Retinoic Acid Induces Myogenin Synthesis and Myogenic Differentiation in the Rat Rhabdomyosarcoma Cell Line BA-Han-1C

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Abstract. Two clonal rat rhabdomyosarcoma cell lines BA-Han-1B and BA-Han-1C with different capacities for myogenic differentiation have been examined for the expression of muscle regulatory basic helix-loop-helix (bHLH) proteins of the MyoD family. Whereas cells of the BA-Han-1C subpopulation constitutively expressed MyoD1 and could be induced to differentiate with retinoic acid (RA), BA-Han-1B cells did not express any of the myogenic control factors and appeared to be largely differentiation-defective. Upon induction with RA, BA-Han-1C cells expressed also myogenin, in contrast to BA-Han-1B cells which never activated any of the genes encoding muscle bHLH factors. The onset of myogenin transcription in BA-Han-1C cells required de novo protein synthesis and DNA replication suggesting that RA probably did not act directly on the myogenin gene. Although MyoD1 was expressed in proliferating BA-Han-1C myoblasts, muscle-specific reporter genes were not activated indicating that MyoD was biologically inactive. However, transfections with plasmid expressing additional MyoD1 protein resulted in the transactivation of muscle genes even in the absence of RA. mRNA encoding the negative regulatory HLH protein Id was expressed in proliferating BA-Han-1C cells and disappeared later after RA induction which suggested that it may be involved in the regulation of MyoD1 activity. The myogenic differentiation of malignant rhabdomyosarcoma cells strictly correlated with the activation of the myogenin gene. In fact, stable transfections of BA-Han-1C cells with myogenin expressing plasmids resulted in spontaneous differentiation. Taken together, our results suggest that the transformed and undifferentiated phenotype of BA-Han-1C rhabdomyosarcoma cells is associated with the inactivation of the myogenic factor MyoD1 as well as lack of myogenin expression. RA alleviates the inhibition of myogenic differentiation, probably by activating MyoD protein and myogenin gene transcription. BA-Han-1B cells did not respond to RA and the differentiated phenotype could not be restored by overexpression of MyoD1 or myogenin.

TRANSFORMATION of normal cells to a malignant phenotype is believed to involve multistep mechanisms which alter regulatory processes controlling growth and differentiation. In many tumors individual cells can be found that spontaneously undergo partial or complete differentiation or can be induced to differentiate by various agents (Pierce, 1974). These observations have indicated that not all cells in a given tumor are necessarily in the same state of transformation and some can be reverted to a benign phenotype. Induction of differentiation has actually been proposed as an alternative approach to conventional therapy of cancer (Metcalf, 1983; Spremulli and Dexter, 1984; Sartorelli, 1985; Sachs, 1987). To investigate factors and conditions which may influence the balance between the transformed state and the differentiated phenotype, we have used clonal cell lines isolated from a dimethylbenzanthracene-induced rat rhabdomyosarcoma which were shown to exert different capacities to differentiate in vitro (Gerharz et al., 1988). In contrast to normal myogenic cells in which differentiation can be induced by the mere removal of serum components, only a minute proportion of the rhabdomyosarcoma cell line BA-Han-1C forms myotubes under these conditions. The majority of cells requires retinoic acid (RA)[1] or a variety of other inducing agents for effective differentiation (Gabbert et al., 1988; Gerharz et al., 1989). The BA-Han-1B cell line isolated from the same tumor can not be triggered to differentiate to the complete muscle phenotype by inducing agents, although it appears to be of skeletal muscle origin as judged by its morphology and the expression of desmin and muscle creatine kinase (Gerharz et al., 1988, 1989). In the absence of inducers both cell lines appear as mononuclear, rapidly dividing rhabdomyoblasts.

The development of muscle cells has become a preferred model system to study molecular mechanisms underlying cellular differentiation. A group of myogenic control proteins encoded by the MyoD gene family has recently been identified (for review see Olson, 1990; Emerson, 1990;

1. *Abbreviations used in this paper*: bHLH, basic-helix-loop-helix; MHC, myosin heavy chain; RA, retinoic acid; RAR, retinoic acid receptor.

Weintraub et al., 1991). Four distinct cDNAs, MyoD1 (Davis et al., 1987; Braun et al., 1989*a*), myogenin (Wright et al., 1989; Edmondson and Olson, 1989; Braun et al., 1989*a*), Myf-5 (Braun et al., 1989*b*), and MRF4/herculin/Myf-6 (Rhodes and Konieczny, 1989; Miner and Wold, 1990; Braun et al., 1990) have been isolated and shown to possess the capacity to convert 10T1/2 fibroblasts and a variety of other nonmuscle cells to the myogenic lineage. All of these proteins contain a highly conserved basic region adjacent to a sequence motif that is believed to form two amphipathic helices which are connected by a short intervening loop structure. The basic-helix-loop-helix (bHLH) domain is responsible for heterooligomerization and sequence-specific DNA binding to a DNA sequence referred to as E-box (Davis et al., 1990; Murre et al., 1989*a*,*b*). This E-box motif consisting of the highly degenerate DNA consensus sequence CANNTG was found to be present in control elements of many muscle-specific genes where it mediates transcriptional activation by the interaction with the muscle-specific regulatory bHLH proteins (reviewed in Olson, 1990; Emerson, 1990). The biochemical properties of these proteins and their exclusive expression in skeletal muscle and embryonic muscle progenitor cells suggest that their primary biological activity may be to activate the transcription of typical muscle genes.

To investigate the role of the myogenic control factors in the differentiation-competent rhabdomyosarcoma cell line BA-Han-1C and in the differentiation-refractory counterpart BA-Han-1B, we analyzed the expression and activity of the various members of the MyoD family. We were particularly interested in the effects that RA acting as a differentiation inducing agent may exert on the activity of the myogenic factors and their genes. Here, we report that MyoD1 is constitutively expressed in the inducible cell line BA-Han-1C but not in the uninducible line BA-Han-1B. Administration of RA results in a delayed transcriptional activation of the myogenin gene in BA-Han-1C but not in BA-Han-1B cells. Activation of myogenin gene expression is dependent on protein and DNA synthesis suggesting that RA may act rather indirectly. Our results provide evidence that the block of differentiation in the rhabdomyosarcoma cell line BA-Han-1C may be dependent on the lack of myogenin expression. However, a mechanism that inhibits the transcription activating function of MyoD1 may also play an important role for the inability of these cells to differentiate in the absence of the inducer RA. Both events may in fact be related.

Materials and Methods

Cell Culture and DNA Transfections

The isolation and characterization of the rat rhabdomyosarcoma cell lines BA-Han-1B and BA-Han-1C have been described previously (Gerharz et al., 1988, 1989). Cells were cultured in DME supplemented with 10% FCS, penicillin, and streptomycin. Culture media and sera were purchased from Gibco Laboratory (Eggenstein, Germany). To induce differentiation, RA (Serva Inc.) was added to a final concentration of 1 μM from a 5 mM stock solution prepared in 95% ethanol. Medium was routinely changed after 3 d. Rat L6 and mouse C2C12 cells (Yaffe and Saxel, 1977) were obtained from the American Type Culture Collection (Rockville, MD) and were cultured as described elsewhere (Braun et al., 1992; Salminen et al., 1991).

Rhabdomyosarcoma cells were stably transfected with 1 μg of supercoiled pSV2-neo plasmid conferring geneticin (G418) resistance and 20 μg of pEMSV-Myf4 by use of the calcium-phosphate precipitation method. G418-resistant colonies were selected in medium containing 400 μg/ml G418 (Geneticin; Gibco Laboratories, Grand Island, NY). Transient transfections were performed as described previously (Braun et al., 1989*c*). CAT activity was determined by standard procedures (Gorman, 1985) two days after transfection. Transfection efficiencies were controlled by cotransfection of 5 μg RSV-βgal plasmid and CAT activity was standardized according to β-galactosidase activity obtained in the same cellular extracts.

Reporter Plasmids and Expression Vectors

Myf4L-CAT reporter containing 1.1 kb of the human Myf-4 gene promoter driving the CAT gene has been described previously (Salminen et al., 1991). MCK4R-CAT plasmid containing four oligomerized MyoD1-binding sites upstream of the thymidine kinase TATA-box linked to the CAT gene has been provided by A. Lassar (Harvard Medical School, Boston, MA) (Weintraub et al., 1991). TK-CAT reporter plasmid has been obtained by G. Schütz (German Cancer Research Center, Heidelberg, Germany). MLC-CAT plasmid containing the muscle-specific enhancer of the rat myosin light chain 1/3 gene and the MLC1 core promoter linked to the CAT gene has been published previously (Rosenthal et al., 1990). The expression vector pEMSV-Myf4 has been described (Braun et al., 1989*a*).

Isolation of RNA and Northern Blot Analysis

RNA was isolated from tissue culture cells by the guanidinium method (Chomzynski and Sacchi, 1987). 5–10 $\times$ 10^6 cells yielded between 200 and 300 μg of total RNA. Glyoxylation, agarose gel electrophoresis, RNA transfer, and hybridization conditions have been described previously (Braun et al., 1989*b*). Briefly, 25 μg of total RNA was separated on gels, transferred onto PALL Biodyne Membrane (Portsmouth, UK), and hybridized with 1–3 $\times$ 10^6 cpm/ml of radioactively labeled hybridization probe (sp act 1–2 $\times$ 10^8 cpm/μg obtained through random priming with [^{32}P] dCTP) (3,000 Ci/mmol). Filters were hybridized in 50% formamide, 5$\times$ Denhardt's solution, 5$\times$ SSC (1$\times$ SSC contains 0.15 M sodium chloride and 0.015 M sodium citrate), 50 mM sodium phosphate, 0.1% SDS at 42°C for 18 h. Filters were washed in 0.1% SSC, 0.1% SDS at 55°C for 30 min in several steps.

The following probes were used for hybridization: (*a*) the mouse MyoD1 probe was obtained as a 800-bp HpaII/EcoRI fragment from the cloned cDNA (Davis et al., 1987) representing the 3′ noncoding region; (*b*) the myogenin probe was prepared as a 700-bp fragment from the rat cDNA as described previously (Sassoon et al., 1989); (*c*) the GAPDH probe was a 1,100-bp segment of the mouse glyceraldehyde phosphate dehydrogenase cDNA: (*d*) the Id cDNA probe has been provided by H. Weintraub (Fred Hutchinson Cancer Center, Seattle, WA) and has been described (Benezra et al., 1990); and (*e*) mouse Myf-5 and Myf-6 probes have been described previously (Ott et al., 1991; Bober et al., 1991).

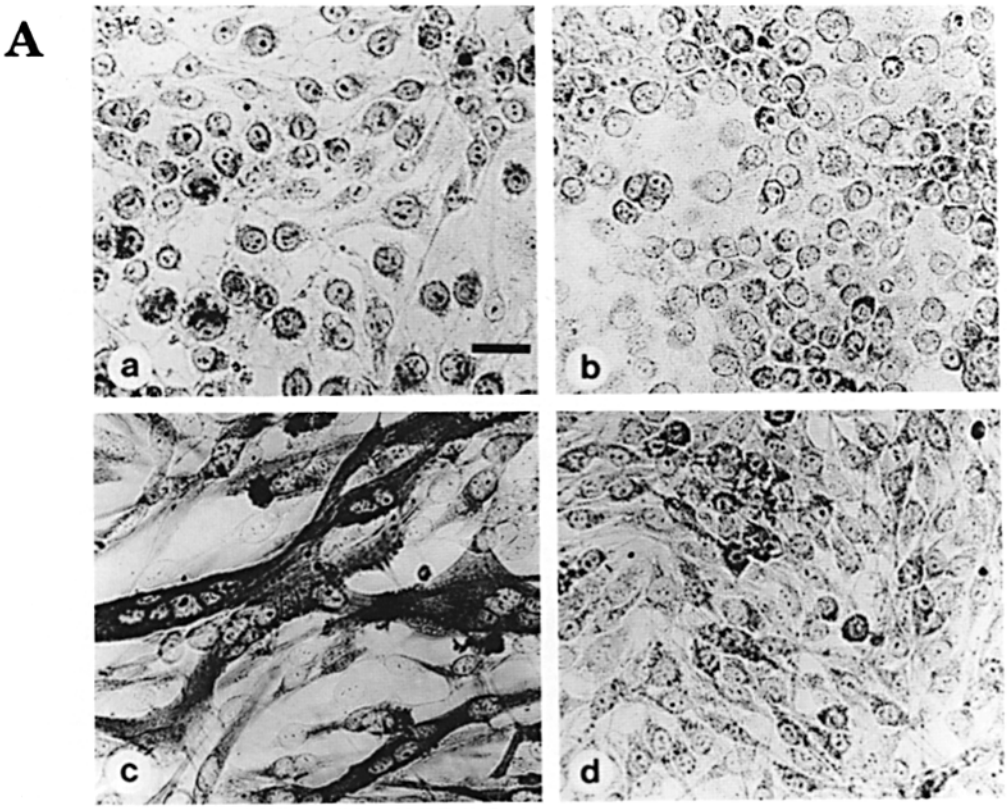
Immunohistochemical Staining of Culture Cells

Cells were seeded on microscopic slides and cultured under the described conditions: cells were fixed in methanol for 5 min followed by acetone treatment at −20°C for 10 s, and air dried. The mAb against rat myogenin, kindly provided by W. Wright (University of Texas, Dallas, TX) was applied for 30 min at room temperature in a moist chamber. Slides were rinsed in PBS solution and incubated with the antimouse IgG second antibody using the avidine-biotin complex and peroxidase staining for detection (Vectastain Kit; ABC, Burlingame, CA).

Results

MyoD1 Is Expressed Constitutively in the Inducible Rhabdomyosarcoma Cell Line BA-Han-1C

RA has been shown previously to induce terminal differentiation in rhabdomyosarcoma cells (Gerharz et al., 1989). In the two clonal cell subpopulations, BA-Han-1B and BA-Han-1C, isolated from a rat rhabdomyosarcoma, administration of RA resulted in a differential effect leading to myogenic differentiation in BA-Han-1C but not in Ba-Han-1B cells (Fig. 1 *A*). To examine the expression of the various genes encoding muscle-specific regulatory bHLH proteins in these two cell lines, we determined the mRNA levels using sequence-



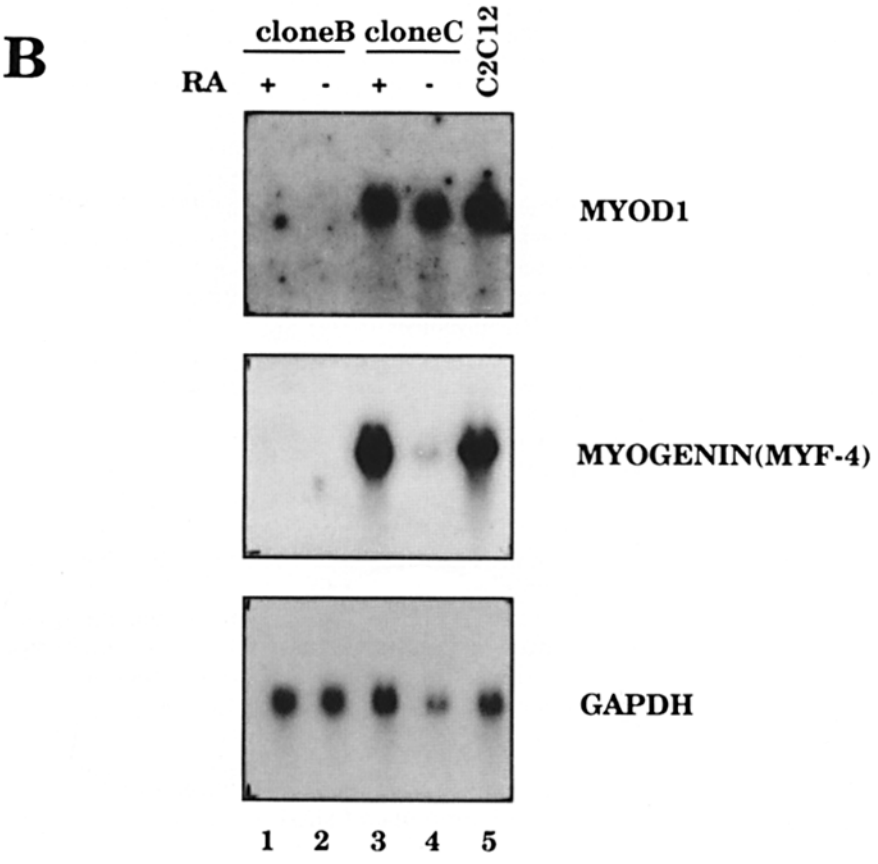


Figure 1. Expression of MyoD1 and myogenin mRNAs in the rhabdomyosarcoma cells BA-Han-1C but not in BA-Han-1B: Induction by retinoic acid. (*A*) Immunohistochemical staining of BA-Han-1B (*a* and *b*) and BA-Han-1C (*c* and *d*) cells with the mAb MF-20 directed against sarcomeric myosin heavy chain. Cells were cultured in the presence (*a* and *c*) and absence (*b* and *d*) of RA for 3 d. Myosin positive cells appear dark by peroxidase staining using the ABC Vectastain system. (*B*) Northern blot analysis of RNA (20 μg) isolated from BA-Han-1B (lanes *1* and *2*) and BA-Han-1C (lanes *3* and *4*) cells grown in the absence (−) or presence (+) of retinoic acid (RA). RNA from differentiated C2C12 myotubes was used as control (lane *5*). The blots were hybridized with mouse MyoD1 cDNA (MyoD1), rat myogenin cDNA (myogenin) and mouse GAPDH cDNA (GAPDH) as described under Materials and Methods. Approximately 2×10^6 cpm/ml of each probe (spec. activity: 1–3 $\times$ 10^8 cpm/μg) were used. Blots hybridized to MyoD1/myogenin and GAPDH were exposed on film for 48 h and 6 h, respectively. Bar, 70 μm.

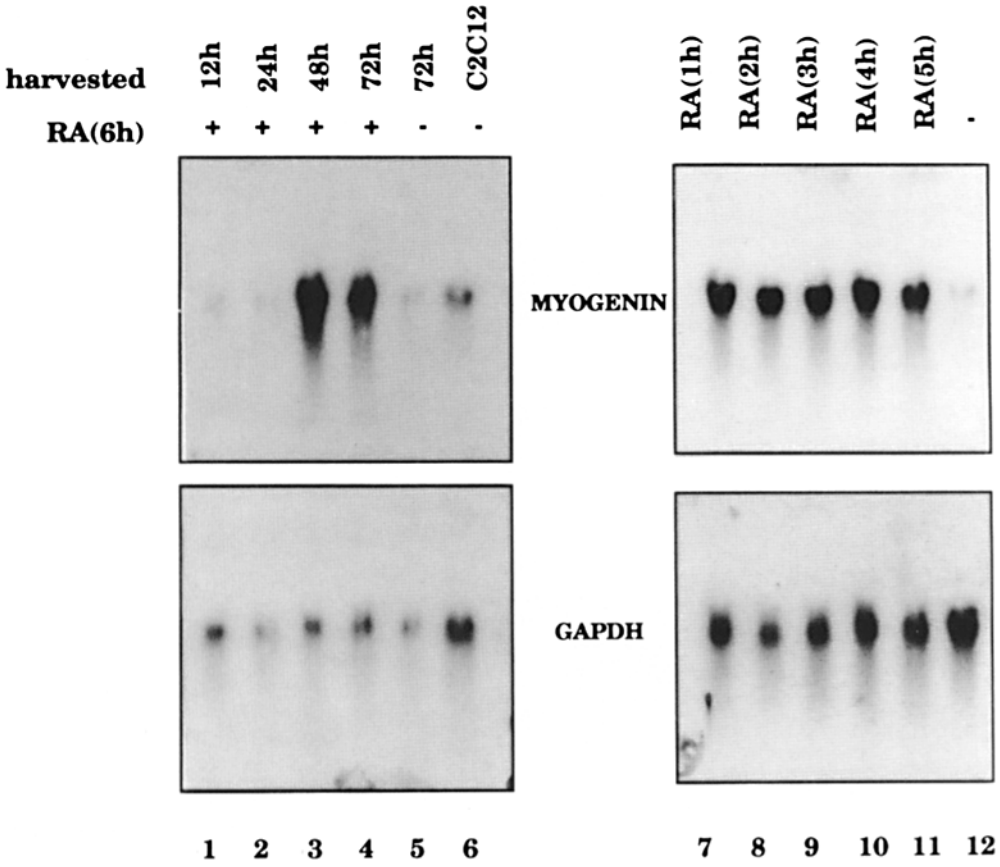


Figure 2. Time course of myogenin mRNA accumulation in BA-Han-1C cells treated with RA. RNA from BA-Han-1C cells treated for 6 h with RA was isolated at various times following RA removal (lanes *1–4*) or from cells growing for 72 h without RA (lane *5*). C2 cells were used as controls (lane *6*). In a parallel experiment RNA was isolated from cells cultured for 48 h following RA treatment for intervals of 1 to 5 h (lanes *7–11*) or from cells cultured for 72 h without RA (lane *12*). Hybridizations were performed with myogenin cDNA and mouse GAPDH cDNA to control RNA loading as described in the legend to Fig. 1.

specific hybridization probes for MyoD1, myogenin, Myf-5, and MRF4. Total cellular RNA isolated from both cell lines which have been cultured for 5 d in the absence or presence of RA, was analyzed on Northern blots. As shown in Fig. 1 *B*, in growing BA-Han-1C cells (clone C) MyoD1 mRNA accumulated to a level comparable to that observed in C2 myoblasts, regardless whether or not RA was present. In contrast, no MyoD1 mRNA was detected in BA-Han-1B cells (clone B). Myogenin mRNA was absent (Ba-Han-1B cells/clone B) or expressed at very low levels (BA-Han-1C cells/clone C) when cells were cultured in the absence of RA. Addition of RA to BA-Han-1C cells, however, resulted in a large increase of myogenin mRNA, whereas it had no effect on BA-Han-1B cells. This differential response to RA was not due to a different expression of retinoic acid receptors (RAR) in BA-Han-1B and BA-Han-1C cells as both lines have been shown to accumulate comparable levels of mRNAs encoding α and g RAR (Ramp et al., 1992). Myf-5 and MRF4 mRNAs were undetectable in both cell lines, in the absence and presence of RA (data not shown). These results indicate, in line with previous observations on several established myogenic cells, that the muscle forming capacity of BA-Han-1C cells is associated with the constitutive expression of MyoD1 and that expression of myogenin only starts at the onset of muscle cell differentiation. In contrast, BA-Han-1B cells which lack all of the myogenic control factors can not be induced to differentiate suggesting that the myogenic potential of rhabdomyosarcoma cells may be determined by the expression of MyoD1.

Activation of the Myogenin Gene Is a Late Response to Induction by Retinoic Acid

We have noticed that morphological differentiation of BA-Han-1C cells required at least 2 to 3 d after the addition of RA. To determine the precise time course of the accumulation of myogenin mRNA, total RNA isolated from cells grown in the presence of RA for 12, 24, 48, and 72 h was analyzed on Northern blots. We found consistently that myogenin mRNA started to accumulate 48 h after the induction by RA reaching levels similar to or exceeding those in differentiated C2 myotubes and persisting at nearly constant levels throughout the rest of the culturing period (Fig. 2, lanes *1–4* and *6*). Cells grown in 10% FBS or in 5% horse serum for up to 72 h did not activate myogenin expression above a marginal level (Fig. 2, lane *5*) and also failed to differentiate except for a minor proportion of cells which formed small myotubes (<1%). To define the time period during which RA was required in order to induce myogenin synthesis, cells were incubated in RA containing medium for intervals of 1 to 5 h after which they were refed with fresh medium without RA. After incubation for an additional 48 h, RNA was isolated and hybridized on Northern blots with the myogenin-specific probe. As shown in Fig. 2, myogenin mRNA accumulated to similar levels in all cultures treated with RA for 1 to 5 h indicating that the inducer was only required for a period of 1 h or less (Fig. 2, lanes *7–12*). Taken together, these results suggested to us that the activation of myogenin transcription may not be a direct effect of the RA receptor complex on the myogenin gene but may rather require additional intermediate processes.

The Induction of Myogenin Transcription by RA Is Dependent on Protein and DNA Synthesis

To test this idea, BA-Han-1C cells were induced with RA and treated with cycloheximide, an inhibitor of protein synthesis.

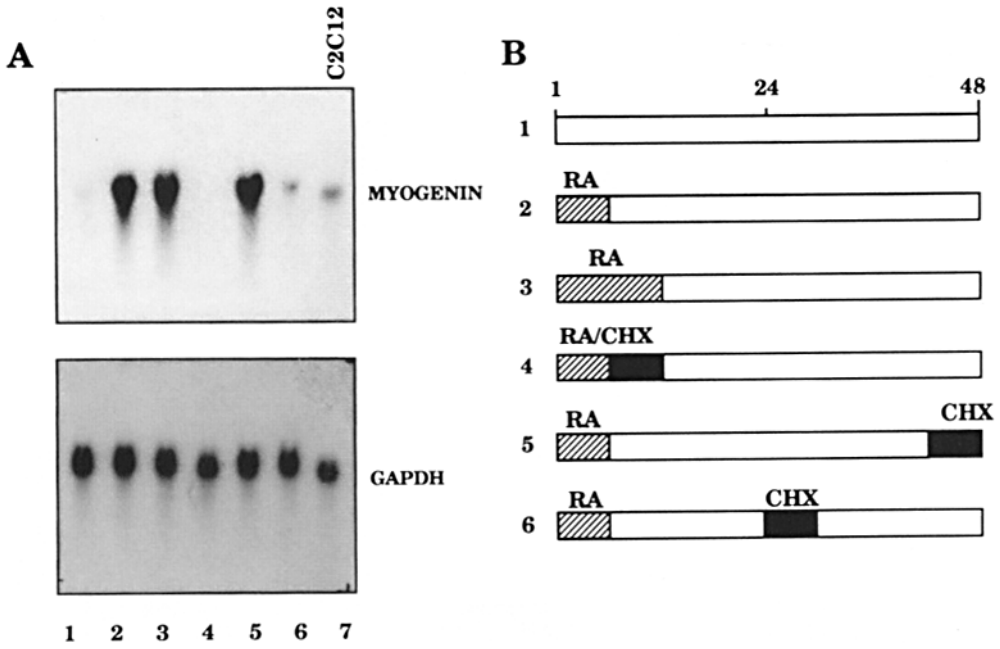


Figure 3. Accumulation of myogenin mRNA in BA-Han-1C cells is inhibited by cycloheximide. Northern blot analysis of uninduced and induced BA-Han-1C cells treated with cyclohexemide (*CHX*) at various times following RA addition (*A*). As illustrated in *B* cells were grown for 48 h either without RA (*1*), or with RA for 6 h (*2*), and 12 h (*3*), or with RA plus CHX for 6 h (*4*), with RA for 6 h and CHX added 42 h later (*5*) or with CHX added 24 h after RA (lane *6*). Lanes *1–6* in *A* correspond to numbers *1–6* in *B*.

Cycloheximide was added either simultaneously together with RA or at various times following the induction. RNA was isolated 48 h after the administration of RA. As shown in Fig. 3, inhibition of protein synthesis prevented the expression of myogenin mRNA when cycloheximide was present at any time during the first 30 h after treatment with RA. However, cyclohexemide had no effect when it was added 42 h after RA treatment. This result clearly indicated that in order to induce myogenin transcription with RA, active protein synthesis is required for at least 30 h. The fact that myogenin gene activation is dependent on the synthesis of additional proteins again argues against a direct interaction of the RA–receptor complex with the myogenin gene.

The onset of myogenin synthesis followed the induction by RA with a lag period of approximately 48 h. As it seemed unlikely that the synthesis of newly made protein would take two days, we wondered whether the activation of the myogenin gene may also require DNA replication. To test this proposition, cells were induced with RA and treated either with aphidicoline or hydroxyurea, both agents that inhibit DNA synthesis. As shown in Fig. 4 *A*, in the presence of either inhibitor, RA failed to induce transcription of myogenin mRNA whereas the accumulation of GAPDH mRNA used as an unrelated control was unaffected. This result indicated that induction of the myogenin gene in BA-Han-1C cells not only required protein synthesis but also DNA replication. As it is known that methylation of DNA may block the expression of genes and demethylation may occur during replication of DNA, we investigated the possible role of demethylation for the activation of the myogenin gene. Cells were pretreated with 5-azacytidine in three cycles for 9 d each. Individual colonies were picked after each round of azacytidine treatment and isolated DNA was digested with the isoschizomeric restriction endonucleases HpaII and MspI to ascertain the degree of hypomethylation (data not shown). The azacytidine treated cells containing maximally hypomethylated DNA were then tested for the induction of myogenin expression by RA. As shown in Fig. 4 *B*, myogenin mRNA accumulated 48 h after the addition of RA in a time pattern identical to the untreated control cells. If hypomethylation of the myogenin or any other gene had been a prerequisite for the induction of myogenin expression, one would have expected accelerated onset of transcription in cells that had been pretreated with azacytidine. However, our result suggests that methylation of DNA did not play a role for myogenin gene expression and therefore can not explain the requirement for DNA synthesis.

MyoD1 Present in BA-Han-1C Cells Fails to Transactivate Muscle-specific Reporter Genes

While BA-Han-1B cells did not express any of the myogenic determination factors (see Fig. 1) which provides a possible reason why these cells may not be induced for terminal differentiation, BA-Han-1C cells constitutively expressed MyoD1 but nevertheless almost completely failed to activate the myogenic differentiation program in the absence of RA. To analyze whether this was due solely to the inability of MyoD1 to transactivate muscle-specific genes or if additional factors might be missing, we examined the activation of reporter genes containing either complex muscle-specific enhancers or a minimal enhancer containing a tetrameric E-box (MyoD1-binding site). The CAT reporter plasmids, Myf4L-CAT containing the human myogenin promoter (Salminen et al., 1991), MCK4R-CAT containing a tetrameric MyoD1 binding site in front of the thymidine kinase TATA-box (Lassar et al., 1991), and MLC-CAT containing the muscle-specific enhancer of the rat myosin light chain gene (Rosenthal et al., 1990), were transiently transfected into BA-Han-1C cells and assayed for CAT activity 48 h later. The TK-CAT plasmid containing only the basal TK-promoter was used as non-muscle control. In parallel cultures, pEMSV-MyoD plasmid which expresses high levels of MyoD1 was cotransfected with each of the CAT-reporter constructs. In cells transfected with the reporter plasmids alone, none of the muscle-specific CAT genes was activated above a marginal background level indicating that the endogenous MyoD1 was unable to support their transcription (see Fig. 5). In contrast, cells cotransfected with the plasmid pEMSV-MyoD leading to forced expression of exogenous MyoD1 showed a

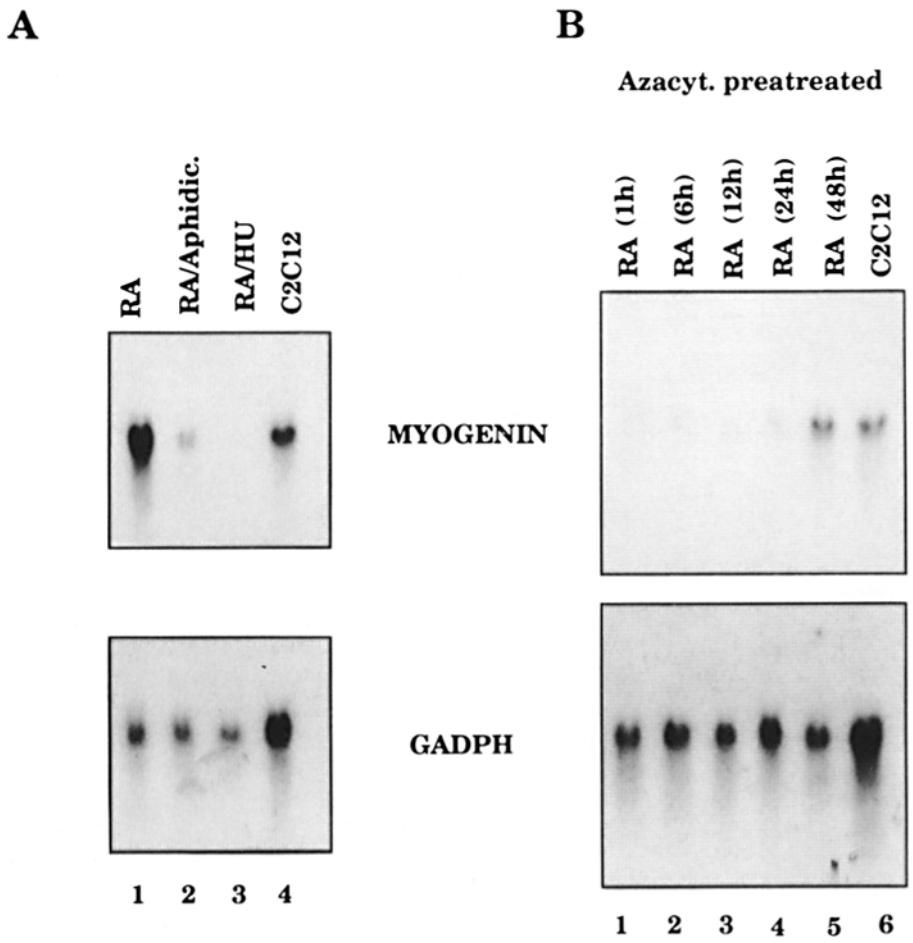


Figure 4. Inhibitors of DNA synthesis prevent RA-induced accumulation of myogenin mRNA; demethylation of DNA does not accelerate RA-induced transcription of myogenin. RNA from BA-Han-1C cells treated for 48 h with RA (lane *1*), RA plus aphidicoline (lane *2*), or RA plus hydroxyurea (lane *3*) was hybridized to myogenin and GAPDH-specific probes (*A*). RNA from BA-Han-1C cells pretreated with 5-azacytidine for three cycles (see text) and induced for differentiation by the addition of RA for 1, 6, 12, 24, and 48 h (*B*, lanes *1–5*) was hybridized to myogenin and GAPDH specific probes. RNA from differentiated C2 cells was used as control (lanes *A4* and *B6*).

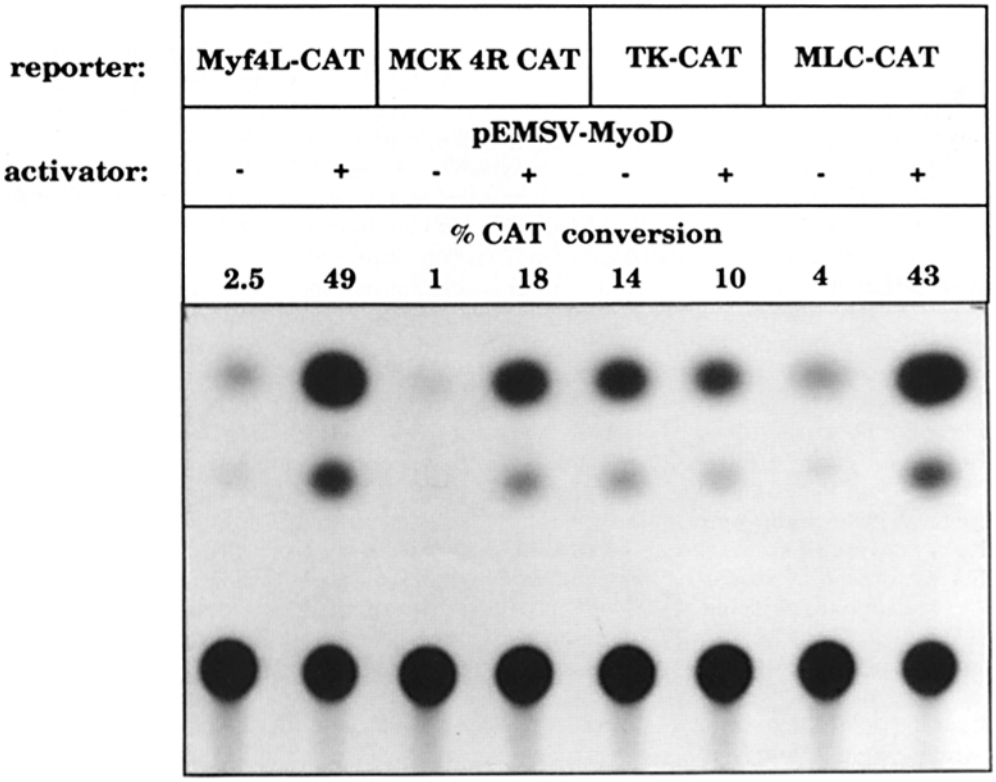


Figure 5. Transactivation of muscle-specific reporter genes by cotransfected pEMSV-MyoD in BA-Han-1C cells. The indicated reporter plasmids (5 μg) were transiently transfected into BA-Han-1C together with pEMSV-MyoD plasmid (5 μg) or pEMSV-α-scribe (5 μg) used as control. CAT activities were determined 2 d following transfection as described under Materials and Methods.

Table I. Acquisition of the Differentiated Muscle Phenotype in BA-Han-1B and BA-Han-1C Cells Stably Transfected with pEMSV-Myf4, or pEMSV-MyoD

Cells	Transfected plasmids*	Analyzed colonies‡	Differentiating colonies§	Percent of conversion
BA-Han-1C	pEMSV-MyoD	15	9	60
	pEMSV-Myf4	12	6	50
	pEMSV-α-scribe	10	0	0
BA-Han-1B	pEMSV-MyoD	12	0	0
	pEMSV-Myf4	12	0	0
	pEMSV-α-scribe	12	0	0

* All transfections were performed with 1 μg pSV2-neo plasmid and 20 μg pEMSV expression plasmids followed by selection of G418-resistant colonies.
‡ Randomly picked G418-resistant colonies were analyzed for spontaneous differentiation in normal growth medium.
§ Colonies showing MHC-positive cells detected by immunostaining with the monoclonal antibody MF20.

large increase of CAT activity driven by the muscle-specific promoters. The TK-CAT control plasmid showed moderate activity independent of additional MyoD expressed from the pEMSV-MyoD construct. It is worthwhile noting that during the time span of these transient experiments (2 d), addition of RA had no positive effect on the lack of muscle-specific transactivation nor did it enhance the activity of exogenous MyoD1 (data not shown). As endogenous MyoD1 was unable to transactivate even the simple MCK4R-CAT gene that presumably does not require additional factors, we interpret this result as evidence that MyoD1 apparently accumulates in BA-Han-1C cells in a biologically inactive form or alternatively is present at a concentration that is not sufficient to activate transcription. Elevated levels of MyoD1 alleviated the block of transcriptional activation, even in the absence of retinoic acid. It should be mentioned that BA-Han-1C cells stably transfected with pEMSV-MyoD also exhibited a large proportion of differentiating and myosin positive colonies (see Table I).

The Inhibitory HLH Protein Id Is Downregulated in Rhabdomyosarcoma Cells Treated with Retinoic Acid

A possible explanation for the inactive MyoD1 in BA-Han-1C cells could be the presence of an inhibitor. The negative muscle regulatory protein Id has been proposed to be involved in the control of MyoD activity in proliferating myoblasts (Benezra et al., 1990). Id is a HLH protein which lacks the basic region and therefore can not bind to DNA. It presumably prevents DNA binding of other HLH proteins as a competitive inhibitor since it effectively dimerizes with E12 and MyoD1 and thereby forms complexes which contain only one active half-site of the DNA-binding domain. The expression of Id is stimulated by serum and withdrawal of serum, for instance in myoblast cultures, results in its downregulation whereby the inhibitory effect on MyoD1 is released (Benezra et al., 1990). To test whether Id may play a regulatory role in the rhabdomyosarcoma cell lines, we analyzed Id mRNA levels following the induction by RA. As shown in Fig. 6, Id mRNA was present at moderate concentrations in both rhabdomyosarcoma cell lines and in normal L6 myoblasts when cultured in medium containing serum. Id mRNA continued to accumulate for 24 h after the addition of RA and then declined to almost undetectable levels at 48 h. The decrease of Id mRNA occurred independent of serum as cells were kept in medium containing 10% FCS throughout the experiment. This result showed that Id expression is downregulated by RA treatment in differentiating BA-Han-1C as well as in differentiation-defective BA-Han-1B cells and also in normal L6 myoblasts which do not differentiate under these conditions. Interestingly, the time course of Id mRNA repression correlated inversely to the induction of myogenin mRNA in BA-Han-1C cells (compare Fig. 1) but the functional significance of this correlation is rather unclear, since Id downregulation also occurred in non-differentiating cells which do not express myogenin mRNA.

Forced Expression of Myogenin in BA-Han-1C Cells Bypasses the Requirement of RA to Induce Terminal Differentiation

From the data presented above, it became evident that the inability of BA-Han-1C cells to differentiate in the absence of RA was associated with inactivation of the MyoD1 protein and the failure to express myogenin. RA-induced differentiation was always accompanied by the expression of myogenin. The functional relationship of these various observations, however, remained obscure. Moreover, it was unclear whether factors different from MyoD and myogenin may also contribute to the suppression of the differentiated phenotype. To address this problem, we examined whether constitutive expression of myogenin would be sufficient to induce terminal differentiation and to bypass the requirement for the inducer RA. BA-Han-1C were cotransfected with the plasmid pEMSV-Myf4 expressing the human myogenin (Myf-4) protein and pSV2-neo conferring geneticin (G418) resistance. Control cells were transfected with pSV2-neo plasmid alone. Colonies were selected in medium containing G418 and assayed for morphological differentiation and the expression of myogenin (Fig. 7) and myosin heavy chain (MHC) by immunostaining (data not shown). The same experiment was also performed in parallel with BA-Han-1B cells. Whereas the majority of G418-resistant BA-Han-1C cells transfected with pEMSV-Myf4 expressed myogenin and fused spontaneously to MHC^+ myotubes, <1% of the pSV2-neo transfected control cells showed myogenin expression and formed myotubes (Table I and Fig. 7, *c* and *e*). The extent of myogenin expression and myotube formation within the pEMSV-Myf4 transfected BA-Han-1C colonies was similar to that obtained in RA-treated cells but decreased successively with each serial passage of the cells. This phenomenon is most likely due to the selective loss of postmitotic myoblasts and differentiated myotubes (Fig. 7, *d* and *e*). In contrast, BA-Han-1B cells transfected with pEMSV-Myf4 completely failed to form myotubes, although 70–80% of the cells showed positive nuclear staining for myogenin (Table I, Fig. 7, *a* and *b*). This result then indicated that the inhibition of differentiation in BA-Han-1C cells could be obliterated by the constitutive expression of myogenin from a viral LTR promoter suggesting that no additional factors acting in parallel or downstream of myogenin may be required. Moreover, it showed that the block in BA-Han-1C cells was probably not due to a dominant-acting negative mechanism. In contrast, BA-Han-1B cells could not be rescued for the muscle phenotype by the expression of myogenin and therefore must be inhibited by another mechanism.

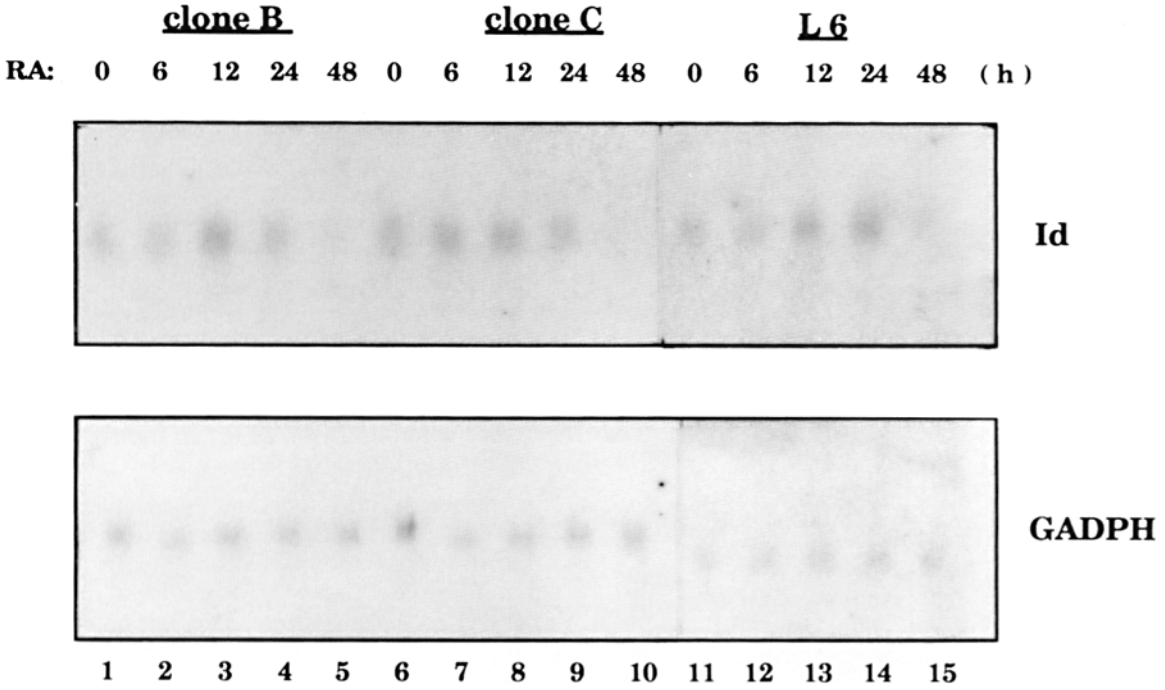


Figure 6. Id mRNA is down-regulated in rhabdomyosarcoma and L6 muscle cells following treatment with RA. Cells were treated with RA for the indicated time periods and RNA was isolated. Northern blots were hybridized to Id and GAPDH cDNAs using probes of comparable specific activity (1×10^8 cpm/μg). Exposure times for Id and GAPDH blots were 6 d and 6 h, respectively.

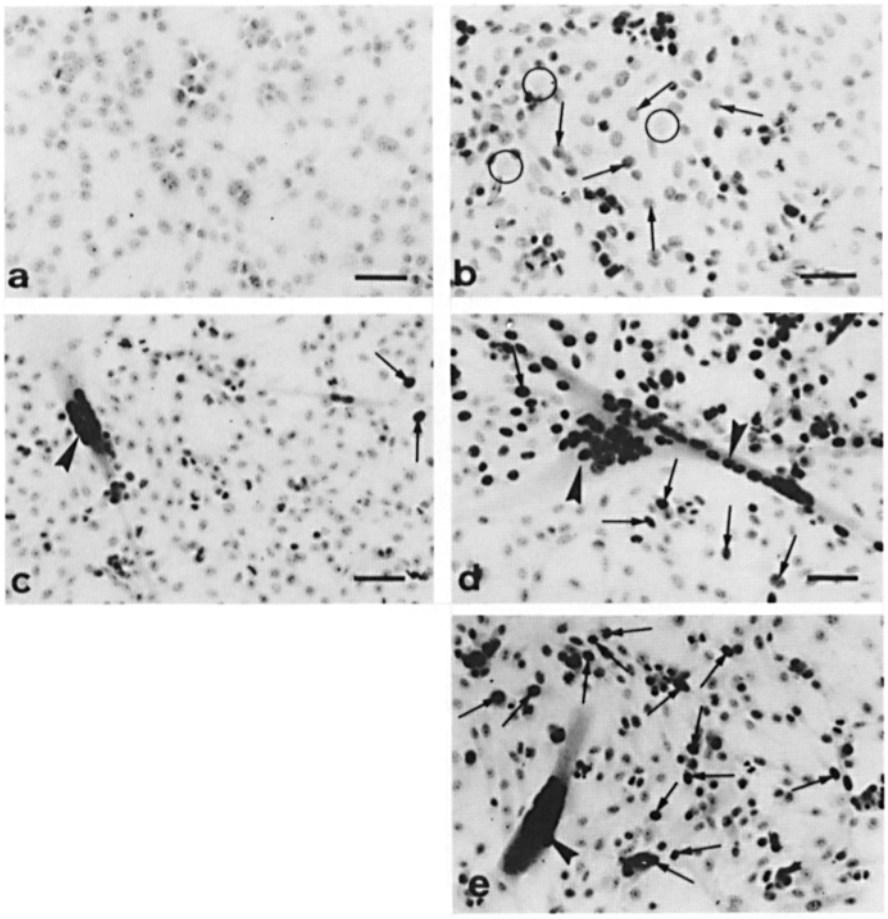


Figure 7. Muscle differentiation occurs in BA-Han-1C but not in BA-Han-1B cells stably transfected with pEMSV-Myf4. BA-Han-1B (*a*) and BA-Han-1C cells (*c*) stably transfected with pSV2-neo plasmid alone or stable colonies of both cell lines transfected with pEMSV-Myf4 (*BA-Han-1B, b*; *BA-Han-1C, e*) were immunostained with a mAb to myogenin (*Myf4*). BA-Han-1B cells show no expression of the nuclear antigen myogenin (*a*), whereas BA-Han-1C cells express myogenin in <1% of the cells, some in mononuclear (*arrows*) and some in myotubes (*arrowhead, c*). Approximately 80% of the pEMSV-Myf4 transfected cells (*b* and *d*) express myogenin (*arrows*), readily distinguishable from the translucent appearance of nuclei lacking myogenin (*circles*). Whereas myogenin expressing cells of the subpopulation BA-Han-1C (*e*) frequently form myotubes, none of the myogenin positive cells of subpopulation BA-Han-1B (*b*) fuse. The pEMSV-Myf4 transfected BA-Han-1C colonies (*e*) resemble the appearance of the BA-Han-1C cells grown in RA (*d*). Note that the particular colony shown here has already been passaged three times and therefore has lost a considerable number of myogenin positive cells. Bar, 50 um.

Discussion

The enhanced proliferative capacity of transformed cells is generally accompanied by a reduced capability to acquire the terminally differentiated phenotype. To begin to investigate possible mechanisms which may explain why tumor cells fail to differentiate, we have used two rhabdomyosarcoma cell lines BA-Han-1B and BA-Han-1C both of which have been isolated from the same dimethylbenzanthracene-induced rat tumor. Although both subpopulations express desmin, a generally accepted marker for myogenic cells, their myogenic potentials are vastly different. As we have shown here, BA-Han-1B cells did not express any of the four known myogenic factors and failed to form myotubes either spontaneously or upon induction with RA. In contrast, BA-Han-1C cells accumulated MyoD1 and showed low level of spontaneous differentiation that was greatly enhanced by RA. These results confirm numerous reports that the muscle phenotype is always associated with the expression of one or several myogenic control genes and absence of myogenic factors abrogates the ability to form muscle cells. Interestingly, many if not all human rhabdomyosarcoma tumors or cell lines derived thereof express also MyoD1 and/or one of the other related myogenic factors (Scrable et al., 1989; Hiti et al., 1989; Clark et al., 1991). Similar to nontransformed, established myogenic cell lines, BA-Han-1C tumor cells begin to accumulate myogenin at the onset of terminal differentiation. However, the activation of myogenin expression requires induction with RA, in contrast to normal muscle cells which start differentiation without any inducing agents when serum has been removed. In fact, BA-Han-1C cells seemed to have lost the responsiveness to the differentiation suppressing effect of serum as they readily formed myotubes in high serum concentrations when RA was also provided. Alternatively, RA may have induced factors that directly or indirectly interfere with signals elicited by serum components, such as growth factors and mitogens. In any case, the induction of muscle differentiation by RA seems to be unique for BA-Han-1C cells as various established myogenic cell lines and other rhabdomyosarcoma cells do not respond to RA with differentiation (H. H. Arnold, unpublished observations). Although we have not analyzed the immediate effects of RA in these cells, we have identified late results, such as the initiation of myogenin gene expression and the subsequent activation of the complete muscle differentiation program. Myogenin gene transcription in these cells was dependent on de novo protein synthesis and surprisingly also on DNA synthesis. It is presently unclear whether myogenin transcription in normal muscle cells also may require protein and DNA synthesis after serum withdrawal, although the relatively long time required for terminal differentiation of all established myogenic cell lines may suggest this. The importance of DNA-synthesis can not be explained by demethylation of DNA as we have shown that extensive pretreatment of the cells with azacytidine did not accelerate the kinetics of myogenin gene activation by RA. One can only speculate that alterations of the chromatin conformation which may occur during DNA replication could be a prerequisite for the activating effect by RA.

Inspection of ~1,100 bp of the 5'-upstream sequence of the human myogenin gene (Myf-4), which were sufficient to mediate muscle-specific and regulated expression (Salminen et al., 1991), revealed no sequence homology to a RA receptor-binding site. This observation supported the interpretation that RA does not activate transcription of the myogenin gene by direct interaction but rather leads to synthesis or activation of other transcription factors which then activate the myogenin gene. Our previous results and those of others (Braun et al., 1989*b*; Thayer et al., 1989) have shown that transfections of nonmuscle cells with plasmids expressing one myogenic factor led to the activation of the endogenous genes and the related genes encoding other members of the MyoD protein family. The auto- and cross-activation of the myogenin gene could also be demonstrated in transient transfection experiments using a CAT reporter construct driven by the Myf-4 promoter and expression vectors for MyoD or one of the related proteins serving as transactivators (Salminen et al., 1991). These experiments indicated that the muscle bHLH proteins directly or through transcription factors activated by them are involved in transcription of the myogenin gene. A potential candidate for an immediate activator is the MEF-2 transcription complex, as the myogenin promoter contains a MEF-2 binding site (Salminen et al., 1991; unpublished results) and the MEF-2 transcription complex was shown to be induced in nonmuscle cells by the expression of MyoD1 (Lassar et al., 1991). If MyoD1 directly or indirectly can activate transcription of the myogenin gene, why then was myogenin not expressed in BA-Han-1C cells?

In the present report, we have demonstrated that MyoD1 was expressed normally in BA-Han-1C rhabdomyosarcoma cells but it obviously lacked the biological activity to function as a muscle-specific transcription factor. It is conceivable that this inactivity is either due to posttranscriptional modifications of MyoD1 or to the presence of an inhibitor. Alternatively, the absence of auxillary factor(s) normally required for MyoD1 activity, could also be the cause. Although we have shown that the inhibitory HLH protein Id was expressed in rhabdomyosarcoma cells and its expression was down-regulated in RA treated cells, we find it unlikely that this contributes specifically to the differentiation block in BA-Han-1C cells for the following reasons: (*a*) Id is expressed to very low levels and its downregulation in the presence of RA does not correlate with differentiation in a positive way (Id mRNA also decreases in L6 and BA-Han-1B cells, nevertheless these cells do not differentiate under these conditions); (*b*) the Id expressing BA-Han-1C cells could be forced to transactivate muscle-specific genes by transfection of pEMSV-MyoD1 which expresses high levels of MyoD1. This indicates that Id is not sufficient to inactivate all E12-like HLH proteins that are required for MyoD to form DNA-binding complexes. The fact that overexpression of MyoD1 restored the muscle phenotype in BA-Han-1C cells also argues against a mechanism that depends on additional positive-acting factors which may be lacking but rather suggests that MyoD1 is probably affected by posttranscriptional modifications in these cells. We have not yet addressed the nature of these modifications, but phosphorylation is a plausible one, since MyoD1 is known to be a phosphoprotein (Tapscott et al., 1988).

Transfection of BA-Han-1C cells with plasmids constitutively expressing myogenin led to strongly increased spontaneous differentiation without induction by RA. Therefore we believe that no events downstream of myogenin expression

affect the differentiation capacity of these tumor cells. In fact, myogenin appears to serve as a switch to initiate terminal differentiation. Taken together, our results demonstrate that both inactivation of MyoD1 protein and the lack of myogenin expression may contribute to the nondifferentiating phenotype. Whether both events are functionally related remains to be seen.

Terminal differentiation of muscle cells has been shown to be inhibited by mutant-activated ras protein (Konieczny et al., 1989; Lassar et al., 1989; Schneider et al., 1987; Gossett et al., 1988; Olson et al., 1987; Payne et al., 1987) and by excess levels of fos (Lassar et al., 1989). Both oncogene products reduce transcription of the endogenous MyoD and myogenin genes. Constitutive expression of MyoD from a stably transfected cDNA can restore the muscle phenotype in cells expressing mutant ras and fos (Lassar et al., 1989). Since MyoD1 expression is not affected in BA-Han-1C rhabdomyosarcoma cells, it appears unlikely that ras and fos gene products are involved in the malignant transformation. Preliminary results on the expression pattern of these oncogenes are in agreement with this interpretation (unpublished observations). Expression of the oncogenic tyrosine kinases v-src and v-fps and transforming growth factor receptors (v-erbB) have also been shown to attenuate muscle differentiation (Falcone et al., 1985; Fiszmann and Fuchs, 1975; Holtzer et al., 1975). A temperature-sensitive v-src protein prevents differentiation of quail primary muscle cells without affecting the accumulation of MyoD1 transcripts (Falcone et al., 1991), similar to the situation observed in BA-Han-1C cells. This observation is interesting because it illustrates that some oncogenes may block differentiation without affecting the synthesis of MyoD protein but rather by interference with its activity. Similarly, forced expression of the nuclear oncogene product c-myc inhibits myogenic differentiation (Schneider et al., 1987; Denis et al., 1987) and the MyoD1 and myogenin-induced muscle conversion of 3T3 fibroblasts (Miner and Wold, 1991). This latter effect can not be overcome by constitutive and high-level expression of MyoD and myogenin, again suggesting a mechanism that inactivates functions of these proteins rather than interfering with their expression. We have recently shown that the transforming region of E1a prevents muscle differentiation and inhibits the transcription activating function of myogenic HLH control proteins but also the expression of myogenin (Braun et al., 1992). Enkemann et al. (1990) have also demonstrated that E1a downregulates the expression of MyoD1 and myogenin. None of the described phenotypes generated by these various oncogenes agrees completely with the phenotype of the BA-Han-1C cells. One can therefore speculate that other mechanisms act in these cells to inhibit differentiation. In this context it is interesting to mention that the bacterial pertussis toxin which interferes with the activity of inhibitory G-proteins can induce differentiation of BA-Han-1C cells (A. Salminen and H. H. Arnold, unpublished observations) suggesting that G_i-protein-dependent signal transduction pathways may be involved. We are currently investigating this possibility. The fact that BA-Han-1B cells never acquire the differentiated muscle phenotype, although they do express desmin, and MCK (Gerharz et al., 1989) argues for a completely different and maybe more complex mode by which these cells have been transformed.

We thank W. Wright, Dallas for the mAb against rat myogenin, H. Eberhardt for excellent technical assistance and A. Broecker-Nagel for skillful secretarial help with the manuscript.

This work was supported by grants from Deutsche Forschungsgemeinschaft, Deutsche Muskelschwundhilfe e.V., and the European Community to H. H. Arnold.

Received for publication 17 February 1992 and in revised form 22 April 1992.

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