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Cloning and Characterization of ori_{L2}, a Large Palindromic DNA Replication Origin of Herpes Simplex Virus Type 2

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An origin of replication within the long unique sequence of herpes simplex virus type 2 designated ori_{L2} has been identified in a position homologous to its type 1 counterpart, ori_{L1}, between map coordinates 0.398 and 0.413. The difficulties encountered in previous attempts to clone both ori_{L2} and ori_{L1} in an undeleted form were surmounted by (i) minimizing the growth of the host *Escherichia coli*, (ii) using a *recBC sbcB E. coli* host, and (iii) purifying the full-length plasmid from deleted forms by using a novel method which exploits the ability of a palindrome-containing plasmid to adopt a cruciform conformation, thereby decreasing its supercoiling. In a previously developed assay for functional origin activity, ori_{L2} was localized to a 241-base-pair *Apa*I-*Sst*II fragment. DNA sequence analysis revealed a 136-base pair, almost perfect palindrome. Comparison with ori_{L1} showed a very high degree of conservation: the two origins differ in only 16 of the 144-base-pair ori_{L1} palindromic region. Most significantly, the differences between ori_{L1} and ori_{L2} mainly occur in pairs so as to generally preserve the potential for intrastrand base pairing. The central region of ori_{L2} is homologous with the shorter palindromic structures detected in origins located within the repetitive sequences of the short component of herpes simplex virus type 1 or 2.

Initiation of replication of herpes simplex virus (HSV) DNA can occur at multiple, widely separated loci. Although this was suggested originally by electron microscopic visualization of the replicating genome (9, 14), subsequent studies which characterized the DNA sequences present in defective HSV type 1 (HSV-1) were more definitive (reviewed by Frenkel [8]). These defective HSVs fall into two classes. The class I defective genome consists of multiple tandem reiterations of a fraction of the full-length genome and always contains a segment of the "c" repeat unit of the S section of HSV-1 DNA. That the class I defective genome bears a replication origin was directly shown by Vlazny and Frenkel (38), who demonstrated that cotransfection of monomer-length class I defective DNA, generated by restriction of the defective genome, with full-length HSV-1 DNA as helper resulted in the replication of the defective DNA and the regeneration of its tandemly repeated form. Origin activity within the "c" sequence was subsequently confirmed by Mocarski and Roizman (22). Stow (35) delimited this so-called ori_S to a 995-base-pair (bp) restriction fragment, and Stow and McMonagle (37) subsequently identified a mere 90-bp segment which sufficed for origin function. In those experiments a recombinant plasmid containing the ori_S segment was shown to be amplified after its transfection into cells which were subsequently infected with HSV-1. Since ori_S resides in a repeat unit, each HSV standard genome contains two copies (or, in the case of HSV-2, four copies [43]) of this replication origin.

Class II genomes contain instead a minimum of 3.4 kilobases (kb) from about the middle of the U_L region of the HSV-1 genome (33). Attempts to clone this region have been frustrated, however, by its marked tendency to suffer deletions when grown in *Escherichia coli* (33, 41, 42). When a plasmid containing the deleted form of this region of the genome is transfected into cells along with HSV-1 DNA, defective genomes indeed arise from the plasmid, but their deleted region has been restored, presumably by recombination with the helper HSV-1 DNA (33). This suggests that the deleted region contains the origin of replication, ori_L, of the class II defective genome. Gray and Kaerner (10), after mapping the region of an HSV-1 (strain ANG) class II defective DNA which deletes in *E. coli*, sequenced this region of the noncloned defective DNA. Similarly, Weller et al. (42) mapped the deleting region of the nondefective HSV-1 (strain KOS) genome to a 425-bp *Bst*EII-*Bam*HI fragment. In addition, Weller et al. were successful in cloning the 2.3-kb *Bam*HI-V fragment, which contains the 425-bp fragment, in a yeast vector in an apparently undeleted form. They showed this construct, p1499, to exhibit origin function since after its transfection into animal cells, infection with HSV-1 caused a dramatic amplification of plasmid. By contrast, deleted clones would not amplify in this assay. Both groups (10, 42) found ori_L of HSV-1 (hereafter referred to as ori_{L1}) to contain a 144-bp (i.e., two arms of 72 bp each) perfect palindromic sequence. As was expected from previous demonstrations of the instability of palindromes in *E. coli* (2, 6, 12, 17, 21, 29), the sequences deleted from p1499 were always at least partially within its palindromic region (42). Thus, the palindrome contains elements required for ori_{L1} function.

ori_{S1} (23, 37, 40) and subsequently ori_{S2} as well (43) were also shown to contain palindromes. These are only about one-third as long the ori_{L1} palindrome, but the middle regions of all three are highly homologous. Knowledge of the sequence of ori_{L2} would complete this picture. Attempts by Spaete and Frenkel to clone ori_{L2} in *E. coli* in an undeleted form have been unsuccessful (34). The present report demonstrates the construction of *E. coli* plasmids containing the undeleted ori_{L2} sequence. We have also demonstrated cloned ori_{L2} to be functional. Finally, we have determined the ori_{L2} sequence. As expected, it is highly homologous to ori_{L1}, yet several important differences are apparent as well. We argue that these differences between the two ori_L sequences further substantiate a physiological role for a cruciform conformation of the palindrome.

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MATERIALS AND METHODS

Cloning procedures. All DNA-modifying enzymes (restriction enzymes, T4 DNA ligase, *E. coli* DNA polymerase I, Klenow fragment of DNA polymerase, DNase I) were from New England Biolabs, Inc. or Bethesda Research Laboratories, Inc. and were used according to the supplier's instructions or established procedures (19). The cloning vectors used were Kos1 (19), pKC7 (27), and pUC19 (45). Electrophoresis was typically done through a 1% agarose (Sigma, medium EEO) gel in submerged "mini" or horizontal (10 by 17 cm) slabs. Preparative gel electrophoresis (used to purify the *Sal*I fragment of pDL017, to separate the deleted and full-length forms of pDL701, and occasionally for DNA sequencing) employed 1% low-melting-point agarose (Bethesda Research Laboratories, Inc.). The desired bands were cut from the ethidium bromide-stained preparative gel, melted at 68°C, extracted with 1 volume of buffer-saturated phenol and then with 1 volume of chloroform, and run over an Elu-Tip column (Schleicher & Schuell, Inc.). The DNA was then eluted from the column as suggested by the supplier and ethanol precipitated. HSV-2 DNA was prepared as previously described (39). The cosmid pBglGJO was constructed by ligation of the product of the partial *Bgl*II digestion of HSV-2 DNA into the Kos1 vector.

The host for all plasmids except those otherwise noted was *E. coli* HB101 (4). *E. coli* JC9387 (*recB21 recC22 sbcB15*; J. R. Gillen, Ph.D. thesis, University of California, Berkeley, 1974) was provided by G. Smith, and strain JC811 (*recB21 recC22 recF143 sbcB15*; 15) was kindly sent by F. Stahl. *E. coli* cells were transformed by established procedures (19). All *E. coli* growth, both on plates and in liquid culture, used L broth (19). After transformation, *E. coli* growth was often kept to a minimum by permitting growth of the transformed bacterium only until it gave a colony of less than 0.5 mm in diameter. Most of the cells in this colony were then removed into 1 to 11 ml of ampicillin-containing broth and grown for 12 to 16 h to a stationary-phase culture, from which plasmid was then purified. Plasmid was prepared from either 1, 10, or 1,000 ml of culture by the alkaline detergent lysis procedure (19). Thorough plasmid purification was achieved using CsCl-ethidium bromide gradients (19).

Replicon assays. The procedure of Stow and McMonagle (37) was closely followed for replicon assays except that the amount of cells used per transfection and the amount of DNA per transfected cell were both scaled down. Briefly, 35-mm-wide wells (six wells per plate) of subconfluent BHK cells, grown on Dulbecco modified Eagle medium containing 5% fetal calf serum, were transfected with equimolar amounts of plasmid (20 to 50 ng of plasmid per well) plus sheared calf thymus DNA (2.4 μg per well) as carrier in 0.2 ml of calcium phosphate precipitate. Cells were refed 1 h after transfection and then shocked with dimethyl sulfoxide 4 h after the DNA was added. At 6 h posttransfection, cells were either mock infected or infected with HSV-2 (strain 333) in Dulbecco modified Eagle medium containing 2% serum at a multiplicity of infection between 5 and 20. After incubation at 37°C for 15 h, all the cells of the infected wells exhibited cytopathic effects. The medium was then aspirated, 0.5 ml of 0.6% sodium dodecyl sulfate–10 mM EDTA–10 mM Tris hydrochloride (pH 7.5)–0.5 mg of pronase per ml was added to the wells, and incubation was continued at 37°C for 4 h. The lysates were transferred to tubes and the wells were rinsed with 0.5 ml of 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA. NaCl was added to 0.2 M, and phenol and chloroform extractions were then performed. The nucleic acid was ethanol precipitated and adjusted to a concentration of 140 μg/ml, as determined spectrophotometrically.

The "minifold" apparatus (Schleicher & Schuell, Inc.) was used as a template for spotting DNA onto nitrocellulose (BA85; Schleicher & Schuell, Inc.). A 50-μl volume of normalized lysate or control sample was combined with 50 μl of 0.4 N NaOH in a 96-well seroculture plate, and 10 μl of this was removed into 90 μl of 0.2 N NaOH for succesive 10-fold dilutions. Before the denatured sample was applied to the nitrocellulose, it was neutralized with 0.35 ml of 0.75 M Tris hydrochloride (pH 7.5)–1 M NaCl. The filter was then gently rinsed, baked, and probed with nick-translated pUC19, using established procedures (19). When pUC19-hybridizable material was analyzed by gel electrophoresis, between 1 and 6 μg of lysate DNA was digested (or not) and electrophoresed through 1% agarose, after which the gel was blotted by the Southern method (32) onto nitrocellulose and the filter was probed as above.

DNA sequence determination. The chemical degradation method of Maxam and Gilbert (20) and the method of dideoxynucleotide extension of synthetic oligonucleotide primers by the large fragment of DNA polymerase using a closed circular DNA template (5, 30) were both used to determine DNA sequence. The sequence of both strands of the 326-bp segment spanning the *Stu*I-*Sst*II sites (Fig. 1) was determined, and any discrepancies were convincingly resolved by additional sequencing runs. The following two fragments were analyzed by the chemical method: (i) pDL601, linearized at the *Kpn*I site in the polylinker sequence of its vector, was 3'-end labeled using terminal tranferase (New England Nuclear Corp.) and cordycepin [α-^{32}P]triphosphate (New England Nuclear Corp.), and the labeled insert was then sequenced after being released by *Hin*dIII digestion and purified on a preparative gel; (ii) after 3' end-labeling of the *Sst*II fragments of pDL601 using terminal transferase and cordycepin triphosphate, the *Stu*I-*Sst*II fragment was made by *Bam*HI digestion and then purified on a preparative agarose gel. The following three determinations using the primer extension method were done: (i) pDL601 was hybridized with d(GTAAAACGACG-GCCAGT) (New England Biolabs, Inc.); (ii) 701A, the full-length subpopulation of pDL701 (see Fig. 3), was hybridized with the "reverse sequencing primer" d(AACAG CTATGACCATG) (New England Biolabs); and (iii) 701B, the deleted subpopulation of pDL701 (see Fig. 3), was hybridized with the same reverse primer.

RESULTS

Cloning of ori_{L2} in an undeleted form. In cloning ori_{L2}, difficulties comparable to those seen when ori_{L1} was propagated in *E. coli* (33, 41, 42) were anticipated. However, in the present study, problems only arose after extensive subcloning. Figure 1 illustrates the series of subcloning steps which purified ori_{L2} from the surrounding HSV-2 sequence. Because the HSV-1 and HSV-2 genomes are known to be colinear, ori_{L2} was anticipated to lie within the O fragment of *Bgl*II-digested HSV-2. Digestion of cosmid pBglGJO with *Bgl*II and ligation of the product into the *Bgl*II site of pKC7 gave pBglO. Comigration of the authentic 4,650-bp *Bgl*II O fragment of HSV-2 (Fig. 2, lane 2) with this insert (Fig. 2, lane 3, lower band) indicates that no detectable deletion had occurred. In fact, however, after purification of the whole insert from pBglO and digestion with *Sal*I (Fig. 2, lane 5), a small amount of deleted fragment was seen in addition to the

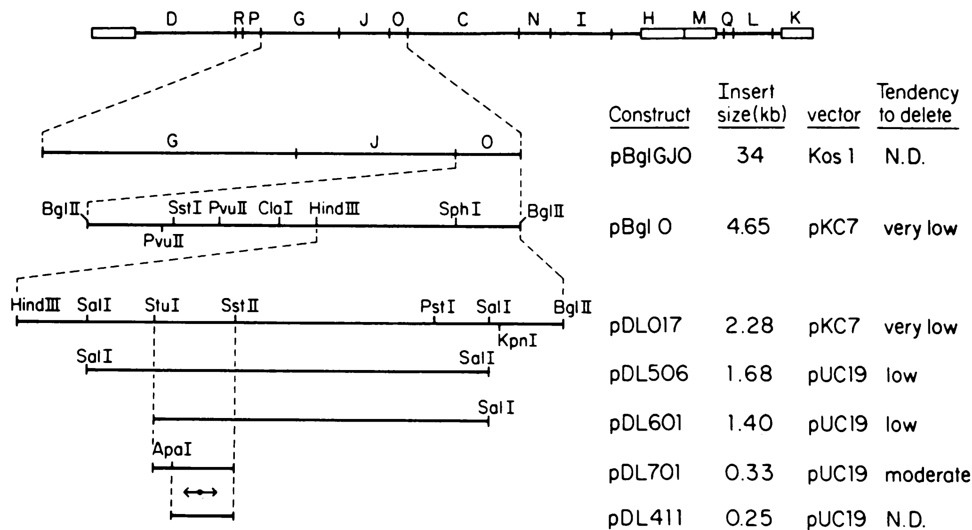

FIG. 1. Restriction enzyme cleavage map of the region surrounding ori_{L2}. The top line portrays the *Bgl*II fragments of HSV-2 (7), and the repeated sections of the L and S regions are indicated by boxes. The lines below portray the successive subclones which contain ori_{L2}. The tendency for the subclones to delete the palindrome, a qualitative assessment described in the text, is summarized in the rightward column. *Cla*I, *Pvu*II, *Sst*I, *Kpn*I, *Stu*I, *Sal*I, *Hin*dIII, and *Sph*I cleave the entire *Bgl*II O fragment only where indicated. *Pst*I cleaves the pDL017 insert only where indicated, but has additional sites in the rest of *Bgl*II O. *Sst*II cleaves the pDL017 insert at least four times, but only the one site which was mapped is shown. The *Cla*I site is cleavable in pBglO grown in *dam*, but not dam^+, *E. coli*. The *Apa*I site was deduced from DNA sequence. The 156-bp palindromic region is represented by a double-headed arrow.

full-length, 1,680-bp internal *Sal*I fragment. The unique *Hin*dIII site within the *Bgl*II O fragment was used to bisect the insert to give pDL017 (containing the rightward half of the *Bgl*II O fragment) and pDL018 (containing its leftward half). In an assay described below, both pBglO and pDL017 contained a functional HSV DNA replication origin, whereas pDL018 did not. The *Sal*I fragment within the insert of pDL017 was then purified and ligated into the *Sal*I site of pUC19, a high-copy-number vector, to give pDL506.

Unlike pBglO and pDL017, it was obvious that pDL506 suffered deletions. Some *E. coli* transformants from this construction yielded homogeneous populations of plasmid from which between 60 and 150 bp of insert was deleted, while other transformants contained a mixture of deleted and apparently full-length molecules. As lanes 7 and 8 of Fig. 2 show, the fraction of deleted material could be kept acceptably small (<10%) by limiting the extent of *E. coli* growth before plasmid preparation. By comparing restriction patterns of plasmid from fully deleted transformants with those of pDL506, the deletions were always found to map between the unique *Stu*I site and the *Sst*II site portrayed in Fig. 1. Thus, the next subcloning step involved the excision of the smaller *Sal*I-*Stu*I segment from the insert by digestion with *Stu*I and *Xba*I, the latter of which cleaves within the vector polylinker. Repair of the ends by DNA polymerase (Klenow fragment) treatment and recircularization by DNA ligase produced pDL601.

pDL601 suffered deletion to about the same extent as did pDL506, and again, the fraction of deleted material in its preparations could be lowered sufficiently (to less than 10% of the population) by minimization of *E. coli* growth (Fig. 2, lane 9). The fragment of insert on the rightward side of the *Stu*I-*Sst*II segment was then excised by digestion with *Sst*II and *Pst*I, the latter of which cuts in the vector polylinker rightward of the *Sal*I site, to produce pDL701. This construct, however, showed an even higher tendency to delete. Even when its *E. coli* HB101 host was grown as little as possible, approximately half of the population of plasmid molecules was deleted (Fig. 2, lane 10). Since DNA sequence cannot be determined from such a heterogeneous population, the deletion problem now had to be solved in other ways.

The elimination of deleted molecules was achieved by two different methods. Initially, the closed circular deleted and full-length populations were purified from each other on a preparative agarose gel. The unfractionated plasmid preparation ("701"; Fig. 3, top) contained, in addition to nicked circles, both a highly supercoiled fraction (comprised of a series of topological isomers which, because of their high supercoiling, were not resolvable on this gel) and a fraction containing the less highly supercoiled topological isomers. These two fractions were separated by cutting a preparative gel through which a large amount of 701 was electrophoresed to give 701A and 701B (Fig. 3, top). Release of the insert by *Bam*HI and *Hin*dIII digestion (Fig. 3, bottom) showed 701 to contain two species of insert, whereas 701A, the less highly supercoiled fraction, contained only the larger insert (shown later to be undeleted) and 701B contained only the smaller, deleted form. The nicked forms which arose during fractionation also illustrated that 701A is larger than 701B (Fig. 3, top).

By changing the *E. coli* host strain, the deletion problem was subsequently more easily solved. A *recBC sbcC* host genotype has been shown to enhance palindrome stability (16, 25, 44). Another study has demonstrated that, in addition, an inactive *recF* gene will allow maintainence of a large palindrome (3). When purified 701A (Fig. 3, top) was introduced into *E. coli* JC9387 (*recBC sbcB*), most transformants, after growth to at least the same extent as the *E. coli* from which the 701 preparation in Fig. 3 was purified, contained a high fraction (>90%) of full-length plasmid (the remaining transformants bore exclusively deleted material). In the same experiment, transformants of *E. coli* JC8111 (*recBC recF sbcB*) had either too low a proportion of full-length material (never more than 60%) or only deleted material (data not shown). Thus the palindromic sequence in ori_{L2}

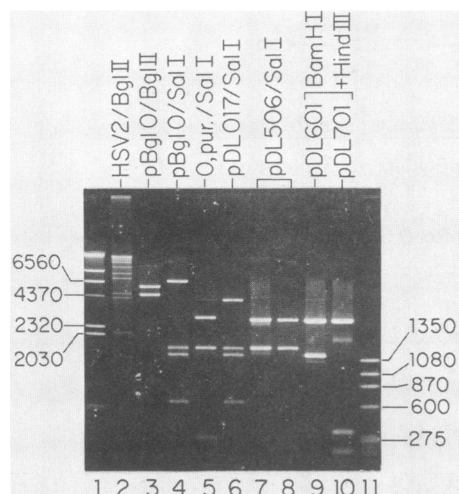

FIG. 2. Restriction enzyme analysis of ori_{L2} subclones. Lane 2, *Bgl*II-digested HSV-2 DNA. Lane 3, Intact insert of pBgl0, released by *Bgl*II digestion. Lane 4, Intact pBgl0, after *Sal*I digestion, gave the 1,680-bp *Sal*I fragment of the insert in addition to two vector-insert junction fragments and a vector-derived fragment. After purification of the insert from pBgl0, digestion with *Sal*I (lane 5) gave the internal 1,680-bp *Sal*I fragment and, in addition, a small amount of deleted *Sal*I fragment migrating slightly faster than the full-length form. *Sal*I digestion of pDL017 (lane 6) again revealed the *Sal*I fragment, but the small amount of deleted *Sal*I fragment was obscured, as in lane 4, by the vector-derived band. Lanes 7 and 8, Two different preparations of pDL506 were digested with *Sal*I to release the full insert. The plasmid in lane 7 was prepared from extensively grown *E. coli*, whereas in lane 8, host growth was kept to a minimum. Lane 9, Release of the complete insert of pDL601 (from minimally grown *E. coli*) by restriction with *Bam*HI and *Hin*dIII, two enzymes whose sites reside in the vector surrounding this insert, providing the 1.4-kb insert and a small amount of deleted material as well. Lane 10, Digestion of pDL701 (prepared from minimally grown HB101 host) with *Bam*HI and *Hin*dIII gave the full-length 335-bp insert and a comparable amount of 190-bp deleted insert. Fragment size markers are *Hin*dIII-digested λ phage DNA (lane 1) and *Hae*III-digested φX174 RF form (lane 11).

appears to be more stable in strain JC9387 than in strain JC8111.

The DNA sequence analysis during the course of the subcloning revealed a unique *Apa*I site within the 326-bp insert of pDL701 and to the left of the palindromic sequence. The final subcloning step was thus the excision of this 85-bp leftward fragment by digestion with *Apa*I and *Kpn*I (another polylinker site) to produce pDL411. In this construction, strain JC9387 served as the immediate host of the ligation product. Again, >90% of the plasmid preparation was full length (data not shown).

Analysis of origin function in vivo. As was shown first with simian virus 40 (24) and later with HSV-1 (35, 37), cloned viral origins can drive the replication of their bacterial vectors when the *trans*-acting viral factors are present in the transfected cell. Using this method, analysis of a series of BAL 31 exonuclease-generated deletants of cloned HSV-1 enabled Stow and McMonagle (37) to delimit sequences necessary for the function of ori_{S1}. We have employed this replicon assay to show that cloned ori_{L2} is functional. In the simplest application of this procedure (Fig. 4), HSV-2 infection was shown to amplify some transfected plasmids but not others. Total DNA from BHK cells, transfected with a plasmid and subsequently infected with HSV-2, was spotted onto a nitrocellulose filter and probed for plasmid vector sequence. There was an enormous increase in dot intensity, upon infection with HSV-2, of DNA from cells transfected with either pBglO, pDL017, pDL601, pDL701, or pDL411 (Fig. 4). In contrast, a much smaller increase in dot intensity was observed when cells were transfected with pDL018 (containing the leftward half of *Bgl*II O) or pUC19. Thus, ori_{L2} is probably contained within the former plasmids, but not the latter.

The vector-hybridizable material in these lysates was also tested for methylation of the N^6 position of adenine in the sequence GATC. This property permits distinction between DNA replicated in *E. coli*, which modifies this sequence using *dam* methylase, from that synthesized in a eucaryotic cell, which is apparently incapable of methylating (or demethylating) the N^6 position of adenine (28). Digestion of lysates with either *Mbo*I, which cleaves only at unmethylated GATC, or with *Dpn*I, which cleaves the same sequence only when methylated, serves to test this property. Figure 5A examines lysates from pDL411-transfected cultures. Lane 1 demonstrates the vector-hybridizable material from the pDL411-transfected and HSV-infected cultures to be of high molecular weight. This was expected, since earlier reports of replication of cloned ori_{S1} (35) and of defective HSV-1 (38) have shown the product to be a tandem repeat of high molecular weight. Digestion with *Mbo*I (Fig. 5, lane 2) completely converted this slow-migrating material into fragments which displayed the same mobility as the digestion products of authentic pDL411 (Fig. 5, lane 12). However, digestion with *Dpn*I had no apparent effect (Fig. 5, lane 3). *Dpn*I was indeed active, since *Dpn*I digestion after addition of *E. coli*-derived pUC19 to the lysate (Fig. 5, lane 5) produced bands characteristic of *Dpn*I-digested pUC19 (lane 10). Lane 6 of Fig. 5 contains undigested lysate from a culture which was not infected with HSV-2 after pDL411 transfection. As demonstrated by the dots of Fig. 4, far less vector-hybridizable material was apparent than in the HSV-2-infected counterpart (Fig. 5, lane 1). The mobility of the uninfected material corresponded to that of authentic pDL411 (lane 11), and none of the high-molecular-weight material of lane 1 was seen. As expected, *Mbo*I digestion (Fig. 5, lane 7) could not alter its mobility, whereas *Dpn*I

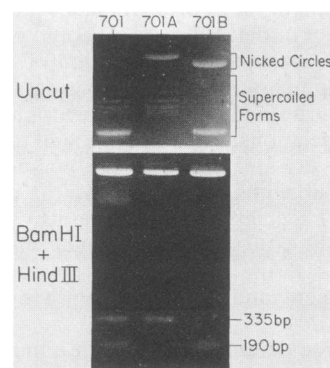

FIG. 3. Separation of the full-length and deleted forms of pDL701. The upper panel contains undigested samples, and the lower shows samples digested with both *Bam*HI and *Hin*dIII, two enzymes which cut on either side of the insert, thereby releasing it. Lane 701, Plasmid pDL701 prepared from minimally grown strain HB101 host. The result of excision of all topoisomers (i.e., excluding the nicked circles) except the highly supercoiled forms from a preparative gel of 701 is shown in lane 701A. Material in lane 701B was purified from a highly supercoiled band of the same preparative gel.

digestion (lane 8) converted it to fragments which corresponded in mobility to authentic *DpnI*-digested pDL411 (lane 12). Thus, Fig. 5A demonstrates that the large amount of vector-hybridizable material which appears upon infection of a pDL411-transfected culture with HSV-2 is indeed synthesized after transfection since (i) it has the characteristic high molecular weight and (ii) it is unmethylated on the N^6 position of adenine in the sequence GATC.

Can HSV-2 infection induce any replication of transfected DNA which lacks HSV origin sequence? This was a possible interpretation of the 10-fold increase in dot intensity of either pDL018- or pUC19-transfected cultures. The methyl group assay was used to test for small amounts of replication of these transfected plasmids (Fig. 5B). Unlike the slow-migrating material seen with pDL411 transfection (Fig. 5A), transfection with pDL018 (Fig. 5B, lane 14) or with pUC19 (lane 23), followed by HSV-2 infection, gave vector-hybridizable material which comigrated with input DNA. Moreover, this material was *DpnI* sensitive (Fig. 5B, lane 15, pDL018; lane 24, pUC19) and *MboI* insensitive (lane 17, pDL018, lane 25, pUC19). Thus, neither pDL018 nor pUC19 was capable of replication. Origin function is therefore quite specific: only when the transfected DNA bears a certain sequence is it capable of replicating after the *trans*-acting factors are provided by HSV-2. The increase in vector-hybridizable material upon HSV-2 infection of cells transfected with plasmids which lack functional HSV origins could perhaps be attributed to an increased permeability of cells to residual calcium phosphate-precipitated DNA.

Sequence of ori_{L2}. When pDL601 and 701A, the material purified from pDL701 growth in HB101 (Fig. 3), were subjected to both the dideoxy triphosphate chain termination (5, 30) and the chemical modification (20) methods, the sequence of the 326-bp *StuI-SstII* segment was determined (Fig. 6). The replicon experiment described above confines the sequence important for ori_{L2} function to bases 86 to 326. The two arms of the palindrome, which extend from base 122 to base 257, are denoted in Fig. 6 by brackets. Note the absence of any intervening nonpalindromic sequence. The AT richness of the ori_{L1} palindrome has been noted previously (42) and is readily apparent for ori_{L2} as well. The 37% A+T content of the entire 326-bp segment is only slightly higher than the 31% A+T content of the entire HSV-2 genome. The 136 palindromic base pairs are 51% A+T, while the 190 bp of flanking nonpalindromic sequence is merely 28% A+T. The middle 96 bp of the palindrome has an A+T content of 64%. Thus, as far as base composition is concerned, ori_{L2}, like its counterpart in HSV-1, is composed of GC-rich domains which flank the AT-rich palindrome. This high AT content is concentrated in the central region of the palindrome.

The deleted subpopulation of pDL701 grown in HB101, 701B (Fig. 3), was also sequenced using the dideoxy triphosphate chain termination method. It was found to be identical in sequence to 701A except for the deletion of bases 94 through 241 of Fig. 6. This directly demonstrates, as had been shown previously in cloning ori_{L1} (42), that deletion occurs at least partially within the palindrome.

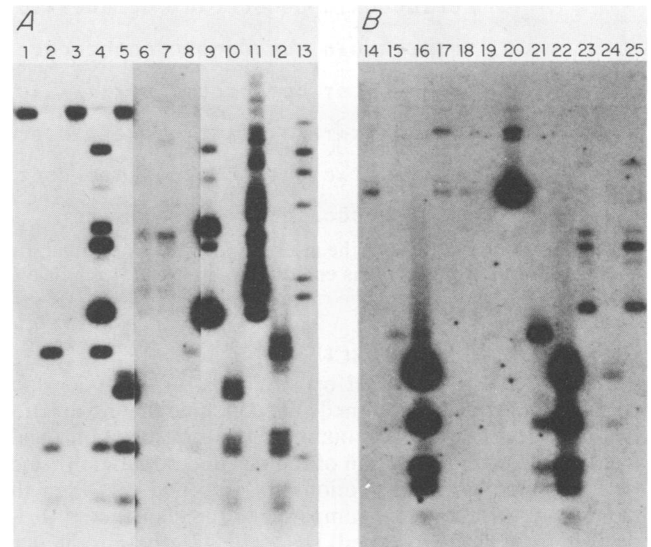

FIG. 5. Digestion of lysates with *MboI* and *DpnI*. pDL411-transfected lysates (A) and pDL018 and pUC19 lysates (B) were analyzed. (A) Lanes 1 through 5 contain lysate from pDL411-transfected and HSV-2-infected cells. Lane 1, Undigested; lanes 2 and 4, *MboI*-digested lysate alone (lane 2) or lysate plus 0.2 ng of pUC19 (lane 4); lane 3 and 5, *DpnI*-digested lysate alone (lane 3) or lysate plus 0.2 ng of pUC19 (lane 5). Lanes 6 through 8 contain lysate from pDL411-transfected and mock-infected cells. A ninefold longer autoradiographic exposure of these three lanes is shown. Lane 6, Undigested; lane 7, *MboI* digested; lane 8, *DpnI* digested. Lanes 9 through 13 contain various markers. Lane 9, pUC19 (0.2 ng), undigested; lane 10, pUC19 (0.2 ng), *DpnI* digested; lane 11, pDL411 (0.3 ng), undigested; lane 12, pDL411 (0.3 ng) *DpnI* digested; lane 13, λ phage DNA, *HindIII* digested and end-labeled with ^{32}P. (B) Lanes 14 through 17 contain lysate from pDL018-transfected and HSV-2-infected cells. Lane 14, Undigested; lane 15, *DpnI* digested; lane 16, lysate combined with 0.2 ng of pUC19 and then digested with *DpnI*; lane 17, *MboI* digested. Lanes 18 and 19 contain pDL018-transfected, mock-infected lysate and are undigested (lane 18) or *DpnI* digested (lane 19). Lanes 20 through 22 bear various markers. Lane 20, pDL018, undigested; lane 21, *DpnI*-digested pDL018; lane 22, *DpnI*-digested pUC19. Lanes 23 through 25 contain pUC19-transfected and HSV-2-infected lysate. Lane 23, Undigested; lane 24, *DpnI* digested; lane 25, *MboI* digested.

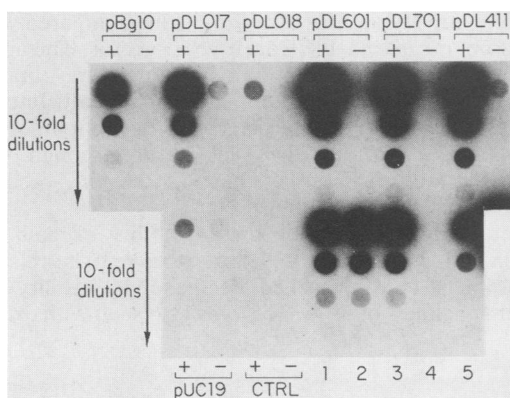

FIG. 4. Hybridization, to vector sequence, of lysates of cells transfected with ori_{L2} plasmids followed by HSV-2 or mock infection. Two wells of BHK cells were transfected with the indicated plasmids combined with carrier DNA or with carrier DNA alone (CTRL). After dimethyl sulfoxide shock, HSV-2 infection (+) or mock infection (−), and overnight incubation at 37°C, cells from 16 wells were lysed, and DNA was prepared. Tenfold dilutions of the lysates were then spotted onto nitrocellulose. In addition, 10-fold dilutions of various controls were spotted. Column 1, 2 ng of pUC19 plus undiluted CTRL, + lysate; column 2, 2 ng of pUC19 plus undiluted CTRL, − lysate; column 3, 2 ng of pUC19 only; column 4, 0.2 μg of HSV-2 DNA; column 5, 2 ng of pKC7. The filter was then probed with nick-translated pUC19.

```
CCTGCGGATG GCGGCCCAGA GCTGGGTATG CTCGGCCGGG GCGGCCGGTA TATGTACGGC   60
GTGCTGGGAG GGGCGGCGTC GGGCCCCGCC CACGGTCCGC CACGCCCCGC GCGTCATCGG  120
C[AGGGGGCGT GGTCGCCCTT CTAAAAAAAG TGAGAACGCG AAGCGTTCGC ACTTTGTCCT  180
AATAGTATA][T ATATTATTAG GACAAAGTGC GAACGCTTCG CGTTCTCACT TTTTTTAGAA  240
GGGCGGCCAC GCCCCCT]TTG ACGTCACGCT CACCCGGGCG GCCGGCCGCC CATAAGCGCG  300
GCCTGCCGGG CCGATAAAAA GAAACC                                       326
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FIG. 6. DNA sequence of the insert of pDL701. One strand, reading 5′ to 3′, of the HSV-2 sequence contained in pDL701 is shown. Each 68-bp arm of the palindrome is enclosed in brackets. *Apa*I cleaves between bases 85 and 86.

DISCUSSION

Cloning of ori_{L2}. A 241-bp fragment of HSV-2 which contains ori_{L2} has been cloned. We conclude that no deletion has occurred during the course of this construction (aside from that in a small fraction of the plasmid population) since (i) the cloned ori_{L2} is functional *in vivo* and (ii) when the cloned ori_{L2} sequence is compared to the sequence of ori_{L1} derived from viral (uncloned) DNA (10, 42), these sequences are highly homologous and colinear, with no indication of sequence present in ori_{L1} that is missing in ori_{L2} (Fig. 7).

Deletion did pose certain problems, but these were surmounted by minimizing the growth of the host, by purifying deleted from undeleted plasmid, and by using a better host strain. The problem is most simply solved by limiting the growth of the *E. coli* transformant. If this is unsuccessful, the use of a different host strain is necessary. Since ori_{L2} is more stable in strain JC9387 (*recBC sbcB*) than in strain JC8111 (*recBC recF sbcB*), whereas the large palindrome from minute virus of mice is more stable in the latter (3), both strains should be tested when cloning palindromes.

The purification of undeleted palindrome-containing plasmid reported here could be used when the simpler approaches prove unsuccessful. This procedure (Fig. 3) exploits the ability of palindromes to adopt the cruciform structure (21, 26). The number of supercoils is determined in part by the number of primary helical turns in a closed circular molecule of DNA. The extrusion of palindromic sequence into a cruciform conformation depletes a molecule of primary helical turns. The negative supercoiling of the plasmid thereby decreases by an amount which depends on the size of the palindrome. In the case of ori_{L2}, about 13 supercoils are removed by cruciform formation (136 bp of palindrome ÷ 10.4 bp per helical turn). The average number of supercoils in noncruciform pDL701 is about 18 (0.096 helical turns per bp × 3,100 bp × 0.06 supercoils per helical turn; 1). Cruciform formation, which presumably occurs only during the course of plasmid purification, therefore causes a substantial reduction in supercoiling. Since electrophoretic mobility depends on the degree of supercoiling, the plasmid which is capable of a decrease in supercoiling by virtue of cruciform formation is separable from that which lacks the palindrome.

Palindromic sequences are known to be poorly propagated in *E. coli* (2, 6, 12, 17, 21, 29). ori_L of HSV was first reported to delete in *E. coli* by Spaete and Frenkel (33), who examined HSV-1. Weller et al. (42) were subsequently able to clone ori_L, but only in a yeast vector since deletion or ori_{L1} in *E. coli* proved to be too severe. Since ori_{L1} cloned in yeast was functional in a replicon assay similar to the one described here, it appears that at least part of the plasmid preparation used was undeleted (42). Previous attempts to maintain ori_L of HSV-2 in *E. coli* have been unsuccessful. Spaete and Frenkel (34) indicate that the *Sal*I fragment (Fig. 1, this paper) suffers "small" deletions. Hayward and Reyes (13) had earlier commented on such deletion in HSV-2, although they claimed that the *Cla*I site, which is 1,200 bp away from the palindrome (Fig. 1, this paper), is always within the 120 bp of deleted sequence. Since we find no evidence of deletion around the *Cla*I site, and since the *Cla*I site is cleavable in plasmid from *dam* but not dam^+ *E. coli* (data not shown), it is likely that the earlier study (14) did not detect a *Cla*I site in the cloned DNA due to *dam* methylation rather than to deletion. The present study, in successfully cloning full-length ori_{L2} in *E. coli*, allows the preparation of relatively large quantities of an ori_L clone compared with the small amounts obtainable from a yeast vector. The ability of the present study, but not previous ones, to obtain full-length ori_L in *E. coli* can be most likely attributable to technical differences and perhaps to an inherent difference in the behavior of ori_{L1} and ori_{L2} in *E. coli*, although the high degree of similarity between the ori_{L1} and ori_{L2} sequences argues against the latter interpretation.

What might account for the instability of palindromic DNA in *E. coli*? One fact which may prove to be relevant to this problem is the marked decrease in stability of the smaller subclones of ori_{L2}. This was also seen with ori_{L1} (42).

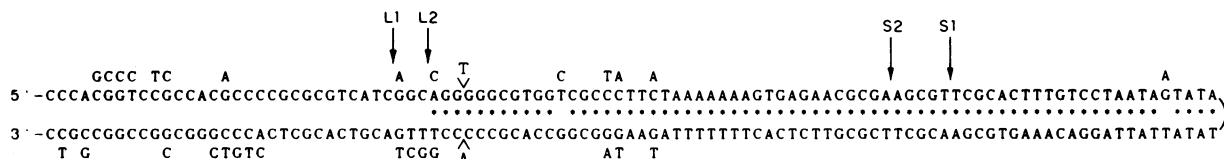

FIG. 7. Palindromic region of ori_{L2} and its comparison with ori_{L1}. One strand of the ori_{L2} sequence (bases 89 through 290 of Fig. 6) is portrayed in a snakelike fashion to illustrate the potential for intrastrand basepairing. The A and T on the rightward ends of the two lines are adjacent in its sequence. Each asterisk denotes a potential intrastrand base pair of ori_{L2}. The bases of ori_{L1} (42) which are different from ori_{L2} are indentified by letters above or below the lines of ori_{L2} sequence. For instance, ori_{L1} contains an A instead of G in the fifth position from the right end of the top line. The carats indicate that the A and T are inserted in ori_{L1} relative to ori_{L2}. The extent of the palindromes of ori_{S1}, ori_{S2}, ori_{L2}, and ori_{L1} are indicated by arrows labeled S1, S2, L2, and L1, respectively.

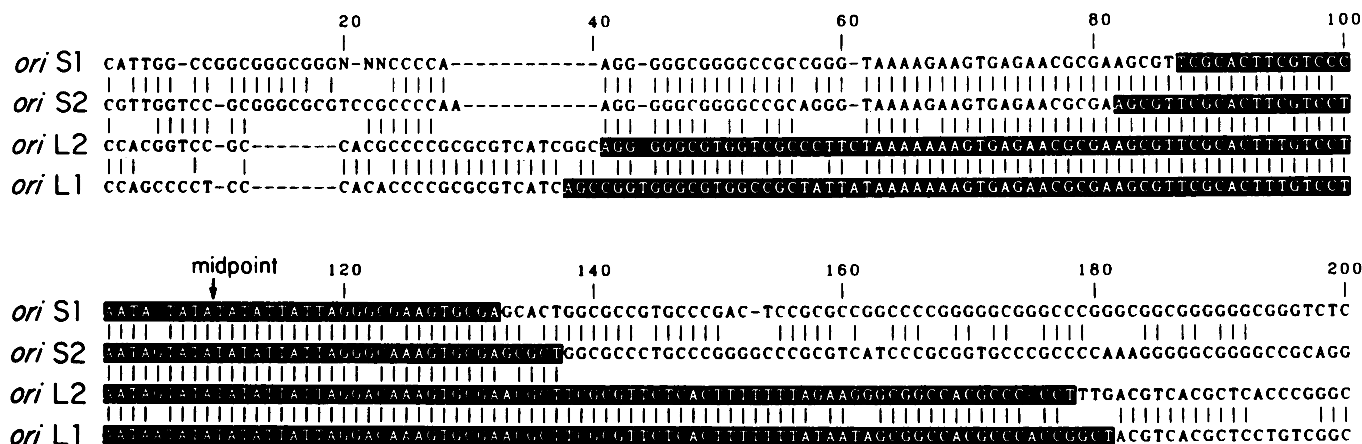

FIG. 8. Four HSV replication origins. Sequences are aligned so that the centers of the palindromes (noted as "midpoint") coincide. The palindromic regions are indicated with white lettering. To allow the optimal alignment of the sequences, gaps (indicated at dashes) are inserted. Thus, for example, G (base 60) and T (base 61) are adjacent in the ori_{S1} sequence. Vertical lines indicate that corresponding bases in adjacent sequences are the same. The ori_{S1} sequence is taken from reference 23 (bases 2345 through 2526; N indicates bases which were undetermined); the ori_{S2} sequence is taken from reference 43 (bases 711 through 896), the ori_{L2} sequence shown is bases 90 through 279 of Fig. 6, and the ori_{L1} sequence is taken from reference 42 (bases 147 through 338).

In the present study, pBglO and pDL017 were initially thought to be refractory to deletion. In retrospect, however, it appears that a small fraction of pBglO prepared from extensively grown *E. coli* is deleted (Fig. 2, lane 5). pDL506 and pDL601 have a higher tendency to delete, and extensive *E. coli* growth had to be avoided to give a sufficiently high fraction of full-length material. Further subcloning to give pDL701 caused an even higher deletion rate. Presumably pDL411 tends to delete to a similar, or perhaps greater extent than does pDL701, although this was not tested. Unfortunately, no simple way to quantitate this phenomenon is apparent. The increase in deletion tendency with smaller insert size is not due simply to a change in plasmid size. When the *Sal*I fragment of pDL017 was ligated into pKC7 (5.8 kb) instead of pUC19 (2.7 kb), the product had as great a tendency to delete as did pDL506 (data not shown). Thus, it appears that some feature of the surrounding HSV sequence has a stabilizing effect on palindromes in *E. coli*. Examination of these flanking sequences would be most interesting if other natural eucaryotic palindromes could be shown to act similarly in this regard.

Features of the ori_{L2} sequence. The most salient feature of ori_{L2}—indeed, the feature which prevented it from being easily cloned—is its 68-bp palindrome. This is illustrated in Fig. 7 by noting with asterisks the 66 potential intrastrand base pairs. Note that aside from two mismatches, the palindrome is perfect, i.e., there are no bases which serve to interrupt the register of the two arms.

Figure 7 also illustrates the differences between ori_{L2} and ori_{L1}, noted as letters above and below the portrayal of ori_{L2} sequence. The 16 differences which occur within the 144-bp palindromic region (rightward of the arrow labeled L1 in Fig. 7) are of two types: those in ori_{L2} which eliminate potential intrastrand base pairs, and those which occur in pairs so as to preserve the potential of intrastrand base pairing. Ten of the 16 differences fall into this latter group of paired changes, as far higher fraction than should occur if there were no selection pressure to maintain the relationship between the two arms of the palindrome. For two changes to constitute a pairwise change, they must not only occur at corresponding positions on the two arms, but they must also be altered to the correct base as well. Four of the pairs of changes are simply the substitution of G and C for A and T or vice versa. The fifth pair of changes, the insertion of T and A in ori_{L1} relative to ori_{L2}, is particularly striking. Thus, despite the 16 differences in the ori_{L1} and ori_{L2} palindrome, the two differ by only six in the number of potential intrastrand base pairs.

At least two functions of palindromes can be envisioned (21). It is possible that the twofold rotational symmetry inherent in a DNA palindrome is important only for its interaction with a dimeric protein complex which also exhibits twofold symmetry. Each half of the dimer would thus recognize one arm of the palindrome. To explain the high degree of perfection of the palindrome, the protein complex would have to be at least as long as the palindrome, binding to it very intimately so as to recognize all the bases over its entire length. If this were not the case, there would be no basis to explain the conservation of palindromic perfection. Considering the extreme structural demands which this model would place on such a hypothetical protein complex, we consider it unlikely.

In the other model, the palindrome exists because of its ability to adopt a cruciform conformation such that the two arms of the palindrome base pair with each other in an intrastrand fashion. This is suggested merely by the existence of these large perfect palindromes. The comparison of the two ori_L sequences even further suggests this model: the differences, occurring mainly in pairs, serve to maintain the potential for intrastrand base pairing. We thus consider it likely that the ori_L palindrome becomes a cruciform during at least part of the viral life cycle. Cruciform formation is well known to be influenced by DNA torsional strain (11, 21, 26), and this is certainly a plausible mechanism for a hypothetical ori_L structural transition. It should be noted, however, that there is no evidence for such strain in HSV-1 in vivo as determined by the rate of trimethylpsoralen photobinding (31). This does not eliminate the possible existence of a small class of replicating viral genomes which have unrestrained supercoils, as has been seen in simian virus 40 (18).

It is unlikely that the base pairing in the cruciform could be maintained all the way to the center of the palindrome, yet there is no nonpalindromic sequence between its two arms. Interestingly, this central region is not simply palindromic but is, moreover, an alternating AT sequence. Perhaps the

unique sequence in this region serves a function different from that of the rest of the palindrome. Its AT composition makes it easily meltable, and the alternating sequence could be necessary to allow this region to melt as easily as possible (11) to allow the initiation of cruciform formation or of replication itself. Stow (36), in fact, has shown the center of the ori_{S1} palindrome to be essential for origin function.

Figure 8 compares the four HSV *ori* sequences. The relationship between ori_{L1} and ori_{L2} has been discussed above. Whitton and Clements (43) have previously discussed the relationships between ori_{S1} and ori_{S2}. As reiterated in Fig. 8, these two sequences are virtually identical. Weller et al. (42) have discussed the relationship between ori_{L1} and ori_{S1}, and these are not directly compared in Fig. 8. Because of the close similarity between ori_{L1} and ori_{L2} and between ori_{S1} and ori_{S2}, general comparisons between ori_L and ori_S can be made. As noted previously (42), the sequences leftward of the centers of the ori_L and ori_S palindromes are highly homologous, whereas rightward of this center, the homology extends only for about 25 bases. Thus, the ori_S palindrome is quite similar to that of ori_L, but is only about 25 bases long.

A comparison of ori_S and ori_L suggests that not all of the more extensive palindrome of ori_L is required for replication. After all, since ori_{S1} is functional with merely a 23-base palindrome (37), and 47 or so additional palindromic bases in ori_L should be nonessential. Furthermore, since Stow and McMonagle have shown that a plasmid (S11 in reference 37) which contains 298 bp of HSV-1 sequence rightward of base 51 (Fig. 8) is a functional ori_{S1} replicon, whereas a plasmid (S13 in reference 37) whose HSV-1 insert extends rightward from base 84 (Fig. 8) is nonfunctional, a sequence essential for ori_{S1} function resides within the sequence homologous to the 25th through the 57th base of the leftward arm of the ori_L palindrome (bases 51 through 83 of Fig. 8). In other words, a sequence which is essential for ori_S function lies outside of its palindrome, while its homolog in ori_L is contained within the ori_L palindrome. If ori_S and ori_L function similarly, an assumption at this point based solely on their homology, this again suggests that the outer region of the palindrome is required for some function other than origin activity. A role for the ori_L palindrome in the control of replication or transcription thus becomes a tempting possibility. If this view were correct, the outer region of the ori_{L2} palindrome should be superfluous with respect to origin function in the replicon assay. We are currently examining the significance of various elements within the ori_{L2} region for origin activity.

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