Cdx Binding Determines the Timing of Enhancer Activation in Postnatal Duodenum*

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In mammalian intestine, adenosine deaminase (ADA) is expressed at high levels only along the villi of the duodenal epithelium. A duodenum-specific enhancer identified in the second intron of the human ADA gene controls this pattern of expression. This enhancer faithfully recapitulates this expression pattern in transgenic mice, when included in CAT reporter gene constructions. Multiple binding sites for PDX-1 and GATA factors were previously identified within the ~300-bp region that encompasses the enhancer. Mutation analyses demonstrated that binding of PDX-1 and of GATA-4 was absolutely essential for enhancer function. In the present study, we have identified additional enhancer binding sites for Cdx factors, for YY1, and for NFI family members. Detailed EMSA studies were used to confirm binding at these sites. This brings the number of confirmed binding sites within the enhancer to thirteen, with five different factors or family of factors contributing to the putative enhancosome complex. Mutation analysis was utilized to examine the specific roles of the newly identified sites. Two sites were identified that bound both Cdx1 and Cdx2. Mutations were identified in these two sites that completely and specifically eliminated Cdx binding. In transgenic mice, these enhancer mutations dramatically changed the developmental timing of enhancer activation (delaying it by 2–3 weeks) without affecting other aspects of enhancer function. In the chromatin context of certain transgenic insertion sites, mutation of the two YY1 sites to specifically ablate binding caused a delay in enhancer activation similar to that observed with the Cdx mutations. No overt changes were observed from mutation of the NFI site.

It has been proposed that increasing complexity in metazoan organisms does not arise from a larger number of genes, but principally from more complex regulation of expression of a similar number of genes (1). The two mechanisms proposed are: (a) a greater number of cis regulatory segments governing specific genes and (b) greater complexity of the multiprotein transcription complexes that regulate gene expression. One of the major types of regulatory sequences in which this complexity resides is the enhancer (2, 3), where a protein complex known as the enhancosome forms and functions (4). These enhancers can be tissue-specific, cell-specific, and inducible and are thought to function by increasing the probability of transcription of an associated promoter within a particular cell. A gene may contain several distinct enhancers, each responsible for part of the total pattern of expression of that gene. A typical enhancer is about 500 bp in length and has ten binding sites for sequence-specific transcription factors (1). Within this array, there may be multiple sites that bind the same factor or family of factors. The function of the enhancer is determined by the combinatorial effect of all the proteins that bind within the enhancer, and each protein may have a distinct role in that function. The proteins may serve as activators, repressors, or play structural roles.

Mammalian adenosine deaminase (ADA) genes are regulated by a set of tissue-specific enhancers located in and around those genes (5–10). One of these enhancers has been shown to activate expression specifically in the duodenal epithelium (8, 11). This enhancer-driven expression has a specific pattern, not only along the anterior-posterior (A/P) axis, but also the crypt-villus (C-V) axis and the axis of developmental time (8). The enhancer activation is not only duodenal-specific, but is limited to villous epithelium (absent in the crypt epithelium) and occurs perinatally (within a 3–5-day window). This enhancer, located in the second intron of the human adenosine deaminase gene, was shown to contain multiple binding sites for proteins from duodenal nuclear extract (11). Five binding sites were identified for the factor PDX-1, and binding of this protein was shown to be essential for enhancer activation and function (12). That study indicates that PDX-1 may be largely responsible for the A/P pattern of expression by the enhancer, since PDX-1 expression is limited to duodenum in the intestinal epithelium (13). Three GATA factor binding sites were also identified and GATA binding was determined to be critical to high level enhancer function (14). In the present study, we characterize enhancer binding sites for the Cdx factors, for Yin Yang-1 (YY1), and for the NFI family of factors. Identification of these five sites brings the total to thirteen known factor binding sites within the duodenal enhancer. Transgenic mouse studies demonstrated that Cdx binding plays a critical role in the timing of enhancer activation during development of the intestinal epithelium.

EXPERIMENTAL PROCEDURES

Oligonucleotides—Oligonucleotides were synthesized at the University of Cincinnati DNA core facility and are as shown in Table I. Consensus oligonucleotides for YY1, NFI, and E2F-1 are as published in the Santa Cruz Biotechnology (Santa Cruz, CA) catalog. The Cdx consensus oligonucleotide sequence is TTTGCGCATAAAATTTATG based on the SIFI site of sucrase-isomaltase (15). Probe oligonucleotides

1 The abbreviations used are: ADA, adenosine deaminase; EMSA, electrophoretic mobility shift assay; wt, wild type; YY1, Yin Yang-1; MDNE, mouse duodenal nuclear extract; CAT, chloramphenicol acetyltransferase; A/P, anterior-posterior axis.
DNA core facility with the following sequences: GTGATGCCCGTAAC-...gene oligonucleotides were prepared at the University of Cincinnati enhancer. Numbers below the sequence ends correspond to residues of the human Asterisks 13196 BsiW1 cut p5 enhancer fragment was then liberated with BsiW1 and ligated into (University of Cincinnati sequencing core) to ensure fidelity of both the tions shown in Table I into the enhancer core fragment. The mutated CGCCAACATGCCACAAGGCCCC (Y2mut-ext), and CATGTCAAGCC-...GTGCTTTATAAATGTCACTTAATG (C2Amut-ext), AATGTGCTCC-...GTGCTTAGTTCAGGGC (C1mut-ext), GCACACACATTAATGTGCTCCA-...Food was removed from 30–40 adult non-transgenic mice 16 h prior to harvest and mouse duodenal nuclear extract (MDNE) was prepared as previously described (12). 5 pmol of gel-purified, duplexed oligonucleotides were labeled as previ-ously described (11). 10 μg of extract was used for each gel shift reaction. Binding reactions were carried out as previously reported (11). For competition studies, a 100-fold molar excess of competitor oligonu-cleotide was added to each reaction. Anti-Cdx1 and anti-Cdx2 antibo-dies were the generous gift of Peter Traber. Anti-NFI antibody was purchased from Geneika Biotechnology (Montreal, Canada). Supershift EMSAs were incubated overnight at 4 °C.

Site-directed Mutagenesis and Transgene Preparation—All enzymes were purchased from New England Biolabs (Beverly, MA). Site-directed mutagenesis was achieved with the Premega Altered Sites protocol (Madison, WI) using pALTER31+ (12) as a template. Mutated trans-gene oligonucleotides were prepared at the University of Cincinnati DNA core facility with the following sequences: GTGATGCCCGTAAC-...CGCCAACATGCCACAAGGCCCC (Y2mut-ext), and CATGTCAAGCC-TGTGCTCCATGCATGTCACTTAATG (C2Amut-ext), AATGTGCTCC-TGTGCTTTATAAATGTCACTTAATG (C2Amut-ext), GCACACATTAAT-GTGCTTAGTTCAGGGC (C1mut-ext), GCACACACATTAATGTGCTCCA-...YY1 site along with an overlapping NFI site. EMSA experi-ments were performed using each of the above oligonucleotides (C2/Y1) Cdx recognition sequence. When labeled Y2/NFI was used as probe, two more specific complexes were observed, and (C2Y1) YY1 and NFI mutant mice were generated (11). Addition of an anti-YY1 antibody prohibited the formation of part of the broad Cdx band observed. Because both Cdx1 and Cdx2 recognize the same se-quence and both have been observed in the small intestine (17), we introduced antibodies specific to each of these proteins in EMSA experi-ments. Similar results with the Cdx complex were observed in competition studies using labeled C1 as probe (data not shown). Because both Cdx1 and Cdx2 recognize the same se-quence and both have been observed in the small intestine (17), we introduced antibodies specific to each of these proteins in order to verify which was responsible for the formation of the observed Cdx complex. Addition of an anti-Cdx1 or anti-Cdx2 antibody inhibited the formation of part of the broad Cdx band as shown in Fig. 2B, verifying that each of these members of the Cdx family bind to both the upstream (C1) and downstream (C2Y1) Cdx recognition sequence. When labeled Y2/NFI was used as probe, multiple shifted complexes were observed, as shown in Fig. 2A. Com-petition studies showed that addition of a 100-fold molar excess of unlabeled Cdx and/or YY1 consensus oligonucleotide prevented the formation of these complexes. These studies were confirmed to involve YY1 and NFI, through competition analysis (Fig. 2C). Addition of an anti-NFI antibody to the reaction significantly reduced the putative NFI band and extracts have previously revealed five distinct protected regions along this enhancer region (11). Potential protein binding sites within these footprinted regions were examined using the TRANSFAC transcription factor data base (16) as well as a data base of sites that our laboratory has assembled from the literature that contains sequences of transcription factors known to be involved in gastrointestinal gene regulation. Pu-tative transcription factor recognition sequences identified within the duodenal enhancer region include five PDX-1 sites, three GATA sites, two Cdx sites, two YY1 sites, and one NFI binding site (Fig. 1). Previous studies have shown that functional PDX-1 and GATA sites are required for high level activation from the duodenal enhancer (12, 14). To assess the binding ability of the Cdx, YY1, and NFI sites, oligonucleotides were synthesized that correspond to the se-quences (C1, C2/Y1, and Y2/NFI in Table I) in the enhancer region and electrophoretic mobility shift assays (EMSA) were performed. Oligonucleotide C1 contains the upstream Cdx site, C2/Y1 contains the downstream Cdx site as well as an overlapping upstream YY1 site, and Y2/NFI contains the downstream YY1 site along with an overlapping NFI site. EMSA experi-ments were performed using each of the above oligonucleotides as a probe. When labeled C2/Y1 was used as probe, multiple shifted complexes were observed, as shown in Fig. 2A. Com-petition studies showed that addition of a 100-fold molar excess of unlabeled Cdx and/or YY1 consensus oligonucleotide prevented the formation of these complexes. Competition was not ob-served when a nonspecific competitor oligonucleotide was intro-duced. Similar results with the Cdx complex were observed in competition studies using labeled C1 as probe (data not shown). Because both Cdx1 and Cdx2 recognize the same se-quence and both have been observed in the small intestine (17), we introduced antibodies specific to each of these proteins in order to verify which was responsible for the formation of the observed Cdx complex. Addition of an anti-Cdx1 or anti-Cdx2 antibody inhibited the formation of part of the broad Cdx band as shown in Fig. 2B, verifying that each of these members of the Cdx family bind to both the upstream (C1) and downstream (C2Y1) Cdx recognition sequence. When labeled Y2/NFI was used as probe, multiple more shifted complexes were observed, and these were confirmed to involve YY1 and NFI, through competition analysis (Fig. 2C). Addition of an anti-NFI antibody to the reaction significantly reduced the putative NFI band and produced a supershifted complex as shown in Fig. 2D. Com-mercial YY1 antibodies were found to be ineffective in our hands. Therefore to confirm the identity of the observed YY1 bands, EMSA experiments were performed with a labeled YY1 consensus sequence oligonucleotide. They showed a complex

**RESULTS**

**The Duodenal Enhancer Region Contains Functional Binding Sites for Multiple Transcription Factors**—Intestinal expression of adenosine deaminase (ADA) is regulated by a 3.4-kb segment located within the second intron of the human ADA gene (8). Found within this segment is a 319-bp region that functions as a duodenum-specific enhancer (11). DNase footprinting studies with human, rat, and mouse duodenal nuclear extract have previously revealed five distinct protected regions along this enhancer region (11). Potential protein binding sites within these footprinted regions were examined using the TRANSFAC transcription factor data base (16) as well as a data base of sites that our laboratory has assembled from the literature that contains sequences of transcription factors known to be involved in gastrointestinal gene regulation. Putative transcription factor recognition sequences identified within the duodenal enhancer region include five PDX-1 sites, three GATA sites, two Cdx sites, two YY1 sites, and one NFI binding site (Fig. 1). Previous studies have shown that functional PDX-1 and GATA sites are required for high level activation from the duodenal enhancer (12, 14). To assess the binding ability of the Cdx, YY1, and NFI sites, oligonucleotides were synthesized that correspond to the se-quences (C1, C2/Y1, and Y2/NFI in Table I) in the enhancer region and electrophoretic mobility shift assays (EMSA) were performed. Oligonucleotide C1 contains the upstream Cdx site, C2/Y1 contains the downstream Cdx site as well as an overlapping upstream YY1 site, and Y2/NFI contains the downstream YY1 site along with an overlapping NFI site. EMSA experi-ments were performed using each of the above oligonucleotides as a probe. When labeled C2/Y1 was used as probe, multiple shifted complexes were observed, as shown in Fig. 2A. Com-petition studies showed that addition of a 100-fold molar excess of unlabeled Cdx and/or YY1 consensus oligonucleotide prevented the formation of these complexes. Competition was not ob-served when a nonspecific competitor oligonucleotide was intro-duced. Similar results with the Cdx complex were observed in competition studies using labeled C1 as probe (data not shown). Because both Cdx1 and Cdx2 recognize the same se-quence and both have been observed in the small intestine (17), we introduced antibodies specific to each of these proteins in order to verify which was responsible for the formation of the observed Cdx complex. Addition of an anti-Cdx1 or anti-Cdx2 antibody inhibited the formation of part of the broad Cdx band as shown in Fig. 2B, verifying that each of these members of the Cdx family bind to both the upstream (C1) and downstream (C2Y1) Cdx recognition sequence. When labeled Y2/NFI was used as probe, multiple more shifted complexes were observed, and these were confirmed to involve YY1 and NFI, through competition analysis (Fig. 2C). Addition of an anti-NFI antibody to the reaction significantly reduced the putative NFI band and produced a supershifted complex as shown in Fig. 2D. Com-mercial YY1 antibodies were found to be ineffective in our hands. Therefore to confirm the identity of the observed YY1 bands, EMSA experiments were performed with a labeled YY1 consensus sequence oligonucleotide. They showed a complex

**FIG. 1. Sequence of the duodenal enhancer region of the human ADA gene.** Shown is the entire sequence of the duodenal enhancer. Numbers below the sequence ends correspond to residues of the human ADA gene sequence (GenBank™ accession number M13792). Asterisks mark the nucleotide residues that were altered to create two BsiW1 restriction sites. Matches to several consensus recognition sequences for potential factor binding sites are boxed (two Cdx-type sites; two YY1-type sites; one NFI-type site). The binding sites of factors for which mutational analysis has been done previously are also boxed (five PDX-1 sites; three GATA sites).
that co-migrates with the YY1 band observed in Fig. 2 (data not shown). The EMSA results as a whole confirm that the shifted complexes observed with the three test oligonucleotides are the result of the binding of Cdx, YY1, and NFI factors to their respective enhancer sequences.

Specific Point Mutations Ablate Binding of Cdx, YY1, and NFI, Independently of One Another—The consensus sequence for Cdx (15), YY1 (16, 18), and NFI (Santa Cruz Biotechnology), as well as the proposed location of the factor binding sites within each oligonucleotide is shown in Table I. A variety of

**Table I**

Sequence of the Cdx, YY1, and NFI binding sites in the human ADA duodenal enhancer

| Oligonucleotides containing the putative Cdx (C1, C2/Y1), YY1 (C2/Y1, Y2/NFI), and NFI (Y2/NFI) binding sites from the human ADA enhancer are shown. Also shown are the consensus binding sites for each transcription factor. Within each wild-type oligonucleotide, the factor-binding site is highlighted in bold letters. Base pair changes from the wild type that eliminate factor binding are shown in bold lowercase letters in the mutant (mut) oligonucleotides C1mut, C2mut, Y1mut, Y2mut, and Nmut. The sequence shown for C2/Y1 grouped with the YY1 oligonucleotides represents the lower DNA strand.

| Sequence | Sequence |
|----------|----------|
| Cdx cs | YATAAAK |
| Y1/NFI | TGGNNNNNAGCCAAT |
| Y2/NFI | CTGGCCTTTGCCCATGTCAAGCCGCCAAC |
| C1 | GGTAAGTGATGCC |
| C2/Y1 | TAAGTGCAATTTATGGAGCACATT |
| C1mut | GGTAAGTGATGCCCGTAA |
| C2/Y1 | AATGTGCTCCAATTTATGG |
| Y1mut | TAAGTGCAATTTATGGAGCACATT |
| Y2/NFI | CTGGCCTTTGCCCATGTCAAGCCGCCAAC |
| Y2mut | CTGGCCTTTGCCCATGTCAAGCCGCCAAC |
| NFI cs | TGGNNNNNAGCCAAT |
| Y2/NFI | CTGGCCTTTGCCCATGTCAAGCCGCCAAC |
| Nmut | CTGGCCTTTGCCCATGTCAAGCCGCCAAC |

* Consensus (cs) binding sites.
Mutations to the Cdx, YY1, or NFI binding regions eliminate the formation of the specific protein complexes. Each lane in all panels contains 10 μg of MDNE. A, lanes 1 and 5 contain labeled wild-type C2/Y1 and C1 oligonucleotides, respectively, as probes. Lanes 2–4 contain a labeled mutant oligonucleotide (C2mut) that eliminates binding of the downstream Cdx site. Lane 3 contains unlabeled Cdx consensus oligonucleotide, and lane 4 contains YY1 consensus oligonucleotide as competitors. Lanes 6–8 contain a labeled mutant oligonucleotide (Y1mut) that eliminates binding at the upstream YY1 site that overlaps the Cdx site. For competition analysis, lane 7 contains YY1 consensus oligonucleotide, and lane 8 contains Cdx consensus oligonucleotide. B, labeled wild-type oligonucleotide incorporating the upstream Cdx site is used as probe in lanes 1 and 2. Lanes 3 and 4 contain a labeled mutant oligonucleotide that eliminates binding of Cdx to this site. Lanes 2 and 4 contain unlabeled Cdx consensus oligonucleotide as a competitor. Evident in this panel is a nonspecific complex (ns) that co-migrates in part with the shifted Cdx complex. C, lanes 1–3 contain labeled Y2/NFI as probe. Lanes 2 and 3 contain YY1 and NFI consensus oligonucleotides, respectively, as competitors. Lane 4 contains a labeled mutant oligonucleotide that eliminates factor binding to the downstream YY1 site, and lane 5 contains a mutant oligonucleotide that eliminates NFI binding.

mutations were introduced into the oligonucleotide sequences and changes in binding were analyzed by EMSA. Mutations that eliminated binding of Cdx, YY1, and NFI without affecting the binding of other protein complexes are designated as C1mut, C2mut, Y1mut, Y2mut, and Nmut in Table I. EMSA experiments with the mutated oligonucleotides that confirm these mutation results are shown in Fig. 3. Gel shifts with the wild-type oligonucleotides are included for comparison. Fig. 3A shows the Cdx and YY1 shifted complexes observed previously when the wild-type oligonucleotide, C2/Y1, was used as probe. When the C2mut oligonucleotide was used as probe, the previously observed Cdx complex did not form. Fig. 3A (right) also displays the Y1mut oligonucleotide used as probe, and the results show that upstream YY1 binding was completely eliminated in the mutated oligonucleotide while Cdx binding to this region remained unaffected. We also ablated binding of the upstream Cdx complex using the C1mut oligonucleotide as shown in Fig. 3B. Here we observed a nonspecific band that co-migrates with the Cdx complex that is seen with both the wild-type and mutated oligonucleotide. This band is specific to the extract used in this experiment and cannot be eliminated by competition with an excess of Cdx consensus oligonucleotide (lanes 2 and 4) or with an excess of nonspecific competitor (data not shown). Using labeled Y2mut oligonucleotide as probe, we show that the downstream YY1 binding was ablated without affecting the overlapping NFI binding (Fig. 3C). Similarly, the Nmut oligonucleotide eliminated NFI binding while YY1 binding was maintained. These mutation studies allowed us to design and create an enhancer fragment devoid of functional Cdx, YY1, and NFI sites independently of one another, while maintaining the integrity of the surrounding protein binding sites.

Cdx Mutant, YY1 Mutant, and NFI Mutant Transgenic Mice Maintain Duodenum-specific Activation—Three separate constructions were created for production of transgenic mice based on the mutated oligonucleotides that resulted in loss of binding of Cdx, YY1, or NFI. The mutations were introduced into the enhancer region such that in one construction, both Cdx binding sites were ablated (Cdx mutant), in another both YY1 binding sites were ablated (YY1 mutant), and in the third, the NFI site was eliminated (NFI mutant). In each, the mutated enhancer core replaced the wild-type enhancer core within a 13-kb intragenic ADA fragment, maintaining the same position and orientation. The basic design of the transgenes used has been described previously (8). The analogous wild-type and mutant transgenes each contain a 3.8-kb human ADA promoter/5' flanking segment attached to the CAT coding sequence and the 13-kb fragment containing the enhancer downstream of the CAT reporter cartridge.

Five independent Cdx mutant lines, seven YY1 mutant lines, and four NFI mutant lines were analyzed. Various tissues from a minimum of two adult F1-F3 mice from each line were analyzed for CAT activity. Protein concentration and copy number were also assessed, and the calculated CAT activity was normalized to each. In every line from each construction, except for the lowest expressing Cdx line (line 1), duodenum had the highest level of transgene activity by at least two orders of magnitude in comparison to 9–17 other tissues (data not shown). This result is very similar to that seen in mice with the wild-type transgene (11, 12, 14). The duodenal CAT activity values are shown in Table II. The CAT activities in mice with the wild-type transgene have been reported previously (8). The analogous wild-type and mutant transgenes each contain a 3.8-kb human ADA promoter/5' flanking segment attached to the CAT coding sequence. In transgenic mice containing the wild-type enhancer the duodenal CAT activity ranges from 1,400–31,000, with a mean activity of 9,100. In contrast, transgenic mice with the enhancer deleted were previously analyzed, and these mice exhibited significantly reduced duodenal CAT activity ranging from 0–1.3, with a mean activity of 0.48 (11). Also, in no line of these enhancerless mice was the duodenum the tissue of highest expression.

As shown in Table II, Cdx mutant mice have a range of duodenal activity from 90–20,500 with a mean of 5,500. Four of the five Cdx mutant lines had duodenal CAT values that fit well within the range established for the wild-type transgene.
Cdx Binding and Duodenal Enhancer Activation

The fifth line, line 1, expressed at 1% of the mean wild-type levels and showed no evidence of duodenal-specific enhancer activity. This probably means that this single copy transgene was inserted into a non-permissive site and was subject to positional variegation. While unusual, this has been observed with this enhancer in a wild-type transgene (11), indicating that some insertion sites are completely refractory to enhancer function.

YY1 mutant mice showed a range of duodenal CAT activity from 180 to 41,000 with a mean activity of 14,000, with most values well within the range of wild-type activity. All seven lines of YY1 mutant mice demonstrated duodenal-specific activation. Unlike the low expressing Cdx mutant line 1, even the low expressing YY1 mutant line 1 exhibited highest activity in duodenum. In that line, the next highest expressing tissue had an activity of 1.3, a value 140-fold lower than the duodenal activity. The duodenal activities in NFI mutant mice ranged from 180 to 41,000 with a mean activity of 14,000, with most values well within the range of wild-type activity. All seven lines of YY1 mutant mice demonstrated duodenal-specific activation. Unlike the low expressing Cdx mutant line 1, even the low expressing YY1 mutant line 1 exhibited highest activity in duodenum. In that line, the next highest expressing tissue had an activity of 1.3, a value 140-fold lower than the duodenal activity. The duodenal activities in NFI mutant mice ranged from 180 to 41,000 with a mean activity of 14,000, with most values well within the range of wild-type activity. All seven lines of YY1 mutant mice demonstrated duodenal-specific activation. Unlike the low expressing Cdx mutant line 1, even the low expressing YY1 mutant line 1 exhibited highest activity in duodenum. In that line, the next highest expressing tissue had an activity of 1.3, a value 140-fold lower than the duodenal activity.

Our laboratory has previously mutated the enhancer binding sites of two other transcription factors, namely PDX-1 and GATA factors, and transgenic mouse analysis provided results markedly different from that of the three constructions discussed here. In both PDX mutant mice and GATA mutant mice, the mutations resulted in complete loss of duodenal activity, suggesting that both of these transcription factors are absolutely required for enhancer function (12, 14). In contrast, our results here suggest that Cdx, YY1, or NFI protein binding sites are not essential for high level enhancer activity in adult transgenic mice.

### Table II

| Transgene   | Transgenic line | Copy number | Duodenal CAT activity pmol/h/100 g/copy |
|-------------|-----------------|-------------|----------------------------------------|
| Wt          | 1               | 9           | 5,900                                  |
|             | 2               | 4           | 31,000                                 |
|             | 3               | 9           | 1,400                                  |
|             | 4               | 41          | 4,100                                  |
|             | 5               | 34          | 13,000                                 |
|             | 6               | 14          | 2,400                                  |
|             | 7               | 5           | 2,000                                  |
|             | 8               | 18          | 2,500                                  |
|             | 9               | 2           | 13,000                                 |
|             | 10              | 23          | 6,000                                  |
|             | 11              | 3           | 19,000                                 |
| Cdx mutant  | 1               | 1           | 90                                     |
|             | 2               | 6           | 1,600                                  |
|             | 3               | 90          | 2,900                                  |
|             | 4               | 11          | 20,500                                 |
|             | 5               | 8           | 2,700                                  |
| YY1 mutant  | 1               | 1           | 180                                    |
|             | 2               | 6           | 7,000                                  |
|             | 3               | 3           | 41,000                                 |
|             | 4               | 1           | 720                                    |
|             | 5               | 1           | 13,000                                 |
|             | 6               | 6           | 2,300                                  |
|             | 7               | 2           | 34,000                                 |
| NF1 mutant  | 1               | 1           | 70,000                                 |
|             | 2               | 19          | 2,400                                  |
|             | 3               | 1           | 105,000                                |
|             | 4               | 10          | 9,200                                  |

### DISCUSSION

The transcription factors Cdx1 and Cdx2 are members of the caudal-related homeobox gene family based on their homology to the factor caudal in *Drosophila melanogaster*. The caudal homeobox factor is required for anterior-posterior regional identity and axial patterning in *Drosophila* (20). During mouse development, the expression of Cdx1 and Cdx2 can be divided into two stages that likely reflect distinct roles (17). During early development, these two factors are expressed in a Home-like manner in the three germ layers and appear to have overlapping functions in anterior-posterior patterning and posterior axis elongation (21–23). The second stage of Cdx1 and Cdx2 expression, from late development into adulthood, is characterized by the loss of expression almost everywhere but the epithelium of small intestine and colon (17). Putative intestine-specific enhancers upstream of the *Cdx1* gene have been identified that may orchestrate the switch in expression patterns for that gene (24).
As might be expected from this pattern of expression, a wide variety of in vivo and in vitro studies have suggested that these two factors are important in controlling epithelial cell differentiation, proliferation, and function in the intestine (25–30). Along the anterior-posterior axis in adult mouse small intestine, both Cdx1 and Cdx2 show a gradient of expression increasing in the posterior direction from moderately low levels in the duodenum to higher levels in the ileum (17, 29, 31, 32). In the colon, significant levels of both Cdx1 and Cdx2 are observed, with higher levels of Cdx2 in proximal colon than in distal colon (17, 29, 31). Observed differences in the patterns of expression of Cdx1 and Cdx2 along the crypt-villus axis in the small intestinal epithelium, led to the proposal of distinct, complementary functions for these two factors in epithelial cells. Along the crypt-villus axis of intestine, gradients of expression are present with Cdx1 expressed principally in the crypts and Cdx2 expressed principally in cells on the villi (17, 29, 34). This distribution is consistent with a role for the two caudal factors in regulating the equilibrium between proliferation and differentiation in epithelial cells, which is critical for intestinal homeostasis. In addition, Cdx2 and, to a lesser extent Cdx1, have also been reported to regulate the intestine-specific expression of a number of genes (15, 35–52).

In this report, we examined the role of Cdx type binding sites in the function of the ADA duodenal enhancer. EMSA studies indicated that the two identified Cdx sites were bound equally well by both Cdx-1 and Cdx-2. Mutation of these sites to prohibit binding of Cdx1 or Cdx2 had no clear effect on the level of enhancer activation achieved in the intestinal epithelium of the adult mouse. However, the temporal pattern of enhancer activation for the Cdx mutant transgene was dramatically altered compared with the wild-type transgene. The observed delay in activation indicates a critical role for the caudal factors in initiating and maintaining enhancer function during the first postnatal weeks, a time when the mouse intestinal epithelium is undergoing dramatic changes in structure and function (53). The study does not distinguish between possible roles for Cdx2 versus Cdx1 in enhancer activation. It is also not clear why the requirement for functional caudal binding sites diminishes with age. It is probably related to the mechanism of enhancer complex (enhanceosome) formation, and the role that Cdx plays in that process. Cdx binding might be required for a seminal

**FIG. 4. Temporal expression of CAT in wild type and mutant transgenetic duodenum.** Changes in the level of duodenum-specific CAT expression in transgenic mice over a developmental time frame from P0 to several weeks of age are shown. Each point represents the average for 2–6 individual mice. A, wild-type pattern of expression is shown. B, temporal CAT expression is shown for Cdx mutant mice from line 5. C, temporal CAT expression is shown for two individual lines of YY1 mutant mice. Line 4 is represented by open circles and line 6 by solid triangles. Two additional lines were analyzed (data not shown). Line 1 showed results very similar to line 4, and line 5 showed results virtually identical to those for line 6. D, temporal CAT expression is shown for NF1 mutant mice from line 1. Two additional lines were analyzed (lines 2 and 4, data not shown) that confirm these results.
event in enhancer function, such as chromatin opening, in the neonatal epithelium. However, it is not clear why this requirement would change with age, since the epithelium is continuously renewed every few days. Previous studies demonstrated that functional binding sites for PDX-1 and GATA factors were required for enhancer function, but were not required for chromatin opening, as assessed by DNase hypersensitive site formation (14).

It is possible that Cdx factors do not have to bind their cognate DNA sites to participate in formation of the putative enhanceosome that forms on the ADA enhancer. They could be localized there by protein-protein interactions with other bound factors. Recent studies have shown that Cdx2 is capable of direct physical interaction with and binding to GATA-4 (35) and PDX-1 (54), both factors that are known to bind the ADA enhancer and are critical to its function (12, 14). In studies with the sucrase-isomaltase promoter, it was demonstrated that GATA-4 could play a role in activation of that promoter without binding its cognate sequence (35).

A wide variety of mouse genes demonstrate significant changes in the level of expression in the intestinal epithelium during the first few weeks of life. This is especially true of the suckling-weanling transition at about 3 weeks of age. Very little is known about the mechanisms that orchestrate these changes. The endogenous mouse ADA gene is activated from low levels to high levels in intestinal epithelium at the suckling-weanling transition (55, 56). The entire human ADA regulatory region used in these studies activates transgene reporter expression precociously, at or near birth, compared with the endogenous ADA gene (8). It was previously proposed that this precocious activation is related to the fact that human ADA expression is activated in intestine in utero, at what might be considered an earlier developmental time point than in mouse (8). Interestingly, the discrete human ADA enhancer alone activates expression at about 3 weeks of age in transgenic mice and additional upstream sequences are required for the precocious activation (11). These upstream segments (including hypersensitive site C) have no inherent enhancer activity, but somehow orchestrate the early activation of the enhancer (located at hypersensitive site D). It will be of interest to determine what role Cdx factors might play in this interaction. Analysis of the analogous intestinal regulatory region and enhancer in the mouse ADA gene would be very informative. To date, that region has not been identified and characterized. Transgenic mouse studies with a fragment of the mouse ADA gene homologous to the human ADA intestinal regulatory region (57), demonstrated that the fragment did not contain intestine-specific enhancer activity.2

Because enhancer activity was significantly perturbed along the temporal axis in the Cdx mutant mice, we questioned whether expression along the other axes was perturbed as well. The reciprocal pattern of expression observed for Cdx1 and Cdx2 along the crypt-villus axis in intestinal epithelium led us to investigate the possibility that the pattern of enhancer activation was perturbed along this axis in the Cdx mutant transgenic mice. Our in situ hybridization studies, however, indicated that this was not the case, as enhancer activation along the C/V axis was virtually identical to that observed with the wild-type transgene. Expression was localized along the villi, past the crypt-villus junction, and was not detectable in the crypts. It has also been suggested that Cdx2 binding of the calbindin D9k promoter might repress or act as a negative factor in regulation of this gene in distal intestine (36). Calbindin-D9k is expressed along the A/P axis of the small intestine in a pattern very similar to that of ADA, with predominantly duodenal expression. However, our analysis of CAT expression by the Cdx mutant transgene along the A/P axis indicated that this was indistinguishable from the wild-type pattern as well.

The YY1 protein is a ubiquitously expressed 68 kDa zinc finger transcription factor that has been shown to have a wide variety of functions (58). It derives its name from the fact that it activates, represses, or initiates transcription, depending on the particular gene, binding site location, and regulatory context in which the YY1 functions (59, 60). In this study, two distinct binding sites for the YY1 protein were identified in the human ADA gene's duodenal enhancer.

Mutation analysis in transgenic mice does not give us a clear idea of what role YY1 might be playing in enhancer function. Five of the seven YY1 mutant transgenic mouse lines demonstrated duodenal-specific activation of CAT reporter gene expression in adults that was within the normal range established for the wild-type transgene. If YY1 is serving as an activator in the duodenal enhancer, it is not essential to enhancer activation. The three YY1 mutant lines (lines 1, 4, and 6) examined all showed wild-type patterns of transgene expression along both the A/P and crypt-villus axes. However, when temporal activation of transgene expression was examined, two lines showed the wild-type pattern and two showed a delayed pattern very reminiscent of the Cdx mutant pattern. The two lines that showed the delayed pattern were lines 1 and 4, the lines that also had the lowest activation levels. This result suggests that the YY1 mutant transgene is more subject to positional variegation related to insertion site location and local chromatin environment. Therefore the absence of YY1 binding can affect both level and timing of enhancer activation in certain chromatin environments. It has been demonstrated that YY1 function is intimately associated with protein acetylation and chromatin modification (61, 62). It has also been shown that YY1 is associated with the nuclear matrix and that YY1 corresponds to the nuclear matrix protein NMP-1 (63, 64). Whether either of these observations is relevant to the role of YY1 in the ADA duodenal enhancer remains to be studied.

The NFI family of site-specific binding proteins function as factors in the regulation of a wide variety of types and classes of genes (33). The products of the four vertebrate NFI genes differ in their ability to either activate or repress transcription from a particular gene. Mutation of the NFI site in the ADA duodenal enhancer had no clearly observable effect on enhancer function. The four NFI mutant transgenic lines all showed significant duodenal-specific activation. It is tempting to speculate that NFI might normally have a repressive function, since two of the NFI mutant lines had among highest CAT/copy duodenal activity that we have ever observed. However, the role of NFI factors in ADA duodenal enhancer function remains unresolved.

Altogether, our studies have identified at least thirteen distinct factor binding sites within the 319-bp segment of the human ADA gene that we have defined as the duodenal enhancer (12, 14). All but one of these sites (the upstream GATA site) lie within the five footprinted regions originally identified within hypersensitive site D in the second intron (11). Five distinct factors, or family of factors, have been shown to bind at these sites: PDX-1, GATA factors, Cdx factors, YY1, and NFI factors. These factors play a combinatorial role in producing enhancer function. Binding of PDX-1 and GATA-4 has been shown to be essential for enhancer activation along the crypt-villus axis during epithelial cell differentiation and migration (12, 14). PDX-1 binding is likely the principal determinant in limitation of enhancer activation to the duodenum along the A/P axis, because its own expression is limited to the proximal

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2 D. A., Wiginton and E. A. Maier, unpublished results.
small intestine in the vast majority of epithelial cells (13). Cdx binding is not essential for enhancer activation in adults, but plays an important role, perhaps with YY1, in determining the developmental timing of enhancer activation. The studies with Cdx demonstrate that the role of a factor may change within the enhanceosome during development. The enhanceosome complex is likely to be a variable structure, whose composition and function change over time.

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