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A Cosmid-Based System for Constructing Mutants of Herpes Simplex Virus Type 1

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Cosmids containing large fragments of herpes simplex virus type 1 DNA were prepared using a vector that allows intact inserts to be excised using the restriction endonuclease *Pac*I. Two independent sets (A and B) of five cosmids were identified whose inserts overlap and represent the entire viral genome, and set C was obtained by replacing two cosmids in set B. Each set gave rise to viral plaques when digested with *Pac*I and transfected into cells in culture. Two cosmids common to sets B and C ostensibly contain one of the origins of viral DNA replication (ori_L) in a region of overlap between inserts, but both actually consist of a minority of apparently intact (ori_L^+) forms and a majority of deleted (ori_L^-) forms. These sets yielded exclusively ori_L^+ viral progeny. When either of these cosmids was replaced by a derivative comprising only ori_L^- forms, ori_L^+ and ori_L^- progeny were obtained, and only ori_L^- progeny were produced when both were replaced. One cosmid in set A contains the ori_L locus in a nonoverlapping region and lacks ori_L^+ forms. This set generated only ori_L^- virus. Viral mutants with lesions in either or both of genes *UL2* and *UL44*, which are not essential for growth in cell culture, were constructed using cosmids containing specifically introduced frameshift mutations. A mutant with a frameshift mutation in an essential gene (*UL33*) was isolated by transfecting a complementing cell line. These results indicate that a cosmid-based system will facilitate isolation of large numbers of defined viral mutants.

INTRODUCTION

The considerable amount of information now available on the biology and molecular biology of herpes simplex virus type 1 (HSV-1), the prototype of the α-herpesviruses, has accumulated largely from genetic studies. Early mutants were generated without detailed knowledge of viral genes and were selected on the basis of host range properties (e.g., Aurelian and Roizman, 1964), plaque morphology (e.g., Ejercito *et al.*, 1968), drug resistance (e.g., Kit and Dubbs, 1963), or temperature sensitivity (e.g., Schaffer *et al.*, 1970). The lesions in drug-resistant mutants map in a very few genes encoding inhibitor-susceptible enzymes (e.g., thymidine kinase and DNA polymerase), and those in temperature-sensitive mutants are in genes required for growth under normal cell culture conditions (so-called "essential" genes). Later, mutants that contain lesions in surface glycoproteins and are resistant to immune cytolysis (e.g., Machtiger *et al.*, 1980) or neutralization by monoclonal antibodies (e.g., Holland *et al.*, 1983) were added to the armory. Determination of the complete DNA sequence of HSV-1, culminating in the paper by McGeoch *et al.* (1988), opened the way for specific mutation of each viral gene, including those that had previously been inaccessible to genetic analysis.

Transfection methods have enabled insertion or deletion mutants in many HSV-1 genes to be isolated. The plasmid-based method usually involves cotransfecting cells with intact viral DNA and a plasmid bearing the target which has been modified by insertion of a marker. Selectable markers include the HSV-1 thymidine kinase gene (Post *et al.*, 1981) and a gene conferring resistance to neomycin (Neidhardt *et al.*, 1987), and detectable markers are exemplified by *E. coli lacZ* (Goldstein and Weller, 1988). Mutants arise by recombination and are isolated from wild-type (*wt*) virus by virtue of expression of the marker. The possibility of reversion can be eliminated by replacing part of the target gene in the plasmid by the marker instead of merely inserting it. Additional manipulations can be carried out to obtain revertants, true deletion mutants lacking the marker or more complex mutants.

An alternative means for constructing mutants was developed by van Zijl *et al.* (1988) for another α-herpesvirus, pseudorabies virus (PRV). Cosmids containing large PRV DNA fragments were constructed, and libraries containing four or five cosmids whose inserts overlap and represent the entire genome were identified. Intact cosmid inserts were obtained by cleaving at flanking *Eco*RI sites (which are absent from PRV DNA) and purified by sucrose-density centrifugation. When the inserts were cotransfected into rabbit kidney cells, viral plaques were produced via recombination between overlapping DNA fragments. The genome profiles and growth properties *in vitro* and *in vivo* of *wt* and

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reconstructed PRV were indistinguishable. Moreover, the utility of this system for producing PRV mutants by replacing the *wt* cosmid with a specifically mutated derivative was demonstrated, and there are several instances where it has been used to produce mutants (e.g., de Wind *et al.,* 1990). Nevertheless, the unavailability of the complete PRV DNA sequence poses a present limitation on genetic studies of PRV by this and other means.

The plasmid- and cosmid-based systems should be viewed as complementary, since each has different merits. The former allows mutations to be introduced easily at specifically targeted loci and can be utilized to generate revertants, thus allowing phenotypic changes to be associated directly with the introduced mutation. This possibility is not inherent in the cosmid-based system, but the association can instead be established by examining several independent mutants. The cosmid-based system does, however, have several advantages that make it very attractive. Most importantly, mutants may be isolated in the absence of *wt* virus. In contrast, transfection of parental DNA in the plasmid-based system may result in cytopathic effect (CPE) or a high background of parental plaques, which may obscure recombinant plaques, particularly those formed by seriously disabled but nonetheless viable mutants. Also, since a marker is not required, the cosmid-based system is a powerful tool for directly generating large numbers of random or quasirandom mutations containing minimally disruptive changes (i.e., insertions or deletions of a few base pairs).

Therefore, development of a cosmid-based system for HSV-1 is a desirable objective, particularly since its application could usefully exploit the available sequence data. We have succeeded in constructing such a system, and demonstrate its utility for generating mutant HSV-1 viruses containing single or multiple lesions in essential or nonessential genes. We have recognized in our strategy that one of the origins of HSV-1 DNA replication (ori_L) consists of a large palindrome that is readily deleted when cloned in bacteria (Gray and Kaerner, 1984; Quinn and McGeoch, 1985; Weller *et al.,* 1985). This sequence is not required for viral growth in cell culture or for establishment and reactivation of latency in mice (Polvino-Bodnar *et al.,* 1987), but its conservation in HSV-1 and HSV-2 suggests that it has a role at some point in the natural infectious cycle. We have found that ori_L can be maintained unstably in cosmid populations, and that viruses possessing ori_L can be constructed without difficulty.

MATERIALS AND METHODS

Cells

BHK C13 cells (MacPherson and Stoker, 1962) were grown in Glasgow modified Eagle's medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.3% (wt/vol) tryptose phosphate broth, and 10% (vol/vol) newborn bovine serum.

Preparation of cosmids

DNA was isolated from cell-released virions of HSV-1 strain 17 (Brown *et al.,* 1973) as described by Wilkie (1973) and cloned into a cosmid vector derived from SuperCos 1 (Evans *et al.,* 1989; Stratagene). SuperCos 1 contains genes conferring resistance to ampicillin and neomycin and two adjacent packaging (*cos*) signals from bacteriophage λ separated by an *Xba*I site. The region for inserting foreign DNA contains a single *Bam*HI site flanked by bacteriophage T7 and T3 transcriptional promoters and *Not*I and *Eco*RI sites in the configuration *Eco*RI–*Not*I–T7 promoter–*Bam*HI–T3 promoter–*Not*I–*Eco*RI. *Not*I and *Eco*RI cleave the HSV-1 genome in several locations, so SuperCos 1 was modified by replacing the *Not*I fragment with a synthetic oligonucleotide duplex to give SuperCos 1MW, containing a cloning region with the configuration *Eco*RI–*Not*I–*Asc*I–*Pac*I–*Bam*HI–*Asc*I–*Pac*I–*Not*I–*Eco*RI, where the sites are contiguous and nonoverlapping. *Pac*I does not cleave HSV-1 DNA and can be used to excise HSV-1 inserts.

HSV-1 DNA fragments were cloned into SuperCos 1MW according to instructions supplied by Stratagene. The vector was treated with *Xba*I and calf intestinal phosphatase and then digested with *Bam*HI. The resulting mixture of two fragments, each terminated by a *cos* site, was ligated to HSV-1 DNA that had been partially digested with *Mbo*I (which recognizes the sequence GATC) to produce large quasirandom fragments and treated with calf intestinal phosphatase. A Gigapack II Plus kit (Stratagene) was employed to package the ligated DNA into λ heads, which were then used to infect *Escherichia coli* NM554. Of the ampicillin-resistant clones obtained, 112 were analyzed by digestion with restriction endonucleases. Sets of cosmids apparently containing the entire genome were characterized further by digestion with additional restriction endonucleases. Bulk preparations of cosmids were obtained by alkaline lysis (Birnboim and Doly, 1979) of bacteria from 200-ml cultures, treated with 100 μg/ml RNase A and purified by precipitating with 11% (wt/vol) polyethylene glycol in the presence of 1.36 *M* NaCl. The concentrations of commercial DNA standards were verified by spectrophotometry, and concentrations of cosmid DNAs (as *Eco*RI digests) were estimated by careful comparison on ethidium bromide-stained agarose gels viewed by uv illumination. Sequences at the ends of all the inserts and in the region of ori_L in one cosmid (cos71) were obtained after subcloning *Eco*RI–*Sma*I fragments and a *Bam*HI fragment, respectively, into bacteriophage M13mp19 RFI

DNA. The average size of the cosmid inserts used in this study is 37.4 kb.

Mutagenesis of cosmids

Individual cosmids were linearized at unique *Xba*I sites, treated with bacteriophage T4 DNA polymerase in the presence of the four deoxynucleoside triphosphates to produce blunt ends, ligated, and transfected into *E. coli* MAX efficiency DH5α (Gibco–BRL). This strategy should insert 4 bp into the *Xba*I site, converting the sequence TCTAGA into TCTAGCTAGA. The resulting clones were screened for loss of the target *Xba*I site, and the precise nature of the insertion mutation was confirmed in one (cos14) by sequencing a *Sma*I fragment subcloned into bacteriophage M13mp19 RFI DNA.

Generation of complementing cell lines

Cell lines were generated by transfecting 40 μg of a cosmid comprising SuperCos 1 and HSV-1 DNA from 43423 to 78287 bp in the published sequence (i.e., from within gene *UL21* to within gene *UL36;* McGeoch *et al.,* 1988) into a 35-mm dish of BHK C13 cells by calcium phosphate precipitation and 20% (vol/vol) dimethylsulfoxide (DMSO) boost (Stow and Wilkie, 1976). After 24 hr at 37°, the cells were diluted in 96-well trays and selected with 400 μg/ml Geneticin G418 sulfate (Gibco–BRL). Surviving colonies were subjected to two further rounds of isolation by limiting dilution in the presence of G418. The complementing cell line used in this study was designated 20A.

Generation of viruses from cosmids

Cosmids were digested with *Pac*I (New England Biolabs) for 16 hr at 37°. Cos17 was also treated with *Xba*I. The DNA was extracted with 1:1 (vol/vol) phenol:chloroform, ethanol precipitated and resuspended in a small volume of 10 m*M* Tris–HCl (pH 7.5), 1 m*M* EDTA, and the integrity of the digests was checked by agarose gel electrophoresis. Equimolar amounts of digested cosmids representing the entire HSV-1 genome were mixed, and the DNA was transfected into 90% confluent BHK C13 cells in 24-well trays by calcium phosphate precipitation and DMSO boost. A volume of 100 μl of calcium phosphate-precipitated DNA, including 2 μg of calf thymus DNA, was added to each well. The cells were incubated at room temperature for 30 min, 1 ml of medium was added, and incubation was continued for a further 3.5 hr at 37°. After boosting at room temperature for 3 min 45 sec, the DMSO solution was replaced with 1 ml of medium and the cells were incubated for 2 hr at 37°. The medium was then replaced with 1 ml of 1.5% (wt/vol) carboxymethylcellulose (sodium salt) in medium. Plaques, which were visible under the microscope after incubation at 37° for about 3 days, were removed from the wells in 5 μl of carboxymethylcellulose overlay using plastic pipet tips, mixed with 0.5 ml of medium, sonicated, and stored at −70°. Viral stocks were prepared by infecting cell monolayers in 6-well trays with 0.25 ml of virus for 1 hr, overlaying with 2 ml of medium and incubating at 37° until CPE was complete (3–4 days). Infected cells were scraped into the medium, sonicated, and stored at −70°. For comparison with viruses generated from cosmids, *wt* virus was obtained by transfecting HSV-1 DNA.

Screening of viral genotypes

Cell monolayers in 24-well trays were infected with 250 μl of viral stock for 1 hr, overlayed with medium, and incubated for 16 hr at 37°. The medium was decanted and infected cell DNA was prepared (Stow *et al.,* 1983). The DNA was digested with appropriate restriction endonucleases, electrophoresed on agarose or polyacrylamide gels, and transferred to nitrocellulose membranes (Southern, 1975). Immobilized DNA was hybridized to [^{32}P]-labeled DNA probes prepared by the random-priming method (Feinberg and Vogelstein, 1983) and autoradiographed. The probes used included HSV-1 DNA and cloned HSV-1 *Kpn*I fragments b, c, d, and v (Preston *et al.,* 1978; Davison and Wilkie, 1983).

RESULTS

Cosmid libraries

As a result of the initial screening procedure, two independent sets (A and B) of five cosmids were identified that contain the entire HSV-1 genome (Fig. 1). Two of the cosmids in set B were subsequently evaluated as posing potential problems for generating virus. Cos18 was found to contain a mutation resulting in an additional *Xba*I site about 700 bp from the *wt* site at 97,667 bp. The precise nature of this mutation, which is located in *UL44* or *UL45,* was not established. Also, by virtue of the presence in viral DNA of four genome isomers differing in the orientations of U_L and U_S, the insert in cos17 consists of the left end of U_L linked to the left end of U_S via the flanking inverted repeats (Fig. 1). Thus, although the cos17 and cos18 inserts are partially homologous and could recombine, the lack of homology between their termini might affect recombination frequency. The likelihood of this occurring was reduced by digesting cos17 not only with *Pac*I but also with *Xba*I, which cleaves at 10,634 bp, in order to excise the majority of nonhomologous terminal sequence. Nevertheless, with these considerations in mind, cos18 and cos17 were replaced by cos56 and cos48, respectively, to give set C (Fig. 1), which thus shares cos48 with set A. Cos56 contains only the *wt*

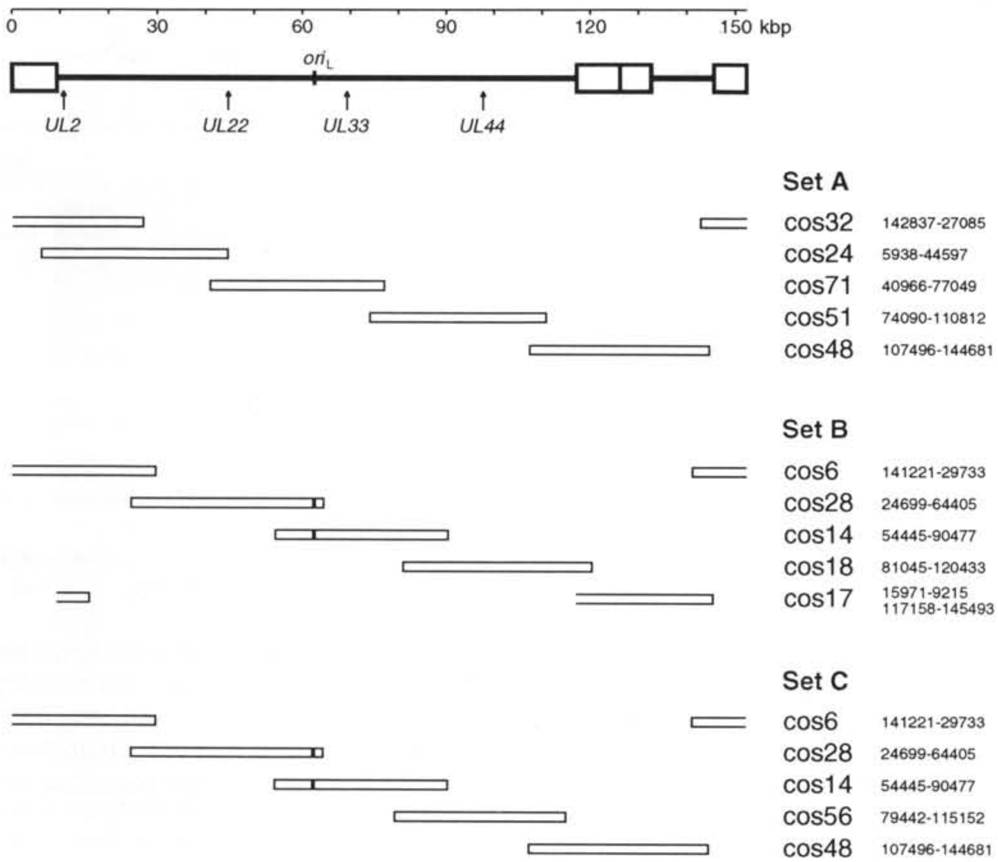

FIG. 1. Locations of cosmid inserts with respect to the HSV-1 genome. The genome is depicted as two unique regions (horizontal lines), the larger U_L and the smaller U_S, flanked by inverted repeats (rectangles). The four *Xba*I sites are indicated by vertical arrows, and the genes within whose protein-coding regions they are located are indicated. Locations of cosmid inserts are indicated by open bars, and their precise locations in the sequence are given in base pairs on the right. The position of ori_L is denoted by vertical lines in appropriate ori_L^+ *cosmids.*

*Xba*I site, and the termini of the cos48 insert are fully homologous to the regions of neighboring cosmid inserts that they overlap.

Generation of viral plaques

When transfected into BHK cells, each of the three cosmid sets generated viral plaques. Thus, the problems anticipated for set B did not materialize. Plaques generated from each set usually took up to 24 hr longer to appear than those from HSV-1 DNA. The absolute numbers of plaques obtained in individual experiments depended on the amount of DNA transfected, and the efficiency of plaque formation by cosmids relative to HSV-1 DNA seemed to be affected by unidentified variables associated with the transfected cells. Table 1 shows the results of three independent experiments in which the DNA input of set C or HSV-1 DNA was varied. Depending on the experiment and DNA dose considered, the cosmids were at least 25% as efficient at generating plaques as an equimolar amount of HSV-1 DNA (e.g., 0.2 μg DNA in Experiment 1), and in some cases the efficiency approached that of HSV-1 DNA (e.g., 0.2 μg DNA in Experiments 2 and 3). The numbers of plaques generated from defined inputs of DNA were of the same order as those reported by van Zijl *et al.* (1988) for the PRV cosmid-based system, and recombination of the cosmid inserts to form viable genomes appears to be a very efficient process for PRV and HSV-1.

Status of ori_L

The region containing ori_L is clearly unstable in the cosmids (Fig. 2). Although cos28 and cos14 contain a minor proportion (approximately 20%) of molecules that produced full length *Kpn*I v, a major proportion generated a deleted form of this fragment (δv). This is characteristic of deletion of the ori_L palindrome (Gray and Kaerner, 1984; Quinn and McGeoch, 1985; Weller *et al.,* 1985) and, although these cosmids are mixtures, we term them ori_L^+. Derivatives of cos28 and cos14 that completely lack ori_L were obtained by colony-purifying twice; these are termed ori_L^-. DNA sequencing of cos71, which is also ori_L^-, showed that the ori_L-associated palindrome has been lost by deletion of 148 bp, from 62,393 to 62,540 bp in the HSV-1 DNA sequence, and that the deletion has occurred between

TABLE 1

RELATIONSHIP BETWEEN THE AMOUNT OF TRANSFECTED HSV-1 OR COSMID DNA AND THE NUMBER OF VIRAL PLAQUES FORMED

Micrograms of genomic DNA/well	Number of plaques					
	Experiment 1		Experiment 2		Experiment 3	
	HSV-1	Cosmids	HSV-1	Cosmids	HSV-1	Cosmids
0	0	0	0	0	0	0
0.02	—[a]	0	—	5	—	1
0.04	6	—	19	—	24	—
0.08	27	—	37	—	~43	—
0.1	—	8	—	25	—	17
0.2	55	14	~51	60	~50	43
0.4	~62	24	CPE[b]	~106	CPE	47
0.8	CPE	38	CPE	~102	CPE	39
2	CPE	42	CPE	~86	CPE	44

[a] Not done.

[b] A general CPE was observed but the plaques were too numerous to be counted. Plaques formed using cosmids in the corresponding experiments were stained when they were slightly smaller and therefore could be counted at higher densities.

8-bp direct repeats, precisely as described for plasmids and bacteriophage λ clones containing the ori_L region (Gray and Kaerner, 1984; Quinn and McGeoch, 1985; Weller *et al.*, 1985; Polvino-Bodnar *et al.*, 1987). Agarose gel electrophoresis analysis of the cosmid library indicated that this is the most common deletion of ori_L, but other smaller or larger deletions were noted. The endpoints of rarer deletions were not determined, but may correspond to forms noted previously by Weller *et al.* (1985) and Polvino-Bodnar *et al.* (1987).

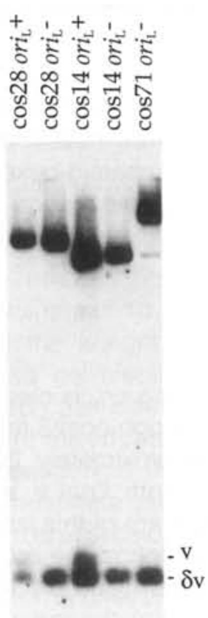

FIG. 2. Status of ori_L in cosmids. *Kpn*I fragments of cos28, an ori_L^- derivative of cos28, cos14, an ori_L^- derivative of cos14, and cos71 were transferred from a 0.7% (wt/vol) agarose gel and probed with a plasmid containing *Kpn*I v, which contains the ori_L region. Fragments representing the full-length (v; 1933 bp) and deleted (δv; approx. 1800 bp) forms of *Kpn*I v are indicated. Hybridization to larger fragments resulted from the presence of vector-containing sequences in the probe.

The genotypes of viral progeny from each cosmid set were analyzed by blot hybridization of infected cell DNA. Eight plaques were examined for sets A and B, and seven for set C. Representative results for sets A and C are shown in Figs. 3–5. The *Kpn*I–*Xba*I profiles of all the progeny from set C were identical to that of *wt* virus, except for fragments that are known to be naturally variable in size (e.g., *Kpn*I j and k), whereas those from set A exhibited *Kpn*I δv rather than *Kpn*I v (Fig. 3). Profiles for the set B progeny were *wt*, except for the presence of the additional *Xba*I site contributed by cos18 (data not shown). Probing with *Kpn*I v confirmed that all of the progeny from set C were ori_L^+ and all of the progeny from set A were ori_L^- (Fig. 4); all of the progeny from set B were also ori_L^+ (data not shown). The resolution of Fig. 4b implies that if ori_L^+ progeny lack any sequence in the vicinity of ori_L, no more than 5 bp are missing. If progeny from the other experiments described below are included, whether mutated or not at other sites in the genome, all of the 26 progeny from sets B or C were ori_L^+ and all of the 16 progeny from set A were ori_L^-. These results show that ori_L^+ molecules in cos14 or cos28 appear exclusively in viral progeny, even though ori_L^- virus is viable.

When cos28 was replaced by an ori_L^- derivative, six of eight progeny analyzed were ori_L^+, one was ori_L^-, and one was a mixture. When cos14 was replaced by an ori_L^- derivative, four of eight progeny analyzed were ori_L^+, one was ori_L^-, and three were mixtures. These results imply that ori_L^- forms of a cosmid are not at a disadvantage in the absence of ori_L^+ forms, and thus become incorporated into viral progeny. When both cosmids were replaced by ori_L^- derivatives, all of the eight progeny analyzed were ori_L^-.

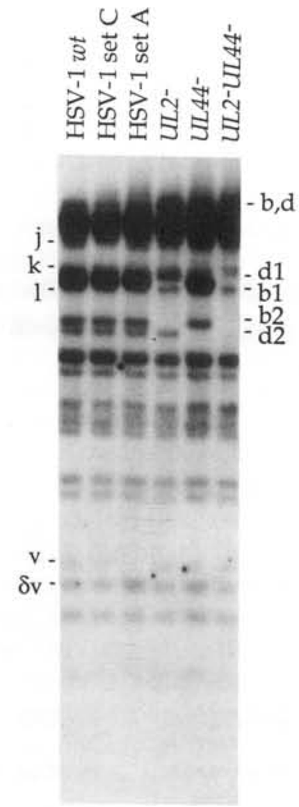

FIG. 3. Restriction profiles of *wt* virus, viruses constructed using cosmid sets C or A, and mutants produced using set C. All the viruses were generated using normal BHK cells. Infected cell DNA was digested with *Kpn*I and *Xba*I, transferred from a 0.7% (wt/vol) agarose gel, and probed with HSV-1 DNA. The positions of *Kpn*I–*Xba*I fragments b1 (6973 bp), b2 (5637 bp), d1 (7286 bp), and d2 (5343 bp) and the fragments from which they originate, *Kpn*I b (12,610 bp) and d (12,629 bp), are indicated on the right. *Kpn*I j, k, l, v, and δv are indicated on the left; j and k are naturally variable in size, k and l comigrated in similar positions to d1 and b1, and δv comigrated with *Kpn*I w and x.

Construction of mutant viruses

As indicated in Fig. 1, the HSV-1 genome contains four *Xba*I sites (Wilkie, 1976), each conveniently located in a single cosmid in at least one cosmid set. These sites are in genes *UL2, UL22, UL33,* and *UL44* (McGeoch *et al.*, 1988). The site in *UL22* when present in cosmids is refractory to *Xba*I because it overlaps a *dam* site (GATC) which is methylated in the bacterial strain used to propagate the cosmids. Frameshift mutations were introduced into the other three genes by linearizing cosmids with *Xba*I, generating blunt ends and religating. Loss of *Xba*I sites was established by restriction endonuclease analysis, and the precise mutation in *UL33* was confirmed by DNA sequencing.

UL2, which encodes uracil-DNA glycosylase, and *UL44,* which encodes glycoprotein C, are known to be nonessential for growth of virus in cell culture (Mullaney *et al.*, 1989; Draper *et al.*, 1984), and single and double mutants were obtained by transfecting normal BHK cells. *UL2*⁻ mutants were produced using sets B and C containing mutated cos6, *UL44*⁻ mutants were generated using set A containing mutated cos51 or set C containing mutated cos56, and the double mutant was obtained using set C containing mutated cos6 and cos56. Six plaque isolates were analyzed from each. All of the set A *UL44*⁻ mutants were ori_L^- and have identical *Kpn*I/*Xba*I profiles, lacking specific *wt* fragments as appropriate (Fig. 3). Similarly, all set C mutants in a particular gene were ori_L^+ (Fig. 4) and displayed identical profiles (Fig. 3), except for one *UL44*⁻ mutant which yielded a novel large *Kpn*I fragment of unknown origin (data not shown). The profiles of set B *UL2*⁻ mutants were also identical, each containing ori_L and exhibiting the additional *Xba*I site present in cos18 (data not shown). The *UL2*⁻ mutants lack the *Xba*I site in *Kpn*I b, resulting in replacement of b1 and b2 by b (Fig. 5a), the *UL44*⁻ mutants lack the *Xba*I site in *Kpn*I d, resulting in replacement of d1 and d2 by d (Fig. 5b),

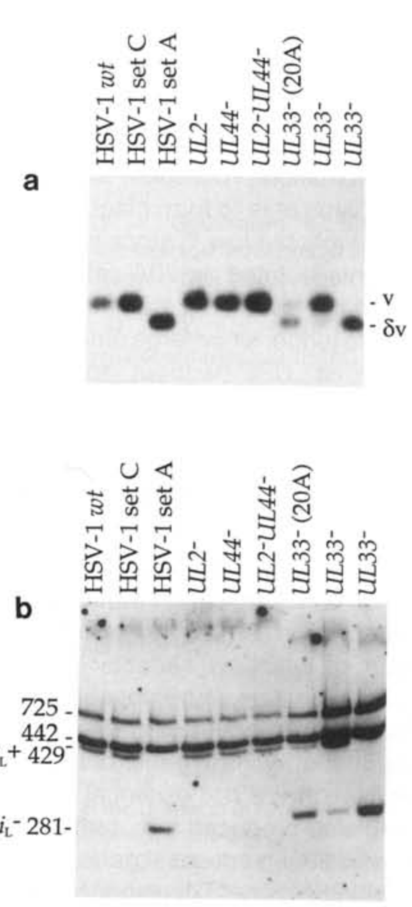

FIG. 4. Status of ori_L in *wt* virus, viruses constructed using cosmid sets C or A, and mutants produced using set C. All the viruses were generated using normal BHK cells except one of the *UL33*⁻ mutants, which was obtained using 20A cells. Infected cell DNA was digested with (a) *Kpn*I or (b) *Bam*HI and *Bst*EII, transferred from (a) a 0.7% (wt/vol) agarose gel or (b) a 5% (wt/vol) polyacrylamide gel and probed with a plasmid containing *Kpn*I v. The positions of *Kpn*I v and δv are shown in (a), and the locations of ori_L^+ and ori_L^- fragments and adjacent fragments are indicated in (b); sizes are in base pairs.

and the *UL2⁻UL44⁻* mutants lack both sites (Figs. 5a and 5b).

UL33 is known to be essential for virus growth in cell culture (Al-Kobaisi *et al.,* 1991), so a complementing cell line (20A) was prepared by transforming BHK cells with a cosmid containing *UL33.* Seven viral plaques obtained using set C containing mutated cos14 were analyzed and each lacks the *Xba*I site in *Kpn*I c, resulting in replacement of c1 and c2 by c (Fig. 5c). Surprisingly, plaques were also obtained using normal BHK cells at an efficiency of up to 10% of that obtained using 20A cells. Analysis of seven plaques showed that they comprise not *UL33⁻* virus alone but *wt* virus containing the *Xba*I site in *Kpn*I c (four plaques) or mixtures of *wt* and *UL33⁻* virus (three plaques, Fig. 5c). One of the plaques isolated on 20A cells and two of the plaques isolated on normal BHK cells were analyzed for the presence of ori_L (Fig. 4). The 20A plaque was a mixture of ori_L^+ and ori_L^- forms, one of the BHK plaques was also a mixture, and the other was ori_L^-. This is consistent with the observed absence of ori_L^+ forms from *UL33⁻* cos14 (data not shown). High-frequency reversion, presumably occurring by loss of 4 bp (CTAG) to regenerate the *Xba*I site, is probably facilitated by active recombination processes, since the proportion of infectious virus able to form plaques on normal BHK cells was variable but low in stocks of 10 independent *UL33⁻* mutants isolated on 20A cells. The upper limit was 10^{-3} but the lower limit (nominally less than 10^{-7}) was difficult to judge, since large amounts of some viral stocks induced CPE without forming detectable plaques.

DISCUSSION

The aim of this study was to develop a cosmid-based system for constructing HSV-1 mutants. We generated three sets of cosmids (two independent and one derived), each of which is capable of yielding HSV-1 plaques after digestion with *Pac*I and transfection into BHK cells. We then constructed viral mutants containing frameshift mutations in either or both of genes *UL2* and *UL44,* which are not essential for growth in cell culture, and also produced a mutant with a frameshift mutation in *UL33,* an essential gene, by transfecting a complementing cell line. These genes were not of specific interest but were chosen because they contain convenient *Xba*I sites. In demonstrating the utility of the system, we have not ruled out the possibility that the cosmids may contain undetected mutations that are transferred to progeny virus. We note, however, that a virus stock obtained using set C showed no growth impairment in comparison with *wt* when human diploid fibroblasts (Flow 2002) cells were infected at

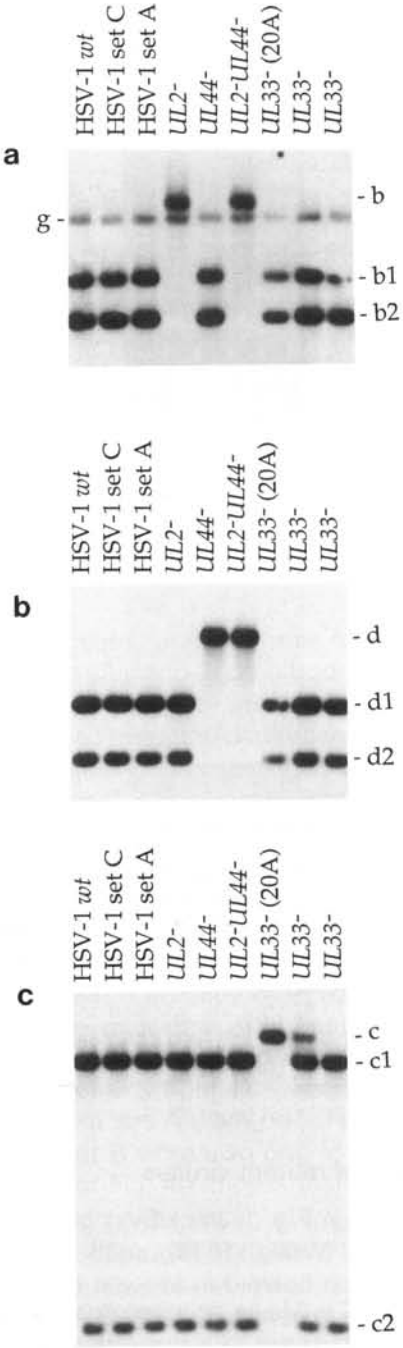

FIG. 5. Status of *Xba*I sites in *wt* virus, viruses constructed using cosmid sets C or A, and mutants produced using set C. All the viruses were generated using normal BHK cells except one of the *UL33⁻* mutants, which was obtained using 20A cells. Infected cell DNA was digested with *Kpn*I and *Xba*I, transferred from 0.7% (wt/vol) agarose gels, and probed with plasmids containing (a) *Kpn*I b, (b) *Kpn*I d, or (c) *Kpn*I c. The positions of *Kpn*I b, d, and c (12,798 bp) when lacking *Xba*I sites and the *Xba*I products of these fragments are shown on the right by b1, b2, d1, d2, c1 (10,212 bp), and c2 (2586 bp). *Kpn*I b contains part of an inverted repeat and thus hybridized in all digests to a fragment (*Kpn*I g, indicated on the left in (a)) from the other repeat.

5PFU/cell and monitored for production of infectious virus during a 24-hr period (data not shown). We intend to compare the *in vivo* growth properties of *wt* and reconstructed viruses in a separate study. The ability of the *UL33*⁻ mutation to revert readily when under selection during the recombination process indicates that transfection of normal BHK cells with cosmids containing small duplications presents a simple method for determining whether the target region is essential for growth in tissue culture. This assay would not necessarily succeed with deletion mutants because they cannot revert genotypically to *wt,* but transfection of cosmids containing minimal deletions into normal cells might form a useful means of selecting phenotypically *wt* viruses produced by mutation at secondary sites.

In developing a cosmid-based system for HSV-1, we investigated the ori_L region in cosmids and viral progeny in some detail. Isolation of viable ori_L^- virus confirms an earlier report that ori_L is not essential for growth of HSV-1 in cell culture (Polvino-Bodnar *et al.,* 1987). The ori_L locus is in a region of overlap between the inserts in cos14 and cos28 in sets B and C and, even though the majority of DNA molecules in these cosmids lack ori_L, only ori_L^+ viral progeny were obtained. ori_L^+ and ori_L^- progeny were isolated when either of these cosmids was replaced by a fully ori_L^- derivative and only ori_L^- progeny were produced when both cosmids were replaced. Production of ori_L^+ virus from cosmids that are predominantly ori_L^- presumably results from the ability of ori_L^+ molecules to replicate and predominate in the viral progeny owing to a dosage effect or an enhanced ability to recombine. The high proportion of mixed progeny obtained when cos14 or cos28 was replaced by ori_L^- forms may have resulted from having picked overlapping plaques. Some isolates (e.g., the UL33⁻ revertants) were obtained from wells that contained few plaques, however, and it is likely that these were a consequence of active recombination occurring during generation of viral genomes in a single infectious center.

Mutants containing ori_L can be obtained easily if the mutated cosmid does not contain the ori_L locus. ori_L^+ mutants can also be isolated using mutated cosmids that contain the ori_L region if the palindrome is maintained (in sets where ori_L is in a single cosmid) or the progeny are screened carefully (in sets where ori_L is in a region of overlap). Of the three cosmid sets produced during this study, set B is unsuitable for further development because of the presence of an additional *Xba*I site in cos18. Set C generated progeny that appeared to be *wt,* and we are now employing it to obtain a range of HSV-1 mutants containing minimal lesions. The availability of a second cosmid set able to yield *wt* virus would facilitate isolation of mutants with lesions in regions of overlap between inserts in set C. To this end, we are currently replacing cos71 in set A with an ori_L^+ alternative.

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