JOURNAL OF VIROLOGY, Sept. 1978, p. 505-512
0022-538X/78/0027-0505$02.00/0

Vol. 27, No. 3
Printed in U.S.A.

Timing of Some of the Molecular Events Required for Cell Fusion Induced by Herpes Simplex Virus Type 1

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Received for publication 10 April 1978

The timing of some of the molecular events that are required for cell fusion was investigated. Cell fusion was produced by a mutant of herpes simplex virus type 1 that causes extensive cell fusion during infection. The timing of molecular events required for fusion was established by the use of blocking agents. Phosphonoacetic acid blocks viral DNA synthesis; actinomycin D blocks RNA synthesis; cycloheximide blocks protein synthesis; 2-deoxyglucose blocks glycosylation of glycoproteins; high temperature, NH_4Cl, and adamantanone block unknown steps required for cell fusion. For cells infected at a low multiplicity of infection, phosphonoacetic acid decreased the rate but not the final amount of fusion, but at a multiplicity of infection of 10 it had no effect on the rate of cell fusion. RNA synthesis was required for fusion until 4 h after infection, protein synthesis until 5.5 h after infection, and glycosylation until 7 h after infection. The temperature-dependent step occurred before 6 h after infection, whereas NH_4Cl and adamantanone acted at steps that occurred until 8 h after infection. Cycloheximide, temperature, NH_4Cl, and adamantanone acted reversibly; actinomycin D and 2-deoxyglucose acted irreversibly. The same order of action of the inhibitors was also determined by using pairs of inhibitors sequentially. These experiments also indicated that the fusion factor was not an α-polypeptide. Virus growth and cell fusion were both found to be highly dependent on temperature in the range of 30 to 40°C. Wild-type infections are apparently characterized by the presence of a fusion factor and a fusion inhibitor. The fusion-blocking agents were added to wild-type-infected cells under a variety of conditions in an attempt to selectively block the production of the fusion inhibitor molecule and thereby cause extensive cell fusion. However, fusion was not observed in any of these experiments.

It now appears that wild-type herpes simplex virus type 1 (HSV-1) infections are characterized by the presence of fusion factor and fusion inhibitor activity (10, 15, 16; Person and Warner, unpublished data). The action of the inhibitor dominates so that only a small fraction of cells fuse (13). We have isolated mutants that cause extensive fusion, and these may be altered in the expression of fusion inhibitor activity (15). Using a qualitative assay for cell fusion and some of the inhibitors employed here, others have examined the requirement and timing of macromolecular synthesis for cell fusion (3, 4, 8). The present study employs a quantitative assay for cell fusion and extends the previous studies.

MATERIALS AND METHODS

Cell cultures and virus stocks. The growth and maintenance of HEL cell cultures and virus stocks were described previously (13). The HSV-1 strain KOS was kindly provided by Priscilla Schaffer (Sydney Farber Cancer Institute, Harvard Medical School, Boston, Mass.). The mutant of KOS used here, *syn* 20, causes extensive syncytia formation. The procedure for mutant isolation was described previously (13).

The growth medium used in these experiments was a modified F12 containing 10% fetal bovine serum. The saline solution used to wash cells and dilute virus suspensions was a tricine-buffered saline. Both were described previously (13). At various times after infection, cells were harvested with trypsin and EDTA and counted with a Coulter Counter. The extent of fusion was determined by measuring the number of cells with pulse heights corresponding to the size of small single cells. Cells disappear from this interval as they fuse with other cells and shift to a larger threshold interval (13).

Metabolic inhibitors. Inhibitors were dissolved in growth medium, and the pH was adjusted to 7.3 when necessary. Cell monolayers were washed once with tricine-buffered saline before the addition of growth medium containing the inhibitor. Metabolic inhibitors were added to cultures at various times and remained in the cultures during subsequent incubation, except

where otherwise indicated.

Phosphonoacetic acid (PAA) was obtained from Richmond Organics, Richmond, Va.; actinomycin D and 2-D-deoxyglucose were from Sigma Chemical Co., St. Louis, Mo.; cycloheximide was from Calbiochem, San Diego, Calif.; 2-adamantanone was from Aldrich Chemical Co., Milwaukee, Wis.; and ammonium chloride was from Fisher Scientific Co., Fairlawn, N.J. In preliminary experiments, actinomycin D, cycloheximide, and 2-deoxyglucose were found to selectively inhibit RNA synthesis, protein synthesis, and glycosylation, respectively, at the concentrations employed. These results are consistent with those reported by others (5, 9, 11, 14).

Equilibrium sedimentation analysis. Duplicate cultures of HEL cells were seeded in 16-ounce (ca. 480-ml) prescription bottles (2.5×10^4 cells per cm^2) and grown for 40 h at 37°C (about 90% confluent). Immediately after virus adsorption, 4 μCi of [^{3}H]thymidine per ml was added to the cell cultures, and PAA was added to one-half of the cultures. After 10 h of incubation at 34°C, the cells were scraped into growth medium, pelleted, and washed twice in the following solution: 10^{-1} M NaCl, 10^{-3} M EDTA, and 10^{-2} M Tris-hydrochloride (pH 7.4). Uninfected cells labeled with [^{14}C]thymidine (0.1 μCi/ml) were harvested and washed in the same way and mixed with either PAA-treated or untreated, [^{3}H]thymidine-labeled, infected cells. The cells were lysed in 12 ml of lysing solution (0.5% of sodium dodecyl sulfate and sodium lauryl sarcosine in 2×10^{-3} M dithiothreitol and 2.5×10^{-2} M EDTA for 15 min at room temperature), and the DNA was deproteinized with autolysed (30 min at room temperature) Pronase (100 μg/ml) for 30 min. The lysate was added to solid cesium chloride (CsCl) to give a mean density of 1.707 g/cm^3 and centrifuged for 64 h at 30,000 rpm in a Beckman type 50 titanium rotor. After centrifugation, fractions were collected, calf thymus carrier DNA was added to each fraction, and DNA was precipitated with 5% (wt/wt) trichloroacetic acid. Precipitates were collected onto membrane filters (0.22-μm pore size; Millipore Corp., Bedford, Mass.), washed with distilled water, and air dried, and the filters were suspended in Omnifluor liquid scintillation fluid. The ^{3}H and ^{14}C radioactivity was determined in a liquid scintillation spectrometer.

Radioactive chemicals and Omnifluor liquid scintillation fluid were from New England Nuclear Corp., Boston, Mass.; sodium lauryl sarcosine, dithiothreitol, and Tris were from Sigma Chemical Co., St. Louis, Mo.; NaCl and EDTA were from Mallinkrodt, St. Louis, Mo.; sodium dodecyl sulfate was from Pierce Chemical Co., Rockford, Ill.; Pronase was from Calbiochem; CsCl was from Metallgellschaft Ag., Frankfurt, West Germany; trichloroacetic acid was from Fisher Scientific Co., Pittsburgh, Pa.; and membrane filters were from Millipore Corp.

RESULTS

Effect of PAA on DNA replication and cell fusion. PAA blocked HSV-1 DNA replication as measured by equilibrium sedimentation of labeled DNA (Fig. 1). Cells were infected with *syn* 20 and labeled with [^{3}H]thymidine before

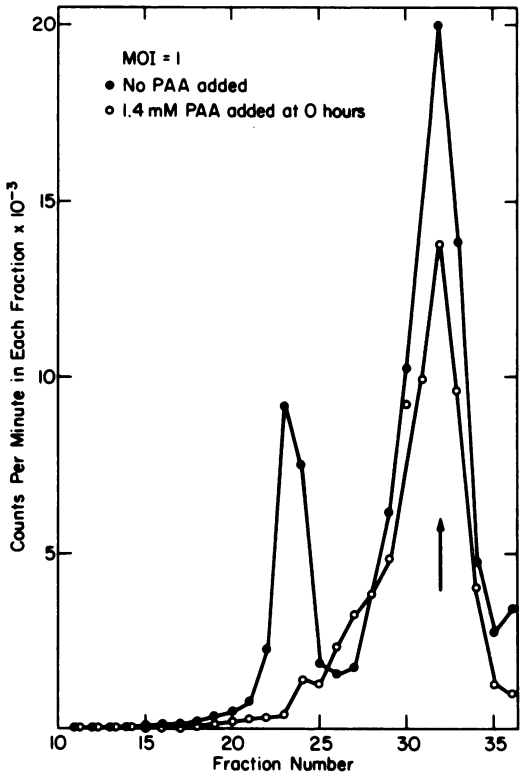

FIG. 1. *Effect of PAA on DNA replication. Cultures of HEL cells were infected with syn 20 at an MOI of 1 and labeled with [^{3}H]thymidine from 0 to 10 h after infection. Infected cultures were lysed, the DNA was centrifuged to equilibrium in CsCl, and trichloroacetic acid-insoluble radioactivity was determined for each fraction. The radioactivity in each fraction is plotted as a function of the fraction number; the direction of sedimentation is from right to left. The arrow indicates the position of cellular DNA which was determined by mixing [^{14}C]thymidine-labeled uninfected cells with infected cells before cell lysis.*

sedimentation in CsCl. Data are shown for virus infections in the presence and absence of PAA. In the presence of PAA, viral DNA synthesis was almost completely eliminated, whereas cellular DNA synthesis was only decreased by about 25% (Fig. 1). A large amount of cellular DNA was observed because the label was added immediately after virus adsorption.

When cells were infected with *syn* 20 at a multiplicity of infection (MOI) of 0.2 or 1.0 and PAA was added immediately after infection, the rate of fusion was decreased (Fig. 2 and 3). After the onset of fusion (about 5 h after infection), a semilogarithmic plot of the fraction of cells in a culture remaining unfused as a function of time after infection yields a straight line whose slope is a measure of the rate of fusion. The percent

inhibition of fusion is defined as 1 minus the ratio of the slopes of the fusion kinetics curves, in the presence and absence of an inhibitor, times 100. At an MOI of 1, the percent inhibition of fusion by PAA was 50% when PAA was added immediately after infection, and decreased to 10% when PAA was added 4 h after infection (Fig. 4).

Data presented in Fig. 4 could reflect a requirement for transcription of some minimum number of DNA genomes, and the translation of the resulting mRNAs, to achieve a maximum rate of fusion. This idea was tested by determining the inhibition of fusion as a function of the adsorbed MOI. The percent inhibition of fusion was found to decrease as the MOI increased, reaching zero at an MOI of 10 (Fig. 5). Furthermore, by extending the fusion kinetics curves to longer times, extensive fusion was ultimately observed even for infection at the lower MOI (data not shown).

Effect of actinomycin D, cycloheximide, temperature shift, 2-deoxyglucose, NH_4Cl, and adamantanone on cell fusion. If one of these inhibitors was added shortly after infection, fusion was completely blocked, but the

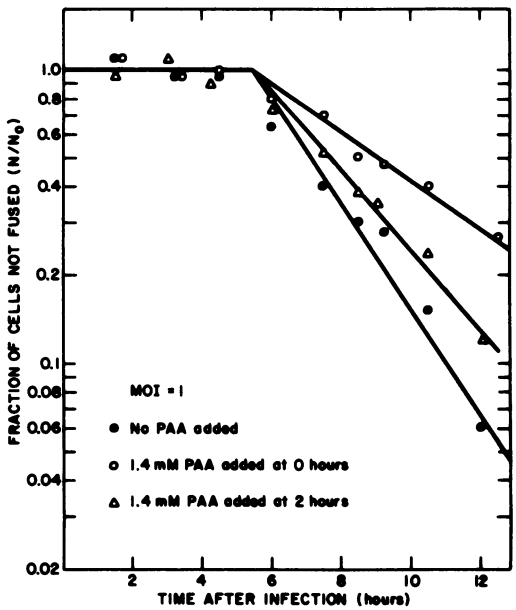

FIG. 3. *Effect of PAA on the kinetics of cell fusion; PAA was added at 0 and 2 h after infection. Cells were infected with syn 20 at an MOI of 1 and assayed for cell fusion as described for Fig. 2. The fraction of cells remaining unfused as a function of time after infection is shown for PAA added at 0 and 2 h after infection.*

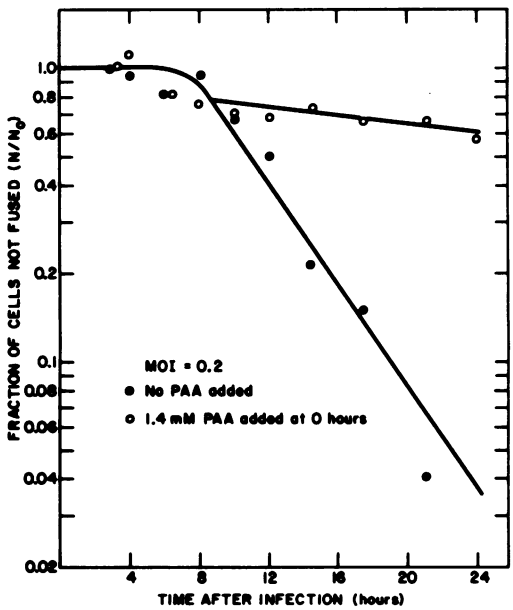

FIG. 2. *Effect of PAA on the kinetics of cell fusion. Cultures were infected with syn 20 at an MOI of 0.2 and incubated for the times indicated. Cells were harvested with a trypsin-EDTA solution, and the number of small single cells was measured by using a Coulter Counter assay described in the text. The fraction of single cells remaining unfused is plotted as a function of time after infection for cultures incubated in the presence and absence of PAA.*

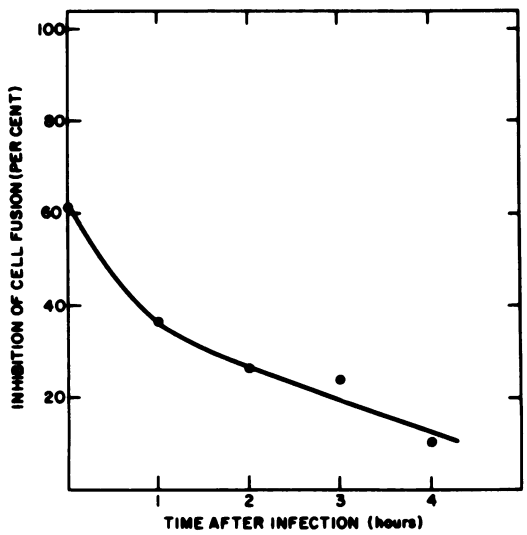

FIG. 4. *Inhibition of cell fusion by PAA as a function of time after infection. Fusion kinetics curves for cells infected with syn 20 at an MOI of 1 were determined as described for Fig. 2 and 3. PAA was added to the cultures at the indicated times after infection and left in the cultures during subsequent incubation. The percent inhibition of fusion is defined as 1 minus the ratio of the slopes of the fusion kinetics curves, in the presence and absence of the blocking agent, times 100.*

fusion blocking was limited to a specific time interval during infection. Data which support this conclusion are shown for infected cells incubated in the presence of cycloheximide (Fig. 6) or 2-deoxyglucose (Fig. 7), and for a temperature shift from 34 to 41°C during incubation (Fig. 8). Similar results were obtained by using actinomycin D, NH_4Cl, and adamantanone (data not shown). Results of many fusion kinetics curves using these blocking agents are most simply expressed in diagrammatic form (Fig. 9). A shaded rectangle is used to show the time interval during which fusion inhibition changed from 100 to 0% for a particular blocking agent. For example, the addition of cycloheximide before 3 h after infection gave complete inhibition of fusion and, when added after 5.5 h, gave no inhibition of fusion. With this criterion, the temporal order of agents that blocked synthetic processes required for fusion was actinomycin D, cycloheximide, temperature shift, and 2-deoxyglucose. Blocking agents, NH_4Cl, and the membrane perturber adamantanone presumably blocked postsynthetic events required for fusion. The accuracy of determination of the timing for 100 and 0% inhibition of fusion in Fig. 9 is probably ±0.5 h. That is, occasionally a single fusion kinetics curve would be obtained that differed from the average values by ±0.5 h (compare curve for 5.5 h after infection [Fig. 8] with average data [Fig. 9]).

The temperature dependence of cell fusion with virus growth was determined and the percentages of the maximum values were plotted as a function of incubation temperatures (Fig. 10).

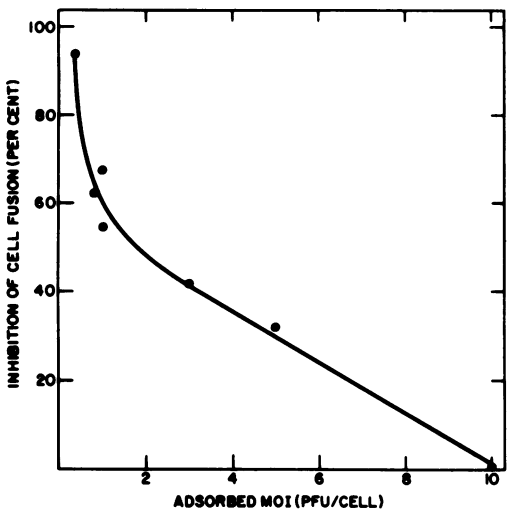

FIG. 5. *Inhibition of cell fusion by PAA as a function of MOI. PAA was added to cultures immediately after infection with syn 20 at the indicated MOI. The inhibition of cell fusion was measured as described for Fig. 4.*

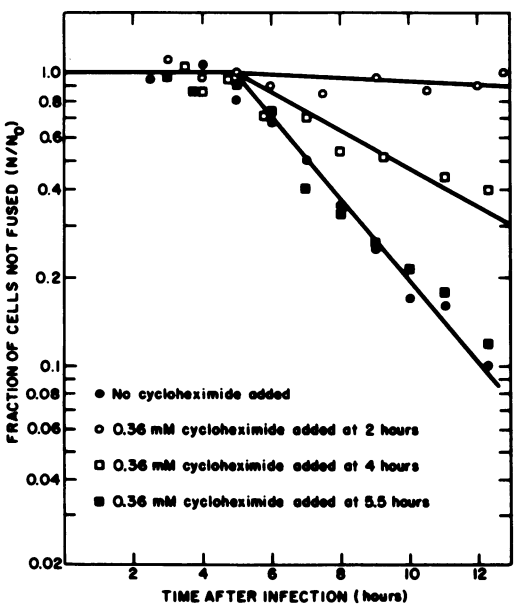

FIG. 6. *Effect of cycloheximide on cell fusion. Cells were infected with syn 20 at an MOI of 10 and assayed for cell fusion as indicated for Fig. 2. The fraction of cells remaining unfused is plotted as a function of time after infection.*

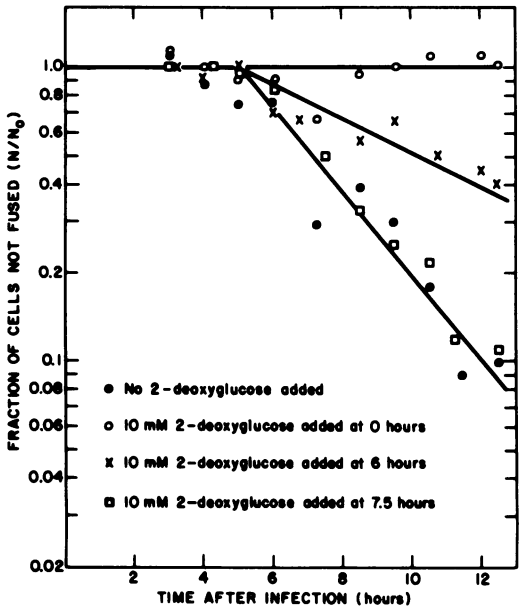

FIG. 7. *Effect of 2-deoxyglucose on cell fusion. Cells were infected with syn 20 at an MOI of 10 and assayed for cell fusion as indicated for Fig. 2. The fraction of cells remaining unfused is plotted as a function of time after infection.*

Both curves have a maximum between 34 and 38°C and decrease rapidly at higher and lower temperatures. Although the overall data are somewhat similar, there was a more rapid decrease in the rate of cell fusion than for virus growth between 38 and 39°C.

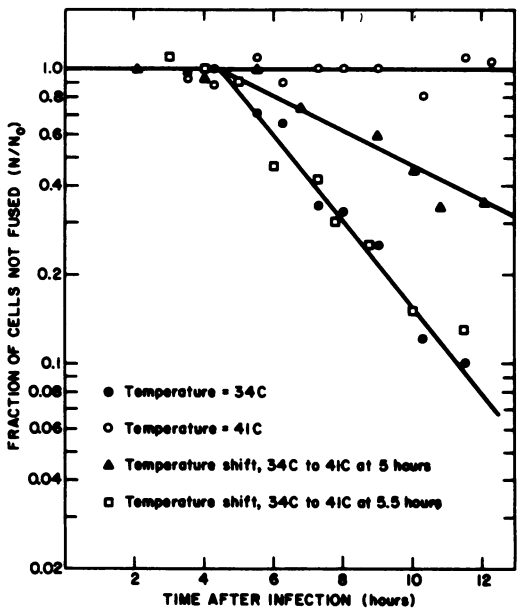

FIG. 8. *Effect of temperature shift on cell fusion. Cells were infected with syn 20 at an MOI of 10 and incubated at 34°C. At the times indicated, cultures were shifted to 41°C. Cells were assayed for cell fusion as indicated for Fig. 2. The fraction of cells remaining unfused is plotted as a function of time after infection.*

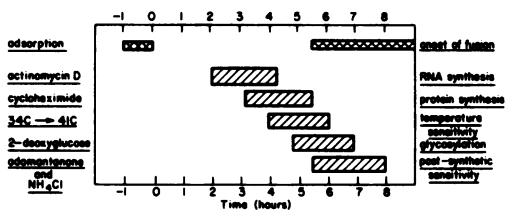

FIG. 9. *Timing of molecular events required for cell fusion. The inhibitors used are shown at the left of the diagram, and the events blocked by the inhibitors are shown at the right. A time scale is plotted as the horizontal axis. The times for virus adsorption and the onset of fusion are shown as shaded rectangles at the top of the figure. For each blocking agent, the left-hand edge of a shaded rectangle represents the latest time after infection at which an agent completely inhibited fusion. The right-hand edge of the rectangle represents the earliest time after infection at which an agent had no effect on fusion. The concentrations of blocking agents used were: actinomycin D, 8 μM; cycloheximide, 0.36 mM; 2-deoxyglucose, 10 mM; NH_4Cl, 20 mM; adamantanone, 7 mM.*

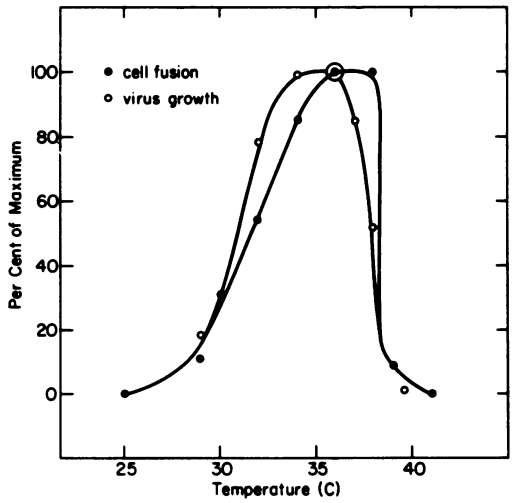

FIG. 10. *Effect of temperature on cell fusion and virus growth. The rate of cell fusion and extent of virus growth are plotted as a percentage of the respective maximum values. For fusion, the slope of the fusion kinetics curve is taken as the rate of fusion; a maximum rate, 100%, was obtained at 36 and 38°C. For virus growth, optimal incubation times for virus growth were determined, and this value was plotted for each temperature. Virus growth was measured by plaque formation on HEL cells; maximum growth (100%) was obtained at 34 and 36°C.*

Independent method of establishing the order of action of blocking agents. Consider a two-step pathway consisting of a reactant A, an intermediate B, and a product C in which the production of B and C may be blocked by the agents *a* and *b*, respectively ($A \xrightarrow{a} B \xrightarrow{b} C$). If *b* is present, the intermediate B will accumulate. Replacing *b* with *a* will then allow the conversion of B to C. If the addition of *a* precedes its replacement with *b*, the product C will never be formed. Pairs of blocking agents were used in this way to establish the order of action of the blocking agents. If two agents block the same step in a pathway, or if the agent that blocks the later step acts irreversibly, one cannot determine the order of action of the blocking agents.

Experiments were performed by infecting monolayers of cells in petri dishes and scoring fusion 24 to 36 h later by a qualitative microscope assay. One blocking agent was present from 1 to 9 h after infection and was replaced by a second blocking agent at that time (Table 1). Cycloheximide, NH_4Cl, adamantanone, and the temperature-dependent step were reversible, whereas actinomycin D and 2-deoxyglucose were not (Table 1, lines 1 to 6). The cycloheximide-sensitive step preceded the 2-deoxyglucose, NH_4Cl, adamantanone, and perhaps the temperature-sensitive step. The temperature-

TABLE 1. *Order of action of fusion-blocking agents*[a]

Blocking agent	Cell fusion						
	WT-infected HEL cells			*syn* 20-infected HEL cells			
	>1	>9	1 → 9	>1	>9	1 → 9	1 → 9, >9
Actinomycin D	−	−	−	−	++	−	
Cycloheximide	−	−	−	−	++	++	
2-Deoxyglucose	−	−	−	−	++	−	
NH_4Cl	−	−	−	−	++	++	
Adamantanone	−	−	−	−	++	++	
Temperature shift	−	−	−	−	++	+	
Actinomycin D, cycloheximide							−
Cycloheximide, actinomycin D							−
Cycloheximide, 2-deoxyglucose							−
2-Deoxyglucose, cycloheximide							−
Cycloheximide, NH_4Cl							−
NH_4Cl, cycloheximide							++
Cycloheximide, adamantanone							−
Adamantanone, cycloheximide							++
NH_4Cl, adamantanone							−
Adamantanone, NH_4Cl							+−
Cycloheximide, temperature shift							−
Temperature shift, cycloheximide							+−
Temperature shift, NH_4Cl							−
NH_4Cl, temperature shift							++

[a] Blocking agents were used at the following concentrations: actinomycin D, 8 μM; cycloheximide, 0.36 mM; 2-deoxyglucose, 1 mM; NH_4Cl, 20 mM; and adamantanone, 7 mM. The temperature of incubation was shifted from 34 to 39.5°C at the times indicated. WT, Wild-type virus; *syn* 20, *syn* 20 virus (MOI, 10). >1, Agent was added to cultures at 1 h after infection and remained in cultures during subsequent incubation; >9, blocking agent was added at 9 h after infection and remained in cultures during subsequent incubation; 1 → 9, blocking agent was added at 1 h after infection and was removed at 9 h; 1 → 9, >9, nomenclature used for pairs of blocking agents. The first agent of a pair was added to the medium at 1 h after infection and was removed at 9 h after infection; the second agent was added at 9 h after infection and remained in the medium during subsequent incubation. In the case of the wild-type infections, blocking agents were also added at a number of other times after infection and at several lower concentrations. Monolayers were scanned under the microscope 24 to 36 h after infection and scored for fusion. ++, Estimated that greater than 75% of the cells were fused; +, about 50% of the cells were fused; +−, estimated that 10 to 20% of the cells were fused; −, estimated that less than 10% of the cells were fused.

dependent step occurred earlier than the NH_4Cl-sensitive step. The overlap between NH_4Cl and adamantanone blocking was not resolved, but a small amount of fusion was observed when adamantanone preceded the use of NH_4Cl. Cycloheximide is known to act at a later step than actinomycin D, and cycloheximide was shown to act reversibly. Nonetheless, when cycloheximide was added before actinomycin D, no fusion was observed. The same results were obtained when the reversible protein inhibitor puromycin was used (data not shown).

From studies of mutants of HSV-1 that affect cell fusion (10, 15, 16; Person and Warner, unpublished data), it is thought that wild-type infections are characterized by the presence of a fusion inhibitor. By adding agents that block fusion at several times after infection and at several concentrations, some of which were less than those required to block fusion, it was hoped to selectively block fusion inhibition in wild-type infections. Then wild-type infections would result in the extensive fusion characteristic of *syn* mutant infections. These experiments were done, but extensive fusion was not observed in any of the experiments (Table 1 and data not shown).

To determine whether PAA blocked the synthesis of the fusion inhibitor, it was added to wild-type infected cells (MOI, 10) at 0, 1, and 2

h after infection. Again, extensive fusion was not observed (data not shown). As a control, *syn* 20-infected cells were similarly treated, and extensive fusion was observed.

DISCUSSION

We have studied the timing of some of the molecular events required for cell fusion by using metabolic blocking agents and a quantitative assay for cell fusion. Our data are largely in agreement with previous data which were based on a qualitative assay for cell fusion. The order of action for the blocking agents used was actinomycin D, cycloheximide, temperature shift, 2-deoxyglucose, NH_4Cl, and adamantanone. There is considerable uncertainty about the assignment of the order of NH_4Cl relative to adamantanone.

The onset of DNA replication and of cell fusion in HSV-1 infections occurred at roughly the same time. Falke found that actinomycin D and cycloheximide depress DNA replication and fusion by similar amounts and speculated that DNA replication might be a trigger for cell fusion (3, 4). However, the inhibition of DNA replication by mitomycin C (12) or cytosine arabinoside treatment (8) did not block cell fusion. By using PAA, which specifically blocks viral DNA polymerase (11), we have shown that the rate but not the final amount of fusion was decreased when a low MOI was used. At an MOI of 10, there was no influence of PAA on cell fusion. We conclude that DNA replication per se is not required for cell fusion but that the presence of about 10 HSV-1 genomes per cell (PFU/cell) is required to achieve the maximum rate of fusion. Since extensive fusion occurred in the absence of DNA replication, virus production was not required for cell fusion.

In contrast to the results for PAA, actinomycin D and cycloheximide added early in infection completely blocked fusion for an extended period of time. RNA synthesis was required for cell fusion until 4 h after infection; protein synthesis was required until 5 h after infection. Considering differences in virus and cell strains, media, and temperature of incubation, these results are in good agreement with results reported by Falke and by Keller (3, 4, 8). The action of cycloheximide is readily reversible, while the removal of actinomycin D does not result in the production of fusion scored 24 h later. It has been reported previously that 2-deoxyglucose blocks cell fusion and the glycosylation of HSV-1 glycoproteins (1, 8, 9). Here we show that it acted irreversibly and that glycosylation was required until 7 h after infection.

Adamantanone and NH_4Cl, which blocked fusion reversibly, appear to act at postsynthetic steps. A small amount of fusion was observed when adamantanone preceded the use of NH_4Cl. This may indicate that the NH_4Cl-sensitive step precedes the adamantanone-sensitive step. Since adamantanone is a known perturber (2), it is possible that both of these agents block fusion by action at the plasma membrane. NH_4Cl has little influence on viral growth (6), but adamantanone reversibly inhibits a late step in virus replication, perhaps at envelopment (T. C. Holland, unpublished data).

We have observed a temperature-dependent step for cell fusion; it occurred early in infection and was reversible. To determine whether the temperature dependence was specific for cell fusion, we determined the temperature dependence of fusion and compared it with the temperature dependence of virus growth. Both had maximum values between 34 and 38°C and declined rapidly at lower and higher temperatures. Although the data for both fusion and growth did not differ strikingly, there was a precipitous decrease in cell fusion between 38 and 39°C. The temperature-dependent step for virus growth may be required for cell fusion. It would be of interest to know more about the effect of temperature for both functions. A temperature-dependent step for the growth of a bovine herpesvirus has been reported (17, 18). It was thought that the critical step was a block in DNA replication, but we note that DNA replication may be somewhat dependent on RNA and protein synthesis (4).

Honess and Roizman have defined three types of peptides following the infection of cells with HSV-1: α, β, and γ (7). If cycloheximide and actinomycin D are used sequentially, as in the experiments reported in Table 1, then only α-polypeptides are made; β- and γ-polypeptides require the prior synthesis of α-polypeptides. Since this same protocol blocks fusion, we conclude that the fusion factor is not an α-polypeptide.

We found that fusion inhibitor production in wild-type-infected cells did not require viral DNA replication, and that fusion inhibitor activity could not be selectively inhibited by the application of a wide variety of concentrations and times of addition of the blocking agents. Apparently, the production of fusion factor and fusion inhibitor activity have similar metabolic and temporal requirements.

ACKNOWLEDGMENTS

This research was supported by grants from the U.S. Department of Energy, the National Science Foundation, and the U.S. Public Health Service.

We acknowledge discussion of experiments, results, and

preparation of the manuscript with A. Bhagwat, V. C. Bond, P. M. Keller, R. W. Knowles, and G. S. Read. We also thank Richard Adler and Myron Levine for communicating unpublished data on the action of PAA on cell fusion, and Wallace Snipes for suggesting that PAA action might depend on MOI. The competent and cheerful technical assistance of Susan C. Warner is gratefully acknowledged. We especially thank J. Kielman for his help with the CsCl gradients.

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