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Id proteins control growth induction in mammalian cells

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ABSTRACT **Id1, Id2, and Id3 (HLH462) dimerize with members of the basic helix–loop–helix protein family, but due to the absence of the basic region, the resulting heterodimers cannot bind DNA. Therefore Id-type proteins negatively regulate DNA binding of the basic helix–loop–helix proteins. Here we report that Id1, Id2, and Id3 are induced shortly after serum stimulation in arrested NIH 3T3. Antisense oligonucleotides against the Id mRNAs delay the reentry of arrested cells into the cell cycle elicited by stimulation with serum or growth factors. Antisense oligonucleotides against all three Id mRNAs are more effective than individual ones. Combined, these results indicate that Id proteins are involved in the control of growth induction.**

The basic helix–loop–helix (bHLH) proteins are a family of transcription factors involved in differentiation (1, 2). They have in common a conserved DNA binding and dimerization domain, designed as the HLH motif (3, 4). Protein–protein interactions can occur between various HLH members to form mostly heterodimers (3, 5, 6). The ability to form heterodimers and the large number of HLH proteins give considerable potential for regulation of various pathways. Heterodimers can be formed between the ubiquitously expressed HLH proteins such as daughterless, E12, E47, E2-2, and those HLH proteins that have a tissue-specific pattern of expression, such as achaete-scute T3, achaete-scute T5, MyoD, myogenin, myf5, and mrf4 (2). The ubiquitously expressed HLH proteins, so called class A, can interact with the tissue-specific bHLH proteins (class B) to form transcriptionally active heterodimers. MyoD is a bHLH protein specifically involved in muscle differentiation; its expression is restricted to muscle tissue and its overexpression in fibroblasts leads to myotubule formation (2, 7). Since differentiation and cell growth are mutually exclusive (8), MyoD can induce growth arrest when it is overexpressed in various cell types. Mutational analysis showed that this ability appears to be independent of the differentiation function (9, 10).

Id1, Id2, Id3 (HLH462), and the product of the extramacrochaetae locus of *Drosophila* (*Emc*) are HLH proteins without a basic domain (11–13). They are ubiquitously expressed and dimerize with members of the class A and B HLH proteins. Due to the absence of the basic region, the resulting heterodimers cannot bind DNA (11, 14). The Id-type proteins may, therefore, negatively regulate DNA binding of the bHLH proteins (2). Since Id1 inhibits DNA binding of E12 and MyoD, it probably inhibits muscle-specific gene expression (11, 15). High concentration of Id proteins might likewise inhibit MyoD or E12 or E47 from forming heterodimers necessary for activation of muscle-specific genes. Under conditions that facilitate muscle cell differentiation the Id protein levels fall, allowing E12 and/or E47 to form heterodimers with MyoD and myogenin, which in turn activates the myogenic program (2, 15).

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The pattern of expression in mouse embryo (16) and during differentiation suggests that there is a correlation between the growth potential of the cells and the expression of Id molecules. In fact expression of both Id1 and Id2 mRNAs is downregulated during differentiation of lymphocytes on the progression from pro-B cells (LyD9) to pre-B cells (HAFTL and PD31) and finally to mature B cells (WEHI-231) (12). Id1 and Id2 mRNA levels are reduced in induced mouse F9 embryonic carcinoma cells (11) and Id2 mRNA levels are hardly detectable in MEL cells induced to differentiate with dimethyl sulfoxide (11, 12, 17). F3 aza-myoblasts induced to differentiate have a lower Id1 expression, and C3H10T½ and NIH 3T3 fibroblasts after 2 days of postconfluence have Id1 mRNA levels lower than those found in proliferating cells (11). Moreover, Id3 is induced in mouse NIH 3T3 cells as part of the immediate early transcriptional response to growth factors (13).

In this report we show that the expression of all the Id molecules is strongly dependent on the presence of growth factors and reduction of the Id mRNA levels by antisense oligonucleotides leads to a delayed reentry of arrested cells into the cell cycle after serum or growth factor stimulation.

MATERIALS AND METHODS

Northern Blot Analysis. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) fetal calf serum (FCS) and arrested by growing them in medium containing 0.5% FCS. Total RNA was extracted 5, 15, 30, 60, and 360 min after adding 0.5% FCS. After 3 days, when <3% of the cell population was incorporating 5-bromo-2′-deoxyuridine (BrdUrd; Sigma, final concentration, 100 μg/ml), arrested cells were induced by addition of medium containing 20% FCS. Samples were collected at the indicated times. RNA was prepared and blotted as described (18). A 15-μg aliquot of total RNA was used per lane; loading was verified by ethidium bromide fluorescence of rRNA. Hybridization was carried out for 16 h at 42°C in hybridization buffer containing 50% (vol/vol) formamide, 10% (wt/vol) dextran sulfate, 1 M NaCl, and 1% SDS. Full-length Id1 (provided by H. Weintraub, Hutchinson Center, Seattle), Id2 (provided by D. Baltimore, Rockefeller University), and Id3 (provided by D. Nathans, Johns Hopkins University) cDNAs were labeled by random oligonucleotide priming; the Boehringer random-priming kit was used according to the manufacturer's instructions. The filters were washed at 65°C for 1 h in 2× standard saline citrate/0.5% SDS. Autoradiography was carried out overnight by using a Molecular Dynamics PhosphorImager and the IMAGEQUANT software provided by the supplier.

Synthesis and Purification of Oligonucleotides. Both modified and unmodified oligonucleotides were synthesized as

Abbreviations: HLH, helix–loop–helix; bHLH, basic HLH; FCS, fetal calf serum; PDGF, platelet-derived growth factor.

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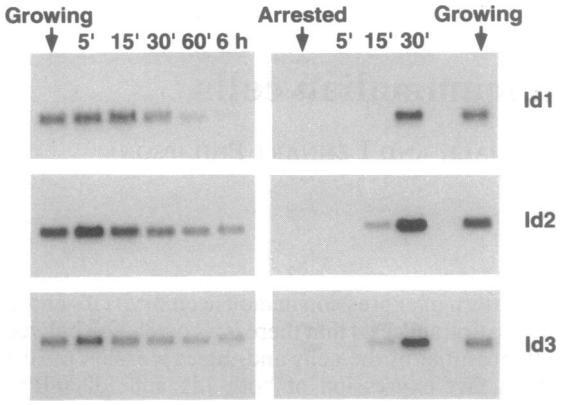

FIG. 1. Expression of Id1, Id2, and Id3 mRNAs in arrested and growing NIH 3T3 fibroblasts analyzed on Northern blots. Total RNA from NIH 3T3 cells actively growing or arrested by growth in 0.5% FCS was extracted at the times indicated after changing the medium to 0.5% FCS (*Left*) or 20% FCS (*Right*). The blots were hybridized with labeled Id1 (5), Id2 (7), and Id3 (9) cDNA labeled probes.

described (16) purified by polyacrylamide gel electrophoresis and ethanol precipitation. Sequences of the oligonucleotides are shown in Fig. 2.

Cell Culture and Immunofluorescence. Approximately 1×10^6 NIH 3T3 cells were seeded on coverslips and cultured for 24 h in DMEM with 10% FCS, and then medium was changed to DMEM with 0.5% FCS. One hour before adding platelet-derived growth factor (PDGF; 5 ng/ml) or FCS (20%), antisense or sense oligonucleotides were added to the medium (final concentration, 100 μg/ml). In the sample with all three sense or antisense oligonucleotides, each oligonucleotide was added at 100 μg/ml. Cells were labeled with BrdUrd between 18 and 24 h after serum stimulation. Coverslips were then washed in phosphate-buffered saline (PBS), fixed for 10 min at room temperature in 3% (wt/vol) paraformaldehyde/PBS, and permeabilized for 5 min in 0.3% Triton X-100/PBS. Incorporation of BrdUrd into cells was detected by immunofluorescence as described (9, 19). Total number of cells was evaluated by staining cell nuclei with Hoechst dye 33258 (Sigma) at a final concentration of 1 μg/ml. A minimum of 200 cells was analyzed for DNA synthesis in each experiment. The results represent the average and standard deviation of three experiments. To confirm that the increased effect of the three oligonucleotides together was not caused by a higher dosage, Id1 antisense oligonucleotide at 300 μg/ml was also used with results similar to those described for 100 μg/ml (data not shown). In the reversion experiment, oligonucleotides were added to the medium 1 h before growth stimulation of cells with PDGF or FCS. After 24 h cells were washed with identical medium not containing oligonucleotides and left growing for an additional 12 h. BrdUrd incorporation after 6 h of labeling was evaluated by immunofluorescence after 24,

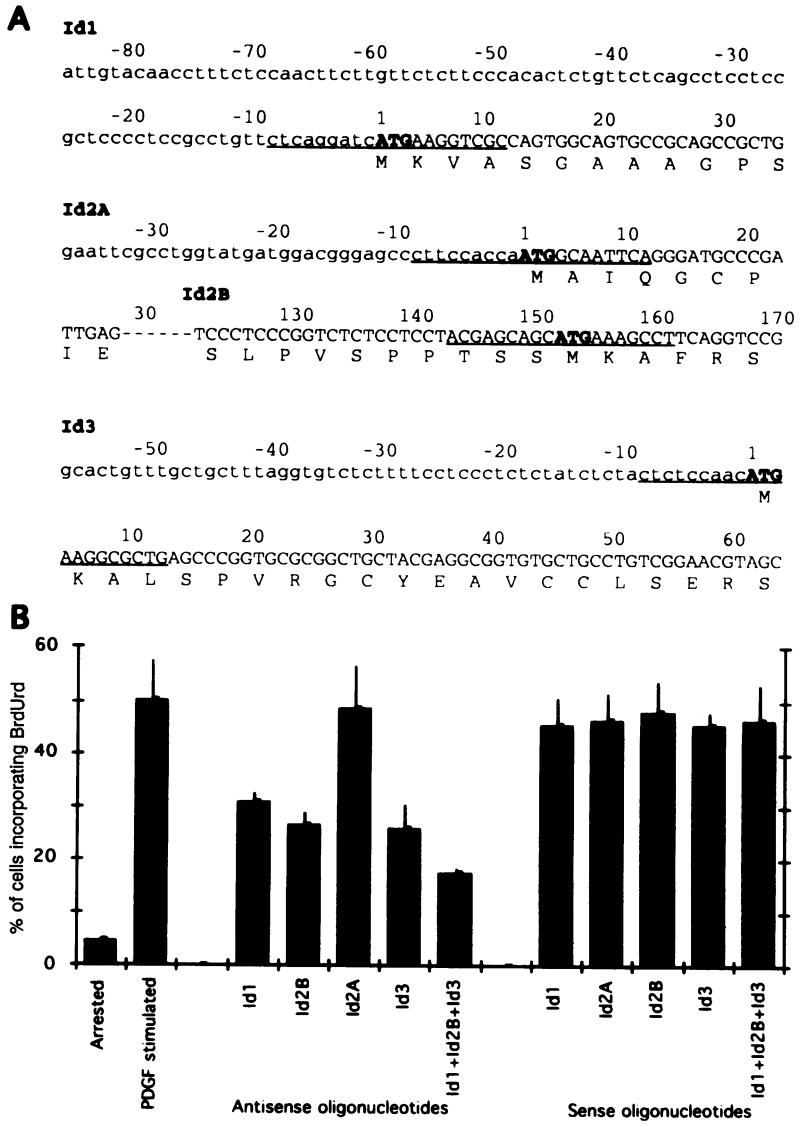

FIG. 2. Antisense oligonucleotides and growth induction. (*A*) The sequences of the 5′ ends of Id1, Id2, and Id3 mRNAs have been reported (6, 7, 9). The oligonucleotides used correspond to the underlined regions around the AUG start codons, indicated in boldface type. Sequences were identical (sense) or complementary (antisense) to the underlined sequences (12). For Id2, two antisense oligonucleotides were synthesized around the two possible initiation codons marked as Id2A and Id2B. (*B*) Effect of Id1, Id2, and Id3 antisense oligonucleotides on growth induction. The histogram reports the percent of cells that incorporated BrdUrd 24 h after PDGF stimulation in the presence of the oligonucleotides indicated. BrdUrd incorporation by arrested and control cells that were PDGF-stimulated (no oligonucleotide added) is also included.

30, and 36 h of culture. The results are the average of two very similar experiments.

RESULTS

Expression of all three Id genes was analyzed in NIH 3T3 fibroblasts. Asynchronously growing cells were arrested in low serum (0.5%) and, subsequently, growth was induced with 20% FCS. Northern blot analysis (Fig. 1) showed that the amount of Id mRNAs was reduced to 50% in <1 h after serum reduction from 10% to 0.5%, indicating that expression of the Id genes is strongly dependent on the presence of serum in the medium and that the half-life of their mRNAs is short. After serum induction, mRNA levels rapidly and transiently increased. At 30 min after induction, their levels were roughly twice the level observed in growing cells. Later RNA levels returned to the same level as in asynchronously growing cells. The pattern was fundamentally the same as reported for Id3 (13). Similar results were obtained when PDGF was used to stimulate the cells (data not shown).

To better understand the role of Id1, Id2, and Id3 in growth induction of arrested cells, we synthesized DNA antisense oligonucleotides covering the region around the AUG initiation codon of Id1, Id2, and Id3 (Fig. 2). For Id2, two possible AUG initiation codons have been reported; therefore, two oligonucleotides Id2A and Id2B were synthesized that were targeted to the two Id2 sites (12). NIH 3T3 fibroblasts were arrested by serum deprivation; 72 h later PDGF was added to induce growth. DNA synthesis, determined by BrdUrd incorporation, was significantly inhibited when antisense oligonucleotides were added to the culture medium immediately before growth stimulation. The Id1 antisense oligonucleotide reduced BrdUrd incorporation to 31%, Id2B to 28%, and Id3 to 27.5%. In PDGF-stimulated control cells with no oligonucleotide added, BrdUrd incorporation was observed in 51% of the cells. Id2A did not have a significant effect on DNA synthesis. Sense Id1, Id2, and Id3 oligonucleotides had no effect on growth stimulation. When the three active antisense oligonucleotide were used together BrdUrd incorporation was reduced to 18.5%. This is obviously not due to a toxic effect of the oligonucleotides since a control, using Id1 antisense at 300 μg/ml, resulted in BrdUrd incorporation similar to the incorporation observed with the Id1 oligonucleotide at 100 μg/ml (see Fig. 2). In fact no difference was

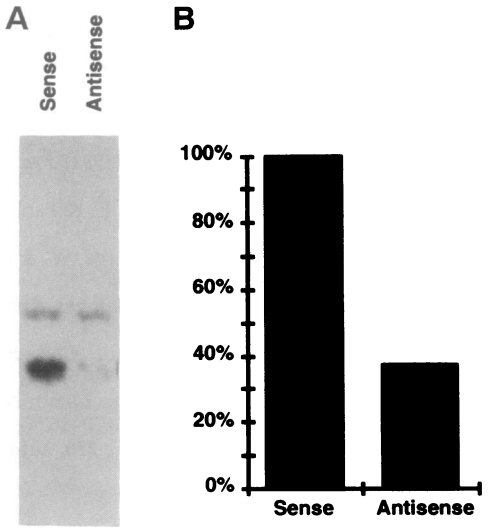

FIG. 3. Effect of Id3 antisense or sense oligonucleotides on mRNA levels. (*A*) Northern blot of RNA extracted from NIH 3T3 fibroblasts arrested by serum starvation and PDGF-induced in the presence of Id1, Id2, and Id3 sense or antisense DNA oligonucleotides. RNA samples were processed 4 h after induction. (*B*) Quantitative analysis of the Northern blot shown in *A*.

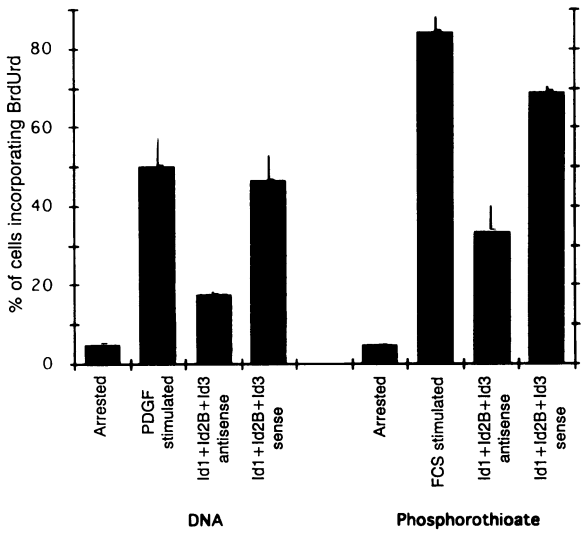

FIG. 4. Effect of Id1, Id2, and Id3 antisense DNA and phosphorothioate-modified oligonucleotides on BrdUrd incorporation. The histogram reports the percent of BrdUrd-incorporating cells 24 h after serum stimulation in the presence of oligonucleotides as indicated. Incorporation by arrested and control (no oligonucleotide added) cells is also shown. Arrested cells were stimulated with 20% FCS for 1 h after adding the three sense or antisense oligonucleotides (each at 100 μg/ml).

observed using each of the sense oligonucleotides at 100 μg/ml or 300 μg/ml. The observed growth inhibition by antisense oligonucleotides was mediated by reduced levels of the corresponding mRNAs. When the three antisense DNA oligonucleotides were added to the cells, Id3 mRNA levels were significantly reduced to 30% of that in cells treated with sense oligonucleotides (Fig. 3). Hybridization with Id1 and Id2 probes gave similar results (data not shown).

In the previously described experiment, FCS could not be used for growth induction because DNA oligonucleotides were unstable in medium containing serum (20). To be able to use FCS, phosphorothioate oligonucleotides with the same sequence were used instead of DNA oligonucleotides. As shown in Fig. 4, the results are similar, except for the level of DNA synthesis, which was higher in FCS- than in PDGF-treated cells. Modified antisense oligonucleotides reduced BrdUrd incorporation to 32%; incorporation in control cells without oligonucleotides was 83%. When the three sense oligonucleotides were used together, a slight inhibition of BrdUrd incorporation compared to nontreated cells was observed, probably due to a toxic effect of higher concentrations of the modified oligonucleotides.

To establish whether the effect of the antisense oligonucleotides was reversible, the ability of cells to reenter the cell cycle after removal of DNA antisense oligonucleotides was investigated. Cells were arrested, stimulated to grow by PDGF, and inhibited by unmodified antisense oligonucleotides. As shown in Fig. 5, 24 h after PDGF stimulation when the cells were blocked, the oligonucleotides were removed and BrdUrd incorporation was scored for a further 12-h period. Six hours after removing the oligonucleotides, BrdUrd incorporation increased. After 12 h, the recovery was complete. These results indicate that the treatment with antisense oligonucleotides was not toxic to the cells and that the inhibition of DNA synthesis was reversible.

DISCUSSION

The pattern of expression of the Id mRNA suggests that there is a correlation between the growth state of the cells (differentiated, arrested, or growing) and the expression of Id

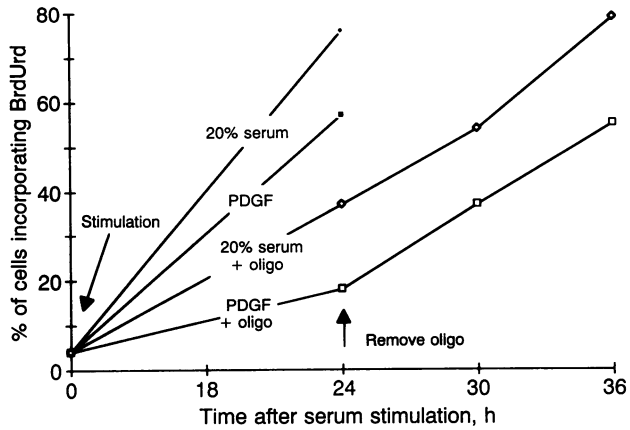

FIG. 5. Antisense inhibition is transient. Fraction of cells incorporating BrdUrd after arrest and serum- or PDGF-stimulation, as indicated. The curves marked + oligo are derived from experiments in which DNA (PDGF induced) or phosphorothioate (FCS induced) oligonucleotides were added 1 h before stimulation and removed 24 h later, as indicated by the arrows.

mRNAs. By Northern blot analysis of NIH 3T3 cells, we show that the expression of all the Id mRNAs is strongly dependent on the presence of serum or growth factors. Only 30 min after induction, Id1, Id2, and Id3 mRNA levels are higher than the basal levels, indicating that they are early responsive genes to growth factor stimulation.

The antisense strategy allowed us to target each of the Id mRNAs one at time or all together. Although when used one at a time the antisense oligonucleotides gave a significant delay of DNA synthesis after growth stimulation, the highest inhibition of DNA synthesis was observed when the three antisense oligonucleotides were added together, suggesting that the role of the Id proteins during growth stimulation is at least partially overlapping. This information should be taken in account when creating null alleles for these genes by homologous recombination. The fact that two sets of molecules like the DNA and the phosphorotioate oligonucleotide gave the same result is a good indication of the specificity of the process. Moreover, addition of the antisense oligonucleotides to cells resulted in a specific reduction of the Id mRNA levels.

Similar experiments have previously established that two other early-responsive genes, c-*fos* and c-*myc*, are also required for growth induction (21, 22). The fact that c-*fos* binds to HLH proteins *in vitro* (23) suggests that regulation of these transcription factors may be more complex than previously anticipated.

In separate experiments (ref. 9 and F.A.P., unpublished data), we have established that several bHLH transcription factors such as MyoD and E12/E47 alone can inhibit DNA synthesis and growth arrest when overexpressed in various cell types. When Id is comicroinjected with one of the arresting proteins such as MyoD or E12/47 (F.A.P., unpublished results), it is able to abolish the arresting function.

These results strongly suggest that in growing cells, Id and the Id homologs are binding and, thereby, inhibiting one or more growth inhibition factors such as E12/E47 or similar unrecognized factors. It is reasonable to propose that the reduction of the Id mRNAs by antisense oligonucleotides during serum stimulation can leave E12/E47 or other arresting partners free to arrest growth. The effect is clearly transient and depends on the presence of the oligonucleotides.

Combined, these results indicate that the Id family of HLH transcription factors plays a role in growth induction of arrested cells. The interplay between the various HLH and other transcription factors, therefore, appears to guide the exit and the entrance of the cell cycle in mammalian cells.

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