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Genetic Studies of Cell Fusion Induced by Herpes Simplex Virus Type 1

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Eight cell fusion-causing *syn* mutants were isolated from the KOS strain of herpes simplex virus type 1. Unlike the wild-type virus, the mutants produced plaques containing multinucleated cells, or syncytia. Fusion kinetics curves were established with a Coulter Counter assay for the mutants and wild-type virus in single infections of human embryonic lung (HEL) cells, for the mutants and wild-type virus in mixed infections (dominance test), and for pairs of mutants in mixed infections (complementation test). In single infections, fusion began 4 to 6 h after infection and proceeded with an exponential decrease in the number of small single cells. At some later time that was characteristic of the mutant, there was a significant reduction in the rate of fusion for all but possibly one of the mutants. Although the wild-type virus did not produce syncytial plaques, it did induce a small amount of fusion that stopped abruptly about 2 h after it started. These data are consistent with the hypothesis that both mutants and wild type induce an active fusion inducer and that the activity of this inducer is subsequently inhibited. The extent of fusion is apparently determined by the length of the interval during which the fusion inducer is active. That fusion is actively inhibited in wild-type infections is indicated by the observation that *syn* mutant-infected cells fused more readily with uninfected cells than with wild type-infected cells. Fusion was decreased in mixed infections with the mutants and wild-type virus, but the mutants displayed a codominant fusion phenotype. Fusion was not decreased in mixed infection with pairs of mutants, indicating that the mutants, with one possible exception, are members of the same complementation group. A linkage map was established for six of the mutants by analysis of recombination frequencies.

Membrane fusion occurs during a number of biological processes, including endo- and exocytosis, neurotransmitter release, and bone and muscle cell development (9, 10). In an effort to understand the molecular basis of membrane fusion, we have been studying the process of cell fusion that occurs during productive infections of cells by some strains of herpes simplex virus type 1 (HSV-1) (1, 2, 6-8, 18, 19). Since HSV-1 apparently uses the machinery of the host cell to synthesize virus-specified glycoproteins and to insert them into cell membranes along with newly synthesized lipids (13, 14), the virus is also a useful probe for studying the synthesis of membranes in eucaryotic cells. An understanding at the molecular level of the HSV-1 fusion process may also provide insights into the interactions of membrane molecules. The use of viruses to study membrane fusion and biosynthesis offers the advantage that both genetic and biochemical techniques can be brought to bear upon the problems. Cell fusion is a particularly good marker activity in studies of the synthesis and interactions of viral membrane molecules because it is the most visible consequence of virus-induced changes in cell membranes.

Recently, two elegant studies of HSV-induced fusion have been reported with recombinant viruses derived from crosses of different syncytial and nonsyncytial virus strains (5, 15). Although these studies have yielded valuable information concerning the number of genetic loci that affect the fusion phenotype (15) and the probable identity of the fusion inducer (5), they are potentially complicated by strain differences that are unrelated to the induction of fusion. As an alternative approach, we have chosen to isolate a large number of fusion-causing mutants from a single wild-type virus strain. The mutants were designated *syn* mutants because they produce plaques that contain multinucleated cells, or syncytia. KOS was selected as the wild-type strain for these studies because it produces nonsyncytial plaques and because it is one of the best geneti-

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cally characterized strains of HSV-1 (16, 17). In this paper, we describe the isolation of eight mutants, kinetic studies of the fusion that they induce, and genetic characterization of the mutants by means of dominance, complementation, and recombination analysis.

MATERIALS AND METHODS

Cells and viruses. Procedures for the growth and maintenance of human embryonic lung (HEL) cell cultures and of virus stocks have been described previously (8). HSV-1, strain KOS, was kindly provided by Priscilla Schaffer (Sidney Farber Cancer Institute, Harvard University, Cambridge, Mass.), and strains mP and F were kindly supplied by Bernard Roizman and Niza Frenkel, respectively (University of Chicago, Chicago, Ill.). The growth medium used in all experiments was a modified F12 supplemented with 10% fetal bovine serum (8).

Isolation of *syn* mutants. *syn* mutants were isolated from mutagenized cultures of KOS. Monolayers of HEL cells in 2-ounce (ca. 60-ml) prescription bottles (21-cm^2 surface area) that were about 75% confluent (6×10^4 cells per cm^2) were infected with KOS at a multiplicity of infection (MOI) of 5 to 10 PFU/cell and incubated at 37°C. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine was added at 2.5 to 10 μg/ml from 2 to 9 h after infection, or 5-bromodeoxyuridine at 0.5 μg/ml was present throughout infection. When cultures were exposed to mutagens from 2 to 9 h after infection, the growth medium was changed to medium lacking the mutagen, and incubation was continued until 20.5 h. Infected cells were scraped into the medium, disrupted by three cycles of freezing and thawing, clarified by centrifugation at $7,500 \times g$ for 5 min, and stored at −75°C as the mutagenized virus stocks. These mutagenic procedures reduced the yields of viable progeny virus to about 10% of control values except for cultures containing 10 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml, which reduced the yields to 1% of the control value. *syn* 1, *syn* 6, *syn* 8, and *syn* 33 were isolated from cultures containing 5-bromodeoxyuridine, and *syn* 20, *syn* 30, *syn* 31, and *syn* 32 were isolated from cultures containing *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

For the isolation of *syn* mutants, mutagenized virus stocks were added to suspensions of HEL cells in growth medium. The mixtures were used to inoculate 96-well tissue culture plates (Falcon Plastics, Oxnard, Calif.) with about 3×10^4 cells and 10 PFU/well. After 2 or 3 days of incubation, the trays were scanned with a microscope. The contents of wells containing syncytia were harvested and used to infect cells in 96-well tissue culture plates at an MOI such that a significant fraction of the wells contained no infected cells. After 2 to 3 days of incubation, the contents of wells that contained syncytia but no visible signs of wild-type infection were picked and cloned twice by a similar procedure before preparation of a stock of the mutant. *syn* 20, *syn* 30, *syn* 31, *syn* 32, and *syn* 33 were all isolated from separate mutagenized stocks, whereas *syn* 1, *syn* 6, and *syn* 8 were isolated from the same mutagenized stock.

The procedure for virus infection of HEL cells was as described previously (8). Under these conditions, the kinetics of adsorption of the mutants and wild type are indistinguishable (V. C. Bond, personal communication). Throughout this paper, the term MOI will be used to signify the number of adsorbed PFU per cell (8).

Fusion of mixed cell populations. Cells with radioactively labeled DNA were prepared by growth in medium containing 1 to 3 μCi of [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.) per ml for 2 to 3 days at 37°C. To start the experiment, we added unlabeled cells at a density of 6.3×10^4 cells per cm^2, or 80% confluency, to 35-mm-diameter petri dishes containing cover slips. After 12 h, these cells and the cells with radioactively labeled DNA were infected at an MOI of 10 with a *syn* mutant or wild-type virus or were mock infected. After adsorption, both sets of cells were incubated in growth medium for 30 min at 34°C to allow penetration of the attached virus. The radioactive cells were then harvested with trypsin and EDTA and added to the cultures of unlabeled cells at a density of 4.2×10^3 cells per cm^2, or about 6% confluency. Incubation was then continued at 34 °C. Cell layers were fixed at various times after infection, autoradiographs were prepared, and the cells were stained as described previously (8). R/R_0, the fraction of labeled nuclei in mononucleated cells, was then determined by examining the cultures with a microscope.

Recombination experiments. Duplicate cultures of HEL cells were simultaneously infected with two *syn* mutants at an MOI of 5 each. Duplicate cultures were also infected singly with each mutant at an MOI of 10. After 24 h of incubation at 34°C, the infected cells were scraped into the medium and subjected to three cycles of freezing and thawing. Duplicate cultures were pooled, clarified, and stored at −75°C. Subsequently, the progeny from each cross and single infection were plated on HEL monolayers to determine the frequency of viruses that produced nonsyncytial plaques.

RESULTS

Plaque morphologies of *syn* mutants and wild-type virus. The mutants that were isolated exhibited a range of plaque morphologies. Wild-type plaques were characterized by their scalloped borders and by the presence of clumps of individual rounded cells throughout the plaques. Plaques of mutants that produced extensive syncytia (*syn* 8, *syn* 20, *syn* 30, and *syn* 33) were 1.5 to 2 times larger than those produced by wild type and contained syncytia resulting from the fusion of as many as several hundred cells. They were characterized by unbroken syncytia with smooth surfaces and swirling borders during day 1 after infection. Later, the plaques contained several large, irregularly shaped syncytia within a lysed-out area. *syn* 31 and *syn* 32 plaques contained large, irregularly shaped syncytia that were somewhat smaller than those produced by *syn* 20. However, *syn* 31

and *syn* 32 plaques often had scalloped borders. Plaques produced by *syn* 1 had scalloped edges and were very similar in appearance to wild-type plaques. They did, however, contain definite small syncytia. *syn* 6 plaques were even more similar to wild-type plaques than were those produced by *syn* 1. They had scalloped borders and contained a few small, round, multinucleated cells produced by the fusion of a few cells at the most. The morphologies of the plaques produced by wild type and the eight *syn* mutants were independent of temperature over a range from 31 to 39°C.

Kinetics of fusion of cells infected with individual *syn* mutant or wild-type viruses. A Coulter Counter assay was used to measure the kinetics of cell fusion induced during productive infections of HEL cells by the various *syn* mutants or by wild type. As has been described previously, this assay uses a Coulter Counter to measure the disappearance of small single cells from parallel cultures of infected cells as a function of time after infection (8). Fusion kinetics curves for the mutants and for wild-type virus are shown in Fig. 1. The kinetics curves for *syn* 8, *syn* 20, *syn* 30, *syn* 31, *syn* 32, and *syn* 33 were similar to the one for *syn* 20 in that they were biphasic, the onset of fusion occurred at 4 to 6 h after infection, and they showed a very similar initial rate of fusion. However, the mutants differed with respect to the time of decrease in the rate of fusion and, therefore, also with respect to the fraction of cells remaining unfused at this time. *syn* 6 differed in that the initial rate of fusion was significantly reduced. For *syn* 1, the initial rate was slow, and it is not possible to tell whether the fusion kinetics curve is biphasic. Similar fusion kinetics curves were obtained for all of the mutants with a fluorescence enhancement assay of cell fusion (4).

Wild-type virus did induce a small but reproducible amount of fusion that halted abruptly about 2 h after it started, giving rise to a biphasic kinetics curve. At this time about 80% of the cells remained unfused. To emphasize the certainty of this small amount of fusion, kinetics curves for wild-type virus were determined over an extended time interval. Points are included to show that other virus strains (F and mP) also caused a similar amount of fusion of HEL cells (Fig. 2). Wild type-induced fusion is also observable upon careful examination of infected cultures with a microscope, as well as by a fluorescence enhancement assay of cell fusion (4).

Test for dominance: fusion kinetics of cells simultaneously infected with *syn* mutants and wild-type viruses. In simultaneous mixed infections with wild type and all of the mutants except *syn* 33, the extent of fusion was greatly reduced relative to single infections with the mutants alone (Fig. 3 and 4 and Table 1). Whereas infection with wild type resulted in 80% of the cells remaining unfused, in mixed infections the fraction of cells not fused at 12 h after infection varied from 0.55 to 0.80. *syn* 1, *syn* 6, and *syn* 20 were almost completely recessive to wild type, whereas mixed infections involving *syn* 8, *syn* 30, *syn* 31, and *syn* 32 resulted in an unfused fraction at 12 h of from 0.55 to 0.70 of the cells.

syn 33 produced substantially more fusion in mixed infections than did any of the other mutants. In infections involving equal numbers of *syn* 33 and wild type, the fraction of cells not fused at 12 h was 0.35 (Table 1). The fusion kinetics of the mixedly infected cells were strongly dependent upon the ratio of wild type to *syn* 33 that was used (Fig. 4). The reduction of fusion that occurred in mixed infections did not appear to be due to interference by wild type with the replication of the *syn* mutants because for all mixed infections the growth of wild type was reduced, whereas the growth of four of the six *syn* mutants was enhanced (Table 2).

Test for complementation: kinetics of fusion of cells infected with two *syn* mutants. Cells mixedly infected with two mutants that are altered in different complementation groups should fuse less readily than cells infected with either mutant alone. Alternatively, fusion kinetics curves equivalent to the ones for either mutant alone or to some intermediate to the curves for either mutant alone would indicate that the two mutants are in the same complementation group. In every mixed infection the latter result was obtained (Fig. 5 and Table 3). The complementation test is not applicable to *syn* 33 because that mutant causes extensive fusion even in mixed infections with an equal number of wild type.

The mutants can be arranged in a dominance hierarchy based upon the extent of fusion produced in mixed infections with other mutants. The order of the mutants, from the most dominant to the least, is as follows: *syn* 33, *syn* 31 and *syn* 32, *syn* 8, *syn* 20 and *syn* 30, and *syn* 6. For example, mixed infections with *syn* 33 plus another mutant resulted in fusion that was approximately characteristic of *syn* 33. Mixed infections of cells with *syn* 1 and another mutant resulted in fusion that was intermediate between that produced by the mutants individually. *syn* 6 is particularly interesting because it produces plaques that are very similar to those of wild type; however, unlike wild type, *syn* 6 has lost the ability to reduce fusion in mixed infections with the other mutants.

Test for active fusion inhibition: kinetics

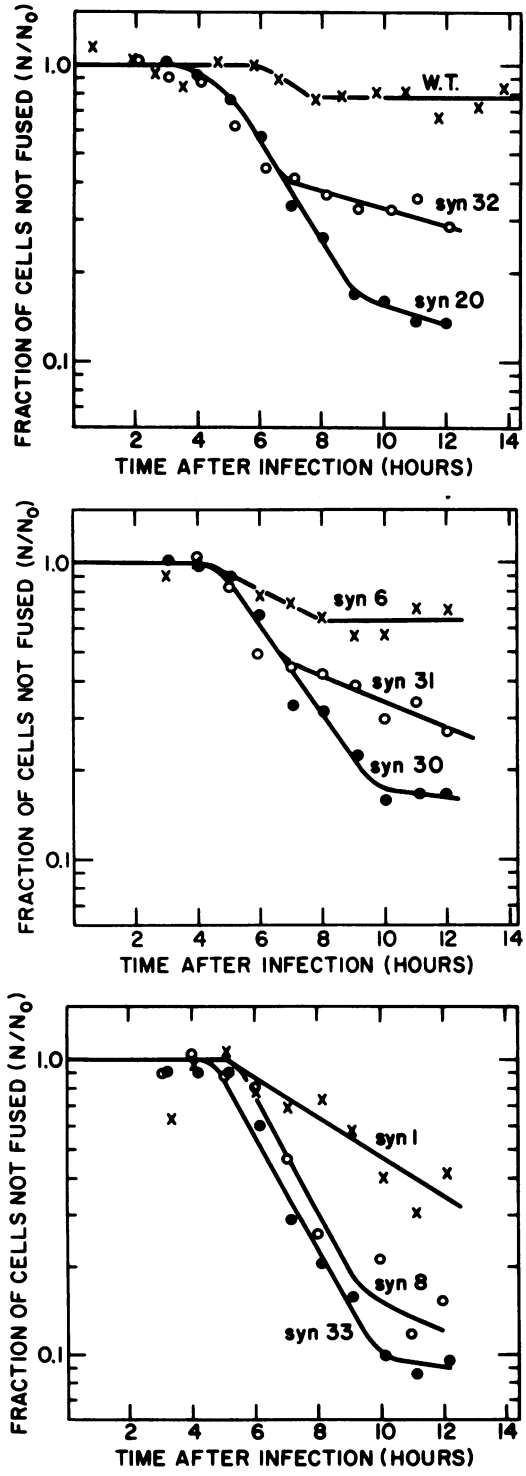

FIG. 1. *Kinetics of fusion induced by the syn mutant and by wild-type viruses. Replicate cultures of HEL cells at a density of 6.7 × 10⁴ cells per cm² were infected with the indicated syn mutants at an MOI of 10 or with wild type (W.T.) at an MOI of 4.1. At various times after the start of incubation at 34°C, cultures were harvested with trypsin and EDTA and counted with a Coulter Counter (8). N is the number of cells in a narrow size interval that corresponds to small, unfused, single cells. N_0 is the average number of cells during the first 4 h after infection. The time of onset and the initial rate of fusion were independent of the cell density and MOI for values of greater than 3 × 10⁴ cells per cm² and 5 PFU/cell, respectively (8).*

of fusion of *syn* mutant-infected cells with uninfected cells and with wild type-infected cells. If wild-type virus does not produce molecules that interact with the plasma membrane to actively block fusion, *syn* mutant-infected cells should fuse equally well with mock-infected and with wild type-infected cells. A finding that *syn* mutant-infected cells fuse less well with wild type-infected cells than with uninfected cells would imply that fusion is actively inhibited late in a wild-type infection.

To test for fusion inhibition, monolayers of HEL cells that were 80% confluent were infected with either *syn* 20 or wild type or were mock infected. Other cells with radioactively labeled DNA were infected with *syn* 20 at the same time as the monolayers. At 30 min after infection, the radioactive cells were harvested and added to the monolayer cultures at a density low enough that, after attachment, each labeled cell was, on the average, surrounded by unlabeled cells. At various times after infection, cell layers were fixed, autoradiographs were prepared, and the extent of fusion between labeled and unlabeled cells was determined. Figure 6 shows that *syn* 20-infected cells fused more readily with uninfected cells than with cells infected with wild type. These data were first presented at the 19th Annual Meeting of the Biophysical Society (G. S. Read and S. Person, Biophys. J. **15**:203a, 1975) and have been repeated under conditions that minimize infection of cells with previously unadsorbed mutant or wild-type virus (unpublished observations).

Test for recombination: construction of a linkage map of the *syn* mutants. To test for recombination between the mutants, *syn* 8, *syn* 20, *syn* 30, *syn* 31, *syn* 32, and *syn* 33 were crossed in all of the possible pairwise combinations. The progeny from each cross were scored as producing either syncytial or wild-type plaques, and recombination frequencies (expressed as percentages) were calculated by the following formula: recombination frequency = 2 (number of wild-type plaques)/(number of wild-type plaques + number of *syn* plaques) × 100. The factor of 2 allows the occurrence of an equivalent number of double mutant recombi-

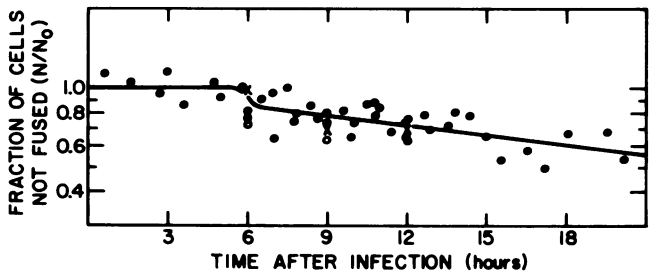

FIG. 2. *Kinetics of fusion induced by wild-type viruses. HEL cells were infected at an MOI of 10, and fusion kinetics curves were determined as described in the legend to Fig. 1. The final slope of the curve was determined by a least-squares fit to data from 7 to 20 h after infection. Symbols: ●, KOS; ○, mP; ×, F.*

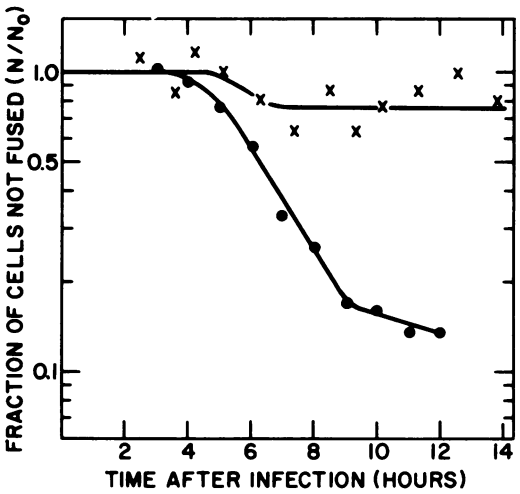

FIG. 3. *Kinetics of fusion of cells infected with syn 20 and syn 20 plus wild-type viruses. HEL cells were infected with syn 20 (●) at an MOI of 10 or with syn 20 plus wild type (×) at an MOI of 5 each and incubated at 34°C. Fusion kinetics curves were obtained as described in the legend to Fig. 2.*

nants which could not be scored if such plaques are syncytial in nature. The number of wild-type plaques was determined for a particular number of total plaques with a single set of petri dishes. The statistical errors (one standard deviation) are, therefore, proportional to the number of wild-type plaques in the population and were determined by the following expression: percent error = 2 (number of wild-type plaques)$^{1/2}$/number of wild-type plaques + number of *syn* plaques) × 100.

Figure 7 is a linkage map of the *syn* mutants, constructed from data obtained by counting the plaques produced by more than 1.5 × 10^5 progeny virus. It is notable that the map distances are approximately additive and that the six mutations are clustered on the virus genome with the largest distance between any two mutations represented by a recombination frequency of between 1.26 and 2.0%. Although the relative order to the most closely linked mutations is not totally unambiguous, the bulk of the data are consistent with the order of the mutations that is shown in Fig. 7. Regardless of the relative order of some of the mutations, the data show that *syn* 33 and *syn* 20 are clustered at the left end of the linkage map, that *syn* 30, *syn* 8, and *syn* 32 are grouped in the right central region, and that *syn* 31 is located at the extreme right end of the map. It is significant that some recombination was observed between each pair of mutants, indicating that the mutants are all nonidentical. *syn* 1 and *syn* 6 were not included in these studies because they produce plaques that are very similar to those produced by wild type. Reversion frequencies of the *syn* mutants were much smaller than the recombination frequencies (Table 4 and Fig. 7). Since the statistical errors and the errors arising from possible spontaneous revertants are independent, they were added to yield the error values shown in Fig. 7. For a particular cross in a recombination experiment, the spontaneous reversion frequencies of the two mutants were averaged. The number of wild-type plaques that could possibly have arisen by spontaneous reversion was obtained by multiplying the average reversion frequency by the number of *syn* mutant plaques counted in that experiment.

DISCUSSION

Eight cell fusion-causing *syn* mutants were isolated from the KOS strain of HSV-1. Although the eight mutants and wild type exhibited a range of plaque morphologies and varied substantially with respect to the final amount of fusion that they induced, they exhibited a striking similarity with respect to the kinetics of fusion. The fusion kinetics curves for wild type and all of the mutants, with the possible exception of *syn* 1, were biphasic. Fusion began 4 to 6 h after infection and proceeded with an exponential decrease in the number of small single cells until it was significantly reduced in

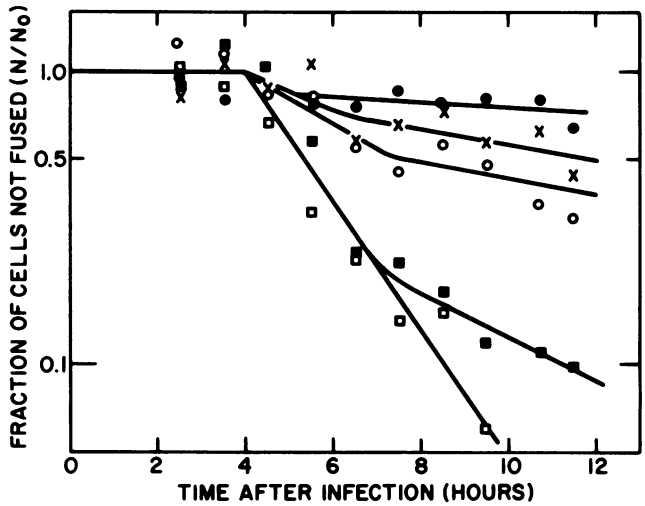

FIG. 4. *Kinetics of fusion of cells infected with syn 33 and syn 33 plus wild-type viruses. HEL cells were infected with syn 33 (□) or wild type (●) at an MOI of 24 or with both viruses at the following MOIs: wild type, 18, plus syn 33, 6 (×); wild type, 12, plus syn 33, 12 (○); and wild type, 6, plus syn 33, 18 (■). Incubation was at 34°C. Fusion kinetics curves were obtained as described in the legend to Fig. 2.*

TABLE 1. *Fusion of the syn mutants and wild type (WT) in single and mixed infections*[a]

Infecting virus	Fraction of cells not fused (N/N_0)
WT	0.80
syn 1	0.35
syn 1 + WT	0.80
syn 6	0.65
syn 6 + WT	0.75
syn 8	0.14
syn 8 + WT	0.60
syn 20	0.13
syn 20 + WT	0.75
syn 30	0.16
syn 30 + WT	0.55
syn 31	0.30
syn 31 + WT	0.65
syn 32	0.35
syn 32 + WT	0.70
syn 33	0.07
syn 33 + WT	0.35

[a] HEL cells were infected with a *syn* mutant or wild type at an MOI of 10 or with a *syn* mutant and wild type at an MOI of 5 each and incubated at 34°C. The ratio N/N_0 was determined at 12 h after infection as described in the legend to Fig. 1. Data in all of the tables were from at least three experiments and yielded a standard deviation equal to or less than ±0.05.

TABLE 2. *Growth of syn mutants and wild type (WT) in mixedly infected cells*[a]

Infecting virus	Titer of progeny virus (PFU/cell)		Ratio of titers (mixed infection/single infection)	
	syn mutant	WT	*syn* mutant	WT
WT		334		
syn 8	64			
syn 8 + WT	86	26	1.4	0.08
syn 20	379			
syn 20 + WT	454	199	1.2	0.6
syn 30	8			
syn 30 + WT	41	66	5.0	0.2
syn 31	402			
syn 31 + WT	224	224	0.6	0.7
syn 32	421			
syn 32 + WT	168	136	0.4	0.4
syn 33	138			
syn 33 + WT	271	182	2.0	0.6

[a] HEL cells were infected with a *syn* mutant or wild type at an MOI of 10 or with a *syn* mutant and wild type at an MOI of 5 each. After 22 h of incubation at 34°C, progeny virus was harvested and titrated on HEL monolayers. In mixed infections the titer of each virus was determined by counting the number of wild-type and *syn* mutant plaques separately.

rate at a later time. The interval between the onset of fusion and the subsequent reduction of its rate was characteristic of the virus. Biphasic fusion kinetics curves are also obtained when fusion is assayed by fluorescent enhancement (4; data not shown) or by a visual microscope assay (data not shown). Since neither of these assays involves harvesting infected cells, the biphasic curve shapes cannot be an artifact of the trypsinization procedure that is used in the Coulter Counter assay.

The *syn* mutants are, to varying degrees, codominant with wild type in simultaneous mixed infections. Although in mixed infections involving seven of the mutants the extent of fusion

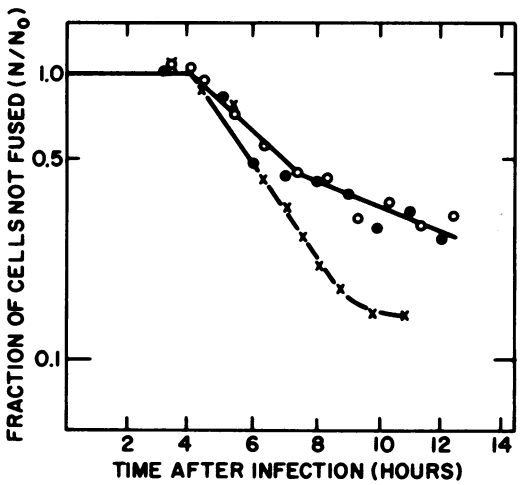

FIG. 5. *Kinetics of fusion of cells infected with syn 31, syn 20, and syn 31 plus syn 20. HEL cells were infected with syn 20 (×) or syn 31 (●) at an MOI of 10 or with syn 20 plus syn 31 (○) at an MOI of 5 each. Fusion kinetics curves were obtained as described in the legend to Fig. 2.*

TABLE 3. *Complementation test for the syn mutants*[a]

Mutant	*syn* 1	*syn* 6	*syn* 8	*syn* 20	*syn* 30	*syn* 31	*syn* 32	*syn* 33
syn 1	0.52	0.40	0.28	0.34	ND[b]	0.48	ND	0.19
syn 6		0.58	0.18	0.17	ND	0.39	ND	0.02
syn 8			0.14	0.12	0.31	0.31	0.32	0.04
syn 20				0.10	0.18	0.35	0.31	0.10
syn 30					0.27	0.39	0.41	ND
syn 31						0.35	0.31	0.14
syn 32							0.36	ND
syn 33								0.07

[a] Triplicate cultures of HEL cells were infected with a *syn* mutant at an MOI of 10 or with pairs of *syn* mutants at an MOI of 5 each. After 10 h of incubation at 34°C, cultures were harvested and pooled, and the number of small single cells, N, was determined with a Coulter Counter. The initial number of small single cells, N_0, was determined by harvesting and counting triplicate cultures at the time of infection. The fraction of cells not fused is given and is an average of the values obtained for three cultures.

[b] ND, Not done.

was significantly reduced, there were quantitative differences among the mutants. These may have been due in part, but not entirely, to small inaccuracies in the ratios of wild type to mutant that were used. However, as has been reported previously (G. S. Read, S. Person, and P. M. Keller, abstract, Third Meeting on Herpes Viruses, Cold Spring Harbor, N.Y., 1976), *syn* 33 caused substantial fusion in mixed infections even when the ratio of wild type to mutant was 3:1. We find that the codominance of the syncytial and nonsyncytial phenotypes in HEL cells is even more striking for a second set of *syn* mutants that has recently been isolated in our laboratory (V. C. Bond, S. Person, S. C. Warner, and D. Bzik, manuscript in preparation). Thus, the *syn* mutations may exert differential effects upon the rates of synthesis on activities of molecules that induce or inhibit fusion.

Our studies are the first observation of the codominance of the syncytial and nonsyncytial phenotypes of HSV-1. Roizman initially reported that the syncytial phenotype is recessive in mixed infections of HEp-2 cells with the syncytial strain, MP, and the nonsyncytial strain, mP, from which it arose (11, 12). Keller later obtained similar results in studies of three syncytial and three nonsyncytial strains of HSV-1 in BHK-21 cells (3). The dominance tests that were used in their studies were qualitative, and no codominance between syncytial and nonsyncytial phenotypes was noted. Recent experiments in our laboratory indicate that dominance is cell dependent since MP is recessive to mP in mixed infections of HEp-2 cells but codominant in mixed infections of HEL cells (D. Bzik and S. Person, submitted for publication).

Seven of the mutants were found to belong to the same complementation group; *syn* 33 was

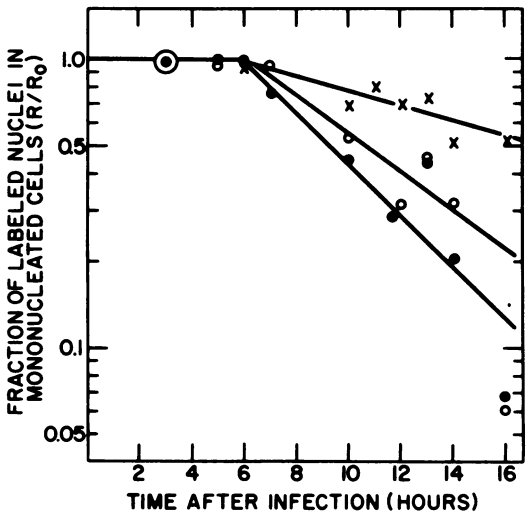

FIG. 6. *Kinetics of fusion of syn 20-infected cells with syn 20-infected, wild type-infected, or mock-infected cells. Cells with radioactively labeled DNA were infected with syn 20, harvested, and added to unlabeled monolayers of syn 20-infected (●), wild type-infected (×), or uninfected (○) cells, as described in the text. After fixation at various times after infection and preparation of autoradiographs, the cultures were scanned with a microscope to determine R_0, the total number of radioactive nuclei, and R, the number of radioactive nuclei in mononucleated cells.*

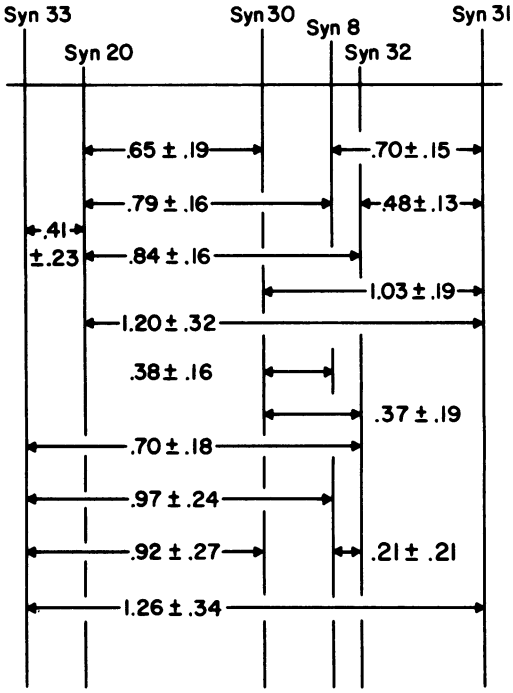

FIG. 7. *Linkage map of the syn mutants. Six syn mutants were crossed in all of the possible pairwise combinations, and the recombination frequencies and errors in the recombination frequencies were computed as described in the text. The recombination frequencies and errors are expressed as percentages. Data are from three experiments.*

TABLE 4. *Estimation of reversion frequencies of syn mutants*[a]

Mutant	Wild-type plaques/ total plaques	Reversion frequency
syn 1	0/2,598	$\leq 3.8 \times 10^{-4}$
syn 6	0/2,386	$\leq 4.2 \times 10^{-4}$
syn 8	0/2,793	$\leq 3.6 \times 10^{-4}$
syn 20	0/120,898	$\leq 8.3 \times 10^{-6}$
syn 30	0/1,814	$\leq 5.5 \times 10^{-4}$
syn 31	2/8,428	2.4×10^{-4}
syn 32	0/12,706	$\leq 7.9 \times 10^{-5}$
syn 33	0/3,152	$\leq 3.2 \times 10^{-4}$

[a] In the course of virus stock preparations and in various experiments, including recombination experiments, *syn* mutants were plated on monolayers of HEL cells, and the number and morphology of the resulting plaques were determined. The only possible wild-type plaques observed were two observed in a single titration of *syn* 31.

not tested since it is dominant in mixed infections with wild type. Furthermore, recombination experiments indicated that six of the mutations map within 2.0 recombinational units of each other. In crosses that were also performed in HEL cells, Schaffer et al. found a recombination frequency of 2.1% between two temperature-sensitive mutants of KOS that are members of the same complementation group (17). Our complementation and recombination data are therefore consistent with the possibility that all of the mutants are mutated in the same gene. However, since *syn* 33 maps at one extreme of the linkage map, it is also possible that it is altered in a different cistron than the other mutants.

There is currently no direct evidence concerning whether the *syn* mutations are point mutations or small additions or deletions. However, the additivity of the map distances in Fig. 7 indicates that the centrally located mutations in *syn* 8, *syn* 30, and *syn* 32 cannot be large additions or deletions. Furthermore, the additivity of distances also indicates that the fusion phenotype of the viruses that contain these mutations cannot be due to multiple mutations that are widely separated.

Our observation that some HEL cells fused after infection with wild-type strains is a direct demonstration that wild-type strains produce at least a partially active inducer of cell fusion. The failure of earlier investigators to observe a small amount of cell fusion induced by wild-type virus strains may have been due to a failure to study in detail the kinetics of fusion or, more likely, to the fact that many of these studies were performed with cells that are not especially sensitive to fusion (Bzik and Person, submitted for publication).

The most compelling evidence that the plasma membranes of wild type-infected cells are altered so as to actively inhibit cell fusion at times later than 6 to 7 h after infection is the observation that *syn* mutant-infected cells fuse less readily with wild type-infected than with uninfected cells. Although it seems likely that the codominance of wild type and the mutants in mixed infections results from the same inhibitory membrane changes, this has not been proven. The similarity in shape of the fusion kinetics curves for wild type and the *syn* mutants further suggests that syncytial strains need not totally lack the ability to induce membrane changes that inhibit fusion. We propose that the fusion inducer is maximally active only during the time interval between the onset of fusion and the subsequent reduction in the fusion rate.

At least two separate genes affect the fusion process. Ruyechan et al. (15), using marker transfer techniques, physically mapped loci that affect fusion. One is at the map position of glycoprotein A/B_2 (*syn* 3), and the other site, which contains multiple loci (*Cr*, *syn* 1, and *syn* 2), is near, but separate from, the map position of glycoprotein C_2 and far from A/B_2. *syn* 1, *syn* 2, and *syn* 3 mutations result in the production

of C_2, whereas the *Cr* mutation does not. *syn* 2 and *Cr* mutations cause fusion of Vero and HEp-2 cells, whereas *syn* 1 and *syn* 3 only cause the fusion of Vero cells. We have shown that *syn* 20 and probably all of the mutants used here complement *tsB5* of the *syn* 3 map position. Also, by recombination, *syn* 20 maps near MP and far from *tsB5* and causes the fusion of Vero but not HEp-2 cells. Therefore, we conclude that *syn* 20 and the other mutations reported here map at the physical genome locus *syn* 1 (Bond et al., manuscript in preparation). The *syn* 1 mutant reported in these studies should not be confused with the physical genome map locus of the same name.

Since there are at least two genes that affect fusion, it is very unlikely, on the basis of random probability, that a set of eight mutants would fall into only one of the complementation groups. The mutants were intentionally selected as representative of a range of plaque morphologies, from near wild type to extensive syncytium formation. Another set of *syn* mutants having uniform and extensive syncytia has been isolated with KOS or HFEM as the HSV-1 parent strain. All six of the mutants isolated from KOS are in the *syn* 20 complementation group, and one, or perhaps two, of the mutants isolated from HFEM are in the same group. One, or perhaps two, of the HFEM mutants are in the *tsB5* complementation group (Bond et al., manuscript in preparation). We have no suggestion as to the lack of occurrence of *syn* mutants in the *tsB5* complementation group when the KOS strain is used. The only *syn* mutant isolated from KOS that we are aware of that may be in the *tsB5* complementation group is one isolated by R. M. Sandri, M. Levine, and J. C. Glorioso by in vitro mutagenesis of isolated restriction fragments (personal communication). The data of Ruyechan et al. may indicate the presence of four complementation groups, but it is also possible that three of the loci are in the same gene. In this connection, one must remember that the different mutants used in these studies are altered in different regions of the same gene and that they give rise to a wide range of fusion phenotypes. It will be necessary to isolate and characterize more *syn* mutants, especially by in vitro mutagenesis of isolated restriction fragments representative of the entire HSV-1 genome, to determine the number of viral genes that can affect fusion.

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