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Heterokaryons of Cardiac Myocytes and Fibroblasts Reveal the Lack of Dominance of the Cardiac Muscle Phenotype

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The molecular characterization of a cardiac determination gene has been an elusive goal for the past several years. Prior to cloning of the skeletal muscle determination factor MyoD, the presence of a dominantly acting skeletal muscle determination factor had been inferred from the observation that the skeletal muscle phenotype was dominant in skeletal muscle-fibroblast heterokaryons (H. M. Blau, G. K. Pavlath, E. C. Hardeman, C.-P. Chiu, L. Siberstein, S. G. Webster, S. C. Miller, and D. Webster, Science 230:758–766, 1985). In these experiments, we have examined cardiac-fibroblast heterokaryons to investigate the existence of a dominantly acting cardiac determination factor. We have employed a novel experimental approach using primary embryonic fibroblasts from transgenic mice as a means of assaying for the activation of a cardiac promoter-luciferase reporter transgene within fibroblast nuclei. This approach provides a potential means of genetic selection for a dominantly acting positive factor and can be generalized to other systems. We have examined the expression of three markers of the cardiac lineage: a myofibrillar protein promoter (MLC2), a secreted protein (ANF), and a transcription factor (MEF2). MEF2 is specific to both cardiac and skeletal muscle cells. Our results indicate that in a majority of heterokaryons with an equal ratio of cardiac to fibroblast nuclei, none of these cardiac markers are expressed, indicating that the cardiac phenotype is not dominant over the embryonic fibroblast phenotype. The distinction from previous results with skeletal muscle is emphasized by our results with MEF2, which is dominantly expressed in skeletal muscle-fibroblast but not cardiac-fibroblast heterokaryons, supporting its divergent regulation in the two cell types.

Cardiac muscle and skeletal muscle are derived from embryonic mesoderm and express many of the same muscle-specific genes. Several transcription factors which regulate tissue-specific expression in skeletal muscle are also involved in tissue-specific expression in cardiac muscle, although several genes expressed in both skeletal and cardiac tissue appear to utilize different *cis* regulatory regions within each context (4, 25). The skeletal muscle program appears to be activated in response to the expression of members of the MyoD family. The MyoD family are members of the helix-loop-helix transcription factor family and include MyoD, myogenin, MRF4, and Myf5 (10). Ectopic expression of each of these factors in cells of diverse embryonic origin results in activation of skeletal muscle genes, which has led to their being described as master regulators. Although many of the skeletal muscle genes which are activated in response to expression of the MyoD family are also those which are expressed in cardiac muscle, the MyoD family does not appear to be expressed in cardiac cells to an appreciable extent, although there is one report of MyoD being expressed at low levels in adult *Xenopus* heart (15).

Prior to the cloning of MyoD, the existence of a dominant regulator of the skeletal muscle phenotype had been inferred from transfection experiments in which genomic DNA from skeletal muscle cells was transfected into a continuous embryonic fibroblast cell line, C3H10T1/2 (10T1/2) cells, and was able to convert them to differentiating skeletal muscle at a frequency compatible with there being one or a few closely linked loci responsible for the conversion (18, 27). Additional evidence for a dominant skeletal muscle determination gene came from cell fusion studies in which polyethylene-glycol (PEG)-mediated fusion of skeletal muscle myotubes with a variety of other cell types resulted in the activation of a broad range of skeletal muscle genes in the nonmuscle nuclei within heterokaryons (1, 2). The extent of activation was dependent on cell type, the nuclear ratio of each cell type within each heterokaryon, and length of time after fusion. Activation of skeletal muscle genes was seen with the greatest frequency in muscle-fibroblast heterokaryons and occurred at high frequencies even when fibroblast nuclei outnumbered skeletal muscle nuclei. One explanation for this facility of conversion was that both cell types are of mesodermal origin.

Although much has been learned about the determination of skeletal muscle, relatively little is known about factors involved in cardiac muscle determination. To date, no cardiac analog of MyoD has been described. Accordingly, in these studies we have performed fusions between differentiated rat cardiac and mouse embryonic fibroblasts to determine whether the presence of a dominantly acting cardiac factor could be detected in the heterokaryon context. In addition, our studies were designed so that if such a factor were detected, we would have a genetic selection approach to cloning the factor.

Within the context of cardiac-fibroblast heterokaryons, we have examined the expression of three markers of the cardiac lineage: a 250-bp promoter fragment of the myosin light-chain 2 (MLC2) gene (36) and the expression of two genes expressed in cardiac cells in a tissue-restricted manner, atrial natriuretic factor (ANF) (16), and muscle enhancer factor 2 (MEF2) (12). MEF2 is a transcription factor whose protein is expressed in differentiated skeletal muscle and cardiac cells, but not in fibroblast cells (12, 35). In skeletal muscle, MEF2 appears to be involved in an autoregulatory loop with members of the

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MyoD family (5, 9). MEF2 appears to be activated by expression of MyoD and myogenin and is also involved in activation of myogenin. As MyoD and myogenin are not expressed in cardiac cells, activation of MEF2 in cardiac cells must be independent of the MyoD family, but might share a common activator in skeletal and cardiac muscle which is downstream of MyoD in skeletal muscle. Results from previous experiments indicated that fusion of myotubes with fibroblasts results in activation of many muscle-specific genes, including those encoding MyoD and myogenin, which are known to activate MEF2 (5, 26). We have examined the expression of MEF2 protein in both cardiac-fibroblast and skeletal muscle-fibroblast heterokaryons to allow direct comparison of results with the two types of fusions, and to determine whether a common element of the regulatory pathway to expression of MEF2 in cardiac and skeletal muscle could be detected.

MATERIALS AND METHODS

Cell culture and fusion conditions. Primary ventricular myocytes were isolated from neonatal rats and purified by Percoll gradient according to published protocols for rat cardiac cells (36). Primary mouse embryonic fibroblast cell lines were prepared from 14- to 17-day embryos, the heart and liver being removed before the cells were isolated as described previously (29). The embryos were obtained from a cross between line 3 MLC250-luciferase parents which were heterozygous for the transgene (20). Individual embryonic hearts were assayed for luciferase activity by luciferase assays or by immunostaining of isolated cardiac cells. Primary embryonic fibroblast cultures were prepared from embryos whose hearts were positive for luciferase activity. To prepare embryonic cardiac cell cultures for luciferase staining, embryonic hearts were isolated, and approximately one-third of the tissue was assayed for luciferase activity. The remaining two-thirds of the tissue was treated with collagenase in the manner described for isolation of rat cardiac cells (36). Cardiac myocytes were not purified from nonmyocytes, and cells from the whole heart were plated directly and maintained in the same culture conditions as those used for primary neonatal ventricular myocyte cultures.

The mouse embryonic fibroblast 10T1/2 cell line was obtained from and grown according to American Type Culture Collection specifications. The rat skeletal muscle cell line RMo was obtained from Paul Gardner of the University of Texas Health Sciences Center at San Antonio and was cultured as described previously (22).

For fusion studies involving cardiac cells, primary ventricular myocytes were coplated with either mouse embryonic fibroblasts from the transgenic animal or mouse 10T1/2 fibroblasts in plating medium consisting of 10% horse serum and 5% fetal bovine serum in Dulbecco's modified Eagle's medium (DMEM)-Medium 199 (4:1; Gibco) onto coverslips precoated with 1% gelatin and 20 μg of laminin per ml (Sigma) in phosphate-buffered saline (PBS) in 35-mm tissue culture plates. Control cultures of each cell type alone were plated with identical conditions. One day later, cells were fused by treatment with 35 to 39% (vol/vol) PEG 1000 (Sigma) in DMEM for 1 min at 37°C, as described previously (6). PEG solution was washed from the cells by dipping coverslips several times in successive solutions of warmed DMEM, and cells were then incubated in plating medium. Cytosine arabinoside (Sigma) was added to the medium at a final concentration of 10 μM 6 h following fusion for cells fixed 24 h after fusion, or 24 h postfusion for cells fixed 48 h or longer after fusion.

For fusion studies between rat RMo cells and mouse 10T1/2 cells, RMo cells were grown to confluence and induced to differentiate in DMEM with 4% horse serum. Cytosine arabinoside was added to the medium to a final concentration of 10 μM 6 h later. The next day, the cells were trypsinized lightly with 0.05% trypsin–EDTA (Gibco BRL) and coplated with mouse 10T1/2 cells onto glass coverslips precoated with 1% gelatin and 0.1 mg of poly-L-lysine (molecular weight > 300,000 [Sigma]) per ml in PBS in 35-mm tissue culture plates. Controls of each cell type alone were also plated in the same manner. To induce fusion, cultures were treated with 42 to 45% (vol/vol) of PEG in DMEM, and the fusion protocol was performed as described above for cardiac-fibroblast cultures.

Nuclei were visualized by Hoechst 33258 staining, which discriminates between mouse and rat nuclei: mouse nuclei have a punctate appearance, whereas rat nuclei stain smoothly. Heterokaryons were determined as clusters of adjacent rat and mouse nuclei within the same cytoplasm. With the above fusion conditions, an average of 42% of cardiac nuclei, 40% of fibroblast nuclei, and 44% of skeletal muscle myotube nuclei formed heterokaryons.

Calcium phosphate transfection. Rat cardiac myocytes were transfected as described previously (11).

Luciferase assays. Luciferase assays were performed as previously described (11).

Immunofluorescent analyses. Antibodies were very generously provided as follows: monoclonal mouse anti-ANF antibody (31) was sent by Chris Glembotski of San Diego State University, monoclonal mouse anti-MyoD antibody (8) was sent by Peter Dias of St. Jude Children's Research Hospital, and rabbit anti-MEF2 antibody was sent by Yie-Teh Yu of Vanderbilt University. The MEF2 antibody was raised against a glutathione *S*-transferase fusion peptide containing predicted amino acids 129 to 253 of the MEF2 cDNA clone (35). Other antibodies were obtained commercially: rabbit antiluciferase antibody was from Cortex Biochemicals; fluorescein isothiocyanate (FITC)-, rhodamine-, and Texas Red-conjugated antibodies were from Jackson Immunochemicals; streptavidin-coupled Texas Red-conjugated antibody was from Amersham.

Luciferase immunostaining. Cells were washed in PBS and fixed in 3.7% formaldehyde in PBS for 10 min at room temperature. After two washes in PBS, they were permeabilized for 10 min at room temperature with 0.25% Triton X-100 in PBS. Cells were washed twice in PBS–0.1% Tween 20 (Sigma) and blocked with 10% goat serum–PBS–0.1% Tween 20 for 10 min at room temperature. The cells were incubated with rabbit antiluciferase antibody diluted 1:400 for 1 h at 37°C and then with either donkey anti-rabbit FITC-conjugated antibody diluted 1:200 or donkey anti-rabbit Texas Red-conjugated antibody diluted 1:800 for 1 h at 37°C. Nuclei were stained with Hoechst 33258 (Sigma, 1 μg/ml) for 5 min before being mounted in gelvatol.

ANF immunostaining. Cells were washed, fixed, permeabilized, and blocked under conditions identical to that for luciferase immunostaining. Cells were then incubated with mouse anti-ANF antibody (diluted 1:200 in 10% goat serum–PBS–0.1% Tween 20) for 1 h at 37°C. After three washes in PBS–0.1% Tween 20, biotin-coupled donkey anti-mouse antibody was added at a 1:100 dilution for 1 h at 37°C. After three washes in PBS–0.1% Tween 20, cells were incubated with streptavidin-coupled Texas Red-conjugated antibody diluted 1:200 for 1 h at 37°C. Before mounting, nuclei were stained with Hoechst 33258.

MyoD immunostaining. Cells were washed with PBS and fixed in 1% paraformaldehyde in PBS for 20 min at room

temperature. After two washes in PBS, 0.25% Triton X-100 was added for 20 min at room temperature to permeabilize the cells. After two washes with PBS, cells were incubated with mouse anti-MyoD antibody diluted 1:400 in PBS for 1 h at 37°C. Cells were then washed twice in PBS before incubation with donkey anti-mouse rhodamine-conjugated antibody diluted 1:200. Nuclei were stained with Hoechst 33258 before being mounted.

MEF2 immunostaining. Cells were washed with PBS and fixed and permeabilized similarly as for MyoD immunostaining. Cells were washed twice in PBS and then incubated with 3% bovine serum albumin (BSA)–PBS–Nonidet P-40 for 30 min at room temperature, and then with rabbit anti-MEF2 (C3) antibody diluted 1:400 in 3% BSA–PBS–0.1% Nonidet P-40 for 1 h at 37°C. After a brief wash in PBS–0.1% Nonidet P-40, cells were incubated with donkey anti-rabbit Texas Red-conjugated antibody diluted 1:800 for 1 h at 37°C. Nuclei were stained with Hoechst 33258 before being mounted.

Cell counting. Stained cells were visualized with a Nikon Diaphot-TMD inverted microscope with epifluorescence equipment. The Hoechst 33258-stained nuclear population was visualized under UV light excitation with a Nikon UV-2A filter. FITC staining results were visualized with blue light excitation with a Nikon B-2A filter; Texas Red and rhodamine staining results were visualized with green light excitation with a Nikon G-2A filter. Microscope fields of heterokaryons were selected from Hoechst 33258-stained nuclei. For each field selected, the Hoechst 33258 as well as the FITC, Texas Red, or rhodamine results were photographed at 40× with Kodak 400 Ektachrome film. The numbers and types of nuclei in heterokaryons or homokaryons were counted and tabulated. Positive expression was defined by comparison of the expression of the marker in positive and negative controls within each experiment. Heterokaryons were categorized on the basis of the ratio of rat nuclei to mouse nuclei.

Statistical analyses. Error bars on graphs indicate the standard error of the proportion, as used previously (23), and were calculated as the square root of p multiplied by q divided by n, where p and q are the respective proportions, and n is the sample size. Where error bars do not overlap, differences are significant at the 0.05 level, with a two-sample Student's t test.

RESULTS

Activation of luciferase expression in cardiac-fibroblast heterokaryons. To determine whether fibroblast nuclei could be activated in *trans* by fusion to cardiac cells, we used embryonic fibroblast cells derived from transgenic mice which harbor a luciferase reporter gene downstream of a cardiac tissue-specific promoter, a 250-bp fragment of the MLC2 promoter. This transgene is expressed at high levels in a tissue-specific manner in the hearts of the transgenic mice, and its expression parallels that of the endogenous MLC2 gene, being expressed very early in embryonic development (19, 20, 24). By assaying for luciferase expression, we could specifically examine activation in fibroblast nuclei within heterokaryons. Cardiac tissue-specific expression of the transgene indicates that the integration site of the transgene, at least in the cardiac context, does not prevent activation by cardiac factors, and would probably allow for activation within the fibroblast nuclei upon fusion to cardiac cells, therefore overcoming some of the problems which have been encountered with stable cell lines in similar studies (14, 32).

As our transgenic lines were heterozygous, embryonic hearts were dissected out as part of the protocol for fibroblast preparation and were subsequently assayed for enzymatic luciferase activity. The embryonic fibroblasts used in the fusion experiments were derived from embryos which had high levels of cardiac luciferase activity (example shown in Table 1), indicating that they harbored the transgene. The cardiac myocytes which were fused to these primary embryonic fibroblasts were rat primary neonatal ventricular myocytes which have withdrawn from the cell cycle and express endogenous MLC2 at high levels. As we intended to assay for luciferase activation in heterokaryons by immunofluorescent antibody staining to detect luciferase protein, we cultured primary cardiac cells from hearts of transgenic mouse embryos to determine whether we could detect luciferase in this manner. The embryonic hearts were also assayed for luciferase activity. Primary embryonic cardiac cultures included both myocytes and nonmyocytes. Cells staining positively for the presence of luciferase protein were observed in cardiac cultures prepared from hearts which were positive for luciferase activity (Fig. 1).

TABLE 1. Luciferase activities in extracts from hearts of MLC2-luciferase transgenic mouse embryos[a]

Embryo	Luciferase activity
1	35,966, 39,110
2	51,162, 50,364
3	22, 16
4	30,704, 33,958
5	22, 14
6	36,521, 31,931
7	19, 17
8	22,526, 21,284

[a] Data shown are from one litter of line 3 × line 3 cross (20). Line 3 is heterozygous for the MLC2-luciferase transgene. Duplicate samples were assayed for each sample. Luciferase activities are in arbitrary units. For further experimental details, see Materials and Methods. As shown in the table, in this litter five of eight embryonic hearts were positive, and embryonic fibroblast cultures were prepared by pooling embryonic fibroblasts from positive embryos 1, 2, 4, 6, and 8.

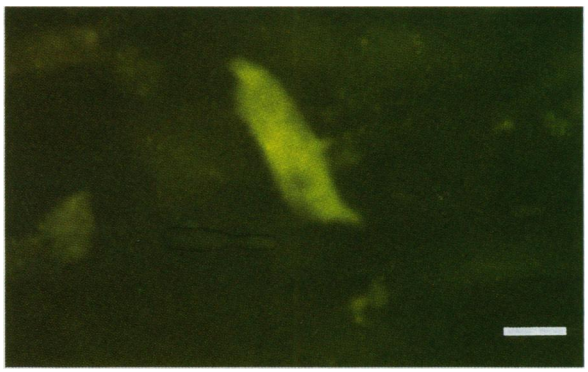

FIG. 1. Immunofluorescent antiluciferase-stained embryonic cardiac cells from MLC250-luciferase transgenic embryo. Cardiac cell cultures were prepared from embryonic hearts, fixed 2 days following plating, and incubated with primary rabbit antiluciferase antibody and secondary donkey anti-rabbit FITC-conjugated antibody (for details, see Materials and Methods). The cultures contain both myocyte and nonmyocyte cells and were plated at low density. The positively staining cell shown in this photograph demonstrates that cardiac cells from these transgenic mice express sufficient luciferase protein to be detected by immunostaining. This result is consistent with previous results which demonstrated specific luciferase staining in hearts of MLC2-luciferase transgenic mice (24). Bar, 15 μm.

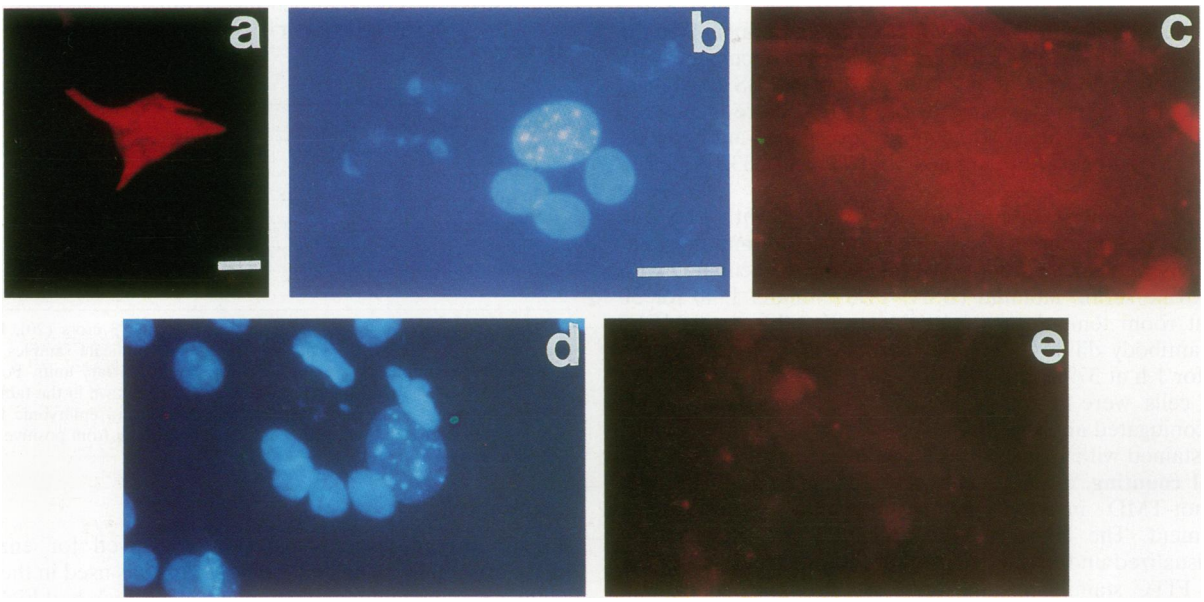

FIG. 2. Immunofluorescent antiluciferase- and Hoechst 33258-stained cardiac-embryonic fibroblast heterokaryons. PEG-treated cocultures of rat primary ventricular myocytes and mouse primary embryonic fibroblasts from MLC2-luciferase transgenic mice are shown. (a) Primary rat cardiocyte transfected with an MLC2-luciferase plasmid, fixed, and stained with antiluciferase antibody and Texas Red-conjugated secondary antibody, as a positive control for antiluciferase antibody staining. (b and c) Hoechst 33258 and antiluciferase staining, respectively, of a 3:1 cardiac-fibroblast heterokaryon. The Hoechst stain identifies the species origin of the nuclei, with rat nuclei staining smoothly and mouse nuclei having a punctate appearance. A faint luciferase-positive signal can be seen outlining and surrounding the four nuclei, indicating that luciferase has been activated in the transgenic fibroblast nucleus by fusion to cardiac cells. (d and e) Hoechst and antiluciferase staining, respectively, of a group of mixed-origin nuclei which are negative for luciferase expression. Bars, 15 μm. For experimental details, see Materials and Methods.

These positively staining cells were not observed in parallel cultures from hearts of littermates which were negative for luciferase activity.

Primary embryonic fibroblasts and primary neonatal ventricular myocytes were fused with PEG and left in high-serum-containing medium containing cytosine arabinoside for 3 days before being fixed and stained for luciferase protein. The cells were also stained with Hoechst dye, which discriminates between mouse and rat nuclei, enabling a determination of heterokaryons and their composition (3). The high-serum medium allows for high levels of expression of cardiac genes, including MLC2. The cytosine arabinoside prevents proliferation of the fibroblast cells and has been used extensively in similar studies with skeletal muscle cells (3), where prevention of DNA synthesis has been shown to have no effect on the transactivation of skeletal muscle genes in heterokaryons. As a positive control for luciferase staining, an MLC250-luciferase plasmid was transfected into neonatal cardiac myocytes, and the transfected cells were stained for luciferase protein (Fig. 2). Negative controls were performed by fusing each cell type alone.

Activation of luciferase expression was observed only in heterokaryons, although the observed luciferase signal was weak relative to that seen with cells transfected with the MLC250-luciferase construct (Fig. 2). The luciferase signal was observed with greater frequency as the ratio of cardiac to fibroblast nuclei increased, but occurred in less than half of the heterokaryons even when the ratio of cardiac to fibroblast nuclei was greater than 3:1 (Fig. 3). These results indicated that the cardiac program is recessive vis-a-vis fibroblast nuclei, unlike the situation with skeletal muscle and fibroblast heterokaryons. Alternately, the lack of expression of luciferase could be a peculiarity of the transgene, or the primary embryonic

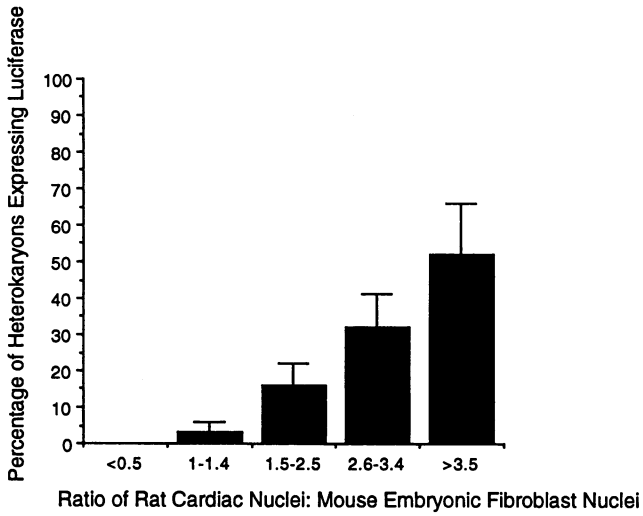

FIG. 3. The effect of nuclear ratio on luciferase expression in heterokaryons. Luciferase protein was detected by immunostaining of fused rat cardiac and mouse primary embryonic fibroblasts, and nuclear composition was determined by Hoechst stain. The results indicate that expression of the MLC2-luciferase transgene in fibroblast nuclei is increasingly activated as the ratio of cardiac nuclei to fibroblast nuclei increases. However, only 55% of heterokaryons express luciferase to detectable levels even at the highest ratios of cardiac to fibroblast nuclei observed, indicating that in the majority of heterokaryons, luciferase is not activated to detectable levels. Error bars indicate the standard error of the proportion. A total of 137 heterokaryons were scored in this experiment. For experimental details and a description of scoring and statistical analyses, see Materials and Methods.

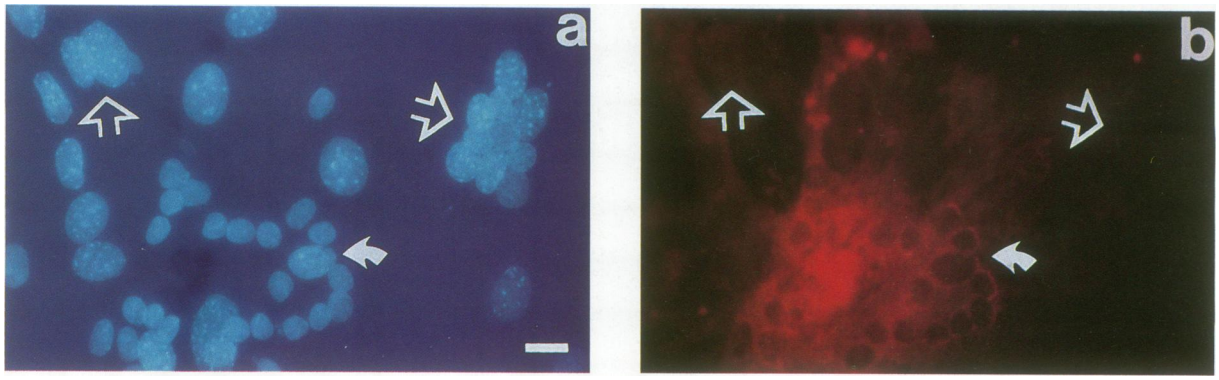

FIG. 4. Immunofluorescent anti-ANF and Hoechst 33258 staining of cardiac-embryonic fibroblast heterokaryons. PEG-treated cocultures of rat primary ventricular myocytes and mouse primary embryonic fibroblasts from MLC2-luciferase transgenic mice are shown. (a and b) Hoechst staining and anti-ANF staining, respectively, of several heterokaryons within one field, indicated by arrows. ANF expression is extinguished in two of the heterokaryons, with 1:3 and 5:5 ratios of cardiac-fibroblast nuclei (outlined arrows). On the other hand, ANF is expressed in a heterokaryon in which the number of cardiac nuclei exceeds the number of fibroblast nuclei (solid arrow). Bar, 15 μm. For experimental details, refer to Materials and Methods.

fibroblasts, or both. Accordingly, we repeated these experiments, examining expression of two endogenous cardiac genes and using a well-characterized continuous embryonic fibroblast cell line, 10T1/2 (28), in addition to the primary transgenic embryonic fibroblasts.

Expression of ANF in cardiac-fibroblast heterokaryons. The ANF gene is expressed at high levels in neonatal ventricular myocytes incubated in high-serum-containing medium (16). ANF is secreted from cardiac cells with a transit time of approximately 5 h (31). The secretion of ANF makes it a suitable marker for examining the question of cardiac phenotype extinction within the time course allowed in heterokaryon studies, although admittedly heterokaryon formation could affect the secretion process itself. Fusions of rat ventricular myocytes and transgenic mouse embryonic fibroblast cells, followed by immunostaining for ANF, indicated that expression of ANF within heterokaryons (Fig. 4 and 5) was consistent with the expression of luciferase within heterokaryons which had been observed in previous experiments (Fig. 3). ANF expression was observed more frequently as the proportion of cardiac nuclei was increased relative to fibroblast nuclei. At 1:1 ratios of cardiac to fibroblast nuclei, more than half of the heterokaryons do not express ANF. This extinction of ANF expression is not owing to the fusion procedure, as homokaryons continued to express ANF at a high frequency (Fig. 5). The percentage of heterokaryons expressing ANF is overall greater than that of those expressing luciferase, which may be a reflection of the greater signal strength of ANF, a peculiarity of the transgene, or an indication that although cardiac genes are expressed in cardiac nuclei, they may not be transactivated as readily in fibroblast nuclei. The data shown in Fig. 5A, B, C, and D are from cells fixed 2 days following fusion. A time course experiment was performed, examining the heterokaryons at 2, 4, and 6 days after fusion. A similar profile of ANF expression was observed in heterokaryons throughout this time course, with ANF expression being extinguished in a majority of equal-ratio heterokaryons (Fig. 5E and F).

These results with luciferase and ANF expression in heterokaryons indicated that the cardiac phenotype, unlike the skeletal muscle phenotype, is not dominant vis-a-vis the fibroblast phenotype. To confirm this observation, we fused neonatal ventricular myocytes to a continuous embryonic fibroblast cell line, 10T1/2, which has been used in many studies of myogenic determination genes, and in cell fusion studies with skeletal muscle (7, 26), and is readily converted to the skeletal muscle phenotype by transfection of MyoD family members and by fusion to skeletal muscle cells. Results from these fusions were consistent with the results from fusions with the primary embryonic fibroblasts (Fig. 6).

Expression of MEF2 in cardiac-fibroblast heterokaryons. Our results indicated that expression of one cardiac promoter failed to be activated and another cardiac gene was extinguished by fusion to embryonic fibroblast cells in heterokaryons with equal genetic material from each parent. As subsets of tissue-specific genes have been shown to be regulated by diverse factors (13), we wanted to examine another cardiac gene to determine whether we could find a member of a subset of cardiac genes which would behave differently. MEF2 is a transcription factor which appears to play an important role in both skeletal and cardiac muscle gene transcription (25), and the protein is not expressed in fibroblast cells, including 10T1/2 cells (12, 35). As with many transcription factors, MEF2 appears to have a relatively short half-life within the cell, approximately 8 to 10 h (34a), which made it an appropriate subject to examine the question of extinction in heterokaryons. In these studies, we have utilized an antibody which preferentially recognizes the MEF2/aMEF2 gene (34a) (see also Materials and Methods).

Results from fusion studies between rat cardiomyocytes and mouse 10T1/2 embryonic fibroblasts indicated that MEF2 expression was affected in the same way that ANF expression was affected. Results from a time course experiment, examining MEF2 expression at 1, 2, and 3 days following fusion, are shown (Fig. 7 and 8). At the 1-day time point, more than half of the 1:1 heterokaryons are expressing MEF2. However, by 2 days, less than half of this group are expressing MEF2, and this situation remains stable after 3 days. These results are consistent with those observed for ANF gene expression and indicate that the expression of several cardiac genes is extinguished in the majority of equal-ratio heterokaryons.

To confirm that our results with cardiac cells differ from those with skeletal muscle cells, we fused rat RMo skeletal muscle myotubes with mouse 10T1/2 cells and immunostained resulting heterokaryons with MyoD and MEF2 in separate experiments. Our results with MyoD immunostaining con-

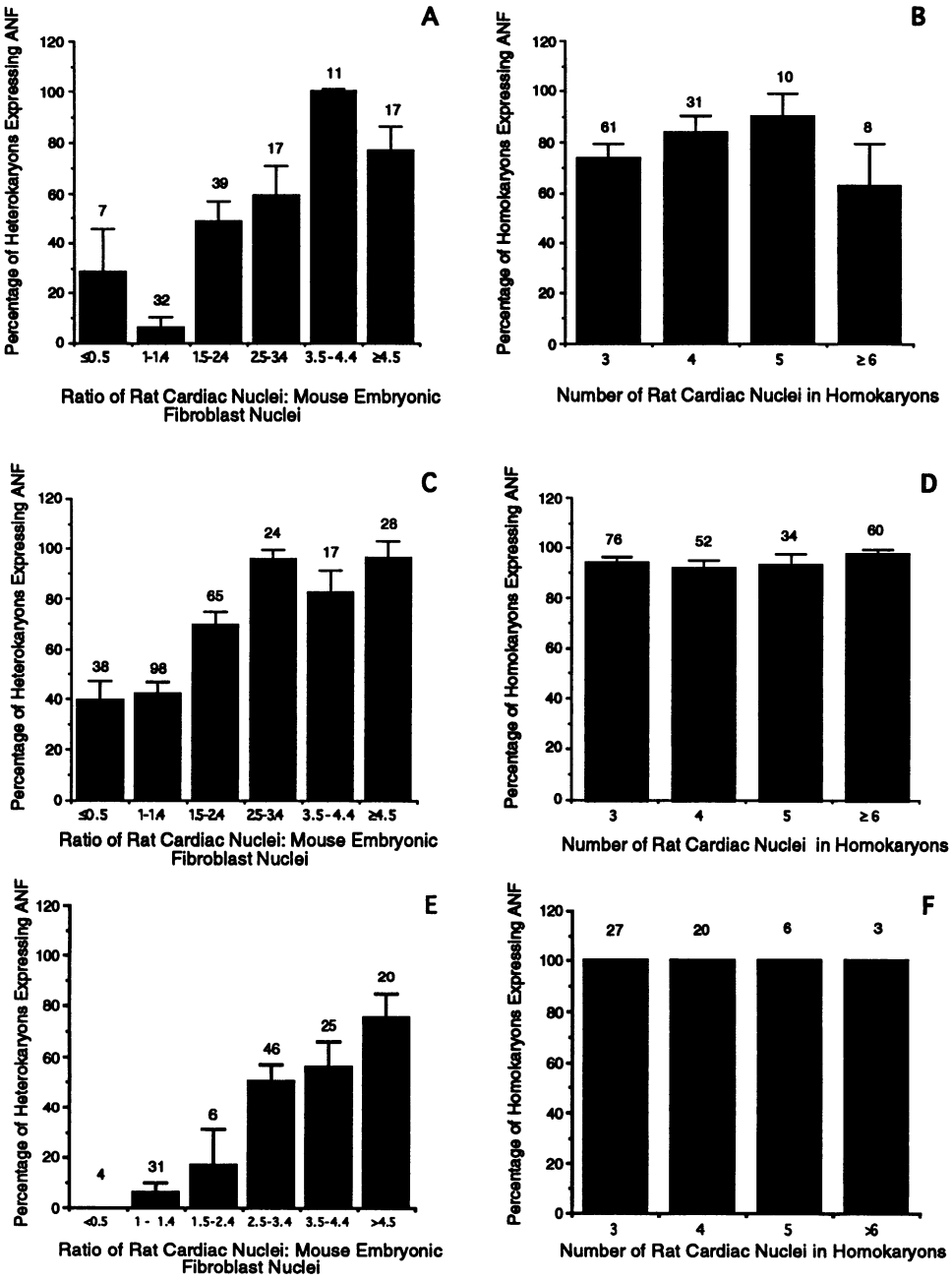

FIG. 5. The effect of nuclear ratio on ANF expression in cardiac-primary embryonic fibroblast heterokaryons. ANF protein was detected by immunostaining of fused rat cardiac and mouse primary embryonic fibroblasts, and nuclear composition was determined by Hoechst stain. (A and C) Heterokaryon results from two separate experiments indicate that expression of ANF is extinguished in a majority of equal-ratio heterokaryons scored 2 days following PEG-mediated fusion. The variability observed in 1-1.4 samples may be a result of heterogeneity in primary cultures, as results with the continuous cell line 10T1/2 were less variable (Fig. 6). (B and D) In both experiments, most homokaryons continue to express ANF, including those with large numbers of nuclei, indicating that the effect observed for heterokaryons is due to the presence of fibroblast nuclei. A total of 393 heterokaryons were scored in these experiments. (E) Extinction of ANF expression in a majority of equal-ratio heterokaryons is still observed 6 days following PEG-mediated fusion. (F) Control homokaryons continue to express ANF 6 days following fusion. A total of 132 heterokaryons were scored in this experiment. Error bars indicate the standard error of the proportion, and numbers above each category indicate the number of individual heterokaryons or homokaryons scored. For experimental details and a description of scoring and statistical analyses, see Materials and Methods.

firmed the results of other investigators, in that MyoD was expressed in a large majority of heterokaryons, including those with a larger proportion of fibroblast than skeletal muscle nuclei (data not shown). Similarly, results of skeletal muscle-fibroblast fusions stained with antibody to MEF2 indicated that all myotube heterokaryons examined expressed MEF2 (Fig. 9 and 10), as expected from results of previous investigators (5, 26).

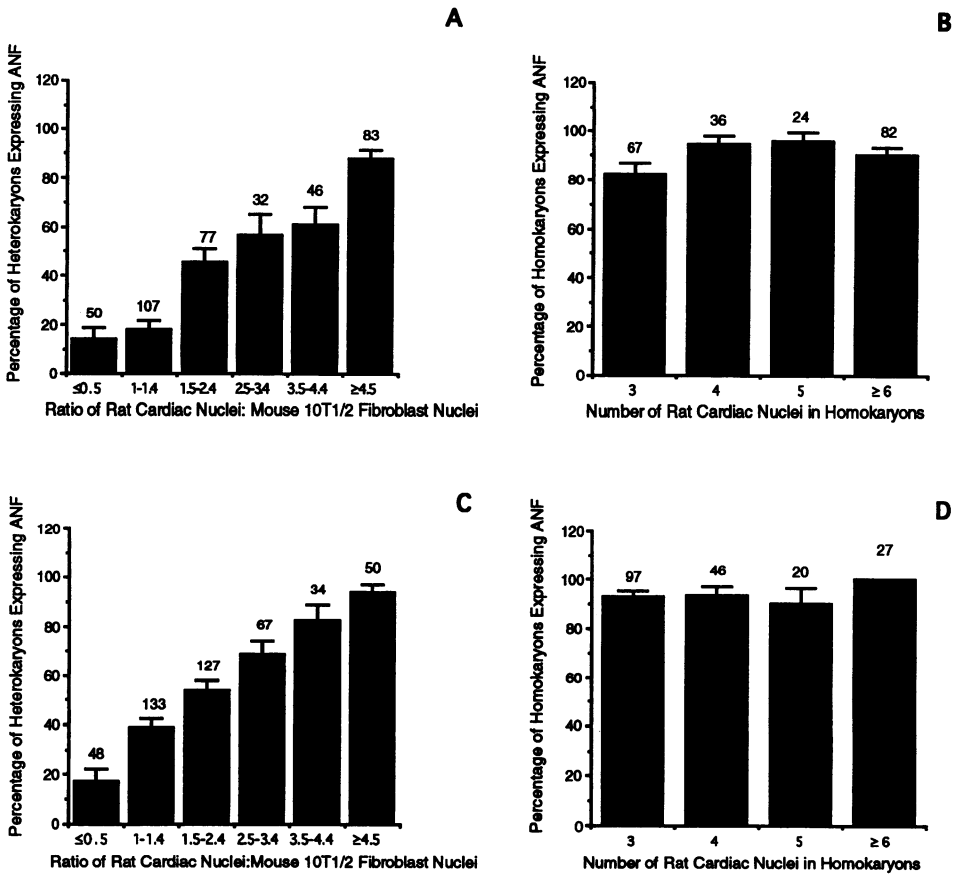

FIG. 6. The effect of nuclear ratio on ANF expression in cardiac-10T1/2 fibroblast heterokaryons. ANF protein was detected by immunostaining of fused rat cardiac and mouse 10T1/2 fibroblasts, and nuclear composition was determined by Hoechst stain. (A and C) Heterokaryon results from two separate experiments indicate that expression of ANF in heterokaryons is progressively extinguished as the ratio of the number of fibroblast nuclei to that of cardiac nuclei is increased, as seen previously with fusions of cardiac cells to primary embryonic fibroblasts. (B and D) ANF expression in homokaryons continues at high levels, including in those with large numbers of nuclei, indicating that the extinction of ANF observed in heterokaryons is due to the inclusion of fibroblast nuclei. Error bars indicate the standard error of the proportion, and numbers above each category indicate the number of individual heterokaryons or homokaryons scored. A total of 854 heterokaryons were scored in the experiments shown here. For experimental details and a description of scoring and statistical analyses, see Materials and Methods.

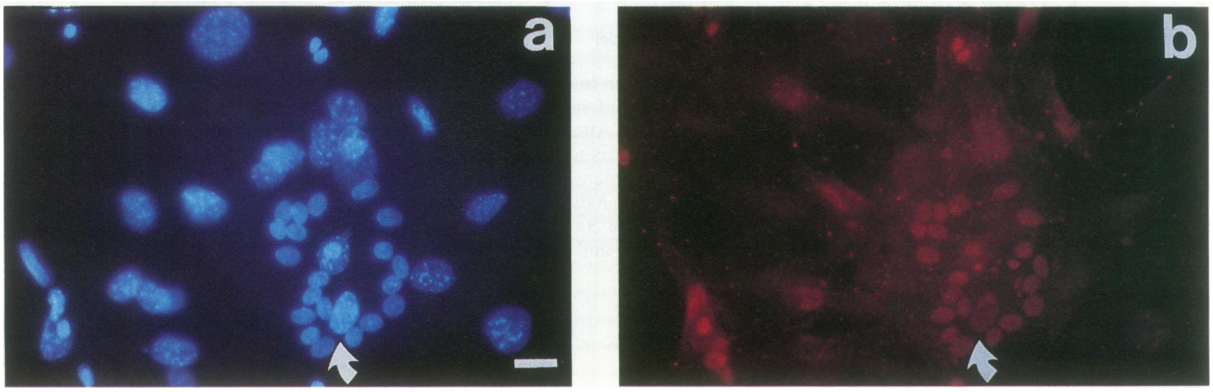

FIG. 7. Immunofluorescent anti-MEF2- and Hoechst 33258-stained cardiac-10T1/2 fibroblast heterokaryons. PEG-treated cocultures of rat primary ventricular myocytes and mouse 10T1/2 fibroblasts are shown. (a and b) Hoechst staining and anti-MEF2 staining, respectively, of a cardiac-fibroblast heterokaryon, indicated by the arrow. In this heterokaryon, cardiac nuclei greatly outnumber fibroblast nuclei, and MEF2 protein is expressed. Bar, 15 μm. For experimental details, refer to Materials and Methods.

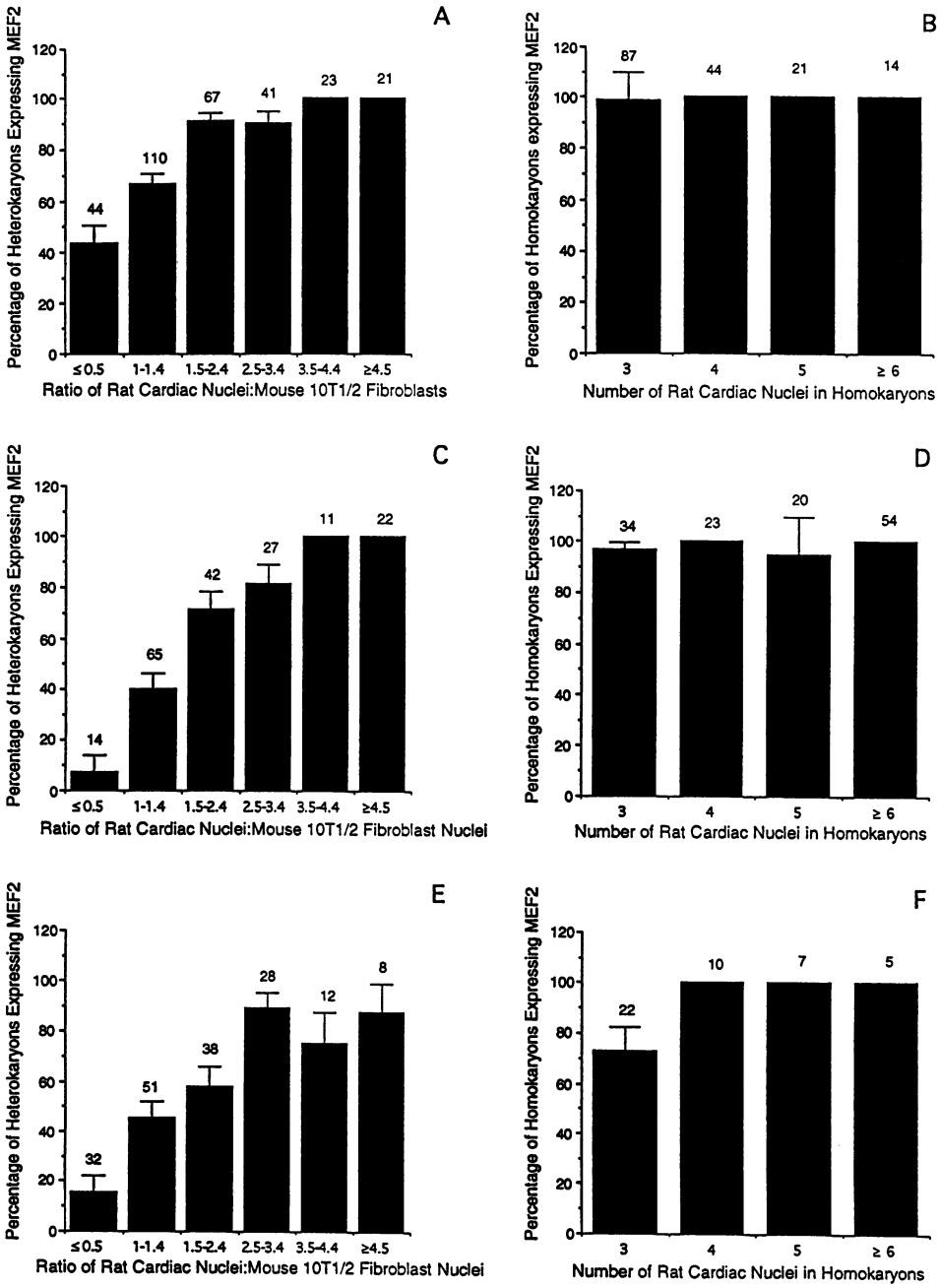

FIG. 8. Time course of the effect of nuclear ratio on MEF2 expression in cardiac-10T1/2 fibroblast heterokaryons. MEF2 protein was detected by immunostaining of fused rat cardiac and mouse 10T1/2 fibroblasts, and nuclear composition was determined by Hoechst stain. (A, C, and E) Heterokaryon results determined 24, 48, and 72 h postfusion, respectively. After 24 h, a majority of equal-ratio heterokaryons are still expressing MEF2; however, after 48 and 72 h this is no longer true. MEF2 expression appears to be stabilized after 48 h. (B, D, and F) In all three experiments, MEF2 continues to be expressed in a large majority of homokaryons, including those with large numbers of nuclei, indicating that extinction of MEF2 expression in heterokaryons results from the presence of fibroblast nuclei. Error bars indicate the standard error of the proportion, and numbers above each ratio category indicate the number of individual heterokaryons scored. Totals of 306, 181, and 169 heterokaryons were scored for the 24-, 48-, and 72-h time points, respectively. For experimental details and a description of scoring and statistical analyses, see Materials and Methods.

DISCUSSION

Cardiac muscle-fibroblast heterokaryons reveal lack of dominantly acting regulators of several cardiac genes. In these experiments, we have examined the activation of an ectopic cardiac tissue-specific promoter-reporter gene (MLC250-luciferase) in primary embryonic fibroblast nuclei in response to fusion with differentiated cardiac cells. Our results indicate that the cardiac promoter can be activated within the fibroblast nuclei, but only to a significant extent in heterokaryons in which the number of cardiac nuclei exceeds the number of fibroblast nuclei. The ectopic cardiac promoter was not activated in heterokaryons in which the number of

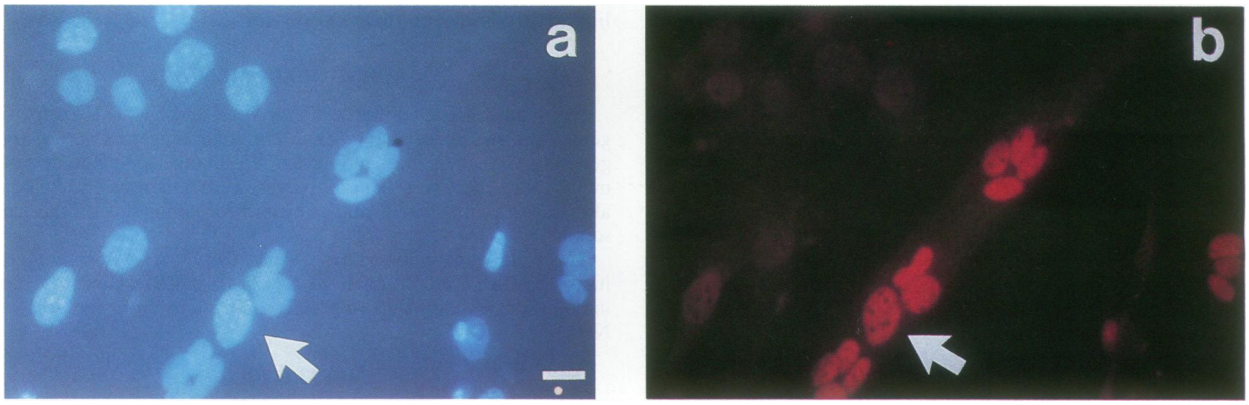

FIG. 9. Immunofluorescent anti-MEF2- and Hoechst 33258-stained skeletal muscle-10T1/2 fibroblast heterokaryons. PEG-treated cocultures of differentiated rat skeletal muscle cells and mouse 10T1/2 fibroblasts are shown. (a and b) Hoechst staining and anti-MEF2 staining, respectively, of skeletal-fibroblast heterokaryon. The heterokaryon is expressing MEF2 protein, with the fibroblast nucleus staining positively for MEF2 protein (arrow). Every myotube-fibroblast heterokaryon observed expressed MEF2, consistent with the dominance of the skeletal muscle phenotype in the presence of fibroblast nuclei. Bar, 15 μm. For experimental details, see Materials and Methods.

fibroblast nuclei was equal to or in excess of that of cardiac nuclei.

To examine the expression of endogenous cardiac genes in cardiac-primary fibroblast heterokaryons, we fused both primary embryonic fibroblasts and embryonic fibroblasts from a continuous cell line to cardiac ventricular myocytes and scored for the expression of two cardiac genes, ANF and MEF2. Fusions with each type of embryonic fibroblasts gave consistent results. Expression of both cardiac markers was extinguished in a majority of heterokaryons which had an equal or excess ratio of fibroblast to cardiac nuclei. Both cardiac markers continued to be expressed in the majority of heterokaryons in which the number of cardiac nuclei exceeded that of fibroblast nuclei. These results are consistent with those obtained with the luciferase reporter and indicate that diverse elements of the cardiac program including a myofibrillar protein promoter, a secreted hormone, and a muscle-specific transcription factor respond in a similar fashion when exposed to embryonic fibroblast cell factors.

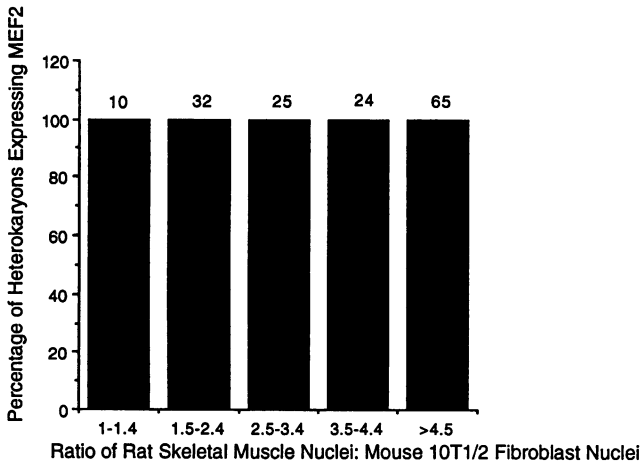

FIG. 10. Nuclear ratio does not appear to affect MEF2 expression in skeletal muscle-10T1/2 cell heterokaryons. MEF2 protein was detected by immunostaining of PEG-fused rat skeletal muscle myotubes and mouse 10T1/2 fibroblasts, and nuclear composition was determined by Hoechst staining. In all myotube-fibroblast heterokaryons observed, MEF2 protein was expressed. In two of the 1:1 heterokaryons, the fibroblast nuclei were not positive for MEF2, but the skeletal muscle nuclei were, and the heterokaryon was scored as positive. Because of large numbers of nuclei in RMo myotubes, it was more difficult to find heterokaryons with equal ratios of fibroblast and skeletal muscle nuclei, and no heterokaryons in which the fibroblast nuclei outnumbered skeletal muscle nuclei were observed. Despite these limitations, a comparison of results with cardiac-fibroblast fusions indicates that, unlike cardiac cells, skeletal muscle cells dominantly express MEF2 when fused to fibroblast cells. Numbers above each ratio category indicate the number of individual heterokaryons scored. A total of 156 heterokaryons were scored in this experiment. For experimental details and a description of scoring and statistical analyses, see Materials and Methods.

The extinction of cardiac gene expression in a majority of cardiac-fibroblast heterokaryons in which the number of fibroblast nuclei is equal to or greater than the number of cardiac nuclei indicates that the fibroblast nucleus is dominant over the cardiac nucleus in the one-to-one situation, unlike the situation previously observed for skeletal muscle vis-a-vis fibroblast nuclei. This dominance could indicate negative factors present in the fibroblast nucleus, limiting positive factors in the cardiac nucleus which become diluted upon fusion, or a combination of the two. An alternative possibility is that a putative rat cardiac factor may function inefficiently in mouse cells.

Skeletal muscle genes are activated in fibroblast nuclei at an extremely high frequency in heterokaryons even when the fibroblast gene dosage is in excess of the skeletal muscle gene dosage. Our results with MEF2 emphasize this difference between cardiac and skeletal muscle gene programs in response to fusion with embryonic fibroblasts. Expression of MEF2 was extinguished in a majority of heterokaryons in which the number of fibroblast nuclei is equal to or in excess of that of cardiac nuclei, yet was observed in every fibroblast-myotube heterokaryon examined, consistent with results of previous investigators (3). These results also confirm that MEF2 is regulated by a different hierarchy of factors in cardiac and skeletal muscle tissues (10).

Relevance of skeletal muscle-fibroblast heterokaryons to the control of the cardiac muscle phenotype. The dominance of the skeletal muscle phenotype in fibroblast-myotube heterokaryons is consistent with the high frequency of conversion of

fibroblasts to skeletal muscle following transfection with genomic DNA, or MyoD family cDNAs (7, 27). In this context, and from our results, it is less likely that ectopic expression of a single locus by genomic DNA transfection could convert the embryonic fibroblast cell to a cardiac cell. Does this indicate that cardiac cells do not have a master regulator like the MyoD family?

The high frequency of conversion of fibroblast cells to skeletal muscle has been postulated to be due to their common mesodermal origin. However, cardiac cells, too, are of mesodermal origin and do not appear to convert these fibroblast nuclei. In fact, the cardiac phenotype is extinguished in the presence of an excess of fibroblast nuclei. This situation is reminiscent of expression of the skeletal muscle phenotype following fusion to hepatocytes, in which an excess of hepatocyte nuclei results in extinction of the skeletal muscle phenotype in a majority of heterokaryons (3, 23). That is, although the skeletal muscle cells have a master regulator, its ability to dominate is influenced by the cellular environment, whether or not complementing factors or negative factors are present (30, 34). Of the many cell types examined, fibroblast cells have been found to most readily convert to skeletal muscle when fused to myotubes, or when transfected with cDNAs of myogenic regulators, although there is heterogeneity in the frequency of conversion by myogenic regulators even among fibroblasts (7). 10T1/2 cells appear to provide an extremely hospitable environment to skeletal muscle (7). 10T1/2 cells, when treated with 5-azacytidine, give rise to muscle, bone, and fat cells (17, 33), which may indicate that they are a dorsal mesodermal stem cell. Perhaps the ready conversion of 10T1/2 cells to skeletal muscle in the presence of a dominantly acting positive regulator is an indication of the close embryonic lineage of these cells.

If cardiac cells have a positively acting dominant regulator, its presence might better be detected by fusions to fibroblast cells of closer embryonic relatedness, that is, of splanchnic rather than dorsal mesodermal origin, as cardiac cells derive from splanchnic mesoderm. An attractive candidate partner, then, would be cardiac fibroblast cells. Primary embryonic fibroblasts probably represent a diversity of fibroblast lineages, including those of splanchnic mesodermal origin, but the latter may be a minority of the population.

Genetic approaches to identifying regulators of the cardiac muscle gene program. In initiating these studies with primary embryonic fibroblasts derived from transgenic mice, we were attempting to develop an assay system for dominantly acting cardiac determination genes. The use of a cardiac reporter gene in a cellular context where it could be activated only in response to cardiac factors which could prevail in a noncardiac environment appears to be an effective assay for such factors. This approach has been explored by previous investigators employing stable cell lines (14, 32). Our approach using primary cell lines derived from transgenic animals offers the advantage that the integration effect is minimalized, in that tissue-specific expression can be assayed in the animal prior to isolation of the cell lines. The current availability of many transgenic animals bearing tissue-specific promoters should allow this approach to be generalized to other systems and different reporter genes, such as β-galactosidase, which could allow for genetic selection. Primary fibroblasts containing a responsive reporter gene could be transfected with pools of cDNA expression clones from an appropriate library and scored for expression of the reporter gene. Positive pools would be further divided, resulting in the eventual purification and isolation of an active clone, as has been reported previously for the cloning of receptor proteins with radiolabelled ligands to assay for their expression (21).

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