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Fusion Protein of the Paramyxovirus SV5: Destabilizing and Stabilizing Mutants of Fusion Activation

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The fusion (F) protein of the paramyxovirus SV5 strain W3A causes syncytium formation without coexpression of the SV5 hemagglutinin-neuraminidase (HN) glycoprotein, whereas the F protein of the SV5 strain WR requires coexpression of HN for fusion activity. SV5 strains W3A and WR differ by three amino acid residues at positions 22, 443, and 516. The W3A F protein residues P22, S443, and V516 were changed to amino acids found in the WR F protein (L22, P443, and A516, respectively). Three single-mutants, three double-mutants, and the triple-mutant were constructed, expressed, and assayed for fusion using three different assays. Mutant P22L did not cause fusion under physiological conditions, but fusion was activated at elevated temperatures. Compared with the W3A F protein, mutant S443P enhanced the fusion kinetics with a faster rate and greater extent, and had a lower activation temperature. Mutant V516A had little effect on F protein-mediated fusion. The double-mutant P22L,S443P was capable of causing fusion, suggesting that the two mutations have opposing effects on fusion activation. The WR F protein requires coexpression of HN to cause fusion at 37°C, and does not cause fusion at 37°C when coexpressed with influenza virus hemagglutinin (HA); however, at elevated temperatures coexpression of WR F protein with HA resulted in fusion activation. In the crystal structure of the core trimer of the SV5 F protein (Baker, K. A., Dutch, R. E., Lamb, R.A., and Jardetzky, T. S. (1999). *Mol. Cell* 3, 309–319), S443 is the last residue (with interpretable electron density) in an extended chain region and the temperature factor for S443 is high, suggesting conformational flexibility at this point. Thus, the presence of prolines at residues 22 and 443 may destabilize the F protein and thereby decrease the energy required to trigger the presumptive conformational change to the fusion-active state.

INTRODUCTION

Simian virus 5 (SV5) is a member of the Paramyxoviridae family of viruses. SV5 encodes three integral membrane glycoproteins, the hemagglutinin-neuraminidase (HN), the fusion protein (F), and the small hydrophobic protein (SH) (Hiebert *et al.,* 1985a,b; Paterson *et al.,* 1984a,b), each of which is a component of the SV5 virion envelope (He *et al.,* 1998; Scheid *et al.,* 1972; Scheid and Choppin, 1974). The HN protein functions to attach the virion to sialic acid, the viral receptor, on the surface of target cells. The F protein is directly involved in virus-mediated membrane fusion, which occurs at neutral pH. The function of the SH protein is unknown. However, the SH protein is not essential for replication of SV5 in tissue-culture cells (He *et al.,* 1998).

The F protein is synthesized as a precursor (F_0) which has to be proteolytically cleaved to generate the biologically active form of the protein, which consists of the disulfide-linked subunits F_1 and F_2 (Homma and Ohuchi, 1973; Scheid and Choppin, 1974). At the newly generated N-terminus of the membrane-bound subunit F_1 is a hydrophobic region known as the fusion peptide. The F protein is a homotrimer (Russell *et al.,* 1994) but little is known concerning the atomic structure of the native F protein. However, the atomic structure of a core fragment of the SV5 F_1 subunit (heptad repeat containing peptides N-1 and C-1) (Joshi *et al.,* 1998) was determined recently at 1.4-Å resolution (Baker *et al.,* 1999). An N-terminal central coiled-coil, which is linked to the fusion peptide, is surrounded by three C-terminal antiparallel helices. This core structure is thought to represent the final most stable form of the protein either at fusion or after fusion has occurred, and it is structurally related to the core structure of other viral-fusion proteins such as the low pH-induced form of the HA_2 subunit of influenza A virus hemagglutinin (HA), gp41 of human immunodeficiency virus, env-TM domain protein of Moloney murine leukemia virus, and GP2 of Ebola virus (Bullough *et al.,* 1994; Chan *et al.,* 1997; Fass *et al.,* 1996; Weissenhorn *et al.,* 1998).

For most paramyxoviruses the available evidence suggests that coexpression of the homotypic HN is required for F-mediated membrane fusion to occur (Cattaneo and Rose, 1993; Deng *et al.,* 1995; Ebata *et al.,* 1991; Horvath *et al.,* 1992; Hu *et al.,* 1992; Morrison *et al.,* 1991; Stone-Hulslander and Morrison, 1999; Tanabayashi *et al.,* 1992;

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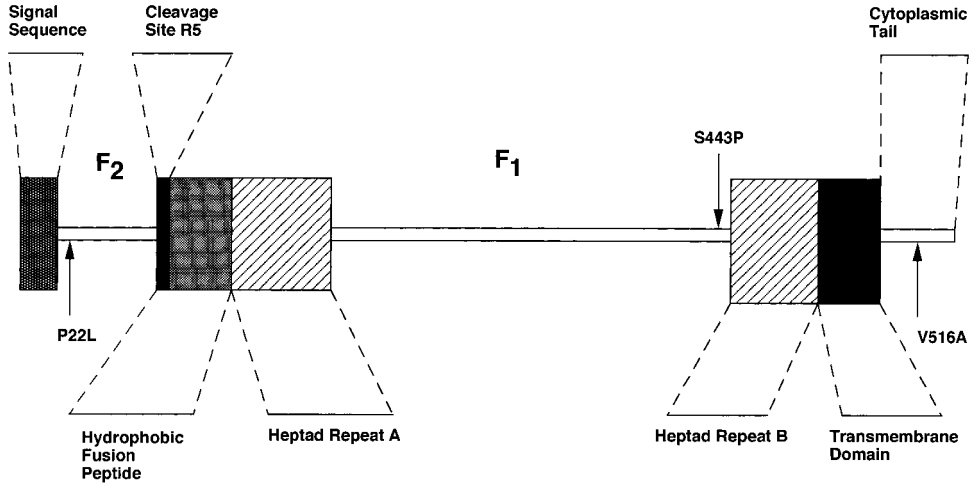

FIG. 1. Schematic diagram of the W3A F protein. The positions of the signal sequence, cleavage site, fusion peptide, heptad repeats A and B, transmembrane domain, and cytoplasmic tail are shown. The three amino acid residues that are not conserved between the W3A and WR strains are also indicated with the single-letter code for the W3A residue shown preceding the residue number and the code for the WR residues following the number.

Tong and Compans, 1999; Wild *et al.,* 1991). However, for the W3A strain of SV5 the F protein can promote fusion in the absence of the homotypic HN and can promote syncytia formation in cell monolayers in the absence of any viral binding protein (Dutch *et al.,* 1998; Horvath and Lamb, 1992; Horvath *et al.,* 1992; Ito *et al.,* 1997; Paterson *et al.,* 1985; Ward *et al.,* 1995). Recently, the fusion requirements of another strain of SV5, WR, were examined and compared with those of the W3A strain (Ito *et al.,* 1997). It was found that when the WR F protein was expressed in BHK cells, syncytia formation was dependent on the coexpression of SV5 HN (derived from W3A or WR strains), and unexpectedly the mumps virus HN could substitute for the SV5 HN protein (Ito *et al.,* 1997). A comparison of the amino acid sequences of the W3A and WR F proteins indicates that only the three residues 22, 443, and 516 differed between the two sequences (Fig. 1) (Ito *et al.,* 1997; Paterson *et al.,* 1984b). Single amino acid substitutions were made in the WR cDNA with residues found in the analogous position in the W3A F protein; i.e., leucine at position 22 to proline, proline at position 443 to serine, and alanine at position 516 to valine (Ito *et al.,* 1997). When the mutant proteins were expressed in tissue culture, with and without HN, it was found that substituting the leucine at position 22 with proline in the WR F protein resulted in an F protein that now could cause syncytia formation in the absence of HN (Ito *et al.,* 1997). Apparently, a single amino acid substitution converted the F protein from an HN-dependent to an HN-independent fusion protein.

We have examined the interplay of residues 22, 443, and 516 of the SV5 F protein in mediating membrane fusion in more detail using single and double amino acid substitutions in the W3A F protein and by using sensitive and quantitative fusion assays. The data obtained are interpreted as being relevant to understanding the transition of the F protein from a presumptive metastable conformation to a more stable conformation, a transition that occurs during the membrane-fusion event.

RESULTS

Expression of the wt and mutant F proteins

The F proteins of the SV5 W3A and WR strains differ by three amino acid residues at positions 22, 443, and 516 (Ito *et al.,* 1997). In Fig. 1 the positions of these residues are shown in relation to the biologically important structural features of the SV5 F protein: the N-terminal signal sequence, the cleavage site, the fusion peptide, heptad repeats A and B (N-1 and C-1) that assemble into the core trimer (Joshi *et al.,* 1998), the transmembrane domain, and the cytoplasmic tail.

The W3A F gene was subjected to site-specific mutagenesis such that amino acid residues P22, S443, and V516 were replaced with the amino acids found in the WR F protein (L22, P443, and A516, respectively). Each residue was changed individually to yield the single-mutant F proteins P22L, S443P, and V516A. The single mutations were then combined to make the three double-mutants—P22L,S443P; P22L,V516A; and S443P,V516A—and the triple-mutant P22L,S443P,V516A. The amino acid sequence of the W3A triple-mutant F protein is identical to that of the WR F protein and thus, for simplicity, it is referred to as WR. To examine the cell-surface expression of the mutant F proteins relative to that of the W3A F protein, the proteins were expressed transiently in CV-1 cells using the recombinant vaccinia virus-T7 RNA polymerase system (vac-T7) (Fuerst *et al.,* 1986) and the cells were stained for flow cytometry using the F-specific monoclonal antibody (MAb) F1a (Randall *et*

TABLE 1

Cell-Surface Expression of Mutant F Proteins

F protein expressed	Relative expression[a]	Relative mean fluorescence intensity
W3A	1.00	1.00
P22L	1.02	0.76 ± 0.04
S443P	1.07	1.08 ± 0.07
V516A	1.07	1.05 ± 0.02
P22L,S443P	1.01	0.90 ± 0.03
P22L,V516A	0.99	1.00 ± 0.01
S443P,V516A	1.07	1.02 ± 0.04
WR (P22L,S443P,V516A)	1.05	0.90 ± 0.03

Note. F proteins were expressed in CV-1 cells using the vac-T7 expression system. Surface expression of the F protein was determined by flow cytometry using the Fla MAb (Randall *et al.,* 1987) followed by fluorescein isothiocyanate-conjugated secondary antibody ($n = 2$).

[a] Number of cells expressing F normalized to W3A: transfection efficiency for W3A was 80–90%, depending on the experiment.

al., 1987). As shown in Table 1 the mutant F proteins were expressed at the cell surface to levels of at least 76% of that of the W3A F protein.

F protein-mediated cell–cell fusion

To analyze in detail the effect of the mutations on the fusion activity of the W3A F protein, the ability of the mutant proteins to mediate the mixing of the outer leaflets of the plasma membrane, often referred to as lipid mixing, was examined. In addition, the ability of the mutant proteins to permit the mixing of the aqueous cytoplasmic contents between two cells was measured. Although the W3A F protein can induce syncytia formation when expressed by itself in monolayers of CV-1 and BHK cells (Horvath *et al.,* 1992; Ito *et al.,* 1997; Paterson *et al.,* 1985), the assays used here to examine lipid mixing and aqueous content mixing involve the binding together of two cell populations, and this requires the coexpression of the F protein and a sialic acid binding protein.

Analysis of lipid mixing by confocal microscopy. The mixing of the outer leaflets of the plasma membrane can be measured by the transfer of the lipophilic dye R18 from the membrane of labeled human erythrocytes (RBCs) to the membrane of F protein-expressing acceptor CV-1 cells. The W3A and mutant F proteins were coexpressed with the SV5 W3A HN protein in CV-1 cells using the vac-T7 expression system, and fusion assays were performed with RBC-cell complexes and the cells examined by confocal microscopy. As shown previously for the W3A F protein (Bagai and Lamb, 1995b; Dutch *et al.,* 1998), fusion of the RBCs with CV-1 cells was not observed without a period of incubation at 37°C (Fig. 2; compare W3A 4°C to W3A 37°C). Expression of mutant P22L caused no lipid mixing (Fig. 2). This result was unexpected given that the WR F protein, which has a leucine at position 22, can cause membrane fusion (Ito *et al.,* 1997) as measured by syncytia formation when coexpressed with HN of the W3A or WR strains. In contrast, expression of mutant S443P induced more extensive lipid mixing than did expression of the W3A F protein (Fig. 2). The double-mutant P22L,S443P exhibited limited fusion activity as measured by R18 transfer (Fig. 2). The R18 transfer activity of the mutant V516A was similar to that of W3A F (data not shown) and the double-mutants P22L,V516A and S443P,V516A exhibited lipid-mixing activity that was indistinguishable from those of the single mutants P22L and S443P, respectively (data not shown). The WR F protein appeared to exhibit less lipid-mixing activity than that of the W3A F protein (Fig. 2).

It has been shown previously that the W3A F protein can induce lipid mixing when influenza virus HA, rather than the homotypic HN protein, is used as the sialic acid binding protein (Bagai and Lamb, 1995a). Therefore, it was of interest to determine whether HA could substitute for HN when coexpressed with the mutant F proteins. Lipid-mixing assays were performed as described earlier using CV-1 cells coexpressing either the W3A or mutant F proteins and HA as the binding protein. As shown in Fig. 3, for most of the mutant proteins, coexpression of F and HA gave results similar to those obtained when the F proteins were coexpressed with HN. For example, R18 transfer observed for mutant V516A was similar to that seen with W3A F (Fig. 3, V516A), mutants S443P and S443P,V516A caused more extensive dye transfer than W3A (compare panels in Fig. 3), and there was no detectable R18 transfer observed with mutants P22L and P22L,V516A (Fig. 3). The exceptions were the double-mutant P22L,S443P and WR F, where coexpression with HN resulted in limited lipid mixing, whereas on coexpression with HA detectable R18 transfer was not observed (compare Figs. 2 and 3). Expression of influenza virus HA in CV-1 cells did not induce lipid mixing at neutral pH (Fig. 3, HA alone).

Spectrofluorimetric analysis of lipid mixing. The results obtained by using the confocal microscope to monitor lipid mixing indicated that the S443P mutant F protein was able to promote lipid mixing more extensively than was the W3A F protein. To determine whether S443P induced lipid mixing at an increased rate and to a greater extent than fusion induced by the W3A F protein, spectrofluorimetric analysis of lipid mixing was undertaken. For these experiments W3A and S443P F proteins were coexpressed with influenza virus HA because, unlike SV5 HN, it does not possess neuraminidase activity, an activity which can lead to rapid elution of the erythrocytes when the cells are in suspension. As shown in Fig. 4 the S443P mutant F protein induced fusion that had an increased initial rate and greater final extent than fusion induced by the W3A F protein.

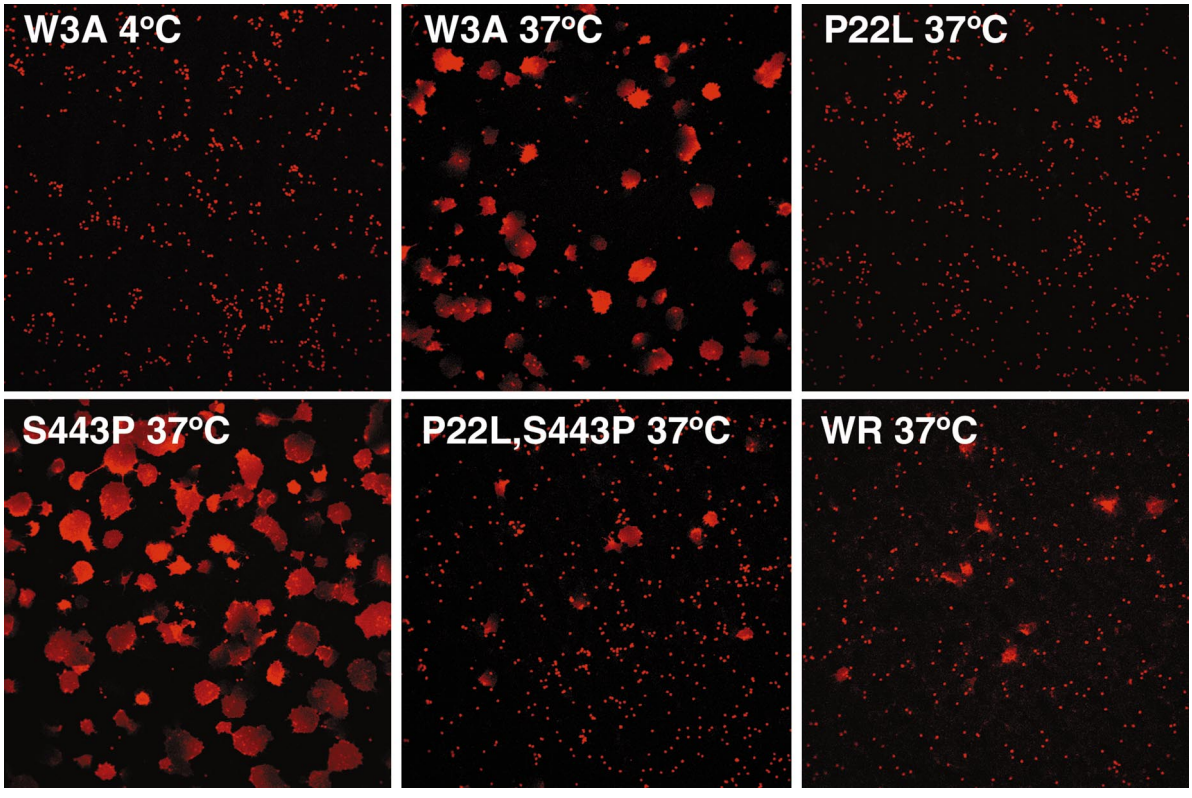

FIG. 2. Lipid-mixing activity of the W3A and mutant F proteins when coexpressed with the SV5 HN protein. R18-labeled RBCs were bound to CV-1 cells coexpressing HN and either W3A F or selected F mutant proteins at 4°C. Fusion was initiated by incubation at 37°C. Fusion was detected by confocal microscopy as described in Materials and Methods. W3A F, 4°C (no incubation at 37°C). The samples in the remaining panels were incubated at 37°C for 10 min.

Analysis of content mixing. To examine the aqueous content mixing resulting from membrane fusion mediated by the W3A and mutant F proteins, a sensitive and quantitative reporter assay was used (He *et al.,* 1998). In this assay monolayers of Vero cells are transfected with plasmids expressing the F and HN genes under the control of the chicken β-actin promoter (pCAGGS) (Niwa *et al.,* 1991) and a plasmid containing the chloramphenicol acetyl transferase (CAT) gene under the control of the T7 RNA polymerase promoter. A separate population of Vero cells was transfected with a plasmid containing the T7 RNA polymerase gene under the control of the chicken β-actin promoter. After overnight incubation to allow expression of the F, HN, and T7 RNA polymerase proteins, the T7 RNA polymerase-expressing cells were overlaid onto the F- and HN-expressing cells. Expression of the CAT protein was obtained on fusion and mixing of the cytoplasms of the two cell populations. The data of a typical experiment, performed in duplicate, are shown in Fig. 5. As observed in the lipid-mixing assay, mutants P22L and P22L,V516A were barely above background levels and the addition of the S443P mutation to the P22L protein restored some fusion activity (P22L,S443P). In addition, in agreement with the R18 outer-leaflet fusion assay, the F mutant proteins S443P and S443P,V516A cause increased cytoplasmic content mixing compared with that caused by W3A (Fig. 5). Interestingly, in this assay the cytoplasmic tail mutant V516A was also capable of causing greater content mixing than was W3A, whereas when lipid mixing was measured the results obtained for the two proteins were similar (Figs. 2 and 5).

Syncytium formation. The ability of the W3A F protein to induce syncytia when expressed in mammalian cell monolayers in the absence of HN is well documented (Horvath *et al.,* 1992; Ito *et al.,* 1997; Paterson *et al.,* 1985). As the F mutant proteins were found to differ according to the efficiency with which they could cause lipid or content mixing, in a relatively short timescale, it was of interest to compare their syncytia-forming activity, which occurs over a longer timescale. However, the extensive nature of SV5 F protein-induced syncytia makes quantification cumbersome. For syncytia assays subconfluent monolayers of BHK 21F cells were cotransfected with pCAGGS plasmids expressing F and HN, or F and HA, or transfected with pCAGGS F alone. The syncytia produced in BHK 21F cells when the F proteins were coexpressed with HN is shown in Fig. 6A. Untransfected or HN-expressing BHK cells show some small areas of spontaneous fusion where three to six cells form a giant cell (data not shown) and such giant cells can be observed for F mutants P22L and P22L,V516A. These two mutants did not cause any syncytia formation over the

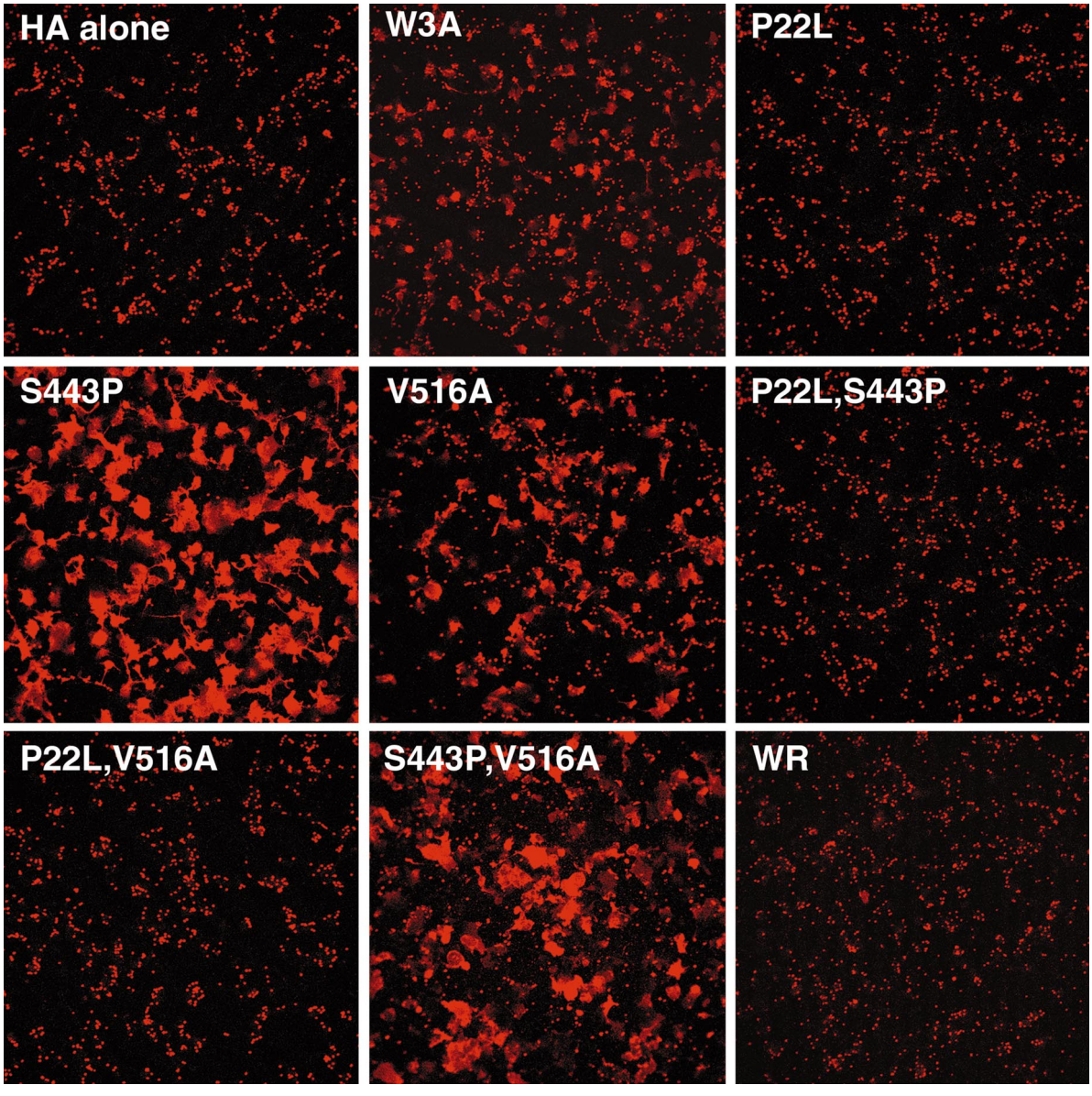

FIG. 3. Lipid-mixing activity of the W3A and mutant F proteins when coexpressed with the influenza HA protein. The lipid-mixing assay was performed as described under Materials and Methods and the legend to Fig. 2. HA alone (uncleaved HA expressed alone and no low pH activation). All the samples were incubated at 37°C for 10 min prior to confocal microscopy.

level of spontaneous fusion. All the other F proteins tested induced the formation of syncytia in BHK 21F when coexpressed with HN.

Although difficult to represent in the photomicrographs shown in Fig. 6A, because of the inherent conflict of showing individual cells versus the entire 6-cm-diameter monolayer of cells, the extent and size of the syncytia observed were consistent with the results of the R18 assay for lipid mixing. Expression of the S443P-containing mutants induced the most extensive areas of syncytia, W3A F and V516A induced less-extensive areas of syncytia formation, and double-mutant P22L,S443P and WR F induced syncytia that were more limited in size. When HA protein was cotransfected with the F proteins, WR F and P22L,S443P were unable to cause syncytia formation; otherwise, the data were similar to those obtained upon cotransfection of F and HN, with mutant V516A inducing syncytia similar in size to those of W3A F protein and mutants S443P and S443P,V516A inducing more extensive syncytia. The data for W3A, WR, S443P, and P22L are shown in Fig. 6B. Finally, all the F proteins were tested for their ability to cause cell fusion when expressed alone in BHK 21F cells. The data for W3A and mutant S443P are shown in Fig. 6B (bottom panel) and it can be seen that there are extensive areas of syncytia formation. It was found that F proteins containing a leucine at position 22 (WR; P22L; P22L,S443P; P22L,V516A) were unable to promote syncytia formation

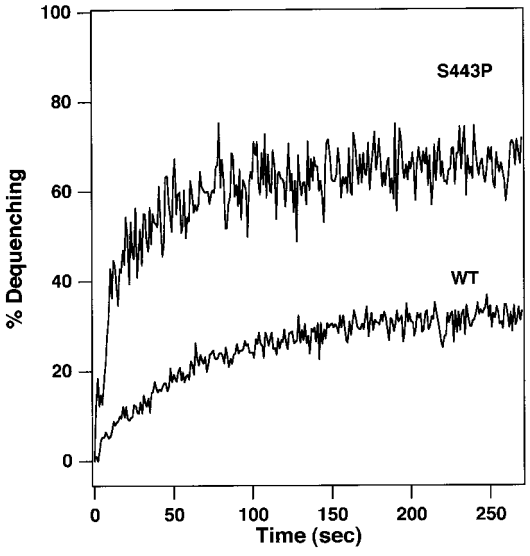

FIG. 4. Kinetics of R18 fluorescence dequenching. R18-labeled RBCs were bound to CV-1 cells coexpressing either W3A F or S443P and influenza HA, and the cell complexes were prepared for spectrofluorimetric analysis as described under Materials and Methods. The traces shown are the results of a typical experiment.

when expressed alone, expression of mutant V516A resulted in syncytia similar in size to those observed for W3A, and S443P,V516A gave rise to syncytia that were similar in size to those seen with S443P (data not shown).

Temperature dependence of fusion of mutants S443P and P22L as compared with that of W3A F protein and dependence of HA requirement of WR F protein

It seemed possible that the difference in phenotype between mutants S443P and P22L may be the result of a difference in energy required for fusion activation; therefore, the effect of temperature on the fusion activity of the W3A, S443P, and P22L F proteins were compared. Human RBCs were double-labeled with the lipid probe R18 and the aqueous cytoplasmic probe 6-carboxyfluorescein (CF) and bound to CV-1 cells coexpressing HN and either W3A, S443P, or P22L mutant F proteins. After washing away unbound RBCs, the F- and HN-expressing cells were incubated for 10 min at various temperatures and returned to ice, and the lipid and aqueous dye transfer visualized using a confocal microscope.

As shown in Fig. 7A, incubation at 37°C resulted in R18 transfer similar to that shown in Fig. 2 for W3A and S443P F proteins with the S443P protein, causing more extensive lipid mixing than that caused by W3A F protein. The CF aqueous dye transfer for the same fields of cells indicated that S443P was also more efficient than W3A F protein at promoting content mixing (Fig. 7A), as shown earlier using the CAT assay for content mixing. When the temperature of incubation was lowered to 29°C a dramatic decrease in the fusion (both lipid mixing and content mixing) mediated by the W3A F protein was observed, whereas the activity of the S443P F protein was unchanged. Lowering the temperature further to 22°C had very little effect on the fusion activity of S443P, whereas W3A F protein-mediated fusion was completely inhibited. At 18°C there was a reduction in both the lipid mixing and content mixing mediated by S443P (data not shown) and at 15°C, S443P fusion was completely inhibited (Fig. 7A). It is also important to note for both W3A and S443P F proteins that the number of cells simultaneously undergoing lipid and aqueous mixing is similar (compare R18 and CF panels, Fig. 7A).

Mutant P22L F protein had the opposite phenotype to S443 P and caused fusion only at elevated temperatures. Fusion of RBCs with CV-1 cells expressing P22L was not detected at 37°C (Fig. 2). However, increasing the temperature to 45°C triggered a limited amount of P22L-mediated fusion (Fig. 7B). At 53°C somewhat more P22L-mediated fusion was observed (data not shown). Interestingly, for some cells only R18 transfer and not CF dye mixing was observed, but at the present time we cannot determine whether the data reflect fusion halted at the hemifusion stage or whether the integrity of some CV-1 cell membranes has been breached at the higher tem-

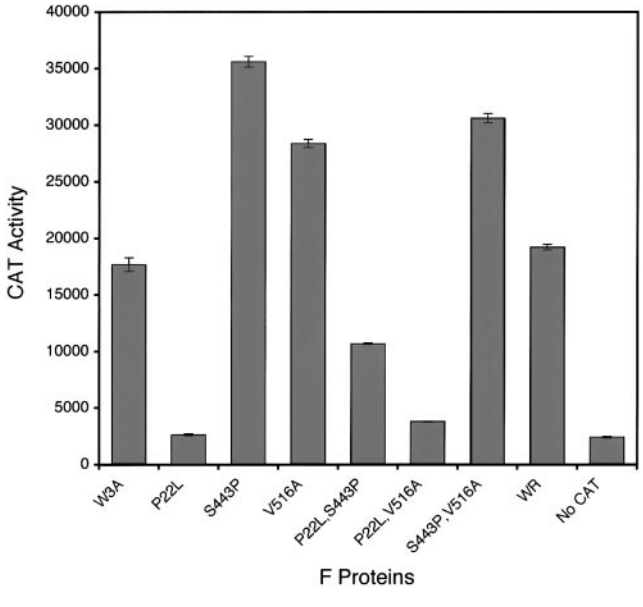

FIG. 5. Content-mixing activity of the W3A and mutant F proteins. Vero cells were cotransfected with plasmids encoding the F and HN proteins under the control of the chicken β-actin promoter and the plasmid encoding CAT under the control of the T7 RNA polymerase promoter. A second set of Vero cells was transfected with the plasmid encoding T7 RNA polymerase under the control of the β-actin promoter. After overnight incubation the T7 RNA polymerase-expressing cells were overlaid onto the F- and HN-expressing cells containing the CAT plasmid. F protein-mediated membrane fusion allows T7 RNA polymerase to gain access to the CAT plasmid, which results in CAT protein expression. The CAT activity was assayed as described under Material and Methods. No CAT = cells transfected with plasmids encoding HN and W3A F only. CAT activity is expressed as [^{14}C] counts per minute; $n = 2$.

peratures, causing the CF label to leach out. No R18 or CF dye transfer was observed at 45°C when HN was expressed without P22L (data not shown). The specificity of the F protein-mediated fusion at 45°C was shown further by the ability to block fusion upon addition of the MAb F1a, an antibody shown previously to block fusion (Bagai and Lamb, 1995b) (Fig. 7B).

The WR F protein would not cause fusion at 37°C using HA as a binding protein (Fig. 3). Therefore, we also tested whether increasing the temperature would trigger WR F protein to cause fusion on coexpression with HA. As shown in Fig. 7B, WR F protein did not cause fusion with HA at 45°C but it did cause fusion when the temperature was increased to 53°C. Similar to the results for HN + P22L at 45°C, more cells showed lipid mixing than content mixing. When HA (uncleaved, no low pH treatment) was expressed alone, no lipid mixing was observed, and fusion was blocked by addition of the MAb F1a, indicating that the fusion observed was mediated by the WR F protein (data not shown).

DISCUSSION

We have introduced mutations into the SV5 W3A F protein corresponding to the three amino acid residues that have been found to differ between the W3A and WR strains of SV5 (Ito *et al.,* 1997; Paterson *et al.,* 1984b) and have analyzed in detail the effect the mutations have on the fusion activity of the W3A F protein. As shown in Fig. 1 the residues that differ between the two strains are located at residue 22, residue 443, and residue 516. By using various sensitive assays for membrane fusion, we have examined the relative ability to promote fusion of proteins that possess single mutations, the three possible combinations of double mutations, and the triple-mutant, which is equivalent to the WR F protein. All the mutant F proteins were expressed at the cell surface and were recognized by a conformation-specific monoclonal antibody. Thus, gross changes to F protein folding are unlikely, but the possibility cannot be eliminated that localized conformational differences exist.

The experiments of Ito and colleagues (1997) were confirmed by finding that a proline at residue 22 is important in defining the HN-independent fusion activity of the W3A F protein. However, in the converse experiment when we substituted proline at position 22 in the W3A F protein with leucine, the resulting F protein was so severely debilitated for fusion that we were unable to detect fusion even in the presence of HN (Figs. 2, 5, and 6). Another striking result was obtained on making the S443P substitution in the W3A F protein. The S443P mutant was found to have greater fusion-promoting activity than that of W3A F in every assay used (Figs. 2–7), and spectrofluorimetric measurement of the lipid mixing induced by the W3A and S443P F proteins showed that S443P had both a faster initial rate and greater final extent of lipid mixing than did W3A F protein (Fig. 4). Combining the P22L and S443P substitutions in the double-mutant P22L,S443P gave rise to a protein that had limited fusion-promoting activity when coexpressed with HN (Figs. 2, 5, and 6), showing that the negative effect of leucine at position 22 can be compensated for, to some extent, by the fusion-enhancing effect of proline at position 443. The opposing effects of these two residues are presumably the reason that the WR fusion protein is less active than the W3A F protein in some assays for fusion (e.g., Figs. 2 and 6A). The V516A mutant F protein had little effect on fusion, except in the CAT assay for cytosolic content mixing, when a reproducibly higher level of activity was observed. We do not know the reason for this increased activity but clearly the cytoplasmic tail of the F protein contributes to fusion activity (Bagai and Lamb, 1996).

By analogy to influenza A virus HA, the SV5 F protein is presumed to exist in a metastable native fusion-inactive state in which the F_1 N-terminal fusion peptide is buried within the interior of the protein (Baker *et al.,* 1999; Lamb, 1993). It is predicted that the F protein will be induced to undergo a conformational change, by an as-yet unidentified trigger, in which the fusion peptide will be exposed and the protein will undergo a conformational change, which may include a refolding event, to a fusion active form (Lamb, 1993). The experiment in which the temperature dependence of F-mediated fusion was tested (Fig. 7) suggests that the energy barrier for the conformational change from the metastable state to the fusion-active conformation is lower for the S443P mutant than it is for the W3A F protein. Conversely, the energy barrier for mutants containing P22L is much higher than that for W3A F protein. Thus, these data are consistent with the notion that the S443P and P22L mutations in W3A F protein are compensating changes and that the residues at these locations in the protein play a key role in the transition to the fusion-active form. These data also emphasize the role of the HN protein in triggering the fusion reaction. In syncytia assays coexpression of the SV5 HN protein augments the extent of syncytia formation by W3A F protein expressed alone (Horvath *et al.,* 1992). In content-mixing assays, when HN is used as a binding protein W3A F protein-mediated fusion occurs to a greater extent than when HA is used as a binding protein (Paterson, Russell, and Lamb, unpublished observations). Furthermore, WR F protein does not cause fusion at 37°C when using HA as the binding protein (Fig. 3); however, when the temperature was raised to 53°C fusion occurred, suggesting that the HN function for WR F protein triggering can be artificially supplanted by providing energy from raising the temperature. A direct protein–protein interaction between F and HN and a conformational change in HN on binding its receptor sialic acid could provide the energy to trigger the F protein under natural conditions.

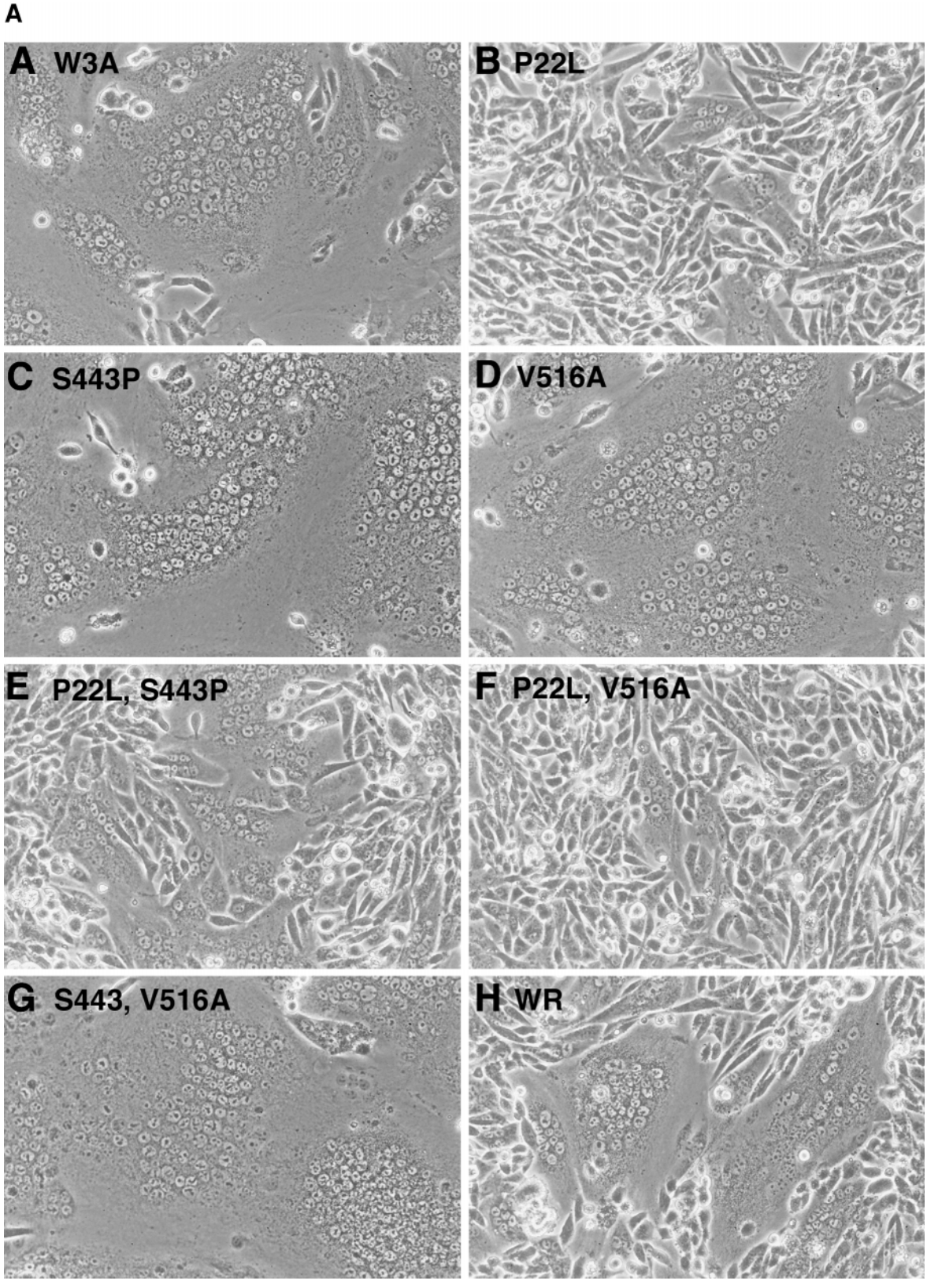

FIG. 6. Syncytia assay. Monolayers of BHK 21F cells were transfected with plasmids encoding F and HN, F and HA, or F alone as described under Materials and Methods. At 18 to 24 h posttransfection, monolayers were examined for the presence of syncytia. Representative photomicrographs are shown. (A) F and HN; (B) F and HA and F alone.

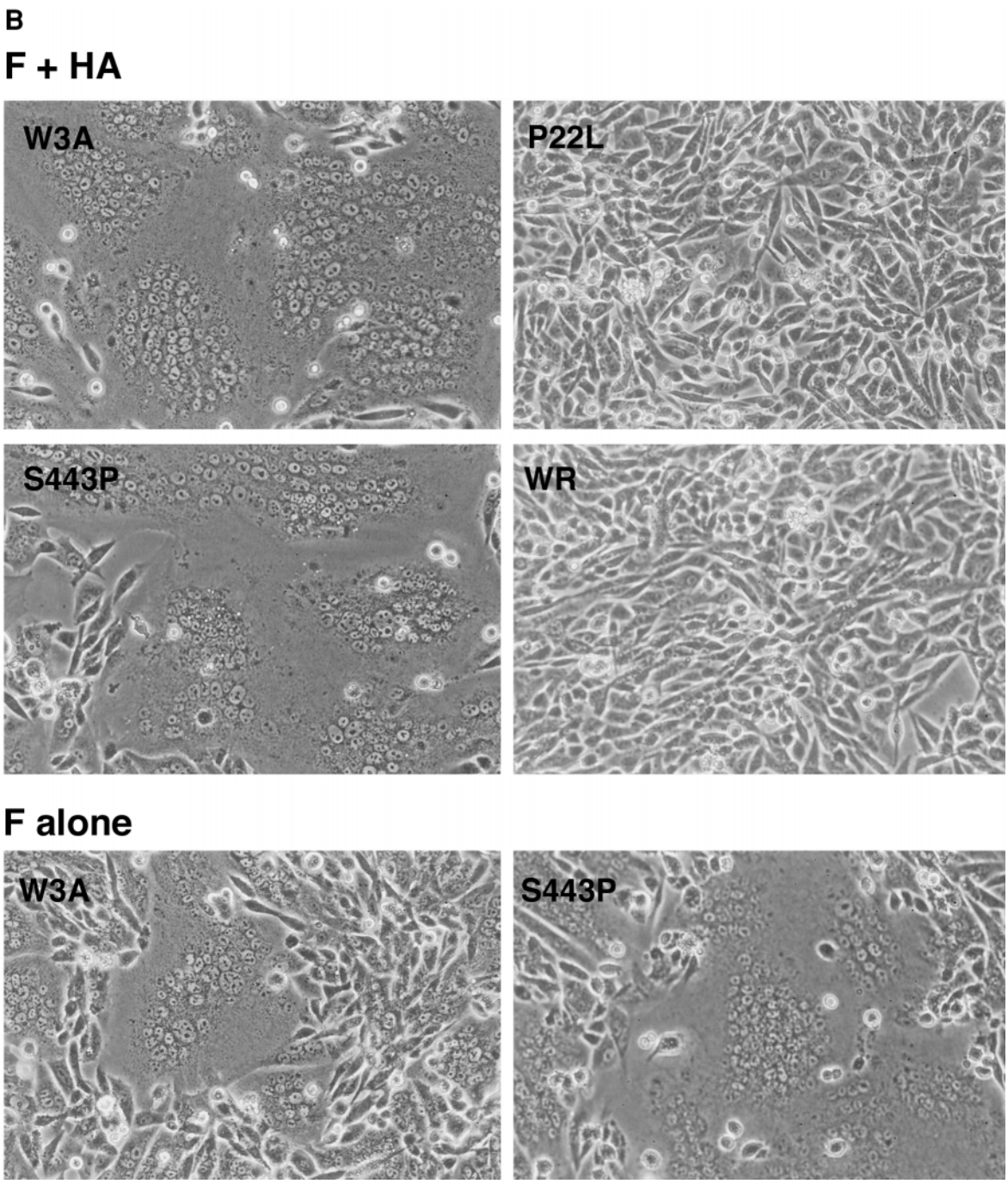

FIG. 6—*Continued*

In some respects the P22L and S443P mutants are related conceptually to mutants in the influenza virus HA protein that were selected for their ability to grow in the presence of amantadine hydrochloride (Daniels *et al.,* 1985). The HA mutants were found to undergo the pH-dependent conformational change at a pH higher than that of the wt HA. Many of the amino acid substitutions that were identified in the mutant HA proteins were recognized as changes that would be likely to destabilize the pH 7 conformation, thus reducing the energy barrier to the low pH-induced conformational change (Daniels *et al.,* 1985).

We have shown previously that the peptide N-1 (F residues 122–184) corresponding to the heptad repeat that abuts the fusion peptide and the peptide C-1 (F residues 435–477), which includes the heptad repeat B that abuts the transmembrane domain, form a highly stable complex which is a trimer of heterodimers (Dutch *et al.,* 1999; Joshi *et al.,* 1998). In the crystal structure of this F_1 core trimer two copies of the N1/C1 heterodimer

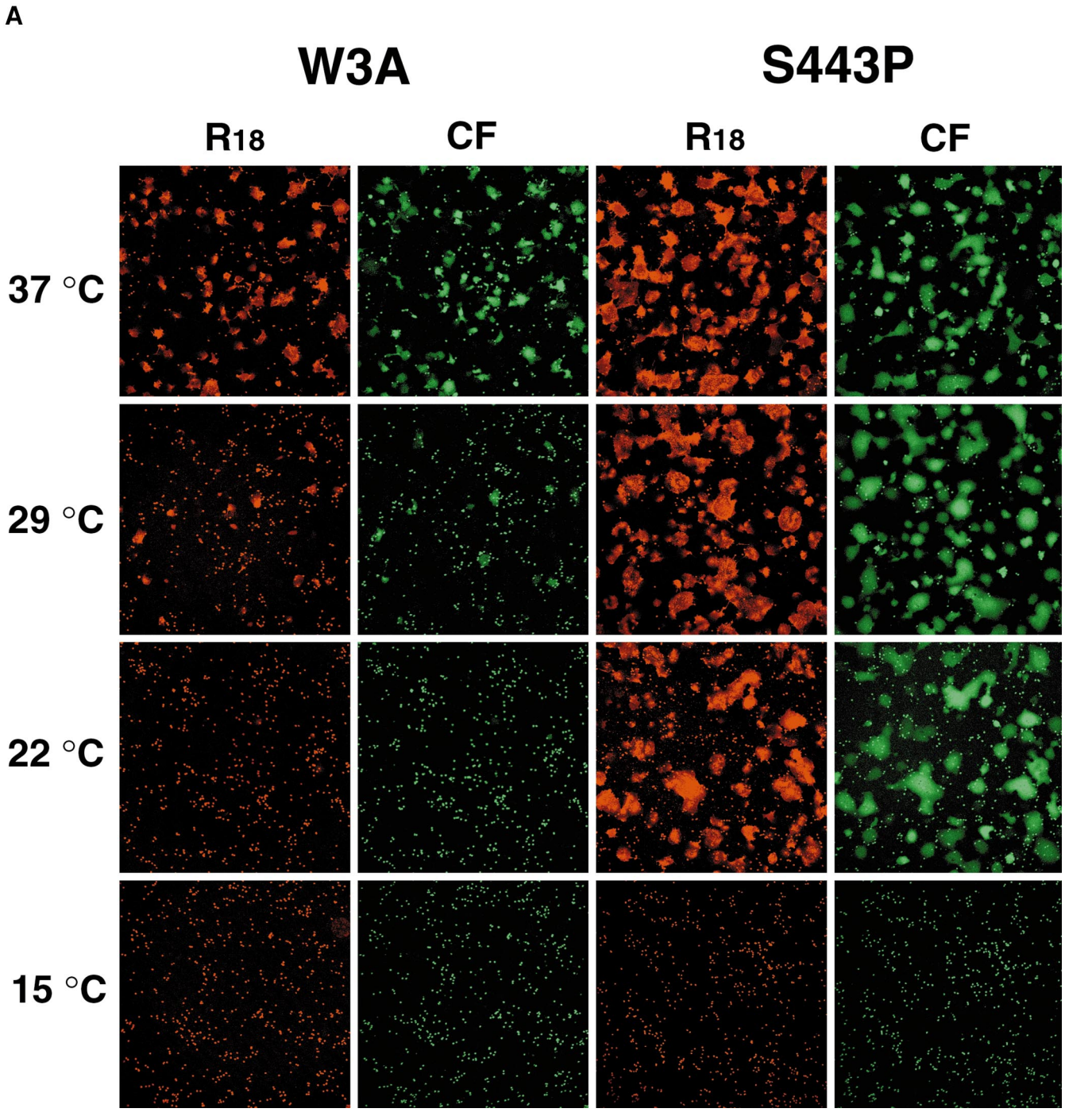

FIG. 7. Temperature dependence of W3A, S443P, and P22L F protein-mediated fusion, and temperature dependence of WR F protein-mediated fusion with HA. Human RBCs double-labeled with the lipid probe R18 and the aqueous probe CF were bound to CV-1 cells coexpressing HN and either the W3A, mutant S443P, or mutant P22L F proteins, or were bound to CV-1 cells coexpressing HA and WR F protein. The cells were incubated for 10 min at different temperatures and examined for both lipid mixing and content mixing by confocal microscopy. The lipid mixing and content mixing are shown for the same field of cells. (A) W3A and S443P F protein-mediated fusion. (B) P22L F protein-mediated fusion, and HA coexpressed with WR F protein-mediated fusion. Temperature of incubation to trigger fusion is indicated. F1a = addition of a 1:100 dilution of ascites fluid of MAb F1a prior to adding the dual-labeled RBCs and raising the temperature.

are observed in the crystallographic asymmetric unit and for one copy the interpretable electron density ends with S443, whereas in the other copy electron density can be interpreted to Q440 (Baker *et al.,* 1999). S443 is situated in the extended chain region of the C-1 peptide and it is beyond the conserved L447 and I449 residues that make hydrogen bonds with the conserved Q169 and N173 residues of the N-1 coiled-coil. The temperature factor of

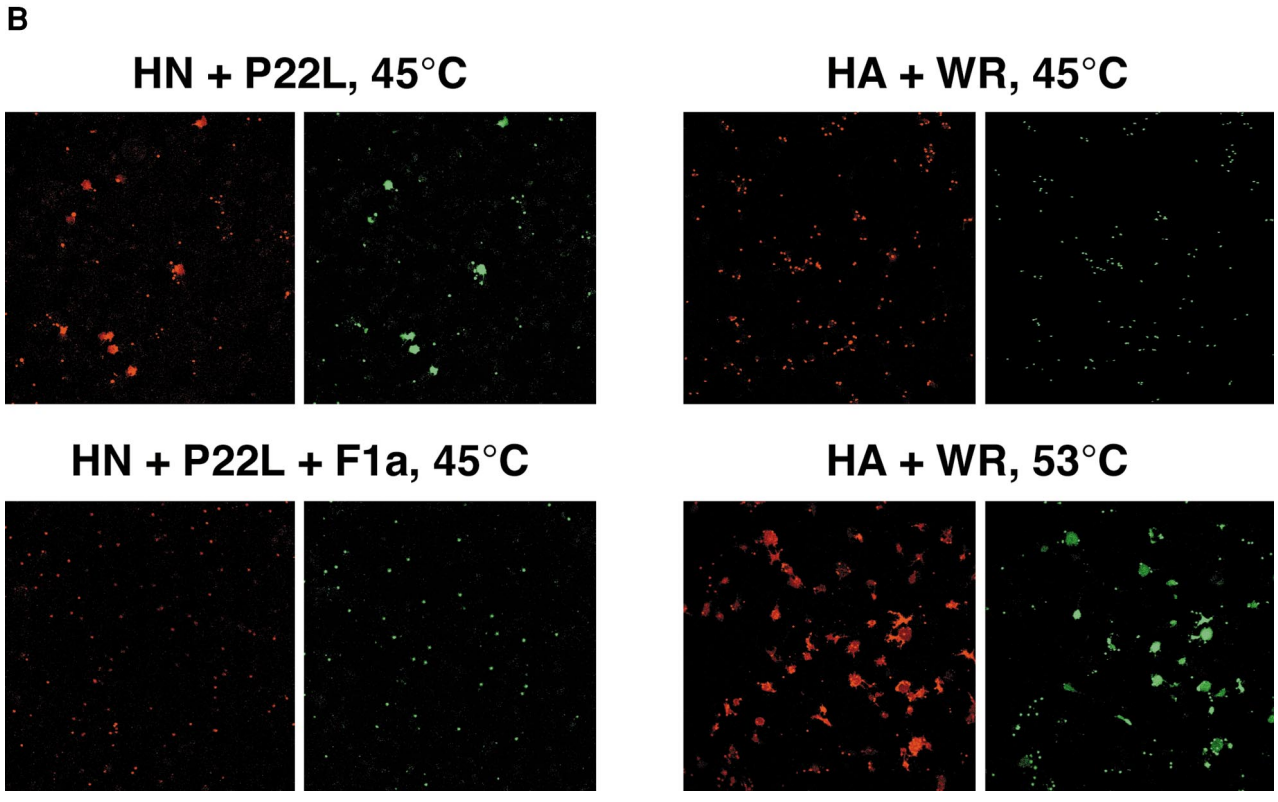

FIG. 7—*Continued*

S443 is high, suggesting conformational flexibility in the structure at this point and it has been suggested that F protein residues between the last residue of N-1 (residue 184) and the beginning of the C-1 peptide (residue 440/443) constitute a separate domain of the F protein (Baker *et al.,* 1999). There is no information on the structure of the F_2 subunit that contains the P22L mutation. However, based on the data presented here on the importance of the nature of residues 22 and 443 in biological activity we now speculate that the presence of proline at residues 22 and 443 destabilize the protein such that it is more readily triggered to undergo the conformational change to the fusion-active state. Conversely, the presence of a leucine residue at position 22 increases the stability of the F protein such that fusion can be triggered only at 37°C if a proline is located at residue 443.

MATERIALS AND METHODS

Cells and viruses

Monolayer cultures of Vero cells and the TC7 subclone of CV-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FCS). BHK 21F monolayers were grown in DMEM supplemented with 10% FCS and 10% tryptose phosphate broth. The recombinant vaccinia virus vTF7–3, which expresses bacteriophage T7 RNA polymerase, and the wild-type vaccinia virus WR were grown in CV-1 cells as described previously (Fuerst *et al.,* 1986).

Plasmids

The SV5 F cDNA (Paterson *et al.,* 1984a,b) was subcloned into pGem2X, a derivative of pGem2 containing an *Xho*I site. The genes encoding the F mutant proteins were constructed by USE site-specific mutagenesis (Amersham Pharmacia Biotech Inc. Piscataway, NJ) according to the manufacturer's directions. The mutant F genes were sequenced in their entirety using a BigDye terminator cycle-sequencing ready-reaction kit (Perkin–Elmer Applied Biosystems [ABI], Foster City, CA) and a ABI 310 automated DNA sequencer. The SV5 HN cDNA (Hiebert *et al.,* 1985a; Paterson *et al.,* 1984b) was subcloned into pGem3X, a derivative of pGem3 containing an *Xho*I site. Plasmid pTF7.5 HA, which contains the HA cDNA of influenza virus A/Udorn/72, was described previously (Simpson and Lamb, 1992) and pGINT7βgal (Dr. Richard A. Morgan, National Center for Human Genome Research) was kindly provided by Edward Berger and Bernard Moss (National Institutes of Health, Bethesda, MD). The cDNAs encoding the W3A F, F P22L mutant, and HN proteins were subcloned into the *Xho*I site in pCAGGS (Niwa *et al.,* 1991). The Udorn HA cDNA was excised from pTF7.5 by *Sac*I digestion and subcloned into pCAGGS/MCS (Niwa *et al.,* 1991). The cDNAs en-

coding the remaining F mutants were amplified by PCR using a 5′ primer containing an *Eco*RI site and a 3′ primer containing a *Cla*I site and were subsequently cloned into pCAGGS/MCS. Plasmid pBH82, which contains the chloramphenicol acetyl transferase gene (CAT) under the control of the T7 RNA polymerase promoter, has been described previously (He *et al.,* 1995). The plasmid containing the T7 RNA polymerase gene was prepared by excising the T7 RNA polymerase gene from pYZ4 (Zhou *et al.,* 1990) by digestion with *Stu*I and *Bam*HI and subcloning it into pCAGGS digested with *Sma*I and *Bgl*II.

Expression of F, HN, and HA proteins

For confocal microscopy and fluorimetry assays the F, HN, and HA proteins were expressed using the recombinant vaccinia virus–T7 RNA polymerase transient expression system (Fuerst *et al.,* 1986). Briefly, monolayer cultures of CV-1 cells in 60- or 35-mm-diameter tissue-culture dishes were infected with recombinant vTF7–3 at 10 plaque forming units (pfu) per cell and incubated at 37°C for 1 h. The cells were then transfected with 2.4 μg of pGem F and 2.4 μg of pGem HN or HA plasmid DNA (made up to 5.0 μg DNA using empty vector DNA) in Optimem (Life Technologies, Gaithersburg, MD) using cationic liposomes prepared as described previously (Rose *et al.,* 1991). At 4 h posttransfection the monolayers were washed with phosphate-buffered saline (PBS) and incubated in DMEM supplemented with 10% FCS overnight at 33°C. For syncytia assays the F, HN, and HA proteins were transiently expressed using the pCAGGS F, HN, and HA plasmids. Monolayer cultures of BHK 21F cells in 60-mm tissue-culture dishes were transfected with 2 μg of DNA using Lipofectamine Plus (Life Technologies) according to the manufacturer's protocol. Syncytia were examined at 18 to 24 h posttransfection using a Nikon Diaphot inverted phase-contrast microscope (Nikon Inc., Garden City, NY). Photographs were taken using a Kodak DCS 420 digital camera (Eastman Kodak Company, Rochester, NY).

Quantification of F protein cell-surface expression

Monolayers of CV-1 cells in 60-mm dishes infected with vTF7–3 and transfected as described earlier were prepared for flow cytometry as described previously (Horvath and Lamb, 1992). The monoclonal antibody F1a, specific for the SV5 F protein (Randall *et al.,* 1987), was used as the primary antibody and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Jackson Immunoresearch Laboratories Inc., West Grove, PA) as the secondary antibody. The cell-surface fluorescence of 10,000 cells was analyzed using a FACSCalibur flowcytometer (Becton Dickinson, San Jose, CA).

Labeling of human erythrocytes with octadecyl rhodamine B chloride and 6-carboxyfluorescein

Freshly drawn human erythrocytes (RBCs) were labeled with the lipid probe octadecyl rhodamine B chloride (R18; Molecular Probes, Eugene, OR) as described previously (Bagai and Lamb, 1995b; Morris *et al.,* 1989). To prepare double-labeled RBCs, R18-labeled RBCs were loaded with 6-carboxyfluorescein (CF; Molecular Probes) as previously described (Melikyan *et al.,* 1995).

Analysis of lipid and content mixing by scanning confocal microscopy

Following overnight incubation at 33°C transfected CV-1 cells grown on glass coverslips and expressing F and HN or expressing F and HA were washed with PBS and incubated for 1 h at 37°C with 50 mU per ml of neuraminidase (*Clostridium perfringens* type V; Sigma Chemical Co., St. Louis, MO). The cells were then washed twice with ice-cold PBS and incubated with 0.5 ml of R18-labeled RBCs (0.05% hematocrit) or 0.5 ml of R18/CF double-labeled RBCs (0.25% hematocrit) at 4°C for 30 min. Unbound RBCs were removed by washing with ice-cold PBS, keeping the cells at 4°C until fusion was initiated by incubation at the desired temperature. After 10 min at 37°C, fusion was terminated by transferring the cells to ice, after which dye transfer was examined using a scanning confocal microscope (Zeiss LSM 410, Zeiss Instruments Inc., Thornwood, NY).

Spectrofluorimetric analysis of lipid mixing

To measure the kinetics of lipid mixing, F- and HA-transfected CV-1 cells in 60-mm dishes were treated with neuraminidase and washed with cold PBS as described earlier. The R18-labeled RBCs were counted using a hemocytometer and diluted. The CV-1 cells were incubated with ~2 RBCs per CV-1 cell in a volume of 1.5 ml at 4°C for 30 min. The unbound RBCs were removed by washing with cold PBS and the RBC-cell complexes were removed from the dishes by incubation at 4°C in PBS (deficient in calcium and magnesium) containing 50 mM EDTA. The complexes were pelleted by centrifuging at 400 *g* for 2 min and resuspended in 100 μl of PBS; 50 μl was added to 3 ml of prewarmed PBS to assay for lipid mixing. The fluorescence was measured continuously using a spectrofluorimeter (series 2; SLM Aminco-Bowman, Rochester, NY) with 1-s time resolution at excitation and emission wavelengths of 560 and 590 nm, respectively. To reduce scattering, a 570-nm cutoff filter was placed in the emission optical pathway. The percentage fluorescence dequenching (%FDQ) was calculated as $100(F - F_0/F_t - F_0)$, where F_0 and F are the fluorescence at time zero and at a given time point and F_t is the fluorescence intensity in the presence of 1% Triton X-100, defined as the fluorescence at infinite dilution of the R18 (Bagai *et al.,* 1993; Sarkar *et al.,* 1989).

CAT assay for content mixing

The analysis of content mixing using a sensitive CAT assay was performed essentially as described previously (He *et al.,* 1998). Briefly, monolayers of Vero cells in 6-well dishes were transfected with 0.8 μg each of pCAGGS F, pCAGGS HN or HA, and pBH82 (CAT) using Lipofectamine Plus according to the manufacturer's instructions. The negative control consisted of transfections in which the CAT plasmid was replaced with pCAGGS vector. Vero cells in 6-cm dishes were transfected with 2 μg each of the T7 RNA polymerase gene-containing plasmid and pCAGGS. Following overnight incubation at 37°C the cells expressing T7 RNA polymerase were trypsinized and overlaid onto the monolayers containing the CAT plasmid and expressing the F and HN or F and HA proteins. Following incubation at 37°C for 7 h, the monolayers were removed from the dishes by scraping, lysed by freeze-thawing, and clarified by centrifugation at 14,000 rpm in an Eppendorf microcentrifuge (Brinkmann Instruments Inc., Westbury, NY). The CAT activity in the cell lysates resulting from fusion of the two cell populations was assayed as described previously (Neumann *et al.,* 1987) using [butryl-1-^{14}C]Coenzyme A (NEN Life Science products, Boston, MA) as the substrate. The CAT activity was quantified using a Beckman LS 6500 scintillation counter (Beckman Instruments, Fullerton, CA).

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