PDX-1 is required for activation in vivo from a duodenum-specific enhancer

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SUMMARY

The purine metabolic gene adenosine deaminase (ADA) is expressed along a defined spatiotemporal pattern in the developing mammalian small intestine where high-level expression is limited to the villous epithelium of the duodenum. This activation is observed in rodents as the intestine completes the final maturation resulting in adult crypt-villus structures at 2-3 weeks post-partum. A regulatory module responsible for this pattern of expression has been identified in the second intron of the human ADA gene. Of the multiple duodenal proteins that can interact with this small duodenal enhancer region, the studies contained in this work describe the identification of five of these proteins as the dispersed homeobox protein PDX-1. This transcription factor exhibits a profile of expression in the small intestine similar to that observed for ADA making it an ideal candidate factor for the duodenum-specific ADA enhancer. Loss of PDX-1 binding, via a PDX-1 mutated enhancer transgenic construction, resulted in complete loss of high-level activation in the duodenum demonstrating the absolute requirement for this factor in vivo. However, co-transfection experiments suggest that other proteins that bind the enhancer are also required for enhancer function, since PDX-1 alone was incapable of significant transactivation.
INTRODUCTION

Many molecular events and interactions are required in the complex process of organogenesis of the gut. All of these events must be regulated within both space and time to result in a functional gastrointestinal tract. The lining of the vertebrate small intestine is an epithelial monolayer of endodermal origin that is folded into finger-like projections called villi and recessed pits called crypts. This monolayer is composed of four differentiated cell lineages. The majority of cells are enterocytes that express a wide range of genes required for digestion and absorption. Also present throughout the epithelium are enteroendocrine cells that secrete peptide hormones and a variety of growth factors, mucous-secreting goblet cells and Paneth cells that express defensins. All four cell types are derived from a small number of anchored stem cells located near the crypt base [1, 2]. As cells migrate away from the stem cells the process of lineage allocation begins. Paneth cells differentiate and migrate to the base of the crypts. The remaining cell types migrate from the proliferative compartment in the crypt onto the surface of the villus as they complete terminal differentiation. The constant production of differentiating daughter cells by the stem cells causes the movement of fully differentiated cells along the length of the villus. At the tip of the villus, cells are either sloughed into the intestinal lumen or undergo apoptosis [3]. Lineage allocation and terminal differentiation occur along this crypt-to-villus (C/V) axis throughout the length of the intestine.

Along its length, the small intestine is traditionally divided into three sections: duodenum, jejunum (sometimes subdivided into proximal and distal), and ileum. Although each differentiated cell type is produced along the length of the intestine, they
manufacture widely differing gene products that result in functional demarcations that correspond to the physical demarcations. Development from duodenum to ileum is often referred to as anterior-to-posterior (A/P) development. Both C/V and A/P development are regulated with respect to developmental time. The A/P positional identity is established early in embryogenesis and is maintained throughout the life of the organism [4]. The intestinal epithelium begins as a cuboidal monolayer of endodermal epithelium devoid of crypt/villus structures. An initial wave of development resulting in primitive crypt/villus structures begins at eight weeks gestation in humans and shortly before birth in mice. Maturation of the epithelium into adult crypt/villus structures is completed by 12 weeks gestation in humans [5, 6] and 2-3 weeks after birth in mice [7].

In order to delineate some of the underlying mechanisms involved in determining positional identity along the A/P axis, lineage allocation along the C/V axis, and developmental timing of both, we have chosen to use a purine metabolic gene, adenosine deaminase (ADA), as a model. ADA is expressed in a well-defined pattern that is conserved among mammals [8, 9]. Within the intestine, high-level ADA expression is limited to the proximal small intestine, the duodenum, along the A/P axis. Within the duodenum of both mice and humans, high levels of ADA are limited to the villous epithelium where expression is found in the enterocyte lineage of terminally differentiated cells along the C/V axis [10, 11]. High-level, duodenum-specific expression also exhibits developmental regulation. In mice, increased ADA levels are observed at the suckling-weaning transition coinciding with terminal formation of adult crypt/villus structures [10, 12, 13]. A similar developmental pattern has been assumed for human, but not shown, as this differentiation event occurs in utero. It is supported by
the fact that duodenal ADA levels are quite high at birth in humans (DAW, unpublished results).

The region responsible for this pattern of expression is located in the second intron of the human ADA gene. Transgenic mice containing an enhancer fragment from this region of intron two express a linked reporter gene in a manner identical to the endogenous ADA gene along both the A/P and C/V axes of development. Both the reporter gene and ADA exhibit high-level expression limited to the duodenal enterocytes of the villous epithelium. The enhancer contains multiple DNase I hypersensitive sites in duodenum, of which one is predominant, hypersensitive site D (HSD) [13]. The region containing HSD has been observed to be absolutely required for enhancer function. Removal of this small region of DNA results in a specific and total loss of duodenum-specific activation. DNase I footprinting analysis showed that this segment of 319 bp was able to interact with multiple proteins from duodenal nuclear extract [14]. Sequence analysis of these footprinted regions for transcription factor consensus binding sites yielded a list of both ubiquitous and intestinally restricted factors as potential candidates. In this study we identify the factor PDX-1 as a protein bound to multiple sites within the ADA enhancer. Studies that demonstrate the absolute requirement of PDX-1 for enhancer function in vivo are also presented. The identification of this protein and investigation of its role in enhancer function is a vital step to modeling how ADA is regulated along each axis of development in the intestine.
EXPERIMENTAL PROCEDURES

Preparation of intestinal nuclear extracts for EMSA experiments- All materials, rotors, solutions, etc. are at 4°C unless otherwise stated. All buffers (except buffer D) were supplemented with 0.5 mM PEFABLOC (Roche Molecular Biochemicals; Indianapolis, IN), 1 µg/ml aprotinin, 1 µg/ml leupeptin, 130 µM bestatin, 1 µg/ml pepstatin A, and 0.5 mM DTT. A 2 cm³ segment of frozen human duodenum or 30-40 FVB/N mouse duodena were harvested, split lengthwise, and washed vigorously in buffer A (50 mM N-acetyl-L-cysteine, 320 mM sucrose, 50 mM HEPES-KOH pH 7.9, 25 mM KCl, 5 mM MgCl₂, 1 mMCaCl₂, 1% milk powder) on ice. Tissues were transferred to a new aliquot of buffer A (20-25 mls) and homogenized briefly (about 5 sec.) with an Ultra Turrax homogenizer after addition of every 10 duodena. Mild homogenization prevented disruption of the muscular layer which is subsequently removed by filtering the homogenate through spectra-por mesh and then homogenized 20-30 strokes in a ground glass homogenizer. Nuclei were pelleted at 2,000 RPM, 4°C in Beckman JS13.1. The pellet was washed twice in 10 mls of buffer A and resuspended in 3 mls of buffer A, which was then mixed with 60 mls of buffer B (2.2M sucrose, 50 mM HEPES-KOH pH7.9, 25 mM KCl, 5 mM MgCl₂ and 1 mM CaCl₂). The nuclei were layered equally over 15 mls of buffer B and spun in an SW28 rotor for 1 hour at 50,000 RPM at 4°C. The supernatant was aspirated and the pellet resuspended in 10 mls buffer C (20 mM HEPES-KOH pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂ and 0.2 mM EDTA). Resuspended nuclei were lysed by homogenization, mixed 30 minutes on ice and spun 15,000 RPM at 4°C for 30 minutes in SW 60. Nuclear proteins were precipitated with NH₄SO₄, collected by centrifugation for 25 minutes at 35,000 RPM at 4°C in SW60. The
protein pellet was resuspended in 0.5-1.0 ml Buffer D (20 mM HEPES-KOH pH 7.9, 20% glycerol, 0.1 M KCl, and 0.2 mM EDTA) and dialyzed against buffer D. Protein concentrations were assessed using BioRad protein assay (Bio Rad Laboratories; Hercules, CA).

**Oligonucleotides**- All oligonucleotides were synthesized at the University of Cincinnati DNA core facility and are as shown in Figure 1. Mutated oligonucleotide sequences are identical to the wild type oligonucleotides except for the double point mutations listed in Table 1. P1 oligonucleotide sequence is GCCCTTAATGGGCCAAACGGCA [15]. Opposite strand DNA’s were resuspended and mixed in equimolar amounts. DNA mixtures were heated to 90°C for 10 minutes and then slow cooled to 25°C to duplex. An aliquot was diluted to 10 pmole/µl and served as 100X competitor in gel shift experiments. Another aliquot was purified through 4% Biogel (Qbiogene; Carlsbad, CA) and the DNA isolated using the Mermaid kit (Qbiogene). This purified oligonucleotide preparation was quantitated, diluted to 0.5 pmole/µl and used as labeling stock.

**In vitro transcription/translation of PDX-1**- The plasmid pKK4 containing the PDX-1 cDNA was a gift from Dr. Helena Edlund [16]. It was linearized with HindIII and used as a template for in vitro transcription/translation using TNT T7 coupled reticulocyte lysate (Promega; Madison, WI) as per the manufacturer’s instructions.

**Electrophoretic mobility shift assay (EMSA)**- 0.5 pmole of gel-purified, duplexed oligonucleotide was labeled using gamma $^{32}$P (6000 Ci/mmol) and polynucleotide kinase. Labeled oligonucleotides were purified using G-25 Sephadex spin columns (Roche Molecular Biochemicals) in a volume of 50 µl. Binding reactions contained 5 µl of 5X
buffer (125 mM Tris pH 8.0, 32.5 mM MgCl₂, 2.5 mM DTT, 2.5 mM EDTA, 250 mM KCl, and 0.6 µg/µl BSA), 0.5 µl of polydI:dC/dA:dT at 2 µg/µl, 10 µl of nuclear extract or extract diluted with D buffer (as in nuclei preparation), competitor oligonucleotide and/or antibody as needed plus water to a final volume of 24 µl. Binding reactions were incubated 15 minutes on ice after which 1 µl of probe (10 fmol at 40,000-80,000 cpm) was added and incubation continued for 15 minutes on ice. The entire reaction mix was loaded onto a 6% polyacrylamide gel and electrophoresed at 4 °C, 30mA, in 1X glycine buffer (50 mM Tris base, 0.38 M glycine, 2 mM EDTA). Gels were dried in vacuo and exposed to X-ray film overnight. Anti-PDX-1 antibody was the generous gift of Dr. Helena Edlund.

Plasmid constructions and mutagenesis- pALTER-1 (Promega) was digested with SmaI and BsiWI linkers (New England BioLabs; Beverly, MA) were ligated to form pALTER-1 BsiWI. The 319 bp BsiWI fragment containing the enhancer was isolated from pALT 3.4(-)Dmut [14] and ligated into BsiWI digested pALTER-1 to form pALTER 319+. Single-stranded DNA was produced from this phagemid and site-directed mutagenesis was performed according to the Altered sites protocol (Promega). Successive rounds of mutagenesis were used to introduce all of the double point mutations shown in Table 1 into the enhancer fragment. The mutated BsiWI enhancer fragment was sequenced in its entirety to ensure fidelity of both the mutated sites and the remaining sequences. The mutated enhancer fragment was liberated with BsiWI and ligated into BsiWI cut p5'acba L117ΔD [14] to generate p5’acba L117 PDX mut. The target vector for co-transfection analysis is the plasmid previously described as p5’acbaΔ3 [13]. An RSV-driven PDX-1 expression vector and the control target
pInsulin-CAT were a generous gift from Dr. Helena Edlund [16]. The empty expression vector pRSV0 was made from the PDX-1 expression vector by removing the PDX-1 cDNA and religating the plasmid. pGEM 4Z was from Promega.

Transgene fragments, mice and analysis- Transgenes listed as wild type and Δ enhancer have been previously described and characterized as TG IV and TG XIII respectively [13, 14]. p5’acba L117 PDX mut was digested with NdeI and PvuI. The resulting 18,471 bp fragment was isolated and purified as described previously [13]. Transgenic mice were made by microinjection at both the Cincinnati Children’s Hospital Research Foundation and the University of Cincinnati core facilities. Analysis of F1 mice was performed between 4-6 weeks. CAT assays, protein determination and copy number analysis was performed as previously described [13]. Tissues assayed included tongue, esophagus, stomach, duodenum, jejunum, ileum, colon, liver spleen, and thymus for each line.

Transient transfection analysis- CHO-K1 (CRL 9618) cells were cultured in HAM’s media with 10% fetal bovine serum, 0.05 units penicillin, and 0.05µg/ml streptomycin. DNA was introduced into cells at 60-70% confluence by lipofectamine transfection (Life Technologies; Rockville MD) according to the manufacturer’s instructions using 7 ul of lipofectamine/sample. 0.1 µg of the target plasmid (p5’acbaΔ3 [13] or pInsulin-CAT) was added alone, with 0.1 µg of the PDX expression vector (pRSV-PDX), or with 0.1 µg of the empty expression vector (pRSV-0). A constant amount of DNA (2 µg) was added to each transfection by making up the difference using pGEM 4Z. Transfections were performed twice in duplicate. Cells were washed 5 hours
post-transfection and harvested 48 hours after transfection. The cell extract was assayed for CAT and protein concentration as described previously [17].
RESULTS

All the information necessary to direct high-level duodenum-specific expression in a pattern similar to the endogenous ADA gene along each axis of intestinal development (cephalocaudal, crypt/villus and temporal) is contained within a 3.4 kb enhancer fragment from intron two of the human ADA gene. A sub-region of 319 bp within this segment that is absolutely required for enhancer function in duodenum is shown in Figure 1A. Regions protected in DNase I footprinting experiments [14] are shown on the sequence in Figure 1A as the gray shaded boxes. Sequences within the footprinted regions were examined by TRANSFAC transcription factor data base search [18] to identify putative binding sites for common transcription factors. Sequences from these protected regions were also subjected to analysis using a search file created in our lab containing DNA sequences implicated in the transcriptional regulation of other genes expressed in the intestine. Among the potential binding sites identified within the footprinted regions are five sequence motifs similar to the known binding site for the pancreatic-duodenum homeobox factor PDX-1 (YHTTAATK) [15, 19] as well as to known PDX-1 binding sites such as one from the rat insulin promoter (P1) [15]. The sequence of these PDX-like motifs and the P1 sequence are shown in Figure 1B.

Five independent PDX-1 binding sites are located in the ADA enhancer - The ability of the PDX-1 motifs within the footprinted regions to bind PDX-1 and possibly other proteins was assessed by electrophoretic mobility shift assay (EMSA). Oligonucleotides from both DNA strands encompassing either the entire footprint or a subregion of each footprint were synthesized, duplexed and gel purified to use as EMSA probes. The location of each oligonucleotide probe is shown as a solid black line in
Figure 1A. Footprint region 2 contained three PDX-type motifs that were separated into oligonucleotides FP 2a, FP 2c, and FP 2d. Footprint region 4 is within the oligonucleotide called FP 4. Likewise, footprint region 5 is located within the oligonucleotide FP 5. This oligonucleotide contains one of the double point mutations created to remove the enhancer [14]. The FP 5 oligonucleotide was synthesized with both the wild type and BsiWI sequences and used as a probe in gel shift experiments. EMSA’s showed no differences in complexes formed using either oligonucleotide (data not shown). Gel shifts presented in this work use the wild type oligonucleotide.

Initial experiments were designed to examine the ability of PDX-binding motifs found in footprint regions 2 (3 sites), 4 and 5 to produce shifted complexes using mouse duodenal nuclear extracts. Production of these extracts was designed to enrich epithelial contribution and minimize the contribution from the underlying intestinal musculature. Results of these gel shift experiments using oligonucleotides containing putative PDX sites, FP 2a, FP 2c, FP 2d, FP 4, and FP 5 are shown in Figure 2. Each of these oligonucleotides and the P1 oligonucleotide was labeled with \(^{32}\)P and used as probe. As mentioned above, P1 contains a known PDX-1 binding site. Each probe was allowed to react with either 10 µl of buffer only (Panel A; Lanes 1, 3, 5, 7, 9, and 11) or 10 µl of buffer containing 10 µg of mouse duodenal nuclear extract (Panel A; Lanes 2, 4, 6, 8, 10, and 12). A trio of shifted complexes is observed using the P1 probe (Lane 2). A similar trio of shifted complexes can be observed for each of the ADA enhancer PDX-motifs, although the intensity of these complexes varies among the sites. Other shifted complexes were observed as well, often superimposed over the three PDX-like complexes. These complexes were subjected to competition experiments (Panel B) as
well as antibody supershift experiments (Panel C). As can be seen clearly in Panel B, the addition of 100-fold molar excess of unlabeled P1 oligonucleotide to the binding reaction containing each of these probes prevents (Lanes 2, 4, 6, and 10) or significantly reduces (Lane 8) formation of these three complexes. This competition is specific for the P1 oligonucleotide and was not observed when a non-specific oligonucleotide or an oligonucleotide with a binding site for a different homeodomain protein (Cdx) was used as competitor in the binding reactions (data not shown). Addition of an anti-PDX-1 antibody to the reactions prohibited formation of the complexes with each probe (Panel C; Lanes 3, 6, 9, 12, and 14). Since the PDX-1 antibody is directed against the DNA binding domain of PDX-1, a DNA–protein interaction is prevented and no complexes or supershifted complexes are formed. A similar effect was not observed upon addition of a non-specific antibody (Panel C; Lanes 2, 5, 8, and 11). These results confirm that the three shifted complexes observed are due to PDX-1 binding to these enhancer sequences. Each of the three complexes seems to react like PDX-1 alone in gel shift experiments. All three complexes were always observed and always in roughly equal amounts despite amount of nuclear protein added and from a number of different preparations of duodenal nuclear extract. This suggests that each complex is the result of a single protein bound to the oligonucleotides and that protein is PDX-1. PDX-1 phosphoproteins have been reported in cells from pancreatic lineages with apparent molecular weights of 30, 42, 45, and 50 kDa. Relative proportion of each phopho-PDX-1 is consistent within a given cell type, but varies between different lineages [20]. Similar studies have not been done with PDX-1 from the duodenum. Yet, it seems likely that the three PDX-1 complexes observed in gel shift using duodenal nuclear extract are due to the presence of three
variously sized phosphorylated species of PDX-1 from the duodenum. This would explain the presence of three protein complexes that behave like PDX-1 alone and are always present in the same relative ratio.

*Mutations specifically ablate PDX-1 binding –* A number of different mutations were introduced into the oligonucleotide sequences at various positions and assessed by gel shift for the effect each had on complex formation (data not shown). Mutations that eliminated only the PDX-1 complexes but not the non-PDX-1 complexes are shown in Table 1 as the bolded lower case letters in the sequences labeled 2a mut, 2c mut, 2d mut, 4 mut, and 5 mut. Gel shifts using these mutated oligonucleotides, in comparison to wild type oligonucleotides are shown in Figure 3. PDX-1 protein was produced by in vitro transcription/translation from a linearized plasmid containing the PDX-1 cDNA [16] and used in the gel shift experiment shown in Figure 3A. Each wild type oligonucleotide was used as probe and was able to form a PDX-1-specific complex upon addition of the PDX-1 protein (Lanes 1, 3, 6, 8, and 11) that co-migrated with the PDX-1 complex produced by the P1 oligonucleotide and PDX-1 protein (Lanes 5 and 10). Additional non-PDX-1 complexes were also observed with some of the oligonucleotides. In contrast, the oligonucleotides containing the PDX-1 binding site mutations shown in Table 1 were unable to interact with the PDX-1 protein and hence did not produce this complex (Lanes 2, 4, 7, 9, and 12) confirming the ability of these mutations to ablate PDX-1 binding.

The final objective of these mutational studies was an enhancer fragment devoid of PDX-1 sites, but with all other potential binding sites intact. As described above, studies using translated PDX-1 protein identified mutations that eliminated PDX-1
binding. In order to assess possible alterations to other protein binding sites, a gel shift using the mutated oligonucleotides as competitor and/or probes was repeated using mouse duodenal nuclear extract (Figure 3B). This extract can generate a more complex set of bands that is more representative of the wide range of proteins, in addition to PDX-1, that can interact with these sequences in vivo. Each lane in Figure 3B contains 10 ug of mouse duodenal nuclear extract. Shifted complexes formed with wild type FP 2c oligonucleotide are shown in Lane 1. The faster migrating complexes are PDX-1. The slower mobility complex marked with an asterisk is not PDX-1. Addition of 100 fold excess of unlabeled the wild type oligonucleotide reduces all complexes (Lane 2). Addition of excess unlabeled mutant oligonucleotide, 2c mut, does not compete the PDX-1 complexes, but does compete the slower mobility * complex (Lane 3). Comparison of the complexes formed when the wild type FP 2c and the mutant, 2c mut, oligonucleotides are used as probes (Lane 4 versus 5) show that both form the slower mobility * complex, while only the wild type forms the PDX-1 complexes. Examination of the sequence in this oligonucleotide reveals a GATA type binding site, WGATAR [21]. This site has recently been shown to bind a GATA protein from duodenal nuclear extract that results in formation of the * complex (MRD, unpublished results). Multiple shifted complexes were also produced with the FP 2d oligonucleotide (Lane 6). PDX-1 complexes could be competed by the addition of excess unlabeled P1 oligonucleotide (Lane 7) that contains a wild type PDX-1 binding site, but not by the addition of excess unlabeled mutant oligonucleotide, 2d mut (Lane 8). The shifted complexes produced by the mutant oligonucleotide 2d mut (Lane 9) look identical to those in Lane 7 produced by the wild type oligonucleotide in the presence of excess unlabeled P1 oligonucleotide (i.e. in the
absence of PDX-1 complexes). This suggests that the mutation to ablate the PDX-1 binding site has not affected the binding sites for these other unknown proteins. Analysis of the sequences within these oligonucleotides suggests that these bands may be the result of another bound homeodomain protein. Shifted complexes produced by FP 4 oligonucleotide (lane 10) are competed by self (Lane 12), but not by the mutant oligonucleotide 4 mut (lane 11). The mutant oligonucleotide no longer binds PDX-1 but retains the faint non-PDX-1 complexes like those observed with the wild type oligonucleotide (Lane 13). Of the complexes formed with FP 5 oligonucleotide (lane 14) only the non-PDX-1 complexes are competed by the addition of excess unlabeled mutant oligonucleotide 5 mut (Lane 15). This oligonucleotide, 5 mut, does not bind PDX-1 (lane 16) but also retains other non-PDX-1 complexes. FP 2a interacted with duodenal nuclear extract to produce only the PDX-1 complexes (Figure 2). 2a mut did not compete or form these complexes (data not shown). It is clear from these results that the mutations introduced into the PDX-1 binding sites do eliminate PDX-1 binding without disrupting other observed protein complexes. While these mutations were chosen with great care, it remains possible that these mutations might eliminate binding of other factors absent from this extract such as factors that bind these sequences during earlier stages of development or in other regions of the intestine that act as repressors.

Transgenic mice containing a PDX-1 mutated enhancer do not maintain duodenum-specific activation- In order to examine the effects of loss of PDX-1 binding at the enhancer in vivo a transgene containing a PDX-1 mutated enhancer was constructed. The 319 bp BsiWI fragment shown in Figure 1 containing the duodenal enhancer was subcloned into pALTER-1 (Promega) and subjected to multiple rounds of site-directed
mutagenesis to introduce the mutations from Table 1 into the enhancer. Mutations were confirmed by sequencing the enhancer in its entirety and the enhancer was subcloned back into its native location and orientation within a 13 kb intragenic fragment previously analyzed in other transgene experiments [13, 14]. The location of the duodenal enhancer relative to the human ADA gene is shown in Figure 4A where the enhancer is represented as an open bar in intron 2. The transgene designated as wild type (wt) contains the enhancer in its native orientation and location within this 13 kb segment. The transgene designated as Δ enhancer contains the same fragment with the 319 bp enhancer fragment deleted. The transgene designated as PDX mutant is identical to the wt transgene with the exception of the PDX-1 binding site ablation mutations (denoted by the asterisks in open box). This transgene also contains the base pair changes used to create the BsiWI sites flanking the enhancer. Each transgene also contains 3.8 kb of human ADA promoter/5′flank and the CAT coding sequence.

A minimum of two adult F1 mice for each independent transgenic line were analyzed for CAT activity in various tissues. Protein concentrations and transgene copy numbers were also assessed. All transgenic CAT activities reported are normalized to both protein concentration and transgene copy number and are expressed in units of pmol/hr/100μg protein/transgene copy and are shown in Figures 4B and Table 2. Duodenal CAT activity/copy for two previously reported transgenes are included for comparison [13, 14]. Duodenal CAT activity/copy (Figure 4B) in mice containing the wild type enhancer (wt; black bars) ranges from 1,400-31,000 with a median activity of 9,100. In every line of these transgenic mice duodenum is the site of highest reporter gene activity, usually by 100-1000 fold (Table 2). Deletion of the enhancer (Δ enhancer;
open bars) resulted in a transgene with significantly reduced duodenal CAT activity/copy in every line. Duodenal CAT activity/copy for these transgenic mice range from 0-1.3 with a median activity of 0.48. This represents only 0.005% of the median wild type enhancer activity. In these mice, duodenum was never observed to be the tissue of highest expression. In fact, duodenum was often 100-1000 fold lower than the tissue with the highest CAT activity (Table 2). Values for other tissues were similar to those seen for other non-duodenal tissues in mice with the wild type transgene (Table 2). Transgenic mice containing the mutated enhancer (PDX mutant; gray bars) also exhibit very reduced duodenal reporter gene activity ranging from 0-180 with a median CAT activity/copy of 18.5. This value represents 0.2% of the median wild type activity. For all but two lines, duodenal CAT activity/copy is 100-10,000 fold lower than the activity in the tissue with the highest expression (Table 2). One line (PDX mutant, Line 5) had duodenum as the tissue of highest expression by only a slight margin (Table 2). In this line all tissues had very similar low levels of activity (data not shown). This expression pattern is not one of true duodenal activation such as was observed in the wild type transgenes (1.6 fold versus 100-1000 fold), but rather seems to be evidence of very low level expression in every tissue. In another line (PDX mutant, Line 3) duodenum had much higher activity (180 units/copy) than was observed in duodenum from any other PDX mutant line. However, all tissues examined in Line 3 had similar moderate levels of activity ranging from 83-220 units/copy (Table 2). This pattern of expression could easily be the result of transgene insertion near a ubiquitous enhancer or in a region of chromatin permissive to expression. Regardless, the results observed clearly show that the mutations introduced that ablate PDX-1 binding in vitro, and by inference in vivo as
well, eliminate the enhancer’s ability to activate consistent, high-level, duodenum-specific transcription, demonstrating an absolute requirement for PDX-1 in vivo.

*Co-transfection of PDX-1 with the enhancer does not result in activation* - In co-transfection transient assay, PDX-1 has been shown to modestly activate transcription through a PDX-1 binding site in the proximal region of the insulin promoter [16, 22]. The ability of PDX-1 to function similarly from the ADA enhancer binding sites was tested in co-transfection experiments. A target plasmid containing 3.8 kb of the human ADA promoter/5’ flanking sequence, the CAT reporter gene and a 3.4 kb fragment of intron 2 including the duodenal enhancer (Figure 5A, [13]) was produced. This construction places the enhancer and associated PDX-1 binding sites at a remote location somewhat analogous to the position it occupies in the ADA gene. An RSV driven PDX-1 expression vector was obtained [16]. The PDX-1 cDNA was removed to create an empty expression vector. Multiple transfections into CHO-K1 cells were performed in duplicate with the target plasmid only, the target plasmid plus the empty vector, or the target plasmid plus the PDX-1 expression vector. Samples were harvested 48 hours after transfection and the extracts were assayed for CAT activity and protein concentration. All CAT activities reported are normalized to protein concentration and expressed in pmol/hr/100µg protein. The results for the ADA-CAT target plasmid are shown in Figure 5A. No significant difference was observed among the target alone (gray bar), the target plus the empty expression vector (open bar), and the target plus the PDX-1 expression vector (black bar). By contrast, in co-transfection experiments using an insulin promoter-CAT construction [16] as the target plasmid (Fig. 5B), a consistent 2-3 fold increase in CAT activity was observed upon co-transfection with the PDX-1
expression vector (black bar) over either the insulin-CAT target alone (gray bar) or the target plus the empty expression vector (open bar). These results are similar to those published for other transfection studies using these plasmids [16]. PDX-1 protein was readily discernible by gel shifts using extracts made from cells containing the expression vector (data not shown). Therefore, it was concluded that PDX-1 alone was insufficient to cause a detectable activation, much less the sizeable activation that is characteristic of this enhancer.

These studies have identified five sites for nuclear protein binding within a duodenum-specific enhancer that bind the homeodomain protein PDX-1. PDX-1 alone was incapable of activating the ADA enhancer in co-transfection experiments. A number of other nuclear proteins also have the ability to interact with this enhancer. It seems likely that some of these bind the enhancer to modulate transcription in either concordance with or opposition to PDX-1. Specific removal of PDX-1 from this potential multi-protein complex bound at the enhancer via site-directed mutagenesis results in either severely limited activation or complete enhancer inactivation in vivo. So while insufficient to activate transcription by itself, PDX-1 clearly plays an integral role in the transactivation mediated by this enhancer in the duodenum.
DISCUSSION

Many genes expressed in the intestine share similar expression profiles along the various axes of development. Along the A/P axis some show rather homogeneous expression throughout the intestine, while others are more spatially limited. The same observation can be made along the C/V axis. Transporters and metabolic enzymes, such as ADA, are absent from the proliferative crypt compartment and only become expressed at the C/V juncture as cells complete differentiation. Others are expressed only in the crypts and are extinguished at the C/V juncture. A similar phenomenon occurs with respect to developmental timing of gene expression. Many embryonic genes are silenced around the time of birth. In rodents, activation at the suckling-weaning transition is also common for many genes including ADA. Common underlying mechanisms are thought to be responsible for these similarities in expression pattern observed among genes in intestine. It has been proposed that mechanisms controlling A/P, C/V, or developmental timing are independent of one another in intestine [23, 24]. The most likely mechanistic basis for these differences is the delicate interplay of factors bound to tissue-specific cis-regulatory elements within each respective gene.

We have begun to identify proteins that interact with a duodenum-specific enhancer module that defines high-level transcription, villous epithelial-specific expression, and activation simultaneous with the final maturation of the small intestine at about two weeks of age. A disproportionately high number of binding sites for one specific factor are present within this enhancer. Within the entire 40 kb of ADA gene sequence only a few poor matches to this sequence exist outside the 319 bp enhancer. The presence of five good site matches within such a small piece of DNA seemed
significant at the outset. We have shown here that the homeotic protein PDX-1 binds these sites and that these sites are required in vivo for enhancer function. PDX-1 is found in only a few tissues in adults including pancreas and duodenal villous epithelium [16, 25-27]. Evidence from targeted mutagenesis experiments eliminating PDX-1 in mice [28, 29] show that it plays a key role in pancreas development, as null mice are apancreatic. These mice also contain numerous defects of the small intestine limited to the duodenum where crypt/villus structures fail to form and the epithelium maintains an immature cuboidal phenotype. Within the pancreas expression of PDX-1 is limited to the islet cell lineage where it has been implicated in the regulation of numerous islet-specific genes including insulin [19, 30, 31], somatostatin [25, 32], elastase [33-35], and islet amyloid polypeptide [36]. A similar role for PDX-1 in intestinal gene regulation has not been established.

Along not only the A/P axis of the intestine, but also the C/V axis of the duodenum as well, there is a good correlation between the location of high-level ADA expression and the location of PDX-1 expression. Both are limited to the duodenum along the A/P axis. Expression of both is also limited along the C/V axis of development to the villous epithelium and is absent from the crypts, lamina propria, and underlying musculature [27]. There are many other genes expressed in the intestine with profiles similar to ADA. Two of these, elastase I [33, 34, 37] and calbindin D9K [38, 39] have regulatory sequences that have been defined. Both genes are expressed at high levels in the duodenum. Calbindin also shares the same C/V [38] and developmental [39] patterns of expression as ADA with minor exceptions. The promoter sequences for each have also been observed to contain sites that can bind PDX-1 in vitro [33, 34, 37, 40]. As yet
no functional studies for either of these sites have been published. But it seems likely that PDX-1 plays a role in defining the expression pattern for each of these genes in the intestine along the A/P and perhaps the C/V axes of development.

Despite the absolute requirement for PDX-1, it alone is not sufficient for the profile observed. There are tissues or developmental time points where PDX-1 is expressed and ADA remains at basal levels such as in islet cells or in embryonic proximal gut [29] suggesting that PDX-1 alone is not sufficient to drive the expression observed in duodenum. Co-transfection experiments clearly demonstrate that PDX-1 alone is incapable of transactivation from the ADA enhancer. Little has been observed regarding the role of PDX-1 in intestinal gene regulation, however much can be inferred from studies examining how PDX-1 operates in islet-specific gene expression. More recent studies of various pancreas-specific promoters show that high level activation is dependent on the presence of PDX-1 and numerous other proteins [41-44]. A distinct but analogous group of factors may share the same role in PDX-1 activation of duodenal-specific genes. In pancreas, PDX-1 has been observed to interact synergistically with a number of other homeodomain DNA binding proteins such as MEIS, PBX, and PAX [32, 35, 43-45] and basic helix-loop-helix, bHLH, E-box proteins E47 and E2 [41, 42, 44] in the process of lineage allocation in the pancreas. In these cases it is the absence or presence of specific binding partners that determine gene expression within a given cell type. High-level activation is only observed when PDX-1 and one of these factors are both bound [44]. Analogous binding partners for PDX-1 in the duodenum have yet to be identified. Within the ADA enhancer there are no consensus E-box binding sites, CANNTG, so interaction with these type of proteins would have to occur independent of
their binding to the DNA. A potential Pbx-1 type binding site exits in footprint 2 within the FP 2d oligonucleotide used in gel shift experiments and an unidentified complex was produced with this probe. However, the Pbx-1 site overlaps the PDX-1 site and is on the other face of the DNA strand. This location does not intuitively seem like one that would foster protein–protein interaction. This sequence contains a long AT tract resembling other sequences known to bind the ubiquitous factor HMG I Y [46-48]. HMG I Y has recently been shown to stabilize the protein-protein interaction between PDX-1 and E47 thereby increasing synergistic activation [44]. Perhaps it performs a similar function in the ADA enhancer between PDX-1 and an as yet unidentified binding partner.

At least one factor known to play a role in intestinal gene transcription has been identified that can interact with PDX-1. PDX-1 has been shown to interact with the intestine-specific homeodomain protein Cdx 2 [49]. Interestingly, within the ADA enhancer there is one Cdx-type binding site that is closely juxtaposed to the multiple PDX-1 sites within FP 2 between oligonucleotides 2a and 2c [13, 14]. The relative spacing of the Cdx and three PDX-1 binding sites to one another suggests that they would all reside on the same face of the DNA strand such that protein-protein interactions could be facilitated. This same region also contains the identified GATA binding site. The multiplicity of these binding sites, three PDX-1, one Cdx and one GATA site, closely juxtaposed within 50 bp might foster an interaction between these proteins in a manner analogous to those observed with PDX-1 and numerous other proteins in pancreas-specific genes. None of the PDX-1 complexes formed in gel shift experiments with the duodenal extract are the result of simultaneous binding of both PDX-1 and Cdx or PDX-1
...and GATA as addition of excess Cdx or GATA consensus oligonucleotides had no effect on formation of these complexes (data not shown).

Studies designed to address the identity of the remaining enhancer binding proteins are underway. The specific functions of these proteins and how they interact with each other and PDX-1 will give us invaluable information about intestinal gene regulation along each of its developmental axis. This knowledge will be useful for deciphering what is involved in intestinal patterning of the ADA gene and potentially many other intestinal genes as well.

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FIGURE LEGENDS

FIG. 1. **Sequence of the ADA duodenal enhancer.** The sequence of the human ADA duodenal enhancer is shown in A. Number above the sequence correspond to residues of the ADA gene sequence (GenBank accession number M13792). Asterisks mark the nucleotide residues altered from the published sequence to create two BsiWI restriction sites. The sequence between these sites was determined to contain a duodenum-specific enhancer [13]. Sequences within the enhancer protected from DNase I digestion by duodenal nuclear extracts in footprinting experiments are shown as gray shaded boxes, FP1-FP5. Oligonucleotides used as probes for electrophoretic mobility shift assays (EMSA) are indicated as black bars. Oligonucleotides are named after the footprint in which they reside. Oligonucleotides FP 4 and FP 5 are found in footprint regions 4 and 5 respectively. Oligonucleotides FP 2a, FP 2c, and FP 2d are all found in footprint region 2. The oligonucleotide FP 5 was synthesized with both the endogenous sequence and the BsiWI altered sequence. In Panel B are the sequences of the five PDX-1 type motifs compared to a known PDX-1 binding site from the rat insulin promoter P1. Also shown is the name of the oligonucleotide containing each site.

FIG. 2. **EMSA with mouse duodenal nuclear extract.** Oligonucleotides containing each of the ADA PDX-1 site motifs (FP 2a, FP 2c, FP 2d, FP 4, and FP 5) as well as an oligonucleotide containing the PDX-1 site from rat insulin promoter (P1) were labeled and used as probes for EMSA. Shown in Panel A, is each oligonucleotide as unreacted probe (Lanes 1, 3, 5, 7, 9, and 11) and probe in the presence of 10 µg of mouse duodenal nuclear extract, MDNE, (Lanes 2, 4, 6, 8, 10, and 12). A similar pattern of shifted bands
is observed for each oligonucleotide. The same probes were used in Panel B. Each lane now contains probe and 10 µg of extract. Lanes 2, 4, 6, 8, and 10 also contain a 100-fold molar excess of unlabeled P1 as competitor. All lanes in Panel C contain 10 µg of MDNE. These experiments confirm that these shifted complexes (Lane 1, 4, 7, 10, and 13) are PDX-1 by their ability to react with a PDX-1 specific antibody (Lanes 3, 6, 9, 12, and 14) but not a non-specific antibody (Lanes 2, 5, 8, and 11).

**TABLE 1. Mutations to PDX-1 motifs.** Mutations were introduced into the PDX-type motifs (2a, 2c, 2d, 4, and 5) and are designated by the bolded lower case letters in the sequences beneath (2a mut, 2c mut, 2d mut, 4 mut and 5 mut). These mutations were incorporated into oligonucleotides identical to the wild type sequences except for the base pairs noted.

**FIG. 3.** Mutations to the PDX-1 motif eliminate PDX-1 but not other nuclear proteins from complex formation. In vitro transcribed translated PDX-1 protein was used to test the efficaciousness of mutations. The resulting EMSA is shown in Panel A. Each lane contains an equal amount of PDX-1 protein. The wild type oligonucleotides (Lanes 1, 3, 6, 8, and 11) produce a PDX-1 shifted complex, marked with an asterisk, which co-migrates with a shifted band produced by the P1 oligonucleotide (Lanes 5 and 10). EMSA with each of the mutated oligonucleotides fails to produce this band (Lanes 2, 4, 7, 9, and 12). 10 µg of MDNE is used in each lane of Panel B. The indicated PDX-1 shifted complexes produced with the wild type oligonucleotides FP 2c (Lanes 1 and 4), FP 2d (Lane 6), FP 4 (Lane 10) or FP 5 (Lane 14) are competed or reduced by the
addition of 100-fold molar excess of unlabeled self (Lanes 2) or unlabeled P1 oligonucleotide (Lanes 7 and 12). The unlabeled mutant oligonucleotide when used as competitor failed to compete the PDX-1 shifted complexes (Lanes 3, 8, 11, and 15), but did compete for other shifted complexes produced as can be seen by the band labeled with an asterisk. Finally the mutant oligonucleotides were labeled and used as probes in EMSA experiments (Lanes 5, 9, 13, and 16). These mutant oligonucleotides were unable to produce the shifted PDX-1 complexes, but retained the ability to bind various other shifted complexes unrelated to PDX-1.

FIG. 4. Loss of PDX-1 binding to the enhancer in vivo results in loss of activation in the duodenum. Panel A shows the location of the duodenal enhancer (designated by an open box) within the second intron of the human ADA gene. Three transgenic constructions are shown which contain the human ADA promoter/5' flanking region attached to the CAT reporter gene. 3' of the CAT coding sequences, each contains an equivalent 13 kb intragenic segment of DNA surrounding the enhancer. The transgene designated as wild type (wt) contains the enhancer in this 13.0 kb fragment as it is found in the human gene. The transgene designated as Δ enhancer contains a 319 bp deletion of the enhancer region shown in Figure 1. The final transgene is identical to the wild type transgene with the exception of point mutations of the PDX binding sites (specified by asterisks) corresponding to those shown in Table 1. Panel B shows a comparison of duodenal reporter gene activity normalized to transgene copy number for multiple lines of mice containing these transgenes (note the log scale). Results with the wt and Δ enhancer have been published previously [13, 14] and are included here for comparison.
Duodenal CAT activity/copy in mice containing the wt transgene (black bars) ranges from 1,400-31,000. Duodenal CAT activity/copy in mice containing the Δ enhancer fragment (open bars) show greatly decreased activity that ranges from 0-0.13. Mice containing the PDX mutant enhancer (gray bars) show a similar and striking reduction in CAT activity/copy that ranges from 0-180.

**TABLE 2.** Tissue CAT activities of wt, Δ enhancer, and PDX mutant transgenic mice. Shown are normalized CAT activities normalized to transgene copy number and expressed as pmol/hr/100ug/transgene copy from various transgenic tissues. Tissues not assayed are designated with a hyphen. Duodenal CAT values for wt and Δ transgene, thymic CAT values for wt enhancer transgene, and the highest tissue CAT values for Δ have been previously reported [13, 14].

**FIG. 5.** PDX-1 Co-transfection. Either an ADA-CAT target plasmid (A) or an insulin-CAT target plasmid (B) were used in transient transfections into CHO-K1 cells using lipofectamine. Each target plasmid was transfected alone (gray bar) or co-transfected with an empty expression vector (open bar) or a PDX-1 expression vector (black bar) and resulting CAT activities (pmol/hr/100ug) from cell extracts are shown.
|     | Sequence       |
|-----|---------------|
| FP 2a | CATTAATG      |
| 2a mut | CATgcATG     |
| FP 2c | ACTTAATG      |
| 2c mut | ACTggATG     |
| FP 2d | TATTAATT      |
| 2d mut | TATTAAcc     |
| FP 4  | CCCTAATG      |
| 4 mut  | CCCTAAcc      |
| FP 5  | CTTTAATG      |
| 5 mut  | CCTgcATG      |
## TABLE 2

| Transgene | Transgenic line | Copy number | CAT activity (pmol/hr/100 µg/copy) |
|-----------|-----------------|-------------|----------------------------------|
|           |                 |             | Tongue | Esophagus | Stomach | Duodenum | Jejunum | Ileum | Colon | Liver | Thymus |
| wt        | 1               | 9           | 2.4    | 0.52    | 3.2    | **5,900** | 3.4    | 0.50  | 0.40  | 0.0020 | 19     |
|           | 2               | 4           | 10     | 9.8     | 7.3    | **31,000**| 18     | 46    | 17    | 0.20   | 8.0    |
|           | 3               | 9           | 2.7    | 0.10    | 1.1    | **1,400** | 4.6    | 3.6   | 0.80  | 0.10   | 1.6    |
|           | 4               | 41          | 3.3    | 0.20    | 1.9    | **4,100** | 1.1    | 1.6   | 0.30  | 0.050  | 3.8    |
|           | 5               | 34          | 12     | 4.6     | 6.1    | **13,000**| 2.4    | 7.5   | 5.2   | 1.8    | 8.0    |
|           | 6               | 14          | 3.9    | 1.4     | 1.2    | **2,400** | 0.80   | 1.5   | 3.3   | 0.040  | 11     |
|           | 7               | 5           | 1.9    | 0.20    | 3.7    | **2,000** | 0.50   | 3.4   | 1.5   | 0.24   | 4.3    |
|           | 8               | 2           | 6.3    | 12      | 8.1    | **2,500** | 0.50   | 1.4   | 0.90  | 0.040  | 8.0    |
|           | 9               | 2           | 2.5    | 2.7     | 0.80   | **13,000**| 2.5    | 2.2   | 0.80  | 0.20   | 0.40   |
|           | 10              | 23          | 3.4    | 3.9     | 2.3    | **6,000** | 1.0    | 1.1   | 1.3   | 330    | 9.6    |
|           | 11              | 3           | 7.0    | 8.3     | 340    | **19,000**| 10     | 260   | 32    | 0.0030 | 8.7    |
| Δ enhancer| 1               | 2           | 0.40   | 0.10    | 0      | **0.90**  | 0      | 0.30  | 0.40  | 0.020  | 0.20   |
|           | 2               | 8           | 23     | 5.2     | 1.1    | **0.25**  | 0.20   | 0.010 | 2.2   | 0.020  | 14     |
|           | 3               | 5           | 6.4    | 1.3     | 0.40   | **0.79**  | 0.30   | 1.1   | 0.70  | 0.10   | 3.2    |
|           | 4               | 10          | 3.0    | 0.50    | 0.10   | **0.020** | 0.30   | 0     | 0.010 | 0.0020 | 2.7    |
|           | 5               | 4           | 28     | 0.80    | 14     | 0        | 0      | 0     | 0     | 0.23   | -      |
|           | 6               | 2           | 10     | 1.4     | 2.5    | **0.13**  | 0      | 0.30  | 0.80  | 0      | -      |
|           | 7               | 210         | 22     | 3.4     | 10     | **1.3**   | 0.10   | 2.5   | 2.5   | 0.050  | -      |
| PDX mutant| 1               | 10          | 74     | 10      | 2.8    | **1.3**   | 0.2    | 7.5   | 6.5   | 0.51   | 57     |
|           | 2               | 84          | 13     | 20      | 10     | **0.24**  | 0.0080 | 5.7   | 1.6   | 0.085  | 12     |
|           | 3               | 2           | 83     | 91      | 87     | **180**   | 140   | 170   | 160   | 73     | 33     |
|           | 4               | 8           | 52     | 7.0     | 88     | **0.010** | 0.64   | 1.7   | 16    | 0.32   | 51     |
|           | 5               | 3           | 1.3    | 0.77    | 0.47   | **3.2**   | 0.24   | 0.13  | 1.3   | 0.060  | 1.5    |
|           | 6               | 32          | 1.3    | 0.21    | 0.45   | **0.070** | 0.19   | 0.43  | 0.67  | 0.25   | 3.5    |
|           | 7               | 3           | 0      | 2.4     | 0      | 0        | 0      | 0.020 | 0.87  | 0      | 0.77   |
|           | 8               | 11          | 12     | 3.2     | 22     | **0.060** | 0      | 6.0   | 2.4   | 0.95   | 16     |
|           | 9               | 26          | 4.7    | 0.77    | 7.4    | **0.070** | 1.1    | 2.9   | 1.8   | 0.041  | 8.0    |
|           | 10              | 2           | 2.2    | 0.20    | 0.020  | 0        | 0      | 0     | 0.26  | 0.024  | 2.3    |
|           | 11              | 29          | 25.0   | 4.1     | 55     | **0.18**  | 0.035  | 0.64  | 2.5   | 0.050  | 18     |
### FIGURE 2

#### A.

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|------|---|---|---|---|---|---|---|---|---|----|----|----|
| MDNE | + | - | + | + | + | + | - | + | - | + | - | + |

#### B.

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|------|---|---|---|---|---|---|---|---|---|----|
| PI oligo | + | - | + | - | + | - | - | - | - | + |
| MDNE | + | + | + | + | + | + | + | + | + | + |

#### C.

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|
| anti PDX-1 Ab | - | + | - | + | - | + | - | + | - | + | - | + | + | + |
| IgG | + | - | + | - | - | - | + | - | - | + | + | - | - | - |
| MDNE | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

**Legend:**
- Lane numbers 1 to 12 correspond to different samples or conditions.
- MDNE and PI oligo indicate specific conditions or inhibitors used in the experiment.
- The presence (+) or absence (-) of bands in the gel indicates specific binding or reaction outcomes.
FIGURE 3

A.

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|------|---|---|---|---|---|---|---|---|---|----|----|----|
| probe| wt m| wt m| wt m| wt m| wt m| wt m| wt m| wt m| wt m| wt m| wt m| wt m|
| PDX-1 | * |  |  |  |  |  |  |  |  |  |  |  |

B.

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|
| mutant | - | - | + | - | - | + | - | - | - | + | + | + | + | + | + | + |
| self |  |  |  |  | - | - | - | - | - | - | - | - | - | - | - | - |
| P1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

| probe | FP 2c | FP 2d | FP 4 | FP 5 |
|-------|-------|-------|------|------|
|       | -2c mut | -2d mut | -4 mut | -5 mut |
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