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Rous Sarcoma Virus Mutant LA3382 Is Defective in Virion Glycoprotein Assembly

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LA3382 is a temperature-sensitive replication-defective mutant of Rous sarcoma virus that contains four active mutations. In this study we performed experiments to determine the biochemical defect that blocks the synthesis of infectious virus late in the replication cycle. At the nonpermissive temperature (41°C) cells infected with LA3382 synthesized virus particles which were noninfectious and exhibited significant reductions in the amounts of gp85 and gp37 present in the virions. An analysis of the intracellular viral polypeptides indicated that the precursor of the viral glycoproteins (Pr95) was synthesized normally but underwent cleavage at a reduced rate at the restrictive temperature. Pr95 did not accumulate in infected cells and was inserted into mutant virions at 41°C; however, Pr95 was cleaved in such a way that gp85 was released from the viruses and could be detected in the supernatant medium by immunoprecipitation. The virus-free glycoprotein was indistinguishable from wild-type gp85 and may have been released due to an anomalous cleavage. Pulse-chase experiments also indicated that the Pr180 polyprotein precursor of the reverse transcriptase was cleaved to the active form of the enzyme more slowly at 41°C in LA3382-infected cells. This accounted for the twofold lower level of polymerase activity found in mutant virions at 41°C, a defect which probably did not account for the observed 20- to 50-fold reduction in infectivity. Furthermore, the replication defect was not complemented by an *env* deletion mutant Rous sarcoma virus [RSV(−)], which should complement a *pol* defect. Therefore, we concluded that the major lesion that impairs replication in LA3382 is within the *env* gene.

The isolation and characterization of temperature-sensitive mutants of avian sarcoma viruses have led to the identification of the molecular functions that are encoded on the viral genome (5, 22, 24). One such mutant of Rous sarcoma virus (RSV) Prague strain subgroup C (PR-C) is LA338, which appears to contain four defects. One of these affects an early function (before integration into the host genome) that impairs both replication and transformation. The remaining defects are in late functions; one is in the *src* gene affecting transformation (29), and the other two are in genes required for virus replication (2, 9, 15, 17, 25). Previously, the defects of LA338 were shown to be due to separate lesions rather than a single coordinate mutation by the generation of recombinants between LA338 and wild-type RSV PR-B (9). One of these recombinants, LA3382, has only the early defect and the lesions that impair virus replication at 41°C. This recombinant has been used for biochemical characterizations of the late replication defect in LA338 since it has a wild-type *src* gene. Therefore, it has the advantage that it transforms cells at both the nonpermissive temperature (41°C) and the permissive temperature (35°C) if the early defect is avoided by initiating infection at 35°C. Thus, the polypeptide compositions of the mutant and wild-type virions produced at the elevated temperature can be compared more accurately since the cells are physiologically similar. The data described here indicated that the major defect which impairs replication of LA3382 at 41°C is in the biosynthesis of the *env* gene products. The mutation appears to result in aberrent cleavage of Pr95, the precursor of the virion glycoproteins, so that gp85 and gp37 are absent from the virus particles but can be identified in the culture supernatants. Although biosynthesis of the polymerase was less efficient in this mutant, the results of complementation experiments indicated that this is not the major defect in the replication of LA3382 at 41°C.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo fibroblasts of the C/O phenotype were prepared from fertilized eggs obtained from Hyline International, Dallas Center, Iowa. Fertilized eggs with the C/E phenotype were

purchased from SPAFAS Inc., Norwich, Conn. C/BE fibroblasts (Kimber) were kindly provided by Carlo Moscovici. Only cells negative for chick helper factor (*chf*$^-$) were used. The cell culture techniques and the *chf* tests used have been described previously (7). The medium used consisted of Hams F10 medium supplemented with 10% tryptose phosphate broth, 5% calf serum, 6.6 mM sodium bicarbonate, 100U of penicillin per ml, and 100 μg of streptomycin per ml. Polybrene (2 μg/ml) was used to enhance virus adsorption, and 1% dimethyl sulfoxide was added for transformed cell cultures.

Cloned RSV PR-C was used as a control in all experiments. Temperature-sensitive mutants of RSV PR-C and avian sarcoma virus B77 (LA335, LA3342, and LA3382) were obtained as described previously (9, 25).

The titers of infectious viruses were determined by focus and replication assays (7). Assays for reverse transcriptase (polymerase) activity were performed as described by Tereba and Murti (20). Competition radioimmunoassays were used to quantitate virus for the polymerase assay (1). Infectious center assays were performed as previously described (23).

Complementation assay. Cells were infected at a high multiplicity with Rous-associated virus-3 and passaged twice before a superinfecting virus was added. At 24 h after superinfection with a mutant virus or a wild-type transforming virus, one set of plates was shifted to 41°C, and a duplicate set was maintained at 35°C. The medium was harvested 48 h later and assayed at 35°C for focus-forming virus.

Radiolabeling of virus. Duplicate chicken embryo cell cultures were infected at 35°C with LA3382 or PR-C and passaged once; when the cells were completely transformed, one group of plates was shifted to the nonpermissive temperature (41.8°C), and a control group was maintained at the permissive temperature (35°C). After 24 to 48 h, the medium was changed, and the cultures were labeled for 24 h with [^{3}H]glucosamine (80 μCi/ml; 6.3 Ci/mmol; ICN, Irvine, Calif.) or [^{3}H]fucose (80 μCi/ml; 26 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) and a ^{14}C-amino acid mixture (10 μCi/ml; 50 mCi/mmol; ICN) in 5.0 ml of medium. For pulse-chase experiments, cultures were labeled with [^{3}H]leucine (1 mCi/ml; 65 mCi/mmol; Schwarz/Mann, Orangeburg, N.Y.), as described previously (18).

Virus purification. The medium was removed from each infected cell culture and clarified by centrifugation with a model J6 centrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) for 10 min at 5,000 rpm. The clarified supernatants were loaded onto discontinuous 40% (wt/wt) potassium tartrate–20% (wt/wt) sucrose gradients, and the viruses were recovered from the gradient interfaces after centrifugation at 40,000 rpm for 90 min in a Beckman SW41 rotor. The virus bands were diluted with buffer, pelleted in an SW41 rotor at 40,000 rpm for 60 min, and suspended in a small volume for electrophoresis (8).

Immunoprecipitation of viral polypeptides. After the labeling period and after the medium was removed, 1 ml of lysis buffer A (1% Triton X-100, 1% sodium deoxycholate, 25 mM Tris-hydrochloride, pH 8.0, 50 mM NaCl) was added to each 60-mm dish, which was left on ice for 15 min. The lysed cells were removed from the dish by pipetting and then centrifuged with a Beckman microfuge for 5 min to pellet the nuclei and debris. The clarified supernatants were removed, sodium dodecyl sulfate was adjusted to 0.1%, and stored at −70°C. Samples (200 μl) were incubated at 37°C for 1 h with 4 μl of antiserum (an empirically determined excess of antibody). Rabbit antiserum to avian sarcoma virus was prepared as previously described by Hunter et al. (8). Then 100 μl of a 10% suspension of Formalin-fixed, heat-killed *Staphylococcus aureus* (3) was added, and the mixture was incubated at room temperature for 30 min. The immune complexes were washed twice in lysis buffer B (0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% sodium deoxycholate, 0.05 M Tris-hydrochloride, pH 8.0, 0.15 M NaCl) and once with 20 mM Tris-hydrochloride (pH 8.0) to remove salt. The immunoadsorbant was dissociated from the antibody-antigen complex by adding sodium dodecyl sulfate and β-mercaptoethanol to final concentrations of 2% and 0.2 M, respectively; then the preparation was boiled for 2 min, and the immunoadsorbant was removed by centrifugation with a Beckman microfuge for 5 min. For immunoprecipitations from culture fluids, 1 ml of medium was processed in the same manner as the cell lystates, except that a 0.25 volume of 5X lysis buffer A was added before clarification.

Polyacrylamide gels. Purified viral polypeptides and immunoprecipitated viral polypeptides were electrophoresed on Tris-glycine slab gels (thickness, 1.5 mm) containing 12% acrylamide or 5 to 20% acrylamide gradients, as described previously (8). Molecular weight standards (Bio-Rad Laboratories, Richmond, Calif.) were located by Coomassie blue staining. Virus-specific polypeptides were located either by slicing and counting in Omnifluor-toluene containing 9% Protosol and 1% water or by fluorography.

RESULTS

Identification of the early and late defects of LA3382. The temperature-sensitive mutant LA3382 appears to contain all of the biological defects of its parent virus LA338 except the late defect in the *src* gene. An early defect should block a function required before integration of the viral genome into the host cell DNA (24) and therefore impair both replication and transformation. However, in previous reports, it was suggested that the early defect of LA338 impaired only transformation, not replication (9, 21). Therefore, we investigated the effect of this lesion in LA3382 by using an infectious center assay (23) to determine whether the defect prevented the establishment of infections at the restrictive temperature. Such an approach measured the efficiency of infection at 41°C directly. In this assay cells were infected at 41°C for 24 h and then treated for 24 h with 1 μg of mitomycin C per ml. The plates were then overlaid with susceptible indicator cells and transferred to 35°C to allow efficient infectious virus

production by infected target cells. Table 1 shows the results of such an assay and a parallel focus assay. It is clear that incubation at 41°C for the first 24 h of infection significantly reduced the efficiency of transformation and replication by LA3382 in a manner similar to the early reverse transcriptase mutant LA335. Wild-type PR-C and LA3342, which has only a late replication defect, transformed and replicated normally under these conditions. Thus, the early defect in LA3382 appeared to prevent the establishment of infection by the virus at 41°C, which would be expected to prevent both replication and transformation. The difference between these results and those reported previously presumably reflected the stringency of the experimental approaches that were used.

LA3382 tranformed chicken cells normally at 41°C if infection was initiated at 35°C, but was not able to produce infectious progeny under these conditions (Table 2). This late reversible defect was particularly interesting, and experiments to define its molecular nature are described below. In these experiments the effect of the early lesion was avoided by initiating all infections at 35°C and shifting to 41°C after all cells were infected in order to assay for biological and biochemical properties.

Production of particles by mutant-infected cells. To determine whether noninfectious particles were assembled and released from mutant-infected cells at 41°C, cells were labeled with [^{3}H]uridine at permissive and nonpermissive temperatures, and the supernatant fluids were analyzed for virus by density gradient centrifugation. With both 35 and 41°C supernatants a sharp peak of uridine label was observed at a density of 1.16 g/ml, which is characteristic of the avian retroviruses (Fig. 1). In contrast, LA334, which has a temperature-sensitive defect in processing of the *gag* gene product, yields virus with a heterogenous density at the nonpermissive temperature (8). Even though infectivity was reduced significantly (20- to 50-fold) at the nonpermissive temperature, normal levels of virus were synthesized at 41°C. Thus, to determine whether the noninfectious particles synthesized by LA3382-infected cells contained a normal complement of viral structural proteins, we compared the polypeptides of the virions produced at 35 and 41°C by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. PR-C, the parent strain of LA338, was used as a control.

LA3382 noninfectious virions are deficient in glycoproteins. Infected cells were labeled with [^{3}H]glucosamine and ^{14}C-amino acids at 35 and 41°C. The virus was purified, dis-

TABLE 1. *Effect of early defect on transformation and replication*[a]

Virus	Titer in focus assay (FFU/ml)[b]		Titer in infectious center assay (FFU/ml)	
	35°C	41-35°C	35°C	41-35°C
PR-C	2.0×10^6	2.5×10^6	2.0×10^6	2.2×10^6
LA3342	4.3×10^6	1.6×10^6	2.1×10^6	8.0×10^5
LA335	1.6×10^6	2.1×10^4	1.7×10^6	1.8×10^4
LA3382	4.2×10^5	1.0×10^2	3.3×10^5	1.0×10^2

[a] An early defect in transformation was determined by a focus assay, in which virus infections were initiated at 41°C and cultures were shifted to 35°C after 24 h. An early defect in replication was determined by an infectious center assay, in which virus infections were initiated at 41°C and cultures were shifted to 35°C after 24 h.

[b] FFU, Focus-forming units.

TABLE 2. *Late replication assay*[a]

Virus	Temp (°C)	Titer (FFU/ml)[b]
PR-C	35	2.0×10^6
PR-C	41	3.5×10^6
LA3382	35	2.0×10^6
LA3382	41	7.0×10^4

[a] C/O cells were infected at 35°C. When the cells were completely transformed, duplicates were shifted to 41°C, and the virus was harvested 48 h later and assayed at 35°C.

[b] FFU, Focus-forming units.

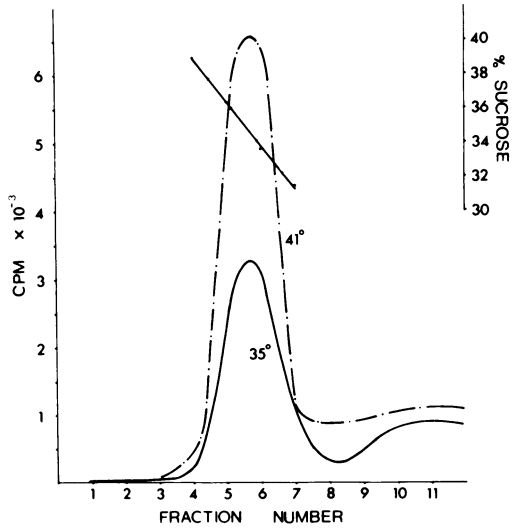

FIG. 1. *Virus particle production at 35 and 41°C by LA3382-infected cells. Chicken embryo (C/O) cells were infected and passaged once, and the transformed cells were shifted to 41°C. Cultures were labeled with [^{3}H]uridine for 16 h, and clarified virus was centrifuged in a 25 to 45% sucrose density gradient at 40,000 rpm for 3 h in a Beckman SW41 rotor. Sucrose densities were determined with a Zeiss refractometer.*

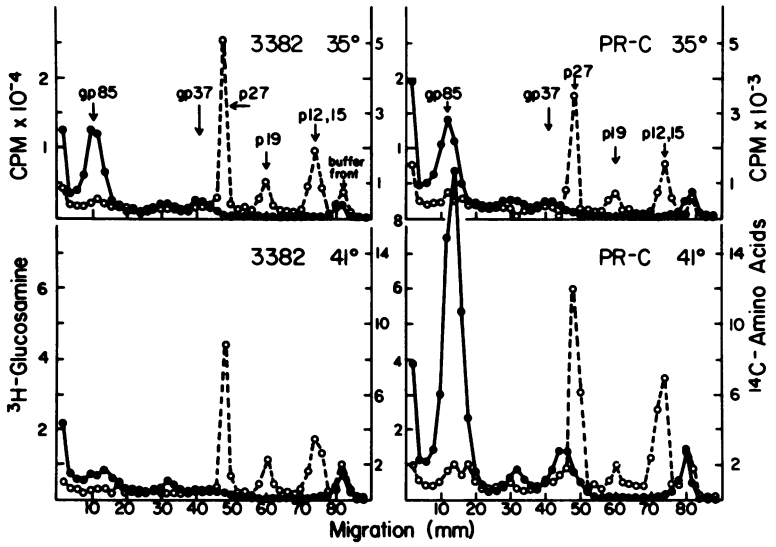

FIG. 2. *Polyacrylamide gel electrophoresis of LA3382 virions released at 35 and 41°C. Virus-transformed C/O cell cultures at 35 and 41°C were labeled with [³H]glucosamine (●) and ¹⁴C-amino acids (○) for 16 h. The virus purified from each culture was electrophoresed on a 12% polyacrylamide gel. ³H and ¹⁴C counts were always plotted at the same ratio.*

rupted, and electrophoresed on polyacrylamide gels (Fig. 2). The polypeptide patterns of LA3382 and wild-type PR-C virions produced at 35°C were very similar. In addition to the major internal structural proteins p27, p19, p12, and p15, two major glucosamine-labeled glycoproteins, gp85 and gp37, were present. At 41°C wild-type PR-C exhibited a similar pattern, except that about four times as much virus was made and there was an enhanced incorporation of sugar label into gp85 and gp37 at the higher temperature. The synthesis of mutant LA3382 virions was also greater at 41°C, but the polypeptide profile was significantly altered. Normal amounts of the non-glycosylated polypeptides were present, but the amounts of gp85 and gp37 appeared to be reduced significantly. Thus, the noninfectious virions produced at the restrictive temperature lacked the glycoproteins necessary for attachment and penetration of target cells. Essentially identical results were obtained when [³H]fucose was used in place of [³H]glucosamine (data not shown). Two other temperature-sensitive mutants of RSV PR-C, PH734 (13) and LA668 (14), have been described, and these mutants also produce virions deficient in envelope glycoproteins. One of these (PH734) is defective in glycoprotein biosynthesis, and the glycoprotein precursor accumulates intracellularly.

Analysis of intracellular viral polypeptides indicates an anomalous processing of mutant glycoproteins. We examined intracellular viral polypeptides to determine whether some aspect of viral glycoprotein synthesis was inhibited or whether the glycoproteins were synthesized but not incorporated into mature virions. The intracellular processing of viral precursor polypeptides was observed in pulse-chase experiments. After leucine deprivation for 1 h, infected cells were pulsed with [³H]leucine for 15 min; then the cells were lysed (pulse) or the labeling medium was removed and fresh medium was added for 2 h before lysing (pulse-chase). The cell lysates were immunoprecipitated with excess antibody to both glycosylated and non-glycosylated virion proteins and were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels (Fig. 3). As expected, we found that Pr76, the precursor of internal structural proteins, was synthesized and processed with equal efficiency in the mutant and wild-type virus-infected cells at both 35 and 41°C. The precursor of viral glycoproteins, Pr95, was also found in pulse-labels of wild-type and mutant virus-infected cells at both permissive and nonpermissive temperatures, with more label incorporated at the higher temperature. Therefore, even though gp85 and gp37 were not found on virions, the glycoprotein precursor was synthesized intracellularly at levels comparable to the levels in the wild type. During a 2-h chase, the amount of Pr95 in wild-type PR-C-infected cells decreased by more than 60%, and a small change in the rate of migration of Pr95 was observed, which may have corresponded to an intermediate processing of the carbohydrate or polypep-

tide moiety. Under these conditions, the majority of the label was released from the cells into the virus, and cell-associated gp85 and gp37 were detected only poorly. Similar results were observed with mutant-infected cells, except that at 41°C significantly less Pr95 was lost from the cells during a 2-h chase. Thus, it appeared that there was a reduction in the rate of processing of Pr95 into virion glycoproteins at the nonpermissive temperature.

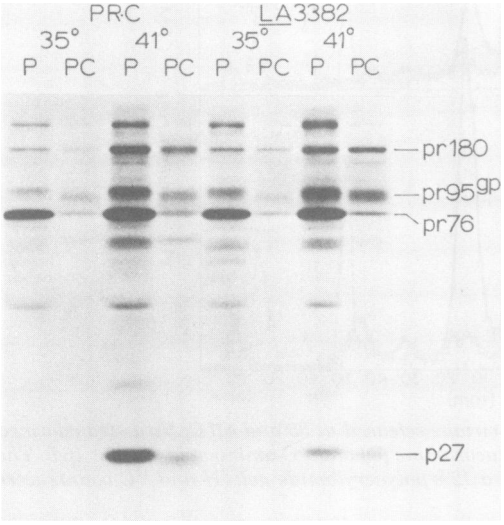

FIG. 3. *Autoradiogram of intracellular viral polypeptides in LA3382- and PR-C-infected cells. Virus-transformed C/O cell cultures at 35 and 41°C were pulsed for 15 min with [^{3}H]leucine (lanes P) or pulsed and then chased for 2 h in unlabeled medium (lanes PC). Each cell lysate was immunoprecipitated with antiserum to whole virus and electrophoresed on a 12% polyacrylamide gel.*

We performed experiments to determine whether the Pr95 polypeptide accumulated in LA3382-infected cells at 41°C under conditions where both radioactive amino acids and sugars were present over longer periods of time. To determine which cell-associated viral polypeptides were present under long-term labeling conditions, infected cells were labeled for 24 h with [^{3}H]fucose; cells were lysed, and viral polypeptides were immunoprecipitated with a specific antiserum (Fig. 4). The polyacrylamide gel patterns of LA3382 and PR-C grown at 35°C indicated that most of the labeled, cell-associated glycoprotein was in the form of the precursor, Pr95. Very little gp85 or gp37 was detected in association with the cells at the lower temperature. On the other hand, at 41°C a significant portion of the cell-associated glycoprotein precursor Pr95 of PR-C was cleaved to gp85 and gp37, which were still associated with the cells. However, this cleavage of the glycoprotein precursor was not observed at the nonpermissive temperature in LA3382-infected cells, where most of the labeled glycoprotein still migrated with Pr95. These results indicated that the gly-

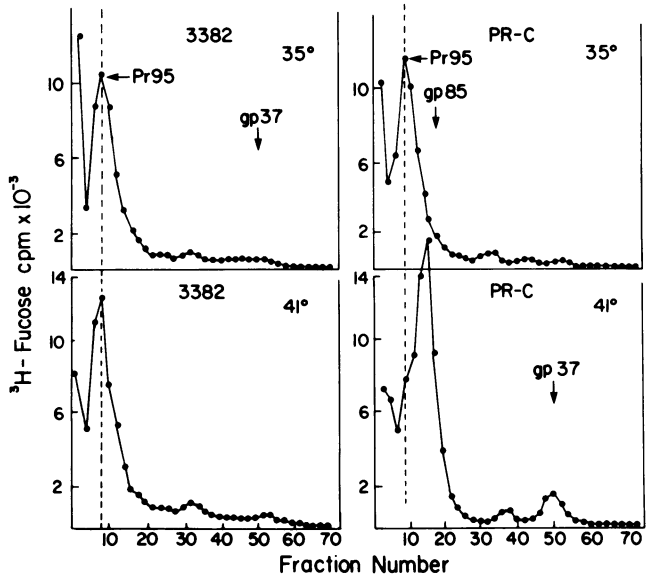

FIG. 4. *Cell-associated viral polypeptides in LA3382- and PR-C-infected cells. Cultures of virus-transformed C/O cells at 35 and 41°C were labeled with [^{3}H]fucose (●) and ^{14}C-amino acids (data not shown) for 16 h. Each cell lysate was immunoprecipitated with antiserum to whole virus and electrophoresed on a 5 to 20% polyacrylamide gradient gel.*

coprotein precursor in LA3382 did not undergo normal cleavage; yet, by comparing the amount of label in the glycoproteins with the amount of label in p27 (data not shown), we found that the glycoprotein did not accumulate in LA3382-infected cells at the restrictive temperature. In this respect LA3382 differed from the glycoprotein mutant PH734, which did accumulate glucosamine-labeled gp85 in infected cells at the nonpermissive temperature (13). However, no such accumulation was observed when [^{3}H]fucose was used to label PH734, presumably because the lesion in this mutant blocked glycoprotein maturation at a stage before fucose was added. We did not observe any accumulation of cell-associated glycoproteins in LA3382-infected cells when we used fucose or glucosamine precursors (data not shown).

Release of defective glycoproteins into the supernatant. Since at the nonpermissive temperature LA3382 Pr95 was not processed as rapidly as wild-type virus Pr95 and yet did not accumulate in infected cells, we postulated that the glycoprotein either might be degraded directly or, perhaps, might be released by an anomalous cleavage event into the supernatant. To differentiate between these two possibilities, infected cells were labeled overnight with [^{3}H]-glucosamine, and samples of labeling medium from which the virus had been removed by high-speed centrifugation were immunoprecipitated and electrophoresed (Fig. 5B). When these patterns were compared with the patterns of immunoprecipitates from noncentrifuged medium containing both virus-associated and free glycoproteins (Fig. 5A), there was a significant difference between the mutant and wild-type virus culture supernatants. All of the glycoprotein found in the supernatant of LA3382-infected cells at 41°C appeared to have been dissociated from the virions, in contrast to supernatants containing mutant virus released at 35°C or wild-type virus produced at either temperature, where most of the gp85 was virion associated. Thus, it appeared that the mutant viral glycoproteins were released into the supernatant medium at 41°C, perhaps due to an aberrant cleavage or to a failure to form disulfide linkages. The glycoprotein which was released into the supernatant was indistinguishable from the gp85 of the virion, as determined by polyacrylamide gel electrophoresis. Whether the mutant glycoprotein was released from infected cells or from nascent virions could not be determined in this experiment.

Pr95 is incorporated into mutant virions. To determine whether the glycoprotein was released from infected cells or nascent virions, cells were pulsed for 15 min with [^{3}H]leucine, and

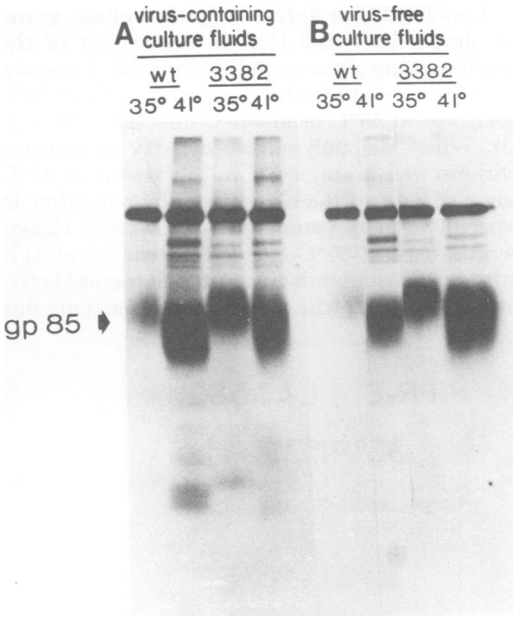

FIG. 5. *Autoradiogram of a polyacrylamide gel showing release of LA3382 gp85 into the culture medium. Virus-transformed cultures of C/O cells were labeled at 35 and 41°C with [^{3}H]glucosamine for 16 h. Culture medium (1 ml) containing free and virus-associated glycoproteins was immunoprecipitated with anti-glycoprotein antiserum (A). After the virus was pelleted, 2.5 ml of medium (containing only free glycoprotein) was immunoprecipitated with the same antiserum (B). wt, Wild type.*

after a 2-h chase released virus was purified and electrophoresed (Fig. 6). The glycoproteins present in virions purified after the 2-h chase differed in the mutant and the wild type. At 35°C the predominant form of the virion glycoprotein was Pr95 for both the mutant (Fig. 6, lane 3) and the wild type (lane 1) (although it was less obvious in the latter); this is in contrast to the long-term labeling conditions (Fig. 2), where gp85 and gp37 predominated. At the higher temperature, however, proteolytic cleavage appeared to occur more rapidly, and a large proportion of the glycoprotein present in wild-type virions was in the mature form (i.e., gp85 and gp37) (Fig. 6, lane 2). However, LA3382 virions contained predominantly Pr95 at 41°C (lane 4), and the maintainance of gp85 and gp37 in these virions at the nonpermissive temperature was impaired significantly. These data indicated that the mutant precursor glycoprotein was synthesized at 41°C and could be incorporated into virions. However the defective step appeared to be at the time of cleavage of the presursor, when gp85 and probably a portion of gp37 were released from the virions into the culture fluids.

LA3382 has a defective polymerase gene. It should be noted that the precursor of the reverse transcriptase (Pr180) in LA3382 was not processed as efficiently in infected cells at 41°C as it was at 35°C or in PR-C infected cells (Fig. 3). When the polymerase activity of purified virions was examined, LA3382 grown at 41°C showed approximately a twofold reduction in specific activity compared with LA3382 virions synthesized at 35°C (Table 3). Panet et al. (17) observed a similar decrease in polymerase activity with LA338, which was at least partially due to the reduced amount of enzyme present in virions synthesized at 41°C; this is consistent with the results of our pulse-chase experiments. A relatively small reduction in enzyme activity such as this may not be biologically significant for the production of infectious virus, since there are normally on the order of 70 to 100 molecules of reverse transcriptase per virion (16). Nevertheless, it was necessary to differentiate between the effect of this lesion and the effect of the *env* gene defect on the physiology of the mutant.

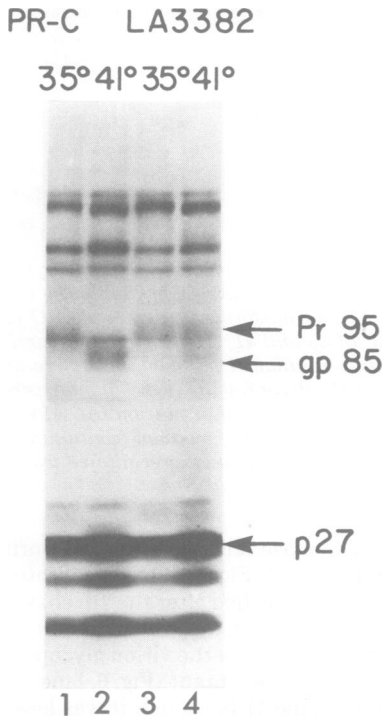

FIG. 6. *Autoradiogram of a polyacrylamide gel showing incorporation of Pr95^{env} into virions. Virus-transformed C/O cell cultures were labeled with [^{3}H]leucine at 35 and 41°C for 15 min and then incubated with fresh medium for an additional 2 h. Virus purified from the medium was electrophoresed on a 5 to 20% polyacrylamide gradient gel.*

LA3382 cannot be complemented by RSV(−), an *env* deletion mutant. To determine whether the predominant late defect that affected replication of LA3382 was within the envelope gene (*env*) or the polymerase gene (*pol*), we performed a complementation assay with a nonconditional *env* mutant. The Bryan high-titer strain of RSV [RSV(−)] is a nonconditional mutant which lacks nearly all of the genetic information for the envelope glycoproteins (4) yet maintains a normal complement of the *gag*, *pol*, and *src* genes. Such a mutant should complement a mutant defective in the *pol* gene by providing an active reverse transcriptase; however, it should not complement a mutant defective in *env*. To establish cells infected with the nonconditional mutant, RSV(−) was fused to C/E cells by using inactivated Sendai virus, as described previously (6). LA3342 and LA3382 and wild-type PR-C were used to superinfect the following three cell types: RSV(−)-infected C/E cells; C/E cells infected with a leukosis virus (Rous-associated virus-3), which should have complemented both *pol* and *env* defects; and uninfected C/E cells. These preparations were used to look for complementation, as typified by the production of infectious virus at the nonpermissive temperature. The temperature-sensitive mutant LA3342 has a late replication defect in the processing of Pr76, the precursor of the internal structural proteins (8). This mutant was clearly complemented by both RSV(−) and Rous-associated virus-3 (Table 4). Although LA3382 was complemented by Rous-associated virus-3, no complementation was observed in RSV(−)-infected cells. The control (PR-C) grew well in all cells at both tempera-

TABLE 3. *Polymerase assay*[a]

Virus	Temp (°C)	Activity (pmol/h per μg)
PR-C	35	1.23
PR-C	41	1.16
LA3382	35	0.68
LA3382	41	0.36

[a] Virus-containing medium harvested at 35 and 41°C was assayed for reverse transcriptase activity on an exogenous template at 30°C.

TABLE 4. *Complementation of LA3382*

Cells preinfected with:	Efficiency of replication at 41°C with:[a]		
	No Virus	RSV(−)	RAV-3[b]
PR-C	2.2	>10.0	>10.0
LA3342	0.0003	>10.0	>10.0
LA3382	0.11	0.016	9.1

[a] Efficiency of replication at 41°C was defined as the ratio of the titer at 41°C to the titer at 35°C.

[b] RAV-3, Rous-associated virus-3.

tures. From these results it seemed evident that the lesion responsible for the major replication defect in LA3382 is located within the *env* gene.

DISCUSSION

The temperature-sensitive mutant LA3382, a recombinant between LA338 and PR-B, retains three of the four defects of LA338 (only the transformation defect in *src* is missing) (9). We used LA3382 to study these defects because it can transform cells at both the permissive temperature (35°C) and the nonpermissive temperature (41°C) and, therefore, can be compared with a wild-type control more accurately. Through the use of an infectious center assay (23), the early lesion was shown to impair both replication and transformation, as expected of an early defect (Table 1). Previously, this had been difficult to demonstrate apparently because of the assay methods used and the leakiness in the expression of this lesion. Moelling and Friis (15) showed that the polymerase of LA338 was about fourfold more thermolabile than the polymerase of wild-type virus when it was assayed at 47°C. These authors concluded that the early lesion was located in the *pol* gene and caused the input viral polymerase to be thermolabile. In contrast, Verma et al. (21) could not demonstrate any thermolabile properties for the LA338 enzyme and, therefore, suggested that the early lesion might be in *env* and might act at a stage before transcription, such as adsorbtion or penetration. This defect in LA3382 has not been defined unequivocally yet, but preliminary results (Hunter, unpublished data) have suggested that it may resemble the defect of another early mutant, LA30, which has a lesion in the *env* gene that affects adsorption and penetration of the virus (19).

The experiments with LA3382 described above strongly suggest that the late defect in replication is due to a lesion in the *env* gene. LA3382 virions produced at 41°C contain significantly reduced amounts of gp85 and gp37 compared with virus released at 35°C or wild-type virus (Fig. 2). When intracellular viral polypeptides were examined by pulse-chase experiments, the processing of Pr76 (the precursor of the internal structural proteins) occurred equally well at 35 and 41°C in mutant-infected cells, but the glycoprotein precursor (Pr95) was processed more slowly in mutant-infected cells at 41°C (Fig. 3). At 35°C, under long-term labeling conditions most of the cell-associated viral glycoprotein in either mutant or wild-type virus-infected cells was found in the form of the precursor, Pr95. This is consistent with a previous report (10) that cleavage of the glycoprotein precursor is extracellular. Whether this cleavage occurs on the surface of the cell or after release of the virus may depend on the culture temperature, since at 41°C most of the cell-associated glycoprotein in PR-C-infected cells is in the form of the cleavage products gp85 and gp37. However, this is not the case in LA3382-infected cells, where at 41°C most of the cell-associated glycoprotein remains uncleaved (Fig. 4). Despite the apparent lack of proteolytic cleavage, we observed no accumulation of the mutant precursor in infected cells. Instead of accumulation or degradation, it appeared that the glycoprotein was released into the supernatant fluid upon cleavage of the precursor (Fig. 5). This could have been the result of an aberrant cleavage of the precursor or could have been due to an instability of the gp85-gp37 complex, since the glycoprotein found in the supernatant was indistinguishable from virion gp85. However, the lack of gp37 in the noninfectious virus released at 41°C favored the former mechanism.

When virus was harvested from cells 2 h after a pulse-label with [^{3}H]leucine, it was clear that both mutant and wild-type virions incorporated Pr95 at 35°C (Fig. 6). At 41°C, wild-type virions contained predominantly gp85 and gp37, whereas virions released from LA 3382-infected cells contained predominantly Pr95. These results strongly suggested that the envelope glycoprotein of LA3382 was synthesized and inserted normally into the viral membrane at 41°C but was cleaved more slowly and in such a way that gp85 was released into the culture medium. Thus, LA3382 is quite distinct from another temperature-sensitive *env* gene mutant of RSV, PH734, which is not able to synthesize a biologically active mature glycoprotein at 41°C. However, LA3382 does resemble SE521, a nonconditional replication mutant of RSV, since SE521-infected cells synthesize a normal *env* gene precursor protein which is not incorporated into virions and is not cleaved to gp85 and gp37 (12). An abnormal cleavage event analogous to that described here for temperature-sensitive LA3382 could explain the phenotype of SE521.

It is thought that gp37 is responsible for anchoring the glycoprotein complex in the viral membrane via disulfide linkages (11; Hunter, unpublished data), but the destiny of gp37 in LA3382 at 41°C is uncertain. No gp37 has been detected in supernatants thus far, but it is also not present in the gel profiles of mutant virions at 41°C; thus, an aberrant cleavage may yield fragments of gp37 that are difficult to resolve in the gel systems which we used. A model for such an anomalous cleavage event is shown in Fig. 7, in which we propose that a second cleavage within gp37 allows gp85 and a glycosylated fragment of the smaller protein to be released into

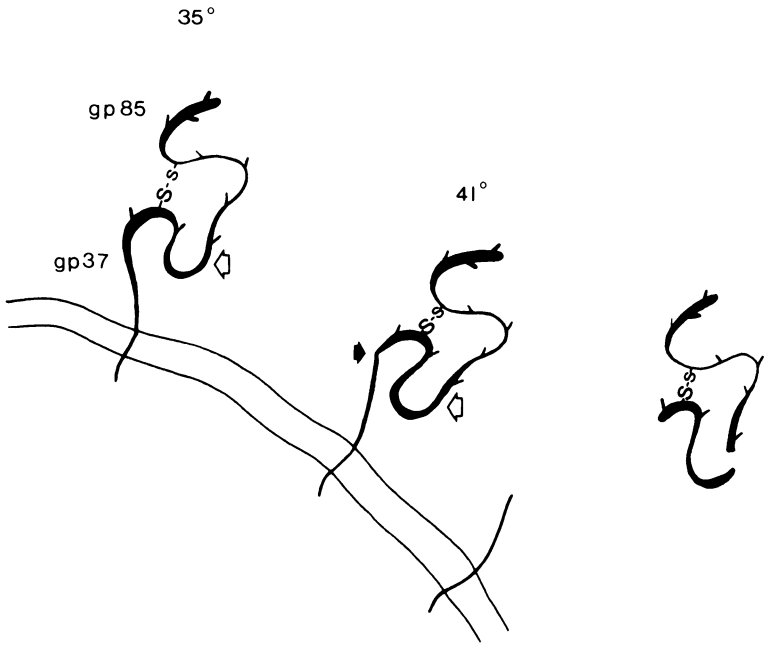

FIG. 7. *Schematic representation of the glycoprotein complex of RSV.*

the culture medium. Previous studies of recombination between LA338 and PR-B in which wild-type recombinants with the host range of the mutant were obtained at a high frequency suggested that the genetic lesion is not linked to the region of the *env* gene that determines host range (9). A mutation in the gp37 region of the gene would be consistent with such results.

LA3382 has an additional defect in the *pol* gene since the polymerase precursor (Pr180) is clearly processed less efficiently at 41 than at 35°C (Fig. 3). Coincident with this observation, a twofold reduction in reverse transcriptase activity was observed in mutant virions synthesized at 41°C (Table 3). Panet et al. (17) also demonstrated that LA338 virions produced at 41°C contained about one-half as much polymerase as virions produced at 35°C, which is consistent with our results. These authors suggested that LA338 contains one lesion in the *pol* gene which is responsible for both the early defect and the late replication defect. Although a complementation assay with a mutant deleted in *pol* alone would be required to quantitate the effect of the polymerase defect, the reduced activity of the mutant polymerase is probably too small to account for the significant reduction in infectivity observed at 41°C. To establish that the major defect is located in the *env* gene, we showed that LA3382 was not complemented by RSV(−), which contains a deletion that spans most of the *env* gene but contains a normal *pol* gene (Table 4). Furthermore, we have observed that revertant clones of LA3382 (those that replicate at 41°C) produce mature glycoproteins but are still deficient in polymerase protein; this is additional evidence that the lesion responsible for the defect in replication is within the *env* gene.

It is of interest that growing LA3382 on C/O chicken cells (Hyline) in which low levels of *chf* expression can be detected results in the loss of the temperature-sensitive phenotype and the appearance of what we presume to be recombinant, glycoprotein-containing viruses at 41°C. Since repeated freezing and thawing of LA3382 stocks results in the gradual loss of infectivity and the temperature-sensitive phenotype, it is possible that even the virus glycoprotein synthesized at 35°C is less stable and that the revertant or recombinant viruses in the stock are selected.

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