *Ucp*-1.23 transgenic and normal wild-type (+/+) mice were also analyzed for comparison. Large differences in signal intensity were due to unequal loading of the gel.

−1,200 bp is essential for expression, since deletion of this 1.8-kb fragment from the *Ucp*-1 minigene results in the loss of detectable *Ucp*-2 transgene expression. It is unlikely that the lack of expression is due to repression by flanking sequences in the insertion sites, because expression was not observed in any of the four transgenic lines.

DNase I-HS site 4 may be critical for *Ucp* expression in vivo.

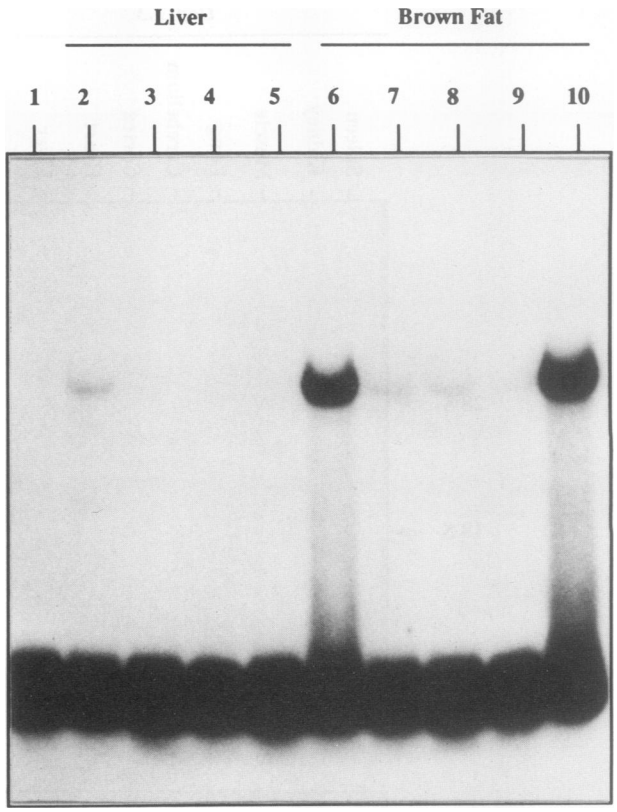

FIG. 11. DNA-binding activity at DNase I-HS site 4. Single-stranded deoxyoligonucleotide 323 containing the intact CRE was end labeled with polynucleotide kinase and [α-^{32}P]dATP and incubated with nuclear extract obtained from liver or brown fat tumor. The binding reaction was loaded on a 6% nondenaturing polyacrylamide gel to resolve the bound and retained DNA as described in Materials and Methods. Lane 1 contained labeled probe only. Lanes 2 to 10 contain 0.5 μg of protein and 1 μg of poly(dI-dC). Competition for binding is indicated in lanes 3 to 5 and 7 to 9 that contain 25, 50, or 100 ng, respectively, of unlabeled oligonucleotide 323. Lane 10 contains 100 additional ng of poly(dI-dC) instead of excess cold oligonucleotide 323.

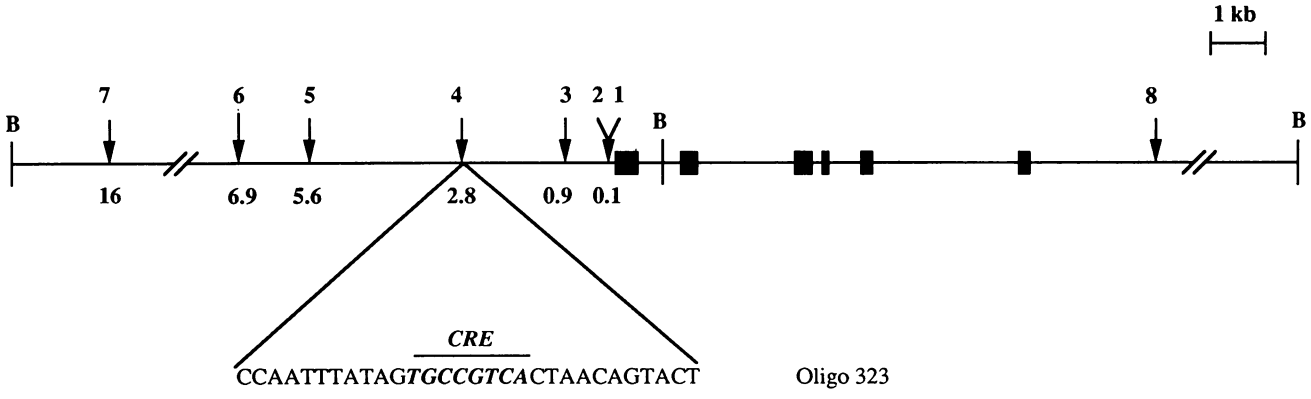

FIG. 10. Oligonucleotide sequence of the CRE at DNase I-HS site 4. The CRE is indicated in italics. Oligonucleotide 323, which contains the intact CRE, was used as a probe and competitor in the gel shift assays. Oligonucleotide 356, which contains only half of the intact CRE, was used as competitor DNA only.

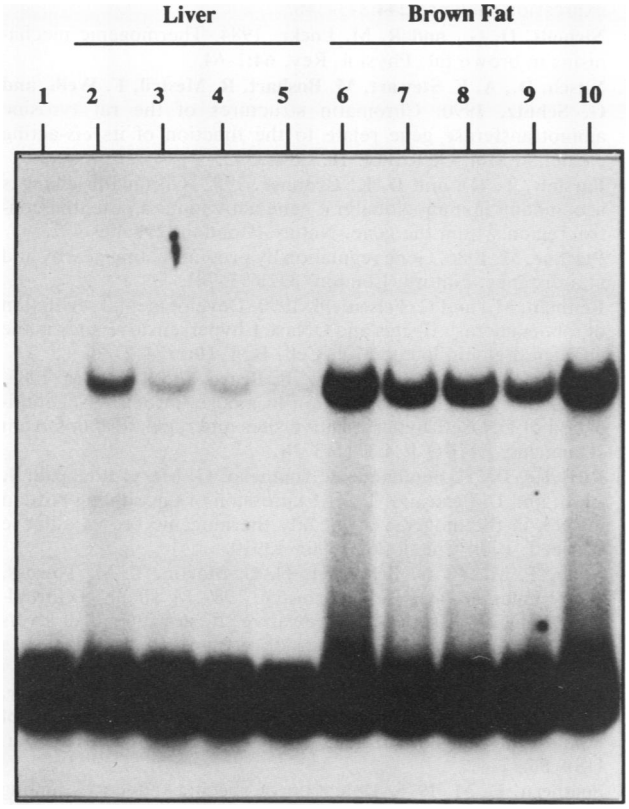

FIG. 12. Competition for DNA-binding activity to the CRE at DNase I-HS site four with oligonucleotide 356. Conditions for DNA-binding activity were identical to those in Fig. 11 except that oligonucleotide 356, containing a partial CRE consensus sequence, was used as the unlabeled competitor DNA. Competition for binding is indicated in lanes 3 to 5 and 7 to 9 that contain 25, 50, or 100 ng, respectively, of unlabeled oligonucleotide 356. Lane 10 contains 100 ng of additional poly(dI-dC) instead of excess cold oligonucleotide 356.

Although the quantitative degree of hypersensitivity can be subjective (4, 38), DNase I-HS site 4 is consistently more HS in brown fat than in any other tissue, regardless of the restriction enzyme used in mapping (4). Furthermore, the deletion of 1.8 kb from the *Ucp*-1 minigene, which includes DNase I-HS site 4, results in the loss of expression of the *Ucp*-2 transgene. This raised the possibility that DNase I-HS site 4 may be involved in the expression of *Ucp* in vivo, possibly through an interaction with a brown fat-specific binding protein.

A CRE has been identified at DNase I-HS site 4, approximately 2.8 kb upstream of the start site of *Ucp* transcription. Gel shift analysis using a single-stranded probe containing the CRE revealed that a *trans*-acting factor binds specifically to this *cis* element. These data support our hypothesis that the *Ucp* HS sites function as access points for DNA-protein interactions. Experiments aimed at determining the tissue specificity and inducibility of this DNA-binding protein are in progress. In addition, it is also unclear whether posttranslational modification via the protein kinase A pathway effects binding of this DNA-binding protein in vivo.

Although additional *cis* elements may be located closer to the transcription start site (at DNase I-HS sites 1 and 2), as is the case for liver-specific (1, 33) and pancreas-specific (32) genes, we suggest that the CRE located at DNase I-HS site 4 and deleted in the *Ucp*-2 transgene may be important for *Ucp* expression in vivo. Through the use of transgenic animals and structural chromatin analysis, we hope to further our understanding of the regulation of *Ucp* gene expression in vivo and provide a foundation for future studies aimed at understanding the sympathetic signalling mechanism regulating tissue-specific gene expression in brown fat.

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