In Vivo System for Characterizing Clonal Variation and Tissue-specific Gene Regulatory Factors Based on Function

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Abstract. The inducibility of stably transfected α-cardiac actin genes differs among L cell clones. We examined the ability of muscle-specific factors to induce the expression of the human muscle α-cardiac actin gene promoter when stably transfected into mouse fibroblast L cells. This promoter is transcriptionally active in L cells at a low level, 2–5% of that in transfected muscle cells. Upon fusion with muscle cells to form heterokaryons, expression of the transfected α-cardiac actin gene promoter can be induced. However, induction is observed with only 10% of transfected L cell clones and the magnitude of this induction varies between 5- and 50-fold. These properties of the transfected L cell appear to be stably inherited. Our results are consistent with the hypothesis that muscle cells contain factors capable of increasing the transcription of the transfected gene, but that differences among L cell clones, possibly in the site of integration in the genome, determine the extent to which the gene can respond. By fusion into heterokaryons, transfectants with responsive genes can be identified. Such clones should prove useful in determining the basis for clonal variation. In addition, they provide an in vivo system for isolating functionally active tissue-specific transcription factors and the genes that encode them.

TISSUE-specific genes must be regulated by diverse mechanisms to accomplish the precise timing, cell specificity, and appropriate level of expression characteristic of development. Genes are often repressed or maintained in a quiescent state by higher orders of chromatin structure such as packaging into nucleosomes by histones, in particular H-1, and by extensive DNA methylation (Schlissel and Brown, 1984; Weintraub, 1984, 1985; Keshet et al., 1986). For certain genes it appears that once this block is released and a gene is rendered active, another level of regulation comes into play (Robins et al., 1982; Charnay et al., 1984; Shaw et al., 1985; Minty et al., 1986). This level involves the interaction of tissue-specific *trans*-acting factors with specific DNA sequences associated with the gene in *cis*. As a result, the transcription of an active gene is markedly increased.

The human α-cardiac actin gene uses several discrete DNA segments within a 485-bp region 5′ of the start site of transcription for accurately initiating tissue-specific expression in muscle cells (Gunning et al., 1984; Minty and Kedes, 1986; Minty et al., 1986; Miwa and Kedes, 1987). A fusion gene containing this tissue-specific sequence and the bacterial chloramphenicol acetyl transferase (CAT)[1] gene is expressed constitutively after transfection into L cells, but at a level 2–5% that in transfected C2C12 muscle cells (Minty et al., 1986). Furthermore, deletion of upstream segments within the 485-bp sequence leads to reduced transcriptional activity in muscle cells, but has no effect on transcription in L cells. These results suggest that the low activity in L cells probably does not result from the interaction of these sequences with repressors. Instead, the gene is transcriptionally active, but L cells appear to lack the tissue-specific transcription factors that muscle cells contain.

Here we examine the inducibility of expression of stably transfected α-cardiac actin genes in L cells. The system we used involves the formation of heterokaryons, nondividing, short-term cell hybrids. Studies with heterokaryons formed by fusing mouse muscle cells and human nonmuscle cells have previously demonstrated the existence of mouse muscle factors that can gain access to and activate previously silent endogenous human muscle genes in several cell types in the absence of changes in chromatin conformation that require DNA replication (Blau et al., 1983, 1985; Chiu and Blau, 1985; Hardeman et al., 1986*a*, Pavlath and Blau, 1986; Miller et al., 1988). These results have been corroborated and extended by others with similar muscle heterokaryons (Wright, 1984*a*, *b*; Clegg and Hauschka, 1987), and heterokaryons formed with other cell types (Enoch et al., 1986; Baron and Maniatis, 1986).

In this report, we show with heterokaryons that muscle cells contain factors that can induce the expression of an ex-

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1. *Abbreviation used in this paper*: CAT, chloramphenicol acetyl transferase.

2. For a preliminary report of these results, see Hardeman et al., 1986*b*.

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ogenous muscle gene stably transfected into L cells.[2] The degree of induction after heterokaryon formation with pools of transfected L cell clones was highly variable. This variability proved to be due to differences among transfected clones. The basal activity of the α-cardiac actin gene differed by as much as 15-fold. Fusion with muscle cells resulted in increased expression in only 10% of the clones analyzed and the range of this induction differed by 10-fold. Both the basal and inducible CAT activities appeared to be stably heritable properties of each L cell clone. As discussed, this novel system is well suited to the study of the basis for the clonal variation in the expression of stably transfected genes.

Our results also suggest that a transfected L cell can be used as a test system for characterizing factors that regulate a stably introduced, well-defined promoter of a tissue-specific gene. However, in contrast to the recent report of Lufkin and Bancroft (1987) in which pooled clones were used, our results suggest that the success of this approach relies on first characterizing individual clones of stable transfectants and identifying those that contain genes capable of responding maximally to regulatory factors in heterokaryons. The transcriptionally responsive clones can then be used as recipients to assay for *trans*-acting regulatory factors and the genes or templates encoding them. A potential advantage of the assay we describe is that it is based on the function of the factors in vivo. The need for such an approach is underscored by recent findings that some regulatory factors may act indirectly and not bind DNA or may function poorly in in vitro transcription assays (McKnight and Tjian, 1986; Jones et al., 1987).

Materials and Methods

Cell Cultures

The mouse muscle cell line, C2C12, is a diploid subclone isolated and karyotyped in our laboratory (Blau et al., 1983) from the C_2 cell line originally isolated from postnatal mouse skeletal muscle after repeated crush injury (Yaffe and Saxel, 1977). The rat muscle cell line, H9c2, is a clonal derivative of cells isolated from embryonic rat heart tissue (Kimes and Brandt, 1976) and was obtained from the American Type Culture Collection, Rockville, MD. Both types of muscle cells were maintained in a mitogen-rich medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (FCS) and 0.5% chick embryo extract (CEE) (Gibco, Grand Island, NY). Muscle cells were allowed to reach confluence in the mitogen-rich medium and then induced to differentiate by exposing them to mitogen-poor fusion medium, DMEM supplemented with 2% horse serum. When grown under these conditions, which promote in vitro skeletal muscle differentiation, the H9c2 cells fuse to form multinucleated myotubes similar to the C2C12 cells.

The origin and culture conditions of the transfected mouse L(TK-) cell line were described previously (Minty et al., 1986). These cells, designated L.pHCA485CAT, are stable transfectants which carry a construct containing 485-bp 5′ to the cap site of the human α-cardiac actin gene plus the first exon and 24 bp of the first intron ligated at the Fnu DII site at position 68 to the bacterial CAT gene. The CAT gene is a Hind III-Bam HI fragment from pSV2CAT (Gorman et al., 1982) which carries a SV-40 intron and polyadenylation site to ensure proper processing of transcripts. The plasmid pSV2neo was cotransfected into these cells to allow G418 selection. After 15 d of selection, ~100 of the surviving colonies were pooled and grown en masse. Subsequently, this pool of stable transfectants was subcloned by serial dilution into 96-well culture dishes. Wells that contained a single cell were identified visually and twenty colonies arising from these cells, designated L.pHCACAT.X, were passaged and analyzed for CAT activity. Clone A is L.pHCA485CAT.4, clone B is L.pHCA485CAT.6, clone C is L.pHCA485CAT.1.G2, and clone D is L.pHCA485CAT.1.H9.

Heterokaryon Formation

In preparation for heterokaryon formation, H9c2 or C2C12 cultures which were maintained in mitogen-poor fusion medium for 2 d and had well-established myotubes, were trypsinized and plated onto collagen-coated culture dishes (1.4 mg/ml of calf skin collagen; Calbiochem-Behring Corp., La Jolla, CA; autoclaved in dH_2O). After attachment of the muscle cells, the transfected L cells were added to the culture dishes and 4 h later the co-cultures were treated with polyethylene glycol 1000 (PEG; BDH Chemicals Ltd., Poole, England) (Blau et al., 1983). Cytosine arabinoside was added to the culture medium at 10^{-5} M immediately after PEG treatment for a total of 48 h to eliminate proliferating myoblasts and unfused L cells. The heterokaryon cultures were harvested after 4 d of maintenance in mitogen-poor fusion medium. The cultures were either harvested for assays of CAT enzymatic activity or stained with Hoechst 33258 (Sigma Chemical Co., St. Louis, MO) (Blau et al., 1983) to quantitate the nuclear composition of a given heterokaryon culture (Hardeman et al., 1986*a*).

CAT Assays

CAT enzymatic activity was determined using a modification of the procedure of Gorman et al. (1982). Cells were harvested in Tris-EDTA-NaCl buffer (0.04 M Tri-HCl, pH 7.4/1 mM EDTA/0.15 M NaCl). The cell pellet was dispersed in 0.25 M Tris HCl, pH 7.8 and lysed by freezing and thawing three times in succession. An aliquot was removed from the lysate supernatant and protein concentration determined. A typical enzymatic assay contained 1–30 μl of cell extract, 0.1–0.2 μCi of [^{14}C]chloramphenicol (New England Nuclear, Cambridge, MA; 40–50 mCi/mmol), 30 μl of 5 mM acetyl coenzyme A, and 0.25 M Tris HCl, pH 7.8 in a final volume of 150 μl. The enzymatic reaction was carried out at 37°C from 4 to 10 h with fresh acetyl CoA added every 4 h. The labeled chloramphenicol was extracted with ethyl acetate and analyzed by thin layer chromatography (chloroform/methanol, 95:5) using Baker-flex, Silica Gel 1B plates (J. T. Baker, Phillipsburg, NJ). The silica plate was exposed to XAR film and the relative levels of CAT enzymatic activity determined by scintillation counting of the appropriate region of the silica plate.

H9c2 Transfections

Plasmids containing various fragments of the human α-cardiac actin gene promoter ligated to the CAT gene were transfected into H9c2 myoblasts that were subsequently analyzed at two stages of differentiation: subconfluent, dividing myoblasts (B) and well-differentiated cultures exposed to fusion medium for 2 d (T). The construction of the plasmids was described in detail by Minty and Kedes (1986). Briefly, pHCAOCAT and pHCA485CAT contain 0 and 485 bp 5′ to the cap site of the gene, respectively. Transfections were carried out by mixing 5 μg of the appropriate DNA in 0.5 ml HEBS buffer (42 mM Hepes/274 mM NaCl/10 mM KCl/1.4 mM Na_2HPO_4/11 mM dextrose) with 0.5 ml of 280 mM $CaCl_2$. Precipitation of the $CaPO_4$–DNA complex proceeded for 30 min and then 1 ml was overlayed on 4 ml of medium per 60-mm dish. The medium was replaced with the appropriate fresh medium 20 h later and the cultures harvested for CAT analysis 36 h after exposure to DNA.

Results

CAT Activity in Transfected L Cells Fused with Mouse Muscle Cells in Heterokaryons

To determine whether mouse muscle-specific transcription factors can gain access to the human α-cardiac actin gene promoter in stably transfected L cell nuclei, we fused mouse muscle cells with transfected L cells to form heterokaryons. The skeletal muscle cells, C2C12, are particularly well suited to these experiments because they can be induced to differentiate rapidly and extensively into multinucleated myotubes that accumulate high levels of cardiac actin transcripts within 24 to 36 h (Bains et al., 1984; Hardeman et al., 1986*a*). The L cells were those previously described by Minty et al. (1986), which had been stably cotransfected with a DNA fragment (pHCA485CAT) containing 485 bp 5′ to

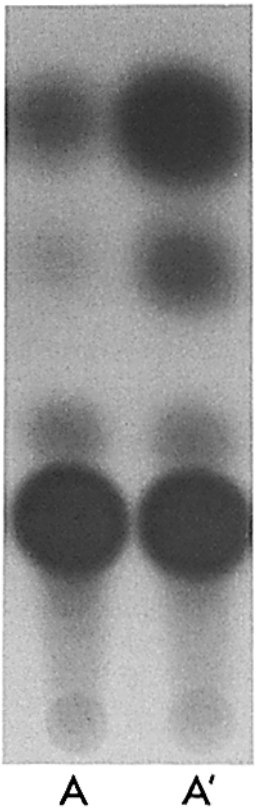

Figure 1. Inducible CAT activity in pooled clones of transfected L cells fused with myotubes to form heterokaryons. Heterokaryons were formed by the PEG-mediated fusion of differentiated mouse skeletal muscle cells, C2C12, and pools of stable L cell transfectants carrying the pHCA485CAT construct. As a control, a culture of L cells only was treated with PEG to form homokaryons. The cultures were harvested 4 d after PEG treatment. Equivalent amounts of protein from cultures of L cell homokaryons (*A*) and from C2C12 × L cell heterokaryons (*A'*) were assayed for CAT enzymatic activity. The 1- and 3-mono-acetylated forms of 14-C-chloramphenicol were separated from the unacetylated substrate by thin layer chromatography and the percent conversion to acetylated products determined by scintillation counting (Gorman et al., 1982). For (*A*) 0.13% and (*A'*) 2.52%, or a relative difference of 19-fold. The silica plate was exposed to XAR film for 2 wk.

the transcription initiation site of the human α-cardiac actin gene linked to the reporter gene, bacterial CAT, and with pSV2neo, which confers resistance to the drug G418. This upstream sequence of the human α-cardiac actin gene promoter is essential to the appropriate initiation and tissue-specific transcriptional activity of the gene in muscle cells (Minty and Kedes, 1986).

Pools of stably transfected L cells were used in our initial experiments. Heterokaryons were formed by exposing cocultures of mouse muscle C2C12 cells and the transfected L cells to PEG, as described by Blau et al. (1983). As a control, a culture of transfected L cells alone was treated with PEG to form homokaryons. 4 d after heterokaryon formation the cultures were harvested and CAT expression determined as described by Gorman et al. (1982). Equivalent amounts of protein were assayed from the two cultures. Clearly CAT expression is induced in the heterokaryon cultures (Fig. 1, *right*) compared with the expression in homokaryons (Fig. 1, *left*). By scintillation counting we determined that the magnitude of the induction in this case was ~20-fold over the basal level of CAT enzymatic activity in the transfected L cells alone. In fact, this constitutes an underestimate of the induction, since not all of the L cells fused with the muscle cells in heterokaryon cultures.

We noted that the magnitude of the induction varied greatly among four experiments performed with pooled L cell clones (data not shown). Nonetheless, the maximal induction observed in heterokaryons was similar to the difference in CAT activity obtained with C2C12 muscle cells and with L cells in transient transfection assays using the same construct (Minty et al., 1986). We concluded from these initial experiments that transcription factors are present in the mouse muscle cells that are capable of gaining access to and inducing the transcription of the transfected human α-cardiac actin-CAT fusion gene stably integrated in the genome of the L cell nucleus.

Basal Levels of CAT Activity in Clones of Transfected L Cells Differ

We determined whether the variability in inducible CAT activity observed with heterokaryons formed with pooled L cells was due to heterogeneity among cells. Possibly the human α-cardiac actin gene promoter in individual stably transfected L cells differed in its ability to interact with

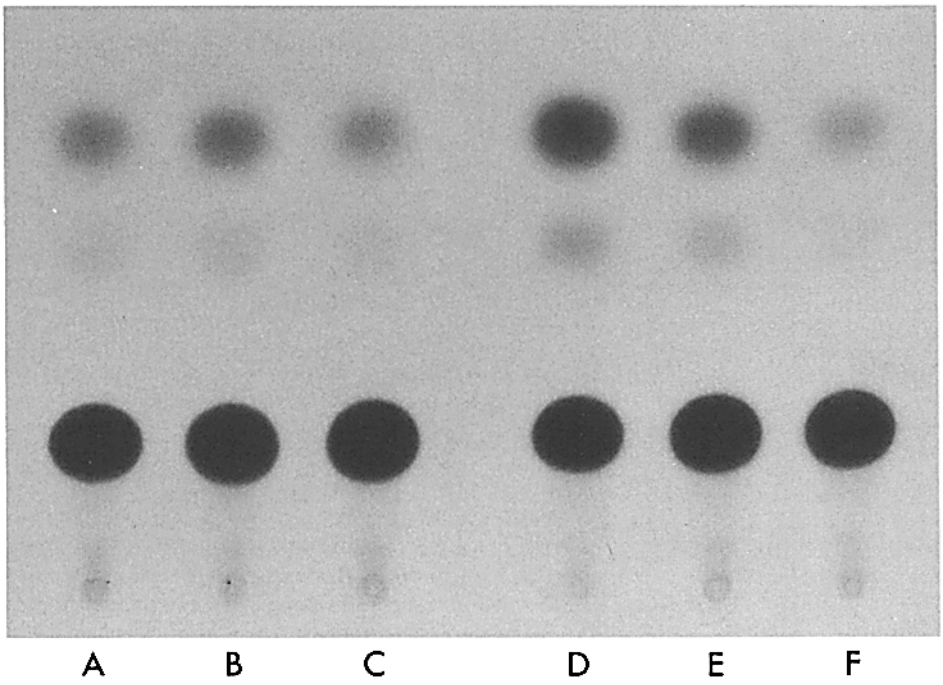

Figure 2. Basal CAT activity in individual clones of transfected L cells. Individual clones of L cells stably transfected with pHCA485CAT were isolated by serial dilution. An equivalent amount of protein from each clonal culture was assayed for CAT enzymatic activity. The results from six representative clones are shown for which the percent conversion to acetylated products was (*A*) 0.12, (*B*) 0.17, (*C*) 0.11, (*D*) 0.47, (*E*) 0.20, and (*F*) 0.06, a range of 8-fold. The silica plate was exposed to XAR film for 1 wk.

muscle-specific transcription factors. Individual clones of L cells containing pHCA485CAT were isolated by serial dilution. Equivalent amounts of cellular protein were assayed and the constitutive level of CAT expression in each clonal population was determined. A total of 20 clones was analyzed and a representative sample of CAT enzymatic activity in 6 of these clones is shown in Fig. 2. Enzymatic activity could be detected in 18 of the clones, but not in 2, regardless of whether the amount of cell lysate assayed was increased or the exposure time of the autoradiograph extended to 1 mo. Furthermore, among those clones in which basal enzyme activity could be detected, significant differences were evident.

The variation in basal levels of CAT activity did not correlate with the number of α-cardiac actin-CAT gene copies integrated per cell. For example, by slot blot analysis we determined that the clone represented in Fig. 2, lane *2* contained 10 stably integrated pHCA485CAT constructs, whereas the clone represented in lane *5* contained only 5 copies, yet its basal CAT activity was higher (data not shown). On average, the clones contained between 5 and 10 transfected gene copies. This twofold difference in transfected gene copy number could not account for the observed eightfold differences in CAT activity (Fig. 2, lanes *D* and *F*). Since the basal level of CAT enzymatic activity in each clone remained constant over a 6-mo period, this property of the L cell appeared to be stably inherited.

Rat Muscle Cells (H9c2) Can Regulate the Transfected Actin Gene and Be Used in Heterokaryons

We quantitated the enhanced transcriptional activity of the transfected human α-cardiac actin gene promoter in individual L cell clones in response to transcription factors contributed by muscle cells in heterokaryons. However, heterokaryon cultures are heterogeneous mixtures of different cell types that include not only myotubes containing both muscle cell and L cell nuclei, but also myotubes containing only muscle nuclei, unfused mononucleated myoblasts, fibroblasts, and homokaryons formed by fusion among fibroblasts. Consequently, quantitation required determining for a given heterokaryon culture, the proportion of total L cell nuclei contained in heterokaryons that was responsible for the enhanced CAT activity. A simple way to determine the nuclear composition of a heterokaryon culture is to take advantage of the differential nuclear staining of the fluorescent dye Hoechst 33258 which preferentially stains adenine and thymine-rich regions of DNA prevalent in the centromeres of mouse but not human or rat chromosomes (Weisblum and Haenssler, 1974; Blau et al., 1983). Since both the muscle C2C12 cell line and L cells used in our initial experiments were derived from mouse, we could not score nuclear composition with Hoechst 33258. Muscle cells of another species, the rat myogenic cell line H9c2 (Kimes and Brandt, 1976), proved suitable for heterokaryon formation in this

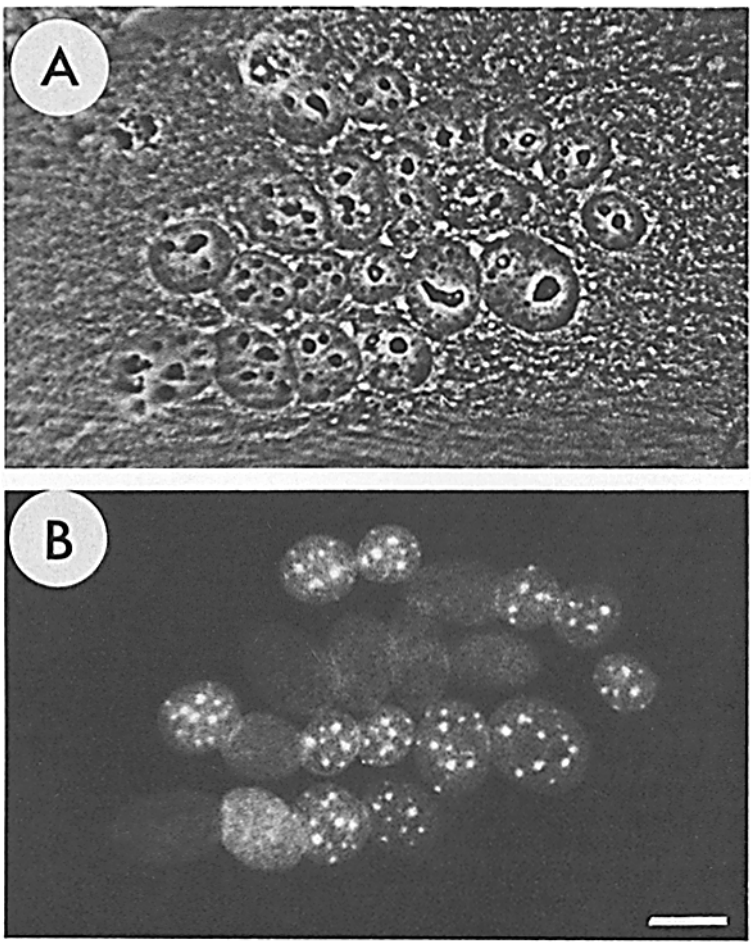

Figure 3. Distinction of rat cardiac muscle and transfected mouse L cell nuclei in heterokaryons. Rat cardiac muscle (H9c2) and transfected L cells (pHCA485CAT) were fused to form heterokaryons and stained with the fluorescent dye, Hoechst 33258. A cluster of nuclei contained in a heterokaryon is shown in phase-contrast microscopy (*A*) and in fluorescence microscopy (*B*). The heterokaryon contains 7 uniformly stained rat muscle nuclei and 13 punctate mouse L cell nuclei. Bar, 10 μm.

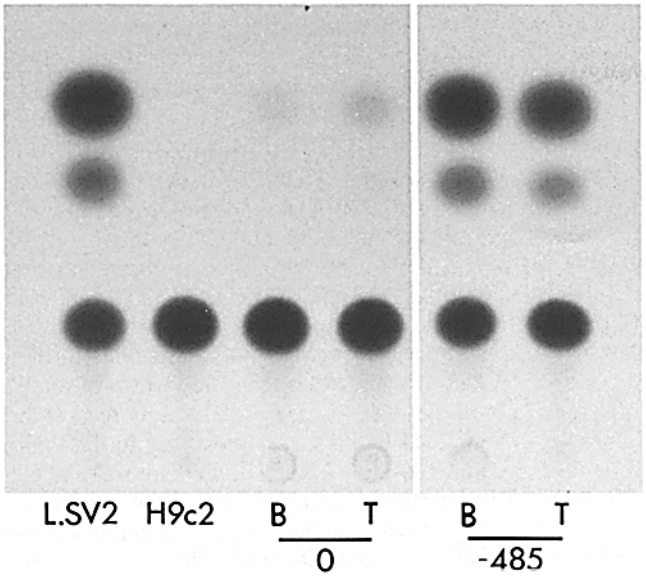

Figure 4. Transcriptional activity of upstream regions of the human α-cardiac actin gene in transiently transfected rat cardiac muscle cells (H9c2). Plasmid DNAs containing 0 (pHCAOCAT) or 485 bp (pHCA485CAT) 5′ to the cap site of the human α-cardiac actin gene were transfected into rat cardiac muscle H9c2 cells. CAT activity was assayed for subconfluent myoblasts (*B*) and for differentiated myotube-containing (*T*) cultures. Cultures were harvested within 36 h of exposure to the DNA and CAT activity determined. Control cultures included L cells stably carrying the pSV2CAT plasmid (L.SV2) and nontransfected, differentiated H9c2 cultures (H9c2). Equivalent amounts of protein were assayed. The silica plates were exposed to XAR film for 24 h.

case. This cell line was originally isolated from rat cardiac tissue, yet it forms multinucleated myotubes typical of skeletal, not cardiac, muscle. As shown in Fig. 3, the rat muscle H9c2 nuclei appear uniformly stained and are clearly distinguished from the punctate mouse L cell nuclei.

It was important to show that the H9c2 cells contained muscle-specific transcription factors capable of acting on the human α-cardiac actin gene promoter. As shown in Fig. 4, the transcriptional activity of H9c2 cultures transfected with the pHCA485CAT plasmid is similar in both the myoblast (B) and myotube (T) cultures indicating that the muscle transcription factor(s) for the α-cardiac actin gene exist at both the determined as well as the differentiated stage. A similar observation was made with the C2C12 cell line (Seiler-Tuyns et al., 1984; Minty et al., 1986). In contrast, basal levels of CAT activity are observed in cells transfected with pHCAOCAT, a plasmid that contains no cardiac actin gene sequences. That these factors are muscle specific was shown in previous experiments in which the same construct transfected into fibroblast L cells and pheochromocytoma PC12 cells yielded only 2–5% the CAT activity observed in transfected C2C12 muscle cells (Minty and Kedes, 1986).

These results show that the factors in the H9c2 muscle cell line derived from rat cardiac tissue interact similarly with the 485-bp upstream promoter sequence as those from skeletal muscle C2C12 cells. Since nuclear composition could be determined with H9c2, this cell line was ideal for the proposed quantitative studies of induction of CAT expression in clones of transfected L cells in heterokaryons.

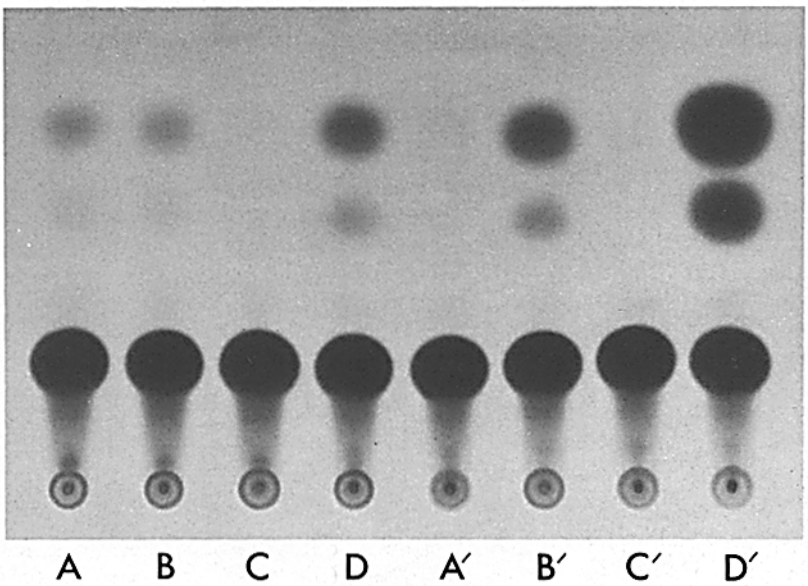

Figure 5. Inducible CAT activity in individual clones of transfected L cells fused with myotubes to form heterokaryons. CAT activity was assayed in heterokaryons of rat muscle (H9c2) and mouse L cell clones (pHCA485CAT) and in homokaryons of the corresponding L cell clones. The L cell clone homokaryons are designated *A*, *B*, *C*, and *D*. The corresponding heterokaryon cultures are designated *A′*, *B′*, *C′*, and *D′*. Note that *D′* is overexposed in this autoradiogram. From scintillation counting, we determined that the increase in CAT activity from *D* to *D′* was 50-fold. The results shown here for clones *A* and *B* are representative of three independent fusion experiments; the result for clone *D* is representative of two experiments. Parallel heterokaryon cultures were stained with Hoechst 33258 and the nuclear composition determined. The total number of nuclei scored per heterokaryon culture ranged from 1,100 to 1,800. To determine the proportion of L cell nuclei capable of being induced on a dish, for each experiment the number of L cell nuclei inside heterokaryons/total number of nuclei on a dish was determined. This value differed less than twofold among experiments with different clones and ranged between 14 and 25%. To establish the proportion of nuclei on a dish capable of contributing CAT activity, the percent of L cell nuclei (inside and outside heterokaryons)/total nuclei (muscle and L cell) on a dish was determined. This latter value ranged between 70 and 80% and was used to adjust the amount of protein assayed from L cell homokaryons so that it was equivalent to the amount of L cell protein assayed from the corresponding heterokaryon culture. The percentage conversion to acetylated products was (*A*) 0.13, (*A′*) 0.03, (*B*) 0.13, (*B′*) 0.83, (*C*) 0.03, (*C′*) 0.03, (*D*) 0.56, and (*D′*) 32.00. The silica plate was exposed to XAR film for 2 wk.

Transfected L Cell Clones Differ in Their Response to Muscle Transcription Factors

We quantitated the induction of the α-cardiac actin gene promoter in individual clones of L cells in response to muscle transcription factors. Heterokaryon cultures were formed between the rat muscle H9c2 cells and distinct transfected L cell clones. As controls, pure cultures of each of the transfected L cell clones were treated with PEG and fused to themselves. 4 d after PEG treatment, replicate cultures were either harvested for the CAT assay or stained with Hoechst 33258 in order to determine nuclear composition. We determined that between 14 and 25% of the nuclei on a dish were from L cells, contained inside heterokaryons. Thus, the proportion of nuclei capable of responding to *trans*-acting factors differed by less than twofold among experiments with different L cell clones and could not account for the differences in CAT activity described below.

To quantitate the transcriptional activity of the human α-cardiac actin gene promoter after exposure to muscle-specific factors, similar amounts of L cell protein from individual clones of PEG-treated pure L cell homokaryon cultures (Fig. 5, *A–D*) and from the corresponding heterokaryon cultures (Fig. 5, *A′–D′*) were assayed and the CAT activities compared. We determined how much protein to assay using the following calculation. First, the protein content of the extracts was determined. Then, to account for the proportion of cells capable of contributing any CAT activity, the percentage of L cell nuclei (inside and outside heterokaryons) relative to the total number of nuclei on the dish was established for a given clone. This value ranged between 70 and 80% and was used to adjust the amount of protein assayed for CAT activity in the corresponding homokaryon culture.

Using this assay, we discovered that the human α-cardiac actin gene promoter in the various L cell clones responded differently to muscle transcription factors supplied by the rat muscle cells. The analysis of four clones is shown in Fig. 5. Two clones demonstrated no responsiveness (compare *A*, *A′* and *C*, *C′*) and the other two displayed increased transcriptional activity of the human α-cardiac actin promoter as evidenced by increased CAT activity after fusion in heterokaryons (compare *B*, *B′* and *D*, *D′*). CAT enzymatic activity was induced in only 2 of the 20, or 10%, of the clones analyzed in this manner. In this small percentage of cells the degree of responsiveness, or increase in CAT activity, ranged from 5- to 50-fold. Furthermore, the basal activity in unfused L cells was not a predictor of inducibility. For example, A and B clones had similar low but detectable basal CAT activities, yet A was not induced at all and B was induced approximately fivefold in heterokaryons. Clone D, which had a relatively high basal CAT activity, was capable of 50-fold induction and expressed the highest levels of CAT activity determined by scintillation counting. This finding suggests that differences among L cell clones affect the ability of the α-cardiac actin CAT promoter to respond to tissue-specific transcription factors.

Inducibility of the α-Cardiac Actin Promoter in L Cells Is a Heritable Property

To determine the reproducibility of the results obtained with different clones, we examined the CAT activity in individual transfected L cell clones in three independent series of heterokaryon experiments performed consecutively over a 2-mo period. In each experiment, the inducible CAT activity proved similar for the cells of a given clone. Examples of the results obtained in three separate fusion experiments with two clones, A and B, are shown in Fig. 6. Note that clone A has a high basal CAT activity and cannot be further induced, whereas clone B has a low basal activity and can be induced fivefold in each experiment. Thus, the inducibility of the transfected cardiac actin gene promoter appears to be a stably inherited property of a given L cell clone.

Discussion

We have developed a generally applicable system for identifying tissue-specific factors that enhance transcription

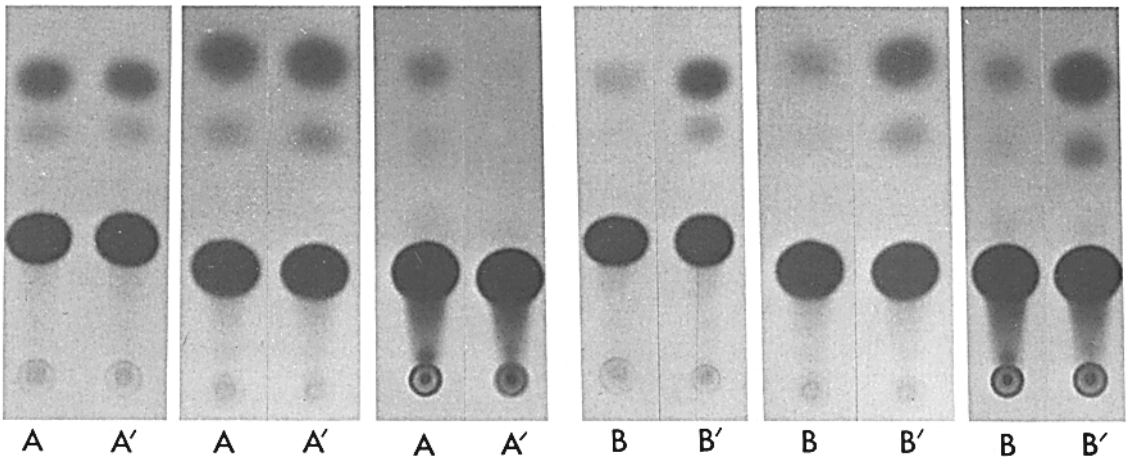

Figure 6. Basal and inducible CAT activities are stably inherited properties of transfected L cells. CAT activity was assayed in three separate fusion experiments with cells derived from L cell clone A (L.pHCA485CAT.4) and from L cell clone B (L.pHCA485CAT.6). Equivalent amounts of L cell protein were assayed for homokaryons of transfected L cells (*A*, *B*) and for heterokaryons formed with the same L cell clones (*A′*, *B′*). The data for the third heterokaryon experiment shown here is also shown in Fig. 5, *A* and *B*. The percentage conversion to acetylated products from left to right was (*A*) 0.29, (*A′*) 0.25, (*A*) 0.35, (*A′*) 0.41, (*A*) 0.13, (*A′*) 0.03, (*B*) 0.07, (*B′*) 0.27, (*B*) 0.07, (*B′*) 0.39, (*B*) 0.13, and (*B′*) 0.83. The silica plates were exposed to XAR film for 2 wk.

based on function in vivo. This system also provides a novel approach to the study of clonal variation in the expression of stably transfected genes. For this purpose, we used nondividing short-term fusion products known as heterokaryons. In this case, muscle cells were fused with fibroblast L cells stably transfected with a muscle-specific gene. The transfected gene was a fusion gene containing 5′ tissue-specific *cis*-acting regulatory sequences of the human α-cardiac actin gene linked to the coding region of the reporter gene, bacterial CAT (Minty and Kedes, 1986; Minty et al., 1986). With pooled clones of transfected L cells, we found that the transcriptional activity of the α-cardiac actin-CAT construct could be induced in heterokaryons by more than an order of magnitude. However, the results varied greatly from one experiment to the next. This variability proved to be due to differences among transfected L cells.

Individual clones of transfected L cells were analyzed and found to differ markedly, both in their basal and in their inducible α-cardiac actin–CAT activity. The human α-cardiac actin gene promoter was transcriptionally active in most L cell clones (90%). However, this basal level of activity differed by as much as 50-fold among clones. In the majority of clones analyzed, muscle factors were not capable of effectively inducing the expression of the actin gene promoter and no increase in CAT activity was observed in heterokaryons. In fact, in only 10% of the clones analyzed was CAT activity induced after fusion and the magnitude of this induction ranged between 5- and 50-fold. The basal level of transcriptional activity in the L cell clone was not a predictor of the magnitude of the induction in responsive cells. Indeed, there were clones with low but detectable basal activity that could not be induced at all (Fig. 5, *A* and *A′*) and other clones with high basal activity that could be further induced 50-fold (Fig. 5, *D* and *D′*). However, both basal and inducible CAT activities appeared to be stably heritable properties of individual L cell clones.

The basis for the clonal variation in the ability to activate stably transfected muscle genes in heterokaryons is of inherent interest. It appears that the variability is not due to gene copy number, since there was no correlation between the level of CAT activity and the number of transfected genes per cell. We cannot rule out the possibility that the differences among clones resulted from stably heritable differences in L cell karyotype. However, the more likely possibility is that the range in basal and inducible CAT activity is due to the random integration of constructs into chromosomal sites of active and inactive chromatin. Sites of integration are known to lead to alterations in methylation patterns, packaging into inactive chromatin, and gene rearrangements (Sweet et al., 1981; Christy and Scangos, 1982; Davies et al., 1982; Gebara et al., 1987). The apparent "*cis* variability" observed here could result from integration of the constructs in concatinated form such that different numbers of genes were transcriptionally active in different cells. Possibly the observed clonal variation could be overcome by transfecting a different gene construct. The role of integration sites in gene activity could be tested by determining whether uninducible clones can give rise to inducible clones after a second transfection with an α-cardiac actin reporter gene construct. On the other hand, nonresponsive clones might give rise to responsive clones after treatment with the hypomethylating agent, 5-azacytidine, if methylation levels play a role in the variable activity of transfected genes. In previous studies of endogenous gene activation, muscle gene expression was not detected in heterokaryons produced with HeLa cells unless the HeLa cells had been exposed to 5-azacytidine before fusion (Chiu and Blau, 1985).

The heterokaryon approach described here could be extended to other types of studies of regulatory factors that induce the expression of transfected genes. For example, it provides a means for studying the tissue-specific regulation of a transfected gene in a cell type that is not itself readily transfected. That a variety of cell types can be fused to form the kind of short-term nondividing heterokaryons described for muscle (Blau et al., 1983, 1985; Chiu and Blau, 1984, 1985; Wright, 1984*a*, *b*; Pavlath and Blau, 1986; Hardeman et al., 1986*a*; Clegg and Hauschka, 1987; Miller et al., 1988), has been demonstrated by Baron and Maniatis (1986), Enoch et al. (1986), and Lufkin and Bancroft (1987). Finally, the utility of heterokaryons is not restricted to transcription-inducing factors. For example, heterokaryons could be used to study the effect of RNA-splicing factors derived from different differentiated cell types on the processing of a gene transfected into an L cell.

Our findings suggest that a genetic approach to the identification of genes encoding tissue-specific transcription-inducing factors may now be possible. The aim of this approach is to introduce DNA into a cell line containing, but not efficiently expressing, a transfected gene of interest, in order to assay for a marked increase in the transcriptional activity of that gene (Episkopou et al., 1984). In contrast to the recent report of Lufkin and Bancroft (1987), our results indicate that if a pool of stably transfected cells is used in such an assay, highly variable results will be obtained. However, both the basal and inducible CAT activities were heritable and stable properties of a given L cell clone. Accordingly, it would now seem possible to identify the appropriate clone of stable transfectants that can respond maximally to the tissue-specific factors of interest required for this approach to work. Although the analysis would be simplified if a single gene product were involved, multiple factors are not precluded. The induction by the transfected gene could be indirect. For example, the gene product could be a factor that modifies preexisting factors rendering them muscle specific.

The need for functional in vivo assays for transcription-inducing factors is underscored by some of the problems encountered with the more traditional biochemical purification approaches. The most stringent criteria for the identification of a transcription factor are that it binds to a specific sequence of DNA and that it alters transcription efficiency in an in vitro assay (McKnight and Tjian, 1986). Yet, increasing evidence suggests that some regulatory proteins such as SV-40 T-antigen or HSV polypeptide 4 can activate transcription without binding directly to a particular *cis*-acting regulatory sequence (McKnight and Tjian, 1986). This raises the possibility that rate-limiting regulatory factors act as modifiers of sequence-specific repressors or activators that are synthesized constitutively. In addition, some well-characterized transcription factors such as CCAAT transcription factor, which binds to the CCAAT sequence, function poorly in in vitro transcription assays (Jones et al., 1987). We suggest that a genetic approach using well-characterized transfected clones should complement biochemical approaches by providing a means for identifying *trans*-acting transcription

regulatory factors or the genes that encode them based on their function in vivo.

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References

Bains, W., P. Ponte, H. Blau, and L. Kedes. 1984. Cardiac actin is the major actin gene product in skeletal muscle cell differentiation in vitro. *Mol. Cell. Biol.* 4:1449–1453.

Baron, M. H., and T. Maniatis. 1986. Rapid reprogramming of globin gene expression in transient heterokaryons. *Cell.* 46:591–602.

Blau, H. M., C.-P. Chiu, and C. Webster. 1983. Cytoplasmic activation of human nuclear genes in stable heterokaryons. *Cell.* 32:1171–1180.

Blau, H. M., G. P. Pavlath, E. C. Hardeman, C.-P. Chiu, L. Silberstein, S. G. Webster, S. C. Miller, and C. Webster. 1985. Plasticity of the differentiated state. *Science (Wash. DC).* 230:758–766.

Charnay, P., R. Treisman, P. Mellon, M. Chao, R. Axel, and T. Maniatis. 1984. Differences in human α- and β-globin gene expression in mouse erythroleukemia cells: the role of intragenic sequences. *Cell.* 38:251–263.

Chiu, C.-P., and H. M. Blau. 1984. Reprogramming cell differentiation in the absence of DNA synthesis. *Cell.* 37:879–887.

Chiu, C.-P., and H. M. Blau. 1985. 5-azacytidine permits gene activation in a previously non-inducible cell type. *Cell.* 40:417–424.

Christy, B., and G. Scangos. 1982. Expression of transferred thymidine kinase genes is controlled by methylation. *Proc. Natl. Acad. Sci. USA.* 79:6299–6303.

Clegg, C. H., and S. D. Hauschka. 1987. Heterokaryon analysis of muscle differentiation: Regulation of the postmitotic state. *J. Cell Biol.* 105:937–947.

Davies, R. L., S. Fuhrer-Krusi, and R. S. Kucherlapati. 1982. Modulation of transfected gene expression mediated by changes in chromatin structure. *Cell.* 31:521–529.

Enoch, T., K. Zinn, and T. Maniatis. 1986. Human β interferon gene activation requires an interferon inducible factor. *Cell. Mol. Biol.* 6:801–810.

Episkopou, V., A. J. M. Murphy, and A. Efstratiadis. 1984. Cell-specified expression of a selectable hybrid gene. *Proc. Natl. Acad. Sci. USA.* 81:4657–4661.

Gebara, M. M., C. Drevon, S. A. Harcourt, H. Steingrimsdottir, M. R. James, J. F. Burke, C. F. Arlett, and A. R. Lehmann. 1987. Inactivation of a transfected gene in human fibroblasts can occur by deletion, amplification, phenotypic switching, or methylation. *Mol. Cell. Biol.* 7:1459–1464.

Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2:1044–1051.

Gunning, P., P. Ponte, L. Kedes, R. J. Hickey, and A. I. Skoultchi. 1984. Expression of human cardiac actin in mouse L cells: a sarcomeric actin associates with a nonmuscle cytoskeleton. *Cell.* 36:709–715.

Hardeman, E. C., C.-P. Chiu, A. Minty, and H. M. Blau. 1986*a*. The pattern of actin expression in human fibroblast X mouse muscle cell heterokaryons suggests that human muscle regulatory factors are produced. *Cell.* 47:123–130.

Hardeman, E. C., A. Minty, L. Kedes, and H. M. Blau. 1986*b*. In vivo assay for trans-acting, tissue-specific regulators of gene transcription. *J. Cell Biol.* 103 (5, Pt. 2):494*a* (Abstr.)

Jones, K. A., J. T. Kadonaga, P. J. Rosenfeld, T. J. Kelly, and R. Tjian. 1987. A cellular DNA-binding protein that activates eukaryotic transcription and DNA replication. *Cell.* 48:79–89.

Keshet, I., J. Lieman-Hurwitz, and H. Cedar. 1986. DNA methylation affects the formation of active chromatin. *Cell.* 44:535–543.

Kimes, B. W., and B. L. Brandt. 1976. Properties of a clonal muscle cell line from rat heart. *Exp. Cell Res.* 98:67–381.

Lufkin, T., and C. Bancroft. 1987. Identification by cell fusion of gene sequences that interact with positive trans-acting factors. *Science (Wash. DC).* 237:283–286.

McKnight, S., and R. Tjian. 1986. Transcriptional selectivity of viral genes in mammalian cells. *Cell.* 46:795–805.

Miller, S. C., G. K. Pavlath, B. T. Blakely, and H. M. Blau. 1988. Muscle cell components dictate hepatocyte gene expression and the distribution of the Golgi apparatus in heterokaryons. *Genes and Development.* In press.

Minty, A., and L. Kedes. 1986. Upstream regions of the human cardiac actin gene that modulate its transcription in muscle cells: presence of an evolutionarily conserved repeated motif. *Mol. Cell. Biol.* 6:2125–2136.

Minty, A., H. Blau, and L. Kedes. 1986. Two-level regulation of cardiac actin gene transcription: muscle-specific modulating factors can accumulate before gene activation. *Mol. Cell. Biol.* 6:2137–2148.

Miwa, T., and L. Kedes. 1987. Duplicated CArG box domains have positive and mutually dependent regulatory roles in expression of the human α-cardiac actin gene. *Mol. Cell. Biol.* 7:2803–2813.

Pavlath, G. K., and H. M. Blau. 1986. Expression of muscle genes in heterokaryons depends on gene dosage. *J. Cell Biol.* 102:124–130.

Robins, D. M., I. Peek, P. H. Seeburg, and R. Axel. 1982. Regulated expression of human growth hormone genes in mouse cells. *Cell.* 29:623–631.

Schlissel, M. S., and D. D. Brown. 1984. The transcriptional regulation of Xenopus 5S RNA genes in chromatin: the roles of active stable transcription complexes and histone H1. *Cell.* 37:903–913.

Seiler-Tuyns, A., J. D. Eldridge, and B. M. Paterson. 1984. Expression and regulation of chicken actin genes introduced into mouse myogenic and nonmyogenic cells. *Proc. Natl. Acad. Sci. USA.* 81:2908–2984.

Shaw, P., B. Sordat, and U. Schibler. 1985. Two promoters of the mouse α-amylase gene Amy-1a are differentially activated during parotid gland differentiation. *Cell.* 40:907–912.

Sweet, R., J. Jackson, I. Lowy, M. Ostrander, A. Pellicer, J. Roberts, D. Robins, G.-K. Sim, B. Wold, R. Axel, and S. Silverstein. 1981. The expression, arrangement, and rearrangement of genes in DNA-transformed cells. *In* Genes, Chromosomes and Neoplasia. F. Arrighi, P. N. Rao, and E. Stubblefield, editors. Raven Press, New York. 205–219.

Weintraub, H. 1984. Histone-H1-dependent chromatin superstructures and the suppression of gene activity. *Cell.* 38:17–27.

Weintraub, H. 1985. Assembly and propagation of repressed and derepressed chromosomal states. *Cell.* 42:705–711.

Weisblum, B., and E. Haenssler. 1974. Fluorometric properties of the bibenzimidazole derivative Hoechst 33258, a fluorescent probe specific for AT concentration in chromosomal DNA. *Chromosoma (Berl.).* 46:255–260.

Wright, W. E. 1984*a*. Expression of differentiated functions in heterokaryons between skeletal myocytes, adrenal cells, fibroblasts and glial cells. *Exp. Cell Res.* 151:55–69.

Wright, W. E. 1984*b*. Induction of muscle genes in neural cells. *J. Cell Biol.* 98:427–435.

Yaffe, D., and O. Saxel. 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature (Lond.).* 270:725–727.