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Regulation of G_1 progression by E2A and Id helix–loop–helix proteins

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In NIH3T3 fibroblasts, the ubiquitous helix–loop–helix (HLH) protein E2A (E12/E47) and the myogenic HLH proteins MyoD, MRF4 and myogenin are growth-inhibitory, while two ubiquitous Id proteins lacking the basic region are not. The dimerization domain mediates inhibition. However, in addition to the HLH region, E2A contains two inhibitory regions overlapping with the main transcriptional activation domains. The growth-suppressive activity of the intact E47 as well as MyoD was counteracted by the Id proteins. When E47 lacking the HLH domain was overexpressed, Id could no longer reverse growth inhibition. By increasing the amount of E47 with an inducible system or neutralizing the endogenous Id with microinjected anti-Id antibodies, withdrawal from the cell cycle occurred within hours before the G_1–S transition point. The combined results suggest that the Id proteins are required for G_1 progression. The antagonism between the E2A and Id proteins further suggests that both are involved in regulatory events prior to or near the restriction point in the G_1 phase of the cell cycle.

Key words: dimerization/G_1 progression/growth arrest/helix–loop–helix/restriction point

Introduction

The E2A (E12/E47) and Id proteins belong to an expanding family of eukaryotic transcriptional regulators characterized by the presence of a highly conserved helix–loop–helix (HLH) motif, which mediates homo- and heterodimerization (reviewed in Visvader and Begley, 1991; Kadesch, 1992, 1993). The majority of the HLH proteins including the E2A proteins contains a basic (b) DNA-binding domain. The ability of the bHLH proteins to bind DNA *in vitro* depends on dimerization and the partner may influence the affinity (Murre *et al.*, 1989a,b; Davis *et al.*, 1990) and sequence preference (Blackwell and Weintraub, 1990) for the E-box binding site, represented by the CANNTG consensus motif (Ephrussi *et al.*, 1985).

The human E2A gene encodes two proteins, E12 and E47, by alternative splicing (Murre *et al.*, 1989a; Sun and Baltimore, 1991). Homologues have been identified in mouse (A1/A2), rat (Pan1/Pan2) and hamster (shPan1/shPan2) (Nelson *et al.*, 1990; German *et al.*, 1991; Aronheim *et al.*, 1993a). The E2A proteins are also closely related to the human ITF2 (E2-2) and HEB proteins (Henthorn *et al.*, 1990; Hu *et al.*, 1992), and *Drosophila* daughterless (da) (Caudy *et al.*, 1988; Murre *et al.*, 1989a). These proteins, collectively called the E-family proteins, show >70% similarity in the HLH region. A functional redundancy may therefore explain the apparent normal phenotype of differentiating mouse embryonic stem cells lacking both copies of the E2A gene (Zhuang *et al.*, 1992).

E2A proteins are widely expressed. They are involved in differentiation of skeletal muscle cells, and in transcriptional activation of pancreatic and immunoglobulin genes (Henthorn *et al.*, 1990; Nelson *et al.*, 1990; Cordle *et al.*, 1991; Lassar *et al.*, 1991; Murre *et al.*, 1991). E2A proteins heterodimerize with tissue-specific bHLH proteins, such as SCL/TAL and MASH, suggesting an involvement of E2A also in differentiation of haematopoietic and neural cells (Hsu *et al.*, 1991; Johnson *et al.*, 1992). Similarly, the *Drosophila* da protein is involved in the control of neurogenesis and sex determination by dimerizing with products of the *achaete–scute* gene complex (Caudy *et al.*, 1988; Cabrera and Alonso, 1991). Together these results support the idea that E family proteins are required for differentiation after heterodimerization with tissue-specific bHLH proteins (reviewed in Jan and Jan, 1993).

On the other hand, the E2A gene is the breakpoint of two translocations associated with lymphoid leukaemia in children. As a consequence, the E2A gene is truncated and the bHLH domain replaced with homeobox (Pbx1) or leucine zipper (Hlf) domains (Kamps *et al.*, 1990; Nourse *et al.*, 1990; Inaba *et al.*, 1992). The E2A portion of E2A–Pbx1 is required for transformation (Van-Dijk *et al.*, 1993). This may suggest a possible involvement of the E2A proteins in growth control, but direct evidence is lacking.

The functions of HLH proteins have been established most clearly in muscle cells in which growth and differentiation seem to be mutually exclusive (Nadal-Ginard, 1978). In cultured myoblasts, myogenic conversion is first dependent upon withdrawal of serum factors from the medium. A family of myogenic bHLH proteins, which includes MyoD, myogenin, Myf5 and MRF4/herculin/Myf6, is subsequently involved in this transition (reviewed in Emerson, 1990; Olson, 1990; Weintraub *et al.*, 1991; Buckingham, 1992). These proteins need to heterodimerize with E2A to induce myogenesis (Lassar *et al.*, 1991). In contrast, MyoD is present as homodimers in proliferating myoblasts and at this stage >90% of the E2A is trapped in a complex with Id (Jen *et al.*, 1992).

The Id-family proteins lack the basic DNA-binding domain, therefore heterodimers containing these proteins are unable to bind DNA *in vitro* and *in vivo* (Benezra *et al.*, 1990; Jen *et al.*, 1992). So far, three Id-like proteins have been isolated in mouse, namely Id1, Id2 and HLH462 (also referred to as Id3) (Benezra *et al.*, 1990; Christy *et al.*, 1991; Sun *et al.*, 1991). They all show considerable similarity in the HLH domain which is also conserved in the *Drosophila* extramacrochaetae (emc) protein (Benezra *et al.*, 1990; Ellis *et al.*, 1990; Garrell and Modolell, 1990). *In vitro*, Id1 and Id2 (which homodimerize poorly) have higher affinity for the E2A proteins than for MyoD and can make MyoD–E2A complexes dissociate (Benezra *et al.*, 1990; Sun *et al.*, 1991). Moreover, Id1 shows no affinity for other bHLH proteins containing a leucine zipper, like c-Myc, TFE3, USF and AP4 (Sun *et al.*, 1991). Overexpression of Id proteins *in vivo* inhibits the transcription of immunoglobulin, insulin and muscle-specific genes (Benezra *et al.*, 1990; Cordle *et al.*, 1991; Wilson *et al.*, 1991; Jen *et al.*, 1992; Kreider *et al.*, 1992). A human Id gene, HLH 1R21, when transfected into NIH3T3 cells, induces morphological changes similar to but less pronounced than those observed in *ras*-transformed cells (Deed *et al.*, 1993).

Id genes are also strongly expressed in undifferentiated, growing and tumour cells. They are immediately induced upon stimulation with serum, growth factors or phorbol esters but down-regulated in quiescent cells or during differentiation (Benezra *et al.*, 1990; Christy *et al.*, 1991; Biggs *et al.*, 1992; Jen *et al.*, 1992; Kreider *et al.*, 1992; Deed *et al.*, 1993; Barone *et al.*, 1994). Similarly, *Drosophila* emc inhibits the action of HLH products from the *da* and the *achaete–scute* loci in neurogenesis and sex determination (Cabrera and Alonso, 1991; Parkhurst *et al.*, 1993). Accordingly, Id-family proteins may function as general inhibitors of differentiation (reviewed in Jan and Jan, 1993) and possibly control growth induction.

A reverse correlation between proliferation and differentiation has been established from studies with growth factors, oncogenes on the one hand and the myogenic HLH proteins on the other (reviewed in Olson, 1992; Emerson, 1993; Weintraub, 1993). MyoD can reduce the colony-forming efficiency (CFE) of transfected fibroblasts (Davis *et al.*, 1987). Overexpression of MyoD inhibits growth in the G_1 phase. The arrest is mediated by the HLH domain and is independent of myogenic differentiation (Crescenzi *et al.*, 1990; Sorrentino *et al.*, 1990). The HLH domain of MyoD and E2-2 mediates the binding to the pocket domain of the retinoblastoma (Rb) protein *in vitro*. Importantly, both growth arrest and differentiation induced by MyoD require an intact Rb function (Gu *et al.*, 1993).

Since MyoD expression is restricted to skeletal muscle cells (Tapscott *et al.*, 1988), we hypothesized that the observed growth arrest of MyoD simulated the action of HLH proteins expressed in other cell types, structurally or functionally related to MyoD. Therefore we decided to analyse the role of members of the E and Id families in growth control. Our results indicate that Id proteins are required for progression through the G_1 phase of the cell cycle up to or near the restriction point, possibly by antagonizing the growth arrest mediated by the E proteins.

Results

Inhibition of cell proliferation by bHLH proteins

We set out to examine whether other HLH proteins shared the growth-inhibitory property of MyoD1, in a CFE assay (Crescenzi *et al.*, 1990).

Five independent experiments for each gene showed that two closely MyoD1-related proteins, such as myogenin (Edmondson and Olson, 1989; Wright *et al.*, 1989) and MRF4 (Edmondson and Olson, 1989; Rhodes and Konieczny, 1989; Miner and Wold, 1990) reduced the CFE as efficiently as MyoD1, suggesting that overexpression of skeletal muscle-specific HLH genes induces growth inhibition (Figure 1A). The more distantly related HLH proteins, E12 and E47 of the E-family (Murre *et al.*, 1989a), also strongly reduced the CFE to only 8% and 7% of that observed in the controls (Figure 1A). In contrast, Id1 and HLH462 (Benezra *et al.*, 1990; Christy *et al.*, 1991), hereafter called Id3 (Sun *et al.*, 1991) of the Id-family did not affect CFE (Figure 1A). The expression

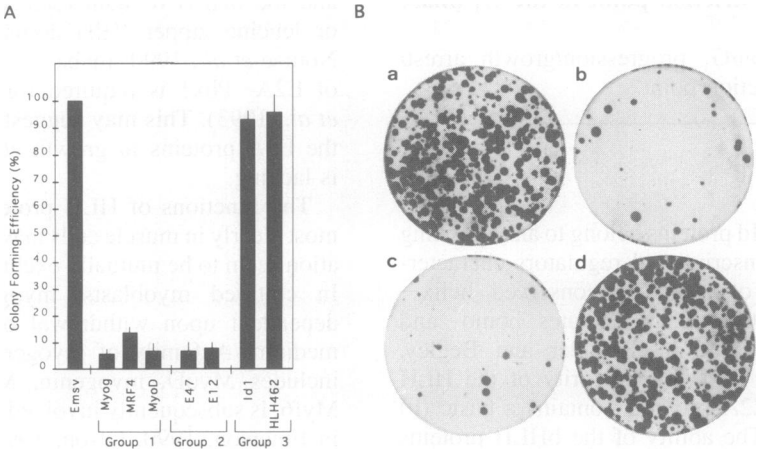

Fig. 1. Muscle-specific HLH (group 1) and E-family (group 2) but not Id-family (group 3) proteins inhibit the proliferation of NIH3T3 mouse fibroblasts. (**A**) The histogram shows the average CFE in five independent experiments for each HLH gene. NIH3T3 cells were transfected with 0.3 μg of pIPB1 or pSV2neo, 10 μg of each HLH-expressing vector or empty vector and 15 μg of NIH3T3 high molecular weight DNA carrier (see Materials and methods). Standard deviation (SD) is indicated by a thin line at the top of the bars. (**B**) Representative Coomassie-stained plates of the CFE of one member of each group of HLH proteins are shown. (a) pEmsv control vector; (b) pEmsv-myogenin; (c) pEmsv-E47; (d) pEmsv-Id1.

of the HLH proteins was verified by immunostaining of transiently transfected cells.

HLH proteins can thus be divided into three groups based on their expression pattern and ability to inhibit growth. Growth-inhibitory HLH proteins may be divided into muscle-specific (group 1) or ubiquitously expressed genes (group 2). HLH proteins lacking a basic domain constitute the third group (Figure 1A). Transfection experiments showing the CFE of a member of each group are shown in Figure 1B.

The role of the HLH region in growth inhibition

Growth inhibition by MyoD1 is dependent on the HLH region (Crescenzi *et al.*, 1990; Sorrentino *et al.*, 1990). To investigate whether this also applied to other HLH proteins, we deleted the second α-helix of the HLH domain of myogenin, MRF4 and E47 and assessed the CFE in four independent experiments. The corresponding mutant of MyoD1 (DM:143–162) (Davis *et al.*, 1990), here called MyoD:ΔH2, was also included.

No significant reduction of CFE was observed for myogenin, MRF4 and MyoD mutants, suggesting that muscle-specific HLH proteins require an intact dimerization domain to induce growth inhibition (Figure 2). Surprisingly, E47:ΔH2 gave a strong reduction in CFE (11%) (Figure 2). To confirm these results, we deleted helix 1, the loop in the dimerization domain, or the entire HLH region of the E47 cDNA. A strong reduction in CFE was observed for all constructs (Figure 2). These results indicate that a difference exists between muscle-specific HLH proteins and the E2A proteins. The latter contain growth-inhibitory regions outside the HLH domain (see below).

Growth-inhibitory regions in E47 cDNA

To identify other inhibitory regions of E47, we prepared a set of truncation mutants which progressively lack amino acids from the C-terminal region, namely E47:Δ601–647, E47:ΔHLH, E47:T523, E47:T493, E47:T371 and E47:T174. At least three independent cotransfection assays in NIH3T3 cells demonstrated that all mutants were growth-inhibitory (Figure 3). From this analysis it appears that a region in the first 174 amino acids of E47/E12 proteins is sufficient to inhibit cell proliferation.

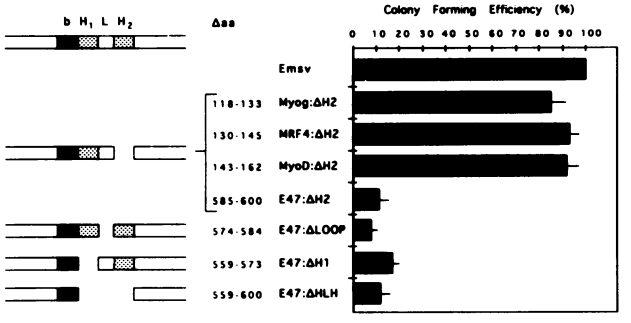

Fig. 2. Deletion of the dimerization domain of muscle-specific HLH but not of E proteins affects the growth-inhibitory ability. A scheme of the HLH region [see Figure 2 in Benezra *et al.* (1990)] and the analysed deletion mutants of the HLH proteins is shown, together with a listing of the amino acids deleted. CFE assays of the indicated deletion mutants were performed as described in Materials and methods. The histogram represents the average of four independent experiments; the SD is also shown.

Another set of five mutants (E47:Δ16–226, E47:Δ16–226/T523, E47:Δ16–226/T493, E47:Δ16–226/T371 and E47:Δ16–226/ΔHLH) carrying internal deletions between amino acids 16 and 226 and progressively truncated from the C-terminus was constructed (Figure 3). These mutants, with the exception of E47:Δ16–226/T371 which was not inhibitory, reduced the CFE to 2%, 53%, 48% and 8%, respectively, indicating that another growth-inhibitory region seems to be located between amino acids 371 and 415 (Figure 3 and see below).

These growth-inhibitory regions overlap with the transcriptional activation domains recently localized between amino acids 1 and 153 of mouse E2A and between amino acids 281 and 339 of the human ITF1 protein corresponding to amino acids 349–407 of human E2A (Aronheim *et al.*, 1993b; Quong *et al.*, 1993), respectively (Figure 3). The low reduction of CFE among mutants carrying the central inhibitory region may be due to defects in protein folding, since we observed a comparable level of expression by immunostaining of transiently transfected cells.

We then examined whether the C-terminal portion of the E47 protein, which contains the bHLH region but no transactivation domain (Henthorn *et al.*, 1990), could by

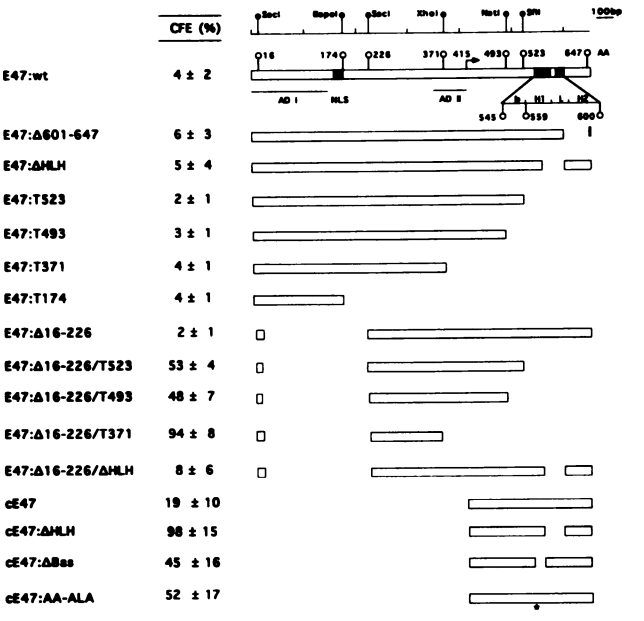

Fig. 3. E47 inhibitory regions overlap the transcriptional activation or the dimerization domains. The indicated mutants were transfected in NIH3T3 cells as described in Materials and methods. Average CFE and SD were calculated from three independent experiments. A schematic view of the restriction enzyme map of the E47 cDNA (black symbols) used in the construction of the deletion mutants is shown at the top. The amino acids which delimit the regions involved in the deletions are indicated by white symbols in the wild-type protein of E47. ADI and ADII are the locations of the main transcriptional activation domains (see Results). The positions of the nuclear localization signal (NLS) and the basic−HLH domains (b-H_1LH_2) are also shown. The internal methionine at position 415, from which all cE47 mutants start, is indicated by a black arrow. The amino acid deletions are indicated beside the name of each mutant, together with the respective CFE and SD. Amino acid refers to site of the termination in the deletion mutants. The asterisk below the cE47:AA-ALA mutant map shows the location of the five amino acid substitutions in the basic domain.

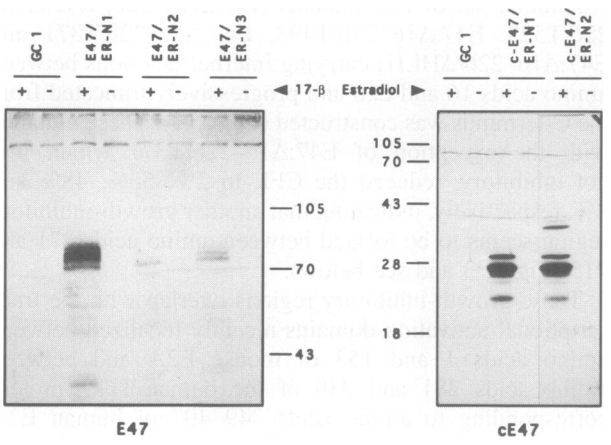

Fig. 4. Immunoblot analysis of oestrogen-dependent E47 proteins. The cells of each E47-inducible clone (**left**), cE47-inducible clones (**right**) or GC1 control clone were incubated with (+) or without (−) 17-β-estradiol (1 μM) for 12 h, after which they were lysed in sample buffer. The lysate (100 μg) was separated by 10% (left panel) or 15% (right panel) SDS−PAGE, transferred to Immobilon filters and probed with an antibody directed against the E47 N-terminal (left panel) or C-terminal region (right panel). The inducible bands were visualized with an anti-rabbit IgG alkaline phosphatase-conjugated antibody. Pre-stained molecular weight markers were included.

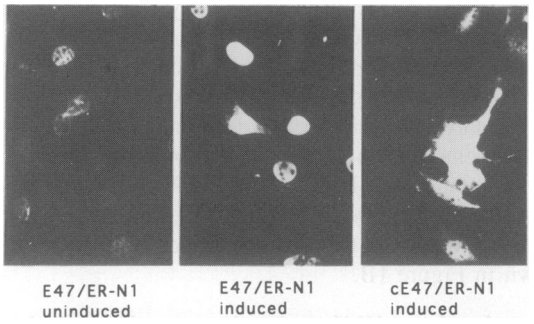

Fig. 5. Immunofluorescence staining of stable NIH3T3 cells expressing oestrogen-inducible E47 or cE47 proteins. Representative fields of the clones presented in Figure 4. The cells were treated and the inducible proteins stained as described in Figure 6.

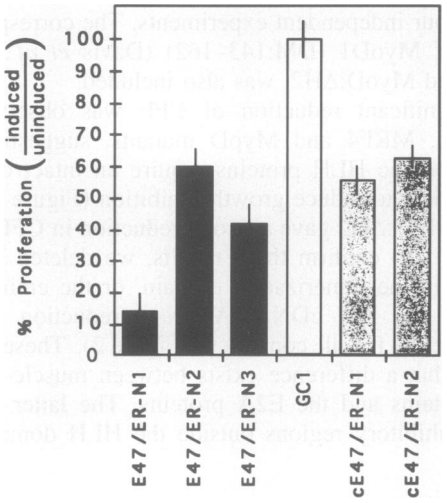

Fig. 6. The re-entry of NIH3T3 cells into the cell cycle is inhibited in a dose-dependent manner by E47 or cE47 oestrogen-induced proteins. The clones were arrested by serum starvation (0.5%) for 48 h and treated with 17-β-estradiol (1 μM), or left untreated, for 8 h before addition of 20% serum and BrdU (30 μM). Twenty-four hours after serum stimulation the cells were immunostained for the inducible protein and BrdU incorporation. The histogram shows the percentage of cell proliferation in oestrogen-treated relative to untreated cells for each clone in two separate experiments. SD is also indicated. Black or grey bars indicate the proliferation for clones expressing the E47 or the cE47-inducible protein, respectively. GC1 (empty bar) is the control clone.

itself prevent cell proliferation. Using an internal starting point of translation corresponding to methionine 415 of the full-length E47 protein, we expressed the C-terminal region of E47 cDNA (cE47). After transfection in NIH3T3 cells, cE47 was still able to reduce CFE to 19% (Figure 3). A deletion mutant of the basic domain, cE47:Δbas, still retained the growth-inhibitory function, reducing CFE to 45% of the control, indicating that DNA-binding is not mandatory for induction of growth arrest (Figure 3). Reduction of CFE was also observed with the cE47:AA-ALA mutant (Figure 3). This mutant contains substitutions of five amino acids in the basic domain which affect DNA-binding and transactivation of a reporter gene (Chakraborty *et al.*, 1991). Similarly, a MyoD mutant, B2proB3, that is unable to bind DNA can still reduce the CFE to ~50% (Crescenzi *et al.*, 1990; F.A.Peverali, unpublished observations). In contrast, a mutant with the HLH domain deleted, cE47:ΔHLH, did not affect the CFE (Figure 3). Thus a common characteristic of E2A and the muscle-specific proteins is that only the dimerization domain is involved in growth inhibition (Figures 2 and 3).

An inducible system for expression of E47

To examine the function of E47 in more detail we established cell lines conditionally expressing the E47 protein by using an inducible system developed by Braselmann *et al.* (1993).

By anti-E47 immunostaining, 50% of the stable transfected clones picked showed oestrogen-dependent E47 expression. Three clones expressing ~5, 15 and 50 times the basal level of E2A in NIH3T3 cells were chosen for further studies (Figure 4, left). The two clones with the highest expression level showed multiple forms of E47, suggesting that phosphorylation sites exist in the protein. The higher molecular weight bands of E47 appeared later than the initial inducible E47 band detectable 4 h after oestrogen stimulation (compare Figures 4 and 7C). Basal levels without oestrogen were at the endogenous E47 expression level in all clones.

Since one inhibitory domain of E47 was located in the C-terminal region corresponding to the single inhibitory domain of the muscle-specific bHLH proteins, we also isolated inducible clones of the mutant cE47 protein (Figure 3). The inducible expression of cE47 in two cell extracts is demonstrated in Figure 4, right panel.

The subcellular distribution of the induced E47 and cE47 was analysed by immunofluorescence. As expected, the full-length protein localized to the nucleus, whereas cE47 partially accumulated in the cytoplasm (Figure 5). The cE47 mutant lacks the nuclear localization signal present in the N-terminal region between amino acids 158 and 175. Nevertheless, 50% of the protein was observed in the nucleus. This may be due to dimerization with other

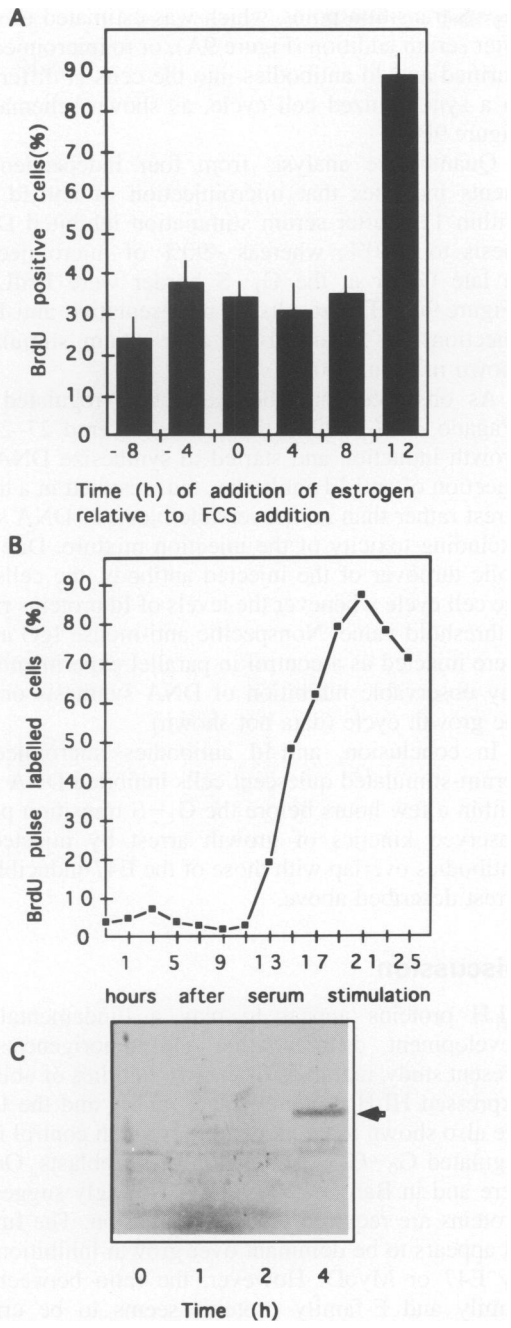

Fig. 7. E47 protein arrests proliferation of serum-stimulated NIH3T3 cells within a few hours before G_1-S transition of the cell cycle. (**A**) The E47-ER/N1 cells were arrested by serum deprivation for 48 h and reactivated with medium containing 20% serum together with BrdU (30 μM). At the indicated times relative to the addition of serum, 17-β-estradiol (1 μM) was added to the medium. The cells were stained for BrdU incorporation 24 h after serum stimulation. The histogram represents the average of two independent experiments where at least 200 cells were counted for each time point. SD is indicated by thin lines at the top of the bars. (**B**) The G_1-S transition of E47-ER/N1 cells was determined in parallel experiments with BrdU (100 μM) incorporation (pulse of 2 h) after serum stimulation in hormone-free medium. (**C**) Growing E47-ER/N1 cells were treated with 17-β-estradiol (1 μM). At the indicated time of treatment (in hours) the cells were lysed and the cell extracts were separated by 10% SDS−PAGE. The expression of the oestrogen-inducible E47 protein (indicated by an arrow) was analysed as described in Figure 4.

nuclear HLH proteins; in fact, mutations that affect the dimerization region reduced the amount of cE47 in the nucleus (data not shown).

Inducible growth inhibition mediated by E47

Serum-stimulated E47-inducible clones showed inhibition of DNA synthesis after treatment with 17-β-estradiol whereas the growth of clones expressing only the fusion protein without the E47 was not affected (Figures 4 and 6). The percentage of BrdU-positive cells, between 15 and 60%, correlated with the degree of E47-expression (Figure 6). Similarly, cE47-inducible clones showed inhibition of DNA synthesis although to a lower extent (Figure 6). The level of E47 induction varied from cell to cell. When cells with high levels of E47 were scored, <20% of them were BrdU-positive. A similar pattern was observed for cE47-expressing clones. These results suggest that a threshold level of E47 protein is required for growth inhibition.

E47 protein arrests cell growth within hours before G_1-S transition

To establish the temporal effect of E47 relative to S-phase entry, quiescent E47-inducible cells were treated with 17-β-estradiol at different times before or after serum induction (Figure 7A). Oestrogen-induced E47 protein inhibited S-phase entry when hormone was added within 8 h after the addition of serum, but not when added at 12 h (Figure 7A). Since Western blot analysis of cell extracts revealed maximal expression of E47 protein ~4 h after addition of oestrogen (Figure 7C), the inhibitory effect of E47 on cell growth probably occurs within the first 12 h after induction. A similar profile of growth inhibition was observed with the cE47 inducible protein (data not shown). In parallel experiments, the time required for the cells to enter S phase was estimated to be ~15 h after serum stimulation (Figure 7B).

In conclusion, E47 can mediate growth arrest within a few hours before the G_1-S transition point.

Id counteracts the E47 inhibition of cell growth

We then asked whether Id1 could counteract the growth arrest mediated by the bHLH proteins. Equal amounts of the Id1 together with either E47 or MyoD1 vectors were introduced into growing NIH3T3 cells by microinjection (Figure 8). The amount of DNA injected was kept constant by adding the empty vector whenever only one HLH was investigated. BrdU was added to the culture 16 h after injection and 6 h later the cells were fixed and immunostained to ascertain whether exogenous HLH proteins were present and to monitor DNA synthesis. When two exogenous HLH proteins were coexpressed in the same cells, we stained to detect the growth-inhibitory HLH protein.

As shown in Figure 8, both E47 and MyoD1 alone inhibited DNA synthesis to 23% and 35%, respectively, whereas in the presence of Id1 reduction in DNA synthesis was minimal (93% and 87%, respectively). When injected alone, Id1 did not inhibit growth (98%). The E47:ΔHLH mutant lacking the dimerization motif inhibited cell growth both when injected alone (48%) or with Id1 (54%). The expression of Id1 was followed by staining with anti-Id antibodies. Both proteins were localized in the nucleus.

In accordance with previous observations (Benezra *et al.*, 1990; Christy *et al.*, 1991; Barone *et al.*, 1994), we observed a strong induction of Id1 mRNA shortly after serum stimulation. No significant variation in the levels

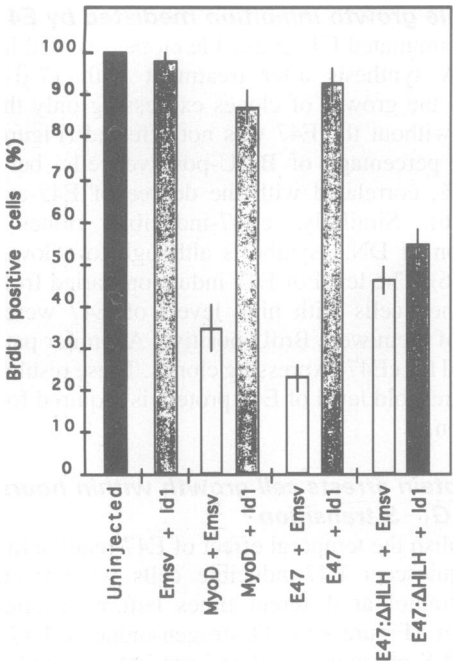

Fig. 8. Id counteracts the growth inhibition of E47 and MyoD1. pEMSV vectors expressing E47 (E47) or MyoD1 (MyoD1) cDNAs were co-injected with pEMSV-Id1 (Id1) or with the pEMSV control vector (Emsv) into exponentially growing NIH3T3 cells. The cells were incubated with BrdU 16 h later for 6 h, then processed for immunofluorescence and BrdU staining. The HLH deletion mutant of E47 (E47:ΔHLH) was also analysed in this manner. The injected cells were stained with polyclonal anti-MyoD1 (MyoD1+Emsv and MyoD1+Id1), anti-E47 (E47+Emsv, E47+Id1, E47:ΔHLH+Emsv and E47:ΔHLH+Id1) or anti-Id1 (Id1+Emsv) antibodies. The histogram shows the average of four independent experiments and the respective SD. The bars indicate DNA synthesis (%) of cells injected with (grey bar) or without (white bar) Id-encoding plasmid. The DNA synthesis in injected cells was normalized to that in uninjected cells (black bar).

of E12 or E47 mRNA was observed in quiescent or serum-stimulated NIH3T3 cells (data not shown).

These results suggest that Id proteins which are only expressed in growing cells (Figure 9D) can specifically counteract the growth arrest induced by E47 or MyoD through heterodimerization via the HLH regions. Growth arrest can probably only be observed in cells expressing high levels of E47 that may out-compete the high endogenous levels of Id (Jen *et al.*, 1992). Interestingly, Id1 can also suppress growth inhibition mediated by the N-terminal regions of E47 when they reside in a molecule with an intact dimerization domain.

Id proteins are required for G_1 progression until a few hours before S phase entry

To assess the role of the Id family proteins in growth regulation, we prepared a polyclonal antibody against the dimerization domain of the Id protein. The affinity purified anti-Id antibodies recognize a 15 kDa band in growing NIH3T3 and C3H10T1/2 fibroblastic cell extracts (Benezra *et al.*, 1990; Jen *et al.*, 1992; Deed *et al.*, 1993), while no signal above background was detected in quiescent cells (Figure 9D). Moreover the anti-Id antibody stains the nucleus of proliferating C3H10T1/2 cells, stably transfected or microinjected with Id cDNA (data not shown).

Quiescent NIH3T3 cells were used either to mark the G_1-S transition point, which was estimated to start 15 h after serum addition (Figure 9A), or to microinject affinity purified anti-Id antibodies into the cells at different times in a synchronized cell cycle, as shown schematically in Figure 9B.

Quantitative analysis from four independent experiments indicates that microinjection of anti-Id antibody within 12 h after serum stimulation inhibited DNA synthesis to <50%, whereas ~90% of microinjected cells in late G_1 or at the G_1-S border were BrdU-positive (Figure 9C). The results of representative anti-Id microinjections at 12 and 14 h after serum stimulation are shown in Figure 10.

As observed for other cell cycle regulated proteins (Pagano *et al.*, 1993), the cells recovered 27–28 h after growth induction and started to synthesize DNA. Microinjection of anti-Id antibodies thus resulted in a temporary arrest rather than an irreversible block of DNA synthesis, excluding toxicity of the injection mixture. Due to metabolic turnover of the injected antibody, the cells re-enter the cell cycle whenever the levels of Id proteins rise above a threshold value. Nonspecific anti-mouse IgG antibodies were injected as a control in parallel experiments without any observable inhibition of DNA synthesis or delay in the growth cycle (data not shown).

In conclusion, anti-Id antibodies microinjected into serum-stimulated quiescent cells inhibited DNA synthesis within a few hours before the G_1-S transition point. The observed kinetics of growth arrest by injected anti-Id antibodies overlap with those of the E47-inducible growth arrest described above.

Discussion

HLH proteins appear to play a fundamental role in development, differentiation and tumorigenesis. In the present study, members of the two families of ubiquitously expressed HLH proteins, the E-family and the Id-family, are also shown to be involved in growth control in serum-regulated G_0-G_1-S transition of fibroblasts. Our results here and in Barone *et al.* (1994) strongly suggest that Id proteins are required for G_1 progression. The function of Id appears to be dominant over growth inhibition induced by E47 or MyoD. However, the ratio between the Id-family and E-family proteins seems to be critical for keeping the cells in G_1 progression. Increasing the amounts of E proteins or neutralizing the Id-like component leads to inhibition of proliferation in the G_1 phase until 3–4 h before entry into the S phase.

E2A-mediated growth inhibition

In fibroblastic NIH3T3 cells, not only bHLH proteins of the MyoD1 family such as myogenin and MRF4, but also the E2A (E12/E47) proteins of the E-family induce growth inhibition (Figure 1).

Whereas the HLH region is required for growth inhibition induced by the MyoD1 family, the E2A proteins also harbour two additional inhibitory regions that are active in the absence of the HLH domain (Figures 2 and 3). These regions are located between amino acids 16 and 174 and between amino acids 371 and 415 in the N-terminal and central parts of E12/E47, respectively, and correspond to the location of the identified transactivation

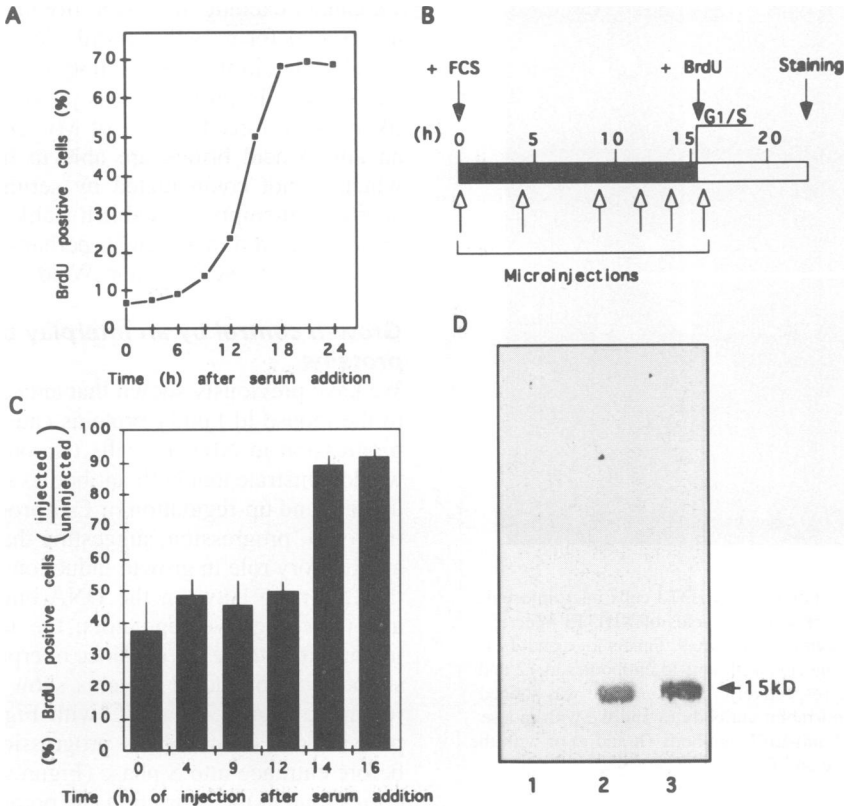

Fig. 9. Microinjected anti-Id antibodies inhibit G_1 progression of serum-stimulated G_0 resting cells within a few hours before G_1-S transition point. (**A**) Quiescent cells were stimulated with 20% serum and incubated for the indicated number of hours in the presence of BrdU, then fixed and stained with an anti-BrdU antibody. The curve shows the percentage of cells stained for BrdU at the indicated times. Each value represents at least 200 cells. (**B**) Schematic representation of the experiments shown in C designed to evaluate the effect of anti-Id antibodies on growth induction. The length of the G_0-G_1 phase is ~15 h as estimated in A. The empty arrows indicate the time points of injection of the anti-Id antibodies. The black arrows indicate the time points of serum and BrdU addition, respectively, as well as termination of the experiment. (**C**) Cells on parallel coverslips as used for experiments shown in A were injected with 2.5 mg/ml anti-Id1 antibodies at the indicated times, in hours, after serum stimulation; 15–16 h later the cells were incubated in BrdU for 6 h, fixed and stained for BrdU. The percentage of BrdU-positive cells among the anti-Id injected cells is shown in the histogram which represents the average of four independent experiments and respective SD. 100–130 cells were injected for each time point. (**D**) Crude lysates of (1) quiescent, (2) growing NIH3T3 or (3) growing C3H10T1/2 cells were separated by 15% SDS–PAGE and probed with the same affinity purified anti-Id antibodies used in C. The molecular weight of the specific band is indicated by an arrow.

domains of this protein (Aronheim *et al.*, 1993b; Quong *et al.*, 1993). The growth inhibition of the N-terminal regions may be caused either by 'squelching' (Ptashne, 1988) of proteins required for the basal transcriptional machinery or by a specific mechanism which is a part of the normal regulatory function of E2A proteins. Both possibilities assume that E12/E47 interact with other proteins. With glutathione S-transferase (GST)–E47 constructs we have preliminary evidence for a specific interaction between the N-terminal region containing the first inhibitory domain and unidentified proteins of ~35 and 130 kDa (F.A.Peverali, unpublished results). Experiments are in progress to characterize these proteins. Regardless of the mechanism of growth inhibition of the N-terminal region, it is important to note that the transactivation domains of the muscle-specific bHLH proteins do not cause a similar growth inhibition (Figure 2).

Nevertheless, the N-terminal region of the E47 is required for the transforming activity of E2A–Pbx protein, associated with leukaemias (Dedera *et al.*, 1993; Van-Dijk *et al.*, 1993). The transactivation domains of E47 may therefore bind proteins required for growth regulation. By analogy, the transactivation domain of Myc is required for the transformed phenotype and cell growth (Kato *et al.*, 1990).

The inhibition of cell growth obtained with the C-terminal bHLH region of E47 correlates well with the inhibition obtained with the MyoD1 family. In both cases the HLH region is required whereas the basic DNA-binding region appears unnecessary for growth inhibition (as described in Results). This opens the interesting possibility that these factors may be part of a nuclear multiprotein complex which regulates growth (see below). Although this complex may not bind DNA through the bHLH region to induce growth arrest, we cannot exclude completely the possibility that overexpression of bHLH proteins lacking the DNA-binding activity, such as cE47:ΔBas, cE47:AA-ALA and MyoD:B2proB3, may titrate out the endogenous Id proteins, allowing a small fraction of endogenous bHLH dimers to bind DNA and arrest proliferation. However, the role of the HLH domain in growth control is intriguing and needs to be further investigated.

The inhibition observed with the C-terminal part of E47 lacking the basic domain is less pronounced than that observed with the intact C-terminal region. Whether this is due to additional activities of the basic domain or to

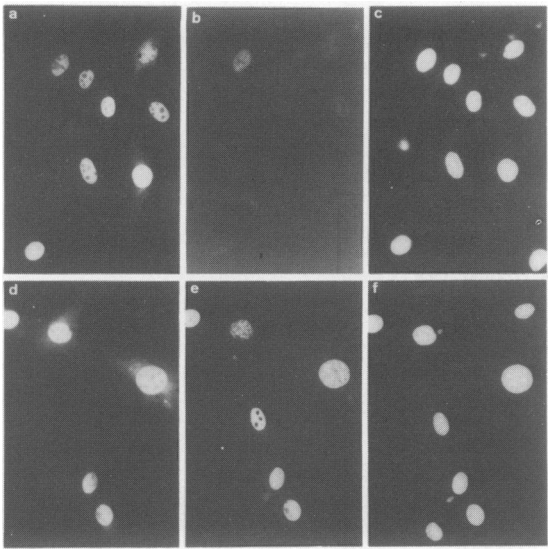

Fig. 10. Immunofluorescence staining of NIH3T3 cells microinjected with the anti-Id antibodies. Representative fields of NIH3T3 injected with anti-Id antibodies as described in Figure 9. Panels a–c and d–f show the same field of cells injected with anti-Id antibodies at 12 and 14 h after serum stimulation, respectively. The same field was stained with Texas red-conjugated anti-rabbit antibody (**a** and **d**), with mouse monoclonal FITC-conjugated anti-BrdU antibody (**b** and **e**) or with the nuclear dye, Hoechst 33258 (**c** and **f**).

changes in the conformation of the nearby HLH region is not known at present. It is of interest in this respect that a conserved sequence element downstream of the HLH region may have some functional significance. The reduced growth-inhibitory efficiency of the C-terminal region may also be due to there being less of the protein in the nucleus since cE47 lacks a nuclear localization signal (Figures 3 and 5).

The E47 oestrogen-inducible cell lines allowed us to determine that overproduction of E47 may negatively regulate the progression through the G_1 phase within hours before the G_1–S border (Figure 7). The relatively high overexpression of E47 required for the growth arrest is not surprising, since a high endogenous level of Id proteins in proliferating NIH3T3 cells has to be overcome by the induced E47. It should be noted that although the endogenous level of E47 is low in fibroblasts, there is nevertheless enough to support MyoD-induced differentiation and the associated growth arrest.

The Id-family proteins reverse growth inhibition

As long as the HLH dimerization domain is preserved in the growth-inhibitory proteins, the Id-family proteins can combine with them and thereby reverse growth inhibition (Figure 8). This is most clearly revealed by the E47 lacking the HLH domain whose N-terminal growth-inhibitory activity cannot be reversed by overexpression of Id proteins (Figure 8). The ability of Id to counteract all the inhibitory domains of intact E47 is possibly due to heterodimerization with E47 which could pull E47 out of a protein complex involved in negative growth control, or mask all the inhibitory regions including those located outside the HLH domain. Both of these hypotheses agree with the observation that all E2A present in growing cells is dimerized with Id proteins (Jen *et al.*, 1992). However, we cannot exclude the possibility that only the Id proteins are crucial for growth control. Overexpression of MyoD or E2A may in that case simply result in a titration of the endogenous Id proteins. This possibility is, however, less likely since forced dimers of MyoD and E47 tethered by an amino acid bridge are able to induce differentiation which is not counteracted by serum growth factors. In addition, attempts to establish stable cell lines expressing these tethered dimers failed, perhaps because they caused growth arrest (Neuhold and Wold, 1993).

Growth control by an interplay between HLH proteins

We have previously shown that antisense oligonucleotides to the mouse Id-family proteins cause a retardation of G_1 progression in NIH3T3 cells (Barone *et al.*, 1994). Here we demonstrate that both antibodies to the Id-dimerization domain and up-regulation of E47 proteins cause an inhibition of G_1 progression, suggesting that HLH proteins have a regulatory role in growth induction. It may be postulated that the ratio between the DNA-binding bHLH proteins and their negative regulators, the Id-family proteins, is important in these events since overproduction of E47 and suppression of the Id proteins show the same phenotype (compare Figure 7A and C with Figure 9C). E47 and Id proteins can influence G_1 progression until a few hours before entrance into S phase (Figures 7 and 9) which may correspond to the restriction (R) point, defined as the point when cells become irreversibly committed to progression into S phase (reviewed in Pardee, 1989). Recently, it has been proposed that Rb proteins also become hyperphosphorylated at the R point and not at the G_1–S border as previously supposed (Mittnacht *et al.*, 1993). Phosphorylation eliminates the growth suppressor effect of Rb. Cyclin–cdk complexes are probably involved in the phosphorylation of Rb. Cyclin D appears to be the most plausible candidate. Cyclin D and Id are both required for entry into S phase and both are encoded by serum-responsive genes. Even more suggestive is the temporal profile of growth inhibition following injection of anti-cyclin D antibody (Baldin *et al.*, 1993) which superimposes upon that observed after injection of anti-Id antibodies (Figure 9).

The recent finding that MyoD proteins may bind to the Rb and p107 proteins may provide a hint to a function (Gu *et al.*, 1993; Schneider *et al.*, 1994). This binding obviously engages part of the same binding pocket used by a cellular transcription factor required for growth induction, E2F. When associated with Rb, E2F represses several promoters of genes required in late G_1 and S phase (Weintraub *et al.*, 1992). In the absence of serum growth factors, it is feasible that MyoD, E12/E47 and other bHLH bind to Rb-family proteins locking the cells in a growth arrest stage. During this stage, E2F is probably associated with the bHLH–Rb complex. Upon growth induction, the Id proteins may remove the bHLH proteins from the complex in a stoichiometric fashion leading to activation of the E2F. The overall fine adjustment of this basic mechanism is then controlled by phosphorylation of the Rb proteins. In transformed cells, the viral oncogenic proteins irreversibly trap the Rb proteins, which become unavailable for growth control. The recent findings that c-Myc can also bind to p107 leading to suppression of

transcriptional activation (Gu *et al.*, 1994) and that p107 may also substitute for Rb as a cofactor for muscle differentiation (Schneider *et al.*, 1994), suggest that the Rb-family may have a pivotal role in growth control. The interrelationship between all these components must be analysed further. Nevertheless, our results imply that the ubiquitous HLH proteins like E47/E12 and Id play a role in G_1 progression which has not previously been observed.

Materials and methods

Plasmids and site-directed mutagenesis

The expression vector pEMSV (Davis *et al.*, 1987) was used to express all HLH-encoding cDNAs and their mutants by microinjection and CFE assays in NIH3T3 cells. The cDNAs of MyoD1 (Davis *et al.*, 1987), myogenin (Edmondson and Olson, 1989), MRF4 (Rhodes and Konieczny, 1989) and Id1 (Benezra *et al.*, 1990) had already been cloned in the pEMSV vector, whereas E12, E47 (Murre *et al.*, 1989a) and HLH462 (Id3) (Christy *et al.*, 1991) cDNAs were inserted into the *Eco*RI site of this vector. The deletions introduced in E47:Δ601–647 and in the bHLH domain of E47, myogenin and MRF4 as well as the point mutations in the cE47:AA-ALA mutant were generated by standard polymerase chain reaction (PCR) using appropriate primers followed by sequencing. The mutant MyoD1:DM143–162, here called MyoD1:ΔH2, was described by Davis *et al.* (1990). The five amino acid substitutions of the E47 basic domain (cE47:AA-ALA, single amino acid code) correspond to those described by Chakraborty *et al.* (1991) for the E2-5/BS2-3 mutant. For expression of proteins from the plasmids, the endogenous translation stop codons were utilized, except for the truncation (T) mutants of E47 for which a stop codon was inserted in the vector just downstream of the cloning site.

The pSV2neo (Southern and Berg, 1982) or pIPB1 (Biamonti *et al.*, 1985) plasmids conferring G418 resistance to the recipient cells were chosen for cotransfections.

DNA purification

All plasmids were prepared by the Quiagen procedure (Diagen), followed by one purification on CsCl density gradients at 95 000 r.p.m. for 6–10 h at 18°C in a TL-100 Beckman ultracentrifuge. The plasmid DNA was extracted four times with water-saturated butanol and dialysed in TE buffer (10 mM Tris–HCl pH 8, 1 mM EDTA) for at least 3 h at room temperature in Collodion bags (Sartorius). DNA was recovered by ethanol precipitation and resuspended in 0.1 × TE buffer. High molecular weight DNA was purified as described by Wigler *et al.* (1979).

Colony forming efficiency assay

NIH3T3 cells (2×10^5 cells/10 cm plate) were seeded 1 day before transfection, then transfected with 0.3 μg of pIPBI or pSV2neo, 3–10 μg of pEMSV-expression vector containing an HLH cDNA, and 15–20 μg of high molecular weight NIH3T3 DNA carrier by a modification of the calcium phosphate precipitation technique (Wigler *et al.*, 1979). The cells were exposed to the precipitated DNA for 16–20 h and split 1:10 in G418 (700 μg/ml Geneticin, Gibco) selective medium 24 h later. The medium was changed every 3–4 days and after 12–15 days the colonies were fixed and stained in 50% methanol, 10% acetic acid containing 0.06% Coomassie brilliant blue R250 (Sigma). The CFE was calculated by dividing the number of colonies on HLH by those on empty vector plates, and was the average of results from at least three separate experiments.

Antibody production and purification

Glutathione S-transferase (GST) fusion proteins were produced by subcloning specific E47 fragments into the expression vector pGEX-2T (Pharmacia). The sequences encoding the first N-terminal 174 amino acids (*Bsp*EI site on E47 cDNA, see Figure 3) or the last C-terminal 281 amino acids (from the *Xho*I site, see Figure 3) of human E47 were cloned in-frame with the GST moiety. The *Rsa*I–*Hin*fI fragment of mouse Id1 cDNA, which encodes residues 29–105 containing the Id1 HLH domain, was cloned in the same vector. The expression and purification of the fusion proteins were performed as described in Smith and Corcoram (1987).

Rabbits were injected with 400 μg of fusion protein in complete Freund's adjuvant and reinjected every 2 weeks thereafter with the same amount of protein in incomplete Freund's adjuvant until a signal was detected by immunoblotting.

Affinity purified anti-Id antibodies used in microinjections were purified as follows: GST–Id1 recombinant protein (1 mg/ml) in 0.1 M MOPS–HCl pH 7 buffer was mixed with 1 ml of Affi-gel 15 (Bio-Rad) suspension, incubated overnight at 4°C and then transferred to a 10 ml Bio-Rad column. An equal volume of 1 M ethanolamine–HCl pH 8 was added to the column. The column was subsequently washed first with PBS, then 0.2 M glycine–HCl pH 2.3 and finally with PBS. To eliminate antibodies against GST, Id1 polyclonal antiserum was first precleared by agitating for 8 h at 4°C with GST protein bound to glutathione–Sepharose beads. The flowthrough was then affinity purified on a GST-Id column. The anti-Id1 antiserum was applied three times to the column. The column was extensively washed with PBS, and affinity purified antibodies were eluted from the column with 0.2 M glycine–HCl pH 2.3. The fractions were immediately neutralized with 1 M Tris–HCl pH 8. Fractions containing the antibodies were pooled and the buffer was replaced with PBS by centrifugation in Centricon 30 tubes (Amicon). The affinity purified antibodies (2.5 mg/ml) were aliquoted, frozen at −80°C and thawed only once.

Oestrogen-inducible E47 cell lines

The oestrogen induction system (Braselmann *et al.*, 1993) was used to generate cell lines expressing the E47 protein under an inducible promoter. Briefly, the pMV-Gal–ER–VP16 plasmid encodes the Gal–ER–Vp16 fusion protein which binds and transactivates the Gal4-responsive promoter in a pGC vector in the presence of 17-β-estradiol. Therefore, the *Xho*I–*Eco*RI fragment encoding cE47 (C-terminal region of E47) or the full-length E47 cDNA were cloned into the *Xba*I site of the pGC plasmid to obtain pGC-cE47 and pGC-E47 plasmids, respectively.

NIH3T3 cells (2×10^5 cells/10 cm plate) were transfected with 0.3 μg/ml of the pMV-Gal–ER–VP16 plasmid (which also confers G418 resistance) linearized with *Pvu*I, together with 3–10 μg/ml of pGC-cE47 or pGC-E47 linearized with *Sph*I and 15 μg/ml of NIH3T3 high molecular weight DNA carrier as described for the CFE assay. After G418 selection, 20–30 randomly picked clones were seeded in 24 multiwells containing coverslips. The cells were grown in phenol red-free DMEM medium (Gibco) containing 10% fetal calf serum (FCS).

To identify clones expressing the inducible protein, coverslips from each clone were either untreated or treated with 17-β-estradiol (Sigma) (0.1–1 μM) for 10–20 h, then fixed and stained with fluorescent E47 antibodies. Clones showing E47 protein after 17-β-estradiol treatment and low basal levels of E47 were chosen for further studies. To derive stable cell lines, these clones were subcloned 1 month later.

Western blot analysis

NIH3T3 cells were lysed in sample buffer and 100 μg of protein applied in each lane on 10–15% SDS–PAGE. The proteins were transferred to Immobilon (Millipore) or nitrocellulose filters and probed with antibody. The blots were then developed with alkaline phosphatase-conjugated anti-rabbit IgG antibody (Promega).

Immunofluorescence

Cells growing on glass coverslips were washed once with PBS, fixed for 10 min at room temperature with 3% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. Antibody reactions were carried out at room temperature for 45–60 min in a moist chamber.

Fixed and permeabilized cells were incubated with specific rabbit polyclonal antibody (dilution 1:800) (this step was skipped for anti-Id1 injected antibodies), washed three times with PBS and incubated with Texas red-conjugated goat anti-rabbit IgG antibodies (dilution 1:100, Amersham) and washed again in PBS.

For BrdU staining, fixed and permeabilized coverslips (already immunostained as above) were further incubated in 2 M HCl in PBS for 10 min at room temperature, then carefully washed in PBS and incubated with monoclonal FITC-conjugated mouse anti-BrdU antibodies (dilution 1:10, Boehringer)

After these treatments the coverslips were incubated for 5 min at room temperature with a 1 μg/ml solution of Hoechst dye 33258 (Sigma) in PBS for nuclear staining of the cells.

The cells were finally washed once in PBS, rinsed quickly in distilled water and mounted on glass slides with Mowiol (Hoechst). The cells were examined on a Zeiss Axiophot microscope using a Neofluar 63× lens. Photographs were taken on Kodak TMZ-3200 or Fuji P1600 films.

Microinjection

Caesium chloride purified plasmids (see above) containing the vector with or without cDNA inserts were mixed together at a final concentration of 100 μg/ml (unless otherwise specified) for each plasmid and microinjected into the nucleus of asynchronously growing NIH3T3 cells using an automated microinjection system (AIS, Zeiss) (Ansorge and Pepperkok, 1988). More than 85% of the cells were successfully microinjected (Pepperkok *et al.*, 1988). After 16–18 h the cells were incubated with 100 μM BrdU and 6–8 h later fixed and processed as described in the immunofluorescence section. DNA synthesis of microinjected cells was evaluated after at least four independent experiments in which 120–140 cells were injected each time.

Affinity purified anti-Id1 antibodies (2.5 mg/ml) or purified control antibodies (3 mg/ml) were microinjected as above in NIH3T3 cells which were synchronized in the G_0 phase by cultivation in low serum (0.5% FCS) for 36–48 h and then induced to grow by addition of 20% FCS. At each experimental time point, 100–130 cells were injected. BrdU was added 15–16 h after serum stimulation and 6–7 h later the cells were fixed (see also Figure 10).

Double immunofluorescence was performed using Texas red-conjugated goat anti-rabbit IgG antibodies (dilution 1:100, Amersham) to recognize the injected anti-Id1 antibodies and monoclonal FITC-conjugated mouse anti-BrdU antibodies (dilution 1:10, Boehringer) as described above.

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