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Identification of *mar* Mutations in Herpes Simplex Virus Type 1 Glycoprotein B Which Alter Antigenic Structure and Function in Virus Penetration

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Analysis of six monoclonal antibody-resistant (*mar*) mutants in herpes simplex virus type 1 glycoprotein B identified two type-common (II and III) and two type-specific (I and IV) antigenic sites on this molecule. To derive additional information on the location of these sites, *mar* mutations were mapped and nucleotide alterations were identified by DNA sequencing. Each mutant carried a single amino acid substitution resulting from a G-to-A base transition. Alterations affecting antibody neutralization were identified at residues 473, 594, 305, and 85 for mutants in sites I through IV, respectively. Two clonally distinct site II antibodies each selected *mar* mutants (Gly to Arg at residue 594) that exhibited a reduction in the rate of entry (*roe*) into host cells. A site II *mar* revertant that regained sensitivity to neutralization by site II antibodies also showed normal entry kinetics. DNA sequencing of this virus identified a single base reversion of the site II *mar* mutation, resulting in restoration of the wild-type sequence (Arg to Gly). This finding demonstrated that the *mar* and *roe* phenotypes were the result of a single mutation. To further define structures that contributed to antibody recognition, monoclonal antibodies specific for all four sites were tested for their ability to immune precipitate a panel of linker-insertion mutant glycoprotein B molecules. Individual polypeptides that contained single insertions of 2 to 28 amino acids throughout the external domain were not recognized or were recognized poorly by antibodies specific for sites II and III, whereas no insertion affected antibody recognition of sites I and IV. *mar* mutations affecting either site II or III were previously shown to cause temperature-sensitive defects in glycoprotein B glycosylation, and variants altered in both these sites were temperature sensitive for virus production. Taken together, the data indicate that antigenic sites II and III are composed of higher-order structures whose integrity is linked with the ability of glycoprotein B to function in virus infectivity.

Glycoprotein B (gB) of herpes simplex virus type 1 (HSV-1) was the first viral glycoprotein shown to be required for virus infectivity (31). This conclusion was based on analyses of conditional lethal mutations that mapped to the gB gene (10, 17, 23, 31). Such temperature-sensitive (*ts*) mutants replicate normally at 34°C, but virus produced at higher temperature (39°C) is noninfectious due to a block in virus penetration (31). Two *ts* mutations have now been identified by DNA sequencing; both result in a single amino acid substitution in the external domain of gB (3). Two other mutant phenotypes have been shown to result from single amino acid changes in the gB coding sequence. These include a rate-of-entry (*roe*) mutation located in the external domain (4, 10) and a syncytial (*syn*) mutation residing in the cytoplasmic anchor which induces fusion of infected-cell membranes (4, 10).

Recently, plasmids carrying *Hpa*I linker-insertion mutants in gB have been tested for the ability to complement a gB^- virus in transient expression assays (5). Most mutant polypeptides lacked terminal carbohydrate modifications and were unable to support virus replication, further illustrating the requirement of this molecule for virus infectivity (5). Two insertions resulted in the production of gB molecules that failed to complement a gB^- virus at high temperature and were located near reported *ts* mutations (5). Taken together, these data indicate that gB is essential to virus replication at the level of virus penetration.

Previously, we described the use of a panel of gB-specific monoclonal antibodies (MAbs) to select a series of neutralization escape variants referred to as MAb-resistant (*mar*) mutants (24). These mutants grew normally at 37°C and produced a gB protein that was functionally indistinguishable from wild-type gB. Each mutant was tested for resistance to complement-dependent neutralization by 12 clonally distinct MAbs. Five unique reactivity patterns were seen, indicating the presence of at least five distinct epitopes on this antigen (24). Using a series of transiently expressed chain-terminating gB mutants in radioimmune precipitation assays, antibody recognition sites have been physically mapped (13). The results showed that residues required for recognition of three sites (I, III, and IV) were contained in the amino-terminal half of the gB molecule, whereas recognition of site II involved residues more proximal to the transmembrane domain.

Because of the existence of *ts* defects resulting from point mutations in gB (3), *mar* B mutants were each tested for their ability to process gB normally and support virus replication at the nonpermissive temperature (39°C). Although *mar*

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mutants altered in a single site grew normally at an elevated temperature, radioimmune precipitation of infected-cell extracts demonstrated that single *mar* mutants altered in sites II or III had reduced carbohydrate processing of the major gB precursor (pgB) to its mature form at high temperature (24). Furthermore, multiple *mar* mutants, possessing both site II and III alterations, produced no mature gB at 39°C (24) and showed reduced titers when grown at 39°C. With these mutants, and *ts* mutants in gB isolated from other HSV-1 strains, it was shown that site II and III antibodies failed to recognize pgB produced by these mutants at high temperature. Thus, it appeared that some gB-specific MAbs could recognize and select for changes in thermolabile structures involved in the function of gB in virus replication (24).

In agreement with the above prediction, it has been shown that the binding of some MAbs can affect virus replication. A site III-specific MAb neutralized virus in the absence of complement (24). This antibody had no effect on virus adsorption, indicating that neutralization occurred by blocking of virus penetration (13). Although antibodies specific for sites I and IV required complement for neutralization, they also reduced the rate of virus penetration in the absence of complement (13). In contrast, antibodies specific for site II had no effect on entry, suggesting that this site was functionally distinct from other sites.

This report extends our analysis of the antigenic structure of gB and further defines the extent to which these domains contribute to gB function. Data on the location of *mar* mutations that cause functional defects in gB identified regions involved in virus penetration. In addition, examination of the nature of antigenic determinants associated with *ts* defects in replication suggests that their integrity is dependent on the maintenance of higher-order structures that are required for the proper function of gB in virus infection.

MATERIALS AND METHODS

Cells and virus strains. Vero cells were cultured in Eagle minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with non-essential amino acids, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, and 5% fetal calf serum (GIBCO). Wild-type HSV-1 (KOS-321) and antigenic variants were grown and titers were determined on Vero cells as described earlier (16). Neutralization resistant mutants were isolated from KOS-321 as described previously (16) and were designated monoclonal antibody resistant (*mar*). Multiple *mar* mutants were generated by mixed infection and sequential antibody selection in each epitope (24). A revertant virus, R3 *mar* B4.1, was isolated from the temperature-sensitive double mutant *mar* B2/4.1 by growth in Vero cells at 39°C and was shown to be sensitive to neutralization by site II antibodies (24). The generation of the gB^- virus K082 and its propagation on the gB-expressing cell line D6 has been described elsewhere (6).

MAbs. The production of HSV-specific MAbs has been described in detail elsewhere (16). MAbs were produced as mouse ascites fluid, clarified by ammonium sulfate precipitation, and suspended in phosphate-buffered saline. In immunoprecipitation experiments, the anti-gB pool contained equal amounts of MAbs B3, B4, B5, and B6, whereas the anti-gC pool contained MAbs C1, C3, C7, C8, C11, C 13, C15, C16, and C17 (25).

Marker rescue of *mar* B mutants. Cotransfection of *mar* mutant DNA and cloned KOS-321 sequences corresponding to gB gene fragments was performed as described by Graham and van der Ebb (12) and modified by Homa et al. (19). The black plaque assay for detection of marker rescued virus was performed as described earlier by Holland et al. (17). Recombinant viruses forming black plaques were detected by immunostaining with the selecting MAb and secondary goat anti-mouse horseradish peroxidase-conjugated immunoglobulin (Sigma Chemical Co., St. Louis, Mo.) (17). The percent rescue of the *mar* phenotype was determined by dividing the number of recombinant black plaques by the total number of plaques formed, multiplied by 100.

Sequencing of HSV-1 gB antigenic variants. Both chemical cleavage (26) and dideoxy (30) sequencing techniques were employed. In sequencing *mar* mutants, the entire region shown to marker rescue each mutant was sequenced with the exception of *mar* B4.1, for which the entire coding sequence was determined. All sequences were compared with the wild-type gB sequence of KOS-321 (29). Sequencing of the revertant, R3 *mar* B4.1, was performed by the dideoxy method, substituting genomic viral DNA for yeast genomic DNA as a template (21; J. M. Huibregste, D. R. Engelke, and D. J. Thiele, Proc. Natl. Acad. Sci. USA, in press) by using the modified T7 polymerase (33) Sequenase (United States Biochemicals, Cleveland, Ohio). After electrophoresis, sequencing gels were exposed to Kodak XAR5 film (Eastman Kodak Co., Rochester, N.Y.) for 72 h at −70°C with intensifying screens. Revertant sequencing was limited to the regions encoding the mutations found in the parental single mutants *mar* B2.1 and B4.1.

***mar* mutant rate of entry.** The rate of entry for KOS-321 was determined (14) by using an acid restricted entry assay adapted from Huang and Wagner (20). Briefly, virus was adsorbed to Vero cell monolayers at 4°C, at which temperature penetration is prevented (18). Monolayers were washed with phosphate-buffered saline–$MgCl_2$, overlaid with medium, and incubated at 37°C. At various times after the temperature shift, monolayers were treated for 1 min with a low-pH citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl [pH 3.0]) and then washed promptly with phosphate-buffered saline–$MgCl_2$. Monolayers were overlaid with methylcellulose (2% fetal calf serum; GIBCO), and plaque formation was scored after 3 days. Treatment of monolayers with the low-pH buffer causes the inactivation of all extracellular virus. Therefore, virus that entered cells was resistant to such treatment and formed plaques on the infected monolayer. At selected intervals, the percentage of intracellular virus was calculated as the number of surviving PFU on citrate-treated monolayers divided by the number of plaques produced on untreated monolayers, multiplied by 100.

Radioimmune precipitation of insertion mutants in HSV-1 gB. The generation of insertion mutants in HSV-1 gB (KOS) has been published elsewhere by Cai et al. (5). Transfection of mutant gB plasmids and their expression in Vero cells by stimulation with a gB^- virus (K082) was described previously (5, 13). Each mutant plasmid was individually transfected into cells and infected with K082 48 h later. Cultures were labeled with [^{35}S]methionine for 1 h, solubilized, and precipitated with antibody pools or individual MAbs. After recovery with protein-A Sepharose beads, polypeptides were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels and fluorographed as described previously (16).

Western blotting. Infected cell lysates were subjected to electrophoresis on 10% polyacrylamide slab gels as described previously (16). Transfer to BA85 nitrocellulose

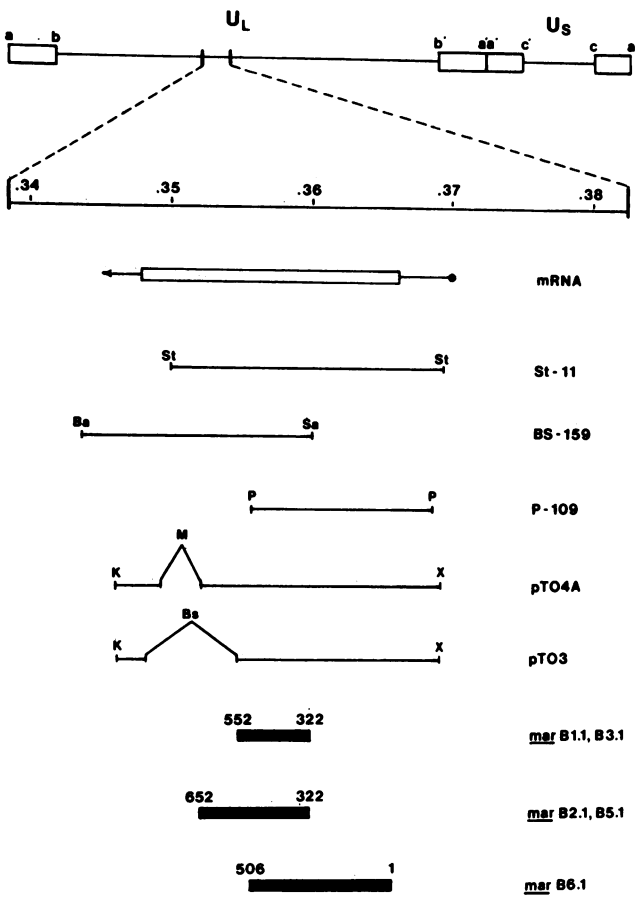

FIG. 1. gB marker rescue fragments. Five different cloned gB fragments were used to marker rescue *mar* B mutations in cotransfection assays. All were derived from the *Eco*RI-F fragment of strain KOS (15). The locations of the gB message (←●) and the coding region (▭) in relation to the rescuing fragments (⊢⊣) are indicated. The regions shown to contain *mar* mutations (▬) are derived from the results in Table 1. Key: Ba, *Bam*HI; Bs, *Bst*EII; K, *Kpn*I; M, *Mlu*I; P, *Pst*I; Sa, *Sal*I; St, *Sst*I; X, *Xho*I.

(Schleicher & Schuell Co., Keene, N.H.) was performed overnight at 10 V/cm in blotting buffer (15 mM Tris, 125 mM glycine, 20% methanol) at 15°C. The transferred filter was blocked with 5% bovine serum albumin in phosphate-buffered saline for 1 h at 37°C, followed by incubation with primary and secondary antibody for 1 h each at 37°C. Between incubations, filters were washed with phosphate-buffered saline at 37°C. Detection of specifically bound antibody with 4-chloro-1-napthol (Sigma) was performed as described previously (17).

RESULTS

Mapping of gB *mar* mutations. To define discrete regions of the gB gene to be sequenced for each of the six single *mar* mutants, marker rescue experiments were performed with the gB containing plasmids diagrammed in Fig. 1. St-11, BS-159, and P-109 were all subcloned from the KOS *Eco*RI F fragment as described previously (15). These plasmids contain sequences of the HSV-1 KOS gB gene corresponding to amino acid residues 1 through 816, 321 through 904, and 1 through 506, respectively. pTO3 and pTO4A are also *Eco*RI-F subclones that have internal deletions corresponding to regions in the external, transmembrane, and cytoplasmic domains of gB. They contain coding sequences for the entire gB protein exclusive of amino acid residues 653 through 824 in pTO4A and 553 through 875 in pTO3.

TABLE 1. Marker rescue frequencies of gB *mar* mutants[a]

mar Mutant	Rescue frequency of gB plasmids				
	BS-159	St-11	P-109	pTO3	pTO4A
B1.1	1.0	2.2	<0.1	0.9	NT[b]
B2.1	2.2	3.5	<0.1	<0.1	2.0
B3.1	1.1	1.5	<0.1	4.1	NT
B4.1	<0.1	<0.1	<0.1	<0.1	<0.1
B5.1	4.8	8.5	<0.1	<0.1	3.1
B6.1	<0.1	1.0	3.5	NT	NT

[a] Rescue frequency as determined by percent black-plaque recombinants (17).
[b] NT, Not tested.

Cotransfection experiments with genomic *mar* mutant DNA and linearized plasmid DNA were performed as described previously (19). Marker rescue of the *mar* phenotype was determined on the basis of black plaques in an immunoperoxidase plaque assay (17). All *mar* B mutants failed to be recognized by the antibodies to which they are resistant (24). Therefore, plaques formed by recombinants in which the *mar* mutation was replaced by wild-type KOS sequences could be identified by their reactivity with the selecting MAb (16).

Site I mutants *mar* B1.1 and B3.1 were rescued by St-11, BS-159, and pTO3 (Table 1), indicating the presence of a mutation in the region coding for residues 322 through 552. Similarly, site II mutants *mar* B2.1 and B5.1 were rescued by St-11, BS-159, and pTO4A (Table 1), localizing the mutation to the sequences corresponding to residues 322 through 652. *mar* B6.1 was rescued with St-11 and P-109, indicating an alteration in the region encoding residues 1 through 506 (Table 1). These results are depicted at the bottom of Fig. 1.

The site III mutant, *mar* B4.1, was not successfully rescued with any of the fragments shown in Fig. 1. However, it was rescued (4.0%) with the entire *Eco*RI F fragment (map coordinates 0.315 to 0.421), placing the mutation in the same general region of the genome as the gB gene. Thus, we were unable to confirm that the mutation causing the *mar* B4.1 phenotype lies solely within the coding sequence for gB. However, gB alone is specifically precipitated from detergent-solubilized infected-cell extracts by antibody B4 (13), making it unlikely that another virus- or host-encoded molecule is involved in maintenance of antigenic site III.

DNA sequencing of *mar* mutants in gB. Both enzymatic (30) and chemical cleavage (26) sequencing strategies were employed in *mar* mutant sequencing. All coding sequences implicated in marker rescue experiments were sequenced. Because we were unsuccessful in attempts to map the *mar* B4.1 mutation, the entire gB coding sequence of this mutant was determined.

A base transition of G to A at nucleotide 2207 resulted in a Ser-to-Asn change at residue 473 for both site I mutants, *mar* B1.1 and B3.1. A transition of G to A at nucleotide 2569 caused the Gly at residue 594 to become an Arg in both *mar* B2.1 and B5.1 (site II). Two G-to-A nucleotide transitions were found in the site III mutant *mar* B4.1. The first occurred at nucleotide 1702 and resulted in a Glu-to-Lys substitution at residue 305. The second, at nucleotide 2419, did not result in a change in the amino acid sequence of the translated product. In antigenic site IV, a transition of G to

SITE I

	469								477
					Asn				
mar B1.1, 3.1	Leu	Arg	Glu	Gln	Ser	Arg	Lys	Pro	Pro
	CTC	CGA	GAG	CAG	AGC	CGC	AAG	CCC	CCA
					A				
	2194								2220

SITE II

	590								598
					Arg				
mar B2.1, 5.1	Ser	Ser	Arg	Pro	Gly	Ala	Cys	Tyr	Ser
	AGC	TCG	CGG	CCC	GGG	GCC	TGC	TAC	AGC
					A				
	2557								2583

SITE III

	301								309
					Lys				
mar B4.1	Tyr	Gly	Tyr	Arg	Glu	Gly	Ser	His	Thr
	TAC	GGC	TAC	CGG	GAG	GGG	TCG	CAC	ACC
					A				
	1690								1716

SITE IV

	081								089
					Asp				
mar B6.1	Pro	Arg	Pro	Ala	Gly	Asp	Asn	Ala	Thr
	CCA	CGC	CCC	GCC	GGC	GAC	AAC	GCG	ACC
					A				
	1030								1056

FIG. 2. Nucleotide and amino acid substitutions in *mar* B mutants. The regions depicted at the bottom of Fig. 1 and the entire coding sequence for *mar* B4.1 were sequenced as described by Maxam and Gilbert (26) or Sanger et al. (30). Individual base changes and the predicted amino acid changes are shown in boldface type for each mutant compared with wild-type KOS-321 sequences (29). Substitutions in *mar* B1.1 and 3.1 and in *mar* B2.1 and 5.1 were identical, although these mutants were isolated with clonally distinct antibodies. The numbering scheme for the DNA sequence assigns the number 1 to the first base of the *Xho*I site at 0.372 map units on the viral genome (15; S. Person, unpublished data). Numbering for amino acids designates the initiation methionine as 1.

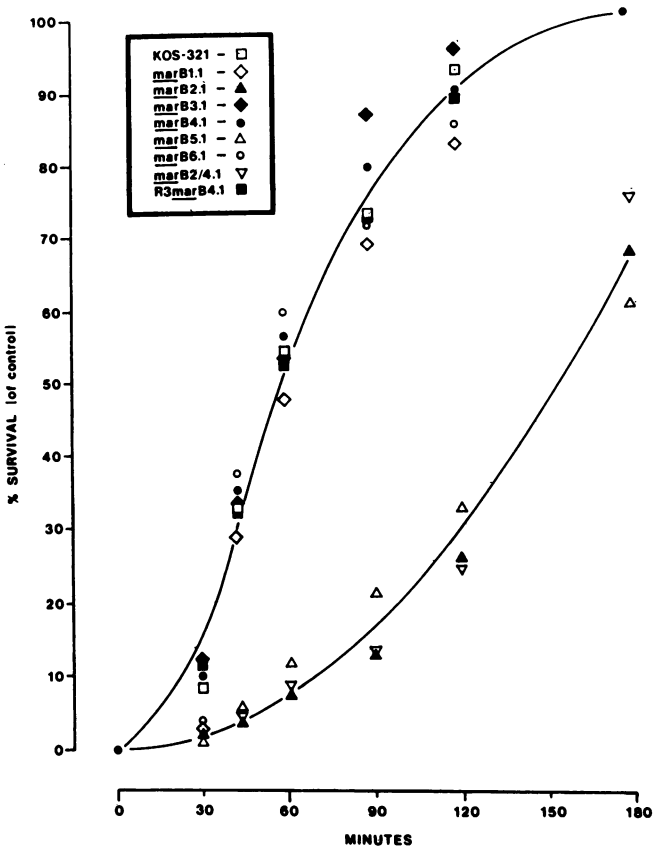

FIG. 3. Rate of entry kinetics for *mar* B mutants. The rate of penetration for KOS-321 and antigenic variants was determined by using a citric acid buffer inactivation assay as described previously (13). Wild-type and mutant viruses were adsorbed to cell monolayers at 4°C for 2 h and then shifted to 37°C. At the indicated times, monolayers were treated with citrate buffer (pH 3.0) for 1 min. The percent survival represents the fraction of input virus that entered cells at a given time, compared with a control sample that received no acid treatment.

A at nucleotide 1043, changed Gly at residue 85 to Asp in *mar* B6.1. Therefore, one amino acid substitution was predicted to occur in the sequenced region of each *mar* mutant (Fig. 2).

Effect of antigenic variation on rate of virus entry. Previously, we showed that antigenic variation in HSV-1 gB can result in impaired glycoprotein processing and function at high temperature (24). The gB rate of entry phenotype (11) was attributed to a mutation at residue 553 in the *ts* B5 variant, which mapped within the limits of the external domain of gB (4, 10). Therefore, the rate of entry for each *mar* B mutant was assayed to determine whether antigenic variation in gB can also cause alterations in the rate of virus entry.

Virus was adsorbed to cell monolayers at 4°C to prevent penetration (18) and then shifted to 37°C to initiate entry. At various times after the shift, monolayers were washed briefly with a low-pH citrate buffer, resulting in inactivation of all extracellular virus (14). Internalized virus was resistant to such treatment and replicated to form plaques. The wild-type virus, KOS-321 (Fig. 3), entered cells slowly up to 30 min after shift to 37°C but began to enter rapidly at 45 min after shift. This rate was maintained until most virus had entered cells (2 h postshift), with all infectious virus becoming resistant to citrate treatment by 3 h postshift. Similar entry kinetics were observed with *mar* mutants B1.1, B3.1, B4.1, and B6.1 (Fig. 3). However, *mar* B2.1 and B5.1 (Fig. 3) entered cells more slowly than did the wild type and did not achieve complete entry until 5 to 6 h postshift. These two mutants were both selected by antibodies recognizing site II, suggesting that the *mar* mutation was responsible for the altered entry phenotype.

In a previous study (24) a *ts* mutant, *mar* B2/4.1, was generated by mixed infection with the mutants *mar* B2.1 and *mar* B4.1 followed by selection with MAbs B2 and B4. Then a revertant of this double mutant was selected on the basis of growth at high temperature. This mutant had also partially regained the ability to process gB and simultaneously regained sensitivity to neutralization with antibody B2, indicating that the *mar* and *ts* phenotypes were the result of the same genetic alteration (24). However, the revertant remained resistant to antibody B4 and was therefore designated R3 *mar* B4.1.

To determine whether the *mar* and slow entry phenotypes were the result of the same mutation, the entry kinetics for the double *mar* mutant and revertant were tested in the assay described above. *mar* B2/4.1 entered cells at a rate similar to the parent, *mar* B2.1 (Fig. 3). The finding that the combination of the B2 and B4 mutations in a single gB gene had no

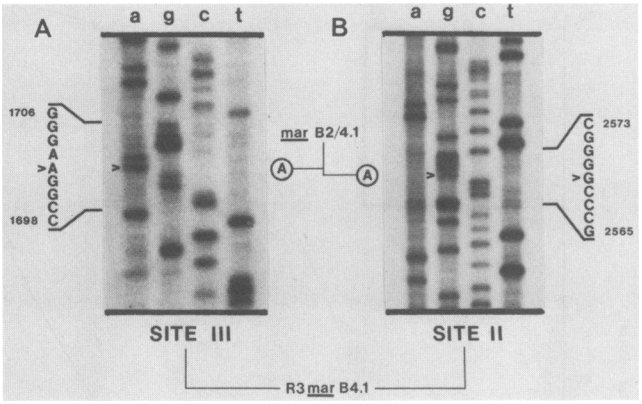

FIG. 4. Sequence of R3 *mar* B4.1 with genomic viral DNA as a template. A ^{32}P-end-labeled 16-base-pair oligonucleotide primer was annealed to 1 μg of CsCl-purified revertant DNA template and sequenced essentially as described previously (21; Huibregtse et al., in press). After ethanol precipitation, samples were suspended in 4 μl of loading dye and subjected to electrophoresis on an 8% denaturing acylamide gel. Panel A shows sequences surrounding the site III mutation, and panel B shows sequences surrounding the site II mutation. Arrows indicate the location of altered nucleotides and circled bases depict the relevant nucleotides in the parental mutant *mar* B2/4.1.

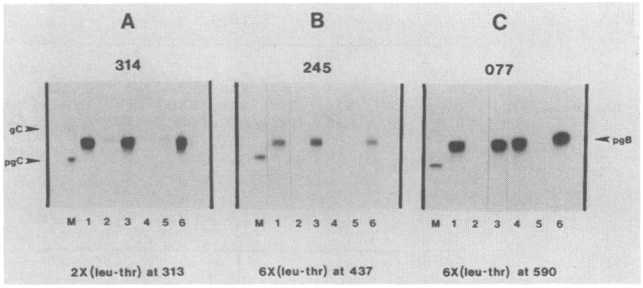

FIG. 5. Radioimmune precipitation of *Hpa*I linker-insertion mutants of HSV-1 gB. Vero cells transfected with insertion mutant plasmid DNA were infected 48 h posttransfection with the gB$^-$ mutant K082. After infection for 7 h, cells were labeled for 1 h with 50 μCi of [^{35}S]methionine per ml. Panels A, B, and C show detergent-solubilized extracts of cells transfected with mutants 314, 245, and 077, respectively. Lane M is an extract of mock-transfected, K082-infected Vero cells precipitated with a pool of anti-HSV glycoprotein C MAbs (25). Lane numbers identify precipitation with the indicated antibody (1 through 6).

additional effect on rate of entry was expected, since only the parental mutant *mar* B2.1, and not the *mar* B4.1 parent, exhibited the slow entry kinetics. As anticipated, the revertant R3 *mar* B4.1 entered cells at a rate similar to that of wild-type virus and of the *mar* B4.1 parent (Fig. 3). Therefore, reversion of the B2 antibody resistance was accompanied by reversion of the slow entry kinetics displayed by *mar* B2.1. This result strongly suggested that, like the temperature-dependent defect in gB processing seen in *mar* B2.1, the reduced rate of virus entry was caused by the site II *mar* mutation.

Identification of nucleotide and predicted amino acid reversion in R3 *mar* B4.1. As described above, the revertant R3 *mar* B4.1 was used to demonstrate that the *ts* and *roe* phenotypes were attributable to the *mar* mutation in site II. To determine the exact genetic nature of the reversion event and to confirm the association of the *mar*, *ts*, and *roe* phenotypes, regions of the gB gene from R3 *mar* B4.1 corresponding to the *mar* B4.1 and *mar* B2.1 lesions were sequenced. This analysis was performed by using a novel sequencing technique where the intact viral genome was used as a template for double-stranded dideoxy sequencing (see Materials and Methods).

The parental mutant *mar* B 2/4.1 had mutations identical to those that resulted in amino acid substitutions in the single *mar* mutants, B2.1 and B4.1, from which it was derived (data not shown). The G-to-A transition causing site III neutralization resistance located at nucleotide 1702 (Fig. 1) was present in the revertant (Fig. 4A). However, the site II G-to-A transition seen at nucleotide 2569 in *mar* B2.1 (Fig. 1) was no longer present, and the revertant contained the wild-type sequence at this location (Fig. 4B). Therefore, precise reversion of amino acid 594 is predicted and confirms that the site II *mar*, *ts*, and *roe* phenotypes result from the same base substitution.

Immune precipitation of HSV-1 gB insertion mutants. Previously, it was shown that selecting antibodies failed to immunoprecipitate gB produced by *mar* mutants (24). In addition, antibodies representing two antigenic sites (II and III) reacted with the gB precursor (pgB) from a number of *ts* mutants grown at 34°C but failed to recognize the same molecule when it was produced by infection at 39°C (24). This result suggested that antibodies specific for sites II and III recognized discontinuous epitopes whose integrity relied on a higher-order structure.

To further identify regions of gB involved in the maintenance of these structures, a series of plasmids containing *Hpa*I linker-insertion mutations in the gB gene (5) were examined. These linkers varied in number and resulted in 2 to 28 in-frame amino acid insertions at different locations throughout the gB product. The effect of insertions on molecular structure was measured by alteration in the ability of MAbs to recognize mutant polypeptides in radioimmune precipitation assays. All insertions in the external domain resulted in altered gB processing and the production of gB molecules that were unable to complement a gB$^-$ virus, indicating a lethal defect in virus replication (5). The single insertion in the cytoplasmic domain had no apparent effect on gB synthesis or virus infectivity.

Experiments were performed by transfecting mutant plasmid DNA into Vero cells, followed by superinfection with a gB$^-$ virus (K082) to activate the expression of the mutant gB gene (5, 13). Infected cells were pulsed for 1 h with [^{35}S]methionine at 7 h postinfection and immunoprecipitated with MAbs B1 through B6. Figure 5 shows the results for three different insertion mutants. Figure 5A shows precipitations from cells transfected with mutant 314, which contains a four-amino-acid insertion after residue 313. Lane 4 lacked the band present in other lanes, demonstrating a loss of recognition by antibody B4 (site III). This result was not unexpected, since the *mar* mutation at residue 305 (Fig. 2) caused a similar loss of reactivity (24), indicating that this region of gB is important for recognition by this monoclonal antibody. Of interest was the observation that this same insertion affected precipitation of gB by two other antibodies, B2 and B5 (Fig. 5A, lanes 2 and 5, respectively), both specific for antigenic site II and for which the corresponding *mar* mutation maps some distance away (Fig. 2). This suggests that sites II and III may share a common structural entity even though residues involved in their recognition by antibodies lie far apart on the primary sequence. Fig. 5B shows another insertion, 245, which adds 12 amino acids after residue 437. Antibodies B2, B4, and B5 all failed to precipitate this molecule (Fig. 5B, lanes 2, 4, and 5, respec-

TABLE 2. Radioimmune precipitation of gB *HpaI* linker-insertion mutants

Plasmid	Residue	No. of inserted amino acids	Precipitation at antigenic site[a]:					
			IV (B6)	III (B4)	I		II	
					B1	B3	B2	B5
265	189	12	+	r	+	+	−	−
185	234	12	+	r	+	+	r	r
314	313	4	+	−	+	+	r	r
147B	380	6	+	+	+	+	r	r
245	437	12	+	−	+	+	−	−
154	463	28	+	+	+	+	r	r
077	590	12	+	+	+	+	−	−
177B	595	6	+	+	+	+	−	−
086	623	14	+	+	+	+	r	r
070	734	2	+	+	+	+	r	r
117	816	12	+	+	+	+	+	+

[a] +, Precipitation of mutant polypeptide; r, significantly reduced precipitation when compared with wild-type gB; −, complete absence of precipitation.

tively). This insertion lies between residues predicted to be critical for recognition by these antibodies (Fig. 2). Figure 5C shows the results for mutant 077, which carries an insertion of 12 amino acids after residue 590. Reactivity of site II antibodies (B2, B5) (Fig. 5C, lanes 2 and 5, respectively) was abolished by this insertion. Again, this result was not unexpected, since the site II *mar* mutation is located at residue 594 (Fig. 2). In addition, this mutant also demonstrated that loss of site II recognition does not necessarily correlate with reduction or loss of site III recognition, as would be suggested by Fig. 5A and B.

Table 2 shows the reactivity of all six antibodies with the entire panel of insertion mutants. Several general trends are apparent. Site II antibody recognition was inhibited, either partially or completely, by each of the 10 insertions in the external domain of gB. Neither the size nor the location of the insertion appeared to correlate with reduction versus complete loss of reactivity. Site III antibody recognition appeared to be somewhat less affected but was altered by insertions throughout the amino-terminal half of the molecule. Site I and site IV antibody recognition was not affected by any insertion, suggesting that these determinants are less dependent than the others on tertiary structure. In fact, mutant 154 had 28 amino acids inserted 11 residues away from the residue affected by site I *mar* mutations (Fig. 2), yet this alteration had no effect on precipitation of pgB by antibodies B1 and B3. Finally, the single insertion in the cytoplasmic domain (mutant 117) did not affect antibody recognition of any site, suggesting that this region has little or no effect on the structure of the external domain.

Determination of antibody recognition in Western blots. Recognition of antigenic sites in gB molecules containing insertion mutations varied widely, suggesting differing contributions of higher-order structure to antibody binding. To further examine the requirements for antibody recognition, the entire panel of antibodies was tested for the ability to react with denatured gB in Western blots. KOS-321-infected cell lysates were subjected to electrophoresis, blotted, and incubated with MAbs B1 through B9. Only antibody B6 (site IV) recognized the denatured gB molecule (data not shown), suggesting that antibody recognition of this site relies on relatively localized structures. Like B6, site I antibodies (B1 and B3) were type specific (24) and inhibited virus penetration (13), and their reactivity with gB was not inhibited by insertional mutagenesis (Table 2). However, unlike B6, these MAbs did not react with denatured gB. Therefore, maintenance of antigenic sites I, II, and III must be dependent on structures in gB that are lost upon denaturation.

DISCUSSION

Our studies of the immunobiology and function of HSV glycoproteins have been greatly facilitated by the use of virus-neutralizing glycoprotein-specific MAbs and *mar* mutants (14, 24, 25). In some instances, antibody binding interfered with infectivity, suggesting that antigenically important structures overlap with regions that directly contribute to virus penetration (13, 14). Consistent with this prediction, some antibody-selected variants were altered in gB function (24) (Fig. 3). Therefore, this type of analysis serves two purposes. First, antigenic sites on HSV glycoproteins can be operationally identified and characterized on the basis of antibody-mutant reactivity patterns, thus providing information on the antigenic structure of the molecule. Second, with MAbs and antibody-selected mutants as complementary molecular probes, large domains, and specific residues can be examined for functional importance. Below, we summarize the results of our immunologic analysis of gB. In addition, mutations that affect the function of gB are discussed in the context of antigenic sites and their respective contributions to virus infectivity.

Antigenic site IV. Antigenic site IV appears to reside near the amino terminus of the gB molecule (13) (Fig. 6). Although it was not possible to accurately define the limits of site IV by using truncation mutants (13), a deletion mutant missing residues 44 to 233 of gB was shown to lack site IV by radioimmune precipitation (S. Highlander, unpublished data). This agrees with the location of the site IV mutation at residue 85. In addition, the single site IV-specific antibody, B6, recognized denatured gB in a Western blot, suggesting that site IV consists of a linear determinant.

The Gly residue at amino acid 85 is type specific since HSV-2 contains an Arg at that same position. This correlates with the finding that antibody B6 neutralized HSV-1 and not HSV-2 and that this region of gB is relatively nonhomologous when compared with other herpesvirus gB homologs (2, 9, 22, 27, 29, 32). Despite the lack of conservation in this region, B6 inhibits the rate of virus penetration (13) and is notable as being our only gB-specific MAb that inhibited cell-to-cell spread of virus (13) and prevented zosteriform spread of virus in vivo (J. Mester, J. C. Glorioso, and B. Rouse, unpublished data).

Antigenic site III. The single site III antibody, B4, required sequences between residues 283 and 380 for immune precipitation of gB (13) (Fig. 6). A base substitution was found in *mar* B4.1 which resulted in a Glu-to-Lys conversion of residue 305. Amino acid 305 was shown to be a Glu in HSV-2 as well, correlating with the ability of antibody B4 to neutralize both serotypes (24). In the absence of complement, neutralization of both HSV-1 and HSV-2 by B4 resulted from prevention of virus penetration (13). The substitution at residue 305 also affected terminal glycosylation in the mutant *mar* B4.1 at a high temperature (24). These findings indicate that structures represented by antigenic site III data are important to the function of gB. In two strain KOS mutants, *ts* J20 and *ts* J12, changes at residues 277 and 373, respectively, have been identified which inhibit gB processing at high temperature and render mutant virus incapable of penetrating cell surfaces (3) (Fig. 6), supporting the notion that this region can affect gB processing and, by extension, virus infectivity. Unlike *mar* B4.1, these mutants

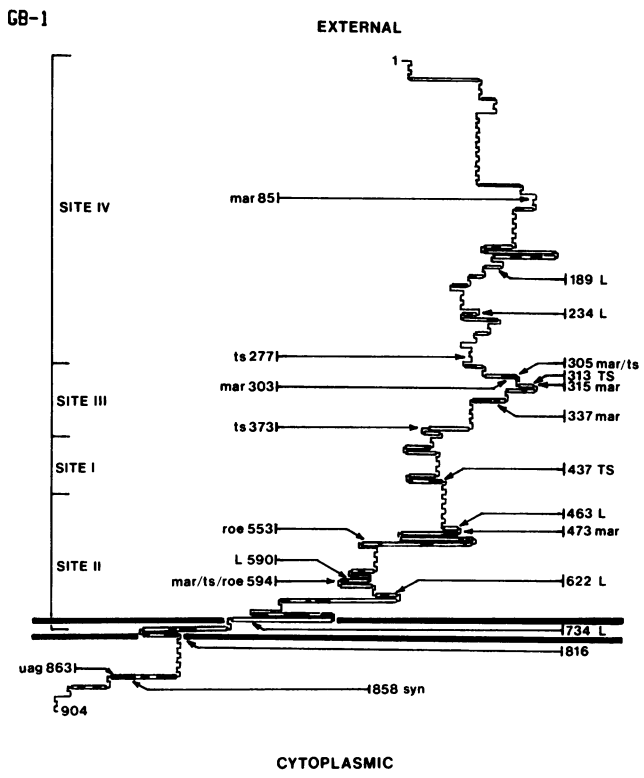

FIG. 6. Predicted secondary structure of HSV-1 KOS-321 gB and location of known mutations. Mutations in HSV-1 KOS-321 gB that have been identified by DNA sequencing are depicted on a schematic representation of the molecule generated by the protein structure analysis program MSEQ (1) with Chou and Fassman parameters (8). Alpha helices (▬), beta sheets (▭), random coil (—), and beta turns (⊏) are depicted. Other published mutations are shown as well (4, 6, 7, 20a, 28). Uppercase letters denote insertion mutations, and lowercase letters denote amino acid substitutions. The amino acid number indicates the altered residue (substitution, termination) or the residue preceding insertion. Antigenic sites are shown as predicted by Highlander et al. (13).

are neutralized by antibody B4, identifying a second type of *ts* mutation in this region that does not alter antigenic recognition of site III (S. Highlander, unpublished data).

As reported earlier, antibody B4 was able to immunoprecipitate pgB produced by a number of *ts* mutants at 34°C but failed to recognize the same molecule at the nonpermissive temperature (24). This finding indicated that site III was dependent on gB conformation, a prediction confirmed by the fact that insertions over a broad region (residues 189 to 437) reduced or blocked recognition of gB by B4 (Table 2, Fig. 6). Moreover, all of these insertion mutations resulted in the production of inactive gB molecules as measured by their failure to complement a gB^- virus in transient expression assays (5) (Fig. 6). Taken together, these observations suggest that a higher-order structure is required to maintain antigenic site III and necessary for gB function in virus penetration.

Pellet et al. reported the location of mutations that result in neutralization resistance in HSV-1 strain F gB (28). Of the three mutants examined, all had changes between residues 303 and 335, close to our site III mutation at residue 305. In addition to being a highly antigenic area of the molecule (Fig. 6), it is almost identical in HSV-1 and HSV-2 (2, 32) and is highly homologous among all herpesvirus gB genes examined (9, 22, 27, 29). This conservation of the primary sequence suggests a tertiary homology as well and indicates that the structure of this region is important for gB function. This is supported by the demonstration that antigenic variation in this site can result in temperature-dependent instability that leads to defects in glycosylation and function at high temperature (5, 24).

Antigenic site I. With truncated gB molecules, site I-specific antibodies were predicted to recognize a structure between residues 381 and 441 (13) (Fig. 6). However, the site I mutation in *mar* B1.1 and B3.1 was located at residue 473. The Ser-to-Asn change at this residue was unexpected in that site I antibodies immunoprecipitate a gB molecule containing only the amino-terminal 441 amino acids, suggesting that an interaction with residue 473 is not required for antibody binding. This could be explained by the introduction of the larger, charged side chain, which prevents antibody contact with residues contained within the first 441 residues of the gB molecule. Alternatively, residue 473 may be required for neutralization, but its loss may not be sufficient to eliminate antibody binding. The first hypothesis is supported by the finding that gB produced by *mar* B1.1 and B3.1 is not precipitated by a site I antibody (24). These molecules contain residues sufficient for antibody recognition (1 through 441) but are not precipitated. Sequence variation in this region in other herpesvirus gB genes (9, 22, 27, 29) suggests little conservation of function. However, site I-specific antibodies did reduce the rate at which the virus entered cells (13), indicating that this general region may play a role in virus infection.

Antigenic site II. In addition to causing *ts* defects in carbohydrate processing at high temperature (24), *mar* mutants selected by site II-specific antibodies also have a reduced rate of virus penetration (Fig. 3). The identification of the site II *mar* mutation at residue 594 not only agreed with the predicted location of site II antibody recognition (13) but also mapped 41 amino acids away from the rate of entry mutation in *ts*B5 at residue 553 (4) (Fig. 6). Because antibodies specific for site II are reactive with both serotypes, the finding that Gly at residue 594 is conserved in both HSV-1 and HSV-2 was not surprising because site II antibodies can neutralize HSV-2. This residue is located in a region (residues 500 through 700) of gB that is highly homologous with other herpesviruses (2, 9, 22, 27, 29, 32), suggesting a conservation of higher-order structure and function.

Like site III antibodies, site II antibodies fail to recognize the pgB produced by *ts* mutants at the nonpermissive temperature (24) and did not bind denatured gB in a Western blot. However, site II was even more susceptible to destabilization than site III since every insertion tested in the external domain either reduced or destroyed antibody recognition of site II (Table 2). Some insertions affected both site II and III recognition, but the corresponding *mar* mutations for these sites lie 289 residues apart (Fig. 2). Although these data are difficult to interpret without detailed information on the tertiary structure of gB, we speculate that these nonoverlapping epitopes are contained within a common higher-order structure created by folding of the molecule. Alternatively, sites II and III could represent structures that are spatially distinct yet sensitive to denaturation by insertions throughout the external domain of gB. Regardless, the finding that at least two of the four sites we have defined are comprised of widely separated sequences is not unexpected. Chapsal and Pereira demonstrated that over half of the

gB-specific MAbs tested were directed against discontinuous epitopes (7).

Conclusion. In considering the data presented here and previously, it seems clear that antigenic sites II and III contain structures important for gB function in virus infection. This conclusion is based on the following evidence. First, mutations that alter the ability of gB to function in virus penetration occur within sequences defined as necessary for the recognition of these sites (13). These mutations can occur naturally (3, 4) or may be selected on the basis of resistance to neutralizing antibodies (Fig. 2). Second, both sites are type common, and the primary sequences that make up these regions are very conserved between many gB homologs. This suggests that their secondary and tertiary structures are also conserved and likely reflect structural constraints imposed by gB function. Third, only these sites are lost from the replication-defective *ts* and insertion mutants. It may be that the structures recognized by these antibodies are required for gB function and are lost in mutant gB molecules. However, only antibodies to site III can neutralize virus infectivity without complement. Antigenic sites III, I, and IV lie in the amino-terminal half of the gB molecule, and antibodies specific for each of these sites inhibit virus infectivity. Antibody binding to this region may sterically inhibit an interaction that is required for penetration. Conversely, antibodies specific for antigenic site II do not inhibit penetration yet select mutants which show altered entry kinetics. Although this determinant is affected by much of the structure of the external domain, it apparently does not contribute to gB function in the same manner as the sequences comprising site III. On the basis of the location of site II *mar* mutations, it appears to be very important to the entry process nonetheless.

It remains to be seen whether these regions have discrete functions and to what degree antigenic sites II and III interact. Using smaller, cloned fragments of the gB gene to express portions of the polypeptide, we hope to determine whether a fraction of the mature molecule can specifically interact with another. If so, it may be possible to use intra- or intermolecular complementation between different mutants to identify distinct functional domains. These experiments, coupled with an extensive mutational analysis of these regions, may lead to a more precise definition of residues and structures that contribute to gB function.

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