Differentiation of Quail Myoblasts Transformed with a Temperature Sensitive Mutant of Rous Sarcoma Virus. I. Relationship between Differentiation and Tyrosine Kinase of src Gene Product

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ABSTRACT. Quail embryonic pectoral myoblasts fuse with each other at 35.5°C and 41°C to essentially equal extents. When the myoblasts were transformed with a temperature-sensitive mutant of Rous sarcoma virus (ts-RSV), their fusion and biochemical processes of differentiation became temperature-sensitive: their fusion occurred at 41°C, the non-permissive temperature, but not at 35.5°C, the permissive temperature, suggesting that the fusion was regulated by the viral transforming gene.

Fusion of the transformed cells proceeded more rapidly and synchronously than that of the parent cells at 41°C, and was completely suppressed at the permissive temperature, unlike that of the parent cells. These transformed cells were used to examine the relationship between myogenic differentiation and the tyrosine kinase activity of the src gene product. In spite of the temperature sensitivity of transformation, results showed that expressions of the src gene at 35.5°C and 41°C were similar. However, the level of tyrosine-phosphorylated protein was decreased at 41°C. Moreover, myoblast fusion could occur at 35.5°C in the presence of herbimycin A, an inhibitor of the tyrosine kinase activity of the src gene product. These results indicate that the tyrosine kinase activity of the src gene product is closely associated with regulation of myogenic differentiation of the cells.

Myogenesis involves various biochemical and morphological differential events. These events are distinct and can be seen even in vitro using established line cells derived from myoblasts. Many workers have thus used myogenic cells as models in studies on the regulation of differentiation (23, 32, 39, 40). Biochemical differentiations of myoblasts have been studied especially extensively from an early stage of muscle research. Myogenic differentiation is of interest not only with respect to characteristic biochemical events, but also with respect to cell fusion. We have been studying artificial cell fusion induced by HVJ (Sendai virus) and have examined changes of the membrane induced by the virus (18-20). For comparison with this artificial cell fusion, we examined the process of myotube formation, one of the sequential processes in muscle differentiation, to obtain more information on the phenomenon of cell fusion at molecular and cellular levels.

During myogenesis, myoblasts proliferate and become aligned and committed to differentiation, and then they fuse simultaneously with each other in a so-called 'fusion burst' (39). There have been many studies on myoblast fusion by various experimental approaches (6, 8, 9, 11, 21, 22, 25, 38), but its mechanism is still obscure. One reason for this seems to be that no suitable experimental system in which the fusion reaction can be controlled is available. Hitherto, the mechanism of myoblast fusion has mainly been studied using established cell lines derived from myoblasts, such as the L6 rat myoblast line, and the fusion reaction has been controlled with calcium. Calcium, however, is associated not only with various physiological reactions in the cell, but also with membrane fusion itself. Moreover, the appearance of myotubes in cultures of these cell lines takes a relatively long time. Therefore, we attempted to develop a better system for investigating myogenic differentiation, especially myoblast fusion, in which fusion occurred more rapidly and could be controlled in some other way than with calcium. For this purpose, we tried to obtain myoblasts in which fusion was temperature-sensitive.

There are reports of isolation of temperature-sensitive myoblasts, but the cells appeared unstable and required a long time to fuse (1, 3, 28, 33). On the other hand, there are reports that differentiation of myogenic cells is affected by transformation of the cells with some retroviruses (2, 35). In particular, the possibility of control of differentiation of myogenic cells by a temperature-sensitive mutant of Rous sarcoma virus (ts-RSV) has been suggested (12, 15, 31). We confirmed this phenomenon, and through detailed examinations under
various conditions, we developed and conditioned a line of transformed myoblasts as a stable experimental system for studying various aspects of myotube formation in which fusion could be controlled by temperature. This line was established by transformation of a primary culture of embryonic quail cells (QM cells) with ts-RSV by a procedure described previously. Using this cell system, we examined the relationship between the src gene product and myotub differentiation. Results showed that the tyrosine kinase activity of the src gene product was closely related with myoblast fusion, suggesting that regulation of myogenic differentiation is controlled by phosphorylation of some protein(s). This paper describes the relationship between differentiation and tyrosine kinase of the src gene product.

**MATERIALS AND METHODS**

**Preparation of primary myoblast cells.** Pectoral muscles were removed from 10-day quail (Coturnix coturnix coturnix) embryos, and washed with calcium-free phosphate buffered saline (PBS(−)); 8 g/1 NaCl, 0.2 g/1 KCl, 1.15 g/1 Na2HPO4, 0.2 g/1 KH2PO4. The muscle was minced with a spatula, and the cells were dissociated by treatment with 0.025% trypsin (Difco, Detroit, MI) at 37°C for 10 min, and filtered through nylon mesh (#200). For removal of fibroblasts, the cells were seeded at a density of 1 × 10^5 cells per 100 mm diameter glass dish in growth medium, and incubated at 37°C for 90 min with gentle shaking every 10 min. The unattached cells were then harvested as myoblasts. For more complete removal of fibroblasts, the cells were incubated in the same conditions once more, and the nonadhering cells were collected by gentle pipetting and centrifugation. These cells were used as primary quail myoblasts (QM cells).

**Transformation of QM cells with a ts-mutant of RSV.** Primary QM cells were seeded in growth medium at a density of 5.9 × 10^5 cells per 60 mm diameter plastic dish (Corning Inc., New York) coated with collagen (Cell matrix, Type I-P, Nitta Gelatin Inc., Yao City, Osaka, Japan) and cultured at 37°C for 3 hr. The cells were then washed with PBS(−), and infected with a temperature-sensitive mutant of Rous sarcoma virus (ts-NY68) at 3 × 10^−2 focus-forming units per cell. After an adsorption period of 30 min on ice, the cells were cultured in growth medium at 35.5°C for 48 hr. After 3–5 passages with 0.02% ethylenediaminetetraaetic acid (EDTA), the cells were collected, frozen in growth medium containing 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen. These cells were used until passage 20 as transformed quail myoblasts (QM-RSV cells).

**Cell culture.** Dulbecco's modified Eagle's medium (DMEM) plus 2 mM glutamine (Nissui Pharmaceutical Co., Tokyo, Japan), penicillin (100 U/ml) and streptomycin (0.1 mg/ml) was used as culture medium. This medium was supplemented with fetal calf serum at 20% and 5% for preparation of growth medium and differentiation medium, respectively. In these conditions, embryo extract was not necessary. The cells were passaged using only 0.02% EDTA. The cells were used until passage 20, and then a new lot was prepared.

For myotube formation, primary QM or QM-RSV cells were seeded at a density of 2 × 10^6 cells per collagen-coated plastic dish (35 mm in diameter). The cells were routinely preincubated at 35.5°C for 24 hr in growth medium, before culture at 35.5°C or 41°C in differentiation medium.

For treatment of QM-RSV cells with herbimycin A (a gift from Dr. Y. Uehara, National Institute of Health, Tokyo, Japan), the cells were precultured as described above, and then cultured at 35.5°C or 41°C in differentiation medium containing a final concentration of 0.05 μg/ml herbimycin A. Herbimycin A dissolved in DMSO was used as stock solution (1 mg/ml).

**Estimation of myoblast fusion.** The cells were allowed to differentiate for 24 hr as described above for myotube formation, fixed with 2% glutaraldehyde at 4°C for 20 min, and stained with 0.02% toluidine blue O (Merck, Darmstadt Germany) at room temperature for 10 min. They were then washed six times with distilled water and air dried. The total number of nuclei in the cells and the total number of cells in 10 fields selected at random were counted under a light microscope. The fusion index was expressed as follows:

\[
\text{Fusion index (\%)} = \left(1 - \frac{\text{Total number of cells}}{\text{Total number of nuclei}}\right) \times 100
\]

**Assay of creatine kinase.** The cells were seeded at a density of 5.9 × 10^5 cells per collagen-coated 60 mm diameter plastic dish, and allowed to differentiate for myotube formation as described above. They were then washed three times with PBS(−), scraped off the dishes with a rubber policeman in the same buffer, and collected in an Eppendorf microtube (Hamburg, Germany) by centrifugation (150 × g) at 4°C for 5 min. The cell pellet was suspended in 50 μl of glycylglycine buffer (0.05 M glycylglycine, 1% NP-40, 0.1% 2-ME, pH 7.5) and extracted by sonication (AstrasonTM Ultrasonics, New York; output 0.5, duty cycle 20, cycle time 1 sec.) three times for 30-sec. periods at 4°C. The cell lysate was centrifuged (8,000 × g) at 4°C for 5 min, and the supernatant was stored at −80°C until use.

CK-NAC reagent (Boehringer-Mannheim, Germany) was used for the assay. A volume of 20 μl of sample solution diluted with glycylglycine buffer was added to 500 μl of CK-NAC reagent which had been preincubated at 30°C for 2 min. After vigorous mixing, the increase in absorbance at 340 nm per minute (Δε/min) of the reaction mixture was measured at 30°C (Beckman, DU-64 Spectrophotometer, Fullerton, CA). Creatine kinase (CK) activity (mIU/ml) was expressed as [Δε/min × 4127].

Protein was measured by the method of Lowry et al. (29) with bovine serum albumin as a standard.
Myogenic Differentiation and src Gene Product

Electrophoresis and Western blotting, QM-RSV cells were cultured and allowed to differentiate as described above. They were then washed with PBS(−) and collected with a rubber policeman, and their protein was extracted with 0.5% sodium dodecylsulfate (SDS) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 1 μg/ml pepstatin, 10 μg/ml trypsin inhibitor, 1 μg/ml antipain).

Fig. 1. Phase-contrast photographs of primary cultures of QM cells at 35.5°C and 41°C. Inocula of 2 × 10⁵ primary QM cells prepared from the pectoral muscle of 10-day quail embryos were plated in collagen-coated plastic dishes (35 mm diameter) and cultured in differentiation medium at 35.5°C or 41°C. Myoblast fusion occurred at both temperatures, resulting in formation of elongated narrow myotubes within 24 hrs (a and c) and long myotubes within 72 hr (b and d). Myoblast fusion proceeded faster at 41°C than at 35.5°C. a, c; cells cultured at 35.5°C and 41°C, respectively, for 24 hr. b, d; cells cultured at 35.5°C and 41°C, respectively, for 72 hr. Magnifications: a, b, c and d, ×100.
at room temperature for 1 hr. Chromosomal DNA was sheared using a 27G-needle, and the cell extract was then centrifuged (320,000 x g) at 4°C for 10 min, and boiled in sample buffer (62.5 mM Tris, 10% glycerol, 2.3% SDS, 0.005% bromphenol blue, 0.05 M dithiothreitol, pH 6.8) for 5 min. Polyacrylamide gel electrophoresis was carried out in 12.5% acrylamide slab gel containing SDS according to the Laemmli method (24). The protein was transferred from the gel to a nitrocellulose membrane (Bio-Rad Laboratories, CA) in a semidry electroblotting apparatus (Nihon Eido, Tokyo Japan). Specific antigen was detected with a Bio-Rad immunoblot assay kit. The monoclonal antibody to the src gene product used as the first antibody was a gift from Dr. M. Hamaguchi (Nagoya University, Nagoya Japan). Horseradish peroxidase conjugated goat anti-mouse IgG antibody (Bio-Rad Laboratories, CA) was used as the second antibody. Non-specific adsorption of the first antibody to the membrane was blocked with 3% gelatin in Tris buffered saline (TBS; 20mM Tris, 500mM NaCl, pH 7.5). The buffer used for washing the membrane was TBS containing 0.05% Tween-20 (TTBS). The first and second antibodies were diluted with TTBS containing 1% gelatin. Protein was determined by the Lowry method (29). The molecular weight of the protein was estimated by use of prestained SDS-PAGE Standards (low range, Bio-Rad Laboratories, CA).

Assay of tyrosine-phosphorylated protein in QM-RSV cells. QM-RSV cells were cultured and shifted up as described above. They were then washed with PBS(−), and total protein was extracted with 0.5% SDS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 1 μg/ml peptatin, 10 μg/ml trypsin inhibitor, 1 μg/ml antipain) and dephosphorylation inhibitors (20 mM sodium pyrophosphate, 10 mM NaF) at room temperature for 1 hr. The chromosomal DNA was then sheared and the cell extract was centrifuged (320,000 x g) at 4°C for 10 min. Proteins were immobilized on a nitrocellulose membrane (Bio-Rad Laboratories, CA) by use of a Bio-Dot apparatus (Bio-Rad Laboratories, CA), and the membrane was incubated with 3% gelatin in TBS at room temperature for 1 hr. It was then washed with TTBS and incubated with anti-phosphotyrosine monoclonal antibody (PY 20; ICN Immuno Biochemicals, Costa Mesa, CA) as the first antibody at room temperature for 2 hr. After washing with TTBS, it was incubated with [125I] labeled anti-mouse Ig (0.1 μCi/ml; Amer-

![Fig. 2. Effect of incubation temperature on fusion of QM cells. QM cells were plated as described in the legend to Fig. 1. They were maintained in growth medium at 35.5°C for 24 hr and then cultured in differentiation medium at 35.5°C or 41°C. Myoblast fusion was assayed at intervals during culture in differentiation medium. The extent of fusion is significantly greater at 41°C than at 35.5°C until 24 hr, but the difference gradually decrease with time. ■■—■; cultured at 41°C. — — — —; cultured at 35.5°C.](image1)

![Fig. 3. Effect of incubation temperature on myoblast fusion of QM-RSV cells. Inocula of 2 x 10⁸ QM-RSV cells were plated (35 mm diameter) and maintained in growth medium at 35.5°C for 24 hr. They were then cultured in differentiation medium at 35.5°C or 41°C. Myoblast fusion was assayed at intervals during culture in differentiation medium. Myoblast fusion was initiated after a lag time of about 12 hr in cultures at 41°C and then gradually increased with the incubation time until 48 hr, whereas no fusion occurred in cultures at 35.5°C. ——; incubation at 41°C, ——; incubation at 35.5°C.](image2)
sham, Buckinghamshire, England) as the second antibody at room temperature for 1 hr. The first and second antibodies were diluted with TTBS containing 1% gelatin. Finally, the membrane was washed with TTBS, dried and exposed to Fuji Medical X-ray film (RX grade, Tokyo, Japan) with an intensifying screen at -80°C. The level of phosphotyrosine was determined by densitometry.

RESULTS

Culture conditions. Chick embryo extract is generally believed to be required for primary culture of embryonic myoblasts, and so has usually been added to the culture medium. As the composition of embryo extract is not, however, well defined, to simplify analyses of differentiation, we attempted to culture embryonic myoblasts without this extract. We found by various examinations that the cells remained in good condition and that their differentiation could be clearly switched on or off under the culture conditions described in "Materials and Methods". Under these conditions, addition of glutamine to DMEM was very important, but addition of horse serum, which is frequently used for differentiation, was not effective in the absence of embryo extract. Thus DMEM containing 2 mM glutamine and 5% fetal calf serum was used as differentiation medium for the myoblasts.

Myotube formation by primary quail myoblast cells. Cell fusion is strongly affected by the incubation temperature (4). Therefore, to ascertain whether myoblast fusion could be controlled by temperature, we first compared the extents of fusion of primary quail myoblasts (QM cells) cultured at 35.5°C and 41°C. Myoblasts prepared from 10-day quail embryos were plated at a density of 2 × 10⁵ cells per 35 mm dish and cultured in differentiation medium at 35.5°C or 41°C. Fig. 1 shows photographs of these primary cultures. The elongated, spindle-shaped cells showed the morphological features of myoblasts, and these features were similar at 35.5°C and 41°C. When the cells were cultured in differentiation medium, they began to fuse forming multinucleated myotubes within about 24 hr both at 35.5°C and 41°C (a, c, in Fig. 1), and many typical myotubes

Fig. 4. Phase-contrast photograph showing microvesicles detected in QM-RSV cells cultured at 41°C for 48 hr. Various sized vesicles (arrows) are seen near myotubes. Magnifications: × 250.
were formed within 72 hr (b, d, in Fig. 1). At first, the extent of fusion appeared significantly greater at 41°C than at 35.5°C, but the difference gradually decreased with time (Fig. 2). This difference seemed to be due to a slower rate of fusion at 35.5°C. Thus, myoblast fusion of QM cells appeared to occur similarly at 35.5°C and 41°C, except that the myotubes formed at 41°C were larger than those formed at 35.5°C. The fusion reached a plateau at a fusion index of 50-60% on day 3, with numerous mononucleate cells remaining unfused. At this stage, contraction of the myotubes was often observed. Similar fusion of cells occurred in cultures in growth medium (data not shown), indicating that the serum concentration had no significant effect on myotube formation by primary QM cells. These results show that myoblast fusion in primary cultures could not be controlled by either the temperature or serum concentration.

Myotube formation of QM cells transformed with a ts-mutant of RSV. As described above, fusion of QM cells in primary culture was not appreciably different at 35.5°C and 41°C. Therefore, we next tried to achieve temperature regulation of myoblast fusion by transforming the cells with ts-RSV. For this, myoblasts were prepared from embryos, and infected with ts-RSV, for which the permissive temperature is 35.5°C, and the non-permissive temperature is 41°C. The cells were then cultured at 35.5°C. In the early stage after infection, the cells appeared morphologically similar to normal QM cells. During serial passages, however, they gradually became polygonal, and somewhat flattened, and clumps of the cells showing the characteristics of transformed cells began to appear after 3-5 passages. The virus used to transform the cells has a genetic defect in the oncogene (src) responsible for transformation, and infected cells are transformed only at the permissive temperature.

Fusion of these transformed myoblasts (QM-RSV cells) differed from that of primary QM cells in being temperature sensitive. When cultured in differentiation medium at 35.5°C, the QM-RSV cells proliferated actively and did not fuse, unlike the parent QM cells. Under these conditions, no myotubes were seen even after culture for more than 48 hr (data not shown). In contrast, when the cells were shifted up to 41°C, the non-permissive temperature, they became similar in morphology to normal primary cells within a few hours, and began to fuse in about 15-18 hr, forming poly nucleated myotubes within 24 hr. On further culture, the myotubes became larger and the degree of fusion reached a maximum in about 48 hr (Fig. 3). This result is essentially consistent with previous observations.
by others (12, 31).

Other differences from fusion of the parent cells were that the fusion of QM-RSV cells was more synchronous, and that it proceeded faster than that of the parent QM cells, reaching a plateau after 48 hr. At this time, about 50% of the cells were fused, with numerous cells remaining mononucleate. Morphologically, the myotubes of QM-RSV cells were thicker and shorter than those formed from the primary QM cells, which were elongated and narrow. Moreover, the myotubes of QM-RSV cells frequently formed branches (Fig. 4), and spontaneous contraction was rarely observed, unlike the case of myotubes formed by QM cells.

Concomitant with myoblast fusion, many microvesicles appeared near fused cells (arrows in Fig. 4). These vesicles varied in size and appeared to have been released from the fused cells, as described previously by others (10, 17). The number of these vesicles reached a peak at 48 hr at 41°C.

**Regulation of biochemical differentiations by temperature.** During muscle differentiation, not only myotube formation by fusion of myoblasts, but also various biochemical differentiations occur, such as synthesis of muscle specific proteins. Therefore, we next examined whether biochemical differentiation of QM-RSV cells was also affected by temperature. The synthesis of acetylcholinesterase and myosin are reported to be temperature-sensitive in chick myogenic cells transformed with ts-LA29, a ts-mutant of RSV (31). We measured creatine kinase (CK) activity as a marker of biochemical differentiation. Cells were cultured at 35.5°C and 41°C, respectively, in differentiation medium and their CK activity was assayed at intervals. As shown in Fig. 5, the enzyme activity gradually increased in parallel with myoblast fusion in cells cultured at 41°C, but not in cells cultured at 35.5°C. These results suggest that CK activity is also controlled by temperature in these cells. For confirmation of this point, cells that had been cultured at 41°C for 24 hr were shifted down to 35.5°C and their CK activity was assayed 24 hr later. As shown in Fig. 6, the enzyme activity was significantly reduced on shift down to the permissive temperature, although it remained higher than that of cells cultured continuously.
at 35.5°C. Desmin and myosin, muscle specific proteins, were also expressed at high levels in myotubes formed at 41°C (data not shown). These results indicate that biochemical differentiations of QM-RSV cells are also temperature sensitive.

**Expression of the src gene product.** Transformation of cells by RSV is associated with the src gene product, p60\sup{vsrc} (16, 36), and the mutant of RSV used in our experiment has a defect of the src gene. Thus, as described above, previous observations by others and our own results indicate that the src gene is closely associated with regulation of differentiation of myoblasts. We next examined the amount of the src gene product in the cells to clarify how expression of the src gene is affected by the incubation temperature. The cells were cultured in differentiation medium at 35.5°C or 41°C, and the extent of src gene product was assayed by immunoblotting with anti-src gene product monoclonal antibody. The product was detected in similar amounts at 35.5°C and 41°C (Fig. 7). Thus the src gene seemed to be expressed equally at 35.5°C and at 41°C, the non-permissive temperature. If the product is expressed only at 35.5°C, not at 41°C, its amount should gradually decrease during incubation time at 41°C due to metabolic degradation. Therefore, to confirm this point, we measured the levels of tyrosine-phosphorylated proteins with time in cells cultured at 35.5°C and 41°C, respectively, using anti-phosphotyrosine antibody. As shown in Fig. 8, the level of phosphorylated proteins decreased during incubation at 41°C. These findings demonstrate that the src gene of the virus is expressed similarly at 35.5°C and 41°C, but the tyrosine kinase activity differs at 35.5°C and 41°C. There is a report suggesting that the tyrosine kinase activity of the src gene product of ts- RSV used in our study is temperature-sensitive, whereas the extent of src gene expression is not, and that the conformation of the enzyme molecule may change with temperature (14). Our results support these conclusions and indicate that the regulation of differentiation is closely dependent on the tyrosine kinase activity of the gene.

![Phase-contrast photographs of QM-RSV cells treated with herbimycin A at 35.5°C.](image)

**Fig. 9.** Phase-contrast photographs of QM-RSV cells treated with herbimycin A at 35.5°C. Inocula of 2 × 10⁴ QM-RSV cells were cultured at 35.5°C for 24 hr in growth medium as described for Fig. 3 and then at 35.5°C for 24 hr in differentiation medium with or without herbimycin A. Myoblast fusion occurred in cultures treated with herbimycin A at 35.5°C (b), but not in control cultures at 35.5°C without herbimycin A (a). Magnifications: a and b, ×50.
product, but not on gene expression itself.

Reversion of fusion at 35.5°C by an inhibitor of the transforming gene product. For direct demonstration of the relationship between differentiation and tyrosine kinase activity of the src gene product, QM-RSV cells were treated with an inhibitor of the gene product, herbimycin A, and myoblast fusion was then examined. Herbimycin A is a benzenoid ansamycin antibiotic that strongly inhibits the tyrosine kinase activity of the src gene product (37). Moreover, it has been shown to reverse cell transformation by RSV (37). QM-RSV cells were cultured at 35.5°C, first in growth medium for 24 hr, and then in differentiation medium which contained the inhibitor for 24 hr to see whether fusion occurred. As shown in Fig. 9, myoblast fusion occurred at 35.5°C in the presence of herbimycin A. After 24 hr, the fused cells in the cultures differed morphologically from those at 41°C in being somewhat flattened. Moreover, the tubes were not elongated, like those in primary cultures at 35.5°C, although they gradually elongated during further culture at 35.5°C. In cultures at 41°C, fusion was not affected by the presence of the drug. These results indicate that regulation of myoblast fusion in QM-RSV cells depends on the tyrosine kinase activity of the src gene product.

The effect of herbimycin A on biochemical differentiation was also examined using CK activity as a marker. As shown in Fig. 10, CK activity was significantly restored by treatment with the inhibitor at 35.5°C in differentiation medium for 24 hr, although it was not completely restored to the level at 41°C. Thus tyrosine kinase activity of the src gene product is not only associated with myoblast fusion, but also with biochemical differentiations.

DISCUSSION

As differentiation of muscle cells involves several processes that can easily be followed in vitro, myoblasts have often been used as experimental models of cell differentiation. Fusion of myoblasts is one of the most distinct processes as a terminal event in myogenic differentiation. However, its mechanism is still unknown. One reason for this is that there is no suitable experimental system in which the fusion reaction can be controlled. As a first step in clarifying the mechanism of myoblast fusion, we tried to develop an appropriate experimental system. The possibility that myogenic differentiation can be controlled by temperature in cells transformed with oncogenic virus has been proposed, as mentioned in the "Introduction", but no clearly defined cell system for this purpose has been reported. In this study we established and characterized a stable experimental system using quail embryonic myoblasts transformed with a ts-mutant of RSV in which myogenic differentiation is controlled by temperature.

In primary culture, QM cells fused equally well at 35.5°C and 41°C, whereas after transformation with a ts-mutant of RSV, their fusion became thermosensitive. In this system fusion proceeded rapidly and synchronously at 41°C, the non-permissive temperature, but was suppressed completely at the permissive temperature. Thus the reaction of myotube formation could be controlled by temperature, rather than calcium. This is a very convenient method for controlling the fusion of myoblasts and facilitates morphologically and biochemical studies on the mechanism of fusion.

The fact that the virus used in our experimental system causes temperature-dependent transformation suggests that the product of the viral oncogene is involved in myoblast fusion and biochemical differential changes. Myoblast differentiation has been suggested to be affected by the src gene product (35), but no direct evidence for this has been reported. In this study, we demonstrated the relationship between differentiation and the src gene product more directly using herbimycin A, an inhibitor of tyrosine kinase. Results indicated that the tyrosine kinase activity of the src gene product, but not expression of the gene itself, is associated with both myoblast fusion and biochemical differentiation. These findings suggest that some prot ein(s) associated with myogenic differentiation or its expression may be inactivated by the src gene product, probably by phos-
phorylation. The src gene product is reported to act as a transforming factor only on its binding to the cell membrane (7). Furthermore, the cytoplasmic surface proteins of the plasma membrane are known to be important in maintaining the membrane structure and in signal transduction pathways. Growth factor signal transduction pathways have been suggested to be associated with myogenic developmental events (5, 13, 30, 34). Thus myogenic differentiation may be affected by binding of the src gene product to the membrane and its signal action. Binding of the src gene product to the cytoplasmic surface may result in phosphorylation of some protein(s) by tyrosine kinase that induces changes of membrane structure that directly or indirectly suppress myoblast fusion and other cellular differentiations. In this connection, it is noteworthy that Lognonne and Wahrmann recently suggested that phosphorylation of a cell surface protein is associated with fusion competence (26, 27). Studies at the cellular and molecular levels on the precise relationship of the src gene product and myoblast fusion are now in progress.

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