Induction of Syncytia by Moloney Murine Leukemia Virus in Myoblasts Defective in Differentiation

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fu-1 cells, a nonfusing variant of the L<sub>M</sub> line of rat myoblasts, form syncytia upon infection with murine leukemia virus (MuLV) or upon cocultivation with MuLV-infected cells; L<sub>M</sub> cells do not form these syncytia, but do fuse into multinucleate myotubes. Syncytia of fu-1 cells form within 1 h after infection. The number of syncytia formed is proportional to the multiplicity of virus within a range of 4 to 16 and is maximum when the cell density is subconfluent. When either XC or fu-1 cells are productively infected with MuLV, they become resistant to syncytia formation by passage 3. The rapid formation of syncytia in fu-1 cells was found amenable for selection of temperature-sensitive mutants of MuLV and for screening additional variants of the L<sub>M</sub> line.

Both DNA and RNA viruses have been reported to cause fusion of many types of cells (11, 12). Although it cannot be assumed that the mechanisms of normal membrane fusions (e.g., pinocytosis, endocytosis, lysosome fusion, myoblast fusion, etc.) are the same as those promoting fusion in pathological situations, it is believed that these fusion reactions share common essential features (12, 14). In virus-induced fusion, the initial interaction between the cells seems to be mediated by the virion. The specificity of this interaction is determined by the ability of the virus to bind to the cell membrane. Thus, defining the mechanism of virus-induced fusion may also be germane to understanding the mechanism of membrane fusion in general. Virus-induced fusion has also had other applications and has provided a model for the study of virus absorption, penetration, and release, as well as virus-cell interactions. More recently, virus-induced fusion has also provided a useful tool for the recovery of oncogenic and pathogenic viruses from transformed or infected cells (19). The rapid cell fusion obtained by high multiplicities of inactivated Sendai virus (9) has also led to the use of virus-induced fusion as an important biological tool for studying somatic cell genetics, gene action and its control, and phenotypic expression.

Another form of naturally occurring cell fusion takes place during the development of skeletal muscle. Individual myoblasts proliferate and spontaneously fuse into multinucleate, elongate filaments or myotubes. A nonfusing variant (fu-1) was isolated from the rat myoblast L<sub>M</sub> line by Kaufman (7). In a comparison of L<sub>M</sub> and fu-1 cells, Kaufman (7; Kaufman, manuscript in preparation) showed that the nonfusing cells have several characteristics of transformed cells. Recently, it has also been demonstrated by Kaufman and Wong (manuscript in preparation) that the fu-1 cells liberate into the culture media a type C particle that has reverse transcriptase activity and that sediments at a density of approximately 1.15 g/cm<sup>3</sup>. This virus could not be detected in the parent L<sub>M</sub> cells by these criteria. Since cells infected with Moloney murine leukemia virus (MuLV), or MuLV alone, can cause XC cells (a rat cell that harbors a latent avian sarcoma virus) to fuse into syncytia (8), we sought to determine whether MuLV would fuse fu-1 cells. We have found that fu-1 cells form syncytia when brought in contact with MuLV or MuLV-infected TB cells. These observations suggested that fu-1 cells could be a useful tool for studying the mechanism of virus-induced fusion. We now report the characteristics of fu-1 and L<sub>M</sub> cells upon infection with MuLV. A comparison is also made with MuLV-infected XC cells.

MATERIALS AND METHODS

Cell cultures. TB cells established from mixed cultures of bone marrow and thymus CFW/D mouse cells by Ball et al. (2) were obtained from J. K. Ball. XC cells were established from a tumor induced in Wistar rats with the Prague strain of Rous sarcoma virus (17). XC cells contain the Rous sarcoma virus genome but do not release infectious virus (8). Our XC cells were supplied by J. W. Hartley.

The fu-1 cell line is a nonfusing variant of the L<sub>M</sub> line of rat skeletal muscle myoblasts isolated by
Kaufman (7) and has been cloned and maintained in culture for more than 2 years by one of us (S. J. K.). The Lₐₚ cells were kindly provided by D. Yaffe.

The cell lines were grown (except where indicated) in Eagle minimal essential medium containing 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.), 50 U of penicillin per ml, and 50 μg of streptomycin per ml. All cell lines were maintained in Falcon plastic T-75 bottles by serial passage of 5 × 10⁶ cells in 20 ml of complete medium. Cultures were recovered from frozen stocks as needed and were not maintained in excess of 30 consecutive passages to minimize any spontaneous production of endogenous virus. Control cultures were routinely tested for the presence of leukemia virus as described by Wong et al. (22). All cell lines were regularly tested for mycoplasma. Only mycoplasma-free cell cultures were used for the experiments reported.

**Viruses stocks and virus assays.** The wild type (wt) and temperature-sensitive (ts) mutants of the Moloney strain of mouse leukemia virus were grown in TB cells as described by Wong and McCarter (20). MuLV strains were assayed for their ability to promote formation of syncytia by the modified XC assay of Wong et al. (22).

**Syncytia assays.** (i) Direct viral assay. Unless otherwise stated, 1.5 × 10⁶ to 2 × 10⁶ cells were plated on 35-mm Falcon plastic tissue culture dishes and incubated at 37°C for 20 to 24 h. Medium was then removed and the cell monolayers were infected with MuLV at a multiplicity of infection (MOI) of 15 to 20. A 0.2-ml aliquot of the virus, in medium lacking serum and containing 28 μg of DEAE-dextran per ml, was added per plate. After 60 min at 37°C, unabsorbed virus was removed, fresh complete medium was added, and the cultures were incubated at 37°C. At appropriate times, the cells were fixed in 70% methanol and stained with a 5% Giemsa solution. Multinucleate cells containing three or more nuclei were counted in four or five randomly selected fields. Duplicate plates were counted, and the mean number of syncytia per square centimeter were calculated.

(ii) **Cell cocultivation assays.** Cocultivation assays were performed as indicated in Tables 1 and 4.

**RESULTS**

**Virus-induced fusion of fu-1 cells.** The fu-1 cells were shown to form syncytia when directly infected with MuLV (Fig. 1 and Table 1); the parent Lₐₚ cells, however, were not fused by the virus. (XC cells will also form syncytia upon direct infection with MuLV.) In addition, when TB cells were infected with MuLV and cocultivated with XC, fu-1, or Lₐₚ cells, only the XC and fu-1 cells formed syncytia around the colonies of TB cells producing MuLV (Fig. 1 and Table 1).

The fact that MuLV could induce syncytia formation in both fu-1 and XC cells prompted further comparisons of these two cell lines. The fu-1 and XC cells were found to have similar growth characteristics; both exhibit doubling times of about 16 h. TB cells grow considerably faster, with a generation time of approximately 12 h.

**Time course of syncytia formation in MuLV-infected fu-1 and XC cells.** Having established that MuLV could induce syncytia formation in fu-1 cells, we compared the rate of syncytia formation in fu-1 and XC cells infected with MuLV. Monolayers of fu-1 or XC cells were inoculated with MuLV at an MOI of 16. The number and size of the syncytia that developed within the next 24 h were determined. In MuLV-infected fu-1 cells, syncytia were observed as early as 1 h after infection. At this time, the mean number of nuclei per syncytium is 3.4; about 3% of the syncytia have 6 or more nuclei. As shown in Fig. 2, the number of syncytia increased rapidly with time; however, the mean number of nuclei per syncytium increased only slightly. By 4 to 6 h postinfection, about 12% of the syncytia contained six or more nuclei. No significant increase in the number of syncytia was observed after 6 h postinfection, although the number of nuclei per syncytium increased slightly with time. Up to 30 nuclei have been observed in a syncytium. By 6 h post-infection some syncytia began to degenerate. In a degenerating syncytium, the cytoplasm becomes vacuolated and the nuclei fuse and may subsequently fragment. By 24 h postinfection, up to 50% of the syncytia may show some signs of degeneration. The gradual decrease in the number of syncytia after 8 h postinfection and the relatively minor increases in the mean number of nuclei per syncytium may be due to fusion of adjacent nuclei, degeneration of the syncytia, or both. Scanning electron microscopy provides further evidence that syncytia development is a continuous process during which cells are recruited. These resultant giant cells, however, are quite different from multinucleate myotubes (Fig. 3).

In XC cells infected with MuLV, essentially the same time course of syncytia formation was observed. Fused cells containing three or four nuclei were abundant within 2 h after infection. As in fu-1 cells, the maximum number of syncytia had formed by 4 to 6 h postinfection, the number of nuclei per syncytium increased with time, and the number of syncytia remained essentially unchanged between 6 and 24 h.

**Effect of MOI on syncytia formation of fu-1 and XC cells.** Virus-induced cell fusion is thought to occur at high MOIs and without the need for replication of the virus. Since syncytia formation in fu-1 and XC cells was observed within 1 h after infection, we could readily test the effect of the MOI of virus on syncytia forma-
Fig. 1. Syncytia formation in fu-1, L8, and XC cells infected with MuLV and cocultivated with infected TB cells. fu-1 (A), L8 (B), and XC (C) cells were grown as control cultures (1), infected with MuLV (2), cocultivated with TB cells (3), or cocultivated with MuLV-infected TB cells (4). Both fu-1 and XC cells formed syncytia upon infection with MuLV or cocultivation with infected TB cells; L8 cells did not form syncytia. Cells were fixed in methanol and stained with Giemsa. Bar equals 100 μm.
and XC cells increased linearly when the monolayers were infected with 4 to 16 infectious units (IU) of virus per cell. 

Effect of cell density. Since contact is obviously required for fusion, the effect of cell density on MuLV-induced syncytia formation of fu-1 cells was tested. Dishes (35 mm) were seeded with various numbers of cells and incubated for 20 h. The monolayers were then inoculated with MuLV, incubated for 6 h, and then fixed and stained. Figure 5 shows that the numbers of syncytia that developed were proportional to the number of cells plated. The maximum number of syncytia was formed when the cell monolayer was nearly confluent (i.e., when \(1.5 \times 10^4\) cells were plated). Little increase in the number of syncytia was obtained at higher densities.

Replication of MuLV in fu-1, XC, TB, and \(L_s\) cells. Our preliminary investigations showed that MuLV not only induced formation

<p>| Table 1. MuLV-induced syncytia formation in fu-1, (L_s), and XC cells |
|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Cell lines</th>
<th>No. of syncytia/cm(^2*)</th>
<th>Colonies with syncytia (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>fu-1</td>
<td>10.7</td>
<td></td>
</tr>
<tr>
<td>fu-1 + MuLV</td>
<td>(1.5 \times 10^3)</td>
<td></td>
</tr>
<tr>
<td>(L_s)</td>
<td>29.3</td>
<td></td>
</tr>
<tr>
<td>(L_s) + MuLV</td>
<td>41.1</td>
<td></td>
</tr>
<tr>
<td>XC</td>
<td>50.0</td>
<td></td>
</tr>
<tr>
<td>XC + MuLV</td>
<td>(1.4 \times 10^3)</td>
<td></td>
</tr>
<tr>
<td>fu-1 + TB</td>
<td>0</td>
<td>15.5</td>
</tr>
<tr>
<td>fu-1 + TB-MuLV</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>(L_s) + TB</td>
<td>0</td>
<td>17.3</td>
</tr>
<tr>
<td>(L_s) + TB-MuLV</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>XC + TB</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>XC + TB-MuLV</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* A total of \(1.8 \times 10^4\) cells were plated on 35-mm dishes. After 20 h the medium was removed and the monolayers were infected with MuLV at an MOI of 16, as described in the text. After 6 h the cells were fixed and stained, and syncytia containing three or more nuclei were counted.

* TB cells were trypsinized before cultures became confluent and transferred in complete medium to Spinner flasks. After 4 h of stirring at 37°C, the concentration of cells was adjusted to \(1 \times 10^6/\)ml. Virus diluted in medium lacking serum and containing 28 \(\mu\)g of DEAE-dextran per ml was used to infect the cells at an MOI of 0.2. Cells were infected in suspension as described by Wong et al. (22), and 500 cells were plated and incubated at 37°C for 3 days. The medium was then removed, \(5 \times 10^4\) fu-1, \(L_s\), or XC cells were added, and the cultures were incubated at 37°C for 1 more day. The cells were fixed and stained with Giemsa, and the proportion of TB cell colonies surrounded by syncytia was determined.
of syncytia, but also replicated in fu-1 cells. Therefore, we determined the replication of MuLV in fu-1 cells and compared it with that in Ls, XC, and TB cells. A total of $5 \times 10^5$ cells were plated in 75-cm$^2$ bottles. After 24 h the monolayer was inoculated with MuLV (MOI of 4). At 1, 2, 3, and 4 days after infection, samples were removed and assayed for IU using the modified XC assay. Growth of MuLV in fu-1 cells and TB cells was very similar (Fig. 6). In both fu-1 and TB cells, the maximum MuLV titers of $2 \times 10^6$ IU/ml were reached in 96 h. MuLV replicated much less efficiently in XC cells than in fu-1 and TB cells; by 96 h a titer of only $2.8 \times 10^6$ IU/ml was obtained. Ls cells also supported the growth of MuLV; however, the maximum titers obtained in these cells were approximately 10-fold less than in fu-1 cells. This difference may reflect the continued proliferation of fu-1 cells after confluence is reached (Kaufman, manuscript in preparation).

The genotype of the virions produced from MuLV-infected fu-1 cells was tested by infecting fu-1 cells at 34°C with ts3, a ts mutant of MuLV, and assaying the viral progeny at the permissive (34°C) and nonpermissive (39°C) temperatures. The results of this assay (Table 2) clearly show that the ts genotype was maintained in the virus replicated in fu-1 cells.

Loss of syncytia-forming ability in MuLV-infected fu-1 and XC cells. Chan et al. (3, 4) have shown that XC cells infected with MuLV lose their ability to form syncytia after three passages in culture; however, these infected cells continue to produce and liberate virus into the culture media. Therefore, experiments were carried out to determine whether fu-1 cells and our XC cell line would become resistant to syncytia formation upon productive infection with MuLV. The fu-1 and XC cells infected with MuLV (MOI of 4) were subcultured every 2 or 3 days. The numbers of syncytia formed were counted (Table 3); both MuLV-infected XC and fu-1 cells lost their ability to form syncytia by passage 2 or 3. Nevertheless, both fu-1 and XC cells continued to produce infectious virus after they had become refractory to syncytia formation. Whereas by passage 2 the numbers of syncytia decreased to about the same level as that in uninfected fu-1 or XC control cultures, no significant differences in the titers of the infectious particles were obtained after the repeated passages. As cited earlier, fu-1 cells produce more virus than do XC cells. The fu-1 and XC cell lines which do not form syncytia, but which are productively infected with MuLV, will be referred to as fu-1(M) and XC(M).

**Fig. 2. Time course of syncytia formation in MuLV-infected fu-1 and XC cells.** A total of $1.8 \times 10^5$ fu-1 or XC cells were plated on 35-mm petri dishes. After 20 h the cell monolayers were inoculated with MuLV, at an MOI of 16, as described in the text. At the time intervals indicated, plates were removed, fixed, and stained and the numbers of syncytia were counted. Symbols: — O — , fu-1 syncytia; — Δ — , XC syncytia.

To further demonstrate that fu-1(M) cells were productively infected, monolayers of uninfected fu-1 or XC cells were overlaid with fu-1(M). Whereas neither cell type grown alone produced appreciable numbers of syncytia (Table 4), cocultivation of fu-1 or XC cells with fu-1(M) resulted in extensive cell fusion. However, in control cultures of TB cells overlaid with fu-1(M), the number of syncytia was not significantly greater than that produced by the fu-1(M) control. This result is as expected since MuLV-infected TB cells do not form syncytia.

**Rapid method for screening ts mutants.** Since MuLV-infected fu-1 or XC cells form syncytia within 1 h after addition of the virus, these cells were tested for their usefulness in screening for ts viral mutants. TB cells infected with the ts and wt MuLV (MOI = 2) were grown for 2 days at permissive (34°C) and nonpermissive (39°C) temperatures. The medium was then removed and replenished with fresh medium. After an additional day at their respective temperatures, the supernatants were harvested, diluted 1:4 with medium containing DEAE-dextran, inoculated onto subconfluent monolayers of fu-1 cells, and incubated at 39°C. After 6 h the plates were fixed and stained. Supernatants from TB cells that were infected with ts1 and ts3 and grown at the permissive temperature promoted the formation of 7- and 100-fold more syncytia than the respective
supernatants from cultures grown at 39°C (Table 5). wt MuLV grew equally well at both temperatures and, thus, promoted the development of equivalent numbers of syncitia. These results suggest that the capability of MuLV to rapidly form syncytia of fu-1 and XC cells can be used to rapidly screen for further MuLV ts mutants.

**DISCUSSION**

In contrast with the parent L8 cells, fu-1 cells form syncytia upon infection with MuLV or upon cocultivation with MuLV-infected cells. The fact that MuLV could not induce syncytia formation in L8 cells is not due to the inability of L8 cells to support the growth of MuLV. We have shown that both L8 and fu-1 cells can be productively infected with MuLV. However, primary cultures and subsequent serial passages of newborn rat thigh muscle cells neither formed viral-induced syncytia nor supported the growth of MuLV (Wong and Kaufman, unpublished data).

fu-1 cells are of interest in that they have lost their ability to differentiate and spontaneously fuse into multinucleate myotubes and possess several characteristics of transformed cells (7; Kaufman, manuscript in preparation). In addition, fu-1 cells have also been found to produce a type C-like virus (Kaufman and Wong, manuscript in preparation). Whether the expression of this virus is related to the failure of these cells to differentiate into myotubes is not presently known. Whether the endogenous or latent virus plays a role in type C virus-induced fusion is also not understood. Thus far, type C virus-induced fusion has only been reported in cells that harbor a resuscitable latent virus (1, 5, 8, 14, 15). The ability of fu-1 cells to form syncytia upon infection with MuLV, in contrast to the inability of the parental L8 cell line to form syncytia upon MuLV infection, suggested that the activation of a latent or endogenous virus may play a role in virus-induced syncytia for-

**Fig. 3.** Comparison of virus-induced syncytia and spontaneously fused myotubes. (A) Scanning electron micrograph of a fu-1 syncytium. A total of 1.5 × 10^5 cells were grown on glass cover slips for 20 h and infected with MuLV at an MOI of 16. After 6 h the cells were fixed in glutaraldehyde and processed for scanning electron micrography as described by Wong and MacLeod (21). Bar equals 15 μm. (B) L8 myotubes were fixed and stained with Giemsa. In contrast to the virus-induced syncytia, myotubes form elongate straps that are often branched and contain dozens of nuclei that remain distinct and do not degenerate. Bar equals 100 μm.
The mechanism of MuLV-induced syncytia formation in the fu-1 cell line will be the subject of a further communication (Wong, Yuen, and Kaufman, manuscript in preparation).

We have compared the XC cell line with the fu-1 cell line. These cell lines are morphologically distinct. fu-1 cells are larger and have much shorter processes than do XC cells (Fig. 1, A1 and C1). Both cell lines support MuLV growth (albeit to different levels) and both lines form syncytia as early as 1 h upon infection with MuLV. Furthermore, when productively infected with MuLV both cell lines become resistant to syncytia formation upon repeated passage. Similar findings have also been reported in other cell lines infected with the appropriate type C virus (1, 5, 8, 13-16). It is not yet known why cell lines that are productively

![Graph](image1)

**Fig. 4.** Effect of MOI on syncytia formation in fu-1 and XC cells. fu-1 and XC cells were plated and 20 h later the cell monolayers were infected with various amounts of MuLV. At 6 h after infection the cell monolayers were fixed and stained, and the numbers of syncytia were counted. Symbols: ○, fu-1 syncytia; △, XC syncytia.

![Graph](image2)

**Fig. 5.** Effect of cell density on syncytia formation in fu-1 cells. Various numbers of cells were plated on 35-mm petri dishes. After 20 h the cell monolayers were inoculated with MuLV at an MOI of 16. The infected cultures were incubated for 6 hr at 37°C, fixed, and stained, and the numbers of syncytia were counted.

![Graph](image3)

**Fig. 6.** Growth of MuLV in TB, fu-1, L5, and XC cells. A total of $5 \times 10^5$ cells were plated in 75-cm² plastic bottles and incubated at 37°C. After 20 h the medium was removed and the monolayers were infected with MuLV (MOI of 5) in medium lacking serum but containing 29 μg of DEAE-dextran per ml. After 50 min at 37°C, the medium was removed, the cells were washed, and fresh medium was added. Cells were incubated at 37°C and samples of media were periodically removed and assayed by the modified XC procedure.

**Table 2.** Growth of wt MuLV and ts3 in fu-1 and TB cells

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cell line</th>
<th>Titer (IU/ml) at:</th>
<th>Ratio of titers at 34°C and 39°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>34°C</td>
<td>39°C</td>
</tr>
<tr>
<td>wt</td>
<td>fu-1</td>
<td>$8.2 \times 10^7$</td>
<td>$2.7 \times 10^7$</td>
</tr>
<tr>
<td></td>
<td>TB</td>
<td>$1.4 \times 10^8$</td>
<td>$6.1 \times 10^7$</td>
</tr>
<tr>
<td>ts3</td>
<td>fu-1</td>
<td>$9.4 \times 10^7$</td>
<td>$5.4 \times 10^7$</td>
</tr>
<tr>
<td></td>
<td>TB</td>
<td>$1.2 \times 10^8$</td>
<td>$6.2 \times 10^7$</td>
</tr>
</tbody>
</table>

*TB and fu-1 cells were grown and infected with wt or the ts3 mutant of MuLV. The infected cultures were incubated at 34°C for 72 h; the medium was then removed and replenished with fresh medium. After 24 h the supernatants were collected and filtered, and the virus was assayed at 34 and 39°C by the modified XC assay.
infected with type C virus become refractory to syncytia formation.

XC cells are used extensively as an assay for MuLV because they form syncytia upon cocultivation of XC cells with cells producing this virus. It has also been reported (6, 18) that syncytia with 5 to 10 nuclei are formed in XC monolayers within 6 h after the addition of MuLV. We were able to detect syncytia in MuLV-infected XC cells as early as 1 h after infection. We do not believe that our XC cell line is more sensitive than those used in other laboratories because the majority of the syncytia we observed at 1 h postinfection consisted of three to four nuclei. The number of syncytia formed depends on incubation time, cell density, and MOI. The optimal conditions for the formation and scoring of syncytia are the use of subconfluent monolayers of cells, infection with MuLV within a multiplicity range of 15 to 20, and observation of syncytia 6 h postinfection. The fixation of the assay plates at 6 h postinfection avoids the fusion of neighboring syncytia and shortens the time required for this assay. The rapidity in syncytia formation in both XC and fu-1 cells makes these systems useful as quick assays for MuLV and for screening ts mutants of MuLV. Of these two systems, the larger size of fu-1 cells facilitates the detection of syncytia. The rapid induction of syncytia by MuLV in fu-1 cells may also be used to screen for additional variants of the L9 cell line. The characterization of these L9 variants will hopefully lead to a fuller understanding of the mechanisms of spontaneous myoblast fusion as well as a clarification of the relationship between transformation and differentiation.

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LITERATURE CITED


