Selective Effect of Rous Sarcoma Virus src Gene Expression on Contractile Protein Synthesis in Chick Embryo Myotubes

Christopher M. West¹ and David Boettiger²

ABSTRACT

Myogenic precursor cells were infected with a temperature-sensitive mutant of Rous sarcoma virus and maintained at the permissive temperature for transformation. Following subculture, a population of cells was produced which failed to differentiate at the permissive temperature but produced a high proportion of myotubes in sister cultures shifted to the nonpermissive temperature. The myotube-containing cultures were further enriched for this cell type by the addition of 1-β-D-arabinofuranosylcytosine to kill replicating mononucleated cells. This myotube population was suitable for testing the effect of viral-transforming gene expression in a postmitotic, terminally differentiated cell which expresses relatively low levels of the cellular homologue of the viral-transforming gene and is resistant to infection by the virus. The consequence of shifting these Rous sarcoma virus-infected myotube cultures to the permissive temperature was assessed in terms of protein synthesis. The total rate of incorporation of exogenous radioactive leucine, supplied at a concentration which saturated the intracellular pool, was similar between cultures held at the two temperatures, suggesting that the absolute rate of total protein synthesis was not affected by expression of viral-transforming gene. In contrast, the rate of synthesis of eight proteins the expression of which is specific for myotubes was suppressed reversibly. The rate of synthesis of five other proteins which are not selectively concentrated in myotubes was either unaffected or stimulated. Thus, the expression of viral-transforming gene in myotubes leads to differential effects on developmentally regulated proteins without inducing several properties of the transformed state classically observed for fibroblastic cells.

INTRODUCTION

RSV² encodes a v-src gene which is essential for its ability to transform (36) cells but does not play a role in normal virus replication (23). The protein product of this viral transformation gene has been identified in RSV-infected cells as a M, 60,000 phosphoprotein with a protein kinase activity (10). A similar gene, c-src, is expressed at low levels in normal, uninfected cells (15). While an enzymatic activity is associated with the transforming protein (v-pp60⁵⁺), the effects of the virus-induced transformation on the host cell are pleiotropic, and its mechanism of action remains obscure. One approach to understanding the mechanism has used specific differentiated cell types in which the response of differentiated cell functions to the action of v-pp60⁵⁺ may be investigated. In some terminally differentiated cells, such as chondroblasts (30) and melanoblasts (6), there is a selective suppression of the expression of typical cell differentiation markers. In contrast, infection of several precursor cell types seems to maintain the expression of their differentiation markers and prevents or inhibits the normal appearance of terminally differentiated cells or their functions in cultures of these precursor cells (13, 14, 17). In some cases, where there is a selective effect on the expression of specific proteins, it is necessary to demonstrate whether there is an effect on synthesis, or degradation, or both. In view of the various developmental models which link the regulation of expression of developmental markers, or the expression of the transformed state, with cell proliferation, it is also of interest to determine whether virus-induced effects require cell cycling. This question cannot be addressed directly in the above systems, for infection was accomplished by continued cell proliferation. Multinucleated myotubes which are incapable of further cell proliferation will differentiate in vitro from embryonic breast tissue (19, 20). These cells may be infected prior to differentiation with a temperature-sensitive v-src mutant of RSV and permitted to differentiate at the nonpermissive temperature. Shifting the cultures to the permissive temperature activates the v-pp60⁵⁺ protein in mature myotubes incapable of further proliferation. Hence, the effects of v-pp60⁵⁺ on a panel of normal differentiation markers can be investigated in the absence of cell proliferation. This study examines the consequences of this particular interaction in terms of the rates of synthesis of several myotube contractile proteins.

MATERIALS AND METHODS

Myogenic cells were obtained by trypsinization of breast muscle anlagen from 11- or 12-day-old, virus-free (specific-pathogen-free-COFAL-negative) embryos (SPAFAS, Inc., Norwich, Conn.) and cultured in 100-mm Falcon or 35-mm Corning tissue culture dishes coated with denatured rat tail collagen, according to procedures described by Bischoff et al. (5). Some cultures were inoculated with the temperature-sensitive LA24 mutant of Prague-stain RSV during the first 2 days of culture (18). Thereafter, all cultures were fed daily with a medium (8:11) consisting of 8 parts minimum essential medium (with Earle's salts and supplemented with l-glutamine), 1 part horse serum, 1 part embryo extract (prepared from virus-free eggs), and antibiotics, and they were maintained at 36⁰, the permissive temperature for transformation. After 6 days, the cultures were passaged by trypsinization. In secondary or tertiary culture, the temperature was shifted up to 41⁰ to allow differen-
tation into myotubes. Unless parallel cultures retained at 36°C failed to show greater than 5% of their nuclei in myotubes, they were not used. Mononucleated cells were depleted by treatment with ara-C (2.0 or 3.0 μg/ml) (12) within 1 to 2 days (depending on cell density) following shift-up. After 3 additional days, greater than 90% of the nuclei appeared to be contained in myotubes, and the ara-C concentration was reduced to 1.0 μg/ml or was removed altogether.

Fibroblastic cells infected with temperature-sensitive RSV were obtained from infected primary cultures described above which were grown and subcultured at 41°C to remove myogenic cells, which do not passage once they differentiate (1). By tertiary or quaternary culture, these cells were free of detectable multinucleated cells (<5% of nuclei) and ready for use. Cell cultures were transformed by shifting down to 36°C for appropriate intervals of time. Cultures were fed daily with 8:1:1 medium as described above.

Total protein synthesis was measured by the incorporation of [3H]-leucine into acid-precipitable material as described (39). Also detailed in that reference is the method by which the absolute rate of total protein synthesis was estimated. This involved measuring uptake under conditions where the intracellular leucine precursor pool was saturated with extracellular radioactive leucine.

Relative rates of synthesis of individual protein species were also inferred from the rate of uptake of radioactive leucine. A potential problem of variable recovery yield for purified proteins was circumvented by pooling experimental and control cultures, labeled with [14C]- and [3H]-leucine, respectively, prior to extraction. The data appeared as the 14C dPM:3H dPM ratio which, when corrected by the experimentally determined ratio of specific activities, yielded the relative rate of incorporation of the precursor between the 2 cultures.

The metabolic labeling protocol and protein isolation procedures are described in detail elsewhere (39) and will only be summarized here. Labeling was performed for a period of 4 hr in leucine-free media. Following labeling, cells were extracted either for total protein and DNA or for purification of individual protein species. The method of Hinegardner (16) was used to precipitate protein and DNA and to assay DNA mass. Individual proteins were purified using either of 2 methods. The first method consisted of solubilizing the entire culture in a sodium dodecyl sulfate-containing isoelectric focusing dissociation cocktail (4, 29) followed by electrophoresis on 2-dimensional gels (see Ref. 39, Fig. 3). Proteins were identified by published reports and comigration with purified standards. To confirm that individual species were purified, cultures grown and labeled in 100-mm dishes were extracted to produce an actomyosin pellet (3) and a high-salt insoluble fraction. The actomyosin fraction was subjected to isoelectric focusing to produce α-, β-, and γ-actins or passed through a gel filtration column to produce myosin heavy chain and MLCs (31) which were then purified on a 1-dimensional gel (33). MIF and FIF subunits were purified directly from the high salt-insoluble fraction by 2-dimensional gel electrophoresis as described above (21). Tropomyosins were purified by 2-dimensional gel electrophoresis of the actomyosin fraction prior to gel filtration.

RESULTS

RSV-infected myotube cultures were produced as described above and maintained in ara-C for 4 days to reduce the proportion of mononucleated cells. Then one-half of the cultures was shifted to 36°C for 2 additional days. The dependence of total protein synthesis on the final temperature of incubation was evaluated as the incorporation of [3H]-leucine into acid-precipitable material. This measurement was performed over a range of extracellular leucine concentrations in an attempt to saturate the intracellular pool. Chart 1 shows that incorporation was independent of temperature except at the highest concentration, 0.62 mM, where the lower temperature stimulated incorporation by about 15%. Incorporation of [3H]-leucine appeared to plateau at the highest concentration used. In contrast, the total intracellular pool of extracellularly derived leucine was proportional to the extracellular leucine concentration. These observations suggest that, at the highest concentration, extracellular leucine nearly saturated the intracellular precursor pool. Using a model described by Schultz et al. (34) and applied in Ref. 39, the precursor pool was calculated to consist of 97% extracellularly derived radioactive leucine when leucine was supplied at 0.62 mM. Since the specific activity of intracellular precursor leucine was the same in cultures at both temperatures, the accumulation of isotope into acid-precipitable material probably reflects the absolute rate of total protein synthesis. Thus, the absolute rate of protein synthesis was very similar between the 2 cultures, differing by not more than 15%.

The data also suggest that the RSV-infected myotubes remained healthy during the course of the experiment, since the uptake of [3H]-leucine was similar to that of the control cultures. Occasional cross-striations were maintained at 41°C, and spontaneous twitching was observed. However, the frequency of these events was abnormally low, and electron microscopic examination showed the myofibrils to be less well organized in infected myotubes at 41°C, in comparison to those in uninfected controls (not shown). There was no evidence for cell vacuolization or detachment (see below), which has been observed after longer periods of temperature shift-down (18), and cells retained their refractility in the phase-contrast microscope.

Uptake of radioactive leucine into individual protein species was examined at extracellular leucine concentrations below 0.07 mM. The proteins studied were of 2 types: (Group A) α-actin (21, 40), MLC1 and MLC2 (33), α-MIF and β-MIF (11), and α-tropomyosin and β-tropomyosin (8, 32), which are detectable in myotubes but not in their precursor, the PMB, or in other fibroblastic cell types; and (Group B), β-actin and γ-actin (21, 40), FLC and FLC2 (33), and α-FIF and β-FIF (28), which accumulate in all fibroblastic cells as well as in 6- to 10-day myotubes. The levels of these Group B proteins may diminish in more mature myotubes, but in our cultures their synthesis is maintained at a level.
similar to that in fibroblasts on a per DNA basis (39).

RSV-infected, ara-C-treated myotube cultures were shifted to 36° and were labeled with [14C]leucine, while parallel cultures maintained at 41° were labeled with [3H]leucine. Following the label period, each 36°:41° pair was mixed and extracted, and the proteins were purified as described in "Materials and Methods." Excised spots from gels were counted to determine the relative incorporation of [14C] and [3H]leucine and corrected for the specific activity of total protein. The similarity of uptake levels between the 2 cultures at lower extracellular leucine concentrations provided a basis for normalizing the uptake ratios for different isotopes of leucine between 2 culture types to a constant specific activity. The effectiveness of the purification scheme involving 2-dimensional gel electrophoresis is explicitly documented in Ref. 39, Table 1. More involved, multistep procedures were also used for every protein in some experiments as described in "Materials and Methods." In most cases, isoproteins were resolved easily with the exception, in some experiments in which 2-dimensional gel electrophoresis was the only purification step, of FIF and actin, as noted in Table 1. All final values are expressed at 14C:3H (36°:41°), which represents the relative rate of protein synthesis at 36° compared to that at 41°.

By 29 hr after temperature shift-down, the Group A proteins were significantly depressed in synthesis (Table 1). α-Actin, MLC1, MLC2, α-MIF, β-MIF, α-tropomyosin, and β-tropomyosin incorporated [3H]leucine at rates only 41 to 62% that of their counterparts at 41°. In contrast, β-actin, γ-actin, FLC1, and FLC2 incorporated precursor at rates similar (within 19%) to those of controls. Incorporation into α-FIF and β-FIF was slightly stimulated by 28 and 43%. Qualitatively similar results were obtained when the time of culture at 36° was varied from 13 to 65 hr. However, the degree of inhibition increased from an average of 46% at 13 hr to 82% at 65 hr. The degree of synthesis stimulation for α-FIF and β-FIF was not time dependent.

Owing to the fact that the data are expressed as a ratio of 2 ratios, statistical variations are relatively high. We routinely detect values ranging from 20% below to 20% above 1.00 for Group B proteins (other than FIF) in these and other experiments (39), and we do not distinguish these values from 1.00. Results from 2 other abbreviated experimental series were qualitatively similar and are given in the legend to Table 1 for comparison.

The dependence of the effects of temperature shift on ara-C treatment was tested by not treating the cultures with ara-C or by removing the drug during temperature shift-down. In the experiment where ara-C treatment was discontinued when the 29-hr temperature shift-down was initiated, there were no differences compared to cultures in which ara-C treatment was continued (Table 1). In the myotube cultures which were never treated with ara-C, it was not feasible to incorporate sufficient label into all of the proteins because of dilution with fibroblastic

### Table 1

<table>
<thead>
<tr>
<th>Type of culture</th>
<th>Experimental treatment</th>
<th>Actins</th>
<th>Myosins</th>
<th>Intermediate filaments</th>
<th>Troponymosins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tertiary, 5-day, RSV-infected, ara-C treated myotubes</td>
<td>13 hr at 36° + ara-C</td>
<td>1.00</td>
<td>0.53</td>
<td>0.53</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>29 hr at 36° + ara-C</td>
<td>0.90</td>
<td>0.41</td>
<td>0.41</td>
<td>0.81</td>
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<tr>
<td></td>
<td>50 hr at 36° + ara-C</td>
<td>0.48</td>
<td>0.68</td>
<td>0.68</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>65 hr at 36° + ara-C</td>
<td>0.54</td>
<td>0.35</td>
<td>0.35</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>29 hr at 36° (no ara-C)</td>
<td>0.54</td>
<td>0.35</td>
<td>0.35</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>Reversal at 36° at 29 hr + 21 hr at 41° (ara-C)</td>
<td>0.54</td>
<td>0.35</td>
<td>0.35</td>
<td>0.81</td>
</tr>
<tr>
<td>Tertiary, 5-day, RSV-infected myotubes (no ara-C)</td>
<td>30 hr at 36° (no ara-C)</td>
<td>0.58</td>
<td>ND</td>
<td>ND</td>
<td>0.40</td>
</tr>
<tr>
<td>Primary, 5-day, ara-C treated, non-infected myotubes</td>
<td>30 hr at 36° + ara-C</td>
<td>0.80</td>
<td>0.80</td>
<td>0.80</td>
<td>1.12</td>
</tr>
<tr>
<td>Secondary, 3-day, RSV-infected fibroblasts</td>
<td>30 hr at 36°</td>
<td>0.82</td>
<td>0.82</td>
<td>0.82</td>
<td>0.98</td>
</tr>
</tbody>
</table>

* Normalized to a value of 1.00.
* ND, not determined.
* Isoproteins were copurified.
* Myosin heavy chain.

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cell products. Incorporation into α-actin, MLC1t, and β-tropomyosin was measurable and, as expected, was depressed (Table 1). In contrast, the synthesis of α-FIF and β-FIF was not stimulated but was also slightly depressed (Table 1). This failure to observe a stimulation can be explained by dilution with FIF produced by mononucleated cells (see below). The results suggest that the effects of incubation at 36° were not dependent on the presence of ara-C.

The stimulation of synthesis of α-FIF and β-FIF was cell type dependent. RSV-infected fibroblasts incorporated similar amounts of leucine into these 2 proteins at 36° and 41° (Table 1).

The temperature shift itself was not responsible for the changes in synthesis, because they were not observed in uninfected control myotube cultures (Table 1). β-MIF, α-FIF, and β-FIF did incorporate label at rates between 65 and 74% at 41° compared to 36°, which suggests that their synthesis was normally somewhat temperature dependent; however, the synthesis of the latter 2 was stimulated rather than depressed in the infected cultures.

The effect of incubation at 36° was reversible. If cultures maintained at 36° for 29 hr were returned to 41° for an additional 21 hr, the selective effects observed at 36° were abolished in addition, for 4 of the 6 proteins the synthesis of which was inhibited at the permissive temperature, synthesis was stimulated up to 61% above the level in same-age controls maintained at 41° for the entire time when recovery was permitted for 21 hr (Table 1). This suggests that the myotubes responded to shift-up by compensating for the induced hiatus in synthesis at 36°.

The DNA content per culture was monitored to evaluate independently the constancy of the cell population during the course of the experiment. Fifty hr of incubation of RSV-infected cultures at 36° resulted in a loss of DNA of 10% compared to that of parallel control cultures maintained at 41° (Table 2). Thus, there was no significant loss of myotube DNA or increase in mononucleated cell or myotube DNA unless opposing changes cancelled one another. This possibility was tested by comparing ara-C-maintained and ara-C-reversed cultures. Continuing ara-C treatment did not affect the DNA content of the cultures (Table 2) which suggested that there could not have been major losses of DNA, since the drug treatment would inhibit synthesis of new DNA to replace it. Finally, we noted that the residual levels of DNA synthesis observed in the cultures were not mechanistically related to the effects on protein synthesis, since synthesis data were not affected by a discontinuation of ara-C treatment (Table 1), although [3H]thymidine incorporation was stimulated almost 3-fold (Table 2).

**DISCUSSION**

RSV-infected myotubes were produced by infecting myogenic cells with a strain carrying a temperature-sensitive mutation in its v-src. By shifting the temperature, the activity of its product, v-pp60src, could be regulated. Thus, myotubes were allowed to form from infected PMB cultures, and the effects of v-pp60src function were investigated by shifting the cultures to the permissive temperature for transformation. We have observed that, although myotubes are not susceptible to infection by RSV, they are responsive to its transforming function.

Activation of v-pp60src by temperature shift-down resulted in a selective effect on the synthesis of one group of proteins without significantly affecting the absolute rate of protein synthesis or the equilibration of precursor pools with [3H]leucine. The synthesis of α-actin, MLC1t, MLC2t, α-MIF, α-tropomyosin, and β-tropomyosin was inhibited in a time-dependent fashion to levels as low as 10% of the level detected in same-age controls. In contrast, the synthesis of β-actin, γ-actin, FLC1, FLC3, α-FIF, and β-FIF was not affected or was stimulated. The former group of proteins is distinguished by their accumulation uniquely in myotubes and not in their myogenic precursors or other cell types. The latter group of proteins is synthesized at similar rates in fibroblasts, early myogenic cells, and myotubes (39). Active v-pp60src thus appears to cause a selective suppression of synthesis of at least 6 developmentally regulated myotube proteins. This effect is reversible and does not appear to be attributable to a change in proportion of cell types during the course of the experiment.

Evidence has been presented in fibroblasts that the levels of mRNA encoding fibronectin and collagen were suppressed following transformation of the cells by RSV (2). Myotube mRNA and putative myosin heavy-chain mRNA have estimated half-lives of 20 to 50 hr (7, 35). The time course of the observed suppression of myotube-specific proteins is consistent with an immediate switch-off of new mRNA synthesis and subsequent decay in mRNA content. It seems unlikely that the effects of temperature shift-down on RSV-infected myotubes can be explained by nonspecific damage to the cells. Under our conditions, myotubes incorporated [3H]leucine normally into both intracellular pools and protein. Occasional cross-striations and twitching were observed, although myofibrillar organization was somewhat abnormal. This could be explained by residual activity of v-pp60src even at the nonpermissive temperature. No abnormal vacuolization or cell detachment was observed. Finally, the results observed are not typical for stressed cells. For example, in cardiac myocytes and lymphocytes, hypertonic media, starvation conditions, actinomycin treatment, and ischemia all inhibit protein synthesis but do not exert a selective effect on cell-unique proteins synthesized by these cells (22, 27, 37).

The selective effects of RSV on developmentally regulated proteins are consistent with the results of several other myotube studies using either RSV or the tumor promoter TPA. TPA has a very similar effect on protein synthesis compared to that of RSV and, in addition, has an approximately inverse effect on protein degradation (39) and removes myofibrils from the sar-

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**Effect of RSV Infection on Muscle Protein Synthesis**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DNA (µg/dish)</th>
<th>[3H]Thymidine uptake (cpm/dish)</th>
</tr>
</thead>
<tbody>
<tr>
<td>41° (ara-C)</td>
<td>156</td>
<td>ND*</td>
</tr>
<tr>
<td>36° (ara-C)</td>
<td>145</td>
<td>39,600</td>
</tr>
<tr>
<td>36° (no ara-C)</td>
<td>141</td>
<td>107,000</td>
</tr>
</tbody>
</table>

*ND, not determined.

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coplasma (38). Similar results have been reported for regulation of the acetylcholine receptor (24). The similarity of effects of RSV and TPA suggests that, in terms of the 2-stage model for carcinogenesis (25), the promoting activity of RSV is responsible for its effects on myotubes.

The selective effect of v-pp60csrc production on myotube function also parallels the effect of this protein on several other terminally differentiated cell types including melanocytes and chondrocytes (17). However, the nature of this effect is not universal. For instance, macrophages, although infected by RSV and capable of replicating virus, are not phenotypically suppressed by infection (9, 26). Furthermore, the expression of 2 developmental markers found in PMBs but not in myotubes is only slightly suppressed in RSV-infected or TPA-treated PMBs (14). The apparently dissimilar effect of v-pp60csrc production in 2 successive compartments of the same lineage, PMBs and myotubes, indicates that we have not viewed from the point of view of the developmental regulation of myogenesis. What are the relative endogenous levels of c-src expression in uninfected PMBs and myotubes? Gonda et al. (15) have reported a relatively low level of expression in myotubes. Contrastingly high levels of c-src expression in PMBs would be consistent with the ability of v-src expression to preserve a PMB-like state in these cells (14).

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REFERENCES

20. The similarity of effects of RSV and TPA suggests that, in terms of the 2-stage model for carcinogenesis, the promoting activity of RSV is responsible for its effects on myotubes.
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