Regulated Expression of a Transfected Human Cardiac Actin Gene during Differentiation of Multipotential Murine Embryonal Carcinoma Cells

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P19 embryonal carcinoma (EC) cells are multipotential stem cells which can be induced to differentiate in vitro into a variety of cell types, including cardiac muscle cells. A cloned human cardiac actin (C^H -actin) gene was transfected into P19 cells, and stable transformants were isolated. Low levels of C^H -actin mRNA were present in transformed EC cells, but a marked increase in the level of C^H -actin mRNA was found as these cells differentiated into cardiac muscle. The accumulation of C^H -actin mRNA paralleled that of the endogenous mouse cardiac actin mRNA. A chimeric gene, which consisted of the C^H -actin promoter linked to the herpes simplex virus thymidine kinase coding region, was constructed and transfected into P19 cells. In these transformants, the thymidine kinase protein was located almost exclusively in cardiac muscle cells and was generally not detectable in EC or other nonmuscle cells. These results suggest that the transfected C^H -actin promoter functions in the appropriate developmental and tissue-specific manner during the differentiation of multipotential EC cells in culture.

Transfection of cloned genes into cultured cells has allowed the identification of discrete DNA sequences which restrict the transcription of eucaryotic genes to particular cell types. This regulation appears to be exerted by sequences just upstream of the transcription start site (reviewed in reference 36) and by regulatory elements such as enhancers (reviewed in references 25 and 40). The different roles played by promoter and enhancer sequences in the tissue-specific regulation of transcription have been defined primarily by experiments in which the expression of the gene is assayed about 48 h after transfection into cultured cell lines. This "transient expression" approach has several practical and theoretical limitations. (i) The appropriate cell line may not be available. (ii) *trans*-acting regulators may be in limiting concentrations in the recipient cells. (iii) Transfection efficiencies vary between different cell lines. (iv) Plasmid topology can affect transcription (50).

Another approach in the investigation of tissue-specific regulation of gene transcription has been to study the expression of genes which have been integrated into the genome of developing organisms after microinjection of the genes into zygotes (reviewed in references 9 and 34). In the transgenic strains produced, the integration site and copy number of the exogenous gene are identical in all cells and gene expression may be assessed within all tissues at various developmental stages. Although it has been done (18), the production of transgenic mammals can become tedious and expensive when a large number of constructs need to be analyzed to identify regulatory regions.

Embryonal carcinoma (EC) cells are cultured lines of multipotential mouse cells whose differentiation patterns closely resemble those of the normal embryo (11) and thus provide an alternative system to study early developmental processes. Genes can be transfected into EC cells, and the expression of the exogenous genes in the resulting transformed clones may be assessed in a variety of cell types. Thus, the EC system may combine some of the experimental

rigor of transgenic mice with the practical advantages of a cell culture system.

In this study, we used the P19 line of EC cells (29). P19 cells can be induced to differentiate in vitro into different cell types depending on the drug used. After aggregation and treatment with retinoic acid (RA), P19 cells differentiate into neurons, glia, and fibroblastlike cells (23, 24). Aggregates of P19 cells exposed to dimethyl sulfoxide (DMSO) differentiate into a different spectrum of cell types which includes cardiac muscle (6, 28, 44). Since no continuous lines of cardiac muscle cells have been established, and since primary cultures of cardiac muscle can only be maintained with difficulty for short periods, the P19 cell system is unique for investigating gene expression during the *de novo* differentiation of cardiac muscle.

An isoform of actin, called cardiac actin, is synthesized exclusively in cardiac and embryonic skeletal muscle cells. We have transfected the human cardiac actin (C^H -actin) gene into P19 cells and have found the gene expressed with appropriate developmental and cell type specificity.

MATERIALS AND METHODS

P19 cell culture. The P19 cells were maintained and differentiated as described elsewhere (29, 38). To induce differentiation, cells were allowed to aggregate and were cultured in petri-grade culture dishes in the presence of 5×10^{-7} M RA (23) or 1% DMSO (28). After 2 to 5 days, the aggregates were transferred to tissue culture-grade dishes. Differentiated cultures were harvested for RNA isolation or fixed for immunofluorescence 10 days after initiation of differentiation unless otherwise stated.

DNA transfection. P19 cells were transfected with plasmid DNA by the calcium phosphate DNA precipitation method (12, 51). Cultures of 10^6 cells were exposed for 4 to 6 h to $10 \mu\text{g}$ of plasmid DNA, immersed in 15% glycerol for 3 min, and then cultured for 48 h before seeding at 10^6 cells per 100-mm dish. Cultures were supplemented with $400 \mu\text{g}$ of geneticin (G418) per ml on the following day, and medium with G418 was replaced every 2 days. Colonies were either pooled or

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FIG. 1. Construction of plasmids and structure of the C^H -actin gene. (A) The C^H -actin gene (7, 17), along with 5.4 kb of 5'-flanking sequence, is contained within a 13-kb *EcoRI* fragment which was inserted into the *EcoRI* site of pSV2-*neo* (46) and called pNA8. A second plasmid was constructed (pNA- Δ I) with sequences upstream of a *Bam*HI site removed, leaving 0.8 kb of flanking sequences upstream of the C^H -actin transcription start site. (B) Relation of hybridization probes to the C^H -actin gene. The 1.5-kb *Pst*I 5' fragment was used as a specific probe to identify P19(C^H -actin) transformants in Southern blot hybridization experiments, and the 0.45-kb *Hae*III-*Pst*I fragment was used as a cardiac actin isotype-specific probe in Northern blot hybridization experiments. The 0.6-kb *Pst*I fragment was used in nuclease S1 mapping experiments. $\rightarrow$, Transcript; $\square$, coding exons; $\blacksquare$, untranslated regions; UT, untranslated.

individually picked after 8 to 10 days of selection and expanded into cell lines in the absence of G418.

RNA purification and nuclease S1 analysis. Cytoplasmic RNA was isolated by streptomycin precipitation (43), whereas total RNA was isolated by the lithium chloride-urea extraction method (1). The 600-base pair (bp) *Pst*I 3' fragment (Fig. 1B) was 3' end labeled with [32 P]dCTP (>3,000 Ci/mmol) by replacement synthesis in the presence of T4 DNA polymerase and 50 μ M each TTP, dATP, and dGTP

(27). Nuclease S1 analysis was performed as described elsewhere (3, 49). Briefly, RNA (20 μ g) was suspended in 20 μ l of 80% formamide–40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 6.5]–0.4 M NaCl–5 mM EDTA containing 50,000 cpm of probe and covered with a drop of paraffin oil. The mixture was heated at 70°C for 10 min and then incubated overnight at 52°C. Then, 0.3 ml of ice-cold 0.25 M NaCl–0.03 M sodium acetate–0.001 M ZnSO₄–20 μ g of denatured salmon sperm DNA per ml containing 400 to

1,000 U of S1 nuclease (Boehringer Mannheim Biochemicals) per ml was added to the hybridization mix, and digestion was allowed to proceed for 45 min at 45°C. The reaction was stopped by a phenol-chloroform extraction. The heteroduplexes were recovered by ethanol precipitation and suspended in loading buffer. The protected fragments were electrophoresed through 5% acrylamide-8 M urea sequencing gels and visualized by autoradiography.

Immunofluorescence. Immunofluorescence experiments were performed as described elsewhere (38). Cells were grown directly on cover slips. Muscle myosin and muscle actin proteins were detected simultaneously by staining the fixed cells (15 min in -20°C methanol) with a mouse monoclonal antibody, MF20, which reacts with muscle myosin (2) and rabbit antiserum raised against an N-terminal peptide of skeletal actin (4). To detect the herpes simplex virus thymidine kinase (HSV-TK) protein, cells were simultaneously stained with rabbit anti-TK antiserum (16) and with mouse monoclonal antibodies against either muscle myosin (MF20) or muscle actin (B4F8) (J. Lessard, personal communication). Second antibodies were fluorescein-conjugated anti-rabbit and rhodamine-conjugated anti-mouse immunoglobulins G.

RESULTS

Introduction of the C^H-actin gene into P19 cells. Our strategy was to transfect the C^H-actin gene into the P19 line of EC cells, obtain stable transformants, and determine whether the C^H-actin gene was appropriately regulated when the transformed cells were induced to differentiate into cardiac muscle and nonmuscle cell types. The two plasmids used for transfection contained the *neo* gene, which confers resistance to the antibiotic G418 (45), and the entire coding region of the C^H-actin gene with either 5.4 (pNA8) or 0.8 (pNA-Δ1) kilobases (kb) of sequence upstream of the site of transcription initiation (Fig. 1A).

Plasmids were introduced into P19 cells by the calcium phosphate precipitation method of DNA transfection (12). G418-resistant colonies were either pooled or individually picked and expanded into cell lines [designated P19(C^H-actin)]. Southern blot analysis of 28 of these lines (data not shown) indicated that 23 contained between 1 and >200 copies of the C^H-actin gene (Table 1).

Developmental regulation of the C^H-actin gene during cardiac muscle differentiation. We used a nuclease S1 mapping procedure to detect C^H-actin mRNA in P19(C^H-actin) transformants and to distinguish the C^H-actin mRNA from transcripts originating from the endogenous mouse cardiac actin (C^M-actin) gene. The 600-nucleotide (nt) probe used in these experiments spans the last exon of the C^H-actin gene (Fig. 1B and 2A). The C^H-actin mRNA protected a 330-nt fragment from nuclease S1 digestion (Fig. 2B, lane 6), whereas the C^M-actin mRNA protected fragments of between 152 and 140 nt (Fig. 2B, lanes 1, 4, and 6). None of the mRNAs encoding other actin isoforms protected a labeled region of the probe (Fig. 2B, lanes 2, 3, and 5). C^M-actin mRNA was readily detected in DMSO-treated P19 cultures (Fig. 2B, lanes 4 and 6), whereas no such transcript was detected in either EC cells or RA-treated cultures (Fig. 2B, lanes 3 and 5).

The levels of C^H-actin mRNA during the development of cardiac muscle were examined to determine whether transcription of endogenous C^M-actin and transfected C^H-actin genes were subject to similar developmental regulation. The experiment was performed with cultures which consisted of

TABLE 1. Characteristics of P19(C^H-actin) transformants

Cell line	Copy no. ^a	% Muscle cells ^b	Increase in C ^H -actin mRNA ^c
P19	0	15	NA
P19A6	1	0	NA
P19A24	>200	10	4.8
P19A35	45-55	11	6.3
P19AD32	20-30	14	3.8
P19AD43	45-55	ND	4.3
P19AD54	>200	30	2.8

^a Copy number was determined by comparison of dot blots of serial dilutions of DNA from human and P19(C^H-actin) transformants hybridized to the 1.5-kb *Pst*I probe (Fig. 1A).

^b Percent muscle cells in DMSO-treated cultures as determined by indirect immunofluorescence staining of dispersed cells with monoclonal antibody MF20, which specifically reacts with muscle myosin (2). ND, Not determined.

^c The amount of C^H-actin mRNA was determined by scintillation counting of nuclease S1-protected fragments cut out of the sequencing gel. The activity found in DMSO-treated cultures was divided by the activity found in EC cultures. NA, Not applicable.

over 50 pooled clones of P19(C^H-actin) cells transfected with the pNA-Δ1 plasmid. This approach was taken to obtain the average of a large number of transfected clones.

Spontaneously beating cardiac muscle and cells reactive with the muscle myosin-specific antibody, MF20 (2), first appeared on day 6. The C^M-actin mRNA also became evident on day 6, increased rapidly to peak on day 7, and decreased slowly to about 30% of the maximum level by day 10 (Fig. 3). This pattern of C^M-actin expression was identical to that observed in untransfected DMSO-treated P19 cells.

A low level of C^H-actin mRNA was present in P19(C^H-actin) cells, but this level decreased continuously during the first 5 days of the differentiation regime. The C^H-actin mRNA level abruptly increased on day 6 in parallel with the appearance of the C^M-actin transcript, peaked on day 7, and decreased thereafter (Fig. 3). These results suggest that the transfected C^H-actin gene was subject to the same regulatory mechanisms which control the developmental expression of the endogenous C^M-actin gene.

Because the simian virus 40 (SV40) promoter drives the *neo* gene in the plasmid carrying the C^H-actin gene, it was possible that the peak in C^H-actin mRNA seen in Fig. 3 might be due to an effect of the SV40 enhancer. However, this seems very unlikely because the level of the *neo* mRNA actually decreased (by about twofold) during the course of DMSO-induced differentiation of transformed cells (data not shown).

In DMSO-induced differentiation experiments, only about 10% of the cells develop into cardiac muscle. To determine the level of C^H-actin mRNA in differentiated nonmuscle cells, four fibroblastlike cell lines were isolated from cultures induced to differentiate with DMSO and their RNA was assessed for the presence of the C^H-actin transcript. No C^H-actin mRNA was detected in two of three clonal fibroblastlike cell lines derived from one of the P19(C^H-actin) clones, P19AD43 (Fig. 4; compare lane 1 with lanes 2, 3, and 4). In a nonclonal fibroblastlike cell line derived from another clone, P19AD54, the C^H-actin mRNA was present at a level much lower than that found in the parental EC cells (Fig. 4; compare lanes 5 and 6). These fibroblastlike cells were not EC or muscle cells, since they did not react with either anti-EC (AEC3A1-9; 19) or anti-muscle myosin (MF20; 2) antibodies. Thus, the low level of C^H-actin transcript present in EC cells becomes reduced even further

FIG. 2. Discrimination between C^H -actin and C^M -actin mRNAs by nuclease S1 mapping. (A) The 600-bp *Pst*I fragment containing the last coding exon (EXON 7) of the C^H -actin gene was 3' end labeled with T4 DNA polymerase, hybridized to RNA samples containing human and mouse C-actin mRNAs, and digested with nuclease S1. The C^H -actin mRNA protects 330 nt, whereas the C^M -actin mRNA protects a maximum of 152 nt of the 3' end of the probe (*, label). (B) RNA from mouse striated muscle and P19 cells subjected to nuclease S1 analysis. M, Size markers (indicated on the left in nucleotides) consisting of pAT153 digested with *Hpa*II and 3' end labeled with T4 DNA polymerase; P, probe only; 1, mouse heart muscle; 2, mouse skeletal muscle; 3, undifferentiated P19 cells; 4, P19 cells induced to differentiate with DMSO into cultures which include cardiac muscle; 5, P19 cells induced to differentiate with RA into neurons, glia, and fibroblastlike cells; 6, P19AD32 after DMSO treatment. Probe, Human, and Mouse denote the 600-nt input probe, the 330-nt human protected fragment, and the 152-nt mouse protected fragment, respectively.

in most of the nonmuscle cells, suggesting that both activating and repressing mechanisms are responsible for regulating the C^H -actin promoter.

Expression of the C^H -actin gene in clonal lines of P19(C^H -actin) cells. A number of cloned lines of P19(C^H -actin) cells containing either the pNA8 or pNA- Δ 1 plasmid were treated with RA or DMSO, and cultures were allowed to develop for 10 days before being harvested for RNA isolation. Each of the P19(C^H -actin) cell lines differentiated into neurons, glia, and fibroblastlike cells when aggregated and exposed to RA. For reasons not yet clear, only half of these cell lines developed cardiac muscle when aggregated and exposed to DMSO. The proportion of muscle cells in DMSO-treated cultures varied between 0 and 30% (Table 1), was a repro-

ducible property of each clone, and appeared to be unrelated to the number of copies of the transfected C^H -actin gene (Table 1), the length of the C^H -actin promoter, or the level of *neo* mRNA (data not shown).

Nuclease S1 analysis of RNA from P19(C^H -actin) cell lines and their differentiated derivatives indicated that most clones had detectable C^H -actin mRNA in the undifferentiated EC cells, that the level of this mRNA was between 2.8- and 6.3-fold higher in DMSO-treated cultures, and that the C^H -actin mRNA was generally lower after RA treatment (Fig. 5 and Table 1). The increase in C^H -actin mRNA in the P19(C^H -actin) cardiac muscle cells was likely 10 to 100 times higher than the 2.8- to 6.3-fold noted above, because only 10 to 30% of the cells in DMSO-treated cultures were muscle

FIG. 3. Concomitant expression of transfected C^H-actin and endogenous C^M-actin genes during cardiac muscle differentiation. (A) Total RNA, from over 50 pooled P19(C^H-actin) transformants derived after transfection with pNA-ΔI, was isolated at daily intervals throughout the DMSO-induced differentiation regime and analyzed for the presence of C^H-actin and C^M-actin mRNAs by nuclease S1 mapping as described in the legend to Fig. 2. At day 0, EC cells were transferred into petri-grade dishes containing medium with 1% DMSO. After 5 days, aggregates were transferred into tissue culture-grade dishes without DMSO. Beating muscle first appeared on day 6. Lanes 0 to 10, RNA samples isolated on days 0 to 10; for abbreviations, see the legend to Fig. 2. (B) Relative levels of C^H-actin mRNA (■) and C^M-actin mRNA (□) during DMSO-induced differentiation. The levels of C^H-actin and C^M-actin mRNA were determined by scanning densitometry of the autoradiogram shown in panel A. The recorded peaks were then excised and weighed. Cardiac actin mRNA is expressed in arbitrary units as a percentage of the C^H-actin mRNA level found on day 7.

(Table 1), the level of C^H-actin mRNA was determined on day 10 although the peak level of expression was on day 7 (Fig. 3), and the level of C^H-actin mRNA actually decreased in the nonmuscle cells within DMSO-treated cultures (Fig. 3 and 4).

The increase in C^H-actin mRNA after DMSO treatment did not correlate with the proportion of muscle cells (Table 1), probably because of the clone-to-clone variation in levels of C^H-actin mRNA in EC cells. The level of C^H-actin mRNA in EC cells was not related to the level of *neo* mRNA (data not shown) or to the copy number of the transfected gene (Table 1). In addition, the length of the upstream region (5.4 or 0.8 kb) flanking the C^H-actin gene did not appear to affect the levels of C^H-actin mRNA in EC cells or their differentiated derivatives (Fig. 5 and Table 1).

In the two cell lines, P19A24 and P19AD54, which carried more than 200 copies of the C^H-actin gene, only small

amounts of C^M-actin mRNA were present in DMSO-treated cultures containing relatively large amounts of muscle (Fig. 5, lanes 8 and 20). Higher levels of the C^M-actin mRNA were present after DMSO treatment of other cell lines, such as P19AD32, which developed similar amounts of muscle but which contained fewer copies of the C^H-actin gene (Fig. 5, lane 14; Table 1). These results suggest that the C^M-actin and C^H-actin genes may compete for limiting amounts of a transcription factor(s) in muscle cells.

Most RA-treated cultures of P19(C^H-actin) cells contained lower levels of C^H-actin mRNA than those found in the corresponding EC cells (Fig. 5, lanes 9, 15, 18, and 21). Two exceptions were observed in the 10 transformants examined. The C^H-actin mRNA was detectable only after RA treatment of P19A6 cells (Fig. 5, lane 6), whereas the level of C^H-actin mRNA increased after RA treatment of P19A35 cells (Fig. 5, lane 12). The unexpected behavior of the C^H-actin gene in

FIG. 4. Levels of C^H -actin mRNA are reduced within differentiated nonmuscle cells in DMSO-treated cultures. After DMSO treatment, cells were passaged several times to obtain a muscle-free population of differentiated fibroblastlike cells. Total RNA extracted from EC cells and fibroblastlike cells was analyzed for the presence of C^H -actin mRNA by nuclease S1 mapping as described in the legend to Fig. 2. EC denotes undifferentiated cells. C1, C2, and C3 were three clonal fibroblastlike cell lines derived after DMSO treatment of P19AD43 cells. MF was a nonclonal population of fibroblastlike cells with mixed form derived after DMSO treatment of P19AD54 cells. No C^M -actin mRNA was detected in any of these samples. For other abbreviations, see the legend to Fig. 2.

these two lines may be a consequence of rearrangement of the transfected gene or its integration into a region of the genome influenced strongly by neighboring sequences.

Restriction of C^H -actin protein to muscle cells. Detectable levels of C^H -actin mRNA were present in some P19(C^H -actin) transformants and in some of their differentiated nonmuscle derivatives (Fig. 3 to 5). To determine whether the C^H -actin mRNA was translated in these nonmuscle cells, we performed immunofluorescence experiments using a polyclonal antiserum which reacts with the cardiac and

skeletal isoforms of actin protein (4). In four clonal lines of transformants (P19A24, P19A35, P19AD43, and P19AD54), virtually no cardiac actin protein was detected in untreated and RA-treated cultures (Fig. 6, panels A through D). Within DMSO-treated cultures, cardiac actin protein was not detected in cells that did not also contain muscle myosin (Fig. 6, panels E through J). In addition, no cardiac actin protein was detected in the differentiated nonmuscle cells from the four fibroblastlike lines derived after DMSO treatment of P19AD43 and P19AD54 (data not shown). These experi-

FIG. 5. Expression of the transfected C^H -actin gene increased during cardiac muscle differentiation of clonal P19(C^H -actin) cell lines. Cytoplasmic RNA isolated from EC cells and differentiated cells from DMSO- and RA-treated cultures of P19(C^H -actin) cell lines was analyzed for the presence of C^H -actin and C^M -actin mRNAs by nuclease S1 mapping as described in the legend to Fig. 2. P19A and P19AD transformants were transfected with the plasmids pNA8 and pNA-Δ1, respectively. Note that the level of C^H -actin mRNA generally increased after DMSO treatment but not after RA treatment. EC, D, and R denote RNA samples isolated from EC cells, DMSO-treated cultures, and RA-treated cultures, respectively. For other abbreviations, see the legend to Fig. 2.

FIG. 7. Expression of the transfected actin-*tk* chimeric gene increased during muscle differentiation. (A) The actin-*tk* chimeric gene (pPTK) was constructed by linking the 1.5-kb *Pst*I fragment (■) containing 700 bp of sequence upstream from the transcription start site, the first exon (44 bp) containing part of the 5' untranslated region, and the first intron (700 bp) of the C^H-actin gene to a fragment of the HSV genome (□) carrying the coding region of the HSV *tk* gene (▨). The 800-bp *Pst*I insert of pT4 was used as a TK-specific probe in hybridization experiments. ~~, Transcript; exons are indicated as raised areas; P, *Pst*I; E, *Eco*RI. (B and C) Actin-*tk* and actin mRNAs were detected by Northern blot hybridization of total RNA isolated from P19(actin-*tk*) transformants to either TK-specific (B) or actin-specific (C) (0.6-kb *Pst*I probe, Fig. 1B) probes. Shown here are two P19(actin-*tk*) clones. EC, D, and R, RNAs isolated from EC cells, DMSO-treated cultures, and RA-treated cultures, respectively. P19P106 but not P19P2 contained the actin-*tk* mRNA, other bands are due to nonspecific hybridization to rRNA. L6U3 is a rat cell line transformed with pPTK, which expresses high levels of actin-*tk* mRNA.

ments suggest that the C^H-actin protein is synthesized in nonmuscle cells at levels below the limits of detection of our immunofluorescence technique, that the C^H-actin protein is not incorporated into microfilaments, that the C^H-actin protein is rapidly degraded in nonmuscle cells, or that the C^H-actin mRNA is not efficiently translated in nonmuscle cells. Further support for the last possibility will be published separately (M. M. Ruben, M. A. Rudnicki, T. S. Bladon, K. Jardine, J. Craig, and M. W. McBurney, submitted for publication).

Muscle-specific expression of an actin-*tk* chimeric gene. The differentiation-dependent changes in the levels of C^H-actin mRNA in P19(C^H-actin) transformants were likely due to regulation of C^H-actin gene transcription. To investigate the

C^H-actin gene promoter in more detail, we constructed a chimeric gene in which the C^H-actin gene promoter (−700 to +740 bp from the transcription start site) was linked to the HSV *tk* coding region (Fig. 7A). The chimeric actin-*tk* gene was cotransfected with pSV2-*neo* (46) into P19 cells. G418-resistant clones were subsequently selected, and those containing the chimeric gene were identified by Southern blot hybridization (data not shown).

Northern (RNA) blot analysis of P19(actin-*tk*) transformants indicated that in five independent clones, a low but detectable level of actin-*tk* mRNA was present in EC cultures and increased 3 to 13 times after DMSO treatment but not after RA treatment (Fig. 7B, lanes 5 to 7). This increase in actin-*tk* mRNA coincided with the appearance of the

FIG. 6. Restriction of the cardiac actin protein to muscle cells in P19(C^H-actin) transformants. Indirect immunofluorescence was used to determine the distribution of cardiac actin and skeletal actin proteins in P19AD43 cells. Both EC cells (panels A and B) and RA-treated cultures containing neurons, glia, and fibroblastlike cells (panels C and D) expressed some C^H-actin mRNA (Fig. 5) but did not contain detectable cardiac actin protein (panels B and D). After DMSO treatment, the majority of myocytes were cardiac muscle (E) but some skeletal muscle was also apparent (H). The muscle cells contained muscle myosin (G and J) and cardiac actin and/or skeletal actin (F and I) proteins. A, C, E, and H are phase-contrast fields. B, D, E, and I are the corresponding fields stained with rabbit antiserum which reacts with cardiac actin and skeletal actin proteins (4). G and J were stained with monoclonal antibody MF20 (2) reactive with muscle myosin. Second antibodies were fluorescein-conjugated anti-rabbit and rhodamine-conjugated anti-mouse immunoglobulins G. Bar, 50 μ m.

TABLE 2. Distribution of TK protein in DMSO-treated P19(activin-*tk*) cells^a

Cell line	Expt	No. of muscle cells		No. of nonmuscle cells		% Muscle ^b	% Muscle also TK ^c	% TK + also muscle ^d
		TK+	TK-	TK+	TK-			
P19P11	1	32	3	5	531	6.1	91	86
	2	102	18	19	ND	ND	85	84
	3	85	23	19	ND	ND	79	82
P19P13	1	34	2	7	377	8.7	94	83
	2	71	9	14	ND	ND	89	84
	3	29	8	5	ND	ND	78	85
P19P22	1	29	2	4	247	11.2	94	88
P19P26	1	53	5	8	243	19.3	91	87

^a Cultures of DMSO-treated cells were dispersed onto cover slips 6 days after initiation of differentiation, fixed, and stained for TK protein and muscle myosin. Microscope slides were scanned and cells were scored for the presence (muscle cells) or absence (nonmuscle cells) of muscle myosin and for the presence (TK+) or absence (TK-) of TK protein. ND, Not determined.

^b Proportion of cells containing muscle myosin.

^c Percentage of muscle cells which also contained TK protein.

^d Proportion of cells containing TK protein which also contained muscle myosin.

1.7-kb C^M-actin transcript in DMSO-treated cultures (Fig. 7C, lane 6). Many of the other P19(activin-*tk*) transformants did not express the chimeric gene under any conditions (Fig. 7B, lanes 2 to 4).

The HSV-TK protein translated from the activin-*tk* mRNA in P19(activin-*tk*) transformants was detected by immunofluorescence by using a monospecific antibody (16). The TK protein was located almost exclusively within the nuclei of cells which also contained muscle myosin (Fig. 8, panels A through C) and cardiac or skeletal actin (Fig. 8, panels D through F). Table 2 summarizes the results of these double staining experiments. In four P19(activin-*tk*) cell lines, over 86% of the muscle cells contained TK protein, whereas less than 2% of nonmuscle cells in DMSO-treated cultures (Table 2) and less than 0.1% of cells within EC cultures (data not shown) had detectable TK protein in their nuclei. These rare TK-containing cells within EC cultures may have spontaneously differentiated into muscle since they did not stain with anti-EC antibody (AEC3A1-9) and did stain with an antibody (B4F8) reactive with all forms of muscle actin.

Thus, the TK product of the activin-*tk* gene appeared almost exclusively in muscle cells, providing strong evidence for the muscle-specific activity of the C^H-actin promoter after its stable integration into multipotential P19 cells.

DISCUSSION

In this study, we have transfected the C^H-actin gene into multipotential P19 cells and isolated stable transformants. Transcripts from the C^H-actin gene were present at a low level in undifferentiated EC cells and in some differentiated nonmuscle cells. However, a marked increase in the level of C^H-actin mRNA occurred in parallel with transcripts from the endogenous C^M-actin gene during cardiac muscle differentiation. In P19 cells transfected with the activin-*tk* chimeric gene, the TK protein was located almost exclusively in

muscle cells. Thus, the DNA sequences which direct the muscle cell-specific expression of the C^H-actin gene lie between -700 and +740 bp from the transcription start site. These results suggest that the changes in the level of C^H-actin mRNA are due to an increase in the rate of transcription of the C^H-actin gene in cardiac muscle cells.

The activity of the C^H-actin gene promoter has been previously investigated after transient transfection or stable transformation of mouse and rat skeletal myoblast cell lines (31). In cells with integrated copies of the C^H-actin promoters, the level of transcript generated by this promoter either did not increase or increased modestly after differentiation of myoblasts into multinucleate myotubes (20, 30; Ruben et al., submitted). The failure of skeletal myoblasts to efficiently regulate the C^H-actin promoter may be due to the relatively advanced developmental stage of the cell lines used. Alternatively, the regulation of the C^H-actin promoter may be a multistep process which requires "programming" by a cell which is a precursor to the muscle lineage. If such programming is required for the developmental regulation of C^H-actin gene expression, the identification of the relevant regions of DNA will require the use of a developing biological system such as that described in this communication.

In EC cells and RA-treated cultures, transcription initiated from the C^H-actin promoter generated low levels of transcripts, although no C^M-actin mRNA was detected. This inappropriate activity of the C^H-actin promoter may be due to an effect of the vector sequences or to the proximity of the SV40 early promoter and enhancer used to drive the *neo* gene. A role for the SV40 enhancer seems unlikely, however, since this sequence does not function as a strong enhancer in undifferentiated EC cells (10). A more likely explanation for the low level of C^H-actin expression in EC cells derives from the presence in EC cells of a cellular product with E1A-like activity (22). Since the adenoviral

FIG. 8. Restriction of TK protein to muscle cells in P19(activin-*tk*) transformants. P19(activin-*tk*) transformants were treated with DMSO, and after differentiation, cells were dispersed and seeded onto cover slips before fixation and staining with rabbit anti-TK antiserum (16) and either MF20 or B4F8, mouse monoclonal antibodies which react with muscle myosin (2) and muscle actin (J. Lessard, personal communication), respectively. Shown here is P19P11; other results are summarized in Table 2. Panels A and D are phase fields. Cells which contained muscle myosin protein (panel B) or muscle actin protein (panel E) almost always also contained TK protein in their nuclei (panels C and F). Almost no nonmuscle cells contained TK protein. Second antibodies were fluorescein-conjugated anti-rabbit and rhodamine-conjugated anti-mouse immunoglobulins G. Bar, 50 μ m in panels A, B, and C and 30 μ m in panels D, E, and F.

E1A gene products enhance the expression of transfected cellular genes (13), it seems likely that the E1A-like activity in EC cells may be responsible for activating expression of the C^H-actin and actin-*tk* genes in the transformed P19 cells. In addition, the chromatin structure of EC cells is unusually nuclease sensitive (5, 21), perhaps conferring an active configuration upon newly introduced genes.

The C^H-actin mRNA and actin-*tk* mRNA present in non-muscle cells did not give rise to detectable protein. Northern blot analysis of RNA from P19(C^H-actin) transformants with a cardiac actin isotype-specific probe (the 0.45-kb *Hae*III-*Pst*I fragment [Fig. 1A], containing the 3' untranslated region) indicated that the C^H-actin mRNA in EC and RA-treated cells was the expected size, 1.7 kb, and therefore was likely correctly initiated and processed. Since the cardiac actin protein can be incorporated into cytoskeletal structures in mouse L cells (15), our failure to detect this protein in P19(C^H-actin) transformants suggests that the C^H-actin and actin-*tk* transcripts are only efficiently translated in muscle cells. More direct evidence for translational regulation will be reported elsewhere (Ruben et al., submitted).

Other tissue-specific genes have been transfected into mouse EC cells. Cloned globin and crystallin genes have been introduced into multipotential EC cells, but regulated expression could not be demonstrated, perhaps because the EC cells did not differentiate into those cell types expected to express high levels of the transfected genes (26, 35, 48). The F9 cell line, which differentiates only into extraembryonic endodermal cells (47), has been transfected with H-2 (37) and alpha-fetoprotein (39) genes, and transcripts from these transfected genes did increase appropriately after transformed cells differentiated into endoderm.

The experiments reported here demonstrate that the transfected C^H-actin and chimeric actin-*tk* genes were subject to appropriate developmental and tissue-specific regulation during differentiation of transfected P19 cells into cardiac muscle. Sequences located between -700 and +740 bp from the C^H-actin transcription initiation site were able to mediate this muscle-specific regulation. Thus, transfected P19 cell cultures, like transgenic animals (8, 32, 33, 41, 42, 52), are a suitable biological test system for identifying those DNA sequences responsible for muscle-specific gene expression.

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