Infection of terminally differentiated myotubes with Rous sarcoma virus: reduced synthesis of env and v-src proteins

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We studied Rous sarcoma virus (RSV) protein synthesis in RSV-infected, terminally differentiated chicken myotubes ('late-infected' myotubes), in which no viral DNA integration takes place but all three viral mRNAs (38S, 28S and 21S) are transcribed normally. With the use of specific anti-RSV protein antisera, we found that only the viral gag and pol proteins were synthesized at levels similar to those synthesized in RSV-transformed fibroblasts; the synthesis of env and v-src proteins was significantly reduced in these infected myotubes. We concluded that the viral RNA transcribed from the unintegrated RSV DNA was functional but that genes at the 3' end of the RSV genome were translated at a lower level. By contrast, when mononucleated replicating chicken myoblasts were infected with a mutant (tsNY68) carrying a temperature-sensitive v-src gene and maintained at the non-permissive temperature for this gene, they developed into myotubes with viral DNA integrated in their chromosomal DNA. These 'early-infected' myotubes expressed all four viral genes (gag, pol, env and v-src) at a level similar to that in infected fibroblasts. This result ruled out the possible presence of specific factor(s) in myotubes that preferentially inhibit the 3' genes of RSV, and suggested other translational control(s) of viral gene expression in late-infected myotubes.

Introduction

In the life cycle of Rous sarcoma virus (RSV; for reviews see Svoboda, 1986; Varmus, 1988; Coffin, 1990; Varmus & Brown, 1989), viral RNA is reverse-transcribed into DNA, which is then integrated into the host chromosomes. The integrated viral DNA (provirus) is transcribed by the host DNA-dependent RNA polymerase, and the resulting viral RNA is spliced and translated by the host machinery. The primary translation products of the RSV genomic size mRNA (38S) are Pr76 (precursor of gag proteins) and Prl80 (precursor of pol proteins), and those of the two RSV subgenomic size mRNAs (28S and 21S) are gPr92 (precursor of env proteins) and p60 (v-src protein), respectively.

In the accompanying paper (Tanaka et al., 1992), we have shown that RSV can infect terminally differentiated myotubes ('late-infected' myotubes). These late-infected myotubes contain as much viral DNA as infected chicken fibroblasts, but the viral DNA is not integrated into the host chromosome DNA. The unintegrated DNA in late-infected myotubes is transcribed to yield all three species of RSV mRNA. In contrast, viral DNA can integrate into myotube chromosomes if myoblasts are infected with RSV carrying a temperature-sensitive v-src gene, and the infected cells are allowed to develop into terminally differentiated myotubes at the non-permissive temperature ('early-infected' myotubes) (Kobayashi & Kaji, 1978). They also contain all three species of RSV mRNA.

In this communication, we show that viral protein synthesis is differentially regulated in late-infected myotubes. The synthesis of gag and pol proteins is normal whereas that of env and v-src proteins is significantly lower in late-infected myotubes than in infected chicken embryo fibroblasts (CEFs).

Methods

Cell cultures and viruses. Early- and late-infected myotube cultures were prepared as described previously (Tanaka et al., 1992). Three types of cells were used as controls: conventional CEFs prepared from the same embryos used for myotube culture; myogenic fibroblasts (MFs), which were mononucleated cells recovered from the supernatant of a trypsinized suspension of a 5-day-old myotube culture [without
cytosine arabinoside (Ara-C) treatment); Ara-C-treated MFs, (CMFs), which were prepared similarly to MFs except that the myotube culture was treated with Ara-C for 2 days followed by 4 days of culturing without Ara-C to allow the growth of cells which survived the Ara-C treatment. All three cultures were infected with a Schmidt-Ruppin wild-type (wt) (subgroup A) strain of RSV (SRA) or with a temperature-sensitive (ts) mutant of SRA, tsNY68 (Kawai & Hanafusa, 1971), at approximately 1 to 5 f.f.u/nucleus. In some experiments, transformation-defective (td) mutants of SRA were used (tdNY101 and tdNY107A) (Hanafusa et al., 1977; Kawai et al., 1977); tdNY101 and tdNY107A differ in the length of the deletion in the v-src gene. These viruses were generous gifts from Dr H. Hanafusa.

**Immune reagents.** Normal rabbit serum and normal goat serum were purchased from Gibco. Tumour-bearing rabbit (TBR) serum was kindly supplied by Dr L. Rohrschneider (Rohrschneider, 1979). Rabbit anti-avian myeloblastosis virus (AMV) gp85 and rabbit anti-B77 gp85 antisera were generous gifts from Dr D. Bolognesi (Collins et al., 1981). Rabbit anti-avian myeloblastosis virus (AMV) gag protein antiserum was obtained from the Office of Program Resources and Logistics, National Cancer Institute. The Cowan I strain of *Staphylococcus aureus* was used as an adsorbant for immune complexes. For indirect immunofluorescence experiments, all the immunological reagents (including normal rabbit serum, TBR serum and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit Ig) were kindly supplied by Dr L. Rohrschneider (Rohrschneider, 1979).

**Cell labelling and immunoprecipitation.** Normal and RSV-infected myotube cultures as well as control mononucleated cell cultures, were labelled 4 days after infection with 100 μCi [35S]methionine (Amersham, 1000 Ci/mmol) in 1 ml of Earle's balanced salt solution (with Ca2+ and Mg2+) per 10 cm plate. The cultures were labelled for 2 h in a 37 °C, 5% CO2 incubator. In some cases, the early-infected myotube cultures were labelled at 42.5 °C for 2 h. After labelling, cultures were rinsed with Tris-saline buffer (140 mM-NaCl, 50 mM-KCl, 2.5 mM-Tris-HCl pH 7.3, 5-5 mM-glucose), and subjected to cell lysis and immunoprecipitation according to the procedure of Oppermann et al. (1977).

For the detection of phosphorylated v-src protein, cultures on 10 cm plates were labelled with 500 μCi [32P]orthophosphate (New England Nuclear) in 1 ml of Dulbecco's phosphate-free MEM per plate. Labelled cultures were lysed and immunoprecipitated with the TBR serum pre-adsorbed with a saturating amount of gradient-purified RSV (30 μg viral protein per 1 μl TBR serum) according to the procedure described by Rohrschneider (1979).

**Gel electrophoresis.** Polyacrylamide slab gels were made with 12% acrylamide, 0-108% N,N'-methylene-bis-acrylamide and 0-1% SDS in the buffers described by Laemmli (1970). Gels were run at 12 mA for approximately 10 h and subjected to fluorography (Bonner & Laskey, 1974).

**Indirect immunofluorescence microscopy.** Cover glasses (22 × 22 mm) were washed with acid, coated with gelatin (10 mg/ml) and sterilized using u.v. irradiation. Primary myogenic cells were seeded at 105 cells and mononucleated cells at 3 × 105 cells per cover glass in 1-5 ml muscle medium in a 35 mm culture dish. Cultures were maintained in the muscle medium and infected with SRA. On the third day after infection, both uninfected and infected myotubes or MF cultures were washed three times with PBS (8.0 g NaCl, 0-2 g KCl, 1-16 g Na2HPO4, 0-2 g KH2PO4)/l. After a quick rinsing with acetone, cover glasses were transferred into a glass Petri dish and cells were fixed with acetone for 10 min at room temperature. The fixed cells were first incubated at 37 °C for 1 h with an appropriate antiserum which had been diluted 30 fold in foetal calf serum (FCS). After washing twice with PBS and twice with H2O, samples were incubated with FITC-conjugated goat anti-rabbit IgG (diluted 1:8 in FCS) for 1 h at 37 °C according to the procedure of Rohrschneider (1979). Cells were washed again with PBS and H2O and were counter-stained with 0-02% Evans' blue. Slides were viewed with a Zeiss microscope equipped with epi-fluorescent illumination and appropriate filters for fluorescein fluorescence. All photographs were taken with a 63 x planapochromatic objective (numerical aperture 1-40), using an exposure time of 90 s, on Kodak Ektachrome 200 slide films.

**DNA assay.** Nuclei, obtained as a pellet after cell lysis and centrifugation, were resuspended and sonicated in 1 ml deionized water. The DNA content was assayed according to the method of Hill & Whatley (1975). More than 90% of total cell DNA was recovered in the nuclear fraction.

**Results**

**Reduced amount of v-src protein in late-infected myotube cultures**

The late-infected myotube cultures were labelled with [35S]methionine and the virus-encoded proteins were detected by immunoprecipitation. As shown in Fig. 1(a), late-infected myotube cultures synthesized similar amounts of viral gag proteins (Pr76gag and p27gag) as infected CEFs. However, the relative amount of p60v-src protein was significantly reduced. Since p60v-src is a phosphoprotein (Brugge et al., 1978), we labelled late-infected myotubes with [32P]orthophosphate and immunoprecipitated labelled proteins with an anti-v-src protein anti-serum. As shown in Fig. 1(b), late-infected myotube cultures produced very little phosphorylated p60v-src (pp60v-src), whereas infected CEF or MF cultures produced abundant quantities of pp60v-src. The low level of pp60v-src in late-infected myotubes is due to a low phosphorylation capacity of myotubes because RSV-infected myotubes can phosphorylate a large number of proteins (Kobayashi & Kaji, 1980). The observations in Fig. 1(a) and (b) demonstrate that the amount of the v-src protein was reduced in late-infected myotubes compared to that in infected CEFs. It should be pointed out that the total amount of labelled protein per μg of DNA in the late-infected myotube culture was comparable with that in infected CEF cultures (data not shown). In this study, the mononucleated cells originating from those present in the myotube culture, representing 1 to 10% of total nuclei, were also used as an additional control (MF). As shown in Fig. 1(a) and (b), the amount of p27gag, Pr76gag and p60v-src detected in infected MFs was very similar to those in infected CEFs.

To detect Pr180gag-pol (precursor of the pol proteins), goat anti-AMV gag protein antiserum was used. The amount of Pr180gag-pol in late-infected myotube culture was comparable with that in infected CEFs (Fig. 1c). It was noted that very little p27gag is detected in infected CEFs compared to that in late-infected myotubes. For
Fig. 1. Reduced amount of radioactive v-src, but not gag or pol proteins in late-infected myotube cultures. Cultures of CEFs, MFs, and myotubes (MT), either uninfected (lanes 1) or infected with SRA RSV (lanes 2), were labelled for 2 h 4 days post-infection with either $[^{35}S]$methionine (a and c) or $[^{32}P]$orthophosphate (b). Aliquots containing identical TCA-insoluble counts (4 × 10⁷ c.p.m. for a and c, 1 × 10⁷ c.p.m. for b) of each cell lysate were reacted with antiserum and immune complexes were analysed by SDS-PAGE. (a) TBR serum; (b) TBR serum pre-adsorbed with detergent-disrupted RSV; (c) goat anti-AMV gag protein antiserum.

reasons not yet understood, the ratio of p27$^{\text{gag}}$ to Pr76$^{\text{gag}}$ varied depending on the kind of antiserum, type of cell, and presence or absence of the v-src gene. For this reason, the total amount of gag protein (the sum of p27$^{\text{gag}}$, P19$^{\text{gag}}$ and Pr76$^{\text{gag}}$) detected using TBR serum was used to estimate the amount of gag proteins relative to other viral proteins.

Detection of viral proteins in late-infected myotubes by immunofluorescence

To establish that the virus-related proteins detected in Fig. 1 were indeed produced by the myotubes and not by the mononucleated cells present in the myotube culture (1 to 10% of total nuclei), acetone-fixed myotubes were analysed by indirect immunofluorescence. As seen in Fig. 2, infected CEFs (a) as well as late-infected myotubes (d and e) show positive fluorescence (greenish yellow) with TBR serum, which contains antibodies against gag and v-src proteins. These results established the presence of viral proteins in late-infected myotubes. When infected CEFs and late-infected myotubes were reacted with the TBR serum pre-adsorbed with viral structural proteins to remove all but the antibody against the v-src protein, only infected fibroblasts were found to show positive fluorescence (Fig. 2b). The absence of v-src-specific fluorescence from late-infected myotubes (Fig. 2f) was consistent with the immunoprecipitation results in Fig. 1. It also agrees with the lack of morphological changes in late-infected myotubes (Fig. 2d, e and f) because the expression of the v-src protein would have caused a morphological deterioration in myotubes (Holtzer et al., 1975; pathway IV in Fig. 1 of Tanaka et al., 1992). In this experiment, the negative controls were infected CEFs (Fig. 2c), late-infected myotubes reacted with normal rabbit serum (Fig. 2g) and normal myotubes treated with the TBR serum (Fig. 2h). Thus these studies indicated that the gag and pol proteins, but not the v-src protein, were produced normally in late-infected myotubes.

We considered the possibility that the deleterious effect of the v-src protein in late-infected myotubes (Holtzer et al., 1975) may have destroyed myotubes showing strong v-src gene expression, leaving myotubes
Fig. 2. Detection of viral structural proteins in late-infected myotubes by indirect immunofluorescence showing absence of the v-src protein. Cells grown on coverslips were fixed with acetone and analysed by indirect immunofluorescence staining using FITC-conjugated goat anti-rabbit IgG as the second antibody. SRA RSV-transformed MFs (a, h, e) and late-infected myotube cultures (d, e, f, g) were reacted with TBR serum (a, d and e). TBR serum pre-adsorbed with virus (b and f) or normal rabbit serum (c and g). Normal myotubes were also reacted with TBR serum (h).
Reduced amount of env protein in late-infected myotube cultures

The amount of RSV env protein in late-infected myotubes was studied by immunoprecipitation of $[^{35}]$S-methionine-labelled proteins with a rabbit anti-AMV gp85 anti-serum. This anti-serum is known to recognize the carbohydrate moiety of the env protein (Collins et al., 1978). As shown in Fig. 3, the late-infected myotube cultures contained much less $[^{35}]$S-methionine-labelled gPr92$^{env}$ (Fig. 3, lane 2) than infected MFs (Fig. 3, lane 4). This experiment established that the amount of env protein relative to gag protein was less in late-infected myotubes than in infected CEFs. This reduced amount of radioactive env protein was not due to an artefact caused by Ara-C in the myotube culture because infected CMFs (Ara-C-treated MF) and infected MFs produced comparable amounts of env protein (data not shown). Similar results were obtained in an experiment using the rabbit anti-B77 gp85 antisemur (Fig. 3, lanes 5, 6, 7 and 8), which recognizes the polypeptide moiety of the env protein (Van Eldik et al., 1978). This indicated that the reduced amount of gPr92$^{env}$ protein detected in the late-infected myotube culture was not due to the lack of glycosylation of this protein. In addition, we obtained similar results in a separate experiment using two different td mutants of RSV (tdNY101 and tdNY107A), indicating that the reduced env protein expression was expressing v-src weakly in the culture. This possibility was eliminated by time lapse photography of late-infected myotubes. No destruction of myotubes was observed for 5 days post-infection (data not shown).

Reduced amount of env protein in late-infected myotube cultures

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### Table 1. Reduced amount of radioactive env and v-src proteins in late-infected myotube cultures compared to those in infected CEF cultures*

<table>
<thead>
<tr>
<th>Viral proteins</th>
<th>Viral proteins</th>
<th>Internally normalized to total gag protein</th>
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<tr>
<td></td>
<td>per 10 µg host DNA</td>
<td>per 10⁷ c.p.m. total protein</td>
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<tr>
<td></td>
<td>CEF MT</td>
<td>CEF MT</td>
</tr>
<tr>
<td>Pr76ag†</td>
<td>1.09</td>
<td>1.81</td>
</tr>
<tr>
<td>Total gag§</td>
<td>3.29</td>
<td>6.08</td>
</tr>
<tr>
<td>Pr180envv §</td>
<td>0.05</td>
<td>0.12</td>
</tr>
<tr>
<td>gp92env §</td>
<td>7.35</td>
<td>1.51</td>
</tr>
<tr>
<td>p60vsrc</td>
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<td>1.06</td>
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* Late-infected myotube cultures (MT) as well as infected CEFs were labelled with [35S]methionine, immunoprecipitated with the appropriate antiserum and analysed by SDS-PAGE as described in legend to Fig. 1. Radioactive viral proteins detected on the X-ray film were quantified by densitometry tracing. Values are in arbitrary units.

† Standard errors of the mean for (I) could not be calculated because not enough experiments were carried out.

Those for (III) and (IV) were calculated from (II).

§ Cell lysates immunoprecipitated with goat anti-AMV p27 antiserum.

¶ Cell lysates immunoprecipitated with rabbit anti-AMV gp85 antiserum.

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not a phenomenon peculiar to the RSV strain used in this experiment (data not shown).

Table 1 summarizes the results of studies on the amount of RSV protein in late-infected myotube cultures. This table shows that, on the basis of unit weight of host DNA, there were reduced amounts of env and v-src proteins, but normal amounts of gag and pol proteins in the late-infected myotube culture compared to those in infected CEFs. When the amount of each viral protein was determined in relation to the c.p.m. of cellular proteins, reduced amounts of env and v-src proteins, but comparable amounts of gag and pol proteins were again found in late-infected myotubes compared to those in infected CEFs. One can also calculate the relative amount of labelled viral proteins by normalizing to the amount of total labelled gag proteins.

In this case, the relative amount of labelled env protein in the late-infected myotube culture was approximately 10% of that in infected CEFs. Similarly, the relative amount of labelled v-src protein in the late-infected myotube culture represented less than one-quarter of that in infected CEFs. We concluded from these results that the relative amounts of env and v-src proteins are significantly reduced in late-infected myotube cultures compared to those in infected CEFs.

### Normal amounts of radioactive v-src and env proteins in early-infected myotubes

The reduced amounts of radioactive v-src and env proteins in late-infected myotubes could be due to the inability of the myotubes per se to synthesize these proteins. To examine this possibility, we studied early-infected myotubes. Myoblasts were infected with a ts mutant of RSV (tsNY68) and cultured at the non-permissive temperature (42.5 °C) to allow cell differentiation. After these infected myoblasts had developed into myotubes, cultures were labelled with [35S]methionine, and virus-related proteins were analysed by gel electrophoresis. Fig. 4 shows that the amounts of radioactive gag, env or v-src proteins in early-infected myotube cultures (lane 2) were very similar to those in infected CEFs (lane 3). In this experiment, some of the early-infected myotube cultures had been shifted down to the permissive temperature (37 °C) prior to labelling. This procedure induced breakage of myotubes, as has been observed previously (Holtzer et al., 1975; Kobayashi & Kaji, 1978), but the profile of none of the radioactive viral proteins was significantly influenced by this temperature shift.

Densitometric analysis of Fig. 4 and similar experiments revealed that, when normalized to the total amount of labelled gag protein, the amounts of radioactive pel, env or v-src proteins relative to the total amount of gag protein in the early-infected myotube culture were very similar to those in infected CEFs. We concluded from these data that early-infected myotubes, in contrast to late-infected myotubes, are capable of expressing all four viral proteins as one would find in infected CEFs. Therefore, the reduced expression of env and v-src genes in late-infected myotubes was not because myotubes had inhibitory activity on the expression of these genes.
Discussion

In this communication, we have shown that normal amounts of gag and pol proteins, but reduced amounts of env and v-src proteins compared to those in infected CEFs are detected in late-infected myotubes. This finding is somewhat surprising in view of the fact that all species of viral mRNA are present in late-infected myotubes (Tanaka et al., 1992). It is unlikely that these gene products are translated from the parental virion RSV RNA because the amount of viral gag proteins produced in late-infected myotube cultures was comparable with that in infected CEFs. It should be noted that the infecting viral RNA has a very short half-life of about 3 h (Bolognesi & Graf, 1970). Translation of parental virion RNA has been reported in AMV-infected cells, but the level of viral proteins produced is only 1% of that in transformed CEFs (Gallis et al., 1976). Also, it is unlikely that the mononucleated cells, which constituted only 1 to 10% of the nuclei in late-infected myotube culture, could have produced sufficient gag proteins to be comparable to those produced by infected CEFs.

Non-coordinate gene expression of retroviruses has been reported in several other systems (Billele et al., 1974; Teramoto et al., 1980). Similarly, control of viral gene expression by host cell differentiation has been reported in adenovirus-infected human keratinocytes (Laporta & Taichman, 1981) and simian virus 40 (SV40)-infected teratocarcinoma cells (Linnenbach et al., 1981; Segal & Khoury, 1979; Swartzendruber & Lehman, 1975). SV40 and polyoma viruses can infect mononucleated myoblasts but not myotubes (Fogel & Defendi, 1967). In contrast, influenza virus replicates well in postfusion myotubes, but not if the infection takes place at the myoblast stage (O'Neill & Kendal, 1975). Recently, Sharpe et al. (1990) reported a case analogous to ours. They found that the murine neurotropic retrovirus, Cas-Br-E, does not express the env protein in the neuron, its major target, although the spliced env mRNA is synthesized. To the best of our knowledge, the non-coordinate expression of RSV genes or the control of RSV gene expression by host cell differentiation has never been reported.

The reduced amount of env and v-src proteins could be due to either reduced synthesis or increased degradation, or both. However, it is unlikely that degradation is the determining factor in late-infected myotubes because no apparent degradation is observed in early-infected myotubes. In support of this notion, the amount of radioactive viral proteins pulse-labelled for 30 min remained almost constant with a 0-5, 1-5 or 3 h chase, indicating that very little degradation took place under our experimental conditions (data not shown). Therefore, it is reasonable to regard the radiolabelled viral proteins in our experiments as the result of synthesis rather than a combination of synthesis and degradation. The mechanism of reduced synthesis of env and v-src proteins must involve translational control because a normal ratio of viral mRNAs had been observed in late-infected myotubes (Tanaka et al., 1992). Examples of such translational control have been reported in muscle cells (Endo & Nadal-Ginard, 1987; Gross & Merrill, 1989) and in maize cells (Skadsen & Scandalios, 1987). In muscle cells, the association of mRNA with ribosomes is not decreased despite the fact that translation is markedly inhibited. In the case of maize cells, the association of mRNA with ribosomes is markedly inhibited, suggesting that mRNA may be entrapped in ribonuclear proteins and unable to bind to the ribosomes. In the case of late-infected myotubes, the exact mechanism for the reduced synthesis of env and v-src proteins remains to be determined. However, we can conclude that it is not due to a possible presence of differentiation-specific translation machinery in the myotubes because normal amounts of viral proteins are synthesized in early-infected myotubes.

The myotube culture always contains small amounts (usually 1%, occasionally up to 10%) of mononucleated cells (Abbott et al., 1974). Therefore, the viral proteins observed in myotube cultures could have originated from these cells. This possibility was eliminated on the basis of the following observations. (i) Positive immunofluorescence was observed in late-infected myotubes with the antibody to RSV proteins (Fig. 2). (ii) Both gag and pol genes are preferentially expressed in late-infected myotubes, but not in infected CEFs (Table 1). (iii) A CEF-associated protein of Mr greater than 130 000 is not observed in the myotube culture (Fig. 1a). (iv) The rate of [35S]methionine incorporation into viral proteins in infected myotube cultures is identical to that in infected CEFs (data not shown). (v) The amount of viral protein per host cell DNA in the late-infected myotube culture is equivalent to that in infected CEFs. Since only 1 to 10% of total nuclei in the myotube culture are derived from mononucleated cells, one would have to assume 10- to 100-fold more efficient viral protein production by the contaminating cells if the viral protein production observed in late-infected myotube culture was from mononucleated cells only. In addition, findings in the accompanying paper (Tanaka et al., 1992) and unpublished data (H. T. Park et al., further support the notion that the viral proteins observed in myotube cultures originate from the myotubes. (i) RSV RNA is present in late-infected myotubes, as demonstrated by in situ hybridization. (ii) Electron microscopy demonstrates C-type particles budding from late-infected myotubes. (iii) Env protein-deficient virus particles are present in the
culture fluid of late-infected myotube culture, but not in that of infected CEFs. Studies on the defective virus particles produced by late-infected myotubes will be reported elsewhere.

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References


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