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Genetics

Expression of herpes simplex virus glycoprotein C from a DNA fragment inserted into the thymidine kinase gene of this virus

(syncytial mutant)

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ABSTRACT **Previous reports have described mutants of herpes simplex virus type 1 that fail to produce or accumulate one of the major glycoproteins, glycoprotein C (gC). This defect is not lethal in cell culture, has been associated with the syncytial plaque morphology of some mutants, and may result from mutations that map to a region on the genome noncontiguous with the structural gene for gC. To investigate the conditions required for, and consequences of, *gC* expression in a specific genetic background, we have inserted a wild-type allele of the gC gene into the thymidine kinase gene (*tk*) of a gC$^-$ fusion-inducing viral mutant, strain MP. This was accomplished by identifying cloned viral DNA fragments homologous to gC mRNA, inserting the appropriate fragments into the viral *tk* cloned in pBR322, and then cotransfecting cells with the recombinant plasmids and DNA from strain MP, for selection of insertional TK$^-$ mutants. All TK$^-$ mutants containing insertions of appropriate sequences (in either orientation) into *tk* were found to express gC while maintaining the syncytial plaque morphology of strain MP. Elimination of the insertion from one of the TK$^-$ mutants was accompanied by loss of ability to produce gC. Our results permit more precise mapping of the DNA sequence encoding gC, to a subfragment of *Sal* I fragment R (map coordinates 0.620–0.640) and indicate also that promoter sequences for the gC gene may be located in this fragment. Moreover, we can conclude that the previously described regulatory mutation of strain MP does not prevent expression of gC from the DNA inserted into its gene *tk* and that the syncytial phenotype of MP cannot be due solely to absence of gC.**

The genome of herpes simplex virus (HSV) is a linear double-stranded DNA approximately 100×10^6 in molecular weight (1). Because certain viral gene products are not essential for replication of the virus in cell culture (2, 3) and because requirements for packaging of the DNA into virions permit some variation in size of the genome (4), it has proved possible by use of recombinant DNA technology to generate viable mutants containing sizable deletions or insertions of specific genetic information (5–10). For example, mutants containing duplications of HSV-1 DNA sequences inserted into the viral thymidine kinase (TK) gene (*tk*) (resulting in a selectable TK$^-$ phenotype) have yielded information about regulatory signals in the HSV-1 genome (8) and about HSV-1 DNA sequences that mediate the inversion of genome segments (6, 7, 10).

We have extended this approach of inserting duplications of HSV-1 DNA sequences into the viral *tk* and screened for the expression of specific genes from the inserts of the resulting TK$^-$ mutants. The immediate aims of the work reported here were to define more precisely the genomic location and boundaries of the HSV-1 gene for glycoprotein C (gC) and to investigate the requirements for and effects of gC expression in a specific genetic background.

The membrane protein gC is one of several HSV-1 glycoproteins that are constituents both of the virion envelope and of membranes in the infected cell (11). Its function is unknown. Although in most HSV-1 isolates from patients *gC* is expressed (12), viable mutants lacking the ability to produce or accumulate gC spontaneously emerge in cell culture (13, 14), suggesting that gC is dispensable for replication of the virus in cell culture but may be essential for survival of the virus in nature. It has been noted that a fraction of syncytium-forming plaque morphology variants of HSV-1 are gC$^-$ and a causal relationship was suggested (15), although more recent studies (unpublished data) indicate that gC$^-$ viruses may not have enhanced capacity to induce cell fusion.

Analyses of HSV-1–HSV-2 recombinant viruses by Ruyechan *et al.* (16) indicated that the structural gene for HSV-1 gC is located between approximately 0.53 and 0.64 map units on the viral genome (Fig. 1). On the basis of marker transfer experiments done in the same study (16), it was concluded that genes capable of transferring the gC$^-$ and syncytium-forming (SYN) phenotypes of strain HSV-1(MP) were located between 0.70 and 0.83 map units; the locus that apparently influenced gC expression but did not overlap the structural gene for gC was designated *Cr* (Fig. 1).

Here we report the identification of HSV-1 DNA fragments homologous to gC mRNA and the construction of recombinant viruses containing one of these fragments inserted into *tk* of the gC$^-$ strain HSV-1(MP). The recombinant viruses rendered TK$^-$ by the insertion were shown to express gC from the insert but to exhibit the same plaque morphology phenotype (SYN) as the parental strain HSV-1(MP). Our results define the map location and boundaries of the structural gene for gC, within the region previously reported to contain the gene (16), demonstrate that the *Cr* allele of HSV-1(MP) does not adversely affect the expression of gC by the gene inserted into *tk*, and also demonstrate that the SYN phenotype of HSV-1(MP) cannot be due solely to absence of gC.

MATERIALS AND METHODS

Viruses. Viral strains used were HSV-1(F) (21), HSV-1(mP) (13), the gC$^-$ *syn* mutant HSV-1(MP) (3, 13), and the TK$^-$ mutant HSV-1(C1 101)B2006 (2).

Plasmids. Plasmids constructed by Post *et al.* (17) were obtained from B. Roizman (University of Chicago) and contain *Bam*HI fragments of HSV-1(F) DNA as indicated inserted into pBR322: pRB103 (Q), pRB127 (H+L), pRB130 (I+M), pRB125

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Abbreviations: gC, glycoprotein C; *tk*, thymidine kinase gene; TK, thymidine kinase; HSV-1 or HSV-2, herpes simplex virus type 1 or type 2; ara T, arabinofuranosylthymidine; kb, kilobase(s).

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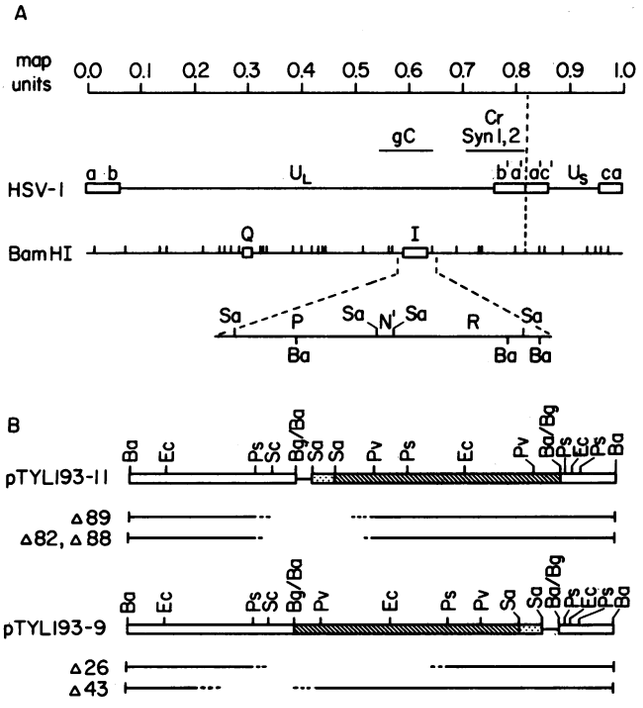

FIG. 1. Maps of the HSV-1 genome and of plasmids used in this study. (*A*) The organization of the genome with respect to repetitive and unique DNA sequences (1) and the positions of *Bam*HI restriction sites in HSV-1(F) DNA (17, 18) and of fragments Q and I are shown along with the positions of *Sal* I sites in and near *Bam*HI fragment I (18). In addition, previously published mapping results are shown for the location of the gC gene, of a gene that influences gC expression (*Cr*), and of *syn* loci present in HSV-1(MP) DNA (16). (*B*) To construct plasmids pTYL193-11 and pTYL193-9, pTYL19 [containing *Sal* I fragment R of HSV-1(F) DNA and a small *Sal* I fragment of unknown origin inserted into the *Sal* I site of pBR322] was cleaved with *Bam*HI and *Hin*dIII, and the *Bam*HI fragment was inserted into the single *Bgl* II site of pRB103, which contains *Bam*HI fragment Q (17). As indicated here the largest *Bam*HI fragment of pTYL193-11 or pTYL193-9 contains DNA sequences from *Bam*HI fragment Q (open bars), pBR322 (line), the *Sal* I fragment of unknown origin (stippled bar) and most of *Sal* I fragment R (striped bar). In pTYL193-11 the *Sal* I fragment R DNA sequence has the same orientation with respect to the *Bam*HI Q sequence as is found in the viral genome whereas in pTYL193-9 the orientation is opposite. Transcription of the unaltered *tk* from the *Bam*HI fragment Q DNA sequence is leftward, starting at a position between the *Bgl* II and rightmost *Eco*RI sites and terminating between the leftmost *Eco*RI and *Pst* I sites; the entirety of the coding sequence for TK is located to the left of the *Bgl* II site (19, 20)—i.e., to the left of the site used for insertion into *Bam*HI fragment Q. Deletion derivatives of pTYL193-11 and pTYL193-9 were generated by *Bal* 31 digestion of the *Sac* I-cleaved DNAs followed by blunt-end ligation. The sizes of the deletions are indicated by the sizes of the gaps in the lines drawn below the maps of the plasmids; the deletions are located within the regions denoted by the dashed lines and gaps, with uncertainty as to the precise positions. Restriction endonucleases: Ba, *Bam*HI; Bg, *Bgl* II; Ec, *Eco*RI; Ps, *Pst* I; Pv, *Pvu* I; Sa, *Sal* I; Sc, *Sac* I.

(M), pRB128 (F), pRB126 (L), and pRB106 (B). Plasmids pTYL01, pTYL12, and pTYL19 contain the P, N′, and R fragments, respectively, of *Sal* I-cleaved HSV-1(F) DNA (Fig. 1) inserted into pBR322. Other plasmids constructed for this study are described in Fig. 1.

Antibodies. We used the anti-gC monoclonal antibody designated HC1 (22) and also a rabbit antiserum designated R#24 which was prepared by M. Sarmiento-Batterson and was obtained after multiple injections of a rabbit with gC isolated by preparative polyacrylamide gel electrophoresis of Nonidet P40 extracts of purified HSV-1 virions.

Selection of gC mRNA for Translation *in Vitro*. Procedures for the preparation of cytoplasmic polyadenylylated RNA, selection of mRNAs by hybridization, translation *in vitro*, and identification of products by immunoprecipitation were as described (23).

Construction, Isolation, and Analyses of Recombinant Viruses. Rabbit skin cells were cotransfected with mixtures of the appropriate viral DNA and pTYL193-9, pTYL193-11, deletion derivatives thereof, or pRB103. Calcium phosphate precipitates were prepared (24, 25) to contain 250 ng of HSV-1 DNA, 100, 250, or 400 ng of *Bam*HI-digested plasmid DNA, and 12 μg of HEp-2 cell DNA per 0.6 ml. After 4 hr of exposure to the precipitates, the cells were treated with 15% (vol/vol) glycerol (26), and then fresh medium was added and the cultures were incubated at 37°C for 5 days. Selection of TK^- mutants from the progeny of these transfections, or enrichment for TK^+ revertants, was done as described (6, 8). Total unselected progeny were screened for gC production by a slight modification of an unpublished procedure (T. C. Holland, R. M. Sandri-Goldin, S. D. Marlin, J. C. Glorioso, and M. Levine, University of Michigan, personal communication). Vero cultures were infected and maintained for 2 days under medium containing 0.5% methylcellulose. The monolayers were then washed with phosphate-buffered saline, fixed with 0.25% glutaraldehyde in buffered saline, and washed again with buffered saline. The monolayers were incubated in sequence for 1 hr at room temperature with HC1 ascites fluid diluted 1:500, biotinylated rabbit anti-mouse IgG (Vector Laboratories, Burlingame, CA) reconstituted as directed and diluted 1:200, and avidin-coupled horseradish peroxidase (Vector) diluted 1:200 (all dilutions were in phosphate-buffered saline containing 3% bovine serum albumin), with buffered saline washes after each incubation. Finally, the monolayers were incubated with substrate (10 mg of 4-chloro-4-naphthol in 1 ml of ethanol added slowly to 100 ml of H_2O followed by addition of hydrogen peroxide to 0.003%) until a purplish black reaction product had deposited on plaques formed by gC^+ viruses (plaques formed by gC^- viruses were colorless but visible under appropriate light and slight magnification).

RESULTS

Identification of HSV-1 DNA Fragments Homologous to gC mRNA. Results shown in Fig. 2*A* demonstrate that the anti-gC monoclonal antibody HC1 and the rabbit anti-gC serum (R#24) both precipitated a single polypeptide, 74,000 in molecular weight, from reaction mixtures in which unfractionated viral and cellular mRNAs had been translated *in vitro*. Electrophoretic analyses of peptides obtained after partial proteolysis (Fig. 2*B*) indicate that the polypeptides precipitated by each antibody preparation were indistinguishable by this test. Antiserum R#24 precipitated the gC polypeptide synthesized *in vitro* more efficiently and was used for all other analyses of *in vitro* products.

Plasmids containing the major *Bam*HI fragments located between map coordinates 0.52 and 0.81 (fragments H, I, F, L, and B) and also *Bam*HI fragment M (coordinates 0.224–0.257) were used to select homologous mRNAs. Only the plasmid carrying fragment I was found to hybridize to a mRNA capable of directing synthesis of gC *in vitro* (Fig. 2*C* and other results not shown). Although this plasmid included fragment M as well as I, another plasmid containing only M did not hybridize to gC mRNA. To refine the mapping further, *Sal* I fragments P, N′, and R (Fig. 1) were cloned into pBR322 and these fragments were used to select mRNAs by hybridization. Only *Sal* I fragment R could be shown to hybridize to gC mRNA (Fig. 2*C*).

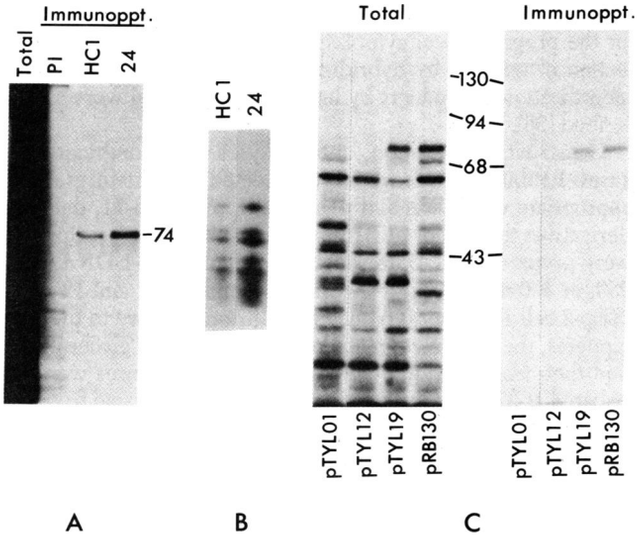

FIG. 2. Electrophoretic analyses of polypeptides synthesized *in vitro* and precipitated by anti-gC antibodies. (*A*) Products obtained by translation of total polyadenylylated RNA extracted from Vero cells 14 hr after infection with HSV-1(mP) and polypeptides precipitated by normal rabbit serum (PI), the monoclonal antibody HC1, and rabbit antiserum R#24. Molecular weight 74,000 position is indicated. (*B*) Peptides obtained, after partial proteolysis with *S. aureus* V8 protease (27), from the molecular weight 74,000 polypeptides precipitated by HC1 and R#24, respectively. (*C*) Total polypeptides translated *in vitro* from mRNAs selected by the indicated plasmids (pTYL01–*Sal* I fragment P; pTYL12–*Sal* I fragment N'; pTYL19–*Sal* I fragment R; and pRB130–*Bam*HI fragments I and M) and also immunoprecipitates obtained from each *in vitro* reaction mixture by use of antiserum R#24. Except for *B*, in which a 15% polyacrylamide gel was used, electrophoretic separations were performed on 10% polyacrylamide gels cross-linked with diallyltartardiamide (3). β-Galactosidase (130,000), phosphorylase *b* (94,000), bovine serum albumin (68,000), and ovalbumin (43,000) were used as molecular weight standards.

Isolation and Characterization of Insertional Mutants with TK⁻ and gC⁺ Phenotypes. The DNA sequences inserted into pRB103 to generate pTYL193-9 and pTYL193-11 interrupt the *tk* gene between the site for initiation of transcription and the coding sequence (19, 20). Therefore, recombination of either of these plasmids with DNA of a TK⁺ virus, by crossovers within *Bam*HI fragment Q sequences on both sides of the inserts, could transfer the insertions to the *tk* of the viral genome and render the recombinant viruses TK⁻ and resistant to arabinofuranosylthymidine (ara T; thymidine arabinoside) as observed previously (6, 7). The results presented in Table 1 demonstrate that transfection of cells with a mixture of HSV-1(MP) DNA and either plasmid usually yielded a significantly higher frequency of ara T-resistant progeny than did transfection with HSV-1(MP) DNA alone although this frequency was variable. Of most importance was the finding that 50–90% of the virus isolates obtained from plaques formed in the presence of ara T were gC⁺ by immunoprecipitation tests, regardless of which plasmid was used to generate the insertional mutants; only 4–8% of the total unselected progeny were gC⁺ by immunoassay of plaques *in situ*. All progeny, whether gC⁺ or gC⁻, were found to express the SYN phenotype on Vero cells and HEp-2 cells.

Thirteen of the ara T-resistant isolates from experiment I, including two gC⁻ isolates, were plaque-purified again in the presence of ara T. All were found to be TK⁻ inasmuch as TK activity (28) in extracts from infected LMtk⁻ cells (29) was the same as that present after infection with the TK⁻ strain HSV-1(B2006) (2), whereas HSV-1(MP) induced levels of TK activity 7–10 times higher than this background (data not shown). Immunoprecipitation experiments done with extracts prepared from Vero cells infected with each of the 13 isolates revealed that all were gC⁺ except for isolates 21-1 and 7-6, the two found originally to be gC⁻ (Fig. 3).

Table 1. Analyses of viral progeny from cells cotransfected with HSV-1(MP) DNA and plasmid DNAs

Plasmid*	Plaque-forming units†		gC⁺ isolates, no./no. of ara T-resistant isolates tested‡
	% ara T-resistant	% gC⁺	
pTYL193-9:			
Exp. I	0.10	8.9	14/18
Exp. II	1.23		13/14
pTYL193-9 Δ26	0.54		0/14
pTYL193-9 Δ43	0.14		9/14
pTYL193-11:			
Exp. I	0.01–0.05	3.8	11/21
Exp. II	2.55		11/12
pTYL193-11 Δ82	2.45		14/14
pTYL193-11 Δ88	1.13		11/14
pTYL193-11 Δ89	2.50		13/14
None	0.015–0.033§	0.7¶	ND‖

* See Fig. 1 for description of the plasmids and their deletion derivatives.

† Virus titrations were done on Vero cells in the presence of ara T (100 μg/ml) or in its absence. Titers in the absence of ara T ranged from 1×10^7 to 3.6×10^8 plaque-forming units/ml. Plaques that formed in the absence of ara T were screened for gC expression by the *in situ* immunoassay described in the text.

‡ Virus isolated from plaques that formed in the presence of ara T was used to infect Vero cells in 24-well culture trays for labeling with [^{35}S]methionine and immunoprecipitation (23) with the monoclonal antibody HC1.

§ Range of values obtained in several experiments.

¶ This value probably represents the background of the assay because gC⁺ revertants have never been isolated from HSV-1(MP).

‖ ND, not done.

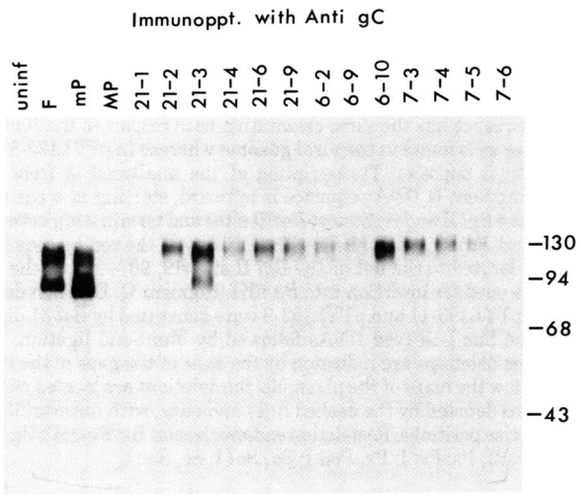

FIG. 3. Production of gC by TK⁻ mutants derived from HSV-1(MP). Each mutant isolate is designated by number; those with prefix 21 were isolated from transfections using pTYL193-9, and the others were from transfections using pTYL193-11. Immunoprecipitation with monoclonal antibody HC1 was performed as described (23) using lysates of Vero cells infected with the TK⁻ isolates or other strains as indicated and incubated with [^{35}S]methionine from 5 to 20 hr after infection. The immunoprecipitates were analyzed by electrophoresis on 10% polyacrylamide gel. The molecular weight of the mature form of gC is approximately 130,000; in some of the immunoprecipitates a partially glycosylated form of gC (molecular weight, approximately 94,000) was detectable. Molecular weights are shown $\times 10^{-3}$.

Southern blot analyses of DNAs from the isolates showed that 10 of the 13 TK^- isolates, which includes all of the gC^+ isolates except 21-3, were lacking *Bam*HI fragment Q [3.6 kilobases (kb)] in their DNAs and instead had a novel 7.4-kb fragment that was homologous to sequences present in *Bam*HI fragments Q and I (Fig. 4). This is the result anticipated for all isolates having insertions into the *tk* gene resulting from recombinations of plasmids pTYL193-9 or pTYL193-11 with the *tk* locus of HSV-1(MP). DNAs from the two gC^- isolates (21-1 and 7-6) and from one gC^+ isolate (21-3) apparently did not have such insertions (Figs. 3 and 4). For the analyses shown in Fig. 4 the DNAs from isolates 21-3 and 7-6 were not digested to completion, which accounts for the extra bands detected by hybridization with the labeled probes.

Additional insertional TK^- mutants were isolated from yields of cells cotransfected with HSV-1(MP) DNA and the deletion derivatives of pTYL193-9 and pTYL193-11 described in Fig. 1. The results presented in Table 1 demonstrate that, for all plasmids except pTYL193-9 Δ26, the TK^- progeny resulting from the transfections included a high percentage that were also gC^+. Therefore, expression of gC from inserts in the TK^- recombinant viruses required only a subset of *Sal* I fragment R sequences located between (and perhaps including) the leftmost *Pvu* I site and the *Bam*HI site (Fig. 1). Because gC could be produced regardless of orientation of the *Sal* I fragment R sequences with respect to *Bam*HI fragment Q sequences, it seems likely that a functional promoter for *gC* gene transcription is located within the subset of *Sal* I fragment R sequences indicated above.

Effect on *gC* Expression of Eliminating the Insertion from a TK^- Isolate. DNA prepared from isolate 6–9 was mixed with pRB103 (containing an intact *tk* gene in *Bam*HI fragment Q) for transfection of cells and the resulting progeny were passaged under conditions designed to favor the replication of TK^+ virus (8). Eleven isolates obtained after two cycles of plaque purification on Vero cells were characterized. Four of the isolates were gC^+ and expressed levels of TK activity that were only 4–14% of the level expressed by HSV-1(MP); their DNAs had the novel *Bam*HI fragment indicative of insertion of *Sal* I fragment R into the *tk* gene. The seven other isolates were gC^- and had levels of TK activity ranging from 6% to 49% of the HSV-1(MP) level, and their DNAs had a normal *Bam*HI fragment Q with no evidence of insertions in this fragment. It is of interest that low levels of TK activity could be detected in isolates that retained the insertion and that recovery of full TK activity did not invariably accompany loss of the insertion. The finding most pertinent to this study, however, was that loss of *gC* expression was invariably associated with loss of the insertion from at least one of the recombinant viruses.

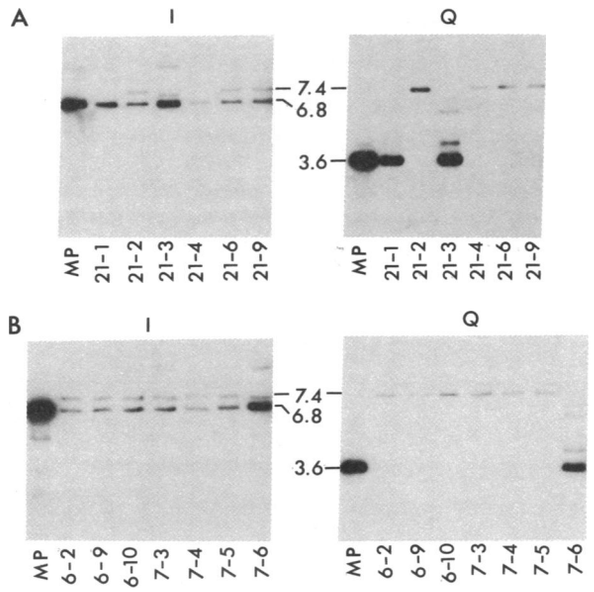

FIG. 4. Analysis of genome structures of TK^- mutants derived from HSV-1(MP). The *Bam*HI restriction fragments of DNAs from the TK^- mutants (those isolated from transfections using pTYL193-9 are shown in *A* and others from transfections using pTYL193-11 are in *B*) or HSV-1(MP) were separated on 0.75% agarose gel by electrophoresis. Fragments containing sequences homologous to *Bam*HI fragment Q or I were identified by hybridization to ^{32}P-labeled probes by using conditions described by Post *et al.* (17). The numbers indicate the sizes of DNA fragments in kb. *Bam*HI fragment Q is 3.6 kb, *Bam*HI fragment I is 6.8 kb, and a novel *Bam*HI fragment detected in most of the mutants and homologous both to Q and I sequences is 7.4 kb.

DISCUSSION

Results presented here show that the sequences coding for the gC polypeptide, and probably also a promoter for gC mRNA transcription, are located in *Sal* I fragment R of HSV-1 DNA, within the region bounded by or just including the leftmost *Pvu* I site and the *Bam*HI site (Fig. 1). This conclusion is based on the finding that gC mRNA hybridizes selectively to the *Sal* I fragment R and on analyses of the progeny virus resulting from cotransfection of cells with DNA from the TK^+ gC^- strain HSV-1(MP) and plasmid DNAs containing *Sal* I fragment R sequences inserted into the viral *tk* gene.

The pertinent findings about these progeny viruses are that most of the TK^- isolates resulted from recombinations that yielded insertions in the *tk* gene, as was shown previously (6, 7), rather than from spontaneous mutations; that all the TK^- isolates containing the appropriate insertions were gC^+; that orientation of the *Sal* I fragment R sequences with respect to *Bam*HI fragment Q sequences did not influence expression of gC; and that elimination of the insertion from one isolate resulted in loss of ability to produce gC. A small fraction of the TK^- gC^+ isolates (<10%) may also express *gC* from the normal locus as well as from the inserted DNA sequences, due to recombination between *Sal* I fragment R sequences of the plasmid and of the viral genome. HSV-1(MP) has a defective structural gene for gC and this defect can be rescued by cotransfection with *Sal* I fragment R sequences from a gC^+ virus (unpublished data). In fact, the results presented in Table 1 and Figs. 3 and 4 show that conventional marker rescue occurred in these experiments [the frequency of gC^+ viruses among total progeny was higher than the frequency of TK^- viruses and one of the TK^- gC^+ isolates (21-3) did not have an insert in *tk*]; gC expression due to marker rescue should probably not be any more frequent among TK^- progeny than among total progeny [4–8% (Table 1)], however.

Frink *et al.* (30) have mapped and characterized a 2.7-kb mRNA that encodes a late HSV-1 polypeptide of molecular weight 69,000 (*in vitro* product). Comparison of our results with theirs suggests that, despite a slight discrepancy in estimated molecular weights of *in vitro* products, this mRNA encodes the gC polypeptide. Their analyses located the 5′ end of the 2.7-kb mRNA 600 base pairs to the left of the *Eco*RI site in *Sal* I fragment R and the 3′ end 340 base pairs outside of fragment R to the right in the prototype arrangement of HSV-1 DNA sequences as shown in Fig. 1. If it is assumed that this mRNA actually does encode the gC polypeptide, it can be concluded from results summarized in Fig. 1 and Table 1 that its 3′ end (approximately 500 bases) is noncoding with respect to this polypeptide and, in addition, is not essential for gC expression.

Coinfection of cells with HSV-1(MP) and nonsyncytial (SYN^+) gC^+ HSV-1 strains can result in the nonsyncytial phenotype (15, 31–33), suggesting that wild-type virus supplies a product capable of preventing or modulating cell fusion. Although it was proposed that this product is gC (15) at least two kinds of experiments indicate that the putative complementing wild-type product is not gC. First, Honess *et al.* (34) showed that the gC^- and SYN markers of a HSV-1 mutant segregated in recombinants derived from the mutant and another strain having wild-type alleles at these loci. Second, the experiments presented here provide a refined complementation analysis and show that expression of *gC* is not sufficient to complement the mutation in HSV-1(MP) that results in its SYN phenotype.

Although presence or absence of gC apparently does not determine plaque morphology with respect to syncytium formation, there are observations pertaining to the gC^- and SYN phenotypes that remain to be explained. First, multiple independent isolates of SYN plaque morphology variants have proven to be gC^- (3, 13, 14). Second, it has been reported that the same fragment of HSV-1(MP) DNA (Fig. 1) can transfer the SYN and gC^- phenotypes to recipient virus; the nonstructural locus affecting *gC* expression was designated *Cr* (16). It remains to be determined how the *Cr* locus affects *gC* expression and whether it is of any significance that some *syn* mutations are accompanied by mutation to the gC^- phenotype.

Because of the ease with which viruses carrying insertions of functional genes into the *tk* gene can be isolated and identified, the kinds of studies described here can be extended for various purposes, including definition of the boundaries of any HSV gene whose product can be differentiated from products of the recipient virus, identification of DNA fragments encoding products that complement temperature-sensitive or other mutations on the recipient viral genome, and identification of regions in viral genes that determine various aspects of synthesis and post-translational processing of the gene products. Clearly, HSV can also serve as a vector for genes of non-HSV origin, although expression of these genes may be inhibited in the environment of the infected cell.

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