JOURNAL OF VIROLOGY, Nov. 1991, p. 6283–6291
0022-538X/91/116283-09$02.00/0

Vol. 65, No. 11

Analysis of an Origin of DNA Replication Located at the L Terminus of the Genome of Pseudorabies Virus

SABINA KUPERSHMIDT,† JEAN M. DEMARCHI,‡ ZHIQIANG LU,§ AND TAMAR BEN-PORAT*

Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Received 28 May 1991/Accepted 6 August 1991

We have localized an origin of DNA replication at the L terminus of the pseudorabies virus genome. This origin differs in location as well as in general structure from the origins of replication of other herpesviruses that have been identified. The 600 leftmost nucleotides of the genome that were found to include origin function have been analyzed. This sequence is composed of an 82-bp palindrome whose center of symmetry is separated by 352 unique bp (U_L2). Within the U_L2, a sequence that fits the consensus sequence of the NF1 binding site, as well as one that has partial homology to the binding site of UL9 of herpes simplex virus, is present. Using truncated fragments of DNA, sequences essential for minimal origin function were delimited to within a fragment that includes the terminal 104 bp of the left end of the genome. Within these 104 bp, two elements essential to origin function have been identified. One of these elements is present within the terminal 64 bp of the L component (within one of the palindromic arms). The other is present within the 22 bp of the UL2 adjacent to this palindromic arm. Other auxiliary elements, although not essential for origin function, contribute to more efficient replication. The NF1 and UL9 binding site homologies were found to be nonessential to origin function.

After penetrating the nucleus of the infected cell, the linear genome of pseudorabies virus (PrV) circularizes and the two genomic termini become covalently linked (6, 15). Replication of the circularized genome is initiated at a specific origin of replication near or within the inverted repeat, producing theta-type structures (6, 15). During later phases, herpesvirus DNA replication occurs in association with concatemeric forms in which genomes are in head-to-tail alignment (4, 5, 14). These observations suggest a rolling circle mode of replication (2), a conjecture that has been supported recently (24).

Two different *cis*-acting sequences (origins) mediating the replication of the herpes simplex virus (HSV) genome were inferred from an analysis of defective interfering particles. One origin is present in the S component (ori_S), and the other is present in the L component (ori_L); the two origins have sequence homologies. These sequences have been cloned from the wild-type HSV genome and have been characterized previously (7, 8, 18, 19, 22, 29, 30, 33, 34).

Two origins of replication have also been identified in PrV, one around the middle of the U_L region and the other thought to be within the S component. Both appear to be enriched for defective interfering particles (35). We had previously concluded on the basis of short pulse-labeling experiments that during the first round of DNA replication, initiation starts within the inverted repeats and proceeds unidirectionally from there (6). However, using plasmid amplification assays, *cis* functions capable of acting as an origin of replication have been found in a region near the middle as well as near the L terminus of the PrV genome (35); a sequence that functions as an origin could not be detected within the S component. It appears possible, therefore, that the origin previously thought to be in the S component resides at the L terminus of the genome and that DNA replication proceeds from there unidirectionally through the concatemeric junction on the circularized DNA into the S component.

Our previous studies dealing with the identification of the origin of replication at the L terminus of the PrV genome (L-ori) have demonstrated that (i) origin activity is contained in a 3.1-kb segment of DNA that spans the concatemeric junction, (ii) sequences within that junction fragment that were derived from the right end of the genome (the S terminus) did not have replication activity but sequences derived from the left end (the L component) did, and (iii) sequences from both ends of the genome were required for efficient cleavage/encapsidation. Thus, the left-terminal 1.4 kb of the genome of PrV include signals necessary for both replication and cleavage/encapsidation (35).

The further analysis of L-ori was deemed interesting because of its position, which differs from that of other origins of replication of herpesviruses. Furthermore, the proximity of the origin of replication to the cleavage/encapsidation site also appeared to be worthy of further attention. In this report, we delimit L-ori of PrV and identify its core and auxiliary sequences.

MATERIALS AND METHODS

Virus strains and cell culture. PrV(Ka) is a strain that has been carried in our laboratory for more than 25 years. The virus was grown in PK or RK cells which were cultivated in Eagle's synthetic medium containing 5% bovine serum.

Enzymes and chemicals. Restriction enzymes, DNA polymerase I, and T4 DNA ligase were purchased from Bethesda Research Laboratories, Inc. T4 polynucleotide kinase was

* Corresponding author.
† Present address: Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232.
‡ Present address: Institute for Molecular Genetics, Baylor College of Medicine, Houston, TX 77030.
§ Present address: Department of Pathobiology, University of Connecticut, Storrs, CT 06268.

purchased from U.S. Biochemical Corp. [α-^{32}P]dCTP was purchased from New England Nuclear Corp.

Purification of virions and of viral DNA. A continuous line of PK cells was infected (multiplicity of infection of 0.5 PFU per cell) and incubated for 24 h in Eagle's medium at 37°C. Virions were purified as described previously (3) and were lysed by the addition of Sarkosyl (final concentration, 2%). The samples were incubated for 15 min at 60°C and digested with nuclease-free pronase (1 mg/ml) for 2 h. The DNA was extracted with phenol-chloroform-isoamyl alcohol (50:48:2, vol/vol/vol) and dialyzed against buffer (0.01 M Tris, 0.001 M EDTA, pH 7.6).

Southern and sequence analysis. Restriction fragments were separated on agarose gels and transferred to nitrocellulose filter paper. The immobilized DNA was hybridized to [α-^{32}P]dCTP-labeled, nick-translated DNA probes (26). Sequencing of the junction fragments was performed as described previously (9, 13, 20).

Characterization of plasmids. All constructs were analyzed by restriction endonuclease digestion for the presence or absence of the appropriate restriction sites. In some cases, they were also sequenced.

Construction of plasmids. Plasmids pJ31, p11, and p4 are the concatemeric *Bam*HI junction fragments of viral DNA obtained from PrV(Ka)- and PrV(USA)7-infected cells cloned into the *Bam*HI site of pBR325. All other plasmids used were constructed by isolating the desired fragments (indicated in the text) from gels, blunt ending them by filling in the overhangs, and ligating them into the *Sma*I site of pTM. (pTM is a derivative of pBR322 into which the multiple cloning site of M13 had been introduced. The construction of pTM is described in more detail elsewhere [24b].)

Replication assays. The assay used to test for origin function in test plasmids is based on the fact that in the presence of helper PrV (which provides viral *trans* functions), plasmids carrying a PrV origin of replication will be amplified. To assay for origin function, test plasmids containing cloned fragments of PrV DNA were cotransfected into RK cells in the presence of helper virus by the calcium phosphate precipitation method (12). The intracellular DNA was harvested 3 days postinfection. The DNA was extracted, digested with *Dpn*I, electrophoresed, transferred to nitrocellulose filters, and hybridized to either nick-translated pBR325 DNA or nick-translated PrV DNA (to ensure that comparable amounts of DNA in all samples had been loaded on the gel). *Dpn*I distinguishes between DNA that has replicated in bacteria from DNA that has replicated in mammalian cells; it cleaves DNA methylated by bacterial methylases, leaving DNA replicated in eukaryotic cells intact. If a test plasmid contains an origin of replication, plasmid sequences will therefore be found in large concatemeric structures while nonreplicated input DNA will be digested by *Dpn*I to smaller fragments.

To quantitate the degree of amplification of the plasmids, appropriately exposed autoradiograms of the Southern blots onto which different amounts of DNA had been loaded were subjected to densitometric scanning and the areas under the curves were determined.

RESULTS

Localization of L-ori function to the terminal 600 bp of the L component of the genome. As a first step in characterization of the sequences necessary for origin function that are present within the L-terminal 1.4 kb of PrV, subfragments of the DNA of the concatemeric junction were tested for their ability to drive the amplification of covalently linked plasmid sequences. pJ31, which contains the *Bam*HI concatemeric junction of PrV(Ka), was cleaved with *Sma*I, and the two fragments contained within the L-terminal 1,400 bp, *Sma*I fragment 4 and *Sma*I fragment 2 (Fig. 1), were subcloned into pBR325. The ability of the two subclones (p529 and p296) to replicate in the presence of PrV helper functions was ascertained (Fig. 2). To obtain results that would allow us to estimate with some confidence the relative degree of amplification of a given plasmid, this as well as all other experiments presented in this report were repeated several times using at least two different plasmid preparations. The results obtained were in all cases similar, although some quantitative (but not qualitative) differences between experiments were obtained with any given plasmid (see below).

pJ31, which contains the concatemeric junction (Fig. 2, lane 2), as well as pD11, which contains the 1.4 kbp derived from the L terminus of the PrV genome (lane 3), as expected, replicated well. pE273, which contains the 1.7 kbp derived from the S terminus, did not replicate (lane 1), confirming previous results (35) which showed that sequences that can function as an origin of replication are present within the L terminus but not the S terminus of the genome.

pJ31, which includes both ends of the genome, was amplified more effectively than was pD11, which includes only the L terminus (Fig. 2; compare lane 2 with lane 3). This can be attributed, at least in part, to the fact that pJ31 contains, in addition to an origin of replication, a complete cleavage/encapsidation signal (35) and can therefore replicate, be packaged into viral capsids, and be recycled. The possibility that the sequences within the S terminus (pE273), even though unable to direct replication on their own, might have an enhancing effect on the L-ori located at the end of the L terminus cannot, however, be eliminated.

In cells cotransfected with PrV DNA and with p529 (*Sma*I fragment 4) but not p296 (*Sma*I fragment 2), amplification of plasmid sequences was observed (Fig. 2, lanes 4 and 5), indicating that p529 includes all *cis* functions necessary for replication. However, even though p529 can act as an origin, whereas p296 cannot, the latter may include sequences that enhance replication because p529 (lane 5) does not replicate as efficiently as does pD11, which includes the sequences of both p529 and p296 (Fig. 2, lane 3; Table 1).

*Sma*I fragment 4 (p529), which has origin function, includes the terminal 584 bp of the L component and 17 bp of the S terminus of the genome. The 17 bp from the S terminus are not, however, essential for origin function since pD11, which consists of the *Bam*HI L-terminal fragment only, i.e., does not include these sequences, replicates well.

Sequence analysis of *Sma*I fragment 4. The nucleotide sequence of *Sma*I fragment 4 was available (9) (Fig. 3) and was inspected for features that may be related to origin function.

An 82-bp perfect inverted repeat, whose two arms are separated by 352 nucleotides, was observed. Because palindromic sequences are a common feature of other origins of replication (for a review, see reference 17), this observation seemed of interest. Within the unique sequence (U_L2) separating the arms of the palindrome, a sequence (TGGCAAG GTGCCAA) that fits the consensus sequence of the NF1 binding site perfectly (16, 27) was observed. Another sequence of interest was one with partial homology to the binding site of HSV UL9, which is a protein required for HSV ori$_S$-directed replication (8, 10, 11, 23). The PrV sequence has 77% homology with the sequence required for the binding of the UL9 protein to HSV type 1 DNA.

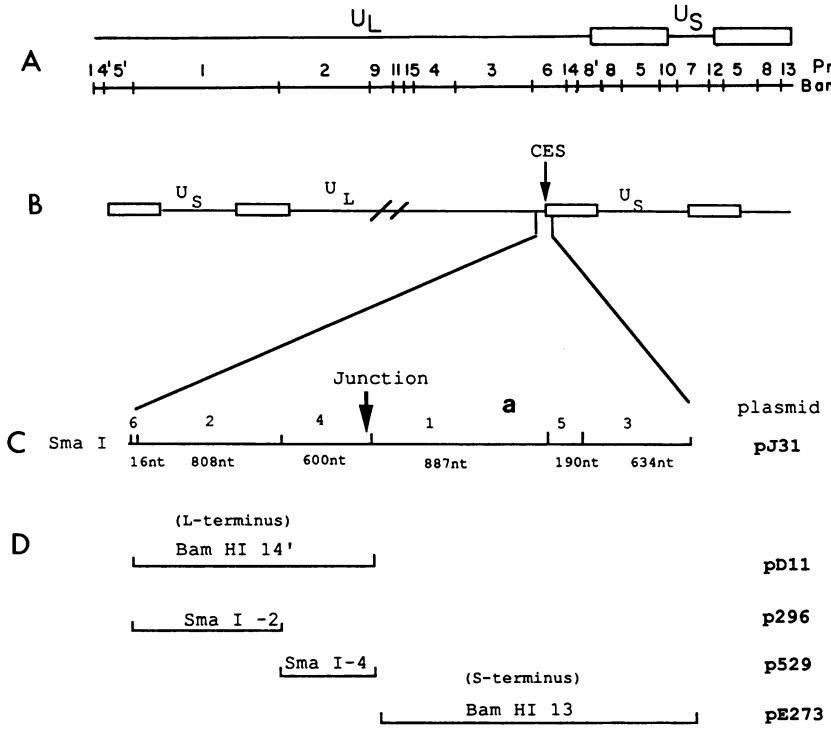

FIG. 1. Map of plasmids constructed to locate sequences with origin function within the concatemeric *Bam*HI fragment junction. (A) *Bam*HI restriction map of the PrV genome. (B) The *Bam*HI concatemeric junction of PrV DNA (pJ31) consisting of the two ends of the viral genome was obtained from intracellular DNA and was cloned in pBR325 (pJ31). CES, cleavage/encapsidation site. (C) *Sma*I map of pJ31. (D) Location of inserts in subclones pD11, p296, p529, and pE273. pD11 and pE273 are the *Bam*HI end fragments of the PrV genome. p296 and p529 are *Sma*I subclones of pJ31.

The sequence characteristics of the L-ori-containing DNA raised several questions concerning the role in origin function of the following: (i) the length and integrity of the unique sequence separating the two arms of the palindrome; (ii) the integrity of the dyad symmetry; (iii) the U_L2 sequence which is flanked by the 82-bp inverted repeat, in particular the sequences with homology to the NF1 binding site; and (iv) sequences within each arm of the palindrome. The experiments described below were designed to answer each of these questions.

Importance of the length and integrity of the unique sequences separating the two arms of the palindrome. Stretches of foreign sequences (M13) of various lengths were inserted into the unique *Sst*II site of *Sma*I fragment 4 (Fig. 4) to define the importance of the distance separating the two arms of the palindrome. The results of an amplification experiment with one such clone (pA3) are shown in Fig. 2. pA3 (lane 8) replicates as well as the parent plasmid p529

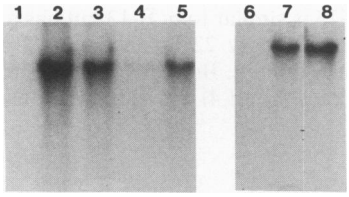

FIG. 2. Amplification of subclones derived from pJ31 in cells cotransfected with PrV DNA. RK cells were cotransfected with PrV DNA and the indicated plasmid. Three days later, the cells were harvested and DNA was extracted, digested with *Dpn*I, and electrophoresed. After transfer to nitrocellulose filters, the DNA was hybridized to nick-translated pBR325 DNA. To ensure that equal amounts of DNA were analyzed in all cases, parallel filters were also hybridized to PrV DNA. Lanes: 1, pE273; 2, pJ31; 3, pD11; 4, p296; 5, p529; 6, pBR325; 7, p529; 8, pA3. (pA3 has an insert of approximately 600 bp of M13 DNA in the *Sst*II site within the U_L2 of p529 [see Fig. 4 for map].)

TABLE 1. Estimate of degree of amplification of different plasmids[a]

Plasmid	L-terminus bp	S-terminus bp	No. of transfections performed	Avg % replication
pD11	1–1391		2	100
p529	1–584	1–17	2	45 (60–30)
pS4L13	328–584		8	0
pS4H4	1–327	1–17	8	27 (35–15)
pCB13	1–190	1–17	4	25 (31–14)
pCF13	1–101	1–17	4	13 (18–11)
pJ31	1–1391	1–1727	5	100
pRR9	1–327	1–661	5	32 (90–20)
pcB3	1–190	1–661	6	29 (35–25)
pcF6	1–101	1–661	6	11 (20–9)

[a] The DNA obtained from transfected cells was digested with *Dpn*I. Different concentrations of DNA were electrophoresed, transferred to nitrocellulose filters, and hybridized with nick-translated pBR325. Appropriately exposed films were scanned, and the amount of plasmid DNA present in the cells was estimated. The results are expressed as the amounts of plasmid DNA found in cells transfected with different plasmids relative to those found in cells transfected with pD11 or pJ31. Numbers in parentheses indicate the variations observed between different experiments.

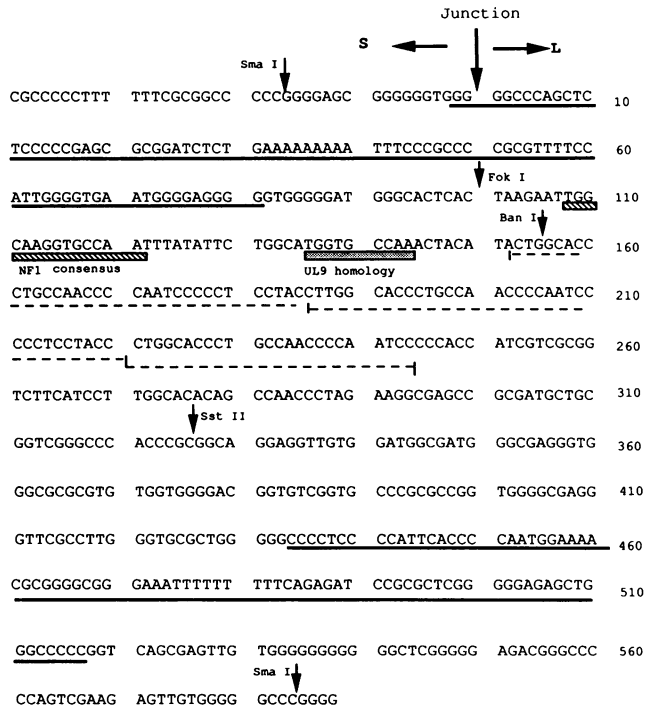

FIG. 3. Nucleotide sequence of *Sma*I fragment 4. The 82-bp arms of the palindrome are underlined by bold lines; the 34-bp repeated sequences are underlined by broken lines. Selected restriction enzyme cleavage sites are indicated. The sequence is numbered with the junction between the S and L component (or the left terminus of the genome) being bp 1.

(lane 7). Thus, the distance between the two arms of the palindrome does not appear to affect the ability of the plasmids to be amplified. Indeed, we previously observed that the copy number of a 34-bp direct repeat sequence (Fig. 3), present in the U_L2 of three different PrV isolates, varied (21). Plasmids carrying the concatemeric junction of these isolates replicate well (see below), indicating that the distance between the arms of the palindrome is not important. Since 34 bp constitute slightly more than three turns of the helix, the cooperation between the arms of the palindrome also does not appear to be necessary for origin function.

Importance of the integrity of the dyad symmetry. To ascertain whether both palindromic arms are essential for origin function, *Sma*I fragment 4 was cleaved with *Sst*II (Fig. 4) and the two resulting fragments were inserted into the *Sma*I site of pTM. pS4H4 (plasmid H) includes the 326 leftmost bp of the viral genome, while pS4L13 (plasmid L) includes the remainder of *Sma*I fragment 4. Each clone contains one complete and equal palindromic arm, but the unique flanking sequences differ.

While plasmid L did not replicate detectably in amplification assays (Fig. 5, lanes 3), plasmid H (lanes 2) replicated well, indicating that the presence of both arms of the palindrome is not essential for origin function. (To illustrate the quantitative variation that is observed, the results of three different experiments are presented.) Plasmid H (lanes 2) did not, however, do quite as well as did the parent p529 (lanes 1), which includes the entire *Sma*I fragment 4 sequence. Thus, the presence of either the second palindromic arm or the unique sequences present in plasmid L appears to augment the level of amplification.

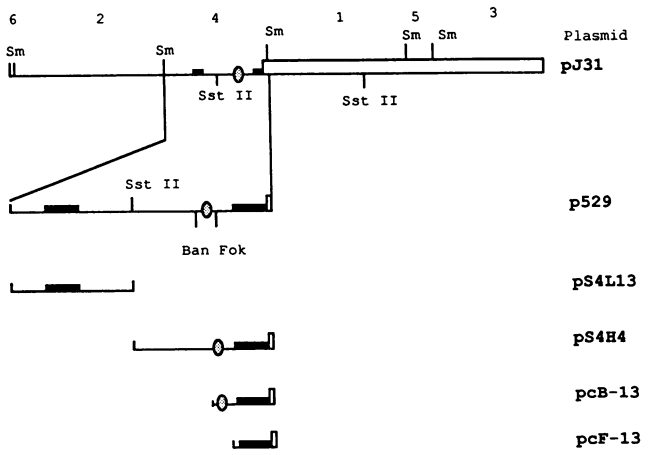

FIG. 4. Plasmids constructed to localize the sequences within *Sma*I fragment 4 that have origin function. Shown are *Sma*I and *Sst*II restriction maps of pJ31, a map of p529 (*Sma*I fragment 4), and maps of four subclones of *Sma*I fragment 4. *Sma*I fragment 4 was cleaved with the indicated enzyme, and the single-stranded overhangs were filled in. The fragments were then ligated into the *Sma*I site of pTM. The open rectangle indicates sequences from the S component; the lines indicate the sequences from the L component. Heavy lines indicate the 82-bp arms of the palindrome in the L component; circles indicate the sequences with homology to the NF1/UL9 binding site. Sm, *Sma*I; Ban, *Ban*I; Fok, *Fok*I.

Since plasmid L did not replicate detectably, the sequences within one arm of the palindrome are clearly not sufficient for origin function, at least not to the extent required to allow its detection in these assays. Thus, the unique sequences that are adjacent to the palindromic arm in plasmid H, but not plasmid L, include *cis* functions essential for replication. It was interesting, therefore, to note that plasmid H includes both the NF1 binding site homology as well as the sequences with partial homology to the HSV UL9 binding site.

Importance in origin function of the unique sequences flanking the palindromic arm. To ascertain whether the putative NF1/UL9 binding sites may play a role in origin function, two clones, pcF-13 and pcB-13, were constructed (Fig. 4). Clone pcB-13 contains the 159 bp of the left terminus (one complete arm of the palindrome, as well as 55 bp of the adjacent U_L2 sequences including the putative NF1/UL9 binding site homologies) as well as the 17 bp of the right terminus of the genome (up to the *Sma*I site; Fig. 3 and 4). Clone pcF-13 is similar to pcB-13 but has a shorter stretch of U_L2 sequences; only 22 bp of the U_L2 adjacent to the palindromic arm remain. Both pcF-13 (Fig. 6, lanes 2 and 5) and pcB-13 (lanes 3 and 4) are amplified. Thus, one arm of

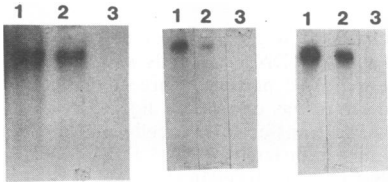

FIG. 5. Amplification of subclones of *Sma*I fragment 4. The experiments were performed as described in the legend to Fig. 2. The cells were transfected with PrV DNA and p529 (lanes 1), pS4H4 (H fragment) (lanes 2), or pS4L13 (L fragment) (lanes 3).

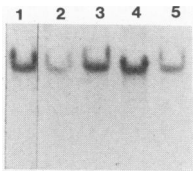

FIG. 6. Amplification of subclones of pS4H4 (H fragment). The experiments were performed as described in the legend to Fig. 2. The cells were cotransfected with PrV DNA and pS4H4 (H fragment) (lane 1), pcF13 (lane 2), pcB13 (lane 3), pcB13 (lane 4), or pcF13 (lane 5).

the palindrome, plus the adjacent 22 bp of the U_L2 as well as the 17 bp derived from the end of the S component, can function as an origin. Since the 17 nucleotides derived from the S component are not essential for replication (pD11 does not include these sequences but replicates well; Fig. 3), we conclude that the sequences within one arm of the palindrome and the adjacent 22 nucleotides contain elements that are sufficient for core origin function.

The results in Fig. 6 also show that while pcB-13 (lanes 3 and 4) was amplified as well as was the parent clone pS4H4 (lane 1), pcF-13 (lanes 2 and 5) was amplified less well. Thus, the 55 bp of the U_L2 (that include the putative NF1/UL9 binding sites) that are present in pcB-13 but not in pcF-13 augment the ability of the plasmids to be amplified. Scans of appropriately exposed radioautograms indicated that pcB-13 was amplified between two and three times better than was pcF-13.

Analysis of sequences necessary for origin function with use of plasmids that include a cleavage/encapsidation site. Because of the relatively low level of replication activity of plasmids with only minimal origin activity, the assays described above, in general, resulted in low-intensity signals. To enhance the amplification of plasmids carrying only minimal origin sequences, plasmids which also include cleavage/encapsidation signals were constructed. In these plasmids, packaging and consequent amplification by recycling of initially replicated plasmids can take place. A major disadvantage of these plasmids is that one cannot, in principle, distinguish between the role of a sequence in cleavage/encapsidation (and the resulting secondary amplification of the DNA in the defective particles produced) from its effect on origin function. The *cis* functions required for origin activity at the L terminus may overlap those required for cleavage/encapsidation. Despite this shortcoming, however, the facts that (i) the presence on the plasmids of a cleavage/encapsidation site might increase the sensitivity of the assay and (ii) the results obtained with these plasmids could confirm those that have been obtained with plasmids that did not include cleavage/encapsidation signals, thereby increasing the degree of confidence with which we can delimit the sequences required for origin function, were sufficient rationales for constructing these plasmids.

Signals required for efficient cleavage/encapsidation are present at both ends of the mature genome of PrV (35) as well as of HSV (32). Therefore, a plasmid carrying the *Bam*HI concatemeric junction fragment (pJ31) served as the parent construct for this series of plasmids. From it, pRR9, which contains the 1-kb *Sst*II subfragment that includes the concatemeric junction and encompasses the sequences in plasmid H plus 661 bp derived from the S terminus of the genome, was constructed (Fig. 7). pRR9 possesses all the *cis* functions necessary for origin function that have been shown above to be located within plasmid H; it also includes sequences from the S component of the genome which provide *cis* functions required for cleavage/encapsidation (35). pRR9 is amplified well (Fig. 8, lane 2) but not quite as well as is the parent plasmid pJ31 (lane 1).

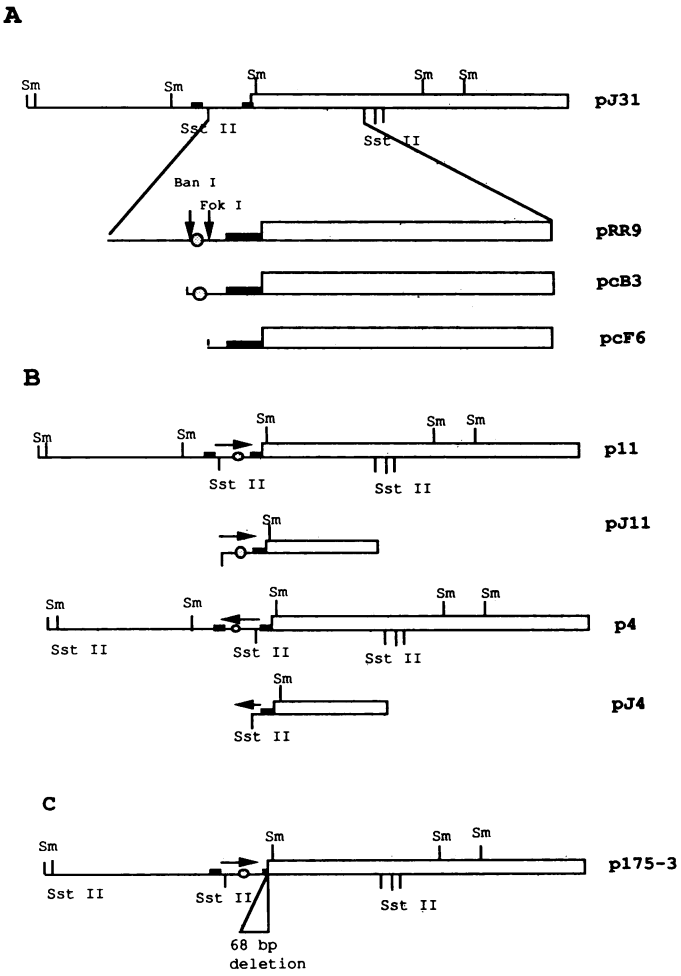

FIG. 7. Maps of plasmids that include sequences from the terminus of the L component as well as from the terminus of the S component. (A) The top line shows a restriction map of pJ31. For pRR9, the *Sst*II fragment which spans the junction between the L and S components of concatemeric DNA was subcloned into pTM. For pcB3, the *Sst*II fragment spanning the junction was truncated by digestion with *Ban*I. For pcF6, the *Sst*II fragment spanning the junction was truncated by digestion with *Fok*I. The single-stranded overhangs were filled in, and the fragments were ligated into the *Sma*I site of pTM. (B) The top line shows a restriction map of p11, which contains the concatemeric junction derived from PrV(USA)7. For pJ11, the *Sst*II fragment spanning the junction of p11 was subcloned as described above. p4 is similar to p11 but the U_L2 bracketed by the arms of the palindrome is in an inverted orientation. In pJ4, the *Sst*II fragment spanning the junction of p4 was cloned as described above. (C) The top line shows a restriction map of p175/3, a concatemeric *Bam*HI junction fragment of PrV(USA)7. This plasmid has a deletion of 68 bp at the junction. Open rectangles indicate sequences from the S component; the lines indicate sequences of the L component. Heavy lines indicate the 82-bp arms of the palindrome; circles indicate the sequences with NF1/UL9 binding site homologies; horizontal arrows indicate the orientation of the U_L2. Sm, *Sma*I.

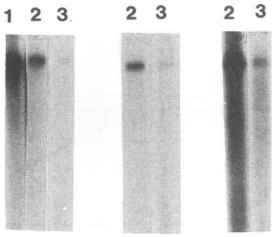

FIG. 8. Comparison of the degree of amplification of plasmids that do or do not include sequences derived from the S component. The experiment was performed as described in the legend to Fig. 2. Lanes: 1, pJ31; 2, pRR9; 3, pS4H4 (H fragment).

A comparison of the degree of amplification of pRR9 (which include the sequences of plasmid H as well as the sequences from the S component necessary for cleavage/encapsidation) with the degree of amplification of plasmid H (pS4H4) allowed us to estimate the contribution made by the presence of the sequences derived from the S component to the ability of a plasmid to be amplified (Fig. 8). While both pRR9 and pS4H4 are amplified, pRR9 (lanes 2) is amplified approximately 5 to 10 times better than is pS4H4 (lanes 3). Similarly, pJ31 (which includes the terminal sequences from both the S and L termini) was found to be amplified approximately three to five times better than was pD11 (which includes only sequences from the L terminus; Fig. 3). The increased amplification of pRR9 and pJ31 over pS4H4 and pD11 can be attributed, at least in part, to the presence of the complete cleavage/encapsidation signal. However, we cannot eliminate the possibility that some sequences within the S component, although incapable of autonomous origin function (Fig. 2), might augment the ability of the core origin to function. Whatever the case may be, it is clear that we can expect the presence of the terminal 661 bp of the S component to increase amplification of the test plasmids by about 3- to 10-fold.

A series of clones similar to the ones used in the experiments described above, but which include the leftmost 661 bp of the S component, was constructed (Fig. 7). pcB3 and pcF6 are both subclones of pRR9 that include the same sequences of the U_L2 as do pcB-13 and pcF-13 (see above), respectively. Figure 9 shows that pcB3 and pcF6 were both amplified but that pcF6 was not amplified quite as well as was pcB3. Quantitation by densitometric scanning of appropriately exposed autoradiograms of six different experiments (Table 1) showed that the intensity of the signal obtained with pcF6 was, on the average, approximately two to three

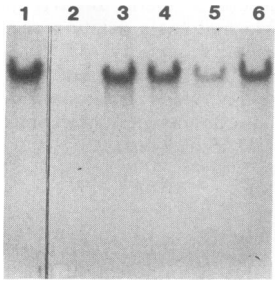

FIG. 9. Amplification of subclones of pRR9. The experiment was performed as described in the legend to Fig. 2. Lanes: 1, pRR9; 2, pBR325; 3, pcB3; 4, pcB3; 5, pcF6; 6, pcF6.

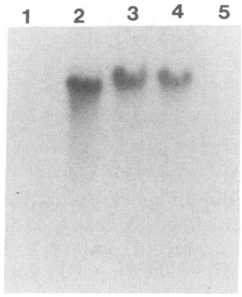

FIG. 10. Amplification of subclones of p4 and p11. The experiment was performed as described in the legend to Fig. 2. Lanes: 1, pBR325; 2, p11; 3, pJ11; 4, p4; 5, pJ4.

times lower than that obtained with pcB3, a result that is similar to that obtained with pcB13 and pcF13 (Fig. 6). Thus, plasmids with only the 104 terminal bp of the L component, i.e., which did not include the NF1/UL9 binding site homology, retained considerable origin function. However, the 55 adjacent nucleotides (that include the putative NF1/UL9 binding homologies) increased the ability of the plasmid to replicate by approximately two-to threefold.

The results described above (Fig. 5) showed that plasmid L did not give a detectable amplification signal. To corroborate this finding in a more sensitive system, a plasmid containing the L fragment adjacent to the terminal 661 bp of the S component, i.e., a plasmid with an intact cleavage/encapsidation signal, was constructed.

Plasmid p11 is the equivalent of plasmid pJ31; while pJ31 contains the *Bam*HI concatemeric junction fragment of PrV(Ka), p11 contains a similar junction obtained from another virus isolate, PrV(USA)7. Plasmid p4 also contains a concatemeric junction fragment derived from PrV(USA)7, but sequence analysis (21) revealed that in this plasmid the U_L2 is in an inverted orientation relative to the S component (Fig. 7). We assume that the inversion of the U_L2 in p4 may have occurred during cloning because we have not detected U_L2 inversion in the viral genome (9). pJ4 and pJ11 were constructed from p4 and p11 (Fig. 7). Both of these plasmids include the sequences derived from the S component of the genome and from the adjacent arm of the palindrome. The difference between pJ4 and pJ11 is that pJ11 contains the same sequences of the U_L2 as does plasmid H, while clone pJ4 contains the U_L2 sequences in plasmid L.

The ability of these plasmids to replicate was ascertained (Fig. 10). No significant difference in the amplification of p11 and p4 was observed. However, while pJ11 replicated well, pJ4 did not replicate detectably. The complete lack of detectable amplification of pJ4 can be ascribed to the absence of the U_L2 sequences that are present in pJ11 but not in pJ4. This finding corroborates the results described above which show that the U_L2 sequences that are present in plasmid H are essential to origin function.

Role of the sequences within the 82-bp palindromic arm in origin function. The results described above showed that both arms of the palindrome are not required for minimum origin function; one arm of the palindrome and some sequences within the U_L2 present in the H fragment are sufficient. The experiments described in this section were designed to ascertain whether minimal origin function might be located entirely within these U_L2 sequences or whether sequences within the arm of the palindrome were also required.

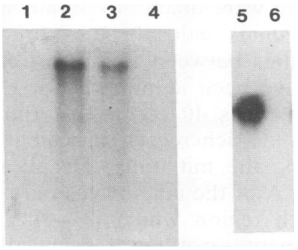

FIG. 11. Amplification of a junction fragment from which the 64 terminal bp of the L component have been deleted. The experiment was performed as described in the legend to Fig. 2. Lanes: 1, pBR325; 2, pJ31; 3, p11; 4, p175-3; 5, pJ31; 6, p175/3.

A plasmid was available that had been serendipitously isolated and that was similar to clones p11 and pJ31 (i.e., contained the *Bam*HI concatemeric junction) but that had suffered during cloning a deletion of 68 bp spanning the junction between the S and L components. In this plasmid (p175/3), 64 bp of the L-terminal sequences, i.e., of the arm of the palindrome adjacent to the S component, as well as the first 4 bp of the S component have been deleted (Fig. 7). The deletion in this plasmid (p175/3) was identified by sequence analysis (21).

The ability of p175/3 to replicate in cells coinfected with PrV was ascertained (Fig. 11). p175/3 had no detectable origin function. If p175/3 were deficient in cleavage/encapsidation signals only (which may be present within the 68 deleted nucleotides) but still replication competent, one would expect to obtain a signal of an intensity similar to that of, for example, pD11 or pS4H4. Since amplification of p175/3 could not be detected in several experiments using different plasmid preparations, we concluded that the 68 nucleotides spanning the junction play an essential role in origin function. Because the sequence in the S component is not essential for origin function, it must be the terminal 64 bp of the L component, i.e., the sequences of the palindromic arm, that are essential.

It is interesting to note that p175/3 has one complete arm of the palindromic sequence as well as an intact U_L2 sequence, i.e., includes all sequences necessary for origin function. However, in p175/3, the intact palindromic arm and the essential U_L2 sequences are in a different orientation and distance from each other than they are in replication-competent constructs. Thus, the appropriate U_L2 sequences must be adjacent to an intact palindromic arm and/or must be in the correct orientation relative to an intact palindromic arm to allow origin function.

Quantitative estimate of the degree of amplification of different plasmids. A comment on the reproducibility of the replication assays is in order. Generally, each plasmid was tested two to eight times using at least two (but usually more) plasmid preparations. The results were completely reliable in identifying plasmids with origin functions, and we were able to distinguish clearly between replication-positive and replication-negative plasmids. Our results showed that two elements are essential, each of them necessary but not sufficient for core origin function. One of these elements resides within one arm of the palindrome, and one resides within the sequences adjacent to the arm of the palindrome. Other auxiliary sequences augment core origin function; a progressive reduction in amplification was observed as these sequences were removed.

A quantitative variation in the signals obtained upon repeated testing of the same plasmids was observed. While the trends obtained were always the same and clearly showed that several elements cooperate to generate optimal origin function, the exact contribution of each of the deleted sequences was more difficult to establish. The averages of the results obtained from the different experiments are summarized in Table 1. A rough estimate of the contribution of the different nucleotide stretches within the sequence that has origin function can be arrived at from these results.

DISCUSSION

The results described in this report deal with the identification of the sequences present near (or at) the L terminus of the genome of PrV that can function in transient assays as an origin of replication (L-ori). This region of the genome had been shown previously to include *cis* functions capable of mediating the amplification of plasmid sequences linked to it (35). The role of sequences within the leftmost 600 terminal bp of the genome (*Sma*I fragment 4) in origin function was ascertained. Sequence analysis of this segment of DNA revealed that the leftmost 82 bp of the viral genome are part of a palindrome that is separated by a 352-bp unique sequence, the U_L2 (7), which includes an NF1 binding site and a sequence with partial homology to the UL9 binding site of HSV.

These observations led us to initiate a series of experiments in which we attempted to identify the sequences that are essential as well as those that have an enhancing effect on origin function. To this effect, progressively truncated constructs derived from segments of DNA that had been shown to include origin function were tested for their ability to promote the amplification of linked plasmid sequences. In particular, we ascertained the importance in PrV L-ori-directed replication of (i) the integrity of the dyad symmetry of the palindrome, since palindromes have been shown to be components of origins of replication in other prokaryotic (see, for example, references 12a and 37) and eukaryotic systems (reviewed by Kelly et al. [17]), (ii) the importance of the sequences within the U_L2, in particular the consensus NF1 binding site as well as the sequence with partial homology to the UL9 protein binding site of HSV (23, 36), and (iii) the sequences within each of the palindromic arms.

The quantitative and qualitative effects of deletions of specific sequences on the ability of the plasmids to replicate were similar in two different types of constructs. One series of plasmids included sequences derived mainly from the left end of the viral genome only and did not include an intact cleavage/encapsidation site. (The 17 terminal nucleotides of the S component do not play a role in cleavage recognition [24a].) The second series of plasmids included, in addition to the sequences derived from the left end of the genome, sequences derived from the right end of the genome. These sequences considerably enhance amplification, probably because of the presence of a functional cleavage/encapsidation site, allowing defective interfering particles that include the plasmid sequences to be recycled autonomously (35). The results obtained with the two families of plasmids were similar; in both cases, the same core origin and auxiliary sequences were identified.

Minimal origin function was found to be present within the last 104 nucleotides of the left end of the genome (pcF6 and pcF13). Within this sequence, there are at least two elements that appear to be absolutely required for minimal origin function. One element is located within the palindromic arms; the other is located within the U_L2 that is adjacent to

the leftmost palindromic arm. Each of these elements is necessary but not sufficient to allow origin function. These two elements have to be present at the correct distance and/or orientation, one relative to the other, to allow origin function.

The importance of the sequences within the U_L2 in minimal origin function can be deduced clearly from the results obtained with pJ11 and pJ4 as well as with plasmids L and H. Plasmids pJ4 and L differ from plasmids pJ11 and H in the content of the unique sequences adjacent to the palindromic arm; only the clones that included the sequences of the U_L2 adjacent to the right palindromic arm (present in pJ11 and plasmid H but not in pJ4 and plasmid L) were replication competent. Since pCF6 and pCF13 were replication competent, bp 83 to 104 must include sequences essential for origin function. The other element that has been identified as being essential for minimum origin function resides between nucleotides 1 and 64 within the palindromic arm. Thus, p175/3, which is equivalent to pJ31 but which has a deletion of the leftmost 64 bp of the palindromic arm, does not replicate. Since p175/3 includes one intact palindromic arm and all of the sequences of U_L2 but does not replicate, the lack of ability of p175/3 to replicate not only implicates the sequences within the palindromic arm as being essential for origin function but also shows that the 22 adjacent essential bp in the U_L2 must be either in the proper orientation or in the proper proximity to the palindromic arm.

In addition to the two essential elements, several auxiliary elements also affect origin function. Thus, although both palindromic arms are not essential for replication, in the presence of the complete palindrome as well as the complete intervening sequence, replication appeared to be enhanced approximately two- to threefold. For example, pS4H4 and pRR9 (which include only one arm of the palindrome and part of the U_L2 sequences) replicated less well than did p529 and pJ31 (which include both arms of the palindrome and the entire intervening U_L2 sequence), respectively. Furthermore, while the sequences in the U_L2 between bp 104 and 327 were not essential for plasmid amplification, they nevertheless enhanced it. While the sequences between bp 159 and 327 did not appear to affect replication, an approximately threefold reduction in replication capacity was found when bp 105 to 159 (which include the NF1/UL9 binding site homologies) were truncated (Table 1). Thus, while neither of these potential binding sites is essential for origin function, they (or other sequences within bp 105 to 159) nevertheless augment the replication of the core origin.

Plasmids that included sequences derived from both the S and L termini were amplified considerably better than were those that included only sequences from the L terminus. It is probable that this is because the juxtaposition of sequences from both termini gives rise to a functional cleavage/encapsidation site (35). The possible overlap of sequences at the end of the L component involved in origin activity and those involved in cleavage/encapsidation is a problem that has been only partially resolved. The addition of the sequences derived from the S terminus to the core L-ori sequences will give rise to a functional cleavage/encapsidation site which will promote encapsidation of the amplified plasmids, thereby increasing the signals. However, the sequences necessary for cleavage/encapsidation, which are present near the end of the S component of the genome, may also include auxiliary origin functions. This problem could, in principle, be overcome by transfecting the cells with the origin-carrying plasmids and subsequently infecting the cells at high multiplicity with PrV. Unfortunately, despite concerted efforts, we were unable to obtain significant amplification of the plasmids under these experimental conditions.

Some similarities between PrV L-ori and the origins of other viruses have been identified. For example, the core origin of simian virus 40 contains a run of A's and T's adjacent to a G+C-rich region at which the initial strand separation during the initiation of replication presumably occurs. Domain A of the adenovirus core origin contains a similar A+T-rich region which is well conserved among otherwise divergent serotypes (reviewed by Kelly et al. [17]). PrV L-ori also contains a stretch of A's followed by a G+C-rich region; a similar sequence is also present in HSV ori (1, 7, 8, 9, 18, 19, 25, 33).

Two sequences with homology to sequences that are protein binding sites and essential to origin function of HSV and adenovirus are also found within L-ori of PrV; a sequence with a 100% homology to the consensus sequence of the NF1 binding site and a sequence with 77% homology to the UL9 binding site are found. NF1 is a cellular factor which has been shown to be essential in adenovirus DNA replication (28). The UL9 binding site is found within ori_L and ori_S of HSV (8, 10, 11, 36) and is essential for replication. It is also found within ori_S of varicella-zoster virus (31) and equine herpesvirus 1 (1). Neither of these binding sites is essential for PrV L-ori function.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-10947 from the National Institutes of Health. Sabina Kupershmidt was supported by training grant 5T32 CA09385 from the National Cancer Institute.

REFERENCES

1. **Baumann, R. P., V. Ramana, R. Yalamanchili, and D. J. O'Callaghan.** 1989. Functional mapping and DNA sequence of an equine herpesvirus 1 origin of replication. J. Virol. **63**:1275–1283.
2. **Becker, Y., Y. Asher, E. Weinberg-Zahlering, and S. Rabkin.** 1978. Defective herpes simplex virus DNA: circular and circular-linear molecules resembling rolling circles. J. Gen. Virol. **40**:319–335.
3. **Ben-Porat, T., J. M. DeMarchi, and A. S. Kaplan.** 1974. Characterization of defective interfering particles present in a population of pseudorabies virions. Virology **60**:29–37.
4. **Ben-Porat, T., and F. J. Rixon.** 1979. Replication of herpesvirus DNA. IV. Analysis of concatemers. Virology **94**:61–70.
5. **Ben-Porat, T., and S. A. Tokaszewski.** 1977. Replication of herpesvirus DNA. II. Sedimentation characteristics of newly synthesized DNA. Virology **79**:292–301.
6. **Ben-Porat, T., and R. A. Veach.** 1980. Origin of replication of the DNA of a herpesvirus (pseudorabies). Proc. Natl. Acad. Sci. USA **77**:172–175.
7. **Deb, S., and S. P. Deb.** 1989. Analysis of ori-s sequence of HSV-1: identification of a functional DNA binding domain. Nucleic Acids Res. **17**:2733–2752.
8. **Deb, S., and M. Doelberg.** 1988. A 67-base pair segment from the ori-s region of herpes simplex virus type 1 encodes origin function. J. Virol. **62**:2516–2519.
9. **DeMarchi, J. M., Z. Lu, S. Kupershmidt, and T. Ben-Porat.** 1990. Structural organization of the termini of the L and S components of the genome of pseudorabies virus. J. Virol. **64**:4968–4977.
10. **Elias, P., and I. R. Lehman.** 1988. Interaction of origin binding proteins with an origin of replication of herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA **85**:2959–2963.
11. **Elias, P., M. E. O'Donnell, E. S. Mocarski, and I. R. Lehman.** 1986. A DNA binding protein specific for an origin of replication of herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA **83**:6322–6326.

12. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–560.
12a. **Grosschedl, R., and G. Hobom.** 1979. DNA sequences and structural homologies of the replication origin of lambdoid bacteriophages. Nature (London) **277**:621–627.
13. **Harper, L., J. M. DeMarchi, and T. Ben-Porat.** 1986. Sequence of the ends of the genome and of the junction between the ends in concatemeric DNA of pseudorabies virus. J. Virol. **60**:1183–1185.
14. **Jacob, R. J., L. S. Morse, and B. Roizman.** 1979. Anatomy of herpesvirus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. J. Virol. **29**:448–457.
15. **Jean, J.-H., and T. Ben-Porat.** 1976. Appearance in vivo of single stranded complementary ends on parental herpesvirus DNA. Proc. Natl. Acad. Sci. USA **73**:2674–2678.
16. **Jones, K. A., J. T. Kadonaga, P. J. Rosenfeld, T. J. Kelly, and R. Tjian.** 1987. A cellular DNA binding protein that activates eukaryotic transcription and DNA replication. Cell **48**:79–89.
17. **Kelly, T. J., M. S. Wold, and J. Li.** 1988. Initiation of viral DNA replication. Adv. Virus Res. **34**:1–42.
18. **Knopf, C. W., B. Spies, and H. C. Kaerner.** 1986. The DNA replication origins of herpes simplex virus type 1 strain Angelotti. Nucleic Acids Res. **14**:8655–8667.
19. **Lockshon, D., and D. A. Galloway.** 1986. Cloning and characterization of ori_{L2}, a large palindromic DNA replication origin of herpes simplex virus type 2. J. Virol. **58**:513–521.
20. **Lu, S., J. M. DeMarchi, L. Harper, G. F. Rall, and T. Ben-Porat.** 1989. Nucleotide sequences at recombinational junctions present in pseudorabies virus variants with an invertible L component. J. Virol. **63**:2690–2698.
21. **Lu, Z.** 1990. The nucleotide sequence of the end and junction fragments of the genomes of different pseudorabies virus variants. Ph.D. thesis. Vanderbilt University, Nashville, Tenn.
22. **Mocarski, E. S., and B. Roizman.** 1982. Herpesvirus-dependent amplification and inversion of cell-associated viral thymidine kinase gene flanked by viral *a* sequences linked to an origin of viral DNA replication. Proc. Natl. Acad. Sci. USA **79**:5626–5630.
23. **Olivo, P. D., N. J. Nelson, and M. D. Challberg.** 1988. Herpes simplex virus DNA replication: the UL9 gene encodes an origin binding protein. Proc. Natl. Acad. Sci. USA **85**:5414–5418.
24. **Rabkin, S. D., and B. Hanlon.** 1990. Herpes simplex virus DNA synthesis at a preformed replication fork in vitro. J. Virol. **64**:4957–4967.
24a. **Rall, G. F., et al.** Unpublished data.
24b. **Reilly, L. M., et al.** Submitted for publication.
25. **Reisman, D., J. Yates, and B. Sugden.** 1985. A putative origin of replication of plasmids derived from Epstein-Barr virus is composed of two *cis*-acting elements. Mol. Cell. Biol. **5**:1822–1832.
26. **Rigby, P. W., J. M. Dieckman, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick-translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
27. **Rosenfeld, P. J., and T. J. Kelly.** 1986. Purification of NF-1 by DNA recognition site affinity chromatography. J. Biol. Chem. **261**:1398–1408.
28. **Santoro, C., N. Mermod, P. C. Andrews, and R. Tjian.** 1988. A family of human CCAAT-box-binding proteins active in transcription and DNA replication: cloning and expression of multiple cDNAs. Nature (London) **334**:218–224.
29. **Spaete, R. R., and N. Frenkel.** 1982. The herpes simplex virus amplicon. A new eucaryotic defective virus cloning-amplification vector. Cell **30**:295–304.
30. **Stow, N. D.** 1982. Localization of an origin of replication within the TR_s/IR_s repeated region of the herpes simplex virus type I genome. EMBO J. **1**:863–867.
31. **Stow, N. D., and A. J. Davidson.** 1986. Identification of a varicella-zoster virus origin of DNA replication and its activation by herpes simplex virus type 1 gene products. J. Gen. Virol. **67**:1613–1623.
32. **Stow, N. D., E. C. McMonagle, and A. J. Davidson.** 1983. Fragments from both termini of the herpes simplex virus type I genome contain signals required for the encapsidation of viral DNA. Nucleic Acids Res. **11**:8205–8220.
33. **Weller, S. K., A. Spadaro, J. E. Schaffer, A. W. Murray, A. M. Maxam, and P. A. Schaffer.** 1985. Cloning, sequencing and functional analysis of ori_L, a herpes simplex virus type 1 origin of DNA synthesis. Mol. Cell. Biol. **5**:930–942.
34. **Whitton, J. L., and J. B. Clements.** 1984. Replication origins and a sequence involved in coordinate induction of the immediate-early gene family are conserved in an intergenic region of herpes simplex virus. Nucleic Acids Res. **12**:2061–2079.
35. **Wu, C. A., L. Harper, and T. Ben-Porat.** 1986. *cis* functions involved in replication of pseudorabies virus. J. Virol. **59**:318–327.
36. **Wu, C. A., N. J. Nelson, D. J. McGeoch, and M. D. Challberg.** 1988. Identification of HSV-1 genes required for origin dependent DNA synthesis. J. Virol. **62**:435–443.
37. **Zyskind, J. W., and D. W. Smith.** 1980. Nucleotide sequence of the Salmonella typhimurium origin of DNA replication. Proc. Natl. Acad. Sci. USA **77**:2460–2464.