Transcription of the Dominant-Negative Helix-Loop-Helix Protein Id1 Is Regulated by a Protein Complex Containing the Immediate-Early Response Gene *Egr-1*[†]

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The expression of Id1, a helix-loop-helix protein which inhibits the activity of basic helix-loop-helix transcription factors, is down-regulated during cellular differentiation and cell cycle withdrawal both in tissue culture models and in mouse embryos. In order to study the mechanism of control of Id1 expression, we have isolated a 210-bp enhancer element in the upstream region of the Id1 gene whose activity recapitulates Id1 expression in C2C12 muscle cells and C3H10T1/2 fibroblasts: i.e., this element is active in proliferating cells in the presence of serum and completely inactivated upon mitogen depletion, cell cycle withdrawal, and (in the case of C2C12) induced myoblast differentiation. Using linker-scanning mutations and site-directed mutagenesis in transient transfection experiments, we have identified two functional elements within the 210-bp enhancer which are required for proper serum responsiveness. One element (A) contains a consensus *Egr-1* binding site and additional flanking sequences required for optimal activity, and the other element (B) fits no known consensus. Gel shift experiments demonstrate that the protein complex binding to the A site contains *Egr-1* and other proteins. This complex as well as a protein complex that binds to the B site is lost within 24 h of serum depletion, correlating with the down-regulation of Id1 expression. On the basis of these findings, we propose that the regulation of the Id1 response to serum is mediated in part by the early response gene *Egr-1* and as such provides a signaling link between the early-growth-response transcription factors and dominant-negative helix-loop-helix proteins.

The differentiation of eukaryotic cells is coordinated by changes in the activity and expression of multiple classes of transcription factors. The helix-loop-helix (HLH) family of proteins is one class of transcription factors that has been shown to play a key role in the differentiation processes of a number of cell lineages (reviewed in references 29, 42, 50, and 72). These proteins contain an HLH dimerization domain composed of two conserved amphipathic α helices separated by a loop and an adjacent region that is rich in basic amino acids and that contacts DNA (16, 21, 43, 46, 47). Basic HLH (bHLH) proteins bind to a DNA sequence known as an E-box (CANNTG) (24, 32, 37) or to the related N-box (34, 68), which is found in the promoter-enhancer of genes expressed in a wide variety of cell lineages, such as muscle cells, B lymphocytes, pancreatic β cells, and nerve cell. In general, cell-type-restricted bHLH proteins such as MyoD (17, 67) and *achaete-scute* (70) form heterodimers with the ubiquitously expressed bHLH proteins which are coded for by the E2A gene (E12, E47 [46], and E2-5/ITF1 [27]) and the E2-2 gene (ITF2 [27]) in the mouse and by the *daughterless* gene (7, 10) in *Drosophila melanogaster*. The formation of heterodimers is essential for DNA binding and transcriptional activation in vivo (38).

The bHLH transcription factors are inhibited by another class of HLH protein, the Id proteins (3, 12, 66). The Id proteins lack a DNA binding domain, and by heterodimerizing with other bHLH proteins, they inhibit their binding to DNA (3, 12, 66). Id acts as a dominant-negative regulator of the

bHLH transcription factors by sequestering the ubiquitously expressed E proteins and preventing them from forming active complexes with the tissue-restricted HLH proteins (30, 48). The down-regulation of Id is necessary for differentiation to proceed in many cell lineages (3, 13, 18, 30, 35, 41, 45, 54, 58–60, 74), and as such, the mechanisms that control Id expression will be important in understanding the molecular events that lead to differentiation.

Four members of the Id family have been identified in the mouse (Id1, Id2, Id3 [HLH462.3], and Id4) (3, 4, 12, 22, 55, 66). All four proteins have highly homologous HLH domains. Id1 and Id3 are the most ubiquitously expressed during mouse embryogenesis (23, 25, 55, 71) and are present in the mesoderm prior to the expression of the myogenic regulatory factors (23, 71). Their dramatic down-regulation during embryogenesis suggests strong regulatory control mechanisms. Importantly, the down-regulation of Id1 and Id3 is recapitulated during induced myogenic differentiation in culture upon serum withdrawal (2, 3). The expression of Id2 and Id4 is more restricted in the embryo (55), but Id2 is present in the presomitic mesoderm (2) and is also down-regulated during myoblast differentiation in tissue culture models (36). It has been shown that an Sp-1 site and a serum-inducible factor binding to an activating transcription factor (ATF)-related site are required for the expression of the human Id2 gene in proliferating muscle cells (36). In addition, a pro-B-cell-specific enhancer that is required for Id1 gene expression in progenitor B cells has been identified (57).

We have chosen to examine the Id1 promoter-enhancer to determine which DNA sequences control the response to mitogen as a means of identifying elements that may be important for proper regulation during embryogenesis. We have identified a 210-bp enhancer-promoter element 1.5 kb upstream of the Id1 gene that confers high-level expression on a

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heterologous reporter gene. Using deletion analysis, linker-scanning mutations, and site-directed mutagenesis, we have identified two functional elements in the 210-bp enhancer which are required for proper Id1 regulation: one element (A) contains an *Egr-1* consensus sequence and the other (B) fits no known consensus DNA binding motif. Electrophoretic mobility shift assays (EMSAs) have shown that specific complexes form on the A and B sites and that both are lost after serum removal, concomitant with the down-regulation of Id1. In addition, it has been shown that the protein complex binding to the A site contains the early growth response gene *Egr-1* as well as other proteins. We propose, therefore, that the response of Id1 to serum is controlled in part by a multiprotein complex containing *Egr-1* and that Id1 therefore is a potential target mediating *Egr-1* activity.

MATERIALS AND METHODS

Cell lines. C3H10T1/2 and C2C12 myoblasts (American Type Culture Collection, Rockville, Md.) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum (FBS) (HyClone). The cells deprived of mitogen were maintained in DMEM containing 10 μ g of insulin per ml and 5 μ g of transferrin per ml without FBS. Protein synthesis was inhibited with 10 μ g of cycloheximide (Sigma) per ml.

Northern (RNA) analysis. Total cytoplasmic RNA was prepared as described previously (3), and 5 μ g of cytoplasmic RNA was used in the Northern analyses unless otherwise indicated. The Id1 probe and the glyceraldehyde-3-phosphate dehydrogenase probe have been described previously (3, 31).

Plasmid constructs. The promoter region of the Id1 gene (−1585 to +88) was amplified by PCR and subcloned into the *HindIII* site immediately upstream of the promoterless luciferase pGL2-basic vector (BV) (Promega, Madison, Wis.) to generate the 1.5 BV plasmid. The sequences of the primers for the PCR were 5'-CCCAGCTTGGGTCTCTGAGACCTAAGTTCGCCCCAGT-3' (upstream) and 5'-CCCAGCTTGGGGATCTGAGAACAGGCGGAGGGGAGCGGAG-3' (downstream), with *HindIII* sites (underlined) for cloning. To insert more 5' sequence into 1.5 BV, the upstream 4-kb *HindIII*-*BglII* fragment was filled in with Klenow fragment and subcloned into a filled-in *BglII* site just 5' of position −1585. Construct integrity was confirmed by restriction analysis and sequencing. 5' deletions were generated by PCR and subcloned into the *HindIII* site of BV. The 3' deletion plasmids, the 210-bp plasmid (−1147 to −934) BV, and the deletions within the 210-bp elements were subcloned into the BV (−134 to +88) which is the 1.5 BV vector digested with *BglII*-*SpeI* still containing the Id1 minimal promoter.

The 210-bp enhancer was PCR amplified with primers 5'*BglII*-1147 (5'-GGAAGATCTTCCAGCGGAGAATGCTCCAGCCCA-3') and 3'*DELSpeI*-934 (5'-GGACTAGTCCAGGCCTCCGAGCAAGCTCTCCCT-3'). To obtain the constructs containing the −1147-to-−937 element upstream of a heterologous promoter, the 210-bp PCR fragment was blunt-ended and subcloned in both directions in the *SmaI* site upstream of the pGL2 promoter vector and upstream of the pGL2 control vector. They both contain the simian virus 40 promoter driving the luciferase reporter gene, and the pGL2 control vector contains a simian virus 40 enhancer at its 3' end. The −1147-to-−934 PCR fragment was also inserted into the *SalI* site downstream from the reporter at the 3' end of the −134-to-+88 BV by blunt-end ligation. The template used in the PCR reaction was the 11-kb *HindIII* genomic fragment subcloned into pBlue-script.

Site-specific mutagenesis. Point mutations were generated by overlap PCRs using synthetic oligonucleotides containing the mutated bases (28). To generate mutation mYY1, the underlined sequence of 5'-GCCGTCTCCATGGCGGACGCGCGCG-3' has been changed to 5'-CAATAG-3'. One reaction mixture contains 5'*BglII*-1147 (5'-GGAAGATCTTCCAGCGGAGAATGCTCCAGC-3'), mutated oligonucleotide 5'-CGCGGGCGGCTATTGTGGAGA-3' as the second primer, and the 11-kb *HindIII* genomic fragment as template. A second reaction mixture contains the same template, the 3'*DELSpeI*-934 (5'-GGACTAGTCCAGGCCTCCGAGCAAGCTCTCCCT-3') primer, and mutated oligonucleotide 5'-CCGTCTCCACAATAGCCGCCC-3'. The two PCR products were gel purified and combined in an equimolar ratio, and five further cycles of PCR were performed without primers. *Taq* DNA polymerase (Perkin-Elmer Cetus) and 5'*BglII*-1147 and 3'*DELSpeI*-934 were then added, and PCR was carried out for an additional 25 cycles. The final product was purified, digested with *BglII* and *SpeI*, and cloned into the −13434-to-+88 BV. In the mEbox promoter vector, the sequence 5'-CCGAGCTGCTGCGCC-3' has been changed to 5'-TGGCCA-3'; in mCREB, the sequence 5'-GGGTGACGT CACGGG-3' has been changed to 5'-GGTAGT-3'; in mEGR, the sequence 5'-GCGACCGCCGCGCGGCGCC-3' has been changed to 5'-CTCGAG-3'. All constructs containing PCR-amplified fragments were verified by sequencing.

Linker-scanning analysis. A two-step PCR method was used to mutate systematically every 6 bp in the 210-bp Id1 enhancer (52). A set of 31-bp mutation

primers was made with 6-base substitutions in the center of the primers (see Fig. 6C). The mutation is a *XhoI* site (CTCGAG) and was introduced in order to facilitate the analysis of mutant clones. In the first PCR reaction, the mutation primers are paired with a 3' primer, 5'-GGACTAGTCCAGGCCTCCGAGCAAGCTCTCCCT-3', from the opposite end of the analyzed sequences to generate a mutated double-stranded PCR product. After gel purification, the mutant PCR products will be used as double-stranded primers and paired with a 5' primer, 5'-GGAAGATCTTCCAGCGGAGAATGCTCCAGCCCA-3', in a second PCR reaction to complete the mutagenesis. The last PCR products are digested with *BglII* and *SpeI* and subcloned into the 1.5 BV vector. By this method, 31 mutants have been generated, and all have been sequenced to confirm the presence of the correct mutations. The template used in the PCRs is the 11-kb *HindIII* Id1 fragment cloned into pBluescript, and titration experiments were performed to determine the optimal amount of template in the reaction mixture. The optimal temperature for the annealing of the primers to the template was determined according to the sequence and the length of the primer. Usually, the PCR cycle was denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, and the cycle was repeated 35 times.

Transfections. Transfections of luciferase reporter plasmids into C2C12 and C3H10T1/2 cells were performed by the DEAE-dextran method (1). Two days before transfection, the cells were plated on six-well dishes at a density of 2×10^5 cells per well and grown to 40% confluence. The cells were refed 2 h before transfection with DMEM plus 15% FBS. The cells were transfected with 2 μ g of luciferase reporter constructs and 2 μ g of RSVCAT as an internal control of transfection efficiency. The cells were harvested 2 days after transfection and assayed for luciferase activity in the presence and the absence of serum (differentiation medium) with a luciferase assay kit (Promega). The amount of luciferase produced by the transfected cells was measured with a luminometer (Berthold Lumat LB9501). Plasmid RSVCAT was generously provided by L. Freedman, and the activity of each luciferase reporter construct was normalized against the chloramphenicol acetyltransferase (CAT) activity measured in the extracts. CAT activity was measured by a nonchromatographic CAT assay (61). Each transfection was repeated at least three times in triplicate.

Whole-cell extracts and EMSA. C2C12 myoblasts and proliferating C3H10T1/2 (40% confluent) cells were grown in the presence of DMEM plus 15% FBS and differentiated C2C12 myotubes were grown in the presence of DMEM, 10 μ g of insulin per ml, and 5 μ g of transferrin per ml for 24 to 48 h. The 150-mm-diameter tissue culture dishes were rinsed twice with phosphate-buffered saline (PBS) before PBS, 5 mM EDTA, and 100 μ g of aprotinin per ml were added. The cells were rocked for 10 min at room temperature and were dislodged from the plate by tapping and harvested into 1.5-ml Eppendorf tubes. The cells were centrifuged for 10 s at $10,000 \times g$. To normalize samples for the number of nuclei, cell pellets from one to two 150-mm-diameter plates were resuspended in 500 μ l of PBS, and 50 μ l of each suspension was diluted into 250 μ l of homogenization buffer (0.3 M sucrose, 10 mM Tris [pH 8.0], 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40, 100 μ g of aprotinin per ml, 10 μ g of leupeptin per ml, 10 μ g of pepstatin per ml) by pipetting and was incubated on ice for 2 min. The cells were vortexed briefly, and the nuclei in each sample were counted. The remaining cell suspension in PBS was centrifuged at $10,000 \times g$, and the cells were resuspended in homogenization buffer plus 400 mM NaCl so that the resulting concentration of nuclei was 5×10^6 /ml. After incubation on ice for 2 min and a brief vortex, the nuclei were pelleted, and the supernatants were decanted and made 20% glycerol. Extracts were stored at −70°C.

EMSAs were performed with double-stranded synthetic oligonucleotides which are described below and which were 5' end labeled with T4 polynucleotide kinase and [γ -³²P]ATP. The binding reactions were carried out in a 20- μ l volume containing 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.6), 50 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, the indicated amount of double-stranded poly(dI-dC), 0.2 ng of probe (10,000 to 60,000 cpm), and 2 μ l of the extracts described above (protein concentrations ranged from 20 to 25 μ g/ml). Binding reactions were carried out at room temperature for 20 min, and the reaction mixtures were then immediately loaded on a 6% polyacrylamide gel (40:1.33 acrylamide-bis-acrylamide) and run in 1× TBE buffer (50 mM Tris, 50 mM boric acid, 1 mM EDTA) for 3 to 4 h at 8 V/cm. The gels were dried and autoradiographed.

Supershift experiments were performed by incubating 2 μ l of the appropriate whole-cell lysate with the appropriate immunoglobulin G fraction (1 to 5 mg/ml). The EGR1,2,3,4 and Sp1 antisera were a gift of Frank Rauscher III (The Wistar Institute, Philadelphia, Pa.) and are directed against the non-zinc finger regions of these proteins and have been shown to abolish the binding of in vitro-translated and recombinant *Egr-1* in gel shift assays (54a). After 20 min at room temperature, the radiolabeled DNA and the poly(dI-dC) were added and incubated for an additional 20 min at room temperature.

The oligonucleotides used as probes were GC-1 (5'-ATGGCGACCGCCGCGCGCGGCCA-3'), GC-2 (5'-CAGTTTGGCGTCTCCATGGCGACCC-3'), GC-3 (5'-CGCGGCGCCAGCCTGACAGCCCGT-3'), mEGR (5'-ATG GCGACCTCGAGGCGGCGGCCA-3'), Egr-1 (5'-CGCCCTCGCCCCCGCG CCGGG-3'), and oligonucleotide A (5'-TTGATAGACGTGCCACCT-3').

RESULTS

Id1 response to serum is rapid. Previous studies have shown that Id1 steady-state mRNA levels decrease about 5- to 10-fold after prolonged mitogen depletion in C3H10T1/2 and C2C12 cells (3). In order to more accurately assess the speed of this response, RNA was extracted from C2C12 and C3H10T1/2 cells grown in the presence of serum and from cells that have been deprived of serum for various amounts of time. The RNA was then analyzed by Northern analysis. As can be seen from Fig. 1A, Id1 is expressed in C2C12 cells grown in the presence of serum (lane 1), but after 2 h of serum deprivation, the expression decreases 10-fold and remains at this low level thereafter (lane 5). Similar results are observed with C3H10T1/2 fibroblasts (data not shown). The decrease in Id1 mRNA expression upon mitogen depletion is almost entirely transcriptional, since the removal of serum reduces the rate of transcription by at least sixfold as measured by nuclear run-on analysis (69). Reexposure to serum restimulates Id1 mRNA levels by fivefold after 1 h, but the RNA levels never recover to a level identical to the level observed before serum deprivation (Fig. 1C; compare lanes 2 and 6).

The rapid down-regulation of Id1 transcription in response to serum deprivation suggests a direct effect of mitogen depletion on Id1 expression. In order to test whether the Id1 response to serum is dependent on new protein synthesis, Northern analysis was performed on RNA from C2C12 and C3H10T1/2 cells that were exposed to cycloheximide before the serum response was measured. As can be seen from Fig. 1B, Id1 mRNA levels are superinduced (fivefold) in cells maintained in serum when the cells were treated with cycloheximide for 3 h (compare lanes 1 and 3 and lanes 5 and 7). A quantitatively similar result is observed for RNA from cells treated for 30 min with cycloheximide, implying that steady state (with respect to cycloheximide treatment) has been reached (data not shown). (Equal amounts of 28S and 18S rRNA are present in each lane, as assayed by ethidium bromide staining [data not shown].) Whether this increase in Id1 mRNA is due to a transcriptional effect is not yet known. Nevertheless, the cells treated with cycloheximide for 3 h fail to down-regulate Id1 mRNA when serum is removed for 3 h (Fig. 1B; compare lanes 3 and 4 and lanes 7 and 8). Thus, the decrease in transcription of Id1 during serum deprivation observed in the nuclear run-on assay does not take place in the presence of cycloheximide in C3H10T1/2 or C2C12 cells. We conclude that the levels of Id1 steady-state mRNA in proliferating cells and the down-regulation of Id1 transcription upon serum withdrawal depend on new protein synthesis.

To determine if restimulation of Id1 mRNA by serum also depends on new protein synthesis, C2C12 cells were restimulated with serum after 2 h of serum deprivation with and without cycloheximide, and Id1 mRNA levels were measured. As was shown previously, in the absence of cycloheximide, Id1 is down-regulated upon serum removal (Fig. 1C; compare lanes 1 and 2), the addition of cycloheximide causes a superinduction in the presence of serum (compare lanes 1 and 3), and the cells treated with cycloheximide fail to down-regulate Id1 (compare lanes 3 and 4). In addition, as stated above, Id1 can be partially restimulated by the readdition of serum in the absence of cycloheximide (Fig. 1C; compare lanes 2 and 6). However, the cells that had been serum deprived for 2 h and then treated with cycloheximide (Fig. 1C, lane 5) failed to be restimulated by serum when cycloheximide was maintained in the medium (lane 7). Assuming that a transcriptional effect above the superinduced state could be observed, it is clear that Id1, unlike Id3 (12), is not an early response gene in that it

FIG. 1. (A) Time course of Id1 mRNA down-regulation after mitogen depletion in C2C12 cells. A Northern blot analysis of total RNA (10 μ g) from C2C12 cells either grown continuously in the presence of serum (lane 1) or switched to differentiation medium (DM) without serum for the times indicated (lanes 2 to 11) is shown. The blot is hybridized to an Id1 cDNA probe and normalized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. (B) Cycloheximide (CH) blocks the down-regulation of Id1 mRNA upon serum removal in C2C12 myoblasts and C3H10T1/2 fibroblasts. The cells were grown in the presence of FBS without cycloheximide (lanes 1 and 5) and with cycloheximide for 3 h (10 μ g/ml) (lanes 3 and 7). Some cells were then switched to differentiation medium without cycloheximide (lane 2 and 6) and with cycloheximide (lane 4 and 8) for 3 h. Equal amounts of 28S and 18S rRNA are present in each lane as assayed by ethidium bromide staining (data not shown). (C) Cycloheximide blocks Id1 restimulation by serum in C2C12 cells. The cells were grown in the presence and in the absence of FBS without cycloheximide (lanes 1 and 2, respectively) or with cycloheximide (10 μ g/ml) for 3 h (lanes 3 and 4). A portion of the cells that had been serum deprived for 2 h (lane 2) were restimulated with serum in the absence of cycloheximide (lane 6). Another portion of the cells that had been serum deprived for 2 h were preincubated with cycloheximide for 30 min (lane 5) and were then restimulated with serum in the presence of cycloheximide for 1 h (lane 7). The RNA was extracted and the blot hybridized with an Id1 cDNA probe and a glyceraldehyde-3-phosphate dehydrogenase probe. The quantitation of Northern blot analysis after normalization is shown. Error bars show standard deviations.

FIG. 2. A 1.5-kb section of upstream sequence confers Id1 expression to a heterologous reporter luciferase gene. The 1.5-kb sequence (–1575 to +88) was fused upstream of a reporter luciferase gene (1.5 BV). C3H10T1/2 fibroblasts were transfected with 2 μ g of 1.5 BV and 5.5 BV (1.5 kb of upstream sequence with an additional 4 kb of upstream sequence fused to luciferase) along with an RSV-CAT construct as an internal control. All luciferase values are normalized to the amount of CAT activity present in the extracts. Cells were either maintained in high serum (+serum) or deprived of serum for 24 h (–serum) prior to harvesting. The results are representative of three to six independent transfection experiments, with the standard deviations being indicated by error bars.

requires new protein synthesis in order to respond to serum restimulation.

Identification of sequences required for Id1 expression. In order to further investigate the transcriptional control of Id1 expression, we isolated and analyzed the Id1 promoter-enhancer region that confers serum responsiveness. A mouse cosmid library was screened with an Id1 intronic probe, and one cosmid clone containing a 40-kb insert was isolated and further characterized. The sequence of this region confirmed the presence of the Id1 gene and demonstrated the presence of one 227-bp intron that begins after the HLH domain (+430) (69). The transcription initiation site was mapped by primer extension to an A residue 25 bp downstream of the TATA box in a region just 5 bp 5' to the end of the published cDNA sequence (69). Direct sequence analysis of 1.5 kb of the upstream promoter sequence reveals a very high density of transcription factor binding sites, including nine E-boxes, three API sites (15), six VDRE 1/2 sites (49), nine AP2 sites (73), one CREB site (6), etc. (69).

To determine the regions that are necessary for the proper control of Id1 expression, we first fused 1.5 kb of the sequence (–1574 to +88) upstream of a firefly luciferase reporter gene (referred to hereafter as 1.5 BV) to determine if this element is sufficient for proper Id1 control. C2C12 muscle cells and C3H10T1/2 fibroblasts were transfected with 1.5 BV and RSV-CAT (as a control for transfection efficiency), and the expression of luciferase-CAT activity was quantified. As can be seen from Fig. 2, the activity of 1.5 BV in C3H10T1/2 cells was high in the presence of serum and decreased 10- to 15-fold in differentiation medium upon serum removal. Results for myoblasts and fibroblasts were identical, although the overall level of luciferase activity is higher in C3H10T1/2 cells than in C2C12 cells (data not shown). Interestingly, when 4 kb of additional 5' sequence was added to the construct (called 5.5 BV), two- to threefold less luciferase activity than that in the cells transfected with the 1.5 BV plasmid was observed (Fig. 2). It is likely that some elements between –1.5 and –5.5 kb have

a repressive effect on the Id1 promoter similar to what has been shown for the B-cell-specific enhancer (57). Nevertheless, these results demonstrate that the 1.5 kb of 5' flanking sequence contains sufficient regulatory sequences to confer proper Id1 serum responsiveness to a reporter gene.

A 210-bp element confers complete serum responsiveness to a reporter gene. In order to characterize the elements within the 1.5-kb sequence responsible for the specific pattern of Id1 expression, we constructed a series of 5' deletions of the 1.5-kb upstream region and fused these deletions to the Id1 minimal promoter (–134-to-+88) luciferase construct. The deletions remove 200 bp at a time and are summarized in Fig. 3A. The constructs were transfected into C3H10T1/2 cells, and the luciferase activity was measured (Fig. 3B). (Note that in all transfection assays reported in Fig. 2 through 6, C3H10T1/2 and C2C12 cells showed quantitatively similar results, but only C3H10T1/2 cell transfection data is shown.) Sequences from –1575 to –1147 can be deleted with little effect on luciferase activity (Fig. 3B; compare 1.5 BV with 5'Del1 and 5'Del2). The further removal of sequences between –1147 to –927, however, results in a dramatic loss of luciferase activity in the presence of serum (compare 5'Del2 with 5'Del3). The level of activity in 5'Del3 is still sixfold above background but is not further down-regulated upon mitogen depletion (data not shown). This experiment defines a 210-bp element that appears to be required for both high-level expression and down-regulation in response to serum depletion.

In order to determine if other sequences between the Id1 promoter and the 210-bp element defined above were also necessary for Id1 expression, plasmids bearing a series of 3' deletions were constructed and transfected into C3H10T1/2 cells, and the luciferase activity was measured. As can be seen from Fig. 4, deletions of sequences from –134 to –930 have no effect on the luciferase activity (compare 1.5 BV with 3'Del1, –2, and –3). In contrast, the removal of sequences between –930 to –1130 drastically diminishes the activity (compare 1.5 BV with 3'Del4), confirming the necessity of the 210-bp element defined above for correct serum responsiveness.

The combined results of the 3' deletion and the 5' deletion series suggest that the 210-bp region was necessary and sufficient for full Id1 serum responsiveness. In order to test this directly, the 210-bp (–1147 to –937) element was placed directly upstream of the luciferase reporter gene, –1147-to-–937 BV. This construct exhibits an activity similar to the 1.5 BV construct, as do all other constructs that include this 210-bp element (data not shown). Moreover, this element behaves like a classical enhancer, exhibiting the same activities in both orientations when placed upstream of the simian virus 40 promoter and at distance when placed downstream of the luciferase gene (data not shown). Therefore, we conclude that the 210-bp fragment in the Id1 promoter sequence is necessary and sufficient for the full serum responsiveness observed with the 1.5-kb upstream region.

An EGR1 consensus sequence is required for promoter activity. Sequence analysis of the 210-bp fragment reveals the presence of a consensus site for Egr-1 (9, 11), YY1 (56), two AP2 sites (73), a CREB/ATF site (6), and an E-box (Fig. 5A). To assess the roles of these putative regulatory elements, point mutations in each site were made. These constructs were transiently transfected into C3H10T1/2 cells, and the results are shown in Fig. 5B. Mutation in the Egr-1 site (mEGR) reduces the enhancer activity 8- to 10-fold. Point mutations in the CREB (mCREB) or the E-box (mEbox) site do not have any significant effect on the expression. Mutation in the YY1 site (mYY1) decreases the expression by about twofold.

To test whether sequences in and around the Egr-1 consen-

FIG. 3. 5' deletion analysis of the Id1 promoter-enhancer region in C3H10T1/2 fibroblasts. (A) Schematic representation of 5' serial deletions of the mouse Id1 promoter. Each construct contains the Id1 basal region from -134 to +88 containing the TATA box, CAAT box, and Sp-1 site. (B) Effect of deletions on Id1 transcription. Cells were transfected with 1.5 BV (-1575 to +88 of Id1 fused to luciferase) and the indicated deletion mutants shown in panel A along with an RSV-CAT construct as an internal control. Each construct (2 μ g) was transfected by the DEAE-dextran method. All numbers are normalized to the amount of CAT activity present in the extracts. Cells were either maintained in high serum (+serum) or deprived of serum for 24 h (-serum) prior to harvesting. The results are representative of three to six independent transfection experiments, with the standard deviations being indicated by error bars.

sus binding site centered at position -1109 are sufficient for promoter activity, a construct that has sequences between -1147 and -1097 and that includes the Egr-1 site was assayed. This construct showed no luciferase activity by itself (data not shown). However, a construct containing sequences from -1147 to -1037 was fully active. Thus, in addition to the Egr-1 site, the region from -1097 to -1037 contains additional sequences required for Id1 expression. These sequences were further localized to the region between -1037 to -1057, since deletion of these sequences severely affected the activity of the reporter construct (data not shown). The presence of an im-

portant regulatory sequence in this region was confirmed by the linker-scanning analysis described below.

A novel site is necessary for full promoter activity. In order to evaluate the role of different DNA sequences within the 210-bp enhancer on Id1 expression, we used a PCR-based linker-scanning mutation analysis. The point mutations generated (m1 to m31) change 6 bp at a time on the 210-bp enhancer and are shown in Fig. 6C. Reporter constructs bearing these mutations were first sequenced to confirm the presence of the correct mutations and were then transiently transfected and assayed for luciferase-CAT activity in C3H10T1/2 cells. As can

FIG. 4. 3' deletion analysis of the Id1 promoter-enhancer region in C3H10T1/2 fibroblasts. (A) Schematic representation of 3' serial deletions of the mouse Id1 promoter. Each construct contains the Id1 basal region from -134 to +88 containing the TATA box, CAAT box, and Sp-1 site. (B) Effect of deletions on Id1 transcription. Cells were transfected with 1.5 BV and the indicated deletion mutants fused to a luciferase reporter gene as described in the legend to Fig. 3.

FIG. 5. (A) Nucleotide sequence of the 210-bp promoter-enhancer region between -1147 and -937. Nucleotides are numbered with respect to the transcription start site. Consensus sequences for known transcription factors (the YY1 site, Egr-1 site, CREB/ATF site, E-box, and two AP2 sites) are boxed. (B) Site-specific mutation analysis of the Id1 210-bp enhancer element. The top panel is a schematic diagram of the 210-bp element -1147 to -937) and a series of site-specific mutants. Each construct contains the Id1 basal promoter region from -134 to +88 containing the TATA box, CAAT box, and Sp-1 site. The bottom panel shows the effect of site-specific mutations on a reporter construct transfected into cells grown continuously in the presence of serum or cells deprived of serum for 24 h prior to harvesting. Transfection efficiencies were normalized with an RSVCAT construct as described in the legend to Fig. 3.

be seen from Fig. 6A, of all the 31 mutations tested, changes in only two regions within the 210-bp fragment dramatically affect the activity of the luciferase reporter construct. The most significant effect is observed with mutation m8, which is adjacent to but does not include the Egr-1 site previously shown (in the mEGR mutant) to be required for enhancer activity. This

result suggested the possibility of more than one protein binding to this region (see below). In addition, the linker-scanning analysis confirms the role of YY1 as a contributing factor, since m6, which creates a point mutation in part of the YY1 site, shows the same twofold inhibitory effect as mYY1. Surprisingly, mutation m7, which falls within the Egr-1 site, has no

FIG. 7. (A) Gel shift assay with the GC-1, GC-2, and GC-3 oligonucleotides. Whole-cell extracts from C2C12 muscle cells grown in the presence of serum (+serum) and switched to medium without serum for 24 h (-serum) were incubated with the radiolabeled probes spanning the region from -1146 to -1084 (the GC-1 sequence [lanes 3 and 4], the GC-2 sequence [lanes 1 and 2], and the GC-3 sequence [lanes 5 and 6]). Oligonucleotide A contains the human IdATF sequence (lanes 7 and 8) as a positive control for the quality of the extracts. Note that the free oligonucleotide probe runs below the region of the gel displayed. (B) EMSAs demonstrating the specificity of the protein complex that binds to the GC-1 sequence. Whole-cell extracts from C2C12 muscle cells grown in the presence of serum (+serum) and switched to medium without serum for 24 h (-serum) were incubated with the radiolabeled GC-1 oligonucleotide probe in the presence of the indicated molar excesses of various unlabeled competitors (GC-1 [lanes 2 and 6], mEGR [lanes 3 and 7], and bona fide Egr-1 consensus sequence [lanes 4 and 8]). The position of the specific protein complex that binds to the GC-1 probe in the presence of serum is indicated by the lower arrow, and the complex that appears after serum deprivation is indicated by the upper arrow. These results were identical to those for whole-cell extracts from C3H10T1/2 fibroblasts. Note that the free oligonucleotide probe runs below the region of the gel displayed.

strong effect on enhancer activity, but this mutation might still be capable of binding Egr-1, perhaps as part of a larger protein DNA complex (see below).

The other dramatic reduction in luciferase activity is found in constructs bearing mutations m16 and m17 (approximately five- to sevenfold reduction). This reduction delineates a second region critical for proper enhancer activity and coincides with the region defined above by deletion analysis (-1037 to -1057) as being important for full activity. The sequence of the m16-m17 region is shown in Fig. 6B. This region does not contain any known binding sites for transcription factors and is currently being further characterized.

These data demonstrate the importance of the GC-rich sequence between -1120 and -1097 containing the Egr-1 and m8 sites for the proper level of Id1 expression in response to serum as well as the importance of a newly identified sequence defined by mutations m16 and m17. Both the putative Egr-1 region and the m16-m17 region appear to be necessary for full enhancer activity.

Identification of a serum-dependent complex containing Egr-1 by mobility shift assay. In order to determine whether the decrease in luciferase activity observed by the mutation of the Egr-1 site in the region from -1146 to -1084 correlates with the loss of binding of a specific protein complex, double-strand-labeled synthetic oligonucleotides spanning this region were used in gel shift experiments with whole-cell extracts from C2C12 cells grown in the presence or absence of serum (differentiation medium). (Similar results are observed with C3H10T1/2 and C2C12 whole-cell extracts in gel shift assays

[see Fig. 7 and 8] [data not shown].) The sequences of the GC-1, GC-2, and GC-3 probes used are listed in Fig. 7A. GC-1 contains the Egr-1, m6, and m8 sites, GC-2 has YY1, and GC-3 contains the m8 site. The GC-1 probe but neither the GC-2 (containing YY1) nor the GC-3 oligonucleotide (m8 alone) forms a serum-dependent DNA-protein complex (Fig. 7A, lanes 1 to 6). [Note that the intense, fastest migrating complex in these assays is probably nonspecific, since it is not inhibited efficiently with cold oligonucleotide (see below) and is inhibited with higher concentrations of poly(dI-dC) (data not shown). Also note that GC-3 does seem to form a complex in a serum-independent manner and that this complex may be required for optimal enhancer activity (see below)]. A new DNA binding complex appears on the GC-1 oligonucleotide when it is incubated with the low-mitogen extracts, suggesting a change in protein components that accompanies the change in transcriptional activity (Fig. 7A; compare lanes 3 and 4). Oligonucleotide A contains sequences from the human Id2 promoter shown to bind an ATF-related factor in proliferating myoblasts (36) and was used as a control for the quality of the extracts (Fig. 7A, lanes 7 and 8). The specificity of the serum-dependent complex is demonstrated by competition with a 100-fold molar excess of unlabeled GC-1 oligonucleotides and a bona fide Egr-1 site (36) but not by competition with a mutant oligonucleotide bearing a mutated Egr-1 site (mEGR) (Fig. 7B, lanes 2, 3, and 4).

In order to directly demonstrate that Egr-1 is a component of the complex that we have identified, gel supershift experiments were performed. Whole-cell extracts from C2C12 myo-

FIG. 8. (A) The protein complex that binds to GC-1 is abolished by an α -Egr-1 antibody. EMSAs were performed with whole-cell extracts from C2C12 myoblasts grown in the presence of serum (+serum) and either the radiolabeled GC-1 or oligonucleotide A probe. The complexes were first preincubated with antibody against Sp-1 (α -Sp1 [lanes 3 and 6]), Egr-1 (α -Egr-1 [lanes 4 and 8]), and preimmune antiserum (lanes 2 and 6) before the addition of the oligonucleotide probe. The specific protein complex that binds to the GC-1 probe is indicated by an arrow. (B) The protein complex that binds to GC-1 in the absence of serum is not abolished by an α -Egr-1 antibody. EMSAs were performed with whole-cell extracts from C2C12 myoblasts grown in the presence of serum (+serum) or serum-free medium (-serum) as indicated and a radiolabeled GC-1 probe. The complexes were first preincubated with antibodies against Egr-1, Egr-2, Egr-3, Egr-4, Sp-1, and preimmune antiserum, as indicated, before the addition of the oligonucleotide probe. The specific protein complexes that bind to the GC-1 probe in the presence and absence of serum are indicated by the arrows on the left and right, respectively. (C) The protein complex that binds to GC-1 and m8 is abolished by an α -Egr-1 antibody. EMSAs were performed with whole-cell extracts from C2C12 myoblasts grown in the presence of serum and the radiolabeled GC-1 or m8 probe (the GC-1 oligonucleotide bearing a mutation in m8). The complexes were first preincubated with antibody against Egr-1 or a preimmune antiserum, as indicated, before the addition of the oligonucleotide probe. The specific protein complex that binds to the GC-1 probe and the m8 probe is indicated by an arrow. (D) Egr-1 binds to both the GC-1 oligonucleotide and to the oligonucleotide bearing the m8 mutation. EMSAs were performed with Egr-1 synthesized in reticulocyte lysates (lanes 2 and 5) or unprogrammed reticulocyte lysates (lanes 1 and 4) or with recombinant Egr-1 protein (lanes 3 and 6). The probes are the GC-1 and the m8 oligonucleotides as indicated. The arrow marks the positions of the complexes formed with in vitro-translated Egr-1 and recombinant Egr-1. (E) The protein complex that binds to the m16-m17 region (B site) is serum dependent. Whole-cell extracts from C2C12 muscle cells grown in the presence of serum (+serum) or from cells switched to serum-free medium for 24 h (-serum) were incubated with the radiolabeled m16-m17 probe in the absence (lane 1) or presence of various amounts of unlabeled competitors (1-fold [lanes 2 and 6], 10-fold [lanes 3 and 7], and 100-fold [lane 4]). The specific protein complex that binds to the m16-m17 probe is indicated by an arrow. These results were identical to those with whole-cell extracts from C3H10T1/2 fibroblasts. Note that the free oligonucleotide probe runs below the region of the gel displayed.

blasts were incubated with antibodies against Sp-1 and Egr-1 and preimmune controls prior to binding to the GC-1 oligonucleotide. As can be seen from Fig. 8A, the antibody against Egr-1 abolishes the binding of the serum-dependent complex to GC-1 (lane 4) but does not abolish the binding of the

complex that binds to an unrelated sequence defined by oligonucleotide A, further demonstrating the specificity of the α -Egr-1 antibody (Fig. 8A, lane 8). However, the binding was not abolished by either α -Egr-2, α -Egr-3, α -Egr-4, or α -Sp1 or preimmune sera from the rabbits inoculated with Egr-1 protein

(Fig. 8B, lanes 4, 5, 6, 7, and 2, respectively). These results indicate that Egr-1 is a component of the protein complex present in serum-stimulated cells that binds to the GC-1 probe.

Mutation m8, which mutates sequences outside of the Egr-1 consensus sequence, exhibits a striking loss of luciferase activity (10-fold). Oligonucleotides bearing the m8 mutation are still capable of binding Egr-1 present in cell lysates, as incubation with an anti-Egr-1 abolishes the binding (Fig. 8C). In addition, recombinant Egr-1 protein alone or Egr-1 protein synthesized in reticulocyte lysates still binds to the oligonucleotide bearing the m8 mutation (Fig. 8D). These results suggest that the binding of Egr-1 alone is not sufficient to activate this element of the enhancer and that Egr-1 probably cooperates with other proteins binding to the site defined by the m8 mutation (see Discussion).

Surprisingly, mutation m7, which generates a point mutation in the Egr-1 site, had very little effect on promoter activity. However, the anti Egr-1 antiserum decreases the binding of the protein(s) complex to m7, suggesting that Egr-1 binding is not completely abolished by the m7 mutation (data not shown). Furthermore, the strong effect of the mEGR mutation on promoter activity, which disrupts the Egr-1 consensus site and causes a complete loss of binding of Egr-1, clearly identifies Egr-1 as a component required for proper Id1 regulation in response to mitogen.

The mobility shift observed after the incubation of GC-1 with nuclear extracts from serum-starved cells does not contain Egr-1 by supershift analysis (Fig. 8B; compare lanes 9 and 10). This complex is specifically inhibited by unlabeled GC-1 probe and partially inhibited by an oligonucleotide bearing a mutated Egr-1 site (twofold decrease in the binding) (Fig. 7B; compare lanes 5 and 7). This latter result suggests that the protein(s) present in the complex formed when serum has been removed binds to sequences outside the putative Egr-1 consensus site. Although we have not yet identified the components of this non-Egr-1-containing complex, the fact that it occupies the Id1 enhancer after serum deprivation suggests the possibility of it being a repressor.

Finally, gel shift experiments with the B site have shown the presence of a serum-dependent complex that is lost within 24 h of serum deprivation (Fig. 8E). This complex is specifically inhibited by a 100-fold of excess unlabeled oligonucleotide (Fig. 8E, lanes 1 and 4). The sequence of the B site fits no known DNA binding consensus and awaits further characterization.

DISCUSSION

The down-regulation of Id1 expression is required for cell cycle withdrawal and differentiation in a number of different cell lineages (3, 13, 18, 30, 35, 41, 45, 54, 58–60, 74) and can be initiated by different signals, including mitogen deprivation, vitamin D, granulocyte colony-stimulating factor, retinoic acid, and transforming growth factor β (5). The mechanisms controlling Id1 expression are therefore likely to integrate signals in these events, leading to cell cycle withdrawal and differentiation. Id1 expression in myoblasts and fibroblasts is markedly decreased by removal of serum, suggesting that Id1 transcription is directly or indirectly strongly dependent on serum growth factors. This component of Id1 transcriptional regulation is explored in the present study.

We have identified a 210-bp enhancer in the Id1 gene which is sufficient to confer proper serum responsiveness to a heterologous reporter gene in both orientations and at various distances from the promoter. This enhancer controls Id1 response to serum in both C2C12 muscle cells and C3H10T1/2 fibro-

blasts, indicating that its activity is not cell type specific. Linker-scanning analysis and site-directed mutagenesis of the Id1 enhancer have shown that two sites (called A and B) within the enhancer are largely responsible for proper Id1 regulation in both C3H10T1/2 and C2C12 cells. The A site of the enhancer contains a consensus Egr-1 binding site flanked by adjacent sequences required for proper regulation, and the B site (defined by mutations m16 and m17 [see above]) contains sequences which fit no known consensus. Both the A and B sites are required for full promoter activity.

It has been shown by gel shift experiments that the decrease in Id1 promoter activity during mitogen depletion correlates with the loss of binding to the A site of a protein complex that contains Egr-1 and with the loss of binding to the B site of an unknown protein complex. Several lines of evidence suggest that Egr-1 is not the only component of the complex formed at the A site. First, mutation of the m8 site adjacent to the Egr-1 site inactivates the enhancer but still leaves the Egr-1 site intact and capable of binding Egr-1 in cell extracts. In addition, we have shown that recombinant Egr-1 alone binds to an oligonucleotide that contains a wild-type Egr-1 site and an m8 mutation. We were not, however, able to detect this other factor under the conditions employed in the gel mobility shift assays, as we fail to see a difference in the serum-dependent mobility patterns of an oligonucleotide with a wild-type Egr-1 site and mutation m8 and an oligonucleotide with an intact Egr-1 site and an intact m8 site. It is formally possible, therefore, that the inactivation of the enhancer by the m8 mutation is due to a *cis* effect on the activity of bound Egr-1. We favor, however, the more conservative hypothesis that there is a factor that cooperates with Egr-1 to fully activate the A site within the enhancer.

The data presented here demonstrate that Id1 is a new physiological target for early response gene *Egr-1*. The Egr-1 protein binds to a consensus sequence, CGCCC(C/G/T)CGC (9, 11), identical to that of the Egr-1 site in the Id1 promoter. *Egr-1* and the closely related *Egr-2* and *Egr-3* belong to a group of immediate-early genes which are rapidly inducible in the absence of new protein synthesis by a variety of stimuli, including such growth factors as platelet-derived growth factor (39) and epidermal growth factor (11, 63), phorbol ester (40, 64), and cellular depolarization (64). Egr-1 mRNA appears within 30 min of stimulation of quiescent fibroblasts with serum (40, 63), reaches a maximal level by about 2 h, and declines rapidly thereafter. The kinetics of Egr-1 expression, therefore, are consistent with Egr-1 playing a role in the Id1 response to serum. Furthermore, the kinetics of Egr-1 protein synthesis after serum stimulation correspond well with the kinetics of its binding to its consensus sequence, suggesting that the newly synthesized Egr-1 binds rapidly to its recognition sequence (9). The observations discussed above make Egr-1 a likely candidate to mediate the Id1 response to serum. In addition, since we have shown that new protein synthesis is necessary for the restimulation of Id1 expression by serum, it is reasonable to propose, in light of the results presented here, that this restimulation depends on newly synthesized Egr-1 protein.

The possibility that a posttranslational modification of Egr-1 may also be required for activation of Id1 is suggested by previous studies. The regulation of Egr-1 DNA binding activity has been demonstrated to be under the control of protein phosphatases and kinases, since the binding of cellular Egr-1 protein to its consensus sequence is strongly but transiently stimulated in fibroblasts by phorbol ester, which stimulates protein kinase C, and by okadaic acid, which is an inhibitor of protein phosphatases (8). Interestingly, it has also been shown that okadaic acid might inhibit myogenesis by increasing the

levels of Id transcription (33, 51). It is therefore reasonable to propose that the effect of serum on Id1 expression could be in part mediated by a posttranslational modification of Egr-1 which stimulates its binding and perhaps increases its activity at the recognition sequence within the Id1 enhancer.

If EGR1 is an important mediator of Id1 expression during mouse embryogenesis, then this fact should be reflected in their relative patterns of expression. In adult mouse organs, Egr-1 is ubiquitously expressed but accumulates to the highest levels in the brain (particularly in the hippocampus), heart, and lung and to lower levels in the kidney and spleen, with very little accumulation in the liver (20, 64). Id1 has also been shown to be expressed most prominently in the lung, kidney, spleen, and hippocampus of the adult mouse (3, 19, 71). During embryogenesis, Egr-1 mRNA is abundant in developing bone and muscle as well as several other mesodermal structures, including tooth mesenchyme and hair follicles (44). Recent *in situ* hybridization analysis has revealed that Id1 is also expressed during bone and cartilage formation and tooth development at later stages of development (29a), again suggesting a significant overlap between Id1 and Egr-1 expression patterns. Taken together, these findings suggest that Egr-1 may play a role in regulating Id1 expression during mouse development. The fact that not all structures expressing Egr-1 express Id1 indicates, as suggested by our cell culture analysis, that Egr-1 is not sufficient for Id1 transcription. Transgenic mice harboring the 210-bp Id1 enhancer and various mutations therein will expand our understanding of the activity of this enhancer in mouse development.

Our results also demonstrate that mitogen removal leads to the loss of the protein complex containing Egr-1 and to the appearance of a new protein complex. This new DNA binding complex is specific and interacts with sequences outside of the Egr-1 site, since an oligonucleotide bearing a mutation in the Egr-1 site competes for the binding. This protein complex does not contain Egr-1 or Sp-1, as shown by the lack of effect of an Egr-1 or Sp-1 antiserum on its binding to the GC-1 sequence. Given the timing of the appearance of this complex, it could represent a repressor. None of the point mutations tested so far, however, have specifically perturbed the binding of this putative repressor, which if required to turn off Id1 transcription in low serum would lead to constitutively high Id1 levels. It is possible that mutations which disrupt the putative repressor-DNA interactions would only affect the kinetics of Id1 down-regulation, since the loss of Egr-1 protein would be expected to eventually lead to the down-regulation of Id1 even without the binding of the repressor. Alternatively, loss of a repressor binding site in the mutants tested could have simultaneously disrupted the activator sequence, and such mutations therefore would not have yielded deregulated expression. Further characterization of this complex at the molecular level is clearly required to resolve these issues.

Kurabayashi et al. (36) have indicated that an Sp-1 site and an IdATF site are required for the expression of the human Id2 gene in muscle cells but are not essential in nonmuscle cells. Although an ATF site is present in the Id1 210-bp enhancer, mutation of that site has no effect on promoter activity. Saisanit and Sun (57) have identified a pro-B cell enhancer that regulates Id1 expression in progenitor B cells and has suggested that it may play a role in the serum response. However, this element fails to augment the activity of the 210-bp element in transient transfection assays in fibroblasts and myoblasts (data not shown) and is therefore unlikely to play a significant role in the mitogen responsiveness of the Id1 gene.

Both Egr-1 and Id1 have been shown to be important for the G₀-G₁ transition in the cell cycle when quiescent fibroblasts are

restimulated with serum and growth factors (26, 39). In addition, the Id1 family of proteins has been shown to antagonize the growth-suppressive effect of bHLH proteins such as MyoD (14, 62) and the E proteins (53). If, as we have suggested, synthesis of Egr-1 leads to the activation of Id1 expression in response to mitogen, then part of the mitogen-stimulated early response mechanism may be the antagonism of the growth-suppressive activity of bHLH transcription factors. This idea can now be tested directly by analyzing the serum responsiveness of fibroblasts derived from mice harboring a homozygous Id1 null mutation (3a) and other Id family mutations.

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