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Herpes Simplex Virus 1 Reiterated S Component Sequences (c_1) Situated Between the *a* Sequence and α4 Gene Are Not Essential for Virus Replication

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The herpes simplex virus 1 genome consists of two components, L and S, each containing unique sequences flanked by inverted repeats. Each of the 6.5-kilobase pair inverted repeats of the S component, designated *a'c'* and *ca*, contains an approximately 700-base pair sequence (designated c_1) located between the *a* sequence and the 3' terminus of the α4 gene. Like the *a* sequence, c_1 consists of direct repeats and unique sequences. Its function is not known. To probe for its function, we constructed a plasmid containing a viral thymidine kinase (TK) gene inserted into the c_1 sequence. The construct was recombined into the genome of a TK^- virus by cotransfection with intact viral DNA and selection for TK^+ virus. As predicted from previous studies (Knipe et al., Proc. Natl. Acad. Sci. U.S.A. 75:3896–3900, 1978), the TK gene was found to be present in both copies of the c_1 sequence in the R3104 virus. To delete the c_1 sequence we constructed a plasmid containing 4 kilobase pairs of pBR322 flanked by an *a* sequence and by structural sequences of the α4 gene. In this instance the cells were transfected with the construct and R3104 DNA; the progeny of the transfection was plated in the presence of 5-bromo-2'-deoxyuridine, and the selection was for TK^- virus (R3158). The pBR322 DNA sequences replaced the c_1 at both termini of the S component in R3158 DNA, but a sequence homologous to c_1 was present in proximity to the 3' terminus of the α4 gene. The results indicate that the c_1 region has no significant role in the replication of the virus in cell culture. The advantage of inserting the pBR322 sequence is that it permits efficient cloning of large herpes simplex virus 1 DNA fragments by simple ligation of digests and transformation of appropriate *Escherichia coli* strains. The effortless selection of recombinants carrying inserts in both copies of the c_1 restates the usefulness of this technique for selection of insertion deletion recombinants and underscores the rapid emergence of sequence identity at both ends of the reiterated regions of the S component as previously reported (Knipe et al., Proc. Natl. Acad. Sci. U.S.A. 75:3896–3900, 1978).

The genome of the herpes simplex virus 1 (HSV-1) consists of two covalently linked components, L (118 kilobase pairs [kbp]) and S (23 kbp). Each component consists of unique sequences (U_1 or U_s) flanked by inverted repeats (17). The inverted repeats of the L component (9 kbp) were designated *ab*, whereas the inverted repeats of the S component (6.5 kbp) were designated *ca* (22). The *a* sequence is the only sequence shared by the two components. In cells infected with the wild-type genome, the two components invert relative to each other to yield four isomeric molecules (3, 5). Relevant to the analyses reported in this paper, and as a consequence of the inversion of L and S components, restriction enzymes which do not cleave within inverted repeats (e.g., *Hin*dIII) yield four terminal fragments and four L-S junction fragments, 0.5 and 0.25 M, respectively, relative to the molarity of the intact DNA. Restriction enzymes which cleave within one pair of the inverted repeats (e.g., *Kpn*I) yield three terminal fragments (one 1 M and two 0.5 M) and two 0.5 M junction fragments.

Studies on the *a* sequence and its adjoining regions revealed considerable sequence conservation (2, 9, 10; E. S. Mocarski, L. Deiss, and N. Frenkel, submitted for publication). The *a* sequence of the HSV-1 F strain [HSV-1(F)] is approximately 500 base pairs (bp) long; it is highly G+C rich (85 G+C mol%) and consists of a 20-bp direct repeat 1 (DR1), a 65-bp unique sequence (U_b), a 12-bp direct repeat (DR2), repeated 20 to 23 times, a 37-bp direct repeat (DR4), repeated 2 to 3 times, a 58-bp unique sequence (U_c), and finally, a DR1 (9, 10). Although the *a* sequences of other isolates differ in length, there is little sequence divergence in both the unique and reiterated regions. The region (c_1) contained within the *c* sequence of the S component and located between the *a* sequence and the 3' terminus of the α4 gene is approximately 700 bp long; it is also G+C rich (72 G+C mol%) and like the *a* sequence contains two sets of reiterated sequences, designated DR5 and DR6, interspersed by unique sequences (2, 9). In this instance, only the reiterated sequences are highly conserved among HSV-1 strains (2, 9).

Studies reported elsewhere have shown that the *a* sequence contains the *cis*-acting sites for the inversion of L and S components relative to each other (9–11) and for the packaging of the DNA (21). The function of the c_1 sequence is not known. Apart from its sequence arrangement and base composition, interest in the c_1 sequence stems from previous studies showing that insertions, deletions, or mutations within the *c* repeat of the S component tend to be identical (6). Although recombinants were isolated which were heterogeneous within the 5' region of the α4 gene coding sequence, the tendency toward identity between corresponding regions of the *c* repeat seemed to be inversely proportional to the distance of the region from the *a* sequence (6, 16) and therefore would be expected to be highest in the c_1 region. To investigate the function of the c_1 region, we attempted to delete it at both ends of the S component. In this paper we report that the c_1 region can be deleted and is therefore not essential for viral replication in cell culture. We also report that all insertions or deletions from the c_1

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sequence were reflected at both ends of the S component, and that the fidelity of the repetitions extended over several kilobase pairs and included nonviral sequences of much lower average G+C content.

MATERIALS AND METHODS

Viruses and cells. Recombinant viruses were derived from HSV-1(F)Δ305 (14, 15), a derivative of HSV-1(F) (4) carrying a 700-bp deletion within the domain of the thymidine kinase (TK) gene. Rabbit skin cells were used for DNA transfections, and Vero cells were used for preparation of viral stocks, for virus titrations, and for preparation of infected cell batches for extraction of viral DNA. Human 143TK$^-$ cells (1) were used for selection of TK$^+$ and TK$^-$ recombinants of HSV-1(F)Δ305. The procedures for cell and virus propagation, virus titrations, and the selection of TK$^+$ and TK$^-$ recombinants were described elsewhere (4, 14, 15). The HAT medium used for the selection of TK$^+$ recombinants consisted of 6 mM thymidine, 1.7 mM hypoxanthine, and 0.2 μg of methotrexate (Lederle-Carolina, P.R.) per ml. For selection of TK$^-$ recombinants, the infected TK$^-$ cells were overlaid with medium containing 5-bromo-2′-deoxyuridine (10 μg/ml; Sigma Chemical Co., St. Louis, Mo.).

Restriction enzymes and hybridization analyses. Restriction endonucleases were purchased from New England Biolabs, Beverly, Mass. T4 DNA ligase was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Restriction endonuclease fragments were separated on SeaKem ME agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) and transferred to nitrocellulose paper (Schleicher & Schuell, Inc., Keene, N.H.) by a modification of the procedure of Southern (18). Hybridization probes were labeled to high specific activity by nick translation with a kit obtained from New England Nuclear Corp., Boston, Mass., by procedures recommended by the manufacturer. Autoradiograms were made with Kodak X-Omat X-ray film and Du Pont Lightning Plus intensifying screens.

Source and construction of plasmids and DNA probes. The DNA cloning procedures were as previously described (13). The preparation of plasmids in *Escherichia coli* C600SF8 or JM103, the extraction, the purification of cloned DNA, and the procedures for transfection of rabbit skin cells with cloned plasmid DNAs and intact viral DNAs were as previously described (13). The DNA probe for the c_1 sequence was derived from pRB373 carrying the *Hae*II L-S junction fragment cloned in pBR322 as an *Eco*RI fragment (9). The complete sequence of the *Hae*II junction fragment was reported elsewhere (9). The probe was prepared as follows. pRB373 DNA was digested with *Eco*RI, and the fragment containing the *Hae*II junction fragment was purified from agarose gels and digested with *Sma*I. The 620-bp *Sma*I fragment containing most of the c_1 sequence was extracted from acrylamide gels and nick translated.

Plasmid pRB3104, containing the TK gene inserted between the c_1 sequence and the 3′ terminus of the α4 gene, was constructed from pRB201 carrying the *Hin*dIII HM junction fragment (cloned by K. Poffenberger) and pRB365 carrying the structural sequences of the TK gene fused in the correct transcriptional orientation to the α27 promoter regulator sequence (8). First, pRB201 DNA was cleaved with *Nar*I under partial digestion conditions in the presence of ethidium bromide (50 μg/ml) (Fig. 1A). Linearized pRB201 DNA was then extracted from an agarose gel, digested to completion with *Cla*I, and religated to generate a family of molecules containing different-size deletions in the *Hin*dIII HM insert. pRB3094 resulted from fusion of the *Hae*II A site (9) of the S component (also a *Nar*I site) with the *Cla*I site of pBR322. The DNA of plasmid pRB3094 containing a deletion of all HSV sequences up to and including the c_1 sequence was then cleaved with *Eco*RI. The DNA fragment containing the coding sequence for the α4 gene and terminating near the beginning of the c_1 sequence was ligated into the *Eco*RI site of pRB365 located 5′ from the α27-TK chimeric gene (Fig. 1B). In the next step, the *Hae*II fragment, isolated as an *Eco*RI fragment from pRB373, was ligated into the *Eco*RI site 3′ from the α27-TK gene in pRB365. The resulting plasmid was designated as pRB3104.

To construct pRB3158, the pRB3094 *Eco*RI fragment containing the coding region of the α4 gene was ligated into the *Eco*RI site of pRB144 (Fig. 1C).

RESULTS

Experimental design. The sequence arrangement of the c_1 and adjacent regions is shown in Fig. 2. The experimental design for the deletion of the c_1 sequence was a variation of that described by Post and Roizman (15) and consisted of two steps. In the first step, a DNA fragment carrying the TK gene linked to the α27 gene promoter (8) was inserted by homologous recombination through flanking sequences into the c_1 region. In the second step, the c_1 region carrying the TK gene was replaced by recombination through homolo-

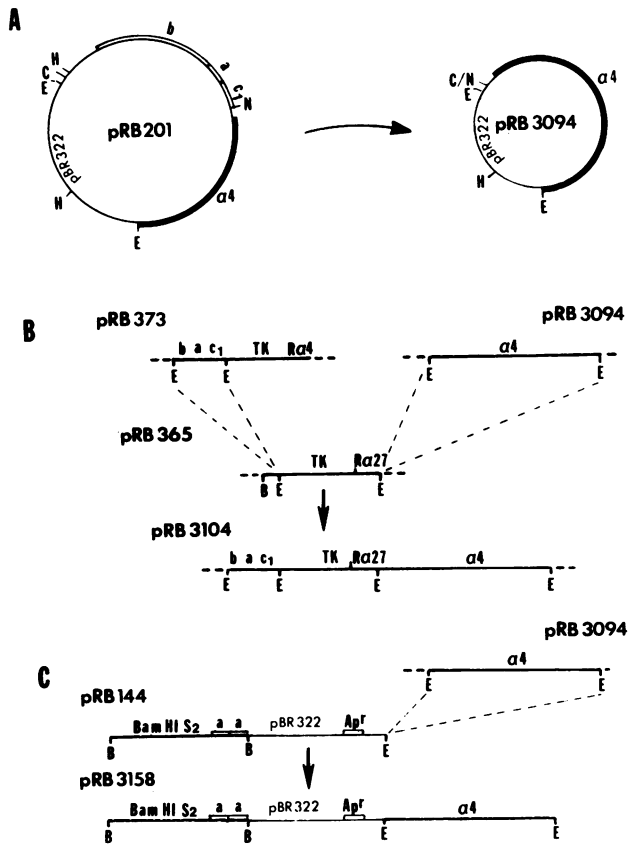

FIG. 1. Construction of plasmids for the deletion of the c_1 sequence. (A) Derivation of pRB3094; (B) construction of pRB3104; (C) construction of pRB3158. The letters *a* identify the two *a* sequences present in the *Bam*HI SP$_2$ fragment spanning the L-S junction. Ap^r identifies the location of the pBR322 ampicillin resistance marker.

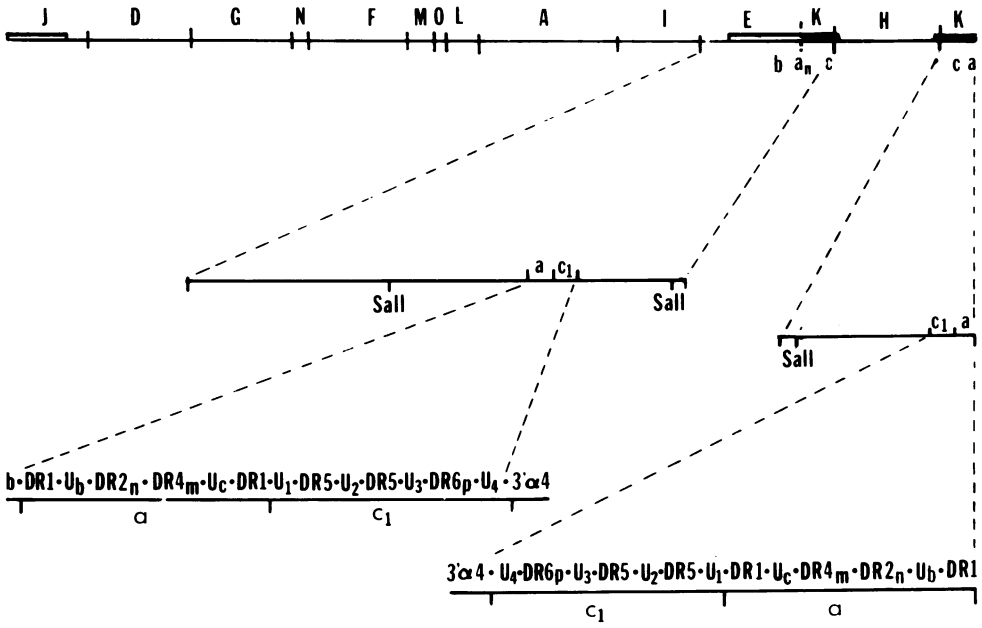

FIG. 2. Sequence arrangement of HSV-1(F) DNA. Top line: *Eco*RI restriction endonuclease map of the prototype (P) isomer of HSV-1(F) DNA. The open and closed rectangles indicate the locations of the inverted repeats of the L component (open) and S component (filled). Middle two lines: location of the *a* and c_1 sequences in the terminal and *Eco*RI junction fragments in reference to *Sal*I cleavage sites. The number of *a* sequences tandemly repeated at the L-S junction and at the terminus of the L component may vary from one to more than five (7, 23). Tandemly repeated *a* sequences share the intervening DR1 (9, 10). Only one *a* sequence is present at the S component terminus, and in this diagram only one *a* sequence is shown at the L-S junction. Because the *Eco*RI cleaves within the S component inverted repeats and within the L component outside the inverted repeats, *Eco*RI generates a single S component terminal sequence, two L component terminal fragments (J and E), and two junction fragments (JK, EK). Bottom lines: arrangement of unique and reiterated sequences within the *a* and c_1 sequences.

gous flanking sequences with an unrelated sequence, i.e., approximately 4 kbp of pBR322 DNA. The desired recombinant viral progeny was obtained by selecting for or against virus carrying a functional TK gene. The α27-TK insert was selected for these manipulations because it lacks the flanking sequences necessary for recombination-insertion into the natural TK site. In each instance the recombinant progeny carried the insertion into the c_1 sequence or deletion-replacement of the c_1 sequence at both ends of the S component.

Insertion of α27-TK gene into the c_1 sequence. The construction of plasmid pRB3104 carrying the TK gene inserted in the c_1 sequence and the 3′ terminus of the α4 gene is described above and illustrated in Fig. 1. To generate the recombinant virus R3104 carrying the chimeric α27-TK gene inserted in the c_1 sequence, intact Δ305 viral DNA was cotransfected with pRB3104 DNA. Virus stocks from the transfection were plated on 143TK$^-$ cells, overlaid with HAT medium, and passaged serially twice under these selective conditions. Progeny TK$^+$ virus (R3104) was then plaque purified on Vero cells.

To delete the c_1 sequence and facilitate analyses of the recombinant, we constructed the plasmid pRB3158 as described above and illustrated in Fig. 1. The HSV-1 sequence arrangement in pRB3158 DNA was such that U_c and adjacent DR1 of the *a* sequence in *Bam*HI S were separated from the 3′ terminus of the α4 gene by approximately 4 kbp of pBR322 DNA, i.e., the DNA fragment extending from nucleotides 375 to 4363 of the Sutcliff map of pBR322 DNA (19). To generate the desired recombinant, intact R3104 DNA was cotransfected with pRB3158 DNA and the progeny of the transfection was then plated on 143TK$^-$ cells in the presence of BUdR. The TK$^-$ recombinant virus, R3158, was further plaque purified on Vero cells.

Two series of experiments were done to analyze R3104 and R3158 DNA. In the first, electrophoretically separated DNA fragments contained in *Kpn*I digests of R3104, R3158, and the parent virus, Δ305, were transferred to a nitrocellulose sheet and hybridized with ^{32}P-labeled c_1 probe DNA prepared as described above. As expected (Fig. 3), the DNA probe hybridized with the submolar S component terminal fragments *Kpn*I and K and the L-S junction fragments *Kpn*I QI and QK (Fig. 4) of Δ305 DNA. These submolar *Kpn*I fragments were replaced by slower-migrating fragments in the electrophoretically separated *Kpn*I digests of the R3104 DNA. Consistent with this observation, hybridizations of the electrophoretically separated *Kpn*I digests with the c_1 probe revealed that both terminal fragments *Kpn*I K and I and the junction fragments *Kpn*I QI and QK were replaced by slower-migrating terminal *Kpn*I fragments 1 and 2 and by the junction fragments *Kpn*I Q1 and Q2. The decrease in mobility reflected an increase in the size of the fragments corresponding to the size of the α27-TK gene insert. Therefore, the results of this experiment indicated that the 2.9-kbp α27-TK chimeric gene became recombined into the c_1 sequences at both ends of the S component.

*Kpn*I digests of R3158 DNA (Fig. 3) revealed additional changes in electrophoretic mobility of submolar fragments. In contrast to those of the R3104 DNA, the junction and S component terminal *Kpn*I fragments of R3158 DNA hybridized weakly with the c_1 probe.

The second series of experiments was designed to verify the absence of c_1 sequences next to the *a* sequence and to locate in R3158 DNA the sequences hybridizing weakly with the labeled c_1 probe. Since the inserted portion of pBR322 contains both the ampicillin resistance gene and the pMB1 replication origin, it could be expected that R3158 DNA

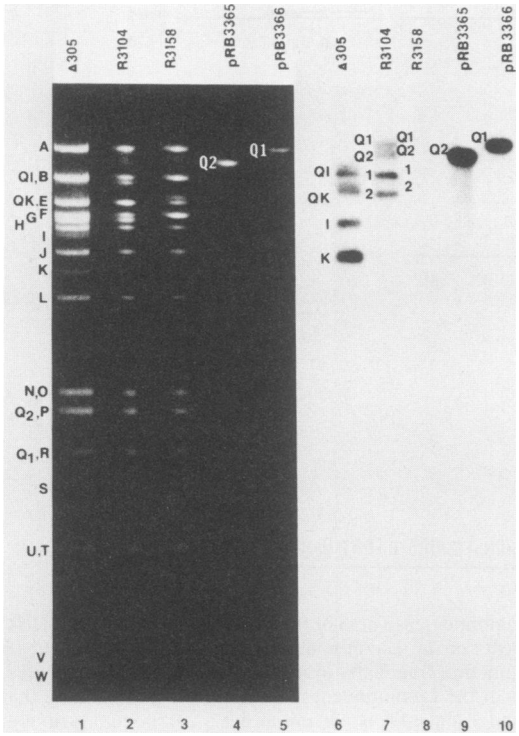

FIG. 3. Photographs and autoradiographic images of electrophoretically separated digests of parental and recombinant viral DNAs and of plasmids derived from recombinant DNAs. Lanes: 1 through 5, photographs of electrophoretically separated *Kpn*I digests stained with ethidium bromide; 6 through 10, autoradiographic images of *Kpn*I fragments electrophoretically separated in agarose gels, transferred to a nitrocellulose sheet, and hybridized with the ^{32}P-labeled c_1 probe. The fragments are designated with the appropriate letter on the left. All junction fragments were designated with a double letter (e.g. *Kpn*I QI, *Kpn*I QK). The novel *Kpn*I terminal fragments of R3104 and R3158 DNAs were designated *Kpn*I 1 and *Kpn*I 2. Inasmuch as *Kpn*I Q was unaltered, the corresponding L-S junction fragments became *Kpn*I Q_1 and *Kpn*I Q_2. In lane 1, Q1 and Q2 identify the submolar L component terminal *Kpn*I fragments containing one and two *a* sequences, respectively.

fragments containing the intact pBR322 insert could be ligated and propagated in *E. coli* without additional manipulations. To test this prediction, *E. coli* C600SF8 was transformed to ampicillin resistance with viral DNA that was purified by NaI density gradient centrifugation from lysates of infected Vero cells, digested with *Kpn*I, and ligated. We obtained two sets of clones. Clones designated pRB3366 contained the *Kpn*I Q1 junction fragment, whereas clones designated pRB3365 contained the *Kpn*I Q2 junction fragment (Fig. 3). The cloned *Kpn*I L-S junction fragments comigrated with the authentic junction fragments present in digests of R3158 DNA (Fig. 3) and hybridized with the c_1 probe. The relevant restriction enzyme maps of the S component of R3158 DNA are shown in Fig. 4. Analyses by hybridization with labeled c_1 probe of the cloned DNAs digested with either *Kpn*I and *Bam*HI or *Bam*HI and *Eco*RI indicated the following (Fig. 5). The labeled c_1 probe DNA hybridized with the *Kpn*I-*Bam*HI fragments 3a and 3b and with the *Bam*HI-*Eco*RI fragments 5a and 5b but not with the *Bam*HI-*Eco*RI fragments 4a and 4b. This indicates that the c_1 sequences were removed from the immediate vicinity of the *a* sequences and that the residual hybridization with the c_1 probe was due to sequences homologous to c_1 located near or within the 3′ terminus of the α4 gene (Fig. 4).

DISCUSSION

The c_1 sequence is of interest from several points of view. Specifically, it has an ordered G+C-rich structure consisting of conserved direct repeats flanked by unique sequences. It has also been reported (20) that sequence alterations at one end of the S component sequence at or near the c_1 region resulted in rapid segregation of genomes in which both ends of the S component contain identical sequences. In this study we showed that the c_1 sequences can be deleted and therefore are not essential for virus replication in cell culture. The ease with which recombinants were selected, i.e., those carrying the TK gene inserted into the c_1 sequence and those carrying an unrelated sequence replacing the c_1 region with the TK insert, underscore the usefulness of the procedure described by Post and Roizman (15) for generation of insertion-deletion recombinants in the genomes of large DNA viruses. We should note that parallel studies (L. Deiss and N. Frenkel, personal communication) also revealed that the c_1 sequence is not essential for the amplification or packaging of defective HSV genomes.

The observation that in both R3104 and R3158 recombinants the inserted and replaced sequences were present in both reiterated sequences (*a′c′* and *ca*) merits further discussion. Since it is unlikely that the recombinant events occurred at both ends of the S component simultaneously, the appearance of the second inserted sequence had to be a secondary event generated by some recombinational process. The recombinational process does not simply bring to sequence identity any two related sequences within HSV-1 DNA. For example, the recombinant R321 was generated by Post and Roizman (15) by insertion of the TK gene into the α22 gene contained in the S component of a virus [HSV-1(F)Δ305] carrying a 700-bp deletion in the natural TK gene. Because the L and S components invert relative to each

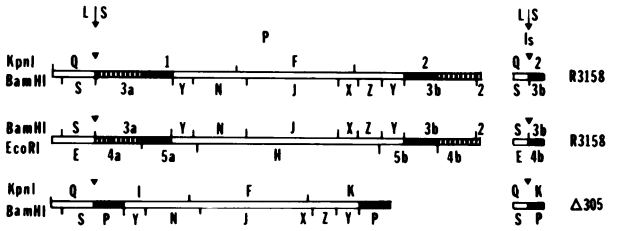

FIG. 4. Restriction endonuclease maps of the P and I_s isomeric arrangements of L-S junctions and S components of R3158 and Δ305 DNAs. Left panel, L-S junction and S component of P isomer; right panel, organization of the L-S junction of the I_s isomer. The remainder of the S component can be readily deduced by inverting the S component maps shown in the left panel. The letters identify fragments present in wild-type and Δ305 DNAs. The numbers identify novel fragments resulting from the insertion of pBR322 DNA sequences near the S component termini in R3158 DNA. The L-S junctions are identified by arrowheads. Cleavage sites are shown by a vertical bar. Note that the construction of R3158 introduced a *Bam*HI cleavage site immediately to the right of the *a* sequence at the L-S junction and, because of recombination, immediately to the right of the terminal S component *a* sequence (fragment 2). Numbers and lowercase letters identify novel fragments that are contained entirely within inverted repeats and are therefore identical except for location. The pBR322 sequences are identified by vertical striations; the *Bam*HI P fragments sequences are filled.

other, the TK gene carrying the deletion and the inserted TK gene spanning the deletion exist in both inverted and direct orientation relative to each. Segregation of viruses containing the deletion at both sites or restoring the intact TK sequence at the natural site occurs rarely (R. Longnecker and B. Roizman, unpublished observations).

The phenomenon of sequence identity was first reported by Knipe et al. (6), who noted that temperature-sensitive mutants in the α4 gene carry the mutation in both copies of the gene and by observing that sequence modifications in the *c* sequence of the S component repeat appear at both termini if the modifications occur near the *a* sequence. Knipe et al. (6) referred to the identity as "obligatory," to differentiate the recombinational events that occur near the *a* sequence in the S component from those that occur elsewhere, and suggested that the identity is mediated by the interaction of the terminal *a* sequence with those at the internal inverted repeat. Varmuza and Smiley (20) objected to the term because of the observation that within a plaque-purified virus population fragments adjacent to the *a* sequence differed in length by 60 bp. A central question is whether the nonidentical fragments were indeed present within the same genome inasmuch as clonal populations of HSV require multiple serial plaque purifications (12). Indeed, mixed intertypic recombinants were detected in stocks plaque purified under agarose overlays as many as five times. However, even these authors (20) found it impossible to maintain the observed heterogeneity on serial passage of the virus.

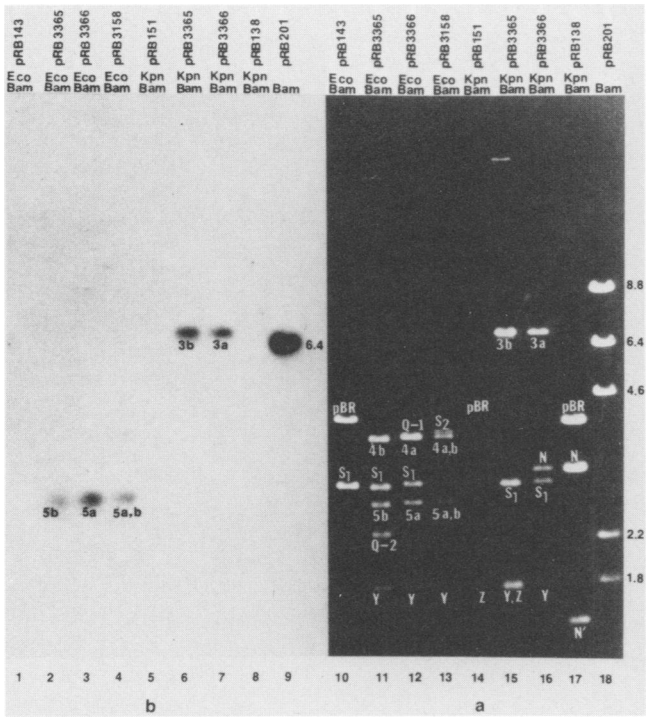

FIG. 5. Photographs and autoradiographic images of pRB3365, pRB3366, pRB151, pRB138, and pRB201 DNA digests electrophoretically separated in agarose gels. Lanes: 1 through 9, autoradiographic images of restriction enzyme digests electrophoretically separated as shown in lanes 10 through 18, transferred to a nitrocellulose sheet, and hybridized with ^{32}P-labeled c_1 probe; 10 through 18, photographs of restriction enzyme digests electrophoretically separated in agarose gels and stained with ethidium bromide. The letter, numbers, or letters and numbers above or below the bands identify the fragments according to the maps shown in Fig. 4. The subscripts in S_1 and S_2 identify *Bam*HI S fragments with one and two *a* sequences, respectively (13). The numbers to the right of the lanes indicate fragment sizes in kilobase pairs. pRB143 carrying the *Bam*HI S_1 fragment, pRB151 carrying the *Bam*HI Y fragment, and pRB138 carrying the *Bam*HI N fragment served as markers. *Bam*HI N is cleaved by *Kpn*I; in lane 17, the large and small products of the *Kpn*I cleavage of *Bam*HI N were designated N and N′, respectively. The bands designated Q-1 and Q-2 represent the products of fusion of the left terminus of the *Kpn*I Q fragment with the right terminus of the *Kpn*I I or *Kpn*I K fragments, respectively, as a consequence of the ligation of the *Kpn*I digest of R3158 DNA before transformation of *E. coli*.

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