Regulation of a Muscle-specific Transgene by Retinoic Acid

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Abstract. Retinoic acid (RA) has been shown to have variable effects on myogenic differentiation in cell culture. The application of RA on primary cultures of embryonic somites, limb buds, and neonatal limbs inhibited myogenic differentiation in a dose-dependent way as indicated by the repression of: (*a*) myotube formation, (*b*) myosin heavy chain protein accumulation, (*c*) myosin light chain (MLC) 1/3, α_{sk}-actin and myogenic factor transcript expression. Expression of retinoic acid receptors (RAR) was also affected by RA treatment, specifically RARγ transcripts were induced. To further understand the pleiotropic action of RA on myogenesis, we took advantage of two muscle-specific transgene markers which consisted of CAT reporter genes driven by regulatory elements either from the myosin light chain 1/3 locus (MLC-CAT) or the α-skeletal actin gene (α_{sk} actin-CAT). RA inhibited MLC-CAT transgene but not α_{sk} actin-CAT transgene expression in primary cultures from these mice. Analysis of MLC-CAT expression in transgenic mouse primary cultures and in stably transfected C_2C_{12} cells demonstrated that repression of MLC-CAT activity by RA was dependent upon diffusible factors in chick embryo extract. We hypothesize that during development, the pleiotropic effects of RA on myogenesis do not depend solely on the distribution and concentration of RA itself, but are also influenced by extracellular signals in the embryonic environment.

D URING development the actions of signaling molecules are required to instruct individual cells when and where to differentiate into their ultimate tissue type. Retinoic acid (RA)[1] has long been known to affect pattern formation in vertebrate embryos (reviewed in Summerbell and Maden, 1990). RA treatment causes perturbation of segment identity along the anteroposterior (AP) body axis (Kessel and Gruss, 1991; Kessel, 1992) and alteration of limb morphogenesis (Tickle et al., 1982). Recent evidence has indicated that Henson's node, a pattern-organizing region at the anterior end of the primitive streak in early embryos (Waddington, 1932; Beddington, 1994) may be an endogenous source of RA (Hogan et al., 1992).

Given the dramatic phenotypic effects of RA on developing embryos, it has been of interest to investigate the nature of its action on specific cell types, such as skeletal muscle precursors. RA induces myogenic differentiation of a rat rhabdomyosarcoma-derived cell line, presumably through induction of myogenin (Arnold et al., 1992). RA has also been reported to induce myogenic differentiation of C_2 myoblast cells and primary adult chicken satellite cells (Albagli-Curiel et al., 1993; Halevy and Lerman, 1993). However, depending on its concentration RA has been shown to either activate or repress myogenesis in both craniofacial mesenchyme (Langille et al., 1989) and in limb buds in early chick development (Momoi et al., 1992).

To further understand the pleiotropic effects of RA on myogenesis and the mechanism underlying its action, we took advantage of two lines of transgenic mice, carrying the chloramphenicol acetyl transferase (CAT) reporter gene under the control of the regulatory elements either from the rat myosin light chain (MLC) 1/3 or chicken α_{sk} actin locus. Both transgenes (MLC-CAT and α_{sk} actin-CAT) are expressed in a strict muscle-specific pattern in mice (Rosenthal et al., 1989; Petropoulos et al., 1989), which reflects the control of the endogenous loci in vivo (Sassoon et al., 1988; Lyons et al., 1990) and in tissue culture (Donoghue et al., 1988; Wentworth et al., 1991; Bergsma et al., 1986). However, the regulatory elements included in the transgenes do not appear to be controlled by the same pathways in cultured muscle cells: activity of MLC-CAT constructs is dependent upon multiple myogenic factor binding sites (E boxes) in the MLC enhancer (Rosenthal et al., 1990; Wentworth et al., 1991) whereas mutation of the single E box in the promoter of the α_{sk} actin-CAT construct has no effect on promoter activity (Chow and Schwartz, 1990).

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1. *Abbreviations used in this paper.* AP, anteroposterior; CAT, chloramphenicol acetyl transferase; CEE, chick embryo extract; dpc, days post coitum; MHC, myosin heavy chain; MLC, myosin light chain; RA, retinoic acid; RARE, RA response element.

The present study documents the effect of RA on primary cultures of limb buds, neonatal limbs, and somites from MLC-CAT and α_{sk} actin-CAT mice. RA repressed myogenic differentiation in a dose-dependent way, and inhibited myogenic factor gene expression. In contrast to the MLC-CAT transgene, α_{sk} actin-CAT transgene expression was not inhibited by RA in these primary cultures, presumably because the activation of the α_{sk} actin-CAT construct is independent of myogenic factors. Interestingly, RARγ transcripts were significantly induced by RA treatment. Further, repression of muscle-specific genes by RA was dependent on culture conditions. Taken together, these results suggest that the pleiotropic action of RA on myogenesis depends not only on the distribution and concentration of RA itself, but is also significantly influenced by diffusible molecules.

Materials and Methods

Transgenic Mouse Lines

The MLC-CAT transgenic mouse line used in this study (line 7), carrying multiple copies of a 1200-bp rat MLC1 promoter-CAT transgene driven by the downstream 920-bp MLC enhancer, was generated and characterized as described previously (Donoghue et al., 1988; Rosenthal et al., 1989). Line 7 was bred to transgenic homozygosity (Grieshammer et al., 1992). Crosses with wild-type animals yielded heterozygous embryos and neonates for dissociated primary cultures. The α_{sk} actin-CAT transgenic mouse line was generated with a construct containing 220 bp of the regulatory elements from the chicken α skeletal actin gene (originally characterized by C. Ordahl, personal communication) driving a CAT reporter gene (Petropoulos et al., 1989).

Preparation of Chick Embryo Extract (CEE)

Chick embryo extract was prepared according to established protocols (J. Powell, personal communication) as follows: embryos from chicken eggs incubated for 9 d (purchased from SPAFAS) were added to an equal volume of Hanks balanced solution (GIBCO BRL, Gaithersburg, MD). The mixture was forced through a 10-ml syringe twice before freezing at −20°C for at least one day. Upon thawing, the mixture was repeatedly passed through pipettes of progressively smaller size until it could be easily passed through a Pasteur pipette. After centrifugation at 1,200 g for 30 min, the supernatant (chick embryo extract [CEE]) was divided into 1-ml aliquots and frozen. To prepare medium, frozen aliquots were thawed and spun for 1 min at full speed in a microcentrifuge. The supernatant was mixed with DME and passed through 0.22-μm filter units. Filter-sterilized DME with CEE was then mixed with other components to a final concentration of 2% CEE.

Primary Cultures

Somite dissections and cultures were described previously (Grieshammer et al., 1992). Limb buds were removed from wild-type, MLC-CAT, or α_{sk} actin-CAT transgenic embryos at 11.5, 12.5, and 13.5 dpc, dissociated by passing through a flame-polished Pasteur pipette, and plated onto 35-mm gelatinized (1% gelatin) Primaria tissue culture dishes (Falcon Plastics, Cockeysville, MD), one dish per pair of limb buds. Neonatal hind legs from wild-type, MLC-CAT, or α_{sk} actin-CAT transgenic mice were skinned and boned, and the dissected muscles were incubated in 0.125% trypsin/0.25% pancreatin in Hanks balanced solution for a total of 12 min, with occasional pipetting during incubation. Cells from the dissociated muscles were preplated on a Falcon petri dish for 1 h to eliminate fibroblasts and thereby enrich the myogenic cell population. The cells were subsequently plated onto 35-mm gelatinized (1% gelatin) Primaria tissue culture dishes, one dish per pair of hind legs. The dissociated cells were cultured in DME with 10% FBS (Sigma Immunochemicals), 10% horse serum (Sigma), 2% chick embryo extract, 2 mM glutamine, 5 U/ml penicillin G, and 5 mg/ml streptomycin sulfate at 37°C, 5% CO_2. The cultures exhibited very limited growth subsequent to plating. Retinoic acid (Fluka

Chemie AG, Buchs, Switzerland, in 100% ethanol) was added to the medium at designated days of culture, resulting in a final concentration of 10^{-7} M or 10^{-5} M. On day 4 (somite and limb bud culture) or day 6 (leg muscle culture) after plating, cells were fixed for histochemical staining or harvested for CAT assays. Neonatal leg muscle cells used for RNA preparations were cultured similarly as described above, but plated onto 60-mm plates to obtain a sufficient number of cells for RNA extraction.

C_2C_{12} Cell Culture, Transfections and CAT Assays

The C_2C_{12} cell line (Blau et al., 1983) is a subclone of the mouse skeletal muscle cell line C_2 (Yaffe and Saxe, 1977). C_2C_{12} myoblasts were cultured in growth medium (DME, with 20% FCS, 2 mM glutamine, 5 U/ml penicillin G, and 5 mg/ml streptomycin sulfate).To obtain a muscle cell line that stably expressed the MLC-CAT gene, C_2C_{12} cells were plated at 2×10^5 cells/100-mm tissue culture plate one day before cotransfection with pMLC1CAT920 (Rosenthal et al., 1989) and pSV2neo constructs, using DNA-calcium phosphate coprecipitation (Gorman, 1985). Transfected pooled clones were selected in G418 and tested for MLC-CAT reporter activity by CAT assays of differentiated cultures as follows: cells resistant to G418 were seeded at similar densities (2×10^5 cells/100-mm tissue culture plates or 2×10^4 cells/35-mm tissue culture plates) and fed every other day with growth medium or the medium specifically defined in the Results section. Upon confluency, which occurred three days after plating at the above density, the growth medium was replaced with differentiation medium (substitution of 2% horse serum for 20% FBS). The cells were fully differentiated within 48 h after the switch in medium, at which point they were harvested and CAT assays were performed as described previously, normalized to total protein content (Grieshammer et al., 1992).

C_2C_{12}-primary Cell Cocultures

Transwell cell culture chamber inserts (Costar) were used for the coculture experiments. Primary cells from wild-type neonatal hind leg muscles were plated in the upper insert chamber, and C_2C_{12} clones stably transfected with an MLC-CAT construct (see above) were plated in the lower culture plate. The two cell cultures were separated by a collagen-treated membrane with 3.0-μm pores. Culture medium was the same as that used for the primary cultures (see above). Differentiation of C_2C_{12} cells and CAT assays were carried out as described above.

RNA Isolation and Northern Analysis

Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform single-step method (Chomczynski and Sacchi, 1987). RNA samples were electrophoretically separated on a formaldehyde agarose gel and transferred to Amersham Hybond-N nylon membrane (Sambrook et al., 1989). The GAPDH cDNA clone was purchased from the American Type Culture Collection (Rockville, MD). Other cDNA probes used included: mouse MLC 1/3 (Lyons et al., 1990), MyoD (Sassoon et al., 1989), myogenin (Sassoon et al., 1989), human RARα (Petkovich et al., 1987), human RARβ (Brand et al., 1988), human RARγ (Krust et al., 1989), and chicken α skeletal actin (Petropoulos et al., 1989). All cDNA fragments were labeled with [α^{32}P]dCTP (DuPont-NEN, Boston, MA) using the multiprime labeling kit from Amersham. Hybridization was carried out in 5× SSC, 50% formamide, 1× Denhardt's, and 0.1% SDS at 42°C overnight after prehybridization with 20 μg/ml herring sperm DNA for 2 h. Blots were sequentially washed under standard conditions (Sambrook et al., 1989). Blots were exposed to Amersham hyperfilm-MP at −70°C with an enhancing screen for 2 h to 1 wk. Autoradiograms were quantitated by densitometry, using a computerized densitometer (Molecular Dynamics, Inc., Sunnyvale, CA).

Histochemistry

Cells were immunohistochemically stained for MHC protein using a Vectastain ABC Kit (Vector Laboratories, Inc.). Cells were washed three times with PBS and incubated with 0.1% Triton in PBS for exactly 1 min. Cells were then fixed on the plates by a 20-min incubation with 100% methanol. After two washes with PBS containing 0.1% BSA, nonspecific proteins were blocked by incubation with normal horse serum for 3 min. Anti-MHC antibody MF20 or F59 (diluted with PBS/0.1% BSA) was then added onto the plates and incubated at 4°C overnight. Subsequent incubation with biotinylated secondary antibody followed by incubation with Vectastain ABC reagent (a preformed avidin and biotinylated horserad-

ish peroxidase macromolecular complex) was carried out according to the standard procedures for the kit. Finally, substrate solution made from DAB tablets (Sigma) following manufacturer's instructions was used to stain the plates for 2-7 min. The degree of staining was recorded with microscopic photography.

Results

Differentiation of Primary Muscle Cell Cultures Is Inhibited by RA

Previous studies using various culture systems to test the effects of RA on myogenesis have yielded inconsistent results. Specifically, the inhibition of myogenesis by relatively high concentrations of RA in the chicken embryo craniofacial mesenchyme (Langille et al., 1989) and chicken limb bud (Momoi et al., 1992) culture systems are in contrast with the induction of myogenic differentiation by similar concentrations of RA reported for the C_2 myoblast cell line (Halevy and Lerman, 1993; Albagli-Curiel et al., 1993) and a rhabdomyosarcoma-derived cell line (Arnold et al., 1992). The concentration of RA used in these studies ranged from 10^{-10}–10^{-6}M, and the RA concentration used to induce differentiation of other malignant cell lines, such as murine melanoma cells, was as high as 10^{-5}M (Niles, 1987). Although variations in RA concentration may be responsible for some of the variability in these results, the data relating RA concentration to myogenic induction or inhibition is still far from conclusive.

The pleiotropic effects of RA on myogenesis were further examined by treating primary dissociated cultures of 13.5 dpc mouse limb buds and neonatal hind leg muscles with 10^{-7} and 10^{-5}M RA. The neonatal cultures were preplated to eliminate fibroblasts and, thereby to enrich for myoblasts. The remaining cells underwent very limited growth subsequent to dissociation. Myogenic differentiation was inhibited at both concentrations of RA tested. In primary cultures treated with 10^{-5}M RA very few fused myotubes were present, whereas untreated cultures contained many myotubes (see Fig. 1). Since both concentra-

tions of RA significantly inhibited myogenic differentiation in the primary cultures but 10^{-5}M RA produced a more dramatic effect, we chose to perform most of the subsequent primary culture experiments at the higher concentration, with selected duplicate experiments at lower concentrations (10^{-7} or 10^{-8}M RA).

As a measure of RA-induced myogenic inhibition, the expression of a muscle-specific structural protein, myosin heavy chain (MHC), was tested by staining the cultures described above with anti-MHC antibodies. Fig. 1 shows a dramatic reduction in MHC staining in the 10^{-5}M RA treated cultures. Treatment with 10^{-7}M RA yielded similar results (data not shown). The reduction in MHC-positive cells in the RA-treated cultures was not due to selective apoptosis, as determined by analysis of DNA ladders in genomic DNA from untreated and treated cultures (data not shown). Thus, RA inhibits both morphological and biochemical differentiation in primary muscle cultures.

Muscle-specific Transcript Accumulation Is Repressed by RA in Primary Muscle Cultures

To investigate the potential mechanism underlying the RA-mediated inhibition of myogenesis, we studied the effects of RA treatment on accumulation of transcripts encoding muscle-specific structural proteins. MLC 1 and 3 are encoded in a single locus and are expressed in a differentiation-dependent manner (reviewed in Rosenthal et al., 1992). In primary muscle cultures prepared from dissociated neonatal mouse hind legs, MLC1/3 transcripts were reduced about 10-fold upon treatment with 10^{-5}M RA (Fig. 2, a and b). Since MLC expression is under the control of myogenic factors (Rosenthal et al., 1990; Wentworth et al., 1991) we examined whether the relative levels of myogenic factor transcripts were changed in these cultures. MyoD and myogenin transcripts were reduced 5- to 10-fold by RA treatment (Fig. 2, a and b), whereas transcripts encoding Myf5 or MRF4 were not detected in ei-
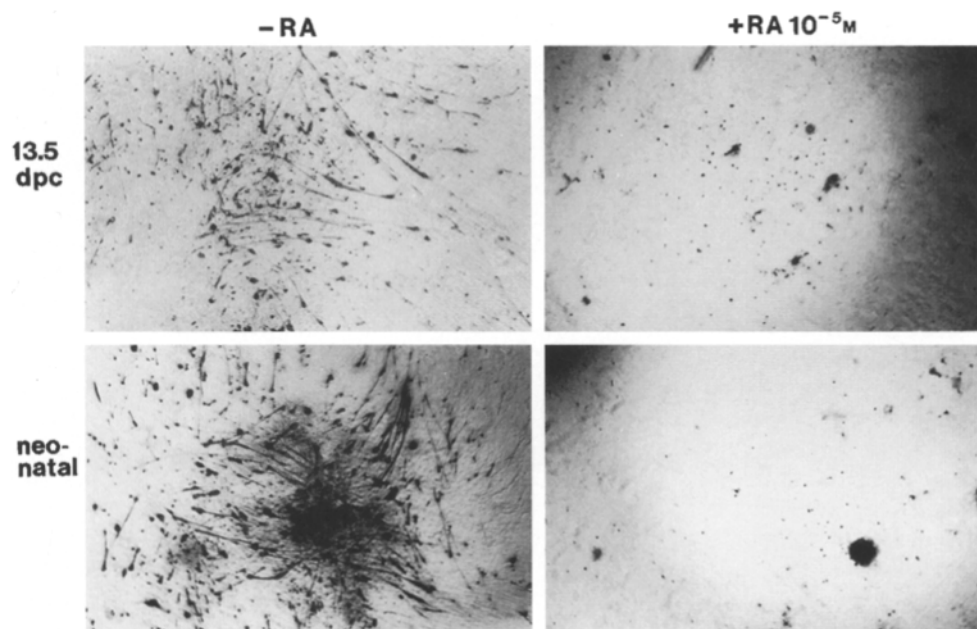

Figure 1. Inhibition of myosin heavy chain protein accumulation by RA treatment of primary muscle cultures. 13.5 dpc limb buds and newborn leg muscles were dissociated and cultured $\pm 10^{-5}$M RA. Cultures were immunohistochemically stained for myosin heavy chain using anti-MHC antibodies F59 (13.5 dpc) or MF20 (neonatal).
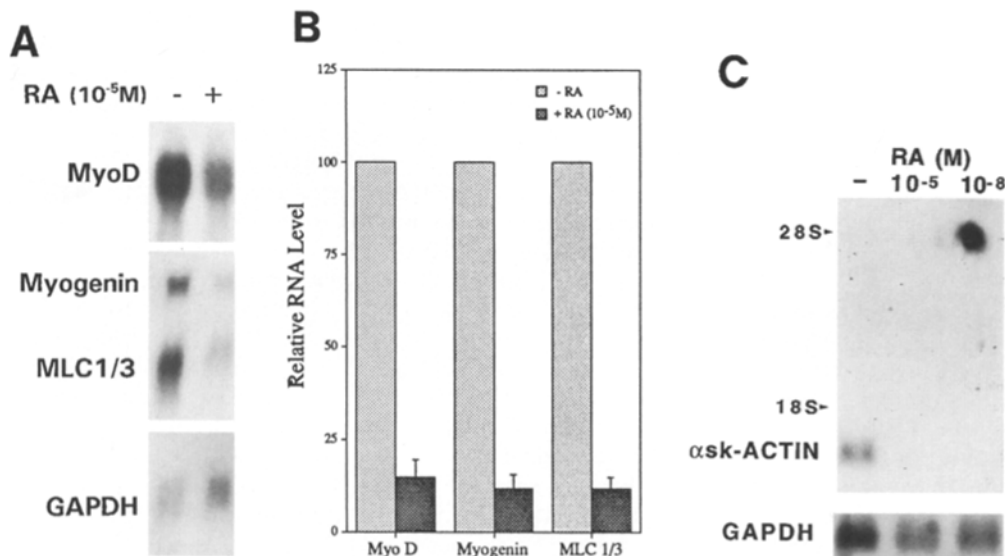
A

RA $(10^{-5}M)$ − +

MyoD

Myogenin

MLC1/3

GAPDH

B

Relative RNA Level

☐ - RA
▨ + RA $(10^{-5}M)$

125
100
75
50
25
0

MyoD   Myogenin   MLC 1/3

C

RA (M)
− 10^{-5} 10^{-8}

28 S►

18 S►

αsk-ACTIN

GAPDH

Figure 2. Reduction of muscle-specific transcripts in RA treated primary cultures. RNA was isolated from neonatal leg muscles dissociated and cultured in the presence or absence of RA and analyzed by Northern blots using probes as indicated. A GAPDH probe was included to control for equal RNA loading. (*a*) Northern analysis of MyoD, myogenin and MLC1/3 transcripts ± $10^{-5}M$ RA. Blots were also probed for Myf5 and MRF4 but no signal was detected (data not shown). (*b*) Histogram showing the relative intensity of the autoradiographic bands in *a*. Densitometric scans of the autoradiographs were normalized to the values obtained for GAPDH. All data points are averages of three independent experiments (with standard error). Data are presented as percentages of untreated controls which were arbitrarily set at 100. (*c*) Northern analysis of α_{sk} actin transcripts ± $10^{-8}M$ or $10^{-5}M$ RA. A GAPDH probe was included to control for equal RNA loading.

ther untreated or treated cultures (data not shown). It is therefore likely that under these conditions, the reduction in MLC transcripts is mediated by a concomitant reduction in MyoD and/or myogenin.

We next examined the effects of RA treatment on the expression of α_{sk} actin, another structural gene whose activation in skeletal muscle is under the control of a combination of positive and negative factors, distinct from the MyoD family of myogenic regulators (Chow and Schwartz, 1990). As seen in Fig. 2 *c*, α_{sk} actin transcripts were dramatically reduced in the presence of either 10^{-8} or $10^{-5}M$ RA. Thus it appears that multiple components of the myogenic program are affected by RA treatment.

RA Inhibits Expression of the MLC-CAT Transgene but Not the α_{sk} Actin-CAT Transgene

To investigate the possibility that RA affects the expression of muscle-specific structural genes at the transcriptional level, we measured the effect of RA on the expression of a transgene which is controlled by DNA regulatory elements known to be responsive to myogenic differentiation. Transgenic mouse lines have previously been generated which contain promoter and enhancer elements from the MLC1/3 locus driving the CAT gene as a reporter (MLC-CAT; Fig. 3 *a*). The expression of this transgene is muscle-specific in the adult and during embryogenesis (Rosenthal et al., 1989; Grieshammer et al., 1992). Embryonic limb buds (11.5, 12.5, and 13.5 dpc) or neonatal hind leg muscles from MLC-CAT transgenic mice were dissociated and cultured with or without $10^{-5}M$ RA. As shown in Fig. 4, MLC-CAT activity was consistently reduced 4- to 15-fold in RA-treated cultures from different developmental stages as compared to untreated cultures. Since the enhancer in the MLC-CAT reporter construct contains myogenic factor binding sites, which are essential for its transcriptional activity in tissue culture (Rosenthal et al., 1990; Wentworth et al., 1991), it seems likely that the RA-
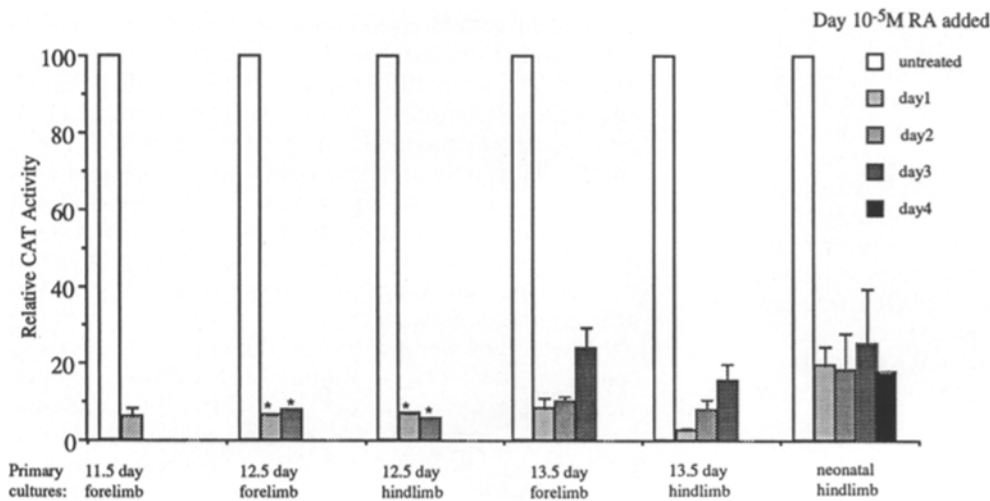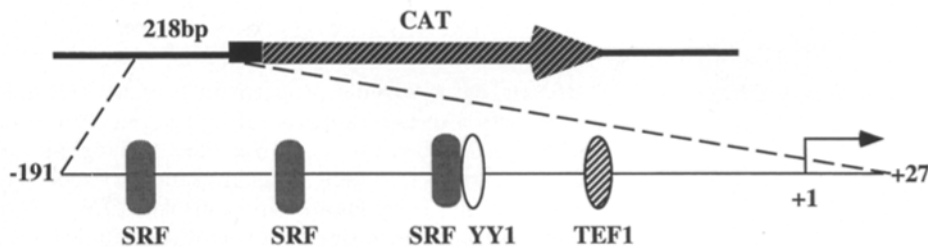
mediated inhibition of transgenic MLC-CAT activity and of endogenous MLC 1/3 expression occurs indirectly in response to a reduction of myogenic factors (Fig. 2, *a* and *b*).

In embryonic somites, the MLC-CAT transgene is expressed in a gradient along the rostrocaudal axis of the embryo: low levels of CAT activity are present in the most mature rostral somites, with increasing CAT activity in more caudal somites as they progressively mature. Graded transgene expression is retained in dissociated somite cultures (Grieshamer et al., 1992, Fig. 5). When 11.5 dpc somites from various rostrocaudal positions were dissociated and cultured with RA ($10^{-5}M$ or $5 \times 10^{-7}M$), a decrease in CAT activity was found at both concentrations when compared to untreated cultures (Fig. 5). These results show that RA inhibits myogenesis in all developing somites, independent of their maturity status, and that RA treatment does not change the graded CAT expression profile in these tissues.

In contrast to the *cis*-acting DNA sequences used for the generation of the MLC-CAT transgene, muscle-specific regulatory elements isolated from the 5' end of the α_{sk} actin gene (Fig. 3 *b*) do not depend on myogenic factor binding sites for their activity in muscle tissue culture (Chow and Schwartz, 1990). Since the endogenous gene is responsive to RA (Fig. 2 *c*), we examined the regulation of a transgene containing a CAT gene reporter driven by a 220-bp α_{sk} actin promoter fragment (Petropoulos et al., 1989). Primary cultures were prepared from dissociated α_{sk} actin-CAT transgenic limb buds (12.5 and 13.5 dpc) or neonatal hind leg muscle, and maintained with or without $10^{-5}M$ RA. In contrast to the response of the endogenous α_{sk} actin locus, expression of the α_{sk} actin-CAT transgene was not inhibited but rather was induced up to threefold by RA at all developmental stages (Fig. 6). Thus it appears that the 220-bp actin promoter fragment used in the transgenic mice lacks regulatory elements present elsewhere in the endogenous α_{sk} actin gene locus that mediate inhibition of actin expression by RA.

A MLC-CAT transgene
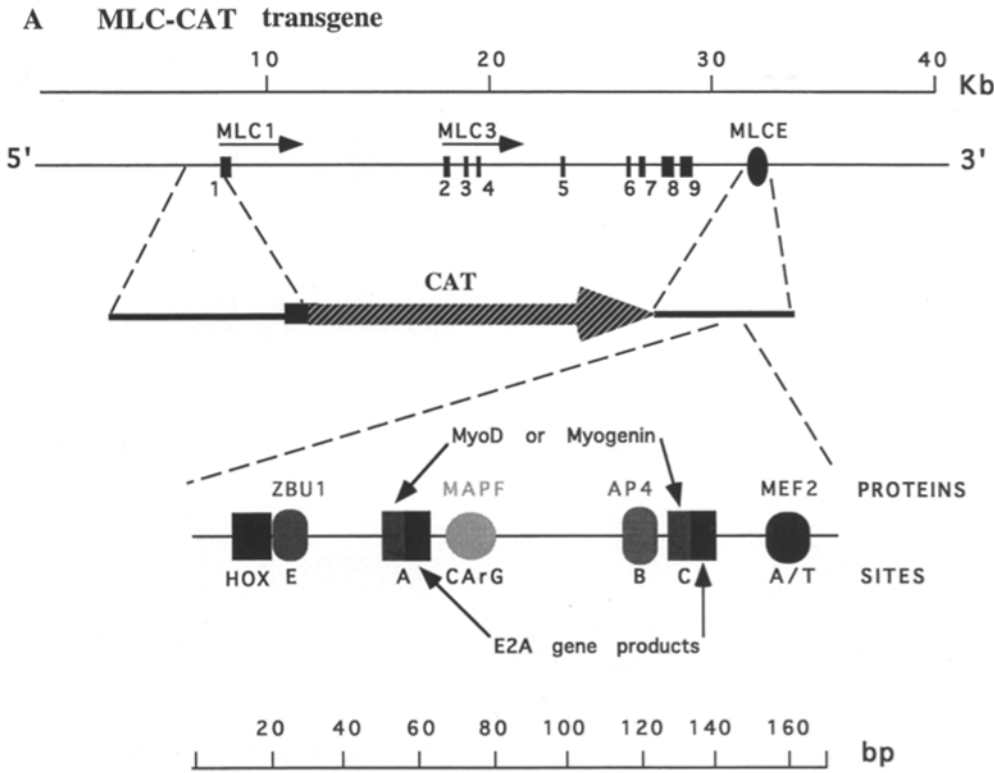
B α-skeletal actin-CAT transgene

Figure 3. CAT constructs used for the generation of transgenic mice. (*a*) The MLC-CAT transgene. Construction of the transgene and generation of transgenic mice has been described (Rosenthal et al., 1989). Protein binding sites in the rat MLC enhancer include a putative Hox binding site, an E box that binds Zbu1, a member of the SWI/SNF family of transcription factors (unpublished data), two E boxes (*A* and *C*) that bind myogenic factors (Rosenthal et al., 1990; Wentworth et al., 1991), a CArG box (Ernst et al., 1991), a fourth E box that binds the bHLH factor AP4 (unpublished), and a MEF2 binding site (Gossett et al., 1989). (*b*) The α_{sk} actin-CAT transgene. Construction of the transgene and generation of transgenic mice has been described (Petropoulos et al., 1989). Protein binding sites in the α_{sk} actin promoter include three SRF binding sites, a YY1 binding site, and a TEF1 binding site (Gualberto et al., 1992 and C. Ordahl, personal communication).
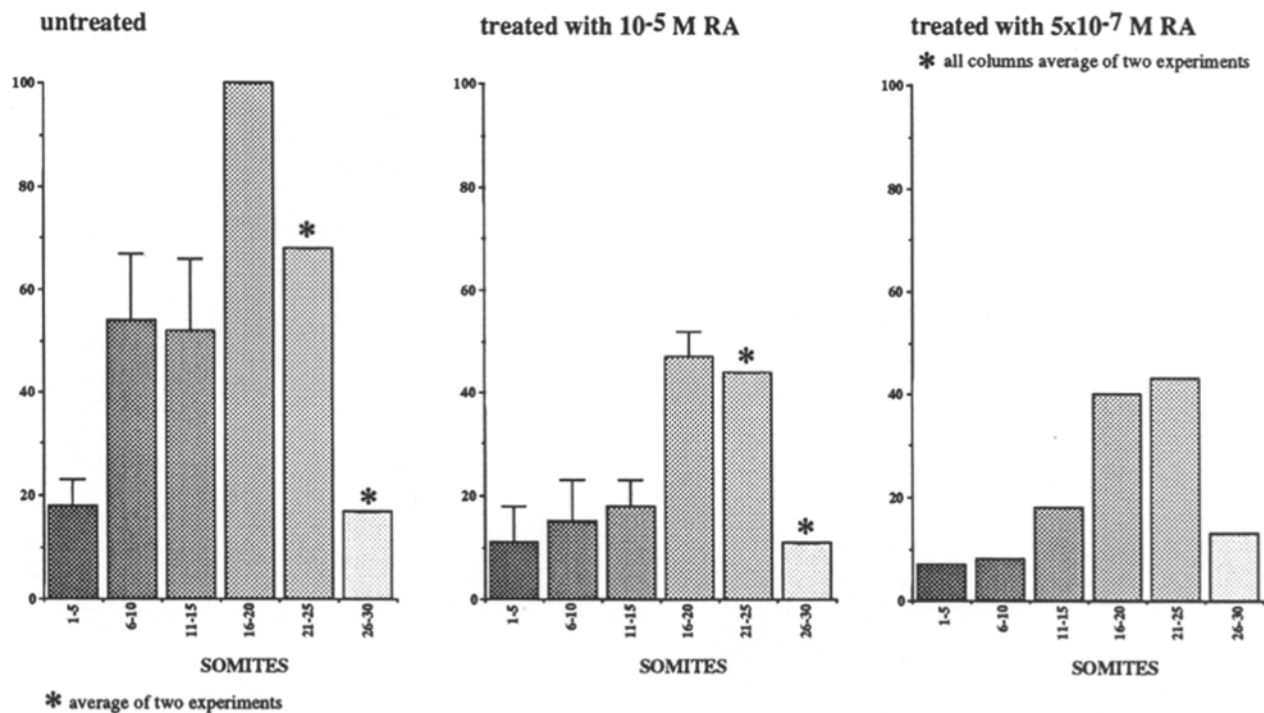

Figure 4. Inhibition of MLC-CAT transgene expression by RA treatment of limb bud and limb muscle primary cultures from different stages. Primary cultures from 11.5, 12.5, and 13.5 dpc limb buds and neonatal leg muscles of MLC-CAT transgenic mice were treated with 10^{-5}M RA at different times after plating as indicated. Cells were harvested 4 d (11.5–13.5 dpc) or 5 d (*neonatal*) after plating and CAT assays were normalized to protein content. CAT activity in untreated cultures increased during development in an approximate ratio of 1(*12.5 dpc*):5(*13.5 dpc*):100(*neonatal*). Values are averages of three independent experiments (with standard error) except the columns marked *, which are an average of two experiments. Data are presented as percentages of untreated controls which were arbitrarily set at 100.

untreated   treated with 10⁻⁵ M RA   treated with 5x10⁻⁷ M RA

Figure 5. Inhibition of MLC-CAT transgene expression by RA treatment of 11.5 dpc somite primary cultures. Triplicate cultures were prepared from pooled five-somite sections isolated from six 11.5 dpc MLC-CAT transgenic embryos as described in Materials and Methods. The most rostral somite sections (1–5) are shown to the left and the most caudal somite sections (26–30) are shown to the right in each panel. 10^{-5}M RA (*middle*) or 5×10^{-7}M RA (*right*) were added to the cultures directly after plating. Cells were harvested for CAT assays 3 d after plating. CAT assays were normalized to protein content for each triplicate set of cultures and are shown relative to the highest value in the untreated somite cultures (set to 100). CAT activity in these triplicate untreated cultures varied less than three-fold. Values are averages of three independent experiments (with standard error), except for the columns marked * which are an average of two experiments.

Retinoic Acid Receptor Gene Transcripts Are Regulated by RA in Primary Cultures

The action of RA on gene expression involves at least three nuclear RA receptors (RAR), RAR α (Gigure et al., 1987; Petkovich et al., 1987; Zelent et al., 1989), β (de The et al., 1987; Benbrook et al., 1988; Brand et al., 1988; Zelent, et al., 1989), and γ (Krust et al., 1989; Zelent et al., 1989). The RAR genes are differentially expressed during development and regulated by RA (de The et al., 1989). To investigate the possible involvement of RARs in RA-mediated repression of myogenesis in cell culture, we examined the effect of RA on RAR gene expression in primary cultures prepared from nontransgenic neonatal hind limb muscles. As shown in Fig. 7, both RARα and γ transcripts were expressed at low levels and RARβ transcripts were almost undetectable in untreated cultures. In response to RA treatment, RARβ and RARγ transcript levels were greatly increased, while levels of RARα transcripts remained unchanged. The differential accumulation of specific RAR transcripts in response to RA suggests that they might be involved in mediating the effect of RA on myogenesis in these primary cultures.

Inhibition of Myogenesis by RA Is Dependent on Diffusible Factors

The results described above show that RA treatment re-

presses the myogenic program in primary cultures. This contrasts with the induction of myogenesis in response to RA that has been reported for the C₂ myogenic cell line and for primary adult chicken satellite cells (Albagli-Curiel et al., 1993; Halevy and Lerman, 1993). To resolve the apparent discrepancy between these studies, we examined the response of the myogenic cell line C₂C₁₂ to RA under different culture conditions. C₂C₁₂ cells were stably transfected with the same construct used for generating MLC-CAT transgenic mice (Fig. 3). These cultures (C₂C₁₂/MLC-CAT) were differentiated in the absence or presence of RA. Treatment with 10^{-5}M RA resulted in a modest twofold reduction in CAT activity (Fig. 8, left), considerably less than the inhibition of the same construct in the transgenic primary culture system (Fig. 4). No change in endogenous MLC or myogenin expression levels was seen in the RA-treated C₂C₁₂ cells (data not shown), suggesting that the C₂C₁₂ line is much less responsive to RA than are primary muscle cultures.

To determine whether the response of C₂C₁₂ cells to RA could be affected by coculture with primary cells, we prepared a mixed culture of C₂C₁₂/MLC-CAT stable transfectants and primary neonatal muscle cells from nontransgenic mice. Expression of the CAT reporter, now exclusively contributed from C₂C₁₂ cells, was reduced about fivefold in response to 10^{-5}M RA (data not shown). The two populations of cells were then cultured in separate chambers, allowing diffusion of media components, to in-
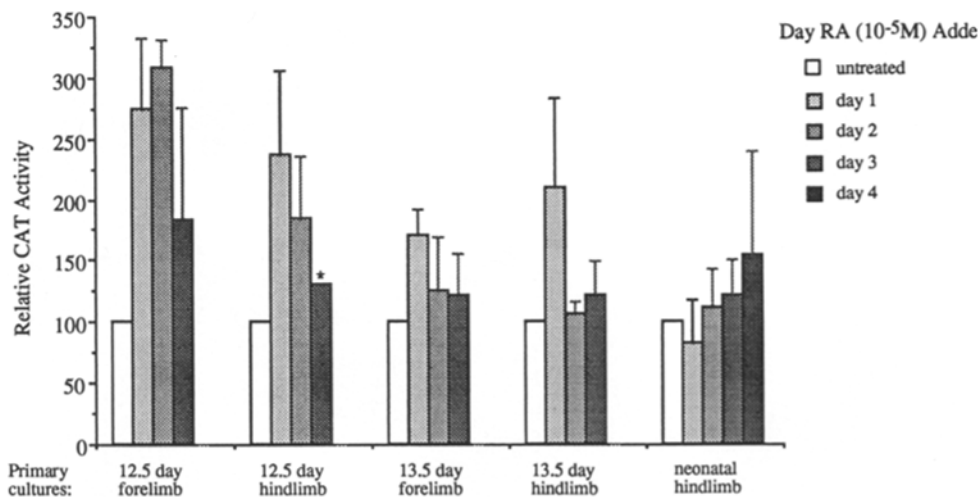
vestigate whether the repression of MLC-CAT activity by RA in the C_2C_{12} background was due to diffusible factors produced by the primary cells. Fig. 8 (*right*) shows that in these separated cocultures, C_2C_{12} transfectants responded to 10^{-5}M RA with a fivefold repression of MLC-CAT activity. As a negative control, C_2C_{12}/MLC-CAT transfectants were cocultured with untransfected C_2C_{12} cells, replacing primary cells in the second chamber. CAT activity was reduced no more than twofold in response to RA treatment (data not shown).

Since different media were routinely used to culture C_2C_{12} and primary cells (specifically the inclusion of CEE in primary cultures) it was important to determine whether

Figure 7. Expression of RAR transcripts in neonatal leg muscle primary cultures. RNA was isolated from neonatal leg muscles dissociated and cultured ± 10^{-5}M RA and analyzed by Northern blots hybridized with RAR α, β, and γ probes as indicated. GAPDH and MLC1/3 probes were included to control for equal RNA loading and myogenic differentiation, respectively. The panel on the right compares the relative increase in RAR γ transcripts with the concomitant decrease in MLC1/3 transcripts in the presence of RA.

culture conditions could affect the response to RA. Primary muscle cells could not be cultured in C_2C_{12} growth media, but C_2C_{12} cells grew normally in primary culture medium. We therefore analyzed the role of different components of the primary culture medium in determining the response of C_2C_{12} cells to RA. C_2C_{12}/MLC-CAT transfectants were cultured in four different combinations of the primary medium components and CAT activity assayed as a measure of myogenic differentiation. As shown in Fig. 9, MLC-CAT expression in C_2C_{12} cultures containing 2% CEE was inhibited by RA, both at 10^{-8}M (2- to 3-fold) and 10^{-5}M (4- to 10-fold). By contrast, MLC-CAT expression in C_2C_{12} cultures lacking 2% CEE was increased up to threefold at 10^{-8}M concentration of RA and remained almost unchanged in the presence of 10^{-5}M RA. These results indicate that the effects of RA upon muscle-specific gene expression are dependent upon diffusible factors present in the culture media.
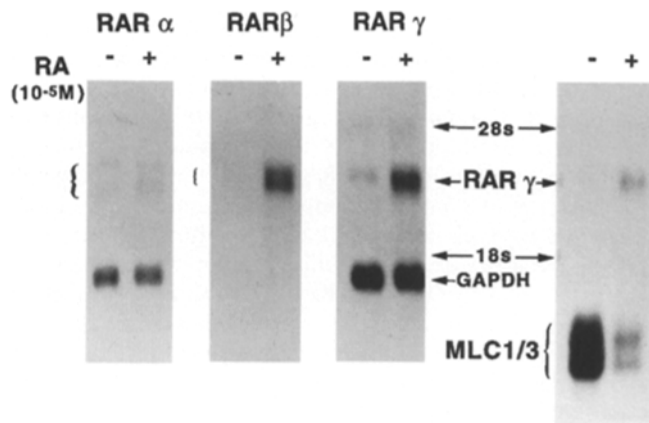
Discussion

This study demonstrates that RA inhibits myogenesis in mouse primary cultures derived from different developmental stages, as indicated by a reduction in MHC protein levels, in transcripts encoding endogenous MLC1/3, α_{sk} actin and two myogenic factors, and in MLC-CAT transgene activity. Further, RA-induced inhibition of gene expression in muscle cell cultures is dependent upon media conditions. This system can now be used to further dissect RA-mediated regulation of gene expression, and may provide a model for the pleiotropic functions of RA in different cellular contexts during embryonic development.

The involvement of diffusible factors in modulating the effects of RA on myogenesis in cell culture may also resolve the discrepancies seen in previous studies. Both activation and repression of myogenic differentiation in response to RA has previously been observed in myogenic
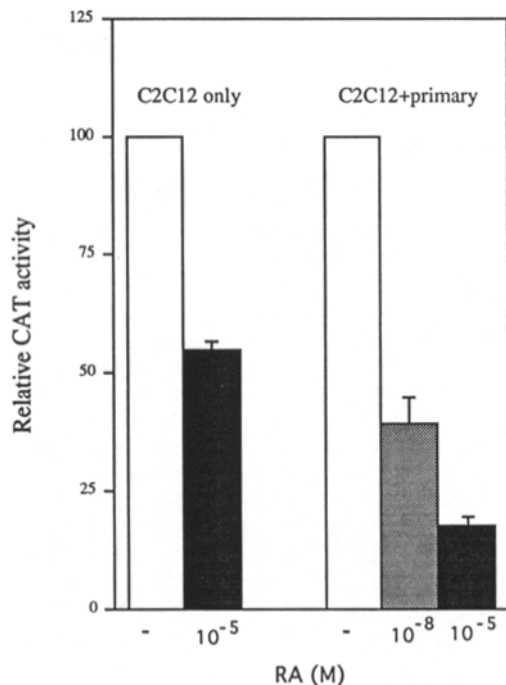
Figure 8. Effects of primary cell coculture on the expression of MLC-CAT in C_2C_{12} cells. (*Left*) C_2C_{12} cells stably transfected with the MLC-CAT construct (see Fig. 3) were cultured ± 10^{-5}M RA. (*Right*) C_2C_{12}/MLC-CAT cells and nontransgenic neonatal leg muscle cells were cocultured ± RA (concentrations as indicated) in two chambers separated by a membrane that permits diffusion of media. All CAT assays were normalized to protein content. CAT activity in untreated cultures varied no more than threefold. Values are averages of three independent experiments (with standard error). Data are presented as percentages of untreated controls which were arbitrarily set at 100.

Figure 9. Effects of culture conditions on the expression of MLC-CAT in RA-treated C_2C_{12} cultures. C_2C_{12} cells stably transfected with the MLC-CAT construct were cultured ± 10^{-8} or 10^{-5}M RA in different media as indicated. CAT assays were normalized to protein content. CAT activity in untreated cultures varied no more than fivefold. Values are averages of four independent experiments (with standard error). Data are presented as percentages of untreated controls which were arbitrarily set at 100.

cells (Langille et al., 1989; Momoi et al., 1992) as well as in other cell types including the P19 embryonic carcinoma cell line. At high concentrations, RA inhibits myogenesis in chicken embryo craniofacial mesenchyme (Langille et al., 1989) and limb buds (Momoi et al., 1992). By contrast, similar RA concentrations have been reported to induce myogenic differentiation in a rhabdomyosarcoma-derived cell line (Arnold et al., 1992), in C_2 myoblasts, and in primary adult chicken satellite cells (Albagli-Curiel et al., 1993; Halevy and Lerman, 1993). It is formally possible that primary muscle cultures may be intrinsically different from tumor cell lines and nontransformed myogenic cell lines, most of which respond to RA with decreased growth rates and/or increased differentiation. Notably, neither endogenous MLC1/3 nor myogenin transcript levels in the C_2C_{12} cultures varied significantly under different RA concentrations or culture conditions (data not shown). Nevertheless, the results of the present study suggest that in addition to RA concentration, culture conditions are critical in determining the effects of RA treatment.

The pleiotropic effects of RA are not limited to myogenic cell types. Many tumor and nontumor cell lines are induced to differentiate in the presence of RA. However, differentiation of some cell lines, such as the Ob17 murine preadipocyte cell line, is impaired by RA treatment (Te-
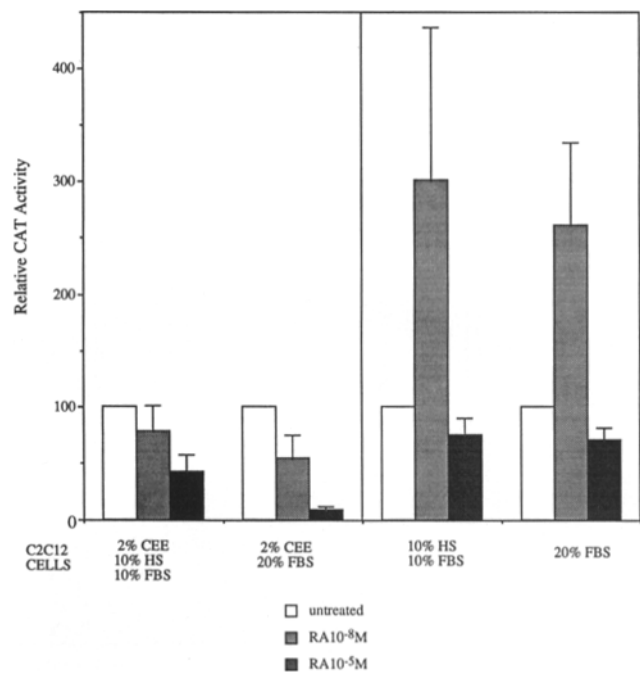
boul et al., 1992) which represses adipsin gene expression at a posttranscriptional level (Antras et al., 1991). P19 cells respond to a low concentration of RA (lower than 10^{-8}M) by differentiating into muscle precursors (Edwards et al., 1983), but undergo neural differentiation at higher concentrations (higher than 5×10^{-8}M; Jones-Villeneuve et al., 1983). In vivo, RA administration has varying effects at different developmental stages. For example, the same RA dose that is highly teratogenic, disturbing segmentation of the central nervous system when administered on 7.5 dpc, has no obvious effects on brain development when administered at 8.5 dpc (Zimmer and Zimmer, 1992). Similarly the type of homeotic transformation of vertebrae caused by RA depends on the developmental stage at which embryos are treated (Kessel and Gruss, 1991; Kessel, 1992). The present report suggests that the concentration-dependent effects of RA are likely to be mediated by other diffusible factors, present at different times and places in the developing embryo.

The unusual RAR gene expression profiles induced by RA treatment of primary muscle cultures (Fig. 7) also may provide insight into the mechanism by which RA functions, and into its role in the regulation of differentiation and development (see de Luca, 1991 for review). RNA localization studies have shown that RARα is ubiquitously distributed whereas RARβ and RARγ transcripts are differentially expressed among cell lines and during development, suggesting that they have specific roles in different

tissues (de The et al., 1989; Mendelsohn et al., 1992). The effects of RA are further modulated by changes in RAR expression in response to RA (de The et al., 1987; Gigure et al., 1987; Petkovich et al., 1987; Benbrook et al., 1988; Brand et al., 1988; Krust et al., 1989; Zelent et al., 1989). The RARβ gene has a RA response element that is involved in a positive auto-regulatory feedback loop (Sucov et al., 1990). In most of the cell lines, the expression of RARβ is induced by RA and impairment of this induction may contribute to neoplastic progression (Hu et al., 1991). In RA-treated mid-gestation mouse embryos, RARβ is the only significantly induced RAR isoform, especially in limb buds (Harnish et al., 1992). The constitutive expression of RARα and induction of RARβ in muscle cultures treated with RA observed in this study (Fig. 7) are consistent with the RAR expression patterns reported previously. However, the significant induction of RARγ transcripts by RA as seen in the muscle cultures presented here (Fig. 7) has been rarely reported in the literature (Wu et al., 1992). It is not clear what role RARγ may play in myogenic determination during development. Distribution of RARγ transcripts during mouse embryogenesis is uniform before 12.5 dpc, but later becomes specifically localized to cartilage and differentiating squamous keratinizing epithelia (Ruberte et al., 1990). The potential role of RARγ in the regulation of myogenic differentiation can now be addressed in culture with the reagents defined in this study.

At present the RA-responsive element(s) in the MLC-CAT transgene remain to be identified. Neither the 1200-bp MLC1 promoter or the 920-bp 3' MLC enhancer fragments included in the transgene construct contain a canonical RA-responsive sequence element, suggesting that RA may operate through repression of upstream regulators of MLC expression, such as the myogenic bHLH factors (see Fig. 2). Myogenic factor binding sites have been implicated in the transcriptional regulation of the MLC 1/3 locus (Rosenthal et al., 1990; Wentworth et al., 1991). It is therefore possible that RA-responsive elements lie within the regulatory sequences of the myogenic factors themselves. In contrast, muscle-specific transcription of the MHC and α_{sk} actin gene promoters is independent of MyoD1 (Chow and Schwartz, 1990; Thompson et al., 1991). Although direct regulation of these genes in vivo by myogenic factors is still a possibility, it is likely that pathways involving other factors also mediate the effect of RA on muscle-specific gene expression. Notably the α_{sk} actin-CAT transgene represents the only muscle-specific marker tested in this study which was not repressed by RA (Fig. 6). The 220-bp α_{sk} actin promoter is not dependent upon myogenic factor binding sites for its activity in muscle cell culture (Chow and Schwartz, 1990), and is highly expressed in proliferating myoblasts (Seiler-Tuyns et al., 1984) suggesting that it may be regulated differently from other muscle-specific promoters. In the present study the persistent expression of the α_{sk} actin-CAT transgene serves as an internal control, confirming that inhibition of myogenesis by RA did not result from selectively killing myogenic precursors in the heterogeneous cell cultures.

The dramatic response of primary cell cultures to RA treatment, accompanied by a reprogramming of RAR expression patterns, may provide important clues to the mechanism of RA action in the context of the developing vertebrate embryo. Retinoids are endogenously produced (Hogan et al., 1992), and unevenly distributed along the embryonic AP limb and body axes at early embryonic stages. The uneven distribution in retinoids in the embryonic limb axis (Thaller and Eichele, 1987) and in the activity of a RA response element (RARE) along the embryonic body axis in RARE transgenic animals (J. Rossant personal communication) is consistent with a concentration-dependent effect on myogenesis. In the simplest model, RA may play a role in preventing myogenic cells from differentiating until the appropriate embryonic stage is reached, consistent with its inhibition of myogenic differentiation in cell culture.

The local extracellular environment, involving cell–cell interactions and diffusible molecules, is probably as important as RA concentration in defining the effects of RA in the intact embryo. RA may therefore act through intermediates, such as the sonic hedgehog morphogen which has been implicated in patterning of both axial structures and limb buds in the embryo (Echelard et al., 1993; Riddle et al., 1993). In addition, while RARα mRNA is ubiquitously distributed in the embryo, transcripts encoding RARβ and RARγ each have a specifically restricted and dynamically changing pattern of expression, which may also play an important role in modulating the effects of RA both spatially and temporally. The present study should provide a valuable model for dissecting the role of differential RAR induction by RA in muscle differentiation, and for identifying and characterizing diffusible molecules which may mediate the specific effects of RA on myogenesis, as well as on the development of other embryonic tissues.

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