Temporal Regulation of Enhancer Function in Intestinal Epithelium
A ROLE FOR ONECUT FACTORS

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An intestine-specific gene regulatory region was previously identified near the second exon of the human adenosine deaminase (ADA) gene. In mammalian intestine, ADA is expressed at high levels only along the villi of the duodenal epithelium, principally if not exclusively in enterocytes. Within the ADA intestinal regulatory region, a potent duodenum-specific enhancer was identified that controls this pattern of expression. This enhancer has been shown to rely on PDX-1, GATA factors, and Cdx factors for its function. Upstream of the enhancer, a separate temporal regulatory region was identified that has no independent enhancer capability but controls the timing of enhancer activation. DNase I footprinting and electrophoretic mobility shift assays were used to begin to characterize temporal region function at the molecular level. In this manner, binding sites for the Onecut (OC) family of factors, YY1, and NFI family members were identified. Identification of the OC site was especially interesting, because almost nothing is known about the function of OC factors in intestine. In transgenic mice, mutation of the OC site to ablate binding resulted in a delay of 2–3 weeks in enhancer activation in the developing postnatal intestine, a result very similar to that observed previously when the entire temporal region was deleted. In mammals, the OC family is comprised of OC-1/HNF-6, OC-2, and OC-3. An examination of intestinal expression patterns showed that all three OC factors are expressed at detectable levels in adult mouse duodenum, with OC-2 predominant. In postnatal day 2 mice only OC-2 and OC-3 were detected in intestine, with OC-2 again predominant.

Development of any functional tissue in a complex eukaryotic organism is determined by the patterns of gene expression within that tissue and the cell types that make it up. For any particular gene involved in that developmental process, there are several questions to be asked. Where is the gene expressed? When is the gene expressed? What controls that pattern of expression? There have been numerous studies that have addressed the regulatory elements and factors that control the tissue-specific or cell-specific expression of a wide variety of genes (1–5). However, only recently have the mechanisms that control the timing of gene expression come under close scrutiny. The dogma has always been that the timing of expression of a particular gene is determined by the availability of the necessary combination of factors that bind and activate the promoter and/or enhancer of that gene. Recent studies from our laboratory have identified an additional level of control over developmental timing of gene expression. An intestinal regulatory region with multiple associated intestine-specific DNase I-hypersensitive sites was previously identified in the human adenosine deaminase (ADA)2 gene near exon 2 (6). A regulatory element that controls developmental timing of the gene activation in the intestinal epithelium was mapped to one of these hypersensitive sites, designated hypersensitive site C (HS-C) (7). This temporal control element is distinct from the previously characterized ADA promoter (8) and intestinal enhancer (6, 7) and has no independent ability to activate alone (7). However, this element is crucial in determining the timing of intestinal enhancer activation. Its presence or absence can change the timing of enhancer activation by 2–3 weeks in newborn transgenic mice. This is true despite the fact that all critical factors that bind the functional enhancer region appear to be available at the earlier developmental time point. We feel that this represents an excitingly novel type of temporal regulator that functions during the crucial early period of development in the intestinal epithelium, when crypts and villi are first forming. Detailed characterization of this novel intestinal regulatory region and the factors that bind there is the focus of the studies described here.

Our working hypothesis is that factors bound in the temporal control region functionally interact with enhancer-bound factors to control the timing of enhancer activation. We have identified three distinct DNase I footprints within the core of the temporal regulatory region (~150 bp) using duodenal nuclear extract. We present evidence that Onecut (OC) transcription factors, YY1, and NFI family members bind to specific sites located within these footprints. Transgenic mouse studies are described that demonstrate that OC factor binding is functional and critical to temporal control. This is especially interesting because OC factors are known to be important in con-

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2 The abbreviations used are: ADA, adenosine deaminase; EMSA, electrophoretic mobility shift assay; OC, Onecut; HS-C, hypersensitive site C; CAT, chloramphenicol acetyltransferase; RT, reverse transcription; Pn, postnatal day n; FP, footprint.
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trolling development and cell differentiation in pancreas and liver (9–14), but almost nothing is known about their function in intestine. Three different members of the Onecut family have been identified in higher vertebrates: OC-1/HNF-6 (15), OC-2 (10, 16), and OC-3 (17). Their expression patterns, relative roles in different tissues, and relationships to one another (including regulation hierarchy and functional redundancy) are just beginning to be worked out (18). We present a preliminary evaluation of expression patterns for these three OC factors in tissues of the mouse gastrointestinal tract.

**EXPERIMENTAL PROCEDURES**

Footprinting—DNase I footprinting was carried out essentially as described previously (19). The plasmid pUC 660 (7) containing HS-C was used to generate radiolabeled DNA for both DNA strands. For upper strand footprinting pUC 660 was digested with NcoI, and the ends were filled using Klenow, [32P]dCTP, and cold free nucleotides. Labeled DNA was then digested with BamHI, and the 359-bp band was gel-purified. For lower strand footprinting pUC 660 was digested with BamHI, and the ends were filled with Klenow, [32P]dGTP, and cold free nucleotides. Labeled DNA was then digested with NcoI, and the 359-bp band was isolated and gel-purified. Specific activity of labeled DNA was 0–25 cpm/ng. DNase I footprinting reactions were performed on each fragment using 0–25 μg of duodenal nuclear extract (see EMSA experiments below).

**Oligonucleotides**—Separate oligonucleotides containing consensus binding sequences for OC factors, YY1, or NFI factors were synthesized at the University of Cincinnati DNA core facility. The OC consensus oligonucleotide sequence is AGGC-CGATATTGATTTTTTTT (20), and the consensus oligonucleotide sequences for YY1 and NFI are as published in the Santa Cruz Biotechnology catalog. Mutant oligonucleotides used in EMSA, as well as wild-type oligonucleotides containing the putative binding sites for OC, YY1, and NFI in HS-C, were synthesized by Integrated DNA Technologies, Inc (Coralville, IA) and are shown in Table 1. Probe oligonucleotides used in the EMSA experiments were gel-purified and quantitated as described previously (21).

**EMSA**—Mouse duodenal nuclear extract was prepared from 30–40 nontransgenic adult mice as previously described (21, 22). Probe oligonucleotides were purified, duplexed, and labeled as described previously (7). Binding reactions were performed as previously reported (7) using 10 μg of extract for each gel shift reaction. Competition studies were performed by adding a 100-fold molar excess of competitor oligonucleotide to each reaction. Anti-OC-1 and anti-YY1 antibodies were purchased from Santa Cruz Biotechnology. Anti-OC-2 and -3 antibodies (17, 23) are the generous gifts of Frederic Lemaigre.

**Plasmid Constructions, Mutagenesis, and Transgene Preparation**—All of the enzymes were purchased from New England Biolabs. The plasmid pALT 3.4 (7) was constructed as described previously (7). This plasmid was digested with BsiWI, and the resulting 376-bp fragment containing HS-C was isolated and purified. This fragment was then ligated into BsiWI-digested pALTER BsiWI (21) to form pALTER 376+. Single-stranded DNA template was prepared from this phagemid, and site-directed mutagenesis was performed according to the Altered Sites protocol (Promega). A mutated OC-binding site was produced using the oligonucleotide TAAATTGAAGAGATGGCGAGTTTCTGG to form pALT-OCmut. The OC mutant 376+ bp HS-C fragment was sequenced to ensure the fidelity of the entire sequence. This fragment was then liberated with BsiWI and ligated into BsiWI-cut p5′/acbaL117ΔC+ (7) to generate p5′/acbaL117+OCmut. This plasmid was digested with NdeI and PvuI, and the resulting 18,471-bp fragment was isolated, purified, and microinjected at Cincinnati Children’s Hospital Research Foundation transgenic core facility to generate OC mutant mice.

**Transgenic Mouse Analysis**—The transgene listed as wild-type has been described and characterized previously as Transgene IV (6). Adult F1-F2 mouse analysis was routinely performed between 4 and 6 weeks of age and, for temporal studies, at other times as noted. CAT assays, protein assays, and transgene copy number analyses were performed as reported previously (6, 19). CAT activity is expressed in units of pmol/h/100 μg of protein/copy number. Tissues routinely assayed for each line include liver, spleen, thymus, tongue, esophagus, stomach, duodenum, jejunum, ileum, and colon.

**Reverse Transcription (RT)-PCR**—Total RNA was prepared as described previously (24) from the following adult and P2 nontransgenic FVB/N mouse tissues: liver, stomach, duodenum, jejunum, ileum, and colon. Poly(A)+ mRNA was then isolated according to the Oligotex mRNA purification system protocol from Qiagen. Reverse transcription of the poly(A)+ mRNA was performed as in the Invitrogen protocol using SuperScript II reverse transcriptase to synthesize cDNA from 500 ng of adult samples and 250 ng of P2 samples. For PCR, 1 μl of undiluted RT DNA was added to a total volume of 50 μl containing 1× Taq PCR buffer-Mg, 1.5 mM MgCl2, 40 μM dNTP mixture, 10 pmol of each primer, and 2.5 units of Taq DNA polymerase (Invitrogen). The contents of each tube were then overlaid with 50 μl of mineral oil, and PCR was performed as follows: 1 denaturing cycle at 94 °C for 5 min; 26 amplification cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and an additional incubation cycle at 72 °C for 5 min. The following primer pairs were used: OC-1/Hnf6 and OC-2 (13), amplifying 350- and 247-bp fragments, respectively, and OC-3, which is comprised of moc3.4-shortened by 10 bases on the 5′ end and moc3.16 (17), amplifying a 322-bp fragment. PCR products were detected by ethidium bromide staining after electrophoresis in a 2% agarose gel.

**RESULTS**

A Novel Temporal Element Located in HS-C Contains Binding Sites for OC, YY1, and NFI Transcription Factors—A 3.4-kb segment located near the second intron of the human adenosine deaminase (ADA) gene has previously been identified that regulates high level intestinal expression of ADA along three axes, including the temporal axis (6, 7). Seven duodenum-specific hypersensitive sites were identified within this 3.4-kb segment and designated as HS-A through HS-G (6) (Fig. 1). Within HS-D, a core region of ~300 bp was identified that acts as a potent enhancer in the duodenum (7). It is this enhancer that is absolutely critical for high level gene activation in the small
intestine, specifically in duodenal enterocytes of the villus epithelium. Previous studies have identified multiple transcription factors that bind to the enhancer, including Pdx-1, GATA, Cdx, YY1, and NFI, and their functional roles within the enhancer have been characterized (21, 22, 25). Directly upstream from the enhancer, a novel element has been identified that controls the timing of activation of this duodenum-specific enhancer.

This temporal element has been mapped to an ~300-bp segment located within HS-C (7).

To begin to identify and characterize potential factors binding to this temporal element, DNase footprinting experiments with human, rat, and mouse duodenal nuclear extracts were performed. A similar pattern of protection was seen with all three extracts on both DNA strands (Fig. 2). The footprints observed on both the upper and lower strand of HS-C are shown in Fig. 2 (A and B, respectively), and their location on the DNA sequence is summarized in Fig. 2C. Three distinct footprints were identified on each strand, with footprint 3 appearing bipartite on the upper strand. Footprint 2 is much more evident on the lower strand in Fig. 2B than the upper in Fig. 2A. The TRANSFAC transcription factor data base (26), along with a data base of factor-binding sites related to gastrointestinal gene regulation that our laboratory has assembled from the literature, were used in homol-
ogy searches to identify potential transcription factor-binding sites within the footprinted regions of HS-C. A number of potential factor-binding sites were identified in this manner, including (a) a single OC site located in FP-2; (b) two YY1 sites, one located in FP-1 and another overlapping the OC site in FP-2; and (c) a single NFI site located in FP-3.

### Table 1: Sequence of the OC-, YY1-, and NFI-binding sites in HS-C

The consensus (cs) binding site sequences for OC, YY1, and NFI are shown. Also shown are oligonucleotides containing the putative OC- (site2), YY1- (site2 and site1), and NFI-binding (site3) sites within the HS-C region of the human ADA gene, with the proposed transcription factor binding sites highlighted in bold type.

| Site | Sequence |
|------|----------|
| OC cs | AAAATCCATAT |
| site2 | GAATTACCAATATGCCAGCTT |
| OCmut | GAATTAAGTAGCCGACCTT |
| YY1 cs | RMCATBTGNC |
| site2 | AGGCTGCCATATTAAATTC |
| YY1m2 | AGGCTGAGGATGTTAMTC |
| site1 | CACTATTCTCGCACCTTTTCAC |
| YY1m1 | CACTATTCTCGGTCATTTCAC |
| NFI cs | AGGCCAAT |
| site3 | CACCTCTGTTGTCGCAAA |

**A.** EMSAs with mouse duodenal nuclear extract demonstrate binding of OC and YY1 to sites in HS-C, and mutations in these binding regions eliminate the formation of specific protein complexes. **B.** OC, YY1, and NFI Bind to Specific Sites in HS-C, and Point Mutations in the OC and YY1 Sites Specifically Ablate the Binding of These Proteins—Shown in Table 1 are the consensus binding sites for the OC family of factors (20), for YY1 (26, 27), and for the NFI family (Santa Cruz Biotechnology), with the core recognition sequence for each of the factors boxed in. Oligonucleotides were synthesized that correspond to the sequence of the footprints in HS-C where OC, YY1, and NFI were thought to bind, so that EMSAs could be carried out. The sequences of these oligonucleotides, as well as the relative position of the putative binding site of each factor, are shown in Table 1. Site2, found in FP-2, encompasses the proposed overlapping binding sites of OC and YY1. Upstream from this region, there is a putative second YY1 binding site, which is located in FP-1 and encompassed by oligonucleotide site1. Finally, in FP-3 there is a proposed binding site for NFI, as indicated in the sequence for oligonucleotide site3.

Oligonucleotides for each of these three sites were duplexed and gel-purified to use as probes, and EMSAs were performed (Fig. 3). As seen in Fig. 3A, three specific shifted complexes are observed with the site2 probe upon addition of 10 µg of mouse duodenal nuclear extract (lane 1). Two of these bands could be specifically competed away by the addition of 100-fold molar excess of unlabeled OC consensus oligonucleotide (lanes 2, 4). The third (middle) band could be competed away with 100-fold excess of YY1 consensus oligonucleotide (lane 3). Formation of all three complexes was ablated by a combination of OC and YY1 consensus oligonucleotides in excess (lane 4). These results support binding of OC factors and YY1 to the proposed

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overlapping sites. Because there are three known members of the OC family of transcription factors, we wanted to identify which member(s) might be involved in the formation of the putative OC-specific shifted complexes. Therefore, EMSA supershift experiments were performed using the site2 probe and antibodies specific to OC-1, OC-2, and OC-3 (lanes 5–7). Supershifted complexes were seen with the addition of anti-OC-1 and anti-OC-2 antibodies, but not with the anti-OC-3 antibody. As well as producing a supershifted band, the anti-OC-2 antibody abrogated formation of both of the shifted OC bands. This strongly suggests that OC-2, along with OC-1, is present in mouse duodenal nuclear extract, and they both bind to this putative OC recognition sequence. To confirm the specificity of the YY1 shifted complex, anti-YY1 antibody was added to the binding reaction, and a supershifted complex was observed (lanes 8 and 9). No supershift was observed with the addition of nonspecific antibody (lane 10).

EMSA experiments with the site1 oligonucleotide probe were used to confirm binding of YY1 to the proposed site in FP-1 (Fig. 3B, lanes 13 and 14). Similar experiments were used to confirm binding of NFI to the proposed site in FP-3 (lanes 17–19). Competition with the NFI consensus oligonucleotide specifically ablated formation of the broad NFI band formed with the site3 oligonucleotide.

We wanted to identify site-specific mutations that independently eliminate binding of OC and YY1 to the site2 oligonucleotide without affecting binding of the other factor. Therefore, a series of specific point mutations were introduced into site2, and changes in binding were analyzed by EMSA. Appropriate mutations were identified, and the resultant mutant oligonucleotides were designated as OCmut and YY1m2 in Table 1. EMSA experiments confirming the ability of the mutations to eliminate binding of the targeted protein without affecting the binding of other proteins are shown in Fig. 3B. In these gel shifts, the mutated oligonucleotides were labeled as probes, and the wild-type site2 oligonucleotide was included for comparison. Lanes 1–3 and 7–9 show the OC and YY1 complexes seen previously with the site2 probe. When the OCmut oligonucleotide was used as probe, the OC-specific complexes did not form (lanes 4–6), but YY1 binding was not perturbed. When the YY1m2 probe was used, the YY1 complex did not form, and OC binding was unaffected (lanes 10–12). It is interesting to note that OC binding is unchanged, even though the OC consensus core TCAAT is altered to TCGAT in the YY1m2 probe, indicating some flexibility in this recognition sequence. To investigate binding of YY1 to FP-1, oligonucleotides were also synthesized with mutations to the YY1 core in site1, and an effective mutation was identified (YY1m1). Lanes 13–16 show that mutation of the YY1 site in the YY1m1 mutant oligonucleotide completely ablates binding of YY1 to the upstream site1 segment of HS-C.

**OC Mutant Mice Exhibit a Delay in the Timing of Gene Activation**—The identification of a putative OC-binding site in the temporal element was especially intriguing, because almost nothing is known about the role of OC factors in intestine. To begin to examine a possible in vivo role of OC factors in temporal regulation, transgenic mice were produced from a construction based on the mutated oligonucleotide (OCmut) that resulted in loss of OC binding to site2 in FP-2. The basic design of the transgene has been used previously and is diagrammed schematically in Fig. 4A (6, 7, 21, 22, 25). In the transgene construction, the mutated HS-C segment replaced the wild-type HS-C segment within a 13-kb intragenic ADA fragment, maintaining position and orientation. The enhancer element, found just downstream of HS-C, was left intact. Various tissues from F1 or F2 transgenic mice from three independent OC mutant lines were analyzed for CAT activity. CAT activity was normalized to protein concentration and copy number. In each line, activity was specific to duodenum, with duodenal CAT activity ~100–800-fold higher than any other tissue. This pattern is very similar to that observed previously with this basic transgenic construction (7, 21, 22, 25). The level of duodenal CAT activity in adult wild-type transgenic mice has been reported previously (6, 7, 21, 22, 25) and ranges from 1,400–31,000 units. Previously, our lab has also analyzed adult transgenic mice with the entire HS-C region deleted, and these mice exhibited levels of CAT activity of 7,100–74,000, demonstrating that this region is not strictly required for enhancer activation (7). Two of the OC mutant lines (Lines 1 and 2) had activity levels well within the normal range, with duodenal CAT values of 3,400 and 37,000 units, respectively (Table 2). In the third line of OC mutant mice (Line 3), the enhancer appeared to never activate significantly, because these mice displayed a low duodenal CAT activity of 200, a value well outside the normal range.

As described above, HS-C is not required for high level CAT activity in adult transgenic mice, but it does control the developmental timing of gene activation. Previous studies have shown that in wild-type transgenic mice, activation of duodenal expression occurs at or near the time of birth (6). Deletion of the HS-C region delays this activation by 2–3 weeks. We examined the temporal patterns of expression in Lines 1 and 2 of the OC mutant mice and compared them to the wild type (Fig. 4B). Both lines of OC mutant mice display a temporal pattern that is clearly delayed compared with that of the wild-type mice. This temporal pattern is quite similar to the pattern observed when the entire HS-C region is deleted (Transgene XII) (7). This result indicates that OneCut factors play a significant role in temporal regulation by the HS-C element.

**Expression Patterns of OC-1, -2, and -3 in Adult and Postnatal Day 2 Mice**—OC factors are known to be expressed in mouse intestine (16, 17). However, because our transgenic mouse results indicate that OC factors may play a critical role in temporal regulation, we wanted to determine the tissue-specific expression of each at crucial developmental time points. To make an initial determination of the tissue distribution of OC-1, -2, and -3 mRNA, RT-PCR was performed on RNA from several adult and P2 organs (Fig. 5). In adult mice, OC-1 mRNA is present in the liver, stomach, and duodenum, whereas in P2 mice expression is limited to liver and stomach and is not detectible in duodenum. In adult tissues, OC-2 mRNA was evident in liver, stomach, duodenum, and jejunum, with by far the strongest signal in the duodenum. With the exception of a lack of detectable signal in jejunum, the expression pattern of OC-2 mRNA in P2 mouse tissues is similar to the adult pattern. OC-3 mRNA is found in adult stomach, duodenum, jejunum, and
ileum, whereas in P2 mice this mRNA is present in the same tissues, as well as in colon. These results confirm that all three of the OC factors are expressed in intestine. However, each demonstrates a distinct tissue-specific pattern of expression. In addition, there are significant changes in the patterns between the two developmental time points examined. All three OC factors are co-expressed in adult duodenum, with OC-2 predominant, whereas in P2 duodenum only OC-2 and OC-3 are detectable. The significance of this pattern in relation to temporal regulation remains to be investigated.

**DISCUSSION**

Dramatic changes in gene expression are associated with development of intestinal epithelium in mice during late fetal and early post-natal stages. In mouse, morphogenesis of the gut epithelium occurs rather late in development, from embryonic day 15 through approximately P21. The mouse gut endoderm undergoes rapid remodeling from embryonic days 15–19 as a proximal-to-distal wave of cytodifferentiation converts it from a pseudostratified to a simple columnar epithelium (28). This monolayer covers nascent villi that are separated from one another by a polyclonal proliferative compartment known as the intervilus epithelium, the precursor of the intestinal crypt. A process of cell selection occurs during crypt formation that converts them to monoclonal compartments by P14. Days P14–P21 are characterized by a dramatic increase in crypt number. The end product is an adult mouse epithelium in which well formed villus structures are covered with columns of epithelial cells emanating from several adjacent crypts. As one might expect, significant changes in enzyme expression patterns along the gut occur that are associated with the developmental sequence of events, before the final adult mouse profile is established.

In humans, similar temporal changes are observed much earlier, while in utero. Villi appear along the length of the human small intestine between 8 and 12 weeks of gestation, developing in a similar caudal wave from duodenum to ileum (29). Primitive crypts appear soon after. Late fetal intestine in humans has a crypt-villus morphology and an enzyme expression pattern very similar to that in adult intestine. The regulatory pathways and fac-

![Figure 4](image-url)

**TABLE 2**

| Tissue-specific CAT activities of OC mutant transgenic mice | Liver | Spleen | Thymus | Tongue | Esophagus | Stomach | Duodenum | Jejunum | Ileum | Colon |
|-----------------------------------------------------------|------|-------|-------|-------|---------|--------|----------|--------|------|-------|
| Line 1                                                    | 3.9  | 5.1   | 9.7   | 11.2  | 25.9    | 3.5    | 3,400    | 3.8    | 4.0  | 5.9   |
| Line 2                                                    | 1.2  | 43.8  | 10.6  | 6.2   | 3.0     | 3.9    | 36,900   | 22.1   | 5.3  | 8.3   |
Our laboratory previously identified an intragenic region in the human ADA gene that regulates ADA expression in the small intestine (6, 7). Transgenic mouse studies were used to identify and map a powerful enhancer within this region that activates expression specifically in the duodenum. Alone, this enhancer is capable of driving reporter gene expression in a pattern that recapitulates exactly, in transgenic mice, all aspects of the endogenous intestinal pattern of mouse ADA. Like ADA, the transgenic CAT reporter is highly expressed in duodenal enterocytes all along the villous epithelium (6, 7). Seven duodenum-specific hypersensitive sites were identified within the ADA intestinal regulatory region (designated HS-A through HS-G), and the essential core of the duodenal enhancer was mapped to HS-D. In addition, temporal control elements were identified that significantly altered the timing of enhancer activation when included in transgene constructions. The resulting pattern of perinatal duodenal activation appears more similar to that observed for the endogenous human ADA gene, from which the entire regulatory domain was isolated, than mouse. One of the temporal elements was mapped to HS-C (7). Detailed characterization of that element is the focus of the studies described in this publication. It seems likely that the factors and mechanisms functioning in the ADA temporal element may also play a more general role in the temporal regulation of other intestinal genes. This is especially true of those, such as lactase and sucrase, that show significant change in expression during perinatal and early postnatal development. Our characterization of the HS-C element should make such analytical comparisons possible. It will also be of great interest to determine whether other enhancers have similar associated temporal elements that control timing of enhancer activation, either in intestine or in other tissues.

Our transgenic mouse results strongly support a critical role for OC factors in the function of the HS-C temporal element. Ablation of OC binding dramatically altered the timing of enhancer activation in two of the three transgenic lines analyzed. The resulting pattern of activation at about 2–3 weeks resembled closely that observed when the entire HS-C temporal domain was deleted from the transgene (7). The third line of mice never showed significant activation of the duodenal transgene. It is not clear whether this is a result of the OC site mutation or is related to the transgene insertion site. However, the latter seems likely, because transgenes with the entire HS-C region deleted still demonstrate normal levels of transgene activation after the delay (7).

Because there are three vertebrate OC factor paralogs, we wanted to begin to assess their potential relative roles in the temporal function of the HS-C element. Previous studies had shown that all three OC factors are expressed in embryonic mouse endoderm and developing gut (16, 17, 18). Adult mouse intestinal expression has also been observed for OC-2 and OC-3 (17). Because of the developmental time frame of temporal element function, we wanted to directly compare OC expression at P2 to adult. OC-2 and OC-3 are each expressed in both adult and P2 duodenum. OC-2 is the predominate OC factor expressed at both time points, although the levels appear more similar at P2. Interestingly, at both times, the extent of OC-3 expression along the intestine was much more posterior...
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than OC-2. In fact, OC-3 expression was evident through all segments of the small and large intestine at P2. Intestinal expression of OC-1 was detected only in adult duodenum, but not P2 duodenum. Previously, OC-1 was reported to be absent from adult mouse intestine (17), but this apparent discrepancy may be due to sampling differences. The previously analyzed tissue was reported only as “gut.” Because we observed OC-1 at low levels and only in the most anterior portion of the small intestine, this might explain the difference.

EMSA binding and competition experiments verified that the putative Onecut binding site in HS-C footprint 2 was bound by OC factors from adult mouse duodenal nuclear extract. OC-2 was the predominant factor bound, as demonstrated by supershift and antibody inhibition, and this probably reflects the higher relative levels of OC-2 present. However, the supershift analysis also confirmed the presence of OC-1, which bound at lower levels. Interestingly, the EMSA analysis did not provide evidence of OC-3 binding. This may reflect the lower abundance of OC-3, the quality of the antibody used for supershift analysis, or differential recognition of the binding site by the OC factors. However, it has been published previously that the binding specificity of OC-1 and OC-3 are similar (17). The OC shifted bands in our EMSA experiments appear as a doublet. Based on the antibody supershift results, these two bands do not result from binding of separate OC factors, because both are significantly inhibited by OC-2-specific antibody. Two discrete isoforms of OC-1/HNF-6 have previously been shown to exist (37). It is likely that discrete isoforms of OC-2, and perhaps OC-3, also exist. Determination of the specific contribution and relative roles of each of the OC factors in HS-C temporal element function will require additional studies. In embryonic endoderm studies, OC-1, -2, and -3 were found to be expressed sequentially, and OC-1 was thought to regulate OC-3 expression directly (18). How many of the OC factor functions are distinct and how many are overlapping or redundant remains to be determined. For example, it is unclear whether the changes in OC-1 and OC-3 intestinal levels observed between P2 and adult are significant in temporal regulation. In future studies, it will be especially important to elucidate the cell type-specific expression pattern of each of the OC factors in the intestinal epithelium, because the ADA enhancer and temporal control element function only in duodenal enterocytes, with activation occurring at the crypt-villus junction (6).

EMSA studies also identified two YY1-binding sites and an NFI site. It will be of great interest to test their in vivo role in transgenic mice in studies similar to those performed for the OC site. This is especially true of the YY1 sites, because the YY1 sites within the enhancer itself have already been shown to play a role in the timing of enhancer activation (22). Cdx factor binding within the enhancer domain has been shown to play a critical role in temporal activation, as well (22). Ablation of Cdx binding in the enhancer resulted in a delay in enhancer activation very similar to that resulting from the OC site mutation in HS-C. Our working hypothesis is that the temporal control element exerts its control through direct protein-protein interactions with the enhancer-bound factors, such as Cdx1/2 and YY1. Our initial hypothesis about the functional nature of the temporal control element was that it might control chromatin activation (opening) in the enhancer domain at specific developmental times. In this way, they would control accessibility of nuclear factors to their binding sites and therefore control the timing of enhancer complex formation at HS-D. To test this hypothesis, we previously carried out nuclear DNase I hypersensitivity studies on transgenic mouse lines derived from constructions in which the HS-C temporal control was removed (21). The complete deletion of HS-C had no effect on formation of HS-D, in duodenal nuclei from either newborn or adult mice, when compared with the wild-type (undeleted) transgene. This result makes it unlikely that the HS-C domain functions in temporal control by regulating factor access to HS-D. Enhancers are currently thought to communicate with their target promoters by looping, tracking, or a combination of both (38–41). The location of the ADA temporal element between the enhancer and promoter suggests the possibility that it might function by facilitating such looping/tracking. At present, there is no direct evidence to support this suggestion. Additional studies will be required to begin to understand the mechanisms by which the temporal domain exerts its control and the role that factors like the OC factors play. However, this temporal element does appear to represent an excitingly novel type of regulatory domain, and such studies should be quite informative.

Acknowledgments—We thank Frederic Lemaigre and Patrick Jacquemin for the generous gifts of anti-OC-2 and-3 antibodies and OC expression vectors used to optimize conditions for the RT-PCR experiments. We also thank Bradford Bemiss for technical assistance and maintenance of the mouse colony.

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