JOURNAL OF VIROLOGY, Apr. 1981, p. 41-49
0022-538X/81/040041-09$02.00/0

Vol. 38, No. 1

Method for Induction of Mutations in Physically Defined Regions of the Herpes Simplex Virus Genome

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Received 10 November 1980/Accepted 29 December 1980

A procedure was developed for inducing mutations in isolated restriction enzyme fragments of herpes simplex virus type 1 (HSV-1) DNA with nitrous acid. The mutations were then transferred to the viral genome by genetic recombination during cotransfection of rabbit kidney cells with the mutagenized fragments and intact HSV-1 DNA. The *HpaI* restriction enzyme fragments LD, B, LG, I, and J were mutagenized. Temperature-sensitive mutants were found at frequencies of 1 to 5% among the progeny of the transfections. Syncytial mutants also were found at high frequency when fragment B or LD was used for mutagenesis. Fifteen of these mutants, 11 temperature sensitive and 4 syncytial, were used for further studies, including complementation analysis, DNA synthesis, and marker rescue. Marker rescue data presented here and in the accompanying publication (A. L. Goldin, R. M. Sandri-Goldin, M. Levine, and J. C. Glorioso, J. Virol. **38**: 50-58, 1981) confirm the map position of some of the newly isolated mutants.

The genome of herpes simplex virus type 1 (HSV-1) is a double-stranded DNA molecule of about 100×10^6 daltons (3, 11, 16). DNA molecules isolated from virions consist of two covalently linked components designated long (L) and short (S), each of which consists of unique sequences (U_L and U_S) flanked by inverted repeats (27). The L and S components invert with respect to each other so that four isomeric arrangements designated P (prototype), I_L (inversion of L), I_S (inversion of S), and I_{SL} (inversion of S and L) occur in equimolar amounts (5, 6, 13, 28).

The size and complexity of the genome thus present problems in elucidating the processes involved in viral replication, latency, and transformation. The availability of conditionally lethal mutants in all parts of the viral genome would greatly aid in defining the mechanisms of viral biosynthesis and virus-host interactions. A number of laboratories have isolated temperature-sensitive (*ts*) mutants, and a complementation analysis of these mutants has led to the identification of 23 complementation groups of HSV-1 (25). Still the genome is far from saturated with mutants, and there are large regions such as U_S which are mutationally silent. Many of the currently available mutants were isolated after random mutagenesis of the genome with thymine-specific mutagens, often resulting in the isolation of mutants in the same complementation group by several different laboratories (25). Chu et al. (4) developed a procedure for the in vitro induction of mutations in preselected regions of the HSV-1 genome by using the cytosine-specific mutagen hydroxylamine. This method resulted in the identification of three new complementation groups (4).

In this report, we describe a similar approach for the isolation of mutants, using nitrous acid as the mutagen. The procedure involves the in vitro mutagenesis of specific restriction enzyme fragments which are subsequently allowed to recombine with the viral genome during cotransfection with intact viral DNA. In this way the mutation is induced within a defined region and then transferred to the viral genome.

MATERIALS AND METHODS

Cells and virus. Primary rabbit kidney (RK) cells were prepared from 3- to 5-day-old rabbits and were passed no more than two times before use. African green monkey kidney (Vero) cells and RK cells were passed routinely in Eagle minimal essential medium (MEM; GIBCO Laboratories) supplemented with nonessential amino acids, 10% heat-inactivated fetal calf serum (GIBCO), 100 μg of streptomycin per ml, and 100 U of penicillin per ml. Stocks of wild-type HSV-1 strain KOS and *ts* mutant virus were grown by infection of Vero cells at low multiplicity and titered by plaque assay on Vero cells as described previously (1). KOS stocks were grown at 37°C. *ts* mutant stocks were grown at 34°C, the permissive temperature. The nonpermissive temperature for *ts* mutants was 39°C.

DNA preparation. HSV-1 (KOS) DNA was prepared from both extracellular virions and infected cell lysates as follows. Vero cell monolayers in roller bottles were infected with KOS at a multiplicity of infec-

tion of 0.05 to 0.1 and incubated at 37°C. When cytopathic effect was generalized, cells were scraped into the medium. The suspension was centrifuged at low speed to pellet the cells. The supernatant fluid was removed and centrifuged at 7,500 × *g* in a GSA rotor at 4°C for 4 h to pellet extracellular virus. The viral pellet was suspended in a small volume of TE buffer (0.01 M Tris-hydrochloride, pH 8.0, and 0.001 M EDTA). The cellular pellet was suspended in a small volume of 0.01 M Tris-hydrochloride and 0.01 M EDTA, pH 8.0, and frozen. The cellular suspension was thawed and mixed with the extracellular virus. Sodium dodecyl sulfate was added to a final concentration of 1%, and *N*-lauroyl sarcosinate (Sarkosyl) was added to a final concentration of 0.5%. RNase A (Sigma Chemical Co.) was added to a final concentration of 500 μg/ml. Solutions of RNase A at 10 mg/ml were heated at 83°C for 20 min before use to inactivate any contaminating DNase activity. The lysate was incubated at 37°C for 60 min. At that time, Pronase was added to a final concentration of 2 mg/ml. Pronase solutions of 20 mg/ml were heated at 83°C for 20 min before use also to inactivate DNase. The lysate was incubated at 37°C for 12 to 16 h. The volume of the lysate was then adjusted to 25 ml with TE buffer, and 32.5 g of CsCl was added. The DNA suspension was mixed very gently, and the refractive index of the suspension was adjusted to 1.4005 to 1.4010. DNA-CsCl solutions were centrifuged at 40,000 rpm for 20 h in a Beckman VTi50 rotor at 20°C. The tubes were punctured at the bottom, and 1.0-ml fractions were collected. The refractive index was read for each fraction, and those fractions comprising the viral DNA peak (densities of 1.740 to 1.720) were pooled and centrifuged two successive times as described above. The viral DNA was dialyzed extensively against TE buffer and then ethanol precipitated by the addition of 1/10 volume of a 3 M sodium acetate–100 mM magnesium acetate solution and 2.5 volumes of absolute ethanol. The suspension was held at −20°C overnight and then centrifuged at 20,000 rpm in an SW27 rotor at 0°C for 60 min. Viral DNA was gently suspended in 1.0 ml of TE buffer.

ts mutant DNA used in marker rescue mapping experiments was isolated from HSV-1 mutant-infected cell lysates. Vero cell monolayers were infected with various *ts* mutants at a multiplicity of infection of 0.05 to 0.1 and then incubated at 34°C until cytopathic effect was generalized. Extracellular virus was pelleted and suspended as described above and then combined with the cellular pellet. The procedures for lysis and digestion of the lysate with RNase and Pronase were as described above. After incubation with Pronase, the DNA was mixed with CsCl (initial refractive index of 1.4005 to 1.4010) and centrifuged in a VTi50 rotor at 40,000 rpm for 20 h at 20°C. Fractions in the density range of 1.740 to 1.715 were pooled and dialyzed against TE and then precipitated with ethanol. The DNA was resuspended in TE buffer. After this procedure, the viral DNA was still contaminated with cellular DNA. The viral DNA was not purified further, however, since the contaminating cellular DNA served as the carrier DNA in the transfection so that no other carrier DNA needed to be added.

Salmon sperm DNA (lyophilized; Sigma Chemical Co.) used as carrier DNA in the mutagenesis procedure was suspended in TE buffer at a concentration of 5 mg/ml. The DNA was extracted with an equal volume of TE-saturated phenol four to six successive times followed by two extractions with chloroform-isoamyl alcohol (49:1) and then extensively dialyzed against TE buffer. The DNA was precipitated with ethanol as described above and resuspended in TE buffer.

Restriction endonuclease cleavage of DNA and purification of DNA fragments. Viral DNA (10 to 15 μg) was digested with *Hpa*I (Bethesda Research Laboratories) at 1 U of enzyme per μg of DNA in 20 mM Tris-hydrochloride, pH 7.2, 20 mM $MgCl_2$, 20 mM KCl, and 1 mM dithiothreitol for 2 h at 37°C. The reaction was stopped by the addition of 1/10 volume of 0.5 M EDTA, pH 8.0, and 1/5 volume of a solution containing 5% bromophenol blue and 25% Ficoll in 30 mM NaH_2PO_4, 36 mM Tris, and 1 mM EDTA, pH 8.0. Fragments were separated by electrophoresis in 0.8% agarose. Electrophoresis was carried out on vertical slab gels (13.5 by 20 by 0.3 cm) in 30 mM NaH_2PO_4, 36 mM Tris, and 1 mM EDTA, pH 8, at 80 V for 15 to 18 h, in tube gels (15 by 0.6 cm) at 40 V for 15 to 18 h, or in horizontal gels (8.5 by 22 by 0.6 cm) at 80 V for 20 to 24 h. Gels were stained with 1 μg of ethidium bromide per ml and visualized under long-wave UV light. Bands were excised from the gel and stored at 4°C overnight in 0.5 ml of TE buffer. DNA was extracted from the agarose by two successive freeze-thaw steps in which the DNA-agarose band was frozen at −70°C and then thawed at 37°C. The agarose was pelleted by centrifugation at 15,000 × *g* for 5 min in an Eppendorf microfuge. The supernatant was carefully removed, and 1.0 ml of TE buffer was added to the agarose pellet and mixed. The freeze-thaw step was repeated, and the agarose was again pelleted. The supernatant was combined with the first supernatant. One-tenth volume of a 3 M sodium acetate–100 mM magnesium acetate solution and about 10 volumes of absolute ethanol were added to the DNA solution, which was then kept at −20°C overnight to precipitate the DNA and to remove the ethidium bromide. The DNA was pelleted by centrifugation in an SW27 rotor at 24,000 rpm for 90 min at 0°C. Control experiments using ^{32}P-labeled KOS DNA showed that 60 to 80% of the ^{32}P counts could be recovered from each band in the agarose gels by this extraction technique.

Mutagenesis of HSV-1 DNA fragments. Purified restriction enzyme fragments extracted from agarose gels as outlined above were resuspended in 100 μl of TE buffer after ethanol precipitation. For each mutagenesis treatment, 10 μg of HSV-1 KOS DNA was digested with *Hpa*I and electrophoresed in agarose. The amount of each DNA fragment recovered from the gel was then added to a 10-fold volume (1.0 ml) of nitrous acid reaction mixture (0.1 M sodium acetate buffer, pH 4.6, containing 0.05 M $NaNO_2$ and 2×10^{-4} M spermine). DNA was incubated in the presence of nitrous acid at 37°C for the times indicated in individual experiments. The reaction was stopped by raising the pH to 7.0 (32) by the addition of 9.0 ml of ice-cold transfection buffer (8 g of NaCl, 0.37 g of KCl, 0.1 g of Na_2HPO_4, 1.0 g of dextrose, and 5 g of HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] per

liter, pH 7.1) (12) containing 10 μg of salmon sperm DNA per ml. The mutagenized DNA fragments were used immediately to cotransfect RK cells.

Transfection procedure. Transfections using mutagenized fragments were carried out by a procedure similar to that described by Graham et al. (12). Intact KOS DNA was added at 0.5 μg/ml to mutagenized DNA fragments in transfection buffer as described above. Calcium chloride was added to a final concentration of 125 mM. The DNA solutions were allowed to stand at room temperature for 30 min for precipitates to form. Two milliliters of this solution was added to a 60-mm petri dish containing a monolayer of subconfluent RK cells which had been seeded into the dish 4 to 5 h earlier. The growth medium was removed from the cells before the addition of the transfection solution. DNA was allowed to adsorb to the RK cells at 34°C for 60 min. The solution was then removed, and 5.0 ml of MEM supplemented with 5% fetal calf serum was added to the dishes. The cells were incubated at 34°C for 4 h, at which time the medium was removed and replaced with 5 ml of MEM supplemented with 5% fetal calf serum. Incubation at 34°C was continued until generalized cytopathic effect was observed in the monolayers. Dishes were then frozen until assayed.

Mutant isolation. Progeny virus from the cotransfections using mutagenized fragments were assayed on Vero cell monolayers in 100-mm petri dishes at 34°C. Approximately 100 to 200 well-isolated plaques were picked from each transfection with each fragment. The virus in each plaque was tested for temperature-sensitive growth by assay at 34 and 39°C. Those isolates which plated at efficiencies at least 10^3 times lower at 39°C than at 34°C were plaque purified three times and used for further study.

Complementation tests. The *ts* mutants isolated were complemented with each other by a procedure similar to the quantitative complementation test of Schaffer et al. (24, 25) as described previously (9).

Measurement of DNA synthesis. Vero cell monolayers in 25-cm^2 flasks (2×10^6 cells) were infected with a multiplicity of infection of 10 of each mutant or wild-type KOS and then incubated at 37°C for 1 h. The inoculum was removed and monolayers were washed twice with phosphate-buffered saline, after which 5 ml of MEM containing 5% fetal calf serum was added. The flasks were incubated at 39°C for 4 h, at which time 10 μCi of [*methyl*-^{3}H]thymidine per ml (specific activity, 6.7 Ci/mmol; New England Nuclear Corp.) was added to each flask, and incubation at 39°C was continued for an additional 20 h. Cells were scraped into the medium and then pelleted by low-speed centrifugation. The cellular pellets were suspended in 1.0 ml of 0.01 M Tris-hydrochloride, pH 8.0, and 0.01 M EDTA. Sodium dodecyl sulfate and Sarkosyl were added to final concentrations of 1% each, and RNase A was added to a final concentration of 500 μg/ml. The lysates were incubated at 37°C for 1 h, at which time 2 mg of Pronase per ml was added, and incubation at 37°C was continued for an additional 12 h. Solutions of RNase and Pronase were heated at 83°C for 20 min before being used to inactivate any DNase. The volume of each lysate was adjusted to 5.0 ml with TE buffer, and 6.5 g of CsCl was added to each tube. The refractive index was adjusted to 1.4005 to 1.4010, and DNA-CsCl solutions were centrifuged at 35,000 rpm in a type 50 fixed-angle rotor at 20°C for 72 h. Fractions (200 μl) were collected by puncturing the bottoms of the tubes. The refractive index of each fraction was read, and then samples were precipitated onto filter paper with 10% trichloroacetic acid. Filters were washed with 5% trichloroacetic acid followed by distilled water and then dehydrated with acetone, all at 4°C. Filters were dried and counted.

RESULTS

Nitrous acid inactivation of intact, infectious HSV-1 DNA. Mutagenesis of nucleic acids by nitrous acid occurs primarily by oxidative deamination of adenine to hypoxanthine and of cytosine to uracil (26, 31, 33), resulting in base substitutions, although a number of other reactions have been shown to occur (for review, see reference 34). Nitrous acid mutagenesis of duplex DNA has been shown to be less efficient than mutagenesis of single-stranded DNA (15, 19, 20, 32). However, duplex DNA is readily mutated by nitrous acid in the presence of various alcohols, phenols, or primary amines (32). Of the above-mentioned compounds, spermine has been found to cause appreciable stimulation of the production of mutants (32). Therefore, we added 2×10^{-4} M spermine to a reaction mix consisting of 0.1 M sodium acetate buffer and 0.05 M $NaNO_2$ at pH 4.6. In an attempt to determine conditions for mutagenesis, inactivation of the infectivity of whole viral DNA in nitrous acid was measured. Intact, HSV-1 (KOS) DNA was added to the nitrous acid reaction mixture and incubated at 37°C. Aliquots were removed at various times, and the infectivity of the DNA was tested by transfection of RK cells. By 25 min, about 99% of the transfecting activity of KOS DNA had been destroyed (Fig. 1). Although this was not a direct measure of the mutagenic activity of nitrous acid on KOS DNA, 25 min was chosen as the time of treatment in mutagenesis experiments, since a 1% survival of transfecting activity indicated that the majority of DNA molecules had received at least one lethal hit.

Mutagenesis of DNA fragments. For mutagenesis of fragments, HSV-1 (KOS) DNA was digested with the restriction endonuclease *Hpa*I. Fragments L_1D, L_2D (13.8 megadaltons [Md]), B (12.4 Md), L_1G, L_2G (10.5 Md), I (5.5 Md), and J (4.6 Md) (Fig. 2) were selected because these fragments represented a variety of sizes and because they spanned regions in the U_L segment, the joint region, and the U_S segment. Therefore, the general usefulness of the technique throughout the genome could be tested. Fragments L_1D and L_2D have the same molecular weight and

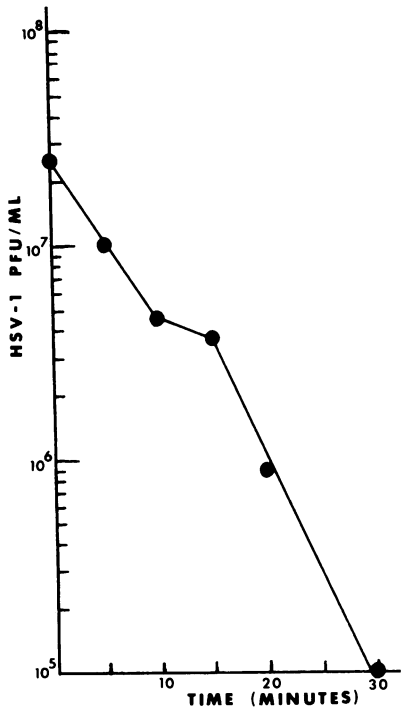

FIG. 1. *Nitrous acid inactivation of the transfecting activity of HSV-1 (KOS) DNA. Ten-microliter samples containing 2.1 μg of KOS DNA were added to a series of tubes containing 200 μl of nitrous acid reaction mixture (see Materials and Methods). Each sample was incubated at 37°C for the times indicated; then 2.0 ml of ice-cold transfection buffer containing salmon sperm DNA (10 μg/ml) was added to inactivate the nitrous acid. $CaCl_2$ (1.25 M) was added to a final concentration of 125 mM, and the suspensions were held at room temperature for 30 min. Aliquots (0.8 ml) of the DNA-$CaCl_2$ suspension were added to each of three 60-mm petri dishes containing 1 × 10^6 to 2 × 10^6 RK cells. After removal of the DNA solutions and addition of fresh growth medium as described in Materials and Methods, the plates were incubated at 34°C for 8 days and then frozen until assayed. Infectious virus first appeared in the monolayers at about 5 days, with maximum titers being achieved in the zero time points at 7 to 8 days.*

therefore could not be separated on agarose gels. This is also true for fragments L_1G and L_2G. For this reason, these fragments will be referred to as LD and LG. DNA was extracted from bands in agarose gels as described in Materials and Methods. The fragments were mutagenized separately with nitrous acid for 25 min at 37°C and then mixed with intact KOS DNA, and the mixture was used to cotransfect RK cells at 34°C. Well-isolated plaques resulting from these transfections were picked and tested for temperature-sensitive growth. Table 1 shows the number of plaques tested from each mutagenized fragment and the number of mutants found. *ts* mutants were isolated at frequencies of about 1 to 5%. Eleven *ts* mutants were plaque purified three times and used in further studies. Nine of these mutants had titers 10^4 times higher at 34°C than at 39°C, and two had titers 10^3 times higher at 34°C than at 39°C. In addition, a number of mutants having syncytial plaque morphology (*syn*) were also isolated (Table 1). Four *syn* mutants were plaque purified three times for further study. Mutants were named according to the *Hpa*I fragment which was mutagenized for their isolation, for example, *tsJ*17 and *synLD*70.

Complementation of mutants. To determine whether mutants isolated from transfections using the same fragment were in different complementation groups, standard quantitative complementation tests (24, 25) were performed between these mutants. Mutants isolated with fragment J, I, or LG complemented (Table 2), demonstrating that it was possible to isolate mutants in different complementation groups from the same fragment. However, three of the mutants isolated with fragment B did not complement. These mutants may be clonally related;

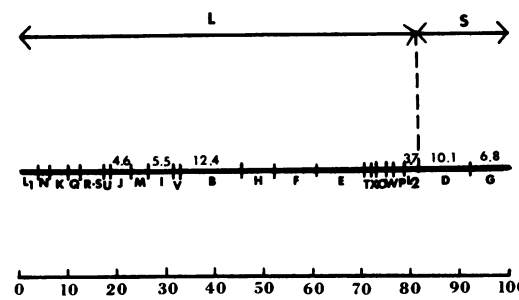

FIG. 2. *HpaI restriction endonuclease map of HSV-1 (KOS) DNA (21). The map represents the P orientation of the genome. The molecular weights (in megadaltons) of the fragments which were mutagenized are indicated above the fragments.*

TABLE 1. *Mutant isolation by nitrous acid mutagenesis of HSV-1 HpaI fragments*

*Hpa*I fragment	No. of plaques tested	No. of mutants	
		ts	*syn*
L_1D, L_2D	120	1	3
B(1)[a]	150	7	
B(2)[a]	100		>10%
L_1G, L_2G	120	2	
I	240	2	
J	215	2	

[a] B(1) and B(2) represent two different transfection experiments.

TABLE 2. *Complementation of temperature-sensitive mutants*

Mixed infections	Complementation index[a]
J17 × J176	7.0
I27 × I232	5.9
LG4 × LG15	6.2
B50 × B54	0.6
B50 × B100	4.8
B50 × B124	0.5
I27 × J17	13.5
I27 × J176	4.9
I27 × B50	5.7
J17 × B50	6.2
J176 × B50	4.2
LD84 × B50	4.0
LD84 × LG4	4.3
LD84 × LG15	7.6
LG4 × B50	6.4

[a] Determined as titer of mutants 1 + 2 at 39°C/(titer of mutant 1 at 39°C + titer of mutant 2 at 39°C). A complementation index of 2 or greater is considered significant (24).

this will be tested by genetic recombination to determine whether that is the case or whether they represent mutations at different allelic sites in the same gene.

Some of the mutants isolated by mutagenizing different fragments were also tested, and all of these mutants complemented (Table 2). In addition, two of the mutants (*tsLG4* and *tsLD84*) were complemented with *ts* mutants representing 10 of the 20 complementation groups in our laboratory collection and were found to complement with all 10 groups (data not shown). We do not know at this time whether any of the mutants represent new complementation groups in HSV-1. However, a standard set of HSV-1 complementation groups (25) has recently become available to us, and we are in the process of testing the new mutants with the standard set.

Viral DNA synthesis phenotypes of *ts* mutants. The amount of viral DNA synthesized at 39°C by each *ts* mutant was measured and compared with the amount synthesized by wild-type KOS virus at 39°C. Mutants *tsJ176*, *tsB100*, *tsLG4*, and *tsLD84* synthesized greater than 20% of the wild-type KOS DNA level, whereas *tsJ17*, *tsI232*, and *tsLG15* synthesized 20% or less of the KOS level (Table 3). Mutants *tsI27* and *tsB50* were DNA negative, since no viral DNA was detected at 39°C. DNA phenotypes based on the percentage of wild-type level were assigned as designated by Aron et al. (2).

Confirmation of map positions of mutants. A major advantage of inducing mutations in specific regions of the genome is that the map positions of the mutants should be delineated from the map positions of the fragments used for mutagenesis. Two approaches were used to confirm the locations of some of the newly isolated mutants: complementation with a mutant which has been mapped previously and marker rescue by cotransfection of RK cells with mutant DNA and wild-type restriction enzyme fragments.

The map position of mutant *synLD70*, which was isolated by using fragment LD for mutagenesis (see Fig. 2), was confirmed by complementation with a previously mapped mutant. *synLD70* had a syncytial plaque morphology on Vero cells different from that of *syn* mutants which were isolated by using fragment B or from mutants *synLD105* and *synLD42*. In addition, unlike the other newly isolated *syn* mutants, *synLD70* does not produce glycoprotein C (T. C. Holland, J. C. Glorioso, R. M. Sandri-Goldin, and M. Levine, manuscript in preparation). The HSV-1 strain mP mutant MP (14) is syncytial and also does not synthesize glycoprotein C (16, 29). The defect has been shown to map between 0.7 and 0.83 units on the HSV-1 map by marker transfer experiments (23). To determine whether MP and *synLD70* are in the same complementation group, Vero cells were mixedly infected with MP and *synLD70* and were analyzed for complementation of the *syn* phenotype and for the synthesis of glycoprotein C. Control infections of *synLD70* and wild-type KOS and of MP and KOS were performed at the same time to be sure that the *syn* phenotype and the glycoprotein C defect of both mutants were recessive and could be complemented. It was found that the KOS phenotype was dominant for both mutants. However, in mixed infection of *synLD70*

TABLE 3. *DNA synthesis phenotypes of ts mutants induced by nitrous acid*

Virus	% Viral DNA at 39°C[a]	DNA phenotype[b]
KOS	100	
tsJ17	8	±
tsJ176	43	+
tsI27	<1	−
tsI232	20	±
tsB50	<1	−
tsB100	51	+
tsLG4	89	+
tsLG15	10	±
tsLD84	36	+

[a] Determined as percent viral DNA in mutant-infected cells at 39°C/percent wild-type KOS DNA at 39°C.

[b] +, 20% of wild-type KOS DNA level; ±, ≤20% of wild-type KOS DNA level; −, no detectable viral DNA.

and MP, the monolayers were still found to fuse, indicating no complementation of the *syn* phenotype. In addition, no glycoprotein C was produced in the mixed infection, indicating that *synLD70* was mutated at the same locus as MP (Holland et al., manuscript in preparation). Fragment LD spans 0.78 to 0.92 map units and covers part of the region in which MP has been mapped. Therefore, the location in fragment LD of the defect in *synLD70* is consistent with the mapping of MP.

Three other mutants were mapped by marker rescue. To isolate the mutants, restriction enzyme fragments were extracted from bands in agarose after one gel purification. Subsequent marker rescue mapping experiments showed, however, that fragments isolated in this way were frequently contaminated with other fragments, resulting in high background levels of rescue of a mutant by more than one fragment. Although a second gel purification minimized this problem, recovery of DNA after two gel purifications was usually very inefficient. We used two methods to increase the purity and yield of DNA restriction fragments for marker rescue. First, we developed a two-dimensional gel electrophoresis procedure which resulted in increased purification of the fragments over that achieved by one single gel purification and still yielded DNA recoveries of 50 to 70%. Figure 3A shows the gel pattern of *Hpa*I fragments which were electrophoresed vertically in a 1% agarose tube gel. The tube gel was then placed horizontally 2 cm from the end of a glass plate, and a 6-mm 0.5% agarose slab gel was poured around the tube gel. After a second horizontal electrophoresis (Fig. 3B), bands were excised, and the DNA was extracted as described earlier.

Table 4 shows the results of a marker rescue experiment using *tsLD84*. *tsLD84* was rescued by fragment B, not by fragment LD. Fragment B (12.4 Md) migrated slightly faster than LD (13.8 Md) (see Fig. 3), so that the LD band on a gel presumably would be contaminated with fragment B after one gel purification. It is likely, then, that it was the contaminating B fragment which was mutagenized in the induction of mutant *tsLD84*. Since this mutant is actually located in B and not LD, it has been renamed *ts B84*.

The two-dimensional gel procedure, although increasing the purity and yield of specific DNA fragments, did not eliminate the problem of contaminating fragments entirely. The second method we used to enable us to obtain large amounts of specific DNA fragments was to clone the HSV-1 genome. The accompanying paper (10) describes the cloning of *Eco*RI fragments of HSV-1 (KOS) in a plasmid vector. These cloned *Eco*RI fragments were used in cotransfection experiments with intact DNA from mutants *tsB84*, *tsLG4*, and *tsJ176*. The results from the marker rescue experiments are presented in the accompanying paper (10). Those data confirm that the location of the mutation in *tsB84* is in the region of *Hpa*I fragment B. The positions of *tsLG4* in fragment LG and *tsJ176* in fragment J are also confirmed in the mapping experiments with cloned fragments (10).

DISCUSSION

This report describes a method for the induction of mutations in specific regions of the viral genome. The procedure involves in vitro mutagenesis of purified restriction enzyme fragments by nitrous acid. The mutations are then transferred to the viral genome by cotransfection of RK cells with the mutagenized fragments and intact HSV-1 DNA so that a recombination event between the fragment and the viral DNA molecule can occur. The efficient marker rescue of mutants by cotransfection of wild-type DNA fragments and mutant DNA (18, 22, 30) suggested to us that mutants could be isolated by this approach at reasonable frequencies. In fact, this proved to be the case, for 1 to 5% of the plaques tested in each mutagenesis treatment were temperature sensitive for growth (Table 1). Syncytial mutants also were isolated with high frequency by this procedure (Table 1).

Chu et al. (4) have described a similar approach for the isolation of HSV-1 *ts* mutants that uses hydroxylamine as the mutagen. We chose nitrous acid as the mutagen because it acts on cytosine and adenine residues (26, 31, 33). HSV-1 has a guanine-plus-cytosine content of 67% (17); therefore, a mutagen which interacts with cytosine would be advantageous in the induction of mutations in guanine-plus-cytosine-rich regions of the genome. Hydroxylamine also interacts with cytosine (8). However, mutagenesis with nitrous acid may have other advantages over mutagenesis with hydroxylamine. Hydroxylamine is mutagenic primarily on single-stranded DNA (8), so that mutagenesis of HSV-1 DNA fragments must be conducted under partially denaturing conditions (4). Because complete denaturation results in loss of infectivity, conditions must be adjusted such that only a small percentage of the HSV-1 DNA is denatured (4). Therefore the mutagenic action of hydroxylamine may be limited to cytosine residues in adenine-plus-thymine-rich regions of the genome. Nitrous acid also mutagenizes duplex DNA less efficiently than single-stranded DNA (15, 19, 20, 32), but the efficiency of mutagenesis

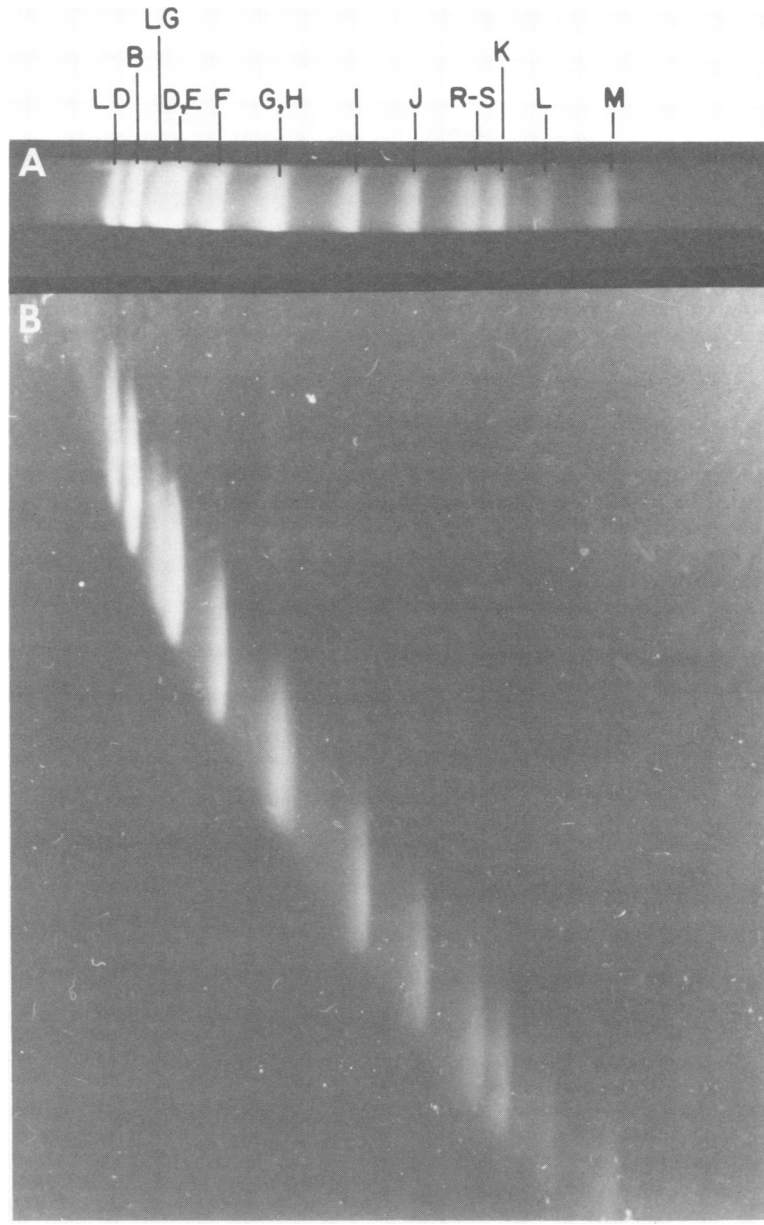

FIG. 3. *Two-dimensional agarose gel purification of HpaI restriction fragments of HSV-1 (KOS) DNA. (A) Ten micrograms of HSV-1 (KOS) DNA was digested to completion with HpaI. The sample was loaded onto a 1.0% agarose tube gel (0.6 by 15 cm) and electrophoresed vertically at 40 V for 18 h. The gel was stained with ethidium bromide to visualize the bands. The origin is on the left-hand side of the figure. (B) The tube gel was placed horizontally about 2 cm from the edge of a glass plate, and a 0.5% agarose slab gel (8.5 by 22 by 0.6 cm) was poured around it. The gel was electrophoresed horizontally at 80 V for 22 h. The origin is at the top of the gel. DNA bands visualized by ethidium bromide staining were cut from the gel, and DNA was extracted as described in Materials and Methods.*

of double-stranded DNA can be increased by adding spermine to the reaction (32), although the mechanism of action of spermine has not been fully elucidated (7). Therefore, it should be possible to mutagenize guanine-plus-cytosine-rich regions of the genome more efficiently with nitrous acid. Finally, the procedure described here for nitrous acid mutagenesis is simple and

TABLE 4. *Marker rescue of tsLD84 by HpaI fragments*

*Hpa*I fragment	Titer		% Rescue[a]
	34°C	39°C	
None	2.52×10^6	1.3×10^4	0.5
LD	7.8×10^6	1.0×10^4	0.2
B	3.65×10^6	1.95×10^5	5.0
LG	1.98×10^6	2.0×10^3	0.1
D, E	6.3×10^6	1.8×10^3	0.03
I	1.55×10^6	1.17×10^4	0.75
RS	2.45×10^5	7.85×10^2	0.3

[a] Determined as (titer at 39°C/titer at 34°C) × 100.

rapid, which should facilitate the isolation of large numbers of mutants.

Another advantage of inducing mutations by in vitro mutagenesis of specific restriction enzyme fragments is that in theory the mutants can be placed on the physical map of the genome by virtue of their induction. However, the use of fragments purified from agarose gels can result in mutation of contaminating DNA fragments instead of the fragment intended. As described above, mutant *tsB84* was isolated by using DNA extracted from band LD but was subsequently found to map in fragment B (Table 4). Although additional gel purifications can reduce contamination of fragments, the problem can be circumvented entirely by using cloned HSV-1 fragments. For this reason, as well as for the easy isolation of large quantities of specific fragments of HSV-1 DNA, we cloned the HSV-1 genome by using HSV-1 *Eco*RI fragments (10). Additional mapping experiments using some of the mutants isolated in this report and cloned *Eco*RI fragments are reported in the accompanying paper (10). We are also mutagenizing specific cloned *Eco*RI fragments by the procedure reported here in an attempt to saturate certain defined regions of the genome with mutants.

In summary, the procedure described here should be generally useful for the induction of mutations in defined regions of any viral genome. This is especially useful for viruses like HSV-1, which have a large genome, since the mutagenesis can be directed to specific fragments.

ACKNOWLEDGMENTS

We thank Alan L. Goldin for valuable discussions and advice and Joseph Bellestri for excellent technical assistance.

This work was supported by grants 07711 and 05860 from the National Science Foundation and by Public Health Service grant 5P40-RR00200 from the National Institutes of Health. R.M.S-G. is the recipient of Public Health Service individual postdoctoral research service fellowship F32-AI05653 from the National Institutes of Health.

LITERATURE CITED

1. **Alder, R., J. C. Glorioso, and M. Levine.** 1978. Infection by herpes simplex virus and cells of nervous system origin: characterization of a nonpermissive interaction. J. Gen. Virol. **39**:9-20.
2. **Aron, G. M., D. J. M. Purifoy, and P. A. Schaffer.** 1975. DNA synthesis and DNA polymerase activity of herpes simplex virus type 1 temperature-sensitive mutants. J. Virol. **16**:498-507.
3. **Becker, Y., H. Dym, and I. Sarov.** 1968. Herpes simplex virus DNA. Virology **36**:184-192.
4. **Chu, C.-T., D. S. Parris, R. A. F. Dixon, F. E. Farber, and P. A. Schaffer.** 1979. Hydroxylamine mutagenesis of HSV DNA and DNA fragments: introduction of mutations into selected regions of the viral genome. Virology **98**:168-181.
5. **Clements, J. B., R. Cortini, and N. M. Wilkie.** 1976. Analysis of herpesvirus DNA substructure by means of restriction endonucleases. J. Gen. Virol. **30**:243-256.
6. **Delius, H., and J. B. Clements.** 1976. A partial denaturation map of herpes simplex virus type 1 DNA: evidence for inversions of the unique DNA regions. J. Gen. Virol. **33**:125-134.
7. **Frankel, A. D., B. K. Duncan, and P. E. Hartman.** 1980. Nitrous acid damage to duplex deoxyribonucleic acid: distinction between deamination of cytosine residues and a novel mutational lesion. J. Bacteriol. **142**: 335-338.
8. **Freese, E., and H. B. Strack.** 1962. Induction of mutations in transforming DNA by hydroxylamine. Proc. Natl. Acad. Sci. U.S.A. **48**:1796-1803.
9. **Glorioso, J. C., M. Levine, T. C. Holland, and M. S. Szczesiul.** 1980. Mutant analysis of herpes simplex virus-induced cell surface antigens: resistance to complement-mediated immune cytolysis. J. Virol. **35**:672-681.
10. **Goldin, A. L., R. M. Sandri-Goldin, M. Levine, and J. C. Glorioso.** 1981. Cloning of herpes simplex virus type 1 sequences representing the whole genome. J. Virol. **38**:50-58.
11. **Grafstrom, R. H., J. C. Alwine, W. L. Steinhart, C. W. Hill, and R. W. Hyman.** 1975. The terminal repetition of herpes simplex virus DNA. Virology **67**:144-157.
12. **Graham, F. L., G. Veldhuisen, and N. M. Wilkie.** 1973. Infectious herpesvirus DNA. Nature (London) New Biol. **245**:265-266.
13. **Hayward, G. S., R. J. Jacob, S. C. Wadsworth, and B. Roizman.** 1975. Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short segments. Proc. Natl. Acad. Sci. U.S.A. **72**:4243-4247.
14. **Hoggan, M. D., and B. Roizman.** 1959. The isolation and properties of a variant of herpes simplex producing multinucleated giant cells in monolayer cultures in the presence of antibody. Am. J. Hyg. **70**:208-219.
15. **Horn, E. E., and R. M. Herriott.** 1962. The mutagenic action on "single-stranded" (denatured) hemophilus transforming DNA. Proc. Natl. Acad. Sci. U.S.A. **48**: 1409-1416.
16. **Keller, J. M., P. G. Spear, and B. Roizman.** 1970. Proteins specified by herpes simplex virus. III. Viruses differing in their effects on the social behavior of infected cells specify different membrane glycoproteins. Proc. Natl. Acad. Sci. U.S.A. **65**:865-871.
17. **Kieff, E. D., S. L. Bachenheimer, and B. Roizman.** 1971. Size, composition, and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. J. Virol. **8**:125-132.
18. **Knipe, D. M., W. T. Ruyechan, R. W. Honess, and B. Roizman.** 1979. Molecular genetics of herpes simplex virus: the terminal a sequences of the L and S compo-

nents are obligatorily identical and constitute a part of a structural gene mapping predominantly in the S component. Proc. Natl. Acad. Sci. U.S.A. **76**:4534-4538.

19. **Kotaka, T., and R. L. Baldwin.** 1964. Effects of nitrous acid on the dAT copolymer as a template for DNA polymerase. J. Mol. Biol. **9**:323-339.
20. **Litman, R. M.** 1961. Genetic and chemical alterations in the transforming DNA of Pneumococcus caused by ultraviolet light and by nitrous acid. J. Chem. Phys. **58**: 997-1003.
21. **Morse, L. S., T. G. Buchman, B. Roizman, and P. A. Schaffer.** 1977. Anatomy of herpes simplex virus DNA. IX. Apparent exclusion of some parental DNA arrangements in the generation of intertypic (HSV-1 × HSV-2) recombinants. J. Virol. **24**:231-248.
22. **Parris, D. S., R. A. F. Dixon, and P. A. Schaffer.** 1980. Physical mapping of herpes simplex virus type 1 ts mutants by marker rescue: correlation of the physical and genetic maps. Virology **100**:275-287.
23. **Ruyechan, W. T., L. S. Morse, D. M. Knipe, and B. Roizman.** 1979. Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. J. Virol. **29**:677-697.
24. **Schaffer, P. A., G. M. Aron, N. Biswal, and M. Benyesh-Melnick.** 1973. Temperature-sensitive mutants of herpes simplex virus type 1: isolation, complementation and partial characterization. Virology **52**:57-71.
25. **Schaffer, P. A., V. C. Carter, and M. C. Timbury.** 1978. Collaborative complementation study of temperature-sensitive mutants of herpes simplex virus types 1 and 2. J. Virol. **27**:490-504.
26. **Schuster, H.** 1960. The reaction of nitrous acid with deoxyribonucleic acid. Biochem. Biophys. Res. Commun. **2**:320-323.
27. **Sheldrick, P., and N. Berthelot.** 1974. Inverted repetitions in the chromosome of herpes simplex virus. Cold Spring Harbor Symp. Quant. Biol. **39**:667-678.
28. **Skare, J., and W. C. Summers.** 1977. Structure and function of herpesvirus genomes. II. EcoRI, XbaI, and HindIII endonuclease cleavage sites on herpes simplex virus type 1 DNA. Virology **76**:581-595.
29. **Spear, P. G.** 1976. Membrane proteins specified by herpes simplex viruses. I. Identification of four glycoprotein precursors and their products in type 1-infected cells. J. Virol. **17**:991-1008.
30. **Stowe, N. D., J. H. Subak-Sharpe, and N. M. Wilkie.** 1978. Physical mapping of herpes simplex virus type 1 mutations by marker rescue. J. Virol. **38**:182-192.
31. **Tessman, I.** 1959. Mutagenesis in phages ϕx174 and T4 and properties of the genetic material. Virology **9**:375-385.
32. **Thomas, H. F., P. E. Hartman, M. Mudryj, and D. L. Brown.** 1979. Nitrous acid mutagenesis of duplex DNA as a three-component system. Mutat. Res. **61**:129-151.
33. **Vielmetter, W., and H. Schuster.** 1960. The base specificity of mutation induced by nitrous acid in phage T2. Biochem. Biophys. Res. Commun. **2**:324-328.
34. **Zimmerman, F. K.** 1977. Genetic effects of nitrous acid. Mutat. Res. **39**:127-148.