Helix-loop-helix transcription factors regulate Id2 gene promoter activity

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Abstract **Id-like helix-loop-helix (HLH) transcription factors are involved in the regulation of proliferation and differentiation of several cell types. We isolated 5′ regulatory region of mouse Id2 gene and demonstrated that it contains several E-box clusters. These E-boxes mediate stimulatory effects of basic-HLH (bHLH) transcription factors ME1, ME2, and NSCL1 on Id2 promoter activity. Co-expression of Id2 blocks the stimulatory effect of bHLH transcription factors which suggests the presence of feedback loops in Id2 transcriptional regulation. Overexpression of NSCL1 in F9 cells blocks the downregulation of Id2 gene expression during retinoic acid induced differentiation. Our data demonstrate that bHLH transcription factors regulate Id2 gene expression.**

Key words: Helix-loop-helix; Id2; NSCL; ME1; ME2; Promoter; Gene expression

1. Introduction

Helix-loop-helix (HLH) transcription factors regulate several developmental processes in different organisms. These factors form an interacting network of transcription factors that regulate many genes including HLH transcription factor genes themselves [1,2]. Different HLH transcription factors potentially interact with each other by forming heterodimers [3]. The HLH transcription factors which form heterodimers are grouped into four classes: class A includes ME1 (HEB, REB, HTF4), ME2 (ITF2), and E2A which are expressed ubiquitously [3–10]; class B includes cell type and tissue specific proteins such as NSCL, NEX, MASH-1, MASH-2, MyoD and others [11,14]; HES-family includes negatively acting transcriptional regulators HES-1, HES-2, HES-3, HES-5 [15–18]; and Id-like family includes transcriptional regulators lacking the basic, DNA binding domain such as Id1, Id2, Id3, and Id4 [19–22]. The activity of each individual HLH transcription factor depends on the complexes formed, and how these complexes bind to DNA and interact with the basic transcriptional complex. For example, class A or class B bHLH transcription factors form non-DNA-binding transcriptionally inactive heterodimers with Id-like molecules [21]. This is the manner in which Id-like proteins regulate onset of myogenesis. Id1 forms inactive complexes with myogenic HLH transcription factors, and, only after the expression of Id1 decreases, the myogenic HLH transcription factors become active [23].

During the nervous system development, the Id2 gene has a changing expression pattern [24]. At early stages of neurogenesis, Id2 is active in proliferating neuroblasts. After the first neuronal populations are born, the expression of Id2 is down regulated in neuroepithelial cells and continues to be high in early borned neurons. Class A bHLH transcription factors ME1 [9] and E12 [25] are expressed in proliferating neuroblasts and their expression is downregulated after cells migrate out from the ventricular zone and initiate differentiation. ME2 (ITF2), a member of class A bHLH family, is expressed in both proliferating neuroblasts and differentiating neurons in several regions of the developing nervous system [10]. Neuronal class B bHLH transcription factor NSCL1 is expressed transiently during the period when the first born neurons start differentiation, and the expression is limited to the subependymal layer of the neuroepithelium [13]. Based on the expression pattern of HLH genes during neurogenesis and on the presence of several E-boxes in the Id2 gene regulatory region, we hypothesize that the expression of Id2 gene is regulated by the HLH transcription factors. In this paper we demonstrate that Id2 gene promoter has two clusters of E-box sequences and that its activity is stimulated by the bHLH transcription factors and suppressed by the Id2.

2. Materials and methods

2.1. Plasmid construction

Mouse Id2 promoter CAT constructs, -2182 CAT, -1283 CAT, -508 CAT and -114 CAT were generated as described [24] using *Bam*HI, *Pst*I, *Hin*dIII, and *Eco*RI restriction enzymes. Promoter fragments were cloned into the *Bgl*II site of the pBLCAT3 vector [26] using *Bgl*II linkers. All the CAT constructs were sequenced using primer in the CAT gene. Full length cDNAs for bHLH transcription factors ME1a, ME2, Id2 [9,10,24], and NSCL1 [13] were cloned into eukaryotic expression vector pRcCMV (Invitrogen). Double stranded mutated E-box cluster A and B oligonucleotides were cloned into the blunted *Xba*I site of the pBLCAT2 [26] reporter plasmid in the front of herpes simplex virus thymidine kinase promoter.

2.2. Cell culture

Neuroblastoma/glioma hybrid NG108–115 cells (gift from Dr. M. Fishman) and teratocarcinoma F9 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco). F9 cells were differentiated with all-*trans* retinoic acid (5×10^{-7} M) and dibutyryl cyclic AMP (1 mM) treatment. To establish cell lines overexpressing ME2 and NSCL1 bHLH transcription factors, ME2 and NSCL1 expression plasmids (pRcCMV-ME2 and pRcCMV-NSCL1, respectively) were transfected into F9 cells followed by selection with G418 (400 μg/ml) for 21–30 days. Expression of ME2 and NSCL1 was analyzed by the Northern blot and cell lines with high expression of transgene were used for further analyses.

2.3. DNA transfection and CAT assays

Cells were transfected with 15 μg of total plasmid DNA using calcium phosphate precipitation method in 60 mm dishes (1–3 × 105 cells/dish). Five μg of reporter DNA construct and 10 μg of eukaryotic expression construct were used for each transfection. The medium was changed to normal growth media 12 to 18 h after transfection. Cells were harvested 48 h later and CAT assays were performed as described [9,24]. Quantitation of acetylation ratios was obtained by PhosphorImager analysis (Molecular Dynamics). To normalize transfection efficiencies, cells

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were cotransfected with 1 μg of the pRcRSVlacZ plasmid. All the CAT activities are normalized to total protein and lacZ activity. The CAT assay values represent the mean of at least three independent transfections.

2.4. Northern blot analysis

Total RNA from untreated and retinoic acid and dBcAMP treated control and ME2 or NSCL1 overexpressing F9 cells was isolated using acid guanidinium/phenol/chloroform extraction procedure [28]. Twenty five micrograms of total RNA were run in each lane and fractionated on 1.2% agarose-formaldehyde gel before transfer onto a nylon membrane (Hybond N, Amersham). The amount and quality of transferred RNA were monitored by methylene blue staining of the filters before hybridization. The full length Id2 cDNA was radiolabeled with [α-^{32}P]dCTP using the Multiprime DNA labeling system (Amersham) and used as a probe. The blots were washed at high stringency (0.2× SSC, 65°C) and exposed to X-ray film for 2 days.

3. Results

3.1. Analysis of the 5′ end of the Id2 gene

A phage clone containing the 5′ end of the Id2 gene that extended for 2,661 nucleotides upstream of the translation initiation codon has been isolated and sequenced (GeneBank accession number U10994). The transcription initiation sites were mapped by primer extension and RNase protection analyses [24,27].

The sequence of Id2 gene proximal promoter contains a consensus TATA box (31 nucleotides from the tanscriptional start site), Sp1 binding site, and two CAAT boxes close to the TATA box. Two E-box clusters and several single E-boxes are localized in the sequenced region of the Id2 gene 5′ end (Fig. 1A). The presence of E-boxes in the 5′ regulatory region raises the possibility that Id2 gene is regulated by the HLH transcription factors and is a subject to negative feedback regulatory loops.

3.2. Effect of basic helix-loop-helix transcription factors on the activity of Id2 promoter

To determine sequences responsible for the effects of bHLH transcription factors on mouse Id2 gene, various portions of the 5′-flanking region were fused to the bacterial chloramphenicol acetyl transferase (CAT) gene as a heterologous reporter gene [24]. The constructs carrying different fragments of the Id2 gene 5′ sequence (Fig. 1A) were transfected into neuroblastoma/glioma NG108–115 hybrid cell line together with NSCL1, ME1, ME2, and Id2 cDNAs cloned into an expression vector under control of CMV promoter. Our previous work demonstrated that the activity of longest Id2 promoter-reporter construct (-2182 CAT) in NG108–115 cells is two times higher compared to the shortest reporter construct (-114 CAT) [24]. Cotransfection of the bHLH transcription factors NSCL, ME1, ME2 with the -2182 CAT construct stimulate the promoter activity around six fold (Fig. 1B). Deletion of the E-box cluster A (construct -1283 CAT, Fig. 2B) reduces the effect of bHLH transcription factors on Id2 promoter about two times (Fig. 1B). Deletion of both E-box clusters, A and B, (construct -508 CAT) further reduces the bHLH transcription factor mediated stimulation of promoter activity (Fig. 1B). Deletion of all E-boxes (construct -114 CAT) eliminates the effect of NSCL and ME1, but ME2 still stimulates 114 bp promoter fragment activity. Co-expression of Id2 suppresses the stimulatory effect of bHLH transcription factors as well as basal CAT activity for

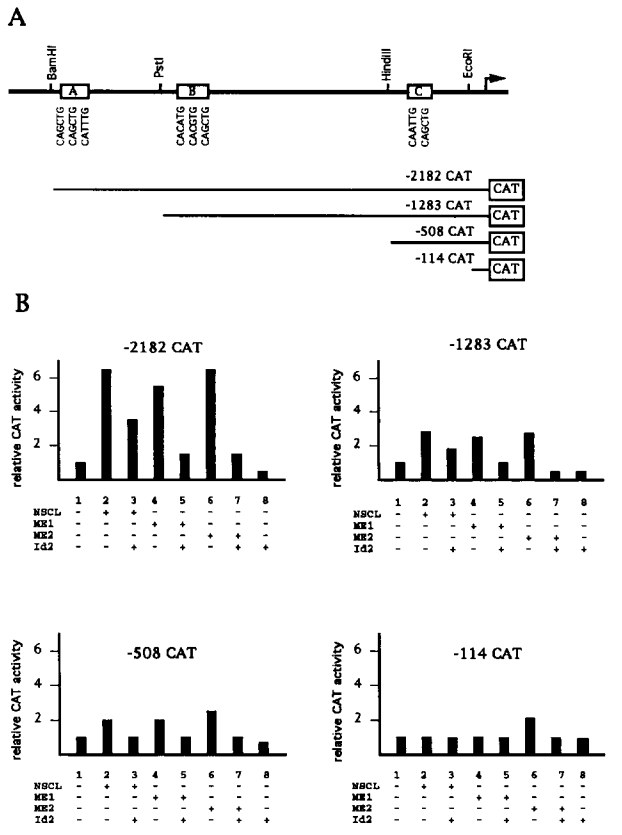

Fig. 1. Effect of different HLH transcription factors on the activity of Id2 promoter in transient assays. (A) Schematic representation of Id2 gene promoter region and CAT constructs. Restriction sites used in generation of CAT constructs and E-box clusters A, B, and C are indicated. (B) Various combinations of NSCL1, ME1, ME2, and Id2 expression constructs (5 μg) were transfected into NG108–15 cells, as indicated below the panel, together with -2182 CAT, -1283 CAT, -508 CAT, and -114 CAT reporter constructs (2 μg). The CAT activities, normalized to the expression of RSVlacZ construct cotransfected as an internal standard, represent the average of three experiments and are expressed relative to the value obtained by transfection of reporter construct and pRcCMV expression plasmid without insert (line 1). Standard deviations were less than 25% from the average values in all experiments.

all the reporter constructs containing E-box sequences. Interestingly, Id2 almost completely blocks the stimulatory effect of ME1 and ME2 but reduces the effect of NSCL by only about 50% (Fig. 1B).

Mutations were introduced into individual E-boxes in clusters A and B to analyze the role of individual E-boxes on the stimulation of Id2 gene promoter by NSCL, ME1, and ME2 bHLH transcription factors. Mutated oligonucleotides containing clusters A and B were cloned in the front of Herpes simplex virus thymidine kinase (HSV TK) gene promoter. Cluster A contains three E-box sequences (Fig. 2A), and we mutated these E-boxes individually (Fig. 2A, oligonucleotides A1, A2, and A3) and all three together (Fig. 2A, oligonucleotide A4). Transient CAT assays using mutated oligonucleotides in front of TK CAT reporter gene demonstrated that mutation in the first E-box (A1) resulted in about a 60% reduction of NSCL, ME1, and ME2 stimulated CAT activity (Fig. 2B). Mutation in the second E-box reduced NSCL1 and ME1 mediated stim-

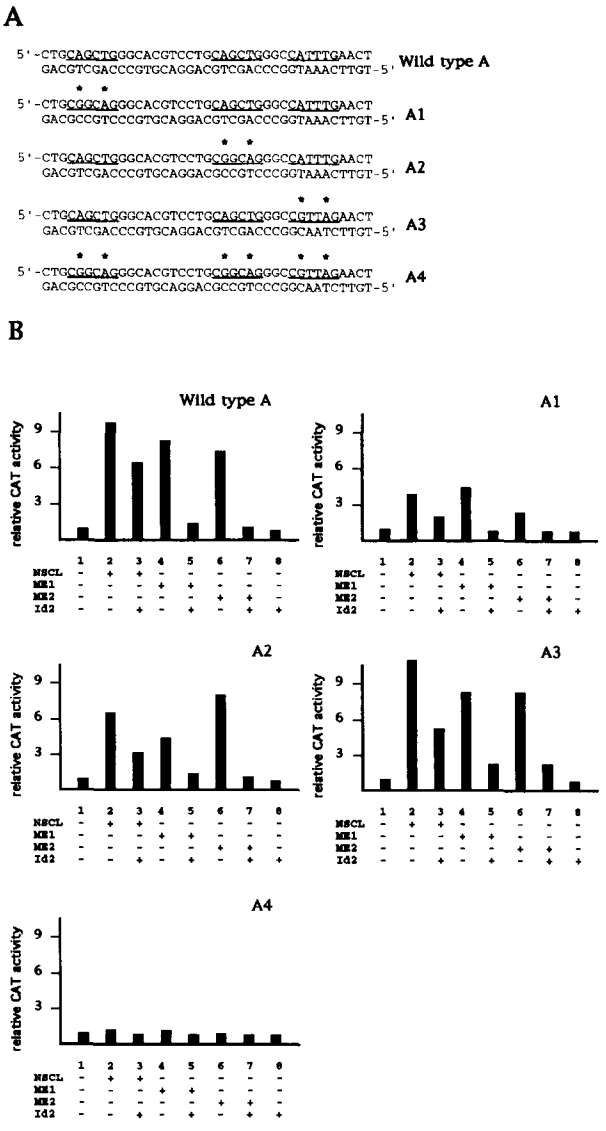

Fig. 2. Effect of mutations in E-box cluster A on heterologous promoter activity. (A) Nucleotide sequences of wild type (Wild type A) and mutant (A1, A2, A3, and A4) E-box cluster A. E-box sequences are underlined and mutations are indicated by asterisk (*). (B) Wild type and mutated E-box cluster A containing oligonucleotides were cloned in the front of TK promoter in pBLCAT2 reporter plasmid. Reporter plasmid containing wild type (Wild type A) and mutated oligonucleotides (A1, A2, A3, and A4) were cotransfected with NSCL1, ME1, ME2, and Id2 expression plasmids into NG108–115 cells. The CAT activities, normalized to the expression of RSVlacZ construct cotransfected as an internal standard, represent the average of three experiments and are expressed relative to the value obtained by transfection of reporter construct and pRcCMV expression plasmid without insert (line 1). Standard deviations were less than 25% from the average values in all experiments.

ulation of CAT activity about 30% and did not affect ME2 mediated induction. Mutation in the third E-box did not affect stimulatory effect of any of the bHLH transcription factors. By contrast, mutations in all three E-boxes (A4) eliminated the stimulatory effect of all bHLH transcription factors. We also introduced mutations into cluster B E-boxes. Mutations in the first or second E-box (Fig. 3A, oligonucleotides B1 and B2) resulted in about a 50% reduction of CAT activity stimulated by the different bHLH transcription factors (Fig. 3B). Double mutation (oligonucleotide B3) completely blocked the effect of bHLH transcription factors. These data demonstrate that Id2 promoter E-boxes function as enhancers in the front of a heterologous promoter, and that bHLH transcription factors NSCL, ME1, and ME2 have different effects on individual E-boxes.

3.3. NSCL blocks downregulation of Id2 in differentiating F9 cells

We generated teratocarcinoma F9 cell lines which overexpressed ME2 and NSCL1 cDNAs under the control of CMV promoter (data not shown) to test the hypothesis that these bHLH transcription factors regulate expression of the Id2 gene. Expression of the Id2 gene is suppressed in normal F9 cells after induction of differentiation with all-trans retinoic acid and dibutyryl cAMP (Fig. 4). Downregulation of Id2 gene is less extensive in ME2 overexpressing cells and does not occur in NSCL1 overexpressing cells (Fig. 4). Although Fig. 4 represents

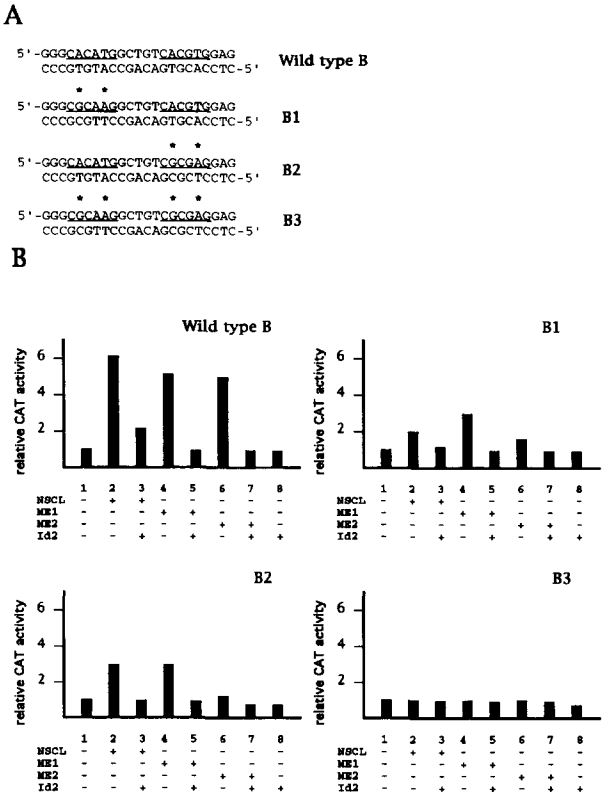

Fig. 3. Effect of mutations in E-box cluster B on heterologous promoter activity. (A) Nucleotide sequences of wild type (Wild type B) and mutant (B1, B2, and B3) E-box cluster B. E-box sequences are underlined and mutations are indicated by asterisk (*). (B) Wild type and mutated E-box cluster B containing oligonucleotides were cloned in the front of TK promoter in pBLCAT2 reporter plasmid. Reporter plasmid containing wild type (Wild type B) and mutated oligonucleotides (B1, B2, and B3) were cotransfected with NSCL1, ME1, ME2, and Id2 expression plasmids into NG108–115 cells. The CAT activities, normalized to the expression of RSVlacZ construct cotransfected as an internal standard, represent the average of three experiments and are expressed relative to the value obtained by transfection of reporter construct and pRcCMV expression plasmid without insert (line 1). Standard deviations were less than 25% from the average values in all experiments.

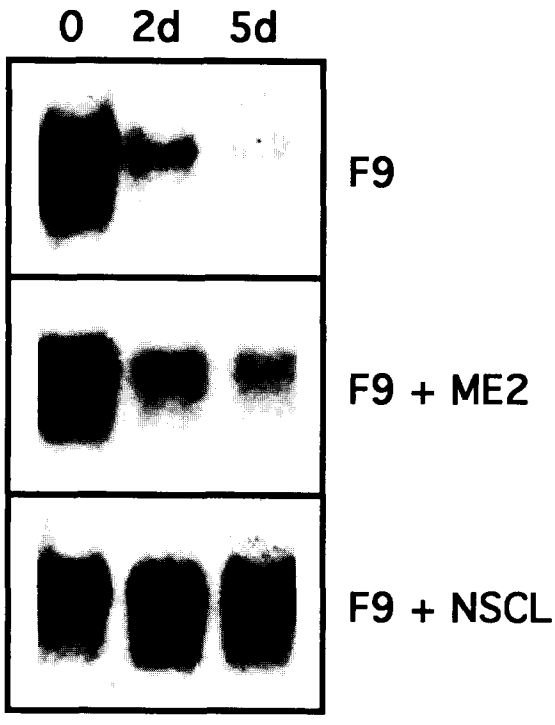

Fig. 4. Northern blot analyses of retinoic acid and dibutyryl cAMP induced downregulation of Id2 expression in ME2 and NSCL1 overexpressing F9 teratocarcinoma cells. Total RNA was isolated from control (0), treated 2 and 5 days (2d and 5d respectively) with retinoic acid and dibutyryl cAMP normal F9 (F9), and ME2 and NSCL1 overexpressing F9 cells (F9 + ME2 and F9 + NSCL, respectively). Overexpression of NSCL1 in F9 cells blocks retinoic acid and dibutyryl cAMP induced downregulation of Id2 gene.

the data obtained from one ME2 and NSCL1 overexpressing clone, identical results were obtained with four additional clones as well as a pool populations of ME2 and NSCL1 overexpressing clones. Additionally, analyses of cell clones and pool populations of cells overexpressing ME2 and NSCL1 cDNAs with mutations that disrupt open reading frame did not result in stimulation of Id2 expression in differentiating F9 cells. These data suggest that NSCL1 is an essential regulator of Id2 expression in differentiating cells.

4. Discussion

In this paper, we explore the possibility that HLH transcription factors are at least partially responsible for the expression of Id2 gene. Recently, it has been demonstrated that human Id2A gene promoter requires presence of Sp-1 and ATF-like sites in the proximal promoter to be expressed in C2 cells, whereas these sites are not essential for the expression in nonmuscle cells, including HeLa and 10T1/2 cells [29]. Whether the proximal promoter Sp-1 and ATF-like sites are important for the Id2 expression in other tissues and cell types is unknown. The presence of E-box sequences in the 2182 bp 5′ regulatory region suggests that HLH transcription factors may regulate expression of Id2 gene. We demonstrated that bHLH transcription factors ME1, ME2, and NSCL1 stimulate the Id2 promoter activity, and that Id2 itself suppresses basal as well as stimulated promoter activity. Suppression of basal promoter activity by Id2 argues that HLH transcription factors present in NG108–115 cells and interacting with Id2 protein, stimulate Id2 gene expression. Previously, we have shown [24] that 508 bp promoter fragment (-508 CAT) which contains E box cluster C is essential for down-regulation of Id2 promoter activity during differentiation of NG108–115 cells. The role of E box cluster C and HLH transcription factors in this down-regulation is unknown.

Expression of several class A bHLH transcription factors and Id-like transcriptional regulators is downregulated during differentiation [9,24,25,30,31] and stimulated when cells are induced to proliferate [32,33]. Also, overexpression of Id2 potentiates proliferation which is likely related to its ability to bind Rb protein [34]. It has been suggested that Id-like transcriptional regulators function as general inhibitors of differentiation and activators of proliferation [23,35]. How these two different functions are coordinated in different cell types remains to be characterized. However, it is clear that the expression level of Id-like proteins is a critical parameter in the regulation of proliferation and differentiation.

In this paper, we demonstrate that bHLH transcription factors regulate Id2 gene promoter activity. In developing nervous system, class A bHLH transcription factors [9,25] and Id2 [24] are expressed in the ventricular zone where proliferating cells are localized. Expression of E12 and ME1 become downregulated in developing neurons when they migrate from the ventricular zone. Also, Id2 expression is reduced in differentiating neurons with the except of first born neurons which continue to express Id2 during their lifetime [24]. This similarity in expression patterns is additional evidence that class A bHLH transcription factors may be necessary for the expression of Id2 in neuroblasts. At the time when first born neurons migrate from the ventricular zone, class b bHLH gene NSCL1 becomes active in these neurons [13]. The presence of NSCL1 transcription factors in differentiating first born neurons may be necessary for the continuous expression of Id2 gene. This hypothesis is supported by our experimental data obtained using NSCL1 overexpressing F9 teratocarcinoma cells. Expression of Id2 gene is downregulated in RA and dBcAMP treated F9 cells but overexpression of NSCL1 blocks the RA and dBcAMP induced suppression of Id2 gene in F9 cells.

At this point, we do not know whether the E-boxes identified in this study are essential for properly directing Id2 gene expression during development or in adult organism. Thus, the physiological role of the E-boxes will require further investigations using mutational analyses of Id2 promoter on transgenic mice.

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