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A Role for Sp and Helix-Loop-Helix Transcription Factors in the Regulation of the Human Id4 Gene Promoter Activity*

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Id family helix-loop-helix (HLH) proteins are involved in the regulation of proliferation and differentiation of several cell types. To identify cis- and trans-acting factors that regulate Id4 gene expression, we have analyzed the promoter regulatory sequences of the human Id4 gene in transient transfections and gel mobility shift assays. We have identified two functional elements, both located downstream from the TATA motif, that control Id4 promoter activity. One element contains a consensus E-box, and we demonstrated that the protein complex binding to the E-box contains the bHLH-zip upstream stimulatory factor (USF) transcription factor. Enforced expression of USF1 leads to E-box-mediated stimulation of promoter activity. The E-box also mediated stimulatory effects of several bHLH transcription factors, and co-expression of Id4 blocked the stimulatory effect mediated by the bHLH factors. A second element is a GA motif, located downstream from the transcriptional start sites, mutation of which resulted in a 20-fold increase in transcriptional activity. Gel-shift analysis and transfections into *Drosophila* Schneider SL2 cells showed that the repressor element is recognized by both Sp1 and Sp3 factors. These data suggest that Id4 transcription control is highly complex, involving both negative and positive regulatory elements, including a novel inhibitory function exerted by Sp1 and Sp3 transcription factors.

The basic-helix-loop-helix (bHLH)[1] family of transcription factors has been shown to play a key role in the differentiation processes of a number of cell lineages (1–3). These proteins contain an HLH domain consisting of two amphipathic helixes separated by a loop, which mediates homo- and heterodimerization, plus an adjacent DNA-binding region rich in basic amino acids (4, 5). The bHLH proteins bind to a DNA sequence known as an E-box (CANNTG) or to the related N-box (CACNAG) (6, 7). There are two major categories of bHLH. The class A are ubiquitously expressed proteins such as those encoded by the differently spliced transcripts of the E2A (E12, E47, and E2–5/ITF1), E2–2/ITF2, and HEB/HTF4 genes (5, 8–10). Class B comprises tissue-specific bHLH proteins such as MyoD, NeuroD, MASH, and TAL (1–3, 11, 12). Dimerization is essential for binding and transcriptional regulation *in vivo*, and in general tissue-specific bHLH form heterodimers with a partner from the ubiquitously expressed class A family (13, 14). These factors form an interacting network that regulate transcription of several genes.

Another class of HLH proteins is defined by the Id genes, which share a highly homologous HLH domain but which lack the basic DNA-binding region. Four members of the Id family have been identified in human and mouse (13, 15–22). Analogous proteins have been identified in *Xenopus laevis* and in *Drosophila* (23, 24). It has been shown that the Id proteins, by heterodimerization with bHLH proteins, inhibit their binding to DNA (13, 15, 21). Thus, the Id proteins act as dominant negative regulators by sequestering the ubiquitously expressed class A proteins and preventing them from forming active heterodimers with the tissue-restricted bHLH proteins. Accordingly, it has been found that the down-regulation of Id is necessary for differentiation to proceed in many cell lineages (13, 21, 25). Conversely, ectopic expression of Id genes inhibits differentiation (25–30). Several lines of evidences also suggest that at least some Id proteins may play a role in controlling proper G_0 to S phase transition in cultured mammalian cell lines (19, 31, 33, 34). Moreover, the expression pattern of dnHLH genes during mouse embryogenesis is temporally and spatially controlled (35). The mechanisms that control Id expression will be important in understanding the molecular events that lead to differentiation. Regions of the Id1 and Id2 promoters controlling the expression of the genes and their response to differentiation or cell cycle withdrawal have been recently identified (36–39). However, very little is known about the expression and regulation of the Id4 gene.

We have chosen to examine the Id4 promoter as a means of identifying elements that may be important for proper transcriptional regulation. Using deletion analysis and site-directed mutagenesis in transient transfection experiments, we have identified in the proximal Id4 promoter two cis-acting regulatory elements. One element is an E-box, and gel shift experiments in the presence of specific antibodies demonstrated that the protein complex binding to the E-box contains the ubiquitously expressed USF transcription factor. Co-transfection experiments demonstrated that the E-box mediates stimulatory effects exerted by USF1. Moreover, the Id4 promoter E-box also represents an effective target site accessible for transcriptional activation mediated by several transcription factors of the bHLH family such as E2A and Myo-D. Co-expression of Id4 blocks the stimulatory effect mediated by the bHLH transcription factors, suggesting the presence of feedback loops in Id4 transcriptional regulation. Conversely, USF1-mediated stimulation was not inhibited by Id4 co-expression. A second element is a GA motif located downstream from the transcriptional start sites. Mutation of this element resulted in a near

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF030295.

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[1] The abbreviations used are: bHLH, basic helix-loop-helix; HLH, helix-loop-helix; kb, kilobase pair(s); CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay; USF, upstream stimulatory factor.

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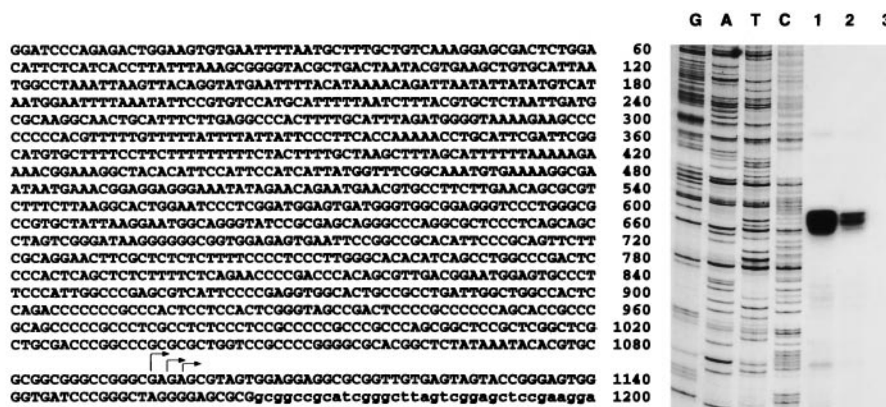

FIG. 1. **Sequences of the 1.2-kb promoter Id4 region and mapping of the transcriptional start sites by primer extension.** On the *left* are reported the DNA sequences of the genomic region encompassing the 5′ end of the published Id4 cDNA. The genomic sequences present in the 5′ end of the previously reported Id4 cDNA are indicated in *lowercase*. On the *right* is reported a primer-extension mapping of the Id4 mRNA. HeLa mRNA (*lane 1*, 100 μg; *lane 2*, 40 μg) and 50 μg of tRNA (*lane 3*) were used in primer-extension experiments using a labeled primer complementary to the 5′-cDNA sequences. The extended products were run on a sequencing gel along with the DNA sequencing reactions performed using the same primer.

20-fold increase in transcriptional activity. Gel-shift analysis and transfections into *Drosophila* Schneider cells showed that this repressor element is recognized by both Sp1 and Sp3 factors. These data suggest that Id4 transcriptional control is highly complex and highlight a novel inhibitory function exerted by Sp1/Sp3 transcription factors.

EXPERIMENTAL PROCEDURES

Reporter Plasmids—The 1.2-kb *Bam*HI-*Not*I genomic fragment encompassing the 5′ end of the Id4 cDNA was subcloned in the *Bam*HI and *Not*I sites of pBluescript KS+ (Stratagene). Subclones were generated using convenient restriction endonucleases and sequenced with the dideoxy sequencing method using the T7 sequencing kit (Amersham Pharmacia Biotech). Luciferase reporter constructs were obtained via cohesive or blunt-end ligation of the pBluescript subclone inserts in pGL2 basic (Promega); details of the construction procedure are available upon request. The pGL-Id#6 and pGL-Id-Id#7 were constructed by cloning into the *Sma*I-*Hin*dIII sites of pGL2 a double-stranded oligonucleotide containing sequences from residues −42 to +32 or −25 to +32, respectively. Similarly, pGL-Id#6 E^-, pGL-Id#6 Sp^- and pGL-Id#8 reporters were constructed using mutated oligonucleotides.

Effector Plasmids—The pCMV-Id4 was constructed by cloning an *Apa*I-*Not*I fragment corresponding to residues 241–808 of the previously published cDNA sequence encoding a full-length Id4 protein (20) into the expression vector pRC-CMV (Invitrogen). The pCMV-USF1 expression plasmid has been described previously (40) and was provided by Dr. R. Roeder (Rockefeller University, New York). The structure of the pCMV/USF-VP16 has been reported (41) as the expression vectors pCMV/E12, pCMV/E47, and pCMV/E2–2, all of which were provided by Dr. T. Kadesch (University of Pennsylvania School of Medicine, Philadelphia).

Primer Extension—Primer extension was performed as described previously (42). In brief, a 31-nucleotide oligomer (5′-TAGCCCACCCGGGTGTCCTAGTCACTCCTTC-3′) corresponding to residues 30–61 of the published Id4 cDNA sequence was 5′-labeled with [γ-^{32}P]ATP and used as a primer. The labeled primer was annealed with different amounts of total mRNA from HeLa cells and processed as described (42). The primer extension products were analyzed by electrophoresis on 6% polyacrylamide, 7 M urea sequencing gel. The length of the fragments obtained were estimated by comparison with sequence reactions loaded on the same gel.

Cell Lines and Transfections—HeLa, C33A, and NIH3T3 cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies, Inc.). Subconfluent cell cultures were transfected by the calcium phosphate method using different amounts of reporter and effector plasmids as described in the text, while maintaining at a constant level the quantity of DNA transfected by the addition of genomic carrier DNA. All transfections included a reference sample with pGL2 basic. Cells were harvested 48 h after addition of the precipitates, and extracts were assayed for luciferase activity. For normalization of transfection efficiencies, 400 ng of renilla (sea pansy) luciferase expression plasmid was included in the transfections (pRL-CMV, Promega). Luciferase assays were performed using the Dual-Luciferase Reporter assay (Promega) according to the manufacturer's instructions. The experimental reporter luciferase activity was calculated by subtracting the intrinsic activity as measured by samples corresponding to the pGL2 basic and then normalized to transfection efficiency as measured by the activity deriving from pRL-CMV.

Drosophila Schneider cells were grown at 25 °C in Schneider medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum and transfected by the calcium phosphate method. The pPac-Sp1 and pPacSp3 expression vectors have been described previously (43, 44). 48 h after addition of the precipitates, the cells were harvested and extracts assayed for luciferase activity using the luciferase assay system (Promega) following the manufacturer's instructions.

Nuclear Extracts and EMSA—Nuclear extracts were prepared according to published procedure (45) using subconfluent HeLa cell cultures. The double-stranded oligonucleotide corresponding to residues −48 to +32 of the Id4 promoter was labeled by filling the terminal 3′ end with the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of α^{32}P-dCTP. Nuclear extracts were preincubated in a 20-μl mix containing 10 mM Hepes (pH 7.9), 25 mM NaCl, 1 mM EDTA, 0.25 mM dithiothreitol, 10% glycerol, 2 mM magnesium spermidine, 25 ng/μl poly(dI-dC) for 5 min on ice with a 50-fold molar excess of the unlabeled competitor specified in the text. The probe (1 μl of 10,000 cpm/μl) was then added and the incubation continued for 20 min at 20 °C. The reaction was stopped by the addition of gel loading buffer, and the mixture was immediately resolved on a 5% acrylamide-bisacrylamide (29:1) 0.5×TBE nondenaturing gel at 15 V/cm. The run was stopped when the bromphenol blue reached the lower margin of the gel. In supershift experiments, the double-stranded oligonucleotides described in the figures were end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Antisera against E2A (gift of Dr. Tomas Look, St. Jude Children's Research Hospital, Memphis, TN), Myc and Max (Santa Cruz), USF (gift of Dr. Piaggio, Laboratorio di Oncogenesi Moleculare CRS-IRE, Rome, Italy), or Sp1 and Sp3 proteins (44) were preincubated with nuclear extracts for 30 min on ice before addition of the probe, and the incubation was prolonged as described above.

RESULTS

Identification of Promoter Sequences Required for Id4 Expression—To investigate the transcriptional control of Id4 expression, we isolated a human sequence containing the 5′ end of the Id4 gene. A human genomic phage library was screened with an Id4 cDNA probe, and one phage clone containing a 17-kb insert was isolated and further characterized. The sequence of relevant portions of this region confirmed the presence of the Id4 gene and demonstrated the presence of two introns, the first located shortly after the HLH encoding region, and the second in the 3′-untranslated region of the cDNA (data not shown). A 1.2-kb genomic fragment, which contained sequences upstream of the previously reported 5′ end of the Id4 cDNA (20), was subcloned and sequenced (Fig. 1). The Id4 transcription initiation site was determined by primer extension (Fig. 1). Three closely spaced transcriptional start sites were identified 26 base pairs downstream of the TATA-box in a region 70 base pairs to the 5′ end of the published cDNA sequences (Fig. 1). Thus, the cloned 1.2-kb genomic fragment contains mainly the Id4 promoter sequence. To identify functional elements contained within the Id4 promoter, the 1.2-kb fragment (Fig. 1) was cloned into the luciferase reporter vector pGL2 (Promega), and a series of 5′ and 3′ deletion constructs was generated (Fig. 2). These deletion constructs were transiently transfected into HeLa cells, and luciferase activity was

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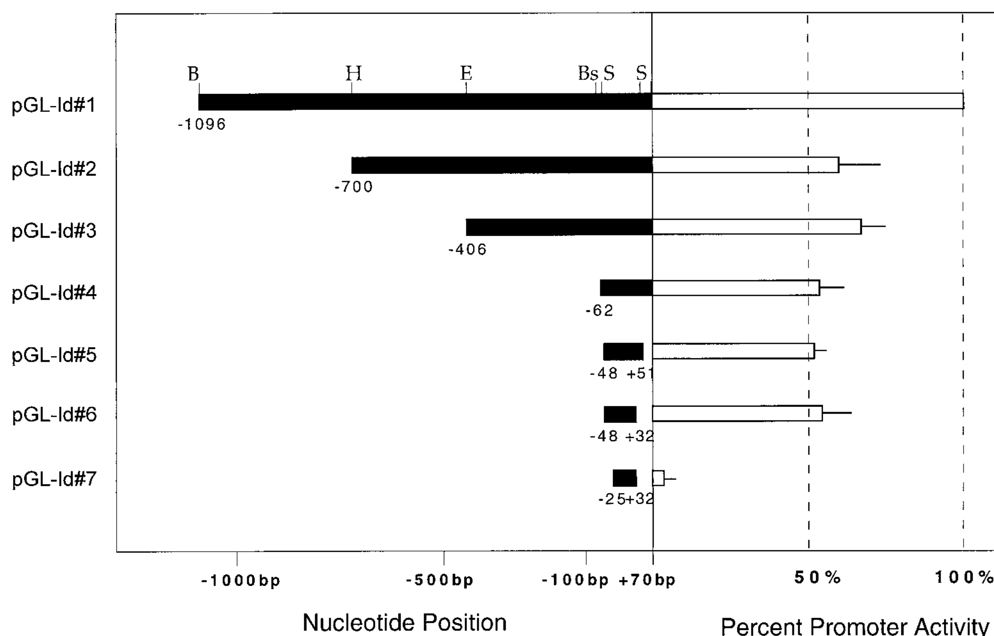

FIG. 2. **Schematic diagram of the deletion mutants of the human Id4 promoter and effect of subclones on the promoter activity.** The *number* indicates the last or the first nucleotide position upstream of the major transcription start site of the Id4 gene. Each pGL-Id construct was transfected into HeLa cells as described in the text. The promoter activity expressed from each subclone relative to the 1.2-kb fragment activity (6.6×10^3 relative light units) is shown on the *right panel*. *B*, *Bam*HI; *H*, *Hin*dIII; *E*, *Eco*RI; *Bs*, *Bss*HII; *S*, *Sma*I.

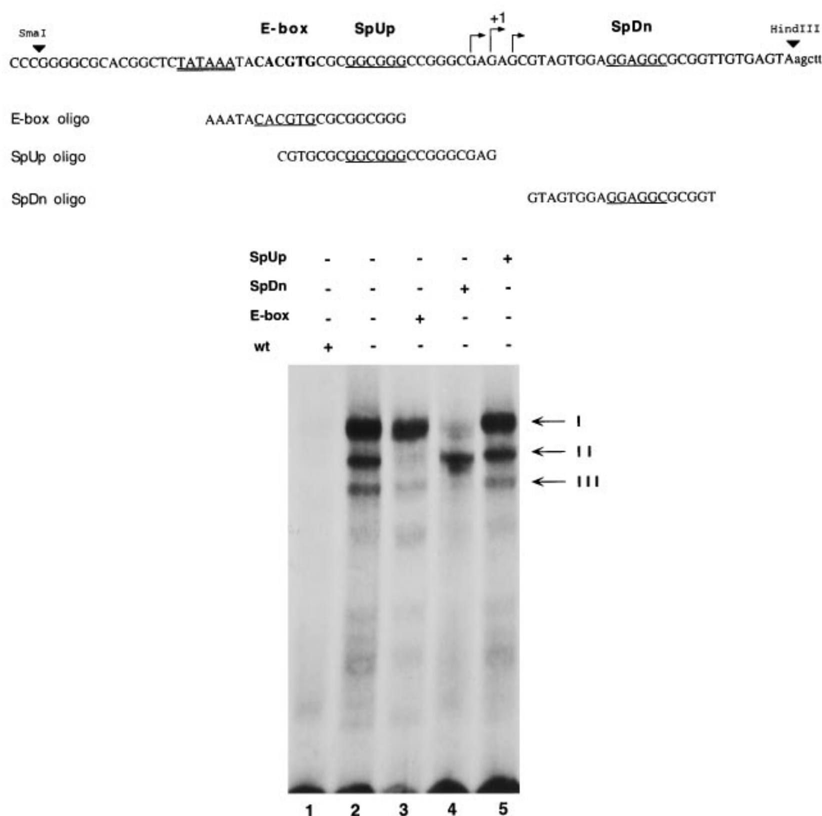

FIG. 3. **Two sites in the Id4 promoter interact with DNA binding activities in EMSA assays.** DNA sequences of the Id4 minimal promoter (−48 to +32) used as a probe in EMSA experiments are shown on the *top*. The TATA-box, the E-box, and two putative Sp binding sites, designated SpUp and SpDn, are in evidence. *Broken arrows* indicate transcriptional start sites, as identified by the primer-extension shown in Fig. 1, with +1 residue attributed to the major start site. The oligonucleotides used for competition in EMSA assays are aligned with the corresponding regions on the promoter. *Bottom*, EMSA analysis was performed by incubating an end-labeled *Sma*I-*Hin*dIII fragment (−48 to +32) with HeLa nuclear extract. Three DNA-protein complexes (I, II, and III) are observed (*lane 2*), and they are specifically inhibited by the unlabeled probe (*lane 1*). Addition of specific double-stranded competitor oligonucleotides encompassing the SpDn (*lane 3*) or the E-box (*lane 4*) resulted in specific inhibition of complexes, whereas no inhibition was observed using the SpUp oligonucleotide as a competitor (*lane 5*).

determined. Fig. 2 shows that deletions up to position −48 did not result in significant changes in the promoter strength, with an activity of the longest Id4 promoter-reporter being only 2 times higher when compared with the pGL-Id#6 construct (−48/+32), and the promoter activity was completely lost using the pGL-Id#7 containing sequences from −25 to +32. These results indicated that the minimal promoter comprises a relatively small region spanning from −48 to +32, and a potential positive regulatory element appear to be located in the sequences from −1096 to −700.

Factors Binding to Id4 Minimal Promoter—The sequence of the Id4 gene proximal promoter contains a TATA-box, an E-box (−23/−18) and two putative Sp1 binding sites at −14 and + 13, designated SpUp and SpDn, respectively (Fig. 3). To identify transcription factors able to bind to these elements, an end-labeled fragment covering residues from −48 to +32 of the Id4

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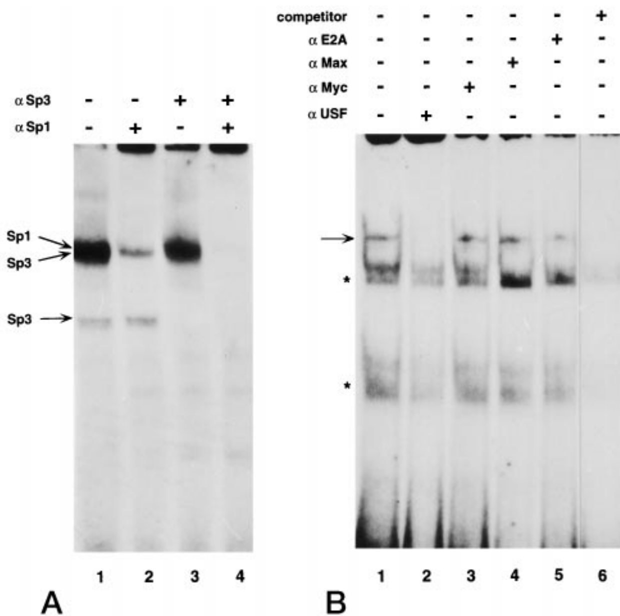

FIG. 4. **Sp1, Sp3, and USF factors binding to elements located in the Id4 promoter.** *Panel A*, EMSA analysis was performed with an end labeled SpDn probe. SpDn oligonucleotide was incubated with HeLa cells nuclear extract only (*lane 1*) or with extracts preincubated with anti-Sp1 (*lane 2*), anti-Sp3 (*lane 3*), or both antisera (*lane 4*). The shifting pattern is indicated by *arrows*; inclusion of cold competitor leads to disappearance of all bands (data not shown). *Panel B*, EMSA analysis was performed with an end labeled E-box probe. E-box oligonucleotide was incubated with HeLa cells nuclear extracts only (*lane 1*) or with extracts preincubated with anti-USF (*lane 2*), anti-Myc (*lane 3*), anti-Max (*lane 4*), anti-E2A (*lane 5*), or unlabeled competitor (*lane 6*). A specific interaction band is indicated with the *arrow*. The *asterisks* denote nonspecific interaction bands, which were not consistently seen with different HeLa nuclear extract preparations.

minimal promoter was incubated with nuclear extracts from HeLa cells, and then subjected to gel electrophoresis. As shown in Fig. 3, the interaction of Id4 promoter fragment with cell nuclear extracts resulted in three specific protein-DNA complexes (*lane 2*). Specificity of binding was shown by a competition assay (*lane 1*). Because the Id4 promoter fragment appears to contain an E-box and two Sp-binding sites, we also used as a competitor specific oligonucleotides spanning these three potential sites, respectively. Using the E-box oligonucleotide as competitor (*lane 3*), complex II was eliminated, while complex I was unaffected and complex III was partially competed. Thus, it appears that complex II is formed by a protein-DNA complex located on the E-box. Moreover, the partial competition of complex III might be due to the presence of a GC-box within the E-box competitor oligonucleotide, which may bind some Sp-like factor(s) in the presence of E-box but not when it is absent (see competition in *lane 5*). Inclusion in the binding reaction of a 50-fold excess of a cold oligonucleotide spanning the SpDn site eliminated complexes I and III, suggesting that at least two specific complexes were formed at the GA-box located downstream from the transcriptional start site. Finally, no effect was observed using the SpUp oligonucleotide. Taken together, these results suggest that complexes I and III are sequence-specific complexes formed at the Sp site located downstream of the start site, and complex II is a specific protein-DNA complex formed at the E-box site.

To identify the protein factors interacting with the E-box and the Sp-site we used oligonucleotides covering the E-box and the SpDn sites in gel mobility shift assays, respectively. As shown in Fig. 4*A*, two distinct protein complexes were observed when the SpDn probe was incubated with HeLa nuclear extracts. It has been shown previously that both Sp1 and Sp3 are able to bind GC- and CT-boxes with similar efficiency, and both proteins are present in many mammalian cell lines (43, 44). To determine the presence of specific members of the Sp family in the complexes formed at the SpDn site, EMSA assays were carried out in the presence of specific antisera raised against bacterially expressed Sp1 and Sp3 proteins, respectively (44). As shown in Fig. 4*A*, it appears that the slow complex also contains an Sp1 binding activity, because the inclusion of Sp1 antiserum in the binding reaction resulted in a reduction of this complex, whereas no effects on the fast migrating complex were seen (*lane 2*). On the other hand, addition of Sp3-specific antisera in the binding reactions resulted in the absence of the fast migrating (compare *lanes 1* and *3*). If both antisera against Sp1 and Sp3 were present in the binding reactions, all three complexes were no longer detected (*lane 4*). Thus, in accordance with previous studies (43, 44), we found the the slow migrating complex represent a doublet of unresolved Sp1 and Sp3 bands. The EMSA data clearly showed that the GA-box located downstream from the start site is a binding site for both Sp1 and Sp3 transcription factors.

The E-box located just downstream of the TATA element may represent a putative binding site for several transcription factors, such as the ubiquitously expressed USF transcription factors, members of the Myc/Max family, and bHLH factors. When the E-box containing oligonucleotide was used as a probe (Fig. 4*B*) a specific complex was observed, which was inhibited by the presence of a 50-fold molar excess of specific competitor. Nonspecific interaction bands were found, as shown by the *asterisks* in Fig. 4*B*. Albeit they were only partially competed by the presence of a 50-fold specific competitor (*lane 6*), these nonspecific complexes were not seen with different HeLa nuclear extract preparations, and a similar partial competition (as reported in *lane 6*) was also observed with a nonspecific oliogonucleotide competitor (data not shown). To help identify the protein(s) present in the complex, we included in the binding reaction antibodies against factors potentially able to bind to this motif. We found that the inclusion of an antibody directed against human USF1 protein, which recognizes all USF family members in the binding reaction, resulted in the absence of the complex (lane 2). Conversely, no effects were found using antibodies raised against human Myc, Max, and E2A, respectively (Fig. 4*B*). Together, these data indicate the complex formed at the E-box site located just downstream of the TATA element, contains the USF1 transcription factor or a protein antigenically related to USF.

Effect of Helix-Loop-Helix Transcription Factors on the Activity of the Id4 Promoter—The presence of a specific USF complex formed at the E-box within the minimal promoter region raised the possibility that Id4 gene transcription might be regulated by the ubiquitously expressed USF1 transcription factor. To determine the functional consequences of ectopic expression of USF on Id4 promoter activity, the Id4 reporter pGLId#6 was co-transfected into HeLa cells together with a USF1 expression vector (40). However, we found that enforced expression of USF resulted in a modest (2–3-fold) activation of the Id4 promoter. Because the transactivation domain of USF1 has been reported to be relatively weak (46), we used a USF-VP16 construct, which contains the activation domain derived from the strong viral activator VP16 inserted in the N-terminal portion of USF1 (41). Using the USF-VP16, we observed a clear E-box-dependent activation of Id4 promoter. The USF-VP16-mediated activation required binding to the E-box as the site-specific E-mutation abolished USF-VP16 stimulation (Fig. 5*A*). The specificity of USF1-mediated activation was further demonstrated by co-transfection experiments with members of the bHLH-zip Myc family of transcription factors. We found that

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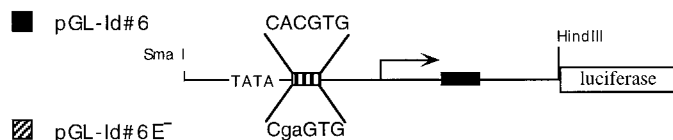

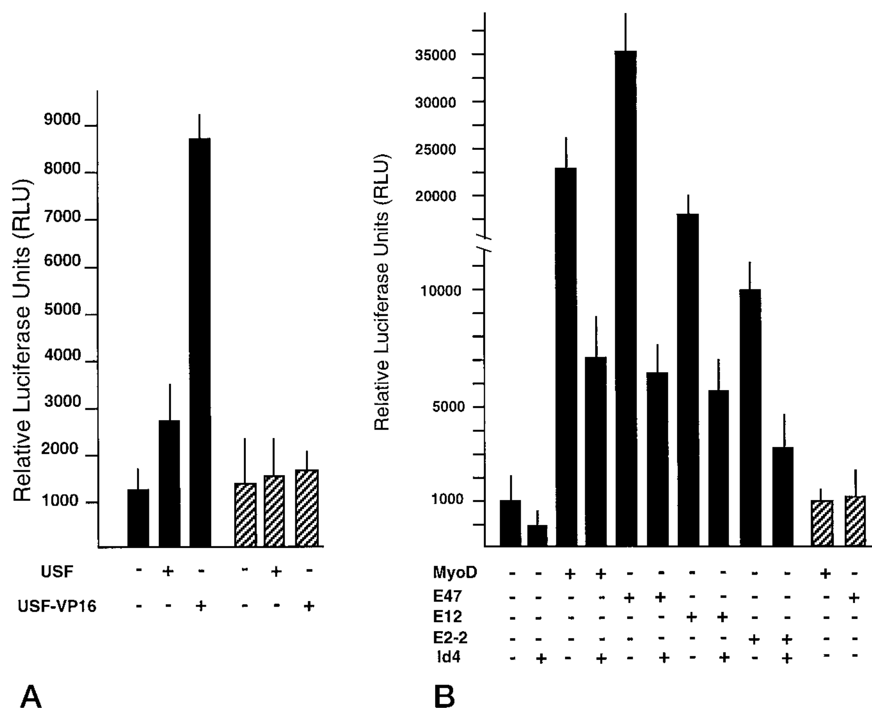

FIG. 5. **The E-box of Id4 promoter mediates transactivation by USF1 and bHLH transcription factors.** *Top*, schematic description of pGL-Id#6 and its derivative pGL-Id#6 E⁻, in which the E-box has been mutated. *Panel A*, pGL-Id#6 and pGL-Id#6 E⁻ (5 μg) were transiently co-transfected into HeLa cells along with CMV-based expression vectors (10 μg) encoding the human USF1 and USF-VP16 transcription factors or with the empty CMV vector, as indicated. *Panel B*, the Id4 luciferase reporters were co-transfected into HeLa along with the pCMV-based expression vectors (10 μg) encoding different bHLH factors in the presence or absence of the Id4 expression vector (10 μg), as indicated. The values presented were normalized with the internal control as described in the text and are representative of three experiments. Standard deviations are shown by *vertical bars*.

co-transfections of the Id4 reporter with expression vectors for Myc, Max, and Mad bHLH-zip factors did not affect the Id4 promoter activity (data not shown).

The presence of the E-box also raised the possibility that the Id4 promoter might be regulated by members of the bHLH family of DNA-binding transcription factors. It has been shown recently that the Id2 gene promoter contains an E-box, which is a target for positive regulation of promoter activity by different bHLH transcription factors. Co-transfection experiments reported in Fig. 5*B* clearly demonstrated that enforced expression of different members of the bHLH family (E47, E12, E2–2, and MyoD) efficiently stimulated Id4 promoter activity, and a mutation of the E-box motif eliminated the stimulatory effect of all bHLH factors (Fig. 5*B* and data not shown). Interestingly, co-expression of Id4 suppresses the stimulatory effect of bHLH transcription factors (Fig. 5*B*), suggesting that Id4 promoter activity might be regulated by bHLH transcription factors, and that such regulation is subject to a negative feedback regulatory loop.

Taken together, our transfection data suggest that the E-box present in the Id4 promoter binds to USF1 and is an effective target site for bHLH transcription factors, accessible for transcriptional regulation by members of the HLH family of transcription factors.

The Id4 Promoter Is Negatively Regulated by the Sp Transcription Factors—The EMSA experiments reported in Fig. 4*A* suggest that the transcription factors Sp1 and Sp3 bind to the GA-box located downstream from the Id4 transcription start site. To further investigate the ability of both transcription factors to bind and regulate the Id4 promoter, we carried out co-transfection experiments using the *Drosophila melanogaster* Schneider SL2 cells, which are devoid of endogenous Sp1-like activity and so are instrumental for examining Sp1-mediated activation *in vivo* (43, 44, 47). The expression vectors for Sp1 and Sp3 (pPacSp1 and pPacSp3) have been described previously (43, 44), and it has been shown that Sp1 and Sp3 proteins are expressed in comparable efficiency in transfected SL2 cells (44). SL2 cells were transfected with expression vectors (pPac) for Sp1 and Sp3 together with the pGLId#6 and pGLId#6 Sp⁻ reporters, respectively (Fig. 6*A*). In these cells, Sp1 enhanced the promoter activity very efficiently, whereas Sp3 showed a relatively modest stimulatory effect. Promoter stimulation was dependent upon the presence of the Sp-binding site, as mutations introduced in this motif abolished any stimulatory effect. Moreover, EMSA using HeLa nuclear extract demonstrated that the mutation introduced into the SpDn site of pGL-Id#6 Sp⁻ reporter prevented binding of Sp1/Sp3 factors and it did not created a new protein binding site (Fig. 6*C*, and data not shown). This set of data clearly demonstrated that both Sp1 and Sp3 can bind the Id4 promoter *in vivo*, and that they both activate the Id4 promoter *in vivo*.

Having defined the GA-box located downstream from the Id4 start site as a genuine Sp1/Sp3 binding site, we then tested the consequences of mutations of this motif on the promoter activity in mammalian cells. As shown in a separate set of experiments (Table I) a specific mutation of the SpDn site present in the pGL-ID#6 Sp⁻ reporter, and a 3′-deletion of the SpDn site present in pGL-Id#8 reporter (containing sequences from −25 to +13) led to strong activation of the Id4 promoter. These data suggest that the wild-type GA-box participates in the partial

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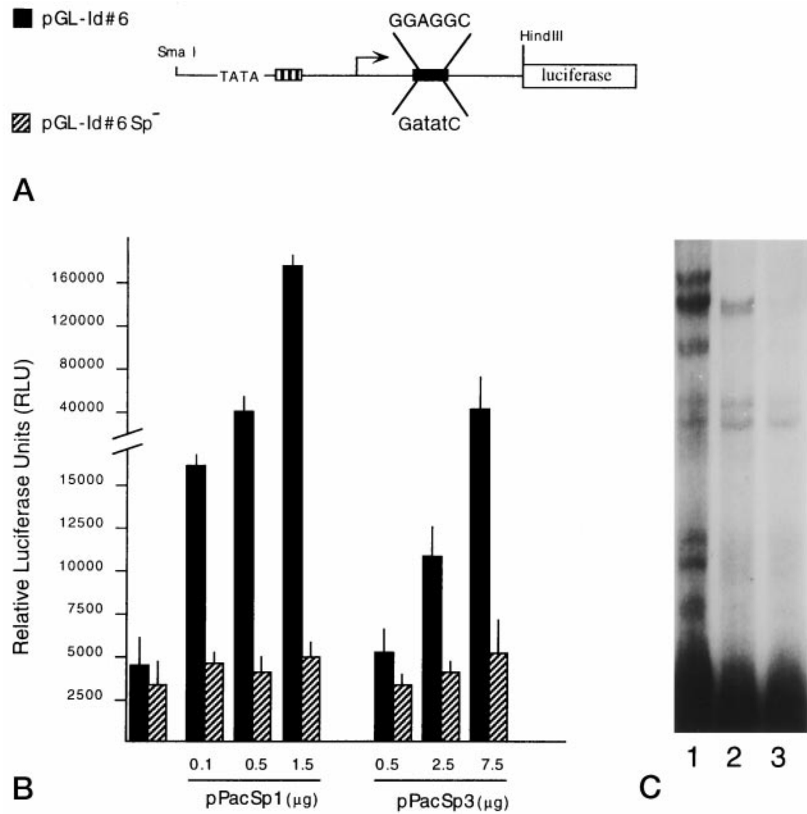

FIG. 6. **Both Sp1 and Sp3 activates Id4 promoter in *Drosophila* Schneider cells.** *A*, schematic representation of the reporter plasmids. *B*, the reporters (4 μg) were transfected into *Drosophila* SL2 cells along with different amounts of either pPacSp1 or pPacSp3 as indicated. Values represent the average of three independent transfections, standard deviations are shown by *vertical bars*. *C*, EMSA analysis was performed as in Fig. 3 with the labeled *Sma*I-*Hin*dIII fragments from pGL-Id#6 (*lane 1*) and pGL-Id#6 Sp- (*lanes 2* and *3*); addition of the E-box oligonucleotide (*lane 3*) eliminates the complex formed by nuclear extracts with the probe with the mutated SpDn site.

TABLE I
Effect of Sp-binding site mutation on transcription initiation from the Id4 minimal promoter

Plasmids pGL-Id#6 and pGL-Id#6 Sp^- were transiently transfected into the indicated cell lines. The mean values ± S.E. are given for five experiments (C33A), two experiments (NIH3T3), or three experiments (HeLa). Activity is normalized for transfection efficiency.

Reporter plasmid	Activity		
	HeLa	NIH3T3	C33A
	%	%	%
pGL-Id#6	100	100	100
pGL-Id#6 Sp^-	3500 ± 153	3500 ± 450	5900 ± 350
pGL-Id#8	2800 ± 210	ND	5500 ± 400

suppression of luciferase expression from the Id4 promoter. Furthermore, because diverse cell lines respond in the same manner, suppression of expression driven by Id4 promoter is probably caused by a factor(s) common to these cell lines.

DISCUSSION

The goal of this study was to identify cis-elements and trans-acting factors important for transcriptional regulation of the human Id4 gene. We have demonstrated that the core promoter of the Id4 gene comprises a relatively small region spanning nucleotide residues from −48 to +32. Apart from the TATA-box, the presence of which is strictly required for promoter activity, we have identified two regions that appear to be important for proper promoter activity, and both cis-acting elements are located downstream from the TATA motif.

The first cis-acting element is an E-box, and we have demonstrated that the USF1 transcription factor, or a protein antigenically related, is present in a complex detected in mobility shift experiments. Enforced expression of USF1 leads to a weak stimulation of promoter activity. These results appear to be consistent with the notion that the transactivation domain of USF1 has been reported to be relatively weak (46). Accordingly, using a chimeric protein comprising the full-length USF1 fused to the VP16 activation domain (41), we found a clear E-box-dependent activation of Id4 promoter. No effects were found using other bHLH-zip factors such as Myc, Max, and Mad. Thus, it appears that the E-box is a target for USF1 transcription activation. Although we have not obtained any evidence of the presence of bHLH factors in the complex formed at the E-box with HeLa cell nuclear extracts, we have determined that the Id4 promoter contains an effective E-box binding site accessible for transcriptional activation by all bHLH factors tested. Interestingly, co-expression of Id4 cDNA in these co-transfections inhibited the bHLH-mediated activation. These results strongly suggest that regulation of Id4 promoter activity may be subjected to a negative feedback regulatory loop. A similar regulatory loop has been demonstrated recently to regulate the Id2 promoter (39). However, the possibility that the bHLH-mediated activation may be indirect cannot be ruled out. In fact, we cannot exclude that overexpression of bHLH factors may activate USF1 gene transcription, which in turn will lead to enhanced Id4 expression.

Another important regulatory element identified in this study is a GA motif located downstream from the transcriptional start site, which functions as a negative cis-acting element recognized by two distinct members of the Sp1 family, Sp1 and Sp3. A number of pieces of evidence support the conclusion that the Sp1/Sp3 GA-binding site is involved in the partial suppression of transcription of the Id4 promoter. First, mutations of the Sp1/Sp3 binding site were found to enhance promoter strength severalfold in transient transfection. Second, the specific SpDn mutation present in pGL-Id#6 Sp^- reporter also prevented binding of HeLa nuclear extracts in mobility shift experiments and it did not create a new protein binding (Fig. 6*B*); thus, elimination of this site is responsible for activation. Finally, enhanced promoter activity of the GA-box mutants was observed with diverse cell lines, indicating that the mechanisms of activation and repression of the Id4 proximal promoter are preserved in different cellular back-

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grounds, an consistent with the ubiquitous nature of Sp1 and Sp3.

Multiple mechanisms can be envisioned whereby Sp1 and Sp3 binding could inhibit transcription. Sp1 has very often been described as an activator, and in many viral and cellular promoters, mutation of the Sp1-binding sites have resulted in decreased transcription. Sp3 was originally defined as an inhibitor of Sp1-mediated activation (43, 44), and it has been suggested that it competes with Sp1 for binding to the same recognition element (43, 44). However, several recent studies strongly suggest that Sp3 is a dual-function regulator, and the context of cognate DNA-binding sites in a promoter appears to be one of the elements that determines the strength of the Sp3-mediated repression (48, 49). Our experiments clearly show that Sp3 acts as a positive regulator in the *Drosophila* cell line SL2. The Sp3-mediated activation of the Id4 promoter is fully consistent with our recent report, indicating that Sp3 may function as an activator where there is a single recognition site and a repressor where multiple tandem recognition sites are present (49).

The level of complexity of promoter bearing Sp1/Sp3-binding sites has been heightened by experiments showing that Sp1-dependent transcription is influenced by specific cell-cycle regulator proteins. It has been reported that RB and p107, two members of the retinoblastoma protein family, activate or repress Sp1-dependent transcription in a cell-type-dependent manner (50–52). Furthermore, it has been suggested that RB indirectly stimulates Sp1 transactivation by liberating Sp1 from a putative Sp1 negative regulator factor Sp1-I (51). In all the above described examples, transcriptional repression is mediated by a physical or functional interference with Sp1 activation. The results reported here support a direct role of Sp1 or Sp3 in mediating transcription repression, although the molecular mechanism underlying this effect is not clear. Interestingly, it has been recently shown that Sp1 inhibits transcription from the core promoter of the human adenine nucleotide translocase 2 (ANT2) (53). In both ANT2 and Id4 promoters, the Sp1-binding repressor elements are located downstream from the TATA-box. It is well documented that TFIID can interact with, and function through, downstream core promoter DNA (54, 55). Therefore, the binding of Sp1 or Sp3 at a site located downstream from the TATA-box may decrease or alter the efficiency with which the transcriptional machinery is recruited or assembled.

Whatever the molecular mechanism responsible for repression, our data demonstrate that, in mammalian cells, Sp1 and Sp3 lower transcription efficiency when bound to the GA motif. However, it remains to be tested whether binding or release of these factors is a physiological mechanism for modulating Id4 expression.

During mouse embryogenesis, Id4 expression is up-regulated between days 9.5 and 17.5 post coitum, and *in situ* hybridizations indicate that Id4 expression is predominantly found in particular neural cells of the developing brain, spinal cord, and cranial ganglions (35). It will be of interest in the future to investigate whether the cis-acting elements identified in this study, as well as their potential binding factors, are involved in the regulation of the Id4 gene during neurogenesis.

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