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Identification of Multiple Regulatory Elements of Human L-Histidine Decarboxylase Gene

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Previously, we reported the structure of human L-histidine decarboxylase gene. To identify the regions that regulate the tissue-specific expression of HDC, we constructed a fusion DNA with the 5′-flanking region from −1003 to +99 of the HDC gene and chloramphenicol acetyltransferase (CAT) gene, which was then transfected into human basophilic leukemia KU-812-F cells or human epithelial carcinoma HeLa cells. The 1102 bp DNA fragment stimulated the CAT activity in KU-812-F cells, but not in HeLa cells. CAT analysis with a series of 5′-deletion constructs of the HDC-CAT gene revealed the existence of two positive and one negative regulatory elements at −855 to −841 and −532 to −497 and −829 to −821, respectively. Sequence analysis showed a nuclear factor c-Myb binding motif, TAACTG, at position −520. Gel mobility shift analysis showed that the nuclear extract of KU-812-F cells, but not that of HeLa cells, contains a factor which can bind to this motif. These results suggest that the 5′-flanking region of the HDC gene contains multiple regulatory elements for HDC gene expression and that at least one element, including a c-Myb binding motif, is responsible for the tissue-specific expression of HDC.

Key words: c-Myb binding motif, human basophilic leukemia cells, human L-histidine decarboxylase gene, nuclear factor, regulatory element.

L-Histidine decarboxylase (HDC [EC 4.1.1.22]) specifically catalyzes the formation of histamine from L-histidine. Histamine plays important roles in various physiological and pathological reactions such as inflammation, immediate-type allergy, gastric acid secretion, and neurotransmission in the central nervous system (*1, 2*). HDC has been reported to be present in a limited number of tissues and cells, such as mast cells and basophils in blood, enterochromaffin-like cells in the stomach, and histaminergic neurons in the central nervous system (*3*).

Many groups, including our own, have characterized HDC proteins (*4-7*) and isolated their cDNAs (*8-11*) from tissues or cells. These studies have revealed that HDC activity is regulated at both transcriptional and translational levels. For instance, various types of stimulants were found to increase HDC activity with accompanying elevation of the HDC mRNA level (*12-14*). Moreover, primary translated HDC was posttranslationally processed at its C-terminus (*15-17*). In contrast to many reports on the regulation of HDC activity, little is known about the molecular mechanism regulating the tissue-specific expression of the HDC gene. Identification of *cis*-acting elements as well as *trans*-acting factors for the transcription of the HDC gene is of great importance for understanding the tissue-specific expression of the HDC gene, and will also facilitate elucidation of the ubiquitous mechanism that regulates the differentiation of various cells.

Previously we isolated genomic DNA clones containing the complete sequence of the human HDC gene and revealed that the HDC gene is composed of 12 exons spanning approximately 24 kb (*18*). In the present study, we analyzed the transcriptional activity of the region upstream from the first exon in the gene to clarify the mechanism(s) underlying the tissue-specific expression of the HDC gene.

EXPERIMENTAL PROCEDURES

Materials—A promoterless plasmid containing the CAT gene (pUC00cat) was obtained from the Japanese Cancer Research Resource Bank (Tokyo). Other materials were obtained from commercial sources, as follows; restriction endonucleases and the Klenow large fragment of *Escherichia coli* DNA polymerase I, Toyobo; 1-deoxy[dichloroacetyl-1-^{14}C]chloramphenicol (*19*), Amersham; [α-^{32}P]dCTP, Du Pont-New England Nuclear (Boston, MA); and acetyl CoA, Sigma Chemical (St. Louis, MO); Sheep polyclonal antibody to c-myb oncoprotein, Cambridge Research Biochemicals (London, UK). The sources of other materials are given in the text.

Cell Culture—The human epithelial carcinoma cell line HeLa S3 (SC) from RIKEN Cell Bank (Tsukuba) were cultured in minimal essential Eagle's medium supplement-

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Abbreviations: HDC, histidine decarboxylase; RSV, Rous sarcoma virus; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction.

ed with 10% (v/v) calf serum and 4 mM glutamine. The human basophilic leukemia cell line KU-812-F (*20*) cells were maintained in RPMI 1640 medium, containing 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) fetal bovine serum.

Preparation of CAT Constructs—The plasmid p1003cat was constructed by inserting a DNA fragment corresponding to the region from −1003 to +99 of the genomic sequence of HDC (*12*) into the *Sma*/*Sph*I site of pUC00cat. Other HDC-CAT fusion mutants, shown in Fig. 2, were constructed using PCR amplification with Vent DNA polymerase (New England Biolabs, Beverly, MA) and specifically designed primers. A series of deletion mutants (p921cat-p782cat) around the position of −850, shown in Fig. 5, was generated with exonuclease III/Mung-bean nuclease as described previously (*21*).

Transient Transfection Assay—Fusion plasmids (10 μg) together with pRSV-β-gal plasmid (5 μg) as an internal control to evaluate the transfection efficiency were transfected into KU-812-F cells or HeLa cells using a Gene Pulser electroporation apparatus (Bio Rad) at 300 V (960 μF) in 0.4 cm cuvettes. After 48 h, cell extracts were prepared for assays as described previously (*19, 22*).

Gel Mobility Shift Assay—Complementary oligonucleotides corresponding to regulatory elements were synthesized for use as probes in gel mobility shift assays. Their sequences are given in the text. Probes were annealed, and radiolabeled by filling-in the 5′-overhangs with Klenow fragment in the presence of [α-^{32}P]dCTP. For the binding reaction, 1-10 μg of nuclear extracts, prepared from KU-812-F and HeLa cells according to the procedure described by Schreiber *et al.* (*23*), was incubated with 3 fmol of α-^{32}P-labeled probe (10,000 cpm) in the presence of 2 μg of poly(dI-dC) as a nonspecific competitor at 30°C for 1 h. Specific competitors were added to each reaction prior to the labeled probes. Samples were analyzed on native 5% polyacrylamide gels in TGE buffer (50 mM Tris-HCl, pH 8.5, 380 mM glycine, and 2 mM EDTA) at 150 V for 2-3 h at 4°C. After electrophoresis, the gels were dried and autoradiographed, then analyzed using a Fujix BAS 2000 imaging analyzer.

RESULTS

5′-Flanking Region of Human HDC Gene Contains Information for Cell-Specific Expression—Previously, we isolated human genomic DNA clones encoding the HDC, and analyzed the 5′-flanking region of the gene (*18*). To examine whether this region contains elements responsible for HDC expression in KU-812-F cells, a 1,102 bp fragment (position −1003 to +99) was tested for its capacity to stimulate the transcription of a heterologous gene. The fragment was inserted upstream of the bacterial CAT gene in pUC00cat to generate the plasmid p1003cat (Fig. 1A). Transcription of the HDC-CAT fusion gene was demonstrated to be initiated at the HDC gene transcriptional start site (data not shown).

Figure 1B shows the result of CAT analysis with KU-812-F or HeLa cells. Plasmids pRSVcat and pUC00cat were used as positive and negative controls, respectively; the former contained the highly active RSV promoter, while the latter carried no eukaryotic promoter region. Plasmid p1003cat induced efficient expression of CAT activity in KU-812-F cells, but not in HeLa cells, although the RSV promoter was active in both cell lines.

The 1,102 bp Fragment Contains Multiple Regulatory Elements for HDC Expression—To analyze the regional function, a series of deletion mutants from −1003 to −371 bp was constructed. Figure 2 shows CAT activities in KU-812-F and HeLa cells, which were transfected with the deletion mutants of HDC-CAT fusion genes. In KU-812-F cells, the plasmids p1003cat, p782cat, p625cat, and

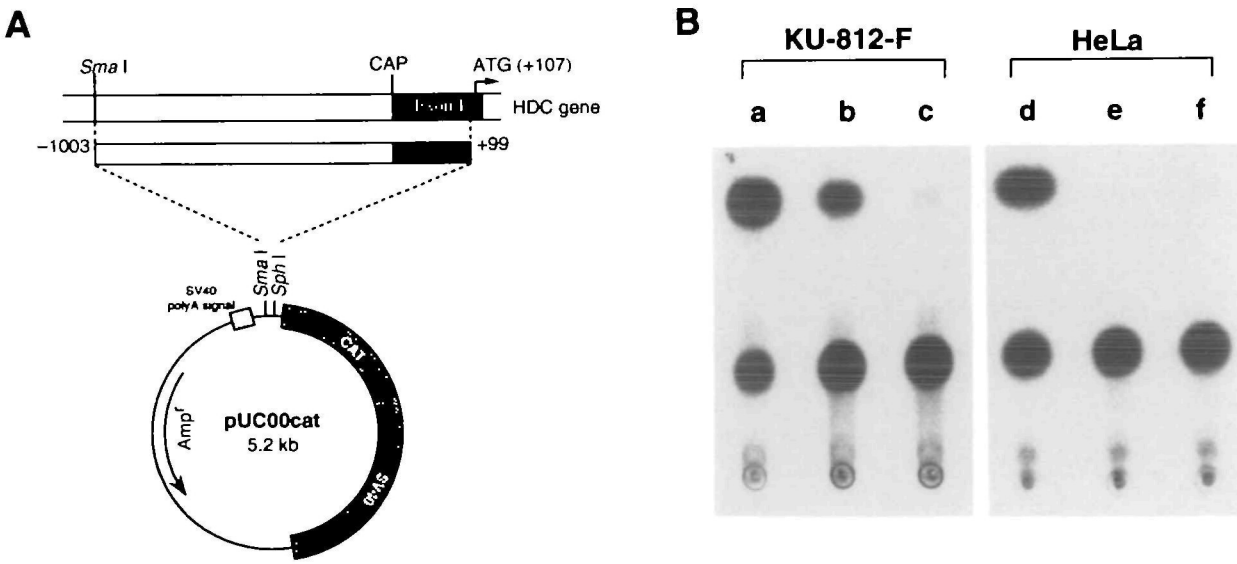

Fig. 1. **Transcriptional activity of the 5′-flanking region of the human HDC gene in KU-812-F and HeLa cells.** (A) A genomic DNA fragment, corresponding to −1003 to +99 of the genomic sequence of HDC, was generated by PCR amplification. The plasmid p1003cat was constructed by inserting the 1,102 bp PCR fragment into the *Sma*I/*Sph*I site of the pUC00cat as described under "EXPERIMENTAL PROCEDURES." (B) KU-812-F cells and HeLa cells were transfected with pRSVcat (a, d), p1003cat (b, e), or pUC00cat (c, f), and CAT activity of these cells was assayed as described under "EXPERIMENTAL PROCEDURES."

p532cat induced significant expression of CAT activities, whereas plasmids p841cat, p497cat, and p371cat were ineffective. In HeLa cells, on the other hand, the CAT activity was not induced by any of the plasmids tested.

The Region from −532 to −497 bp Contains a Consensus Sequence for c-Myb Protein—Sequence analysis of the 5′-flanking region of the HDC gene revealed a v-Myb binding motif, PyAAC(T/G)G (*24*), and a c-Myb binding motif, (Pu/C)(Py/A)AACPyPu (*25*), at around position −520. To examine whether KU-812-F cells contain a DNA binding protein which can interact with the c-Myb binding site, oligonucleotides containing the c-Myb motif were synthesized (Fig. 3B) and used for gel shift analysis. When an oligomer HDC-myb (position −526 to −506) was incubated with a nuclear extract of KU-812-F cells, two major shifted bands were observed (Fig. 3A). The upper band completely disappeared when the nuclear extract was preincubated with a large excess of the unlabeled probe or an oligonucleotide corresponding to the c-Myb binding site (mim-1-A) present in the 5′-flanking region of the mim-1 gene (*26*). However, the upper band was only slightly weakened in the presence of oligonucleotide containing mutated Myb binding site (HDC-μmyb). The lower band was not removed by mim-1-A and HDC-μmyb, but was slightly weakened by oligomer HDC-myb. When oligomer HDC-myb was incubated with nuclear extract of HeLa cells, no shifted bands were detected (data not shown).

Supershift assay was performed with anti v-Myb antibody which raised against the v-Myb peptide corresponding to residues 280–298 of human c-Myb; this has no cross-reactivity with human A-Myb or B-Myb. It was found that Mim-1-A probe produced supershifted bands with the

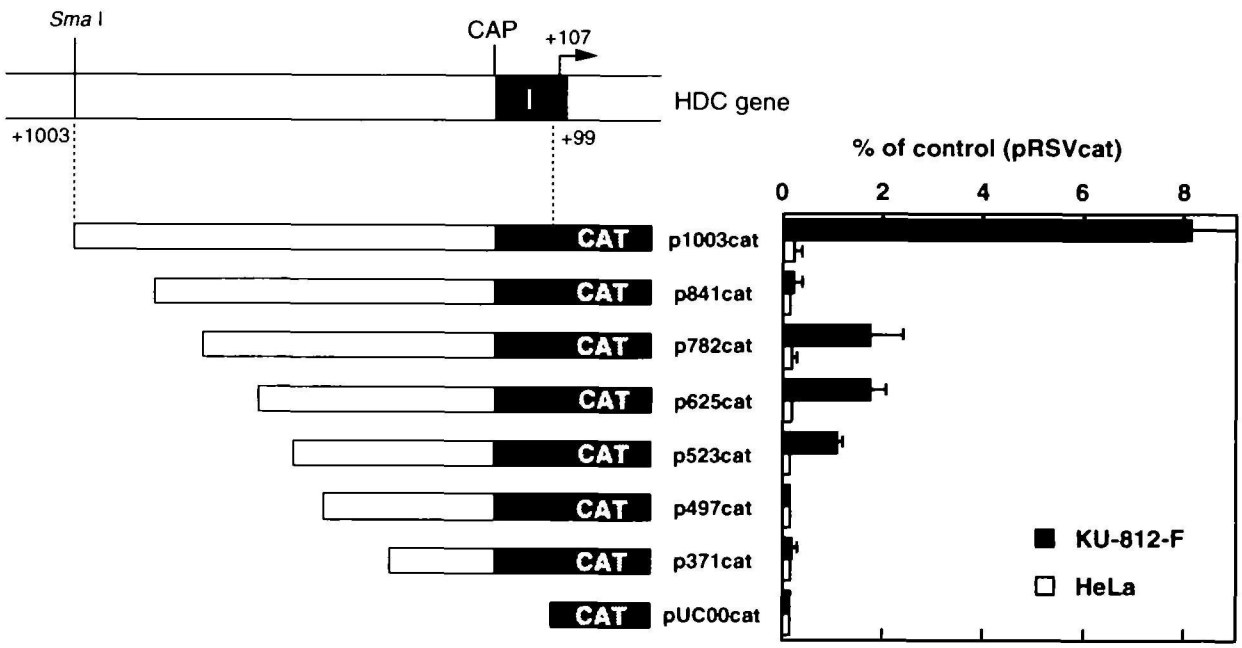

Fig. 2. **Transcriptional activity of a series of deletion mutants generated from p1003cat in KU-812-F and HeLa cells.** A series of 5′-deletion mutants derived from p1003cat was generated as described under "EXPERIMENTAL PROCEDURES." KU-812-F cells and HeLa cells were transfected with each mutant, and CAT activity of these cells was assayed as described under "EXPERIMENTAL PROCEDURES." Results were normalized using the β-galactosidase gene as an internal control and expressed as a percentage of the value produced by pRSVcat. The numbers given for each plasmid represent the length of the promoter region from the transcription initiation site. Thin lines show standard deviations ($n=3$).

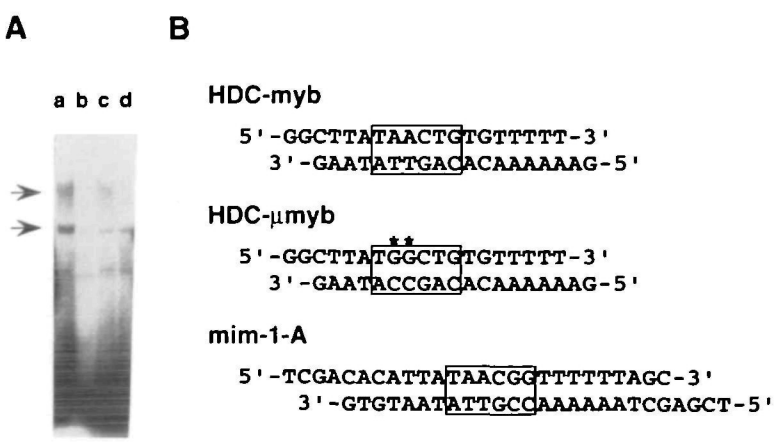

Fig. 3. **Gel mobility shift analysis with an HDC-myb probe.** (A) An end-labeled double-stranded oligonucleotide, corresponding to HDC-myb (position −526 to −506) was incubated with nuclear extracts of KU-812-F cells. Binding reactions were performed in the absence (a) or presence of a 100-fold excess of unlabeled HDC-myb (b), HDC-μmyb (c), or mim-1-A (d). (B) Sequences of the synthesized probes are shown. The boxed sequences show c-Myb binding sites. Asterisks indicate mutated nucleotides.

nuclear extract of KU-812F cells, but not with that of HeLa cells, and HDC-myb probe produced no shifted bands with either extract (data not shown).

When the putative myb binding site was deleted in the p1003cat construct [p1003(⊿myb)cat], the CAT activity was significantly decreased in KU-812-F cells (Fig. 4).

The Sequence around Position −840 Contains Positive and Negative Regulatory Elements—Since it was predicted from the results of CAT analysis shown in Fig. 2 that at least one positive and one negative regulatory element reside in the region between −1003 to −782, a series of detailed deletion mutants in this region was constructed (Fig. 5A). As shown in Fig. 5B, deletion mutants up to position −844 still showed significant CAT activity. However, when cells were transfected with p841cat or p829cat, the CAT activity was significantly diminished. The CAT activity was restored by further deletion of the promoter (p821cat and p782cat).

We next deleted the regions of interest from p1003cat construct and examined whether the same effects were observed. As shown in Fig. 6, p1003(⊿P)cat, which lacks the predicted positive element, showed no CAT activity. On the other hand, the CAT activity of p1003(⊿N)cat in which the predicted negative element was deleted was almost equal to that of p1003cat.

Nuclear Extracts of KU-812-F and HeLa Cells Contain Factors That Bind to the Positive and the Negative Regulatory Elements around Position −840—To characterize DNA binding factors that can bind to the positive and the negative regulatory elements around position −840, gel mobility shift analysis was performed. Four overlapping oligomers were synthesized for use as competitors to determine the respective binding sites in detail (Fig. 7A). When an oligomer containing the positive regulatory element (Probe A) was incubated with nuclear extract of KU-812-F cells, one major shifted band was observed. Nuclear extract of HeLa cells also produced one band, but

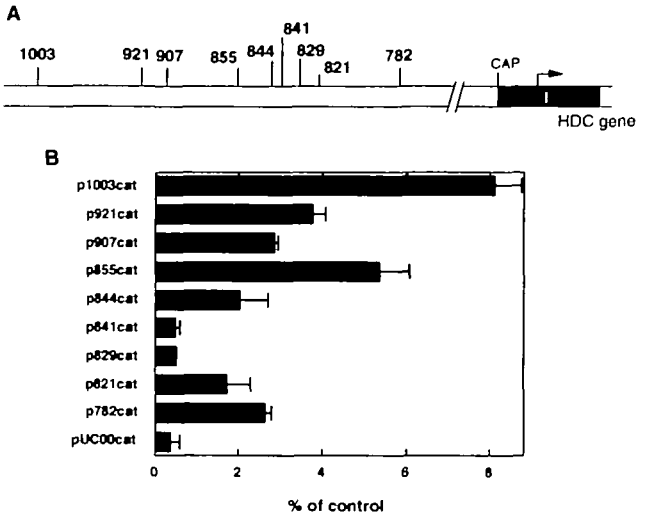

Fig. 5. **CAT analysis with a series of deletion mutants around position −840.** (A) A series of deletion mutants in the region between −1003 to −782 was constructed as described under "EXPERIMENTAL PROCEDURES." (B) CAT activity of KU-812-F cells which had been transfected with each mutant was assayed as described in Fig. 2. Results were normalized using the β-galactosidase gene as an internal control and expressed as a percentage of the value produced by pRSVcat. Thin lines show standard deviations ($n=3$).

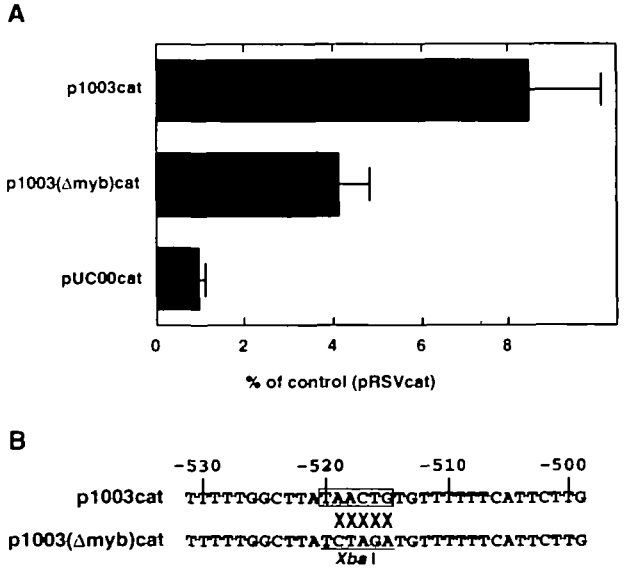

Fig. 4. **CAT analysis with a mutant which lacks c-myb binding site.** (A) The putative c-myb binding site was deleted from p1003cat as described in "EXPERIMENTAL PROCEDURES." CAT activity of KU-812-F cells which had been transfected with each mutant was assayed as described in Fig. 2. Results were normalized using the β-galactosidase gene as an internal control and expressed as a percentage of the value produced by pRSVcat. Thin lines show standard deviations ($n=3$). (B) Sequences of the mutated region are shown. The boxed sequence shows the c-myb binding site. "X" indicates a mutated nucleotide.

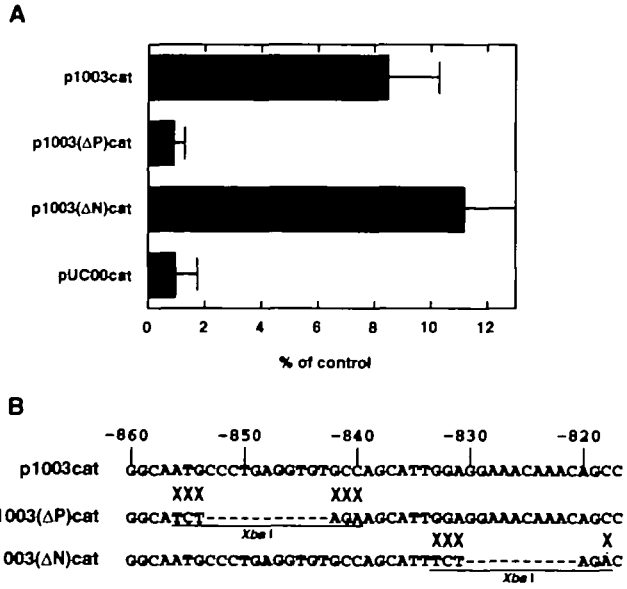

Fig. 6. **Transcriptional activity of mutants which lack positive or negative elements.** (A) Putative positive or negative elements were deleted from p1003cat as described under "EXPERIMENTAL PROCEDURES." Results were normalized using the β-galactosidase gene as an internal control and expressed as a percentage of the value produced by pRSVcat. Thin lines show standard deviations ($n=3$). (B) Sequences of the mutated regions are shown. "X" and "-" indicate mutated and deleted nucleotides, respectively.

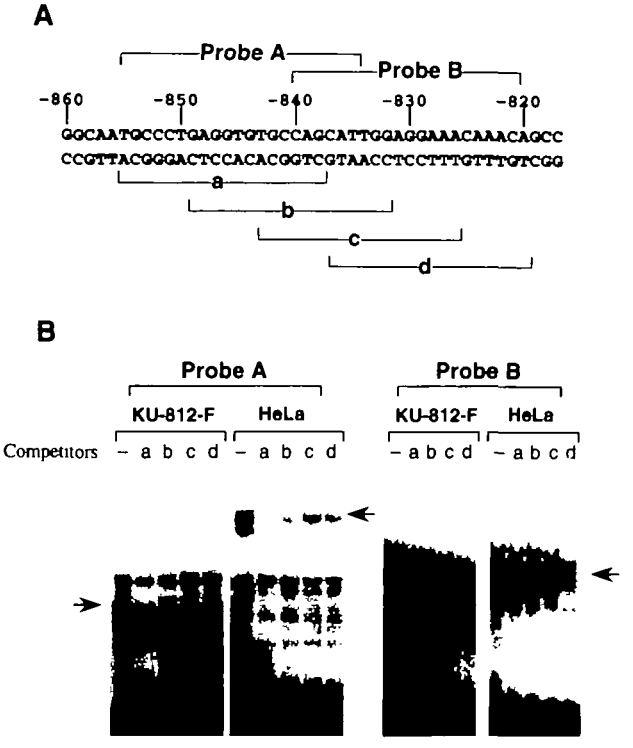

Fig. 7. **Gel mobility shift analysis with probes corresponding to the positive or negative elements.** (A) Annealed oligonucleotides corresponding to the predicted positive and negative regulatory elements, Probe A and Probe B, respectively, were end-labeled. (B) Nuclear extracts of KU-812-F or HeLa cells were mixed with the end-labeled probes A or B. Binding reactions were performed in the absence or presence of competitor probes (a, b, c, or d). Arrows indicate the major shifted bands which disappeared in the presence of excess unlabeled competitor.

its mobility was slower than that formed with the extract of KU-812-F cells. Both bands disappeared completely in the presence of excess unlabeled probe, competitor a, but not competitor b, c, or d. When an oligomer containing the negative regulatory element (Probe B) was used in the gel shift assay, nuclear extract of both cells produced one major band with the same mobility (Fig. 7B), and the band disappeared in the presence of excess unlabeled probe, competitor d, but not competitor a, b, or c.

DISCUSSION

Tissue-specific gene expression is controlled by a combination of ubiquitous *trans*-acting factors interacting with *cis*-acting elements within the promoter region, associated or not with a limited number of cell-specific nuclear proteins (*27*). The exact mechanisms by which these *cis*-acting complexes regulate transcription in a cell-specific manner are not yet understood. Identification and characterization of these factors are critical for understanding such mechanisms. The HDC gene is expressed in a limited number of cell lineages, such as enterochromaffin-like cells in the stomach, histaminergic neurons in the central nervous system, and mast cells and basophils within the hematopoietic system. In the present study, we analyzed the 5′-flanking region of this gene and showed that the region, within the first 1 kb upstream from the transcriptional start site, contains multiple *cis*-acting elements that direct a high level of expression of HDC in a human basophilic cell line, KU-812-F, and that some of the elements might be involved in the cell-specific expression of HDC.

CAT analysis with deletion mutants showed that the region from −1003 to −497 contains at least one negative and two positive regulatory elements for HDC expression. Detailed studies with a series of deletion mutants and the use of gel shift assays revealed that the positive elements reside in positions −855 to −841 and −532 to −497, whereas the negative element exists at position −829 to −821.

The positive element at position −532 to −497 contains a c-Myb binding motif, TAACTG (*25*), centered around nucleotide −520. Gel shift analysis showed that nuclear extract of KU-812-F cells, but not that of HeLa cells, contains a factor that can interact with this motif, suggesting that the c-Myb binding motif is involved in the tissue-specific expression of the HDC gene. Zobel *et al.* (*26*) demonstrated that the gene for F3-H, the protein with the highest binding affinity for the c-Myb protein, contains a palindromic sequence, AACNGTT, flanked on both sides by T clusters. The c-Myb binding site of the HDC gene (AACTGTG) has only one mismatch, and its flanking regions on both sides also contain T clusters.

In gel mobility shift assay, two major shifted bands were observed (Fig. 3A). The upper band but not the lower band was subjected to competition by mim-1-A. This result suggests that the upper band is likely to be a Myb-related protein. The lower band was slightly weakened by oligomer HDC-myb (Fig. 3A), but not by a small amount of competitor which was sufficient to compete out the upper band (data not shown).

The *c-myb* gene is the cellular progenitor of the viral oncogene *v-myb*, and the related genes *A-myb* and *B-myb* are also known (*28*). These *myb* family gene products act as transcription factors (*29-32*), which share the same DNA-binding sequence, PyAAC(C/T)G. It has been shown that the avian leukemia virus E26, which contains the *v-myb* gene, can transform basophilic and/or mast cell-like lineages when chicken bone marrow cells are infected (*33*). It is, thus, possible that the *myb* family of transcription factors participates in the differentiation of these cell lineages through the regulation of HDC gene expression. At present, since there is no available antibody specifically reacting to each of the Myb-related proteins, it remains to be identified which one is involved in the transcriptional regulation of the HDC gene.

Gel shift analysis with nuclear extracts of KU-812-F and HeLa cells (Fig. 7) suggested that the positive element at position −855 to −841, but not the negative element at position −829 to −821, is responsible for the tissue-specific expression of the HDC gene. Although the mechanism underlying the positive and negative regulation of the HDC gene expression through these adjacent elements is unknown, nuclear factors which bind to the respective elements might function by interacting with each other. Further studies are needed to clarify this point.

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