Intestinal Expression of the Calbindin-D9K Gene in Transgenic Mice

REQUIREMENT FOR A Cdx2-BINDING SITE IN A DISTAL ACTIVATOR REGION*

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The calbindin-D9K gene encodes a vitamin D-induced calcium-binding protein that is expressed as a marker of small intestine differentiation. We have shown that 4580 base pairs of its 5’ DNA regulatory region can target reporter transgene expression in the intestine and cause this transgene to respond like the endogenous gene to vitamin D active metabolite and that the homeoprotein Cdx2 is bound to the TATA box in the intestine. We now show that the 4580 base pairs construct confers a differentiated pattern of reporter transgene expression in the intestine and that cooperation between the proximal promoter and a distal element located in an opened chromatin structure is responsible for the intestinal expression and vitamin D responsiveness of the transgene. Gel shift and footprinting assays using duodenal nuclear extracts indicate that this distal element contains a Cdx2-binding site. Finally, a mutation in this distal Cdx2-binding site dramatically decreases intestinal expression in transgenic mice. This report, using an in vivo approach, demonstrates the crucial role of Cdx2 for the transcription of an intestinal gene.

The active cell proliferation and turnover of the intestinal epithelium makes this tissue a good experimental model for investigating the nature of regulatory networks that control events such as cell lineage commitment and differentiation. All mature epithelial cell types found in the adult intestine are derived from a nonmigratory stem cell pool located at the base of the crypts of Lieberkühn (see Ref. 1 for a review). The enterocytes are the most abundant cells in the intestine, accounting for approximately 95% of the mucosal cells of the small intestine. As the enterocyte precursors migrate from the crypts, they stop proliferating and acquire differentiated functions, governed by specific patterns of gene expression, that depend on the cell lineage, state of proliferation or differentiation, and spatial location along the crypt-villus and cephalo-caudal (duodenum/colon) axes. But the molecular basis of this diversity is still poorly understood. Determining the sequences involved in the regulation of genes expressed in a subset of intestinal epithelial cells is a first step toward defining these factors. Traber and co-workers (2) used this approach to identify Cdx2, a homeoprotein involved in the control of the sucrase-isomaltase gene. Cdx2 is one of the very few transcription factors that is active only in the adult intestinal epithelium (3), and it appears to be a key element controlling the differentiation of the intestinal epithelium (4–6).

The complexity of the architecture of the intestinal epithelium is such that transgenic mouse technology is a powerful tool for characterizing the complexity of the cis-acting elements involved in the regulation of intestinal gene transcription (for reviews, see Refs. 7 and 8).

We have used the calbindin-D9K (CaBP9K) gene as a marker of intestinal differentiation in studies on the regulatory elements involved in its intestine-specific pattern of expression. The CaBP9K gene is expressed only in the differentiated enterocytes of the proximal region of the small intestine (mainly in the duodenum) and the large intestine (mainly in the cecum) (9–11). We have shown that the CaBP9K gene expression is under positive transcriptional control of the vitamin D3 active metabolite, 1,25(OH)2D3, in the intestine (10, 12). We used a DNase I hypersensitivity analysis to define two potential regulatory regions (13). The proximal region contains a major intestine-specific DNase I HS,1 HS4, close to the promoter. In vitro footprinting and gel shift assays showed that a combination of an ubiquitous factor (NF1), liver-enriched factors (HNF1, C/EBP, and HNF4), and the Cdx2 homeoprotein bind to this region and may be important for controlling CaBP9K gene transcription in the intestine (14). The distal region, HS1, is a duodenum-specific HS located 3.5 kb upstream from the start site. We have recently shown by transient transfection assays (14), as well as by creating different transgenic mouse lines (15), that 9K/14580 sequences of the CaBP9K gene (from 4580 to +365) containing the two potential HS are necessary to direct active transgene expression in the duodenum in a vitamin D3-dependent manner. In addition, a short construct, 9K/117 (from −117 to +365), containing only HS4, is not sufficient to direct expression of the transgene in the intestine (15).

The present study further examines the mechanisms that restrict CaBP9K gene transcription to the epithelium of the villus and shows that the 9K/14580-chromaphenicol acetyltransferase (CAT) construct confers a correct crypt-villus pattern of transgene expression. We have identified the minimal regulatory elements needed to confer this intestinal expression on the CaBP9K gene and demonstrated that there must be
cooperation between the HS1 distal activator and the minimal promoter containing a Cdx2-binding site for the correct targeting of cephalocaudal transgene expression and vitamin D responsiveness in the small intestine. Last, we have described a potential second Cdx2-binding site in the distal activator HS1 and tested in vivo the functional relevance of this site by mutagenesis in transgenic mice.

**EXPERIMENTAL PROCEDURES**

**Construction of Hybrid Genes and Transgenic Mice**—The plasmid (9K/HS1-4580-CAT) has been previously described (15). Sequence analysis has revealed that it contained 4580 base pairs of the 9′ regulatory sequences of the rat CaBP9K gene (−4580 to +365) placed in front of the coding sequences of the CAT gene. Therefore, this plasmid has been termed 9K/−4580-CAT in this report. The transgene 9K/−117-CAT was isolated by HindIII digestion of the plasmid 9K/−4580-CAT. This fragment contains the promoter region (beginning at −117), the first exon, the first intron, and the beginning of the second exon in front of the CAT gene. A Smal-PvuII CaBP9K fragment (−3735 to −2891) was inserted upstream of a blunted BamHI site of pBLCAT2 to generate 9K/HS1-thymidine kinase-CAT, and insertion upstream of a blunted HindIII site of 9K/−117-CAT generated 9K/HS1−117-CAT. The Kunkel method (16) was used for oligonucleotide-directed in vitro mutagenesis after subsequent SmaI and PvuI cleavage of the KS Bluescript phagemid and transformation in RZ1032 host cells (Promega). The oligonucleotide primer used was 5′-AACATGCCCCATTGTCGAC-3′. The entire wild type and mutant regions were sequenced on both strands (17) to verify that only the desired mutation was present. The mutant fragment was then subcloned in 9K/−4580-CAT-KS instead of the wild type fragment to obtain the −4580Cdxmut-CAT plasmid. Microinjection fragments were linearized and purified. Transgenic mice were generated, identified, and propagated (15).

**Animal Treatments**—Mice were made vitamin D2-deficient and treated with 1,25(OH)2D3 as in (15). The effect of the vitamin D2-depleted diet was verified by Northern blotting using duodenal mRNA hybridized with a CaBP9K probe (data not shown).

**CAT Assays**—Various tissues were dissected out from young adult (5–7–week-old) F1 transgenic mice for CAT assays. Cell lysates were obtained (15). CAT activity was measured according to TLC standard protocols, with 5–300 μg of protein and reaction times of up to 4 h to keep the enzyme activity in a linear range.

**Immunocytochemical Studies**—CAT activity was revealed immunochemically using an anti-rabbit polyclonal affinity-purified antibody to CAT (5 Prime – 3 Prime, Inc., Paoli, PA) (18). The duodena and colon of 9K/−4580-CAT transgenic mice were removed, cut into small pieces and rapidly frozen. Frozen sections (7 μm) were cut with a cryostat and incubated with the anti-CAT antibody (dilution, 1:1000) at room temperature for 1 h. The sections were rinsed in phosphate-buffered saline, incubated with an anti-rabbit IgG-digoxigenin conjugate (dilution, 1:400; Boehringer Mannheim) for 30 min at room temperature, and rinsed three times with phosphate-buffered saline. The sections were then incubated with anti-digoxigenin-fluorescein isothiocyanate (dilution, 1:400; Boehringer Mannheim) for 30 min at room temperature. Sections were rinsed in phosphate-buffered saline, mounted, and examined under a Zeiss microscope equipped with epi-fluorescence optics. Control sections were processed as above but without the anti-CAT antibody.

**Northern Blots**—Total RNA was prepared from several tissues of young adult B6/CBA mice by the guanidium thiocyanate single-step procedure (19) and analyzed by Northern blotting (15).

**Gel Mobility Shift and DNase I Footprinting Assays**—Duodenal nuclear extracts were prepared (14). Gel mobility shift assays and DNase I footprinting assays were performed as described. The DNA fragments used as probes for footprinting assays were prepared by subcloning polymerase chain reaction products from the −5357 to −3281 region of 9K/−4580-CAT and −4580Cdxmut-CAT plasmids to generate wild type (WT HS1) and mutated probe (MUT HS1). The annealed oligonucleotides used as probes or competitors were as follows: SIF1 (−59 to −31), 5′-GAGGATCACAATAAATATTTAGGATTG-3′ from human sucrase-isomaltase promoter (20); 9KdXed (−3441 to −3942); 5′-TGCCATTATGCGCATGTTCC-3′; and mutant dX (5′-TGACCTACATTACTGCATGTGTTCC-3′). The complementary oligonucleotides used for gel shift assays were hybridized and end-labeled using [γ-32P]TP4 polydeoxynucleotide kinase. For supershift assays, binding reactions were incubated for 15 min at room temperature with anti-Cdx2 antibody (a gift from P. G. Traber) with gentle agitation. The radiolabeled DNA was then added (14).

**RESULTS**

**Correct Crypt-villus Patterning Directed by the 9K/−4580-CAT Fragment**—The mouse and rat CaBP9K genes are mainly expressed in the proximal region of the small intestinal epithelium (duodenum) and only in the enterocytes (11, 21, 22) (see Fig. 2E). The CaBP9K gene is expressed in the cecum and weakly in the proximal colon of the large intestine (10) (see Fig. 2E). The 9K/−4580 sequences of the rat CaBP9K gene contain the information necessary for direct transgene expression along the cephalocaudal axis in the small intestine, but this gene causes also ectopic expression of the transgene in the distal colon (15). Immunocytochemical studies on 9K/−4580-CAT mice showed that the transgene was expressed along the crypt-villus axis within the duodenum and the colon (Fig. 1). Analysis of several sections of duodena from transgenic mice revealed that CAT transgene is expressed along the length of the villus, in the epithelial layer but not in the crypt (Fig. 1A). The staining intensity is consistent with the amount of the CaBP9K in the enterocytes, which constitute 95% of the cellular population in the villus epithelium. But the difficulty of immunolocalizing the CAT transgene made it difficult to be sure that transgene expression was restricted to the enterocytes. The same gradient of CAT expression occurred in the distal colon (Fig. 1C). Thus, the 9K/−4580 regulatory sequences of the CaBP9K gene contain the information needed for expression of the transgene in the differentiated epithelial cells in the small and large intestine.

**Cooperation between the Proximal Promoter and a Distal Region (HS1) Is Necessary for Directing Intestinal Expression and Responsiveness to Vitamin D**—The mechanisms underlying the intestine-specific expression of the CaBP9K gene along the cephalocaudal axis were determined by identifying the minimal DNA regulatory regions needed for intestinal specificity in transgenic mice. We created new transgenes with various deletions. As there is a distal intestine-restricted DNase I hypersensitive site (HS1) 3500 bp upstream of the transcrip-
diamonds are as described for the length of the intestine (duodenum to colon).

Bars of transgenes in the duodena of transgenic mice. I hypersensitive sites (HS1 and HS4); lightface arrows indicate minor HSs (HS2, 3, 5). The broken line indicates a DNA deletion. B, CAT expression of transgenes in the duodena of transgenic mice. Bars represent the mean activities of each line, and each black diamond indicates the value of a single expressing mouse line (values are given in Table I and are the means of at least three individuals). C and D, CAT expression pattern along the length of the intestine (duodenum to colon). D, duodenum; J, jejunum; I, ileum; C, cecum; PC, proximal colon; DC, distal colon. Bars and diamonds are as described for B, E. Northern blot analysis of CaBP9K mRNA along the intestine.

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Fig. 2. CAT activities of the distal and proximal CaBP9k promoter in the intestine of transgenic mice. A, diagram showing the transgenes used. The nucleotide positions are numbered from the CaBP9K transcription start site (+1). Boldface arrows denote the major DNase I hypersensitive sites (HS1 and HS4); lightface arrows indicate minor HSs (HS2, 3, 5). The broken line indicates a DNA deletion. B, CAT expression of transgenes in the duodena of transgenic mice. Bars represent the mean activities of each line, and each black diamond indicates the value of a single expressing mouse line (values are given in Table I and are the means of at least three individuals). C and D, CAT expression pattern along the length of the intestine (duodenum to colon). D, duodenum; J, jejunum; I, ileum; C, cecum; PC, proximal colon; DC, distal colon. Bars and diamonds are as described for B. E, Northern blot analysis of CaBP9K mRNA along the intestine.

tion start site (13), we explored its activating potential by generating two types of transgenic mouse lines (Fig. 2A). The 9K/–117-CAT construct was prepared to confirm results previously obtained using the 9K/–117-AgT construct, in which 9K/–117 sequences of the CaBP9K promoter (the minimal promoter) were unable to target expression of the SV40 large T antigen in the intestine (15). We prepared the 9K/HS1–117-CAT construct containing an internal deletion that placed the distal HS1 box just in front of the minimal promoter. We generated seven lines of mice with the 9K/–117-CAT transgene and six lines with the 9K/HS1–117-CAT transgene (Table I and Fig. 2, B and C). The CAT activities in the duodenum and other parts of the intestine were compared with those obtained with mouse transgenic line 94 harboring the 9K/–4580-CAT construct (Fig. 2B). Differences between individuals in each transgenic line were relatively large, so that we were obliged to examine many mice from each transgenic line (Table I).

In agreement with our previous data obtained with 9K/–117-AgT, the 9K/–117-CAT construct did not target any detectable CAT expression in any part of the intestine studied, whatever the transgene copy number (Fig. 2B and C, and Table I). By contrast, juxtaposition of HS1 box and the minimal promoter restored CAT activity in the duodenum, but the activity was weaker than that obtained with the 9K/–4580-CAT construct (Fig. 2B). A compilation of our previous (15) and present data clearly shows that except for one low transgene copy number mouse line (line 84, two copies) that had little CAT activity in the duodenum, the mice of all the transgenic lines harboring the 9K/–4580-CAT construct had high CAT activity in the duodenum, the distribution of which was relatively uniform (Fig. 2B and Table I). By contrast, the CAT activity in the duodena of the mice of the 9K/117-CAT transgenic lines was more dispersed and independent of the transgenic copy number, which is consistent with an integration site effect on transgene expression (Table I and Fig. 2B). This result suggests that the region between –2894 and –117 is required to establish an open chromatin structure for high activity, but not for the duodenal specificity. The anterior-posterior distribution of transgene activity in 9K/–4580-CAT mice (line 94 was representative of the other lines tested (Ref. 15 and data not shown) was maximal in the duodenum and distal colon (Fig. 2C). In contrast, transgene expression in the 9K/HS1–117-CAT mice was maximal in the duodenum and the cecum (Fig. 2D), which correlated more closely with the expression of the endogenous CaBP9K gene (Fig. 2E). We obtained no data on the expression of the transgene along the crypt-villus axis with the 9K/HS1–117-CAT construct; the duodenal CAT activity was below the threshold of the immunocytochemical assay used.

Thus, the distal element HS1 contains sequences that cooperate with the proximal CaBP9K promoter to stimulate specific transcription in the intestine; hence, the HS-1 box can be defined as an intestinal activator region.

We measured the activity of this distal activator element on a heterologous promoter. For this, we cloned the distal HS1 element in front of the ubiquitous minimal promoter of the herpes simplex virus thymidine kinase gene, to obtain the HS1/thymidine kinase-CAT construct (Fig. 2A). The four transgenic lines obtained showed no CAT activity in the intestine or in the other tissues tested (Fig. 2, Table I, and data not shown). Thus the distal activator HS1 did not confer intestinal expression on a heterologous promoter in vivo and acts only on its own promoter. This suggests that there must be cooperation between the distal HS1 box and the minimal promoter to activate the CaBP9K promoter in vivo in the intestine.

As the responsiveness of the CaBP9K gene to vitamin D₃ is an important feature of its intestinal expression, and as the 9K/–4580 sequences confer vitamin D₃-inducible intestinal expression (15), we have looked for a response to this hormone in four transgenic mouse lines harboring the 9K/HS1–117-CAT construct. The 9K/HS1–117 sequences conferred responsiveness to vitamin D₃ on transgenic mice (Table I and Fig. 3A). A single injection of 25 ng of 1,25(OH)₂D₃ caused vitamin D₃-deficient transgenic mice to increase their duodenal expression of CAT 1.5–26-fold (Fig. 3). The CAT activities obtained with the “norm D” mice fed a standard diet were in the same range as those for the “–D” mice fed a vitamin D₃-deficient diet (Table I). This is probably because the standard chow diet (norm D) is naturally deficient in vitamin D₃, because the amount of CaBP9K mRNA in the duodena of mice fed a stand-
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TABLE I
CAT activity in intestinal tissues from 9K/CAT transgenic mice

| Construct     | Line | Copy | n° Duodenum | n° Duodenum | n° Duodenum | Jejunum | Ileum | Cecum | Proximal colon | Distal colon |
|---------------|------|------|-------------|-------------|-------------|---------|-------|-------|----------------|--------------|
| 9k/-4580-CAT  | 94   | 30   | 4 20.3 ± 9.3 5 1643 ± 621 | 5 130 ± 107 6.3 ± 2.7 7.9 ± 4.0 2.3 ± 1.6 0.9 ± 0.6 83.4 ± 37.5 |
| 9k/-117-CAT   | 9    | 20   | ND          | 2 ≤ 0.001* | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ND    | ND            | ND          |
| 5             | 7    | 5    | ND          | 2 ≤ 0.001* | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ND    | ND            | ND          |
| 55            | 60   | ND   | 3 ≤ 0.001   | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ND    | ND            | ND          |
| 65            | 20   | ND   | 3 ≤ 0.001   | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ND    | ND            | ND          |
| 75            | 10   | ND   | 2 ≤ 0.001   | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ND    | ND            | ND          |
| 61            | 20   | ND   | 2 ≤ 0.001   | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ND    | ND            | ND          |
| 9k/HS1-117-CAT| 4    | 20   | 3 1.6 ± 1.5 4 2.8 ± 2.3 7 2.3 ± 2.1 0.005 ± 0.07 0.1 ± 0.3 10.4 ± 15.9 0.2 ± 0.4 0.03 ± 0.05 |
| 24            | 40   | 3 29.3 ± 9.7 2 48.0 ± 20.0 7 30.0 ± 15.3 0.2 ± 0.2 0.3 ± 0.3 77.5 ± 64.0 15.0 ± 10.8 4.1 ± 6.9 |
| 20            | 40   | 9 6.2 ± 3.7 7 34.6 ± 24.7 2 7.3 ± 0.7 0.4 ± 0.1 1.5 ± 0.4 22.8 ± 19.7 16.3 ± 4.3 0.9 ± 0.4 |
| 43            | 15   | 7 3.1 ± 2.2 8 81.6 ± 40.4 10 6.4 ± 7.5 0.8 ± 0.6 2.6 ± 4.0 8.0 ± 6.3 8.3 ± 7.0 4.3 ± 4.6 |
| 25            | 5    | ND   | 6 0.2 ± 0.2 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ND    | ND            | ND          |
| 33            | 100  | ND   | 5 1.0 ± 0.9 | 0.1 ± 0.1 | 0.2 ± 0.2 | 0.4 ± 0.2 | 1.2 ± 1.3 | 0.4 ± 0.1 | ND            | ND          |
| 39            | 20   | ND   | 7 ≤ 0.001   | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ND    | ND            | ND          |
| Mean²         |      |      | 6.7 ± 9.9   | 0.2 ± 0.3 | 0.8 ± 1.3 | 15.9 ± 26.3 | 5.7 ± 6.6 | 0.8 ± 1.4 |      |              |
| 9k/HS1TK-CAT  | 11   | 2    | ND          | 3 ≤ 0.001* | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ND            | ND          |
| 16            | 5    | ND   | 2 ≤ 0.001   | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ND    | ND            | ND          |
| 72            | 15   | ND   | 2 ≤ 0.001   | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ND    | ND            | ND          |
| 73            | 20   | ND   | 2 ≤ 0.001   | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ND    | ND            | ND          |
| 9k/-4580      | 6    | 15   | ND          | 6 0.1 ± 0.1 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ND            | ND          |
| Cdxmut-CAT    | 16   | 15   | ND          | 6 0.3 ± 0.2 | 0.01 ± 0.02 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.1 | ND            | ND          |
| 94            | 150  | ND   | 6 5.7 ± 4.5 | 2.1 ± 0.9 | 0.7 ± 0.5 | 0.5 ± 0.7 | 0.2 ± 0.2 | 6.4 ± 1.8 |              |              |
| 105           | 15   | ND   | 5 0.2 ± 0.2 | 0.03 ± 0.03 | 0.01 ± 0.01 | 0.3 ± 0.5 | 0.2 ± 0.3 | 0.3 ± 0.05 |              |              |
| 200           | 5    | ND   | 6 0.05 ± 0.02 | 0.03 ± 0.01 | 0.03 ± 0.02 | 0.04 ± 0.02 | 0.06 ± 0.02 | 0.07 ± 0.04 |              |              |
| 212           | 20   | ND   | 3 0.2 ± 0.2 | 0.04 ± 0.20 | 0.01 ± 0.02 | 0.02 ± 0.02 | 0.02 ± 0.01 | 0.4 ± 0.1  |              |              |
| Mean²         |      |      | 1.1 ± 2.0   | 0.4 ± 0.8 | 0.1 ± 0.3 | 0.1 ± 0.2 | 0.1 ± 0.1 | 1.2 ± 2.3 |      |              |

a Norm D mice were fed a standard diet, not supplemented in vitamin D3.  
- D mice were fed a vitamin D3 deficient diet, and + D mice were fed a vitamin D3-deficient diet and were injected with 1,25(OH)2D3 16 h before sacrifice.  
* Number of transgene copies integrated.  
* Number of mice analyzed.  
* ≤0.001: no detectable CAT activity.  
* The means ± S.D. of the CAT activities of individual mice from various mouse lines carrying the same construct.

The 9K/HS1–117 sequences thus contain all of the information needed to direct the correct intestinal expression of the CaBP9k gene along the horizontal axis of the small intestine and to mediate responsiveness to vitamin D3. However, a computer sequence analysis of the entire 9K/HS1–117 region (Fig. 4) does not indicate the presence of a consensus vitamin D-responsive element (VDRE), as previously established by an in vitro approach (26), or a VDRE similar to other previously identified VDREs (for a review, see Ref. 27).

A Binding Site for Cdx2 in the Distal HS1 Activator—The HS1 cis-acting sequences that can cooperate with the minimal promoter in stimulating the intestinal activity of the CaBP9k gene promoter were identified by sequencing the CaBP9k DNA regulatory sequences from −4580 to −2232. Published data (13) indicate that HS1 is centered around position −3500 bp and extending 100 bp upstream and downstream this site (Fig. 4).

The caudal-related transcription factors appear to regulate the transcription of several intestinal genes (2, 14, 28–31). We therefore looked for a caudal motif in the distal activator HS1 and found a sequence at −3437 (ATTTATG) that is very similar to known canonical caudal motif (32). We characterized this element by electrophoretic mobility shift assay using the probe 9K/Cdx2 5′-TGCAATTATGGGCGATGGTTCC-3′ (caudal motif is bold). A specific DNA protein complex was revealed with duodenal nuclear extract (Fig. 5A). A 10-fold excess of unlabeled SIF1 element defined as the binding site for Cdx2 in the sucrose-isomaltase gene (2) was sufficient to displace 9K/Cdx2 (Fig. 5A), whereas a mutated 9K/Cdx2 element (Mut, 5′-TGACCTATGGGCGATGGTTCC-3′) competed poorly. In agreement with the competition experiment, the mutated element...
Mutated 9KCdxd (MUT9KCdxd) retained only very faint binding with duodenal nuclear extract (Fig. 5A). The specific 9KCdxd complex was shown to contain Cdx2 using an antibody against Cdx2 (gift from P. G. Traber), which supershifted the entire complex formed. As controls, the SIF1 monomeric and dimeric complexes previously described (2) were also supershifted (Fig. 5B), and an unrelated antibody (anti-IPF1, a gift from Dr. M. Montminy) did not supershift the retarded complex (data not shown). Therefore, the 9KCdxd caudal motif is able to bind to the duodenal Cdx2 homeoprotein in vitro.

**Effect of the Distal Cdx2-binding Site on Intestinal Expression of the 9K/CAT Transgene**—The functional role of the distal Cdx2 motif in vivo was tested by introducing the mutated Cdx2 motif (MUT HS1) into the 9K/24580-CAT construct and by studying the effect of this mutation on the pattern of transgene expression along the intestine (Fig. 7A). Six mouse lines carrying the 9K/24580Cdxmut-CAT fragment were obtained (Table I). Mutation of the distal Cdx2-binding site resulted in a dramatic drop of CAT activity in the duodenum to become about 100-fold decrease than in 9K/24580-CAT mice (Fig. 7B). Only one mouse line harboring the 9K/24580Cdxmut-CAT construct (line 94) with a very high copy number (about 150 copies) had greater transgene expression than the other mouse lines. This CAT activity was only 4-fold that of the transgenic mice (line 84) harboring only two copies of the 9K/24580-CAT construct (15) (Fig. 7B). The very weak expression of the transgene with the 4580Cdxmut-CAT construct probably reflects the residual binding activity of Cdx2 to the 9KCdxdmut site. Thus

FIG. 4. Sequence of the 2.3-kbp DNA fragment upstream from the −2227 EcoRI site of the CaBP9k promoter region. Nucleotides are numbered in the right and left margins. The probable extent of HS1 is denoted by the boxed sequence. The caudal motif is in boldface. HindIII (−4581) is the 5'−end of 9K−4580-CAT fragment. SmaI (−3735) and PvuII (−2891) are the 5'− and 3'−ends of the HS1 fragment subcloned in the 9K/HS1−117-CAT construct. The nucleotide sequence has been updated in the EMBL data library under accession number X16635.

FIG. 5. Binding activity of 9KCdxd caudal motif to duodenal nuclear extracts. A, a double-stranded oligonucleotide containing the distal Cdx2 motif (9KCdxd) was radiolabeled and incubated with nuclear extracts. The complex is shown by an arrow. The competitors of the 9KCdxd complexes, used at 10− and 100−fold excess, were 9KCdxd itself (Cdxd), SIF1 (SIF), and MUT9KCdxd (MUT). The right lane shows binding of the labeled MUT9KCdxd probe to duodenal nuclear extract. B, supershift experiments with an anti-Cdx2 antibody. Black arrows indicate monomeric and dimeric complexes obtained with the SIF1 probe (SIF) and duodenal nuclear extract ( Duo), and the specific complex obtained with 9KCdxd probe and duodenal nuclear extract. Open arrows show the supershifted complexes obtained with an anti-Cdx2 antibody (Ab).
mutation of the distal Cdx2-binding site leads to a strong reduction of transgene expression in the duodenum, suggesting that Cdx2 is critical for the CaBP9K promoter activity in the intestine.

Last, we analyzed the profile of transgene expression along the length of the intestine in the 9K/−4580CAT mice. The CAT activity in these mice was somewhat similar to that of rat duodenal nuclear extracts (DUO). G:A indicates Maxam/Gilbert sequences. The WT HS1-specific protected area is denoted by a box. The two asterisks represent the two mutated thymines (in cytosine) in MUT HS1. The boundaries of the regions are numbered from the transcriptional start site. B, the extent of the DNase I footprint is indicated on the HS1 sequence by a box. The beginning of the protection was difficult to locate, as indicated by the dashed line, because DNase I did not attack these sequences on naked DNA. The caudal motif is in boldface, and the mutated nucleotides are shown.

FIG. 6. DNase I footprinting of the HS1 region of the rat CaBP9K gene. A, Labeled wild type (WT HS1) and Cdx2 mutated (MUT HS1) CaBP9K fragments on the coding and noncoding strands were incubated without protein (DNA) or with 35 μg of rat duodenal nuclear extracts (DUO). G:A indicates Maxam/Gilbert sequences. The WT HS1-specific protected area is denoted by a box. The two asterisks represent the two mutated thymines (in cytosine) in MUT HS1. The boundaries of the regions are numbered from the transcriptional start site. B, the extent of the DNase I footprint is indicated on the HS1 sequence by a box. The beginning of the protection was difficult to locate, as indicated by the dashed line, because DNase I did not attack these sequences on naked DNA. The caudal motif is in boldface, and the mutated nucleotides are shown.

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Last, we analyzed the profile of transgene expression along the length of the intestine in the 9K/−4580CAT mice. The CAT activity in these mice was somewhat similar to that of rat duodenal nuclear extracts (DUO). G:A indicates Maxam/Gilbert sequences. The WT HS1-specific protected area is denoted by a box. The two asterisks represent the two mutated thymines (in cytosine) in MUT HS1. The boundaries of the regions are numbered from the transcriptional start site. B, the extent of the DNase I footprint is indicated on the HS1 sequence by a box. The beginning of the protection was difficult to locate, as indicated by the dashed line, because DNase I did not attack these sequences on naked DNA. The caudal motif is in boldface, and the mutated nucleotides are shown.

DISCUSSION

We have further examined the mechanisms that restrict CaBP9K gene transcription in the intestine. Previously, we showed that nucleotides −4580 to +365 of the rat CaBP9K gene recapitulate most of the intestine-specific CaBP9K profile (15). This study shows that this 5′ flanking region also maintains the crypt-villus expression pattern, limiting transgene expression to the epithelium of the villus. The intestinal specificity and vitamin D₃ responsiveness of the rat CaBP9K gene is conferred by cooperation between the minimal promoter and the distal activator region (HS1). This distal element contains a binding site for Cdx2, mutation of which causes a great reduction in the activating function of HS1 in transgenic mice. Thus, Cdx2 plays a key role in the intestine-specific transcription of the CaBP9K gene.

Cooperation between the HS1 Activator Region and the Inactive Minimal Promoter of the CaBP9K Gene in the Intestine—Transgenic mice have been used to study other intestinal genes and help reveal the complexity of the regulatory processes that govern intestinal gene expression (see Refs. 7 and 8 for reviews). They indicate that multiple functional cis-acting DNA elements are needed to mimic the correct patterns of intestinal genes activities. However, studies on these intestinal genes in transgenic mice (the genes for the liver and intestinal fatty acid binding protein, the ileal binding protein, and sucrase-isomaltase) have shown that the intestinal specificity is conferred by the proximal promoter region of the gene, and that longer fragments of 5′-flanking region of the gene modulate and confer the correct gradient of reporter expression along the various axis of the intestine (33–40). In contrast, our data show that the minimal promoter of the rat CaBP9K gene cannot, alone, direct any significant transgene expression in the intestine, although this minimal promoter contains binding sites for Cdx2 and HNF-1 (14), which are believed to be important transcription factors for many genes expressed in the intestinal epithelium (31).

The regulation for the CaBP9K gene in the intestine may be special because the intestine-specific transcriptional factor Cdx2 is bound to the TATA box (14). This situation is reminiscent of the chicken β²-globin gene. The promoter of this gene binds several transcription factors and the erythroid-specific factor GATA-1 is bound to the TATA box. Thus, for the rat CaBP9K gene, transcription of the β²-globin gene in an erythroid-specific fashion depends on the presence of a distal activator containing a GATA-1 site (41). An in vitro transcription assay was recently used to show that the distal activator acts via derepression of the inactive β²-globin promoter (42). This derepression depends on the specific promoter of the β²-globin gene and does not occur on a consensus TATA box. Thus, the transcription of the CaBP9K gene in the intestine may require the presence of the distal activator HS1 to derepress the inactive CaBP9K promoter produced by binding a tissue-specific factor to the TATA box. Alternatively, the proximal Cdx2-containing transcription complex alone could be by itself insufficient for chromatin opening and significant transcription in the absence of a loop structure, allowing specific cooperation between HS1 and HS4 through elective cooperation between distal and proximal Cdx2. This would explain why the distal activator HS1 cannot be considered to be classical enhancer, because it is unable to enhance the promoter activity of a heterologous promoter or give it intestinal specificity.

Our data show that deleting DNA bases −2891 to −117 does not affect the profile of expression of the transgene in the small intestine and thus imply that the information needed to direct expression of the CaBP9K gene in the duodenum is contained in the 9K/HS1−117-CAT construct. This construct can trigger expression of the transgene in the ecum and decrease transgene activity in the distal colon, which more closely reproduces the pattern of expression of the endogenous gene. This result indicates that the region between −2891 and −117 contains DNA regulatory elements implicated in the spatial control of the CaBP9K gene in the large intestine. Others have suggested such a combination of the DNA regulatory elements to explain the appropriate pattern of expression of intestinal genes (33–40).

An Unusual Vitamin D₃ Activation Pathway in the Intes-
The rat CaBP9K gene is under the positive control of the active metabolite of vitamin D in the intestine (10). This control involves the vitamin D receptor, as mice lacking the vitamin D receptor have much less CaBP9K mRNA than normal and the CaBP9K gene no longer responds to vitamin D (43–45). However, we have found no VDRE implicated in this hormonal control by transfection assays (46). The responsiveness to vitamin D only occurs in transgenic mice (15), (Fig. 3). This result suggests that regulation of CaBP9K gene expression by 1,25(OH)2D3 involves tissue-specific factors. Several examples of tissue-restricted hormonal control have been described (25, 47–49). We find that the 9K/HS1–117 construct, which contains information needed to direct expression in the duodenum, also contains the information needed to confer vitamin D3 responsiveness on the CaBP9K promoter. It will be of interest to see whether the tissue-specific element(s) and the element(s) implicated in the vitamin D regulation, within the 9K/HS1–117-CAT construct, can be separated or whether they act in concert to impart the specific expression of the CaBP9K gene seen in the duodenum.

Careful analysis of the sequence of the regulatory element in the 9K/HS1–117-CAT construct has not revealed any consensual VDRE. This suggests that the regulation of the rat CaBP9K gene by 1,25(OH)2D3 is complex and requires a non-consensual vitamin D-responsive element, as does the c-fos gene. In this gene, a 34-bp DNA element binds vitamin D receptor, 9-cis-retinoic acid receptor, and a member of the NF1 family, which cooperate to produce a full response to vitamin D (50). Nevertheless, a weak VDRE for the rat CaBP9K gene has been found by transfection experiments (51), but its in vivo relevance remains to be determined, because it is not included in our smallest transgene construct that can confer vitamin D3 responsiveness.

Cdx2 and Transcription of the CaBP9K Gene—Our examination of the cis-acting DNA element in the distal activator region, HS1, that confers intestinal specificity on the 9K/CAT constructs, has focused on a Cdx2 binding element in this activator. Several lines of evidence suggest that Cdx2 is crucial for regulating intestinal gene transcription (2, 28–31, 52, 53) and intestinal differentiation: Cdx2 causes the morphological and molecular differentiation of undifferentiated intestinal cell lines (5, 6), and Cdx2-null heterozygote mice develop multiple tumors of the colon (4). This suggests that Cdx2 must be continuously active to keep intestinal cells differentiated, because the intestinal epithelial cells are constantly being renewed from the stem cell pool in the crypts.

We have shown that Cdx2 binds to a caudal motif present in the activator HS1. A mutation in this HS1 distal Cdx2-binding site greatly decreases the activating function of HS1 in transgenic mice. However, Cdx2 alone cannot explain the great expression of the CaBP9K gene in the duodenum. The restricted expression of the transgene of the 9K/CAT constructs in the duodenum is due to a duodenal transcription factor(s) binding to a regulatory site(s) in the proximal promoter that cooperates with Cdx2. This idea is supported by our finding of a duodenal-specific footprint in the proximal promoter region of the CaBP9K gene (footprint B, nucleotides –76 to –46) (14). Cdx2 may require specific coactivators or posttranslational modification before it can activate the CaBP9K gene in the duodenum. Taylor et al. (53) have proposed that cell specific mechanisms determine the ability of Cdx2 to activate transcription when it is present in an enhancer context.

In summary, we have obtained direct evidence that Cdx2 is required for the intestinal expression of the CaBP9K gene by terminally differentiated enterocytes. This is yet another indication that this homeoprotein plays a key role in the maintenance of the differentiated phenotype of the intestinal epithelium.

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