Nucleic Acids Research, 1997, Vol. 25, No. 12 **2293–2300**

Activation of enhancer elements by the homeobox gene Cdx2 is cell line specific

Jennifer K. Taylor, Tao Levy, Eun Ran Suh and Peter G. Traber*

Division of Gastroenterology, Departments of Medicine and Genetics, University of Pennsylvania, 415 Curie Boulevard, Philadelphia, PA 19104, USA

Received March 25, 1997; Accepted April 24, 1997

ABSTRACT

Cdx2 is a caudal-related homeodomain transcription factor that is expressed in complex patterns during mouse development and at high levels in the intestinal epithelium of adult mice. Cdx2 activates transcription of intestinal gene promoters containing specific binding sites. Moreover, Cdx2 has been shown to induce intestinal differentiation in cell lines. In this study, we show that Cdx2 is able to bind to two well defined enhancer elements in the HoxC8 gene. We then demonstrate that Cdx2 is able to activate transcription of heterologous promoters when its DNA binding element is placed in an enhancer context. Furthermore, the ability to activate enhancer elements is cell-line dependent. When the Cdx2 activation domain was linked to the Gal4 DNA binding domain, the chimeric protein was able to activate Gal4 enhancer constructs in an intestinal cell line, but was unable to activate transcription in NIH3T3 cells. These data suggest that there are cell-specific factors that allow the Cdx2 activation domain to function in the activation of enhancer elements. We hypothesize that either a co-activator protein or differential phosphorylation of the activation domain may be the mechanism for intestinal cell line-specific function of Cdx2 and possibly in other tissues in early development.

INTRODUCTION

Homeobox genes are important for developmental pattern formation and organogenesis in multiple species [reviewed in (1,2)], and in the development of neoplasia [reviewed in (3)]. The homeobox encodes for a protein domain that interacts with specific DNA sequences, allowing members of this gene family to regulate transcriptional initiation as either an activator or repressor. The caudal-related homeobox genes are a non-clustered family, including three members in mouse, Cdx1 (4), Cdx2 (5,6) and Cdx4 (7). Each of these genes has a complex pattern of expression in the developing mouse embryo (7–9), but Cdx1 and Cdx2 are highly expressed in the adult mouse only in the epithelium of the small intestine and colon (10,11).

Developmental patterns of Cdx gene expression in mice suggest important roles in early embryonic development. Cdx2 is first expressed at preimplantation stages and at the time of implantation in trophoectodermal cells and extraembryonic ectoderm, and is then expressed in the placenta at later stages (8). Embryonic expression is first seen at day 8.5 p.c. (post coital) in ectoderm, mesoderm and endoderm of the tail bud, and in caudal regions of the neural plate, neural tube and notochord (8). By day 12.5 p.c. expression is limited to the gut endoderm except for expression in the extreme caudal tip of the neural tube (8). Cdx2 expression appears to increase in the endoderm just before the time of the endoderm-intestinal epithelial transition (approximately day 15 p.c.) and continues to be expressed in intestinal epithelial cells of adult mice along the entire crypt-villus axis of the small intestine and in crypts of the colon (6). Consistent with the early developmental expression, preliminary data on mice null for Cdx2 indicates that the homozygous state is associated with preimplantation lethality (12). *Cdx1* is first expressed in mouse embryos at day 7.5 in the region of the primitive streak, predominantly in nuclei of ectoderm and mesoderm and some in visceral endoderm, but not in the definitive endoderm (9). Between embryonic day 8.25 and 12 there is variable expression of Cdx1 in a number of tissues including the neural tube, somites, the mesoderm and limb buds. By day 12 there is marked reduction in expression which lasts until day 14 p.c. when there is a marked induction in endoderm (4). Mice null for Cdx1 have skeletal abnormalities, but the effects on the intestinal epithelium have not yet been published (13). Cdx4 is expressed in posterior structures early in embryogenesis, but is not expressed in the intestinal epithelium (7). In summary, the expression patterns of Cdx genes suggest functional roles in multiple tissue types at different stages of development.

Several lines of evidence suggest that Cdx genes are important in intestinal gene transcription and epithelial cell differentiation. Our laboratory has shown that Cdx2 mediates transcriptional activation of the intestine-specific gene, sucrase-isomaltase (SI), via an evolutionarily conserved DNA promoter element (11). Subsequently, there has been evidence that other intestinal genes are regulated by Cdx2 (14,15). Additionally, we have shown that forced expression of Cdx2 in an undifferentiated intestinal epithelial cell line induces morphologic and molecular differentiation (16). Thus, Cdx2 is the first identified transcription factor, most likely acting in conjunction with a network of other factors, that is responsible for directing development and differentiation of intestinal epithelial cells. There is also some evidence that Cdx2 is important for regulation of pancreatic islet cell promoters

*To whom correspondence should be addressed. Tel: +1 215 898 0154; Fax: +1 215 573 2024; Email: traberp@mail.med.upenn.edu

(17,18), although the level of Cdx2 expression in islet cells *in vivo* is poorly defined.

In addition to tissue-specific promoter elements, there are two putative Cdx binding sites in the Hoxc-8 gene enhancer (19,20). In this study, we show that Cdx2 protein interacts specifically with two well-defined functional enhancer elements in the Hoxc-8 gene. Because of the existence of binding sites in both promoter and enhancer positions and the wide tissue distribution of Cdx2 during development, we examined the ability of Cdx2 to activate transcription from both proximal (promoter) and remote (enhancer) positions and the dependence of these activities on cell type. Using an intestinal cell line that expresses Cdx2 as a model system, we showed that Cdx2 was able to activate transcription in an enhancer context from the SI promoter as well as heterologous promoters. However, this activity was dependent on the cell line used since NIH3T3 cells were unable to activate enhancer constructs when co-transfected with Cdx2 expression vectors, although we have previously shown that Cdx2 is able to activate transcription from a promoter element in NIH3T3 cells. Differences between the ability of these cell lines to activate through enhancer elements was dependent on the activation domain of Cdx2, and not on the DNA binding element. In addition, the differential effect on promoter and enhancer activation was much greater for the Cdx2 activation domain when compared with VP16, a powerful transcriptional activator. Taken together, these results suggest that there are cell-specific mechanisms that determine the ability of Cdx2 to activate transcription from an enhancer context. Cell specificity of function may be mediated by a co-activator protein or via cell specific protein phosphorylation of the activation domain. Further elucidation of this mechanism may be important for understanding cell-specific function of Cdx proteins during development.

MATERIALS AND METHODS

Reporter plasmids

SIF1 is the DNA binding element in the sucrase-isomaltase gene that has been shown to interact specifically with Cdx2 (11). Plasmid constructs containing one or more copies of the SIF1 element were sequentially inserted into several reporter constructs containing various promoters using a previously described method (21). The oligonucleotides used for the SIF1 element incorporated a *Bam*HI recognition site on the 5′-end and a *Bg1*II recognition site on the 3′-end:

sense strand: gatccGTGCAATAAAACTTTATGAGTAa
antisense strand: gatctTACTCATAAAGTTTTATTGCACg

The plasmid pTK-LUC, which contains the herpes simplex thymidine kinase (TK) promoter linked to the luciferase reporter gene (22), was used to insert various numbers of copies of the SIF1 element, either upstream of the TK promoter (pTK-SIF1 promoter) or downstream of the luciferase gene (pTK-SIF1 enhancer). For promoter constructs, pTK-LUC was digested with *Bg1*II and SIF1 oligonucleotides were inserted by ligation, as described (21). Four separate plasmids with one to four copies of SIF1 were confirmed by sequencing [pTK-SIF1 promoter(+1), pTK-SIF1 promoter(+2), pTK-SIF1 promoter (+3) and pTK-SIF1(+4); the (+) indicates the direction of the SIF1 element was in the same direction as transcription from the SI promoter]. For enhancer constructs, pTK-LUC was digested with *Nde*I, the ends filled in with Klenow enzyme, and a blunt-ended SIF1 cassette insert from pTK-SIF1 promoter(+4) was inserted in both orientations. The resultant plasmids were named pTK-SIF1 enhancer(+4) and pTK-SIF1 enhancer(–4).

The sucrase-isomaltase promoter reporter construct was made by amplifying bases –66 to +54 of the human sucrase-isomaltase gene using the polymerase chain reaction, followed by insertion into the *Bam*HI and *Xba*I site in the pGL2 luciferase reporter plasmid (Promega Co.) (phSI(–66)GL2). The four copy SIF1 cassette in pTK-LUC promoter(+4) was excised with *Bam*HI and *Bg1*II and inserted by blunt-end ligation into the *Alw*N1 restriction endonuclease site located immediately downstream of the luciferase reporter gene. Plasmids were sequenced to identify one construct with the SIF1 cassette inserted in each orientation [phSI(–66)SIF1enhancer(+4) and phSI(-66)SIF1 enhancer(–4)].

The SI promoter (–183 to +54) luciferase reporter has been previously described (23,24). The CAT reporter containing five copies of the Gal4 binding site linked to the EIB TATA box has been previously described (25). The Gal4 promoter reporter was created by inserting double stranded oligonucleotides containing the E1B TATA box (GATCTAGGGTATATAATGGCGA) with *Bgl*II and *Hin*dIII linkers into the *Bgl*II and *Hin*dIII sites of the pGL Basic plasmid (Promega Biotech, Madison, WI). A cassette containing five copies of the Gal4 17mer binding site (CGGAGT-ACTGTCCTCCG) was excised from 5xGal4TK CAT (26) using *Bam*HI and *Hin*dIII restriction enzymes. The Gal4 cassette was then inserted into the *Nhe*I site immediately upstream of the E1B TATA in the pGL plasmid by blunt-ended ligation. Plasmids containing 10 copies of the Gal4 binding site were selected by restriction analysis and confirmed by sequencing [pE1B-LUC promoter(Gal4x10)].

The Gal4 enhancer reporter was created by inserting the above described Gal4 cassette into the *Nde*I site located downstream of the luciferase gene in pTK-LUC by blunt-ended ligation. Plasmid containing 10 copies of the Gal4 binding site were selected by restriction analysis and confirmed by sequencing.

Expression vectors

Construction of the pRC/CMV Cdx2 expression vector was previously described (11). The pRc/CMV Cdx2 deletion constructs were generated by PCR. The sequences of the cDNA corresponding to amino acids 1–248 and 164–311 were amplified by PCR with oligonucleotides incorporating a 5′ *Hin*dIII site with a Kozak and methionine sequence and a 3′ primer containing a stop codon and an *Xba*I site.

Cdx2 Δ249–311 primers:
5′-GGGAAGCTTACCATGTACGTGAGCTACCTTCTG
3′-GGGCTGTCTAGATTACTGCTGCTGCTTCTTCTTGAT
Cdx2 Δ1–164 primers:
5′-GGGAAGCTTACCATGATCGGGAAGCCCGCGCAG
3′-CCCTCTAGAGGGGTCACTGGGTGACAGTGGA
The amplified products were subcloned into the *Hin*dIII and *Xba*I sites of pRc/CMV.

Expression constructs for Gal4-Cdx2 fusion proteins were made in the pSG424 vector (27). The sequences of the cDNA corresponding to amino acids 1–180 and 15–180, 247–311 of Cdx2 were amplified by PCR with oligonucleotides incorporating *Sal*I and *Xba*I sites on the 5′ and 3′-ends, respectively.

Cdx2 (1–180)
5′-GGGCGTCGACGGATGTACGTGAGCTACCTTCTG
3′-GGGTCTAGATTACACTTGGCTCCTAGGGACTG

Cdx2 (15–180)
5′-GGGCGTCGACGGCCTAGCTCCGTGCGCCAC
3′-GGGTCTAGATTACACTTGGCTCCTAGGGACTG
The amplified products were subcloned in frame with the Gal4 DNA binding domain into the *Sal*I and *Xba*I restriction sites of the pSG424 vector. All constructs generated by PCR were sequenced for fidelity.

Cell culture and transfection

Caco2 and NIH3T3 cells were maintained and transfected in high glucose Dulbecco's modified Eagle Medium (DMEM) containing 10% fetal calf serum. One hundred mm dishes were transfected by the calcium phosphate precipitation method (28). Briefly, the medium was changed 2 h before transfection. The calcium phosphate and DNA precipitate formed 15 min after mixing 500 µl of 2× HEPES-buffered saline solution (29), 32 µl of 2.0 M calcium chloride and 15–20 µg DNA. The precipitate was added dropwise to the cells and 24 h later the medium was changed. Cells were harvested 48 h later. For transfections using luciferase reporters, the cells were lysed in Triton X-100 buffer and β-galactosidase and luciferase assays were carried out as previously described (11). Results are expressed as activity relative to light units normalized to β-galactosidase activity. Fold activity was calculated as the activity of the Gal4 Cdx2 (15–180) construct divided by the activity of the GAL4 vector expressing the Gal4 DNA binding domain alone. Transfection experiments were performed in triplicate and each experiment was repeated 3–6 times.

For transfections using CAT reporters, the cells were harvested as previously described (30). The β-galactosidase activity of each sample was used to normalize the amount of cell extract used in each CAT assay. The percent acetylation was calculated by analysis using a PhosphorImager (STORM 840, Molecular Dynamics).

Mobility shift assays

Electrophoretic mobility shift assays (EMSA) were performed as previously described (24). Nuclear extracts from transfected cells were prepared by a modification of the method of Dignam *et al.* (31). Reactions contained 10 µg nuclear protein. The Cdx2 antibody used in the supershift experiments has been previously described (11). Oligonucleotides used include:
Hoxc-8 Site A:
top strand: GATCCATGCCACTTTTATGGCCCTGA
bottom strand: GATCTCAGGGCCATAAAAGTGGCATG
Hoxc-8 Site D:
top strand: GATCCTAATTGTTTTATGGTTTAA A
bottom strand: GATCTT AAACCATAAAACAATTAG
SIF1:
top strand: GATCCGTGCAATAAAACTTTATGAGTAA
bottom strand: GCACGTTATTTTGAAATACTCATTCTAG
For competition experiments, the binding reaction was incubated with 100-fold molar excess of unlabelled probe for 10 min and then radiolabeled probe was added. For supershift experiments, 1 µl antibody was added to the binding reaction following the addition of radiolabeled probe.

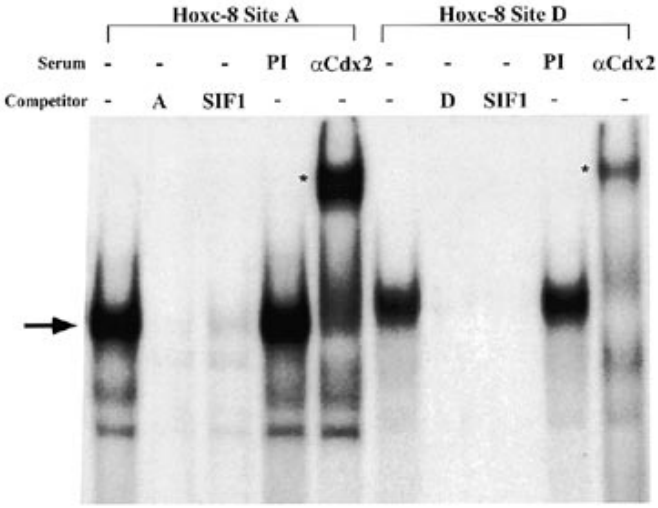

Figure 1. EMSA of Hoxc-8 enhancer elements. Nuclear extracts from NIH3T3 cells transfected with 10 µg pRc/CMV Cdx2 were analyzed for their ability to bind to Hoxc-8 site A and site D. The specific binding complexes formed are marked by the arrow. Competitions were performed with a 100-fold molar excess of the indicated probe. The sequences of the oligonucleotides used (site A, site D, SIF1) are given in the Materials and Methods. For supershift experiments, preimmune (PI) or anti-Cdx2 antibody (αCdx2) was used. Supershifted complexes are indicated by asterisks.

RESULTS

Cdx2 binding sites in enhancer elements

We examined functionally-defined enhancer elements in genes that are expressed in tissues that also express Cdx2 for evidence of Cdx2 binding elements. Only one well-defined intestinal gene enhancer has been identified. An enhancer element in an intron of the apolipoprotein C-III gene regulates intestine-specific transcription of the apolipoprotein A-I gene in transgenic mice (32). Within the 264 nucleotide region of this enhancer there was one sequence in a footprinted region that had weak similarity to a Cdx binding site (element I) (32). However, EMSA using Cdx2 protein failed to show a specific interaction between Cdx2 and this element (data not shown).

We next turned our attention to genes expressed in other tissues that also express Cdx2 during mouse development. An enhancer has been defined in the Hoxc-8 gene that is critical for early developmental expression in posterior neural tube and mesoderm (20). Two potential Cdx binding sites in this enhancer (A and D) were mutated and shown to be essential for expression of transgenes in the posterior neural tube and somites (19). Cdx2 protein binds specifically with both site A and D of the Hoxc-8 promoter (Fig. 1). These data suggest that Cdx2, which is expressed in the same cells that express Hoxc-8 during posterior neuroectodermal and mesodermal development, may be involved in regulation of the Hoxc-8 enhancer.

Enhancer activity of the Cdx2 binding site on the SI promoter

The SI promoter contains a DNA element, SIF1, that has two closely apposed sequences that interact with Cdx2 protein (11).

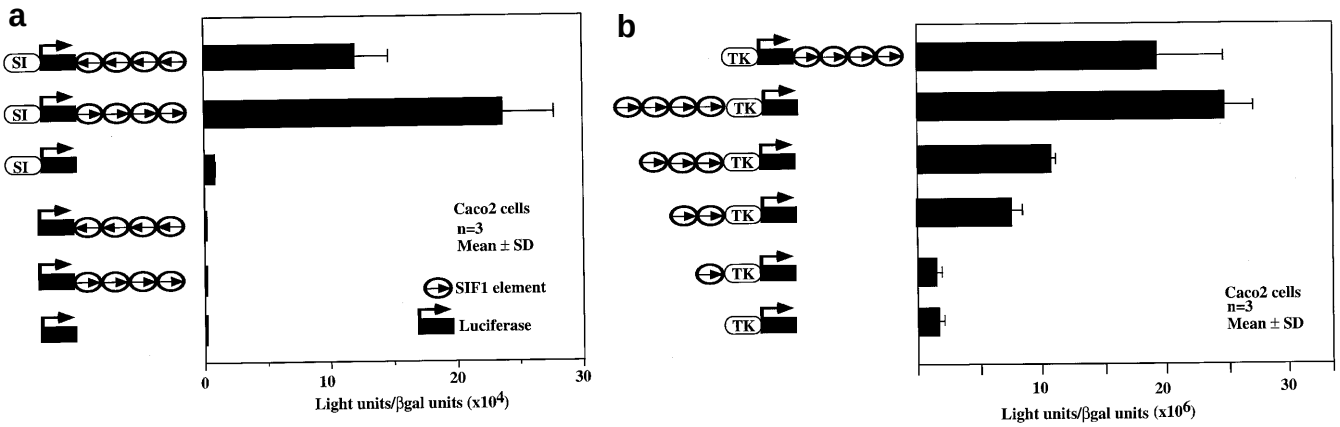

Figure 2. (**a**) Effect of SIF1-enhancer cassette on expression of a sucrase-isomaltase promoter construct in Caco2 cells. Caco2 cells were transfected with the indicated reporter constructs and analyzed for luciferase and β-galactosidase activity as described in Materials and Methods. The amount of plasmids transfected was 8 µg reporter plasmid and 2 µg CMV-βGal plasmid. This experiment was repeated three times with identical results (*n* = 3 for each experiment). (**b**) Effect of SIF1 element on expression of a thymidine kinase promoter construct in Caco2 cells. Caco2 cells were transfected with the indicated reporter constructs and analyzed for luciferase and β-galactosidase activity as described in Materials and Methods. The amount of plasmids transfected was 8µg reporter plasmid and 2 µg CMV-βGal plasmid. This experiment was repeated three times with identical results (*n* = 3 for each experiment).

To evaluate whether the SIF1 element can act as an enhancer on the SI promoter, reporter constructs were made with four copies of the SIF1 element located distant from the SI promoter [see Materials and Methods; phSI(-66)SIF1 enhancer]. A minimal SI promoter that contained only the SIF1 site and the TATA box was used in these constructs. Caco2 cells were used as the cell line in these experiments because in some respects they model an enterocyte phenotype (33), express the SI gene (33) and express Cdx2 which is functional on the SI promoter (23,24). Transfection of Caco2 cells showed that the minimal promoter supported low level transcription when compared with the promoterless luciferase vector, as previously shown (Fig. 2a) (23,24). Transfection of a construct that included four copies of the SIF1 element inserted downstream of the luciferase reporter gene resulted in a 20-fold increase in luciferase activity (Fig. 2a). Moreover, the ability of the SIF1 cassette to induce transcription was independent of orientation (Fig. 2a). As a control for the effect of the SIF1 element cassette on non-promoter dependent transcription, no activation of transcription was seen in the absence of the SI promoter (Fig. 2a).

Enhancer activity of Cdx2 binding sites on a heterologous promoter

Experiments were conducted to determine whether the SIF1 element was capable of activating a heterologous promoter. The herpes simplex virus thymidine kinase (TK) promoter was chosen because it has activity in multiple cell types. The SIF1 binding site increased transcription when placed upstream of the TK promoter, showing greater induction with increasing copy number of the SIF1 element (Fig. 2b). Similar to the results for the SI promoter, the SIF1 cassette when placed in an enhancer context induced expression of luciferase from the TK promoter (Fig. 2b).

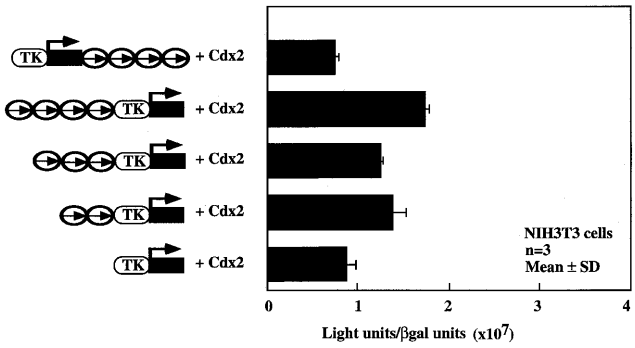

Figure 3. Effect of SIF1 element on expression of a thymidine kinase promoter construct in NIH-3T3 cells. NIH-3T3 cells were transfected with 8 µg reporter, 3 µg pRC/CMV Cdx2, and 2 µg pCMV-β-galactosidase.

Cdx2 is not sufficient for activation of a SIF1 containing enhancer

We examined whether enhancer constructs were able to activate transcription over that of the promoter alone when transfected into NIH3T3 cells. We have previously shown that NIH3T3 cells support transcription of the SI gene via the binding of Cdx2 protein to the SIF1 element in the promoter (11). However, the presence of the enhancer cassette did not affect transcriptional activation of the TK promoter construct in NIH3T3 cells (Fig. 3).

Functional localization of the Cdx2 transcriptional activation domain

To examine the mechanisms of transcriptional activation of both promoters and enhancers by Cdx2 we first identified the transcriptional activation domain of Cdx2. Deletion of amino

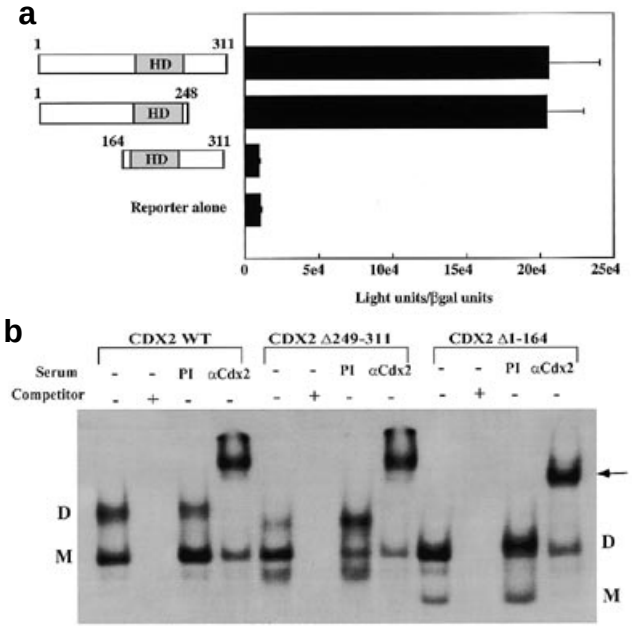

Figure 4. Functional characterization of the transcriptional activation domain of Cdx2. (**a**) Analysis of activation function of Cdx2 deletion proteins. NIH3T3 cells were transfected with 2 µg of the pRc/CMV Cdx2 wild-type (amino acids 1–311) or the indicated deletion constructs and 2 µg of the SI promoter (bases –183 to +54) linked to a luciferase reporter gene. Two µg of pCMV-β-galactosidase was co-transfected to control for transfection efficiency. This experiment was repeated three times with similar results (n = 3 for each experiment). (**b**) Binding of Cdx2 wild-type and deletion proteins to SIF1. Nuclear extracts from NIH3T3 cells transfected with Cdx2 wild-type and deletion constructs were analyzed for their ability to bind to the SIF1 element of the SI promoter. Monomer (M) and dimer (D) complexes are indicated. For competition experiments, a 100-fold molar excess of SIF1 oligo was used. For supershift experiments, preimmune (PI) or anti-Cdx2 antibody (αCdx2) was used. The supershifted complexes are indicated with the arrow.

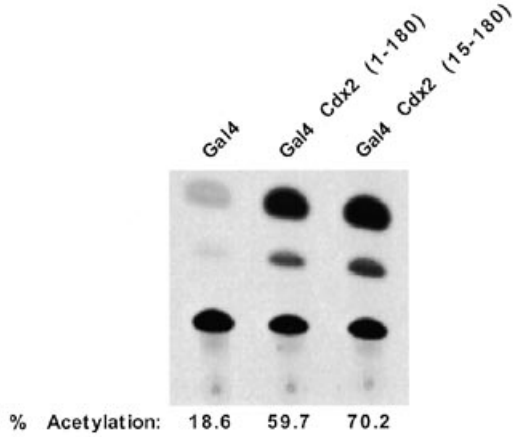

Figure 5. Functional characterization of the transcriptional activation domain of Cdx2 using Gal4 fusion constructs. NIH3T3 cells were transfected with plasmids expressing the Gal4 DNA binding domain alone (Gal4), or the Gal4 DNA binding domain fused to the indicated portions of the Cdx2 protein along with a CAT reporter containing five Gal4 binding sites linked to the E1b TATA box. Two µg of pCMV-β-galactosidase was co-transfected to normalize for transfection efficiency. Percent acetylation was calculated by phosphoimage analysis. The results shown are representative of five independent experiments.

acids N-terminal of the homeodomain eliminated the ability of the protein to activate transcription of the SI promoter, whereas deletion of the amino acids C-terminal to the homeodomain had no effect on transcriptional activation (Fig. 4a). EMSA of the nuclear extracts from transfected cells showed that the deletion constructs were expressed and bound the SIF1 element (Cdx binding element of the SI promoter) (Fig. 4b). To better define and localize the Cdx2 activation domain, chimeric expression plasmids were made linking coding sequence for two lengths of amino acid residues of the Cdx2 protein to the C-terminus of the Gal4 DNA binding domain. The activation domain was found to reside within amino acids 15–180 of the Cdx2 protein (Fig. 5). Immunoblot analysis showed that the Gal4 fusion proteins were expressed in transfected cells (data not shown).

Differential activation of transcription in Caco2 and NIH3T3 cells is not dependent on the SIF1 element

There are multiple possibilities for the observed differences between Caco2 and NIH3T3 cells in their ability to activate a construct with the SIF1 element placed in an enhancer context. One possibility is that there are nuclear proteins in Caco2 cells in addition to Cdx2 that bind to the SIF1 site. Such proteins may act as heterodimers with Cdx2, bind to SIF1 independently, or bind to SIF1 in association with Cdx2. To test this possibility, we examined whether the activation domain of Cdx2 could activate transcription from an enhancer context when tethered to DNA via a different DNA binding domain. Reporter constructs used in these experiments included a minimal E1B promoter with 10 Gal4 sites cloned upstream of the promoter (Gal4 promoter construct) and the TK promoter with 10 Gal4 sites cloned downstream of the luciferase reporter gene (Gal4 enhancer construct).

Expression of Gal4-Cdx2(15–180) in Caco2 cells activated both the Gal4 promoter and enhancer construct (Fig. 6a). When the Gal4 sites were placed in an enhancer position in relationship to the E1B minimal promoter, there was no activation of transcription (data not shown). This indicates that enhancer function requires promoter elements other than the TATA box. We next examined the function of Gal4-Cdx2(15–180) on promoter and enhancer constructs in NIH3T3 cells. As in Caco2 cells, Gal4-Cdx2(15–180) activated transcription from the E1B-Gal4 promoter construct demonstrating that this construct was expressed and functional in NIH3T3 cells (Fig. 6b). In striking contrast however, there was no activation by Gal4-Cdx2(15–180) of the enhancer construct (Fig. 6b).

These results demonstrate two points. First, the findings are consistent with the observation that wild type Cdx2 was unable to activate the SIF1 enhancer construct in NIH3T3 cells. Second, Cdx2 is capable of activating an enhancer element independent of its DNA binding domain, indicating that for Cdx2 to act on an enhancer it is not necessary to postulate that another DNA binding protein is involved.

Comparison of enhancer activity of the Cdx2 activation domain to VP16

In order to determine whether the activation of the enhancer construct in Caco2 cells was a specific property of the Cdx2 activation domain or only the cell line, we compared activation by the Cdx2 activation domain to VP16, a potent transcriptional activator. VP16 activated both promoter and enhancer constructs

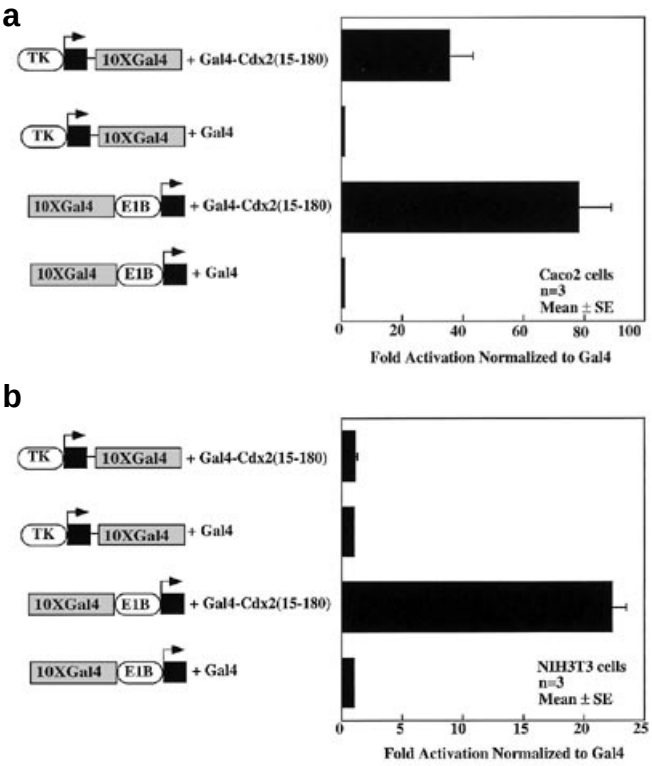

Figure 6. (**a**) Functional analysis of Gal4 Cdx2 (15–180) in NIH3T3 cells. NIH3T3 cells were transfected with 10 µg of either pSG424 Gal4 or pGal4 Cdx2 (15–180) expression plasmid and 10 µg of either Gal4 promoter [pE1B-LUC promoter(Gal4x10)] or Gal4 enhancer reporter [pTK-LUC enhancer(Gal4x10)]. Two µg of pCMV-β-galactosidase was cotransfected to control for transfection efficiency. (**b**) Gal4 fusion constructs Caco2 cells were transfected with 10 µg of either pSG424 Gal4 or pGal4 Cdx2 (15–180) expression plasmid and 10µg of either Gal 4 promoter [pE1B-LUC promoter(Gal4x10) or GAL4 enhancer reporter (pTK-LUC enhancer(Gal4x10)]. Two µg of pCMV-β-galactosidase was cotransfected to control for transfection efficiency.

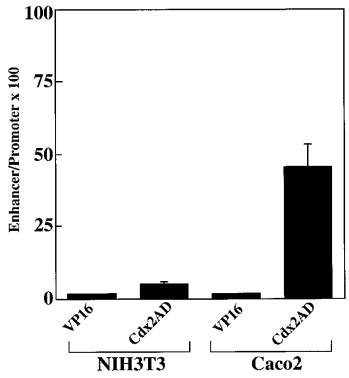

Figure 7. Comparison of activity of Cdx2 activation domain and VP16 in NIH3T3 and Caco2 cells. The graph shows the ratios of enhancer to promoter activity of VP16 and the Gal4 Cdx2 (15–180) (Cdx2AD) in NIH3T3 and Caco2 cells. The values were obtained from Table 1.

in Caco2 and NIH3T3 cells, with overall greater activation seen in Caco2 cells (Table 1). However, the ratio of enhancer to promoter activation in both cell lines for VP16 was very low. For the Cdx2 activation domain, there was also greater expression of both the promoter and enhancer constructs in Caco2 cells than in NIH3T3 cells (Table 1). However, in contrast to the VP16 data, the ratio of enhancer to promoter activation for Cdx2 was much greater in Caco2 cells (Table 1 and Fig. 7). Thus, although the overall transcriptional activity is greater in Caco2 cells, the relative activity on enhancer compared to promoter constructs appears to be specific for the Cdx2 activation domain when compared to VP16.

DISCUSSION

The function of DNA regulatory elements and their cognate DNA binding proteins is dependent on the proximity of other regulatory elements and the position of the cluster of elements with respect to the start of transcription. Native transcriptional regulatory units are composed of multiple regulatory elements that act cooperatively to regulate transcriptional initiation (34). Promoters are regulatory units localized immediately upstream of the transcriptional start site, whereas enhancers are able to regulate transcription in either orientation from locations remote from the transcriptional start site. In this study, we examined the ability of a homeodomain protein, Cdx2, to activate a DNA regulatory element placed in either a promoter or an enhancer context. A reductionist approach was taken by removing the Cdx2 binding site from the more complex environment of a natural promoter or enhancer and by examining the Cdx2 activation domain in isolation. Using this experimental design we showed that there are cell line-specific differences in the ability of Cdx2 to activate transcription from proximal and remote positions.

When the Cdx2 activation domain was tethered to DNA in close proximity to a minimal promoter it activated transcription in both intestinal and non-intestinal cell line. In contrast, when tethered to the DNA in an enhancer context, Cdx2 activated transcription in a cell-restricted fashion. Caco2 cells, a colon cancer cell line with enterocyte-like characteristics, supported enhancer-directed transcription by the Cdx2 activation domain, whereas NIH3T3 cells did not support activation of enhancer constructs by Cdx2. This effect was also specific for the Cdx2 activation domain and not simply a characteristic of these cell lines. Gal4-VP16 activated transcription of promoter and enhancer constructs in both cell lines, but on a relative basis this powerful transcriptional activator was much less active from the enhancer context in Caco2 cells than the Cdx2 activation domain. Thus, the Cdx2 activation domain functions on enhancer elements in a cell-specific manner.

Previous studies have shown that transcriptional activation domains may have different functions depending on whether the domain is tethered to DNA in a proximal (promoter) or a remote (enhancer) position (35). Multiple activation domains are able to activate transcription from both promoter and enhancer positions, although there is considerable variability in the level of activity. Negatively charged domains (VP16, Gal4, p65 of NFκB, TFE3) and serine/threonine rich domains [(ITF-1(E47) and ITF-2(E2-2)], are generally potent activators of both promoter and enhancer elements. Proline-rich domains of AP-2 and CTF/NF1 have marked activity from promoter elements and only weak activity from an enhancer position. In contrast, the glutamine-rich domains of Oct-1, Oct-2 and Sp1 are only able to activate promoter elements and have no effect on enhancer elements when transfected into HeLa cells (35). However, these studies have been performed in a limited number of cell lines and, therefore may not be entirely representative of the functional capacity of these domains.

Table 1.

	NIH3T3			Caco2		
	Enhancer	Promoter	E/P × 100	Enhancer	Promoter	E/P × 100
VP16	8.5 ± 1.5	491 ± 8	1.73 ± 0.3	31.6 ± 2.4	1827 ± 21	1.73 ± 0.2
Cdx2 (15-180)	1.1 ± 0.2	22.3 ± 1.2	5.0 ± 1.1	35.4 ± 8	78 ± 11	45.5 ± 7.9

Other studies have demonstrated that the function of activation domains in proximal and remote positions may be dependent on other cellular factors. Functional diversity of activation domains has been well defined for the POU-homeodomain proteins Oct-1 and Oct-2 in B lymphocyte lineages. The potency of the N- and C-terminal domains of the Oct-2a protein on activation of a promoter construct is greater in B-cell lines than in HeLa cells (35). In contrast, the VP16 activation domain is more active in HeLa cells than in B-cell lines (35). Additionally, an important difference was found between the N- and C-terminal domains with regard to transcriptional activation of sites in an enhancer context (36). The N-terminal, glutamine-rich domain was unable to activate in a remote position in either HeLa or B-cell lines. The C-terminal domain was able to markedly activate transcription from a remote position in B-cells, an activity that was absent in HeLa cells. Taken together, these data suggested that a B-cell specific cofactor, or cofactors, may potentiate transcriptional activation in the B-cell lineage.

Recently an Oct co-activator protein has been cloned and characterized by three groups, OBF-1 (37,38), Bob-1 (39,40) and OCA-B (41). This co-activator is expressed in B-cell lineages, interacts with the POU domain of Oct-1 and 2 proteins, and stimulates transcription in a promoter specific manner. Since OBF-1 has been shown to interact with TBP and TFIIB, one mechanism of action for this protein may involve bridging the connection between Oct proteins and the basal transcriptional apparatus (38). With regard to the activation of enhancers by the C-terminal domain of Oct-2a, the OBF-1 co-activator is not able to reconstitute this activity in non B cells (38). Thus, it is likely that there will be several tissue-specific co-activators binding to separate domains of Oct-2, mediating various functions. OBF-1 is the first tissue-specific co-activator protein that has been well characterized, and raises the likelihood that other tissue-specific co-activators will be discovered for other transcription factors.

The function of the Cdx2 activation domain suggests similarities to the regulatory mechanisms for Oct proteins. Intestinal and non-intestinal cell lines supported activation of promoter constructs by Cdx2, whereas the ability of Cdx2 to activate an enhancer construct was clearly cell line-specific with marked activation in intestinal Caco2 cells and no activation in NIH3T3 cells. One potential explanation of this finding is a co-activator protein in Caco2 cells that interacts with the Cdx2 activation domain, thus mediating increased activity from an enhancer position. There are also other potential mechanisms for these findings including cell-specific phosphorylation of the Cdx2 activation domain.

Our findings may have important implications for function of Cdx2 during development, as well as in adult tissues. In this study, we show that two functional elements in the Hoxc-8 gene enhancer interact specifically with Cdx2 protein. The Hoxc-8 gene is expressed in embryonic tissues that also express Cdx2 and it is a reasonable hypothesis that Cdx2, or other members of the Cdx gene family, may serve to regulate transcription of the Hoxc-8 gene via interaction with these two sites. In this regard, the presence of co-activator proteins that modulate the function of Cdx proteins could play an important role in the function of this enhancer during development. It is important to note that there are functional differences between the two enhancer binding sites, suggesting that the regulation via these elements is more complex than simply interaction with Cdx proteins.

In the intestine, the findings also have potential implications for regulation of intestinal genes. The fact that Cdx2 can act in a position remote from the transcriptional start site extends the range of targets to multiple intestinal genes, including those that do not have a promoter element that binds Cdx2. Moreover, a co-activator protein that interacts with Cdx2 activation domain might provide a second level of regulatory control over Cdx2 responsive genes within the intricate cellular architecture of the intestinal epithelium and during complex transitions occurring in development and differentiation [for reviews, see (42–45)]. A co-activator for Cdx2 would allow cell-specific and spatial regulation of Cdx2-dependent genes in a manner independent of Cdx2 expression.

In summary, we have identified a cell-specific functional difference in the biology of the Cdx2 homeodomain protein. We hypothesize that the ability of Cdx2 to activate transcription in an enhancer context is dependent on either a cell-specific co-activator protein or phosphorylation of the activation domain.

ACKNOWLEDGEMENTS

This work was supported by RO1 DK46704 (P.G.T.). We thank Dr Mitchell Lazar for providing the TK-LUC vector, Dr Thomas Kadesch for providing the plasmid containing the Gal4 sites, and Dr James Alwine for providing pSG424.

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