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Genetics

Application of the mini-Mu-phage for target-sequence-specific insertional mutagenesis of the herpes simplex virus genome

(thymidine kinase selection/random insertion/genetic engineering)

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ABSTRACT **An earlier technique for insertional mutagenesis of large viral genomes involved the insertion of the thymidine kinase (TK) gene at a specific target site, cotransfection of the fragment carrying the insertion with the intact viral genome, and selection of the progeny for viral recombinants expressing the *TK* gene. The inserted *TK* gene could then be replaced by cotransfection of the recombinant DNA with fragments carrying a foreign sequence or a deletion in the target sequence. To enable the probing of larger target domains and facilitate insertional mutagenesis, we extended this technique by insertion of a 2.2-kilobase-pair (kbp) herpes simplex virus 1 (HSV-1) chimeric *αTK* gene into the 7.5-kbp mini-Mu-phage (*αTK*-mini-Mu) and lysogenized *Escherichia coli* with the helper Mu phage and the *αTK*-mini-Mu. Induction of phage multiplication of the lysogenized *E. coli* after transformation with plasmids carrying HSV-1 DNA and subsequent infection of *E. coli* RecA$^+$ lysogenized with Mu phage yielded plasmid populations carrying randomly inserted *αTK*-mini-Mu DNA. Application of this procedure for insertional mutagenesis of the *Bam*HI B fragment, which spans the junction between the unique and reiterated sequences of the L component of viral DNA, yielded two types of recombinants. Viral recombinant designated RBMu1 contained the intact *αTK*-mini-Mu inserted into unique sequences of *Bam*HI B fragment. In recombinant RBMu2, the *αTK*-mini-Mu was inserted at or in the repeated sequences, but ≈14 kbp comprising most of the internal reiterations of the viral genome in the prototype arrangement were deleted.**

Post and Roizman (1) have described a generalized technique for site-specific insertion or deletions in the chromosomes of large viral genomes, applied to herpes simplex virus 1 (HSV-1) DNA. The technique consists of two steps. First, HSV-1 thymidine kinase (TK) gene is inserted into a cloned DNA fragment at the desired site. The amplified chimeric fragment is then cotransfected with intact DNA of a HSV-1 recombinant virus carrying a deletion in the *TK* gene, and the progeny of the transfection is selected for TK$^+$ phenotype. In the second step, an appropriate insertion or deletion is made in the target sequence at the site of insertion of the *TK* gene. The fragment is amplified and cotransfected with the DNA of the recombinant carrying the *TK* gene in the target sequence, and the progeny is selected for TK$^-$ phenotype. In each instance, the recombination of the DNA fragments carrying the *TK* gene or the deletion in the target sequence is facilitated by the presence of homologous sequences flanking the insert or deletion. The *TK* gene was used as a selectable marker because of the availability of powerful selection both for and against the gene. In the case of HSV-1 DNA, a simple modification of the *TK* gene used for insertional inactivation of the target gene can insure that the recombination occurs predominantly if not exclusively into the target sequence rather than restores the activity of the *TK* gene in its natural location in the HSV-1 genome (2, 3). This procedure has been applied to construct recombinants carrying a deletion in an *α* gene (*α22*) of HSV-1 and to delete noninformational sequences located in the internal inverted repeats in the HSV-1 genome (1, 3). Application of this procedure resulted in the isolation of a viable HSV-1 virus from which 15 kilobase pairs (kbp) comprising the internal inverted repeat sequences had been deleted and which is frozen in one of the four isomeric arrangements of the viral genome (2).

Although this technique has been used successfully in several applications, it has two disadvantages. The minor one is that site-specific insertion of the *TK* gene into a target sequence probes only the function of the immediate environment of the inserted site. In many instances there are no readily available sites for insertion, or perturbation of the desired site results in extensive rearrangements that render the insertion technically complex or defeat the purpose of the insertion. A major disadvantage is that to ensure selection of viable recombinants, some knowledge of the underlying gene architecture is essential. Thus, attempts to insert the *TK* gene into the target gene domains containing gene overlaps may inactivate both the target gene and another as yet unidentified or poorly defined gene. In this paper we report a new procedure based on the mini-Mu-phage system of Castilho *et al.* (4). We inserted the selectable marker, an *α*-gene promoter-regulated *TK* gene, into the mini-Mu. Under appropriate manipulation of the system, the *αTK*-mini-Mu (*αTK*-mM) is inserted randomly into a DNA fragment in the bacterial vector. The DNA fragment carrying the selectable marker is then cotransfected with intact viral DNA. Although theoretically all chimeric fragments could recombine into the HSV-1 genome, only viable progeny containing the selectable marker will grow. Therefore, the procedure allows rapid scanning of the genome for genes or sequences that can be interrupted without affecting the growth of HSV-1 in cell culture.

METHODS AND MATERIALS

Cells and Viruses. African green monkey (Vero) cells were used for propagation of virus. A continuous line of rabbit skin cells was used for cotransfection with viral and plasmid DNA. The human 143 *tk*$^-$ cells (5) were used for selection of TK$^+$ viruses. The recombinant virus HSV-1(F)Δ305 was constructed to contain a 700-bp deletion in the *TK* gene (6); it was derived from HSV-1(F), the prototype HSV-1 strain used in this laboratory (7).

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Abbreviations: HSV-1, herpes simplex virus type 1; TK, thymidine kinase; kbp, kilobase pair(s); *αTK*-mM, *αTK*-mini-Mu; Kan^r, kanamycin resistance; Amp^r, ampicillin resistance.

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Bacteria and Plasmids. The following *Escherichia coli* strains were used in this study: MC1040 [F⁻ *araD139 ara*::(Mu *c*ts)3 Δ(*lac*)X74 *galU galK rpsL*]; M8820 [F⁻, *araD139* Δ(*ara-leu*)7697 Δ(*proAB-argF-lacI*POZYA)XIII *rpsL*]; M8820Mucts (M8820 with Mu *c*ts62); M8820TRMucts [M8820Mucts with *recA56* srl::Tn10 Tc^r (tetracycline resistance)]. The plasmid pBC4041 contains the mini-Mu element MudII4041 (Mucts62 A$^+$B$^+$ Kan^r) inserted into a pUC9 vector with the *Hae* II–*lac* fragment removed (4). pRB364 contains the *Bgl* II–*Bam*HI fragment of the HSV-1 *TK* gene fused to the HSV-1 *Bam*HI N fragment from which the S-component origin of HSV-1 DNA replication was deleted by a *Sma* I collapse (8). pRB112 and pRB114 contain the *Bam*HI B and N fragments respectively, of HSV-1 cloned into pBR322 (9).

Cloning of DNA. The procedures for the construction, cloning, screening, and purification of recombinant plasmids were as described (9).

Analysis of Recombinant Virus. Isolation, purification, and restriction enzyme analysis of recombinant virus DNA were as described (1–3). The *Bam*HI N probe used to detect the right terminus of the *Bam*HI N fragment consisted of the 3-kbp *Pvu* II–*Bam*HI subfragment obtained from *Pvu* II and *Bam*HI digests of pRB114. The procedures for labeling DNA fragments with ^{32}P by nick-translation and hybridization to electrophoretically separated DNA fragments transferred to nitrocellulose sheets were as described (8).

RESULTS

Construction of the *αTK*-mM Chimeric Plasmid pRB2500. The *Pvu* II fragment from pRB364 containing a portion of the 5′ transcribed noncoding and coding sequences of the HSV-1(F) *TK* gene fused to the promoter-regulatory domain of the *α4* gene was inserted in the orientation shown in Fig. 1 into the *Bam*HI site of the mini-Mu-phage MudII4041 contained in pBC4041. The resulting *αTK*-mM cloned as pRB2500 was 9.7 kbp in size (Fig. 1). The plasmid was maintained at 30°C in the *E. coli* strain MC1040, which contains a Mu *c*ts prophage preventing transposition of the mini-Mu genome to the *E. coli* chromosome.

Construction of Double Lysogen and Principles of Insertional Mutagenesis of Plasmid Molecules by *αTK*-mM. The procedure for insertional mutagenesis of HSV DNA fragments with *αTK*-mM consisted of three steps as illustrated in Fig. 2 and detailed in the legend. The first step involved (*i*) transfection of *E. coli* lysogenized with both the helper Mu *c*ts phage and the *αTK*-mM phage with plasmids containing specific HSV-1 DNA fragments and (*ii*) induction of phage multiplication. The resulting lysates included phages containing DNA molecules consisting of the transfected plasmid

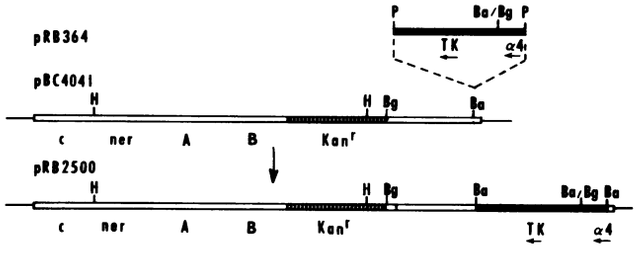

FIG. 1. Restriction endonuclease maps of mini-Mu-phage and construction of *αTK*-mM. The *αTK* chimeric gene from plasmid pRB364 was inserted in the orientation shown into the *Bam*HI site of pBC4041 carrying the mini-Mu. *A*, *B*, *ner*, and *c* are mini-Mu genes; Kan^r refers to the gene for kanamycin resistance, and *α4* refers to the promoter-regulatory domain of the *α4* gene. H, *Hin*dIII; Bg, *Bgl* II; Ba, *Bam*HI; P, *Pvu* II cleavage sites.

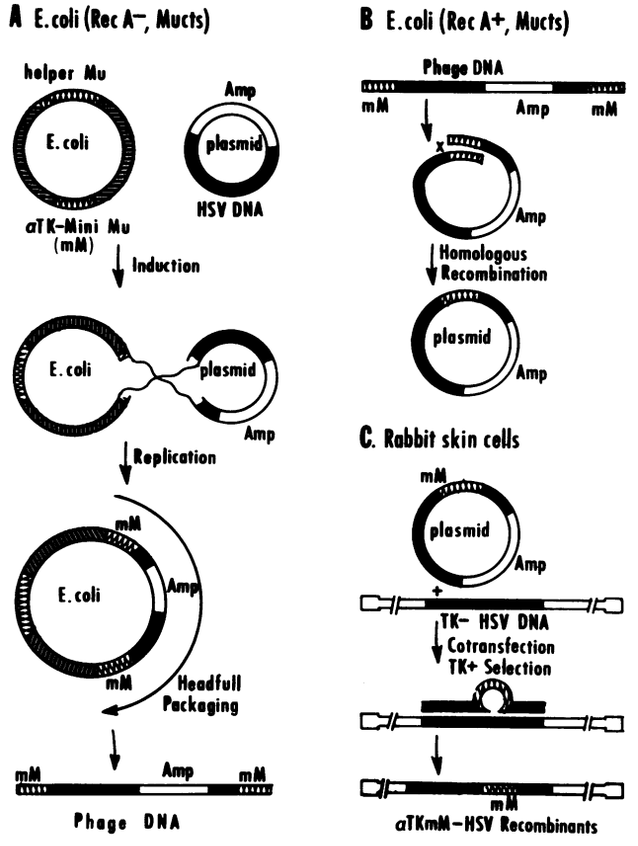

FIG. 2. Schematic representation of insertional mutagenesis of HSV-1 DNA by recombination with plasmid DNA molecules carrying *αTK*-mM. (*A*) Construction of the double lysogen and mini-Mu-phages carrying HSV-1 DNA sequences. *E. coli* strain MC1040 carrying the Mu *c*ts prophage was transformed with pRB2500 as described elsewhere (9). Induction at 42°C (4, 10, 11) resulted in the production of both helper Mu phage and *αTK*-mM phage. The lysate was used to transduce *E. coli* (RecA⁻) M8820TRMucts cells to Kan^r as described (4, 10, 11). Cells containing an integrated *αTK*-mM were selected by screening the transductants for a Kan^r, Amp^s (ampicillin sensitive) phenotype indicative of the presence of the *αTK*-mM prophage and loss of the plasmid DNA sequences. The presence of an integrated Mu *c*ts prophage was confirmed by inducing the cells at 42°C, infecting strain M8820 with the resulting lysate, and screening for plaque formation (not shown). The *αTK*-mM prophage is defective for replication; therefore, only the helper Mu *c*ts prophage is capable of producing plaques. The formal designation of this *E. coli* strain is M8820TRMucts MudII4041::*α*TK abbreviated as Mucts/*α*TK-mM. In the second step, *E. coli* Mucts/*α*TK-mM were transformed with plasmids containing HSV-1 DNA fragments as described (9). Induction at 42°C results in the formation of cointegrate structures (4). The relevant structure would consist of the plasmid molecule flanked by single copies of the *αTK*-mM prophage (Fig. 2*A*). The Mu DNA sequences are packaged into Mu phage heads starting with the left end of Mu and proceeding until a headful of DNA (≈38 kbp) has been packaged. If the length of the cointegrate structure (plasmid and two copies of *αTK*-mM) is <38 kbp, then flanking *E. coli* DNA sequences are packaged. A cointegrate structure whose length is >38 kbp would lack the mini-Mu sequences at the right end. (*B*) Conversion of the phage DNA carrying HSV-1 DNA sequences into plasmids. The lysates containing packaged cointegrate structures, and helper Mu phage were used to infect the RecA$^+$ strain M8820Mucts at 30°C. Infection at 30°C prevents transposition of any infecting Mu phage sequences and results in homologous recombination between the two copies of the *αTK*-mM prophage flanking the plasmid DNA sequences. After infection, ampicillin and kanamycin were added to select cells containing plasmid molecules with *αTK*-mM insertions. Since transposition in Mu phage is essentially a random event (12), plasmid DNA isolated from the Amp^r, Kan^r cells should contain a pool of plasmid molecules with single copies of the *αTK*-mM at different sites within the plasmid

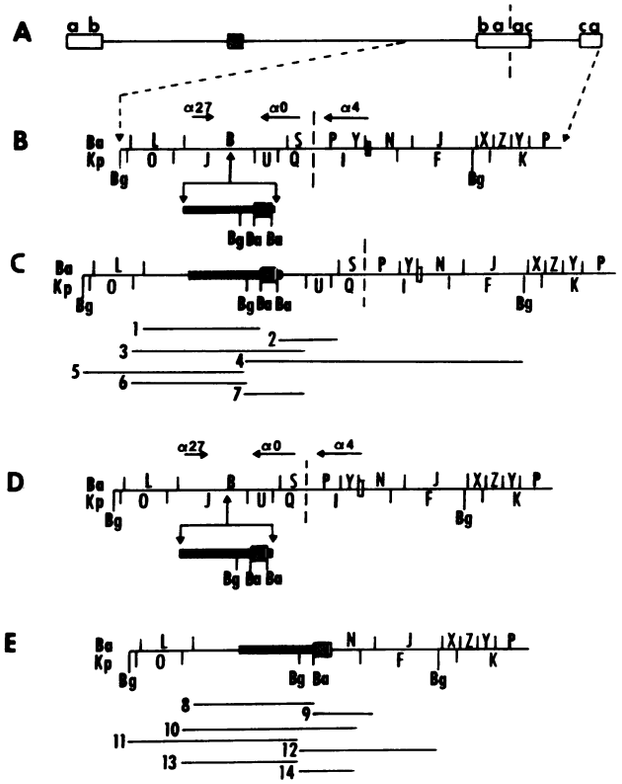

FIG. 3. Sequence arrangement in wild-type and recombinant HSV-1 viruses. (*A*) Sequence arrangement in HSV-1 DNA. The open boxes represent terminal sequences of the L (*ab*) and S (*ca*) components, which are reiterated internally in an inverted orientation and shown as *baac*. The dashed vertical line represents the junction of the L and S components. The hatched box shows the location of the *TK* gene in the prototype arrangement of the HSV-1 DNA. (*B*) Expanded portion of the HSV-1 DNA in prototype arrangement of L and S components showing *Bam*HI, *Kpn* I, and *Bgl* II cleavage sites and the site of the insertion of the *αTK*-mM. The mini-Mu-phage sequences are shown as a thick black line, the *α4* promoter as an open box, and the *TK* sequences as a hatched box. The location of the homologous *α4* promoter sequences in *Bam*HI N fragment are also shown as an open box. The lines above the maps represent the location of *α27*, *α4*, and *α0* genes. (*C*) Site and orientation of *αTK*-mM in the DNA of recombinant RBMu1. The lines below labeled 1–7 represent the novel DNA fragments generated by cleavage with *Bam*HI (lines 1 and 2), *Kpn* I (line 3), *Bgl* II (lines 4 and 5), and *Bgl* II/*Kpn* I (lines 6 and 7). (*D*) Site of insertion of *αTK*-mM in DNA of recombinant RBMu2. (*E*) Orientation of *αTK*-mM and sequence arrangement of the DNA of recombinant RBMu2. The lines below the maps show the novel fragments generated by cleavage with *Bam*HI (lines 8 and 9), *Kpn* I (line 10), *Bgl* II (lines 11 and 12) and *Bgl* II/*Kpn* I (lines 13 and 14).

DNA linearized randomly and flanked by direct copies of the *αTK*-mM DNA. In the second step, these lysates were used to infect *E. coli* RecA$^+$ lysogenized with Mu *c*ts prophage. In this strain, the phage DNA carrying the mini-Mu-phage cannot integrate and forms a plasmid by homologous recombination through the flanking *αTK*-mM DNA sequences. The plasmid confers resistance to kanamycin (Kan^r) and ampicillin (Amp^r), which can be used to select the bacteria carrying the plasmids. In the third step, the amplified plasmid DNA carrying the HSV-1 DNA sequences and the inserted *αTK*-mM were extracted and cotransfected with HSV-1(F)Δ305 DNA carrying a deletion in the *TK* gene. The TK$^+$ progeny was then tested for the presence of *αTK*-mM in the target DNA fragment.

Construction of RBMu1 and RBMu2 Recombinant Viruses. The power of the technique and the consequences of insertion of *αTK*-mM are readily illustrated by the insertional mutagenesis of the 10.4-kbp *Bam*HI B fragment of HSV-1(F) DNA. This fragment spans the junction between the unique and the inverted repeat sequences of the L component near the junction between L and S components of the viral genome (Fig. 3) and contains the entire domain of the *α27* gene and a portion of the domain of the *α0* gene. Fig. 4 shows the electrophoretic profile of the plasmid DNA carrying the *αTK*-mM after cleavage with *Bgl* II and *Cla* I, which cleave only once, within the *αTK*-mM and the plasmid vector sequences, respectively. As would be predicted if *αTK*-mM were to insert randomly, the cleaved plasmid DNA including the HSV-1 *Bam*HI DNA sequences formed a ladder of bands.

Of the HSV-1(F) recombinants carrying the *αTK*-mM, two were selected for detailed analysis. The first, designated RBMu1, carried the entire *αTK*-mM inserted 4.8 kbp from the left terminus of the *Bam*HI B fragment. This was deduced as follows. Digestion of *αTK*-mM with *Bam*HI should yield the 2.2-kbp *αTK* insert and two mini-Mu DNA fragments, 7.4 and 0.1 kbp in size, flanking the *TK* insert. Hybridization of

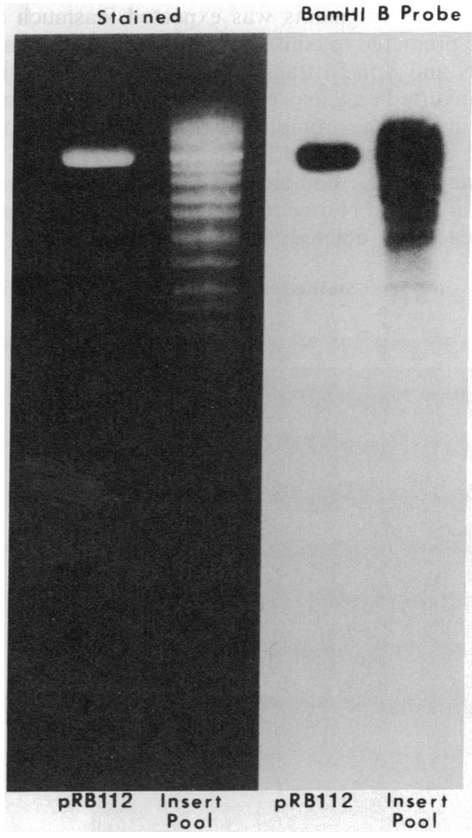

FIG. 4. Photograph of electrophoretically separated fragments in restriction enzyme digests of pRB112 plasmid DNA pools containing randomly inserted *αTK*-mM sequences. pRB112 plasmid DNA and pRB112 DNA containing randomly inserted *αTK*-mM sequences (designated as insert pool) were removed from cesium chloride density gradients, digested with the restriction enzymes *Bgl* II and *Cla* I, and electrophoretically separated in agarose gels. (*Left*) Photograph of ethidium bromide-stained gel. (*Right*) Autoradiogram of the bands transferred to a nitrocellulose sheet and hybridized to ^{32}P-labeled *Bam*HI B fragment DNA as described (8).

DNA sequences. (*C*) Insertional mutagenesis of HSV-1(F) DNA fragments. Plasmid DNA purified from lysates of *E. coli* selected for Amp^r and Kan^r resistance were cotransfected with intact HSV-1Δ305 DNA into rabbit skin cells as described (1). Recombinant TK$^+$ progeny were selected in human 143 TK$^-$ cells overlaid with medium containing hypoxanthine, aminopterin, and thymidine.

electrophoretically separated *Bam*HI fragments with mini-Mu DNA revealed a 12.2-kbp fragment (band 1 in Fig. 3*C* and Fig. 5) that had to carry 7.4 kbp of mini-Mu-phage and 4.8 kbp of *Bam*HI B DNA from either its left or right terminus. The exact orientation, as shown in Fig. 3, was deduced from *Kpn* I and *Bgl* II/*Kpn* I digests. Thus, the entire *αTK*-mM was inserted into the *Kpn* I J fragment (band 3 in Figs. 3 and 5). Cleavage of that fragment with *Bgl* II within the mini-Mu sequences yielded 12.2- and 6.0-kbp fragments (bands 6 and 7 in Figs. 3 and 5), which defined the orientation of the *αTK*-mM and positioned the insert 4.8 kbp from the left terminus of *Bam*HI B fragment inasmuch as, if the *αTK*-mM insert were inverted and located 4.8 kbp from the right terminus of *Bam*HI B fragment, the *Kpn* I/*Bgl* II digest would have yielded 9.4- and 7.3-kbp fragments hybridizing with the mini-Mu DNA probe.

The second recombinant, designated RBMu2, presented an entirely different picture. In this recombinant, the *αTK*-mM was inserted 5.3 kbp from the left terminus of the *Bam*HI B fragment and either near or within the internal copy of the inverted repeats flanking the L component of HSV-1 DNA. In this recombinant, however, all of the sequences to the right of the site of insertion of the *αTK*-mM comprising the internal inverted repeats flanking the unique sequences of the L and S components were deleted. This conclusion is based on the following considerations. The digests of RBMu2 DNA failed to show the fragments *Bam*HI B and N and *Kpn* I J and I at their usual positions (Figs. 3 and 5). The absence of *Bam*HI B and *Kpn* I J fragments was expected inasmuch as they would be predicted to contain the *αTK*-mM. The absence of *Bam*HI N and *Kpn* I I fragments (Fig. 5) suggested that the internal inverted repeats were deleted. Such a deletion could have occurred if the promoter regulatory sequences of the *α4* gene that was fused to the *αTK* gene in the *αTK*-mM were to recombine with the homologous sequences in their natural location in *Bam*HI N fragment such that all of the intervening sequences were deleted. The deleted sequences do not appear to be essential for virus growth inasmuch as a recombinant virus carrying a similar deletion was obtained in an earlier study (2). Fig. 3*E* shows the predicted structure of the region of RBMu2 DNA spanning the novel junction between the L and S components. If this were the case, the *Bam*HI N probe described in *Materials and Methods* should hybridize with the novel bands designated 9, 10, 12, and 14 containing fragments 6.6, 22.2, 17.7, and 7 kbp in size. These fragments are readily seen in Fig. 5. Inasmuch as mini-Mu DNA hybridized with a 12.7-kbp *Bam*HI fragment of RBMu2 DNA, which must contain 5.3 kbp of *Bam*HI B DNA and 7.4 kbp of mini-Mu DNA, the insertion of *αTK*-mM must be as shown diagrammatically in Fig. 3*E*.

DISCUSSION

HSV-1 DNA encodes an excess of 50 genes (13), not all of which appear to be essential for growth in cell culture (14, 15). Another characteristic of herpes-virus DNAs is the presence of numerous reiterated sequences whose function is poorly understood. Examples of such reiterations in HSV-1 DNA are the left-terminal 9 kbp (sequence *ab*, Fig. 3) and right-terminal 6.5 kbp (sequence *ca*, Fig. 3), which are reiterated internally in an inverted orientation. Both detection and analysis of the function of genes not essential for viral growth in cell culture and of the reiterations in the genomic DNA require techniques for site-specific insertions and deletions. As noted in the Introduction, the procedure described by Post and Roizman (1) utilizes a selectable marker, the *TK* gene, for this purpose. This report describes an extension of this technique. Instead of inserting the *TK* gene into a desired site within the target sequence, *E. coli* doubly lysogenized with a helper Mu phage and a mini-Mu-phage carrying the *TK* gene is transformed with a plasmid carrying the target sequence. Straightforward steps in the procedure yield ≈24 hr after the transformation a population of plasmids in which the *αTK*-mM is randomly inserted into the plasmid se-

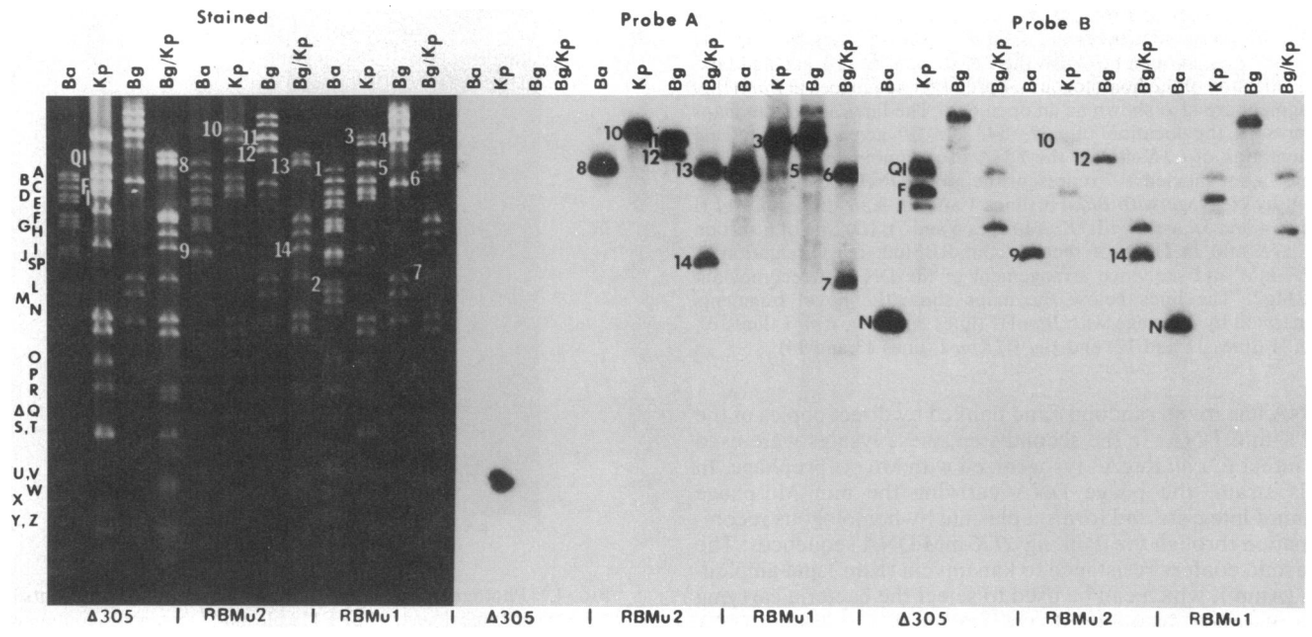

FIG. 5. Photograph of ethidium bromide-stained gels (*Left*) and autoradiograms (*Right*) of electrophoretically separated digests of recombinant virus DNAs hybridized with mini-Mu DNA (probe A) or the *Pvu* II–*Bam*HI 3-kbp fragment from *Bam*HI N fragment DNA (probe B). Ba, *Bam*HI; Kp, *Kpn* I; Bg, *Bgl* II; Bg/Kp, both *Kpn* I and *Bgl* II. The numbers to the left of specific bands refer to the fragments identified by the same number in Fig. 3. The letters to the left of the figure identify the adjacent *Bam*HI bands of the parent virus [HSV-1(F)Δ305)] DNA carrying a deletion in the *TK* gene (Δ*Q*). The letters to the left of the second lane identify the *Kpn* I S component terminal fragment *Kpn* I I, the L–S component junction fragment *Kpn* I Q1, and the fragment *Kpn* I F, whose map location is shown in Fig. 3. The letter N in the autoradiogram of DNA hybridized with probe B identifies the *Bam*HI N fragment.

quences. This population can then be cotransfected into TK^- cells with intact HSV-1 TK^- DNA, and the viral progeny that recombined the *αTK*-mM by homologous recombination through flanking sequences is readily selected under conditions favoring the growth of viruses expressing the TK^+ phenotype. The purpose of fusing the *α4* promoter regulatory sequence to the *TK* coding sequence in the mini-Mu-phage DNA is to reduce the probability that the *TK* gene in the *αTK*-mM will restore the deleted sequences in the natural *TK* gene of HSV-1(F)Δ305 virus inasmuch as the inserted *αTK* carries homologous flanking sequences on only one side of the deleted sequences in HSV-1(F)Δ305. The advantages of this modification of the original technique rest on both the simplicity of the insertion of the marker gene into the target sequence as well as on the fact that the entire domain of the target sequence rather than the specific site of insertion of the *TK* gene is probed by insertional mutagenesis.

Relevant to the procedure described in this report are the following considerations.

(*i*) The size of the *αTK*-mM constructed for this study is 9.7 kbp. Since the size of the DNA packaged by the Mu phage is 38 kbp and since the packaged DNA should contain two copies of the mini-Mu-phage for generation of the recombinant plasmid (Fig. 2*B*), the size of the plasmid should not greatly exceed 18.6 kbp. We should note, however, that smaller mini-Mu constructs are feasible, although they may be less efficient, and that both copies of the mini-Mu need not be intact (unpublished data). Thus, we have obtained insertions of *αTK*-mM in plasmids in excess of 21 kbp.

(*ii*) As would be expected, we obtained a population of HSV-1(F) recombinants differing in the site of insertion of the *αTK*-mM within the domain of the *Bam*HI B fragment. The insertions are within the sequences between the 3′ termini of the *α27* and *α0* genes, indicating that this domain of the *Bam*HI B fragment is not essential for DNA replication. The two recombinants described in this report illustrate the likely outcome of most insertions of *αTK*-mM. Thus, the recombinant RBMu1 contains the entire *αTK*-mM inserted into the target fragment without generating apparent deletions, suggesting that the HSV-1 genome can readily accommodate the additional 9.7 kbp of the *αTK*-mM DNA. The second recombinant carries a spontaneous deletion that appears to extend from the site of the insertion across the L–S component junction. The deletion may have resulted from recombination between the *α4* gene promoter fused to the *TK* inserted into the mini-Mu-phage and the corresponding homologous sequences in the domain of the *α4* gene. The deleted sequences, ≈14 kbp, appear to consist entirely of the internal inverted repeats (≈15 kbp). In this regard, RBMu2 resembles the recombinant I358 carrying a similar deletion (2). That recombinant also was generated by insertional mutagenesis, but into the S rather than L component.

(*iii*) The L and S components of HSV-1 DNA invert relative to each other, giving rise to four isomers present in equimolar concentrations both in the virions and the infected cells (16). Moreover, the *α4* gene is contained entirely within the inverted repeats of the S component (sequence *c*, Fig. 3*A*) and, therefore, is present in two copies per genome (17, 18). The domain of the *α4* promoter-regulatory region fused to the *TK* gene in mini-Mu maps in *Bam*HI N and Z fragments (18). This feature of HSV-1 genome is relevant with respect to two observations. First, although in both recombinants the *αTK* gene in the inserted mini-Mu was in the same, direct, orientation relative to the nearest homologous *αTK* promoter, the spontaneous deletion occurred only in the recombinant carrying the *αTK*-mM nearest or within the L component inverted repeats. It remains to be seen whether the homologous recombination resulting in the deletion of the inverted repeats is favored when the inverted repeats are perturbed by insertional mutagenesis. Second, the deletions occurred between the *α4* sequences fused to the *TK* gene and the corresponding sequences in *Bam*HI N rather than *Bam*HI Z fragment. Because of the inversions of the L and S components, the *α4* sequences in both *Bam*HI N and *Bam*HI Z fragments would be juxtaposed in direct orientation relative to the homologous sequences in the *αTK*-mM. To date, all deletions of internal inverted repeats occurred between sequences in *Bam*HI B and *Bam*HI N fragments—i.e., the one of four isomers designated as the prototype (19). In all of these instances, the DNA was frozen in prototype arrangement (2). The significance of this observation discussed at length elsewhere remains to be determined.

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