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Origin of replication of the DNA of a herpesvirus (pseudorabies)

(genome structure/initiation site/DNA synthesis)

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ABSTRACT **During the first round of the replication of pseudorabies virus DNA, replicating DNA is mainly in the form of circles. The main origin of replication is located in the region of the molecule bearing the inverted repeat. Replication proceeds unidirectionally from the origin.**

The genomes of all herpesviruses thus far analyzed are linear double-stranded DNA molecules with molecular weights ranging from about 80×10^6 to 150×10^6 and with some rather unusual features. The most interesting feature of the DNA of some herpesviruses is the presence within the molecules of large duplicated sequences and the apparent division of the genomes into two distinct segments, which are present in the genome in different orientations relative to one another, so that different isomeric forms of the genomes exist (1). The DNA of pseudorabies (Pr) virus, one of the herpesviruses, consists of a short unique sequence, bracketed by inverted complementary repeats and a long unique sequence (2, 3). Only the short unique sequence inverts itself so that two isomeric forms of the Pr genome exist (D. Powell and N. M. Wilkie, personal communication).

Replication of the genome of Pr virus can be divided into two phases—early and late. Analysis of the DNA synthesized during the first round of DNA replication revealed that replicating DNA sediments with S values up to approximately twice the value of mature DNA (4). At later times after infection, the replicating molecules are in the form of rapidly sedimenting concatemeric molecules, which appear as large "tangles" in the electron microscope (5) and which consist of linear arrays of unit-size molecules in head-to-tail alignment (6). This has been shown to be the case also for the DNA of herpes simplex virus, type 1 (7).

Examination in the electron microscope of the DNA molecules present in the infected cells during the first round of DNA replication revealed the presence of linear unit-size and larger molecules with replicative "eyes" and branches, as well as circular molecules with replicative eyes (8). Analysis of these structures suggested that two origins of replication may exist—one near or at one end of the molecule and another approximately 20 μm from one of the ends. However, because replicating Pr viral DNA (in contrast to mature viral DNA) is extremely fragile (4) and only a few of the molecules with replicative eyes and branches seen in the electron microscope were of genome length, these observations needed to be corroborated by other techniques. Consequently, we determined the origin of replication and the structure of the replicating DNA during the first round of replication by the gel transfer hybridization technique (9), as well as by direct digestion with restriction endonucleases.

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MATERIALS AND METHODS

Virus and Cell Culture. Recently plaque-purified virus (two passages at low multiplicities of infection) was used. The properties of Pr virus and cultivation of rabbit kidney (RK) cells have been described (10).

Media and Solutions. *EDS − PO_4*: Eagle's synthetic medium (11) without PO_4, plus 3% dialyzed bovine serum. *EDS − PO_4 + FUra*: EDS − PO_4 plus 5-fluorouracil (FUra) (20 μg/ml) and thymidine (5 μg/ml). *RSB/2% Sarkosyl*: 0.01 M Tris·HCl at pH 7.4/0.01 M KCl/0.0015 M $MgCl_2$ (12) plus 2% sodium *N*-lauroylsarcosinate-97.

Chemicals and Radiochemicals. FUra was purchased from Calbiochem; inorganic $^{32}PO_4$ (carrier-free), from ICN. Restriction enzymes *Kpn* I and *Hin*dIII and *Bgl* II were obtained from New England BioLabs.

Restriction Endonuclease Digestion and Electrophoresis in Agarose Gels. Digestion of the DNA with restriction enzymes and agarose gel electrophoresis of the fragments were carried out as described (6). Hybridization of ^{32}P-labeled DNA to restriction fragments of DNA fixed to filter strips and the preparation of the DNA filter strips were carried out by the methods of Southern (9), as described (6).

Purification of Viral DNA. The cells were lysed in RSB/2% Sarkosyl and heated at 60°C for 15 min. The samples were then digested with nuclease-free Pronase (2 μg/ml) for 2 hr. Viral DNA was separated from cellular DNA by equilibrium sedimentation in CsCl as described (5) and dialyzed against buffer (0.01 M Tris·HCl/0.001 M EDTA, pH 7.6).

RESULTS AND DISCUSSION

Analysis of the origin of replication of Pr DNA

Because the DNA of the herpesviruses replicates as concatemers and because replication bears no temporal relationship to maturation, it is possible to locate the origin of replication of the viral DNA only if one labels the DNA during its first round of replication.

In the system we use—i.e., rabbit kidney cells infected with a multiplicity of 20 plaque-forming units per cell, Pr viral DNA replication starts at approximately 2 hr and 15 min after infection. The rate of the replication along the Pr virus DNA molecule is about 1 μm/min (13), and the time required to replicate most unit-size Pr virus DNA molecules, which are approximately 48 μm long, is about 48 min. The DNA should be, therefore, in its first round of replication up to about 3 hr after infection.

Figs. 1 and 2 illustrate the relative specific activities of different regions of the viral genome during the first round of replication of Pr viral DNA, as determined by gel transfer hybridization (9). To ensure that correct measurement of the relative specific activities of different regions of the genome was obtained by this technique, several precautions were taken:

Abbreviation: Pr, pseudorabies.

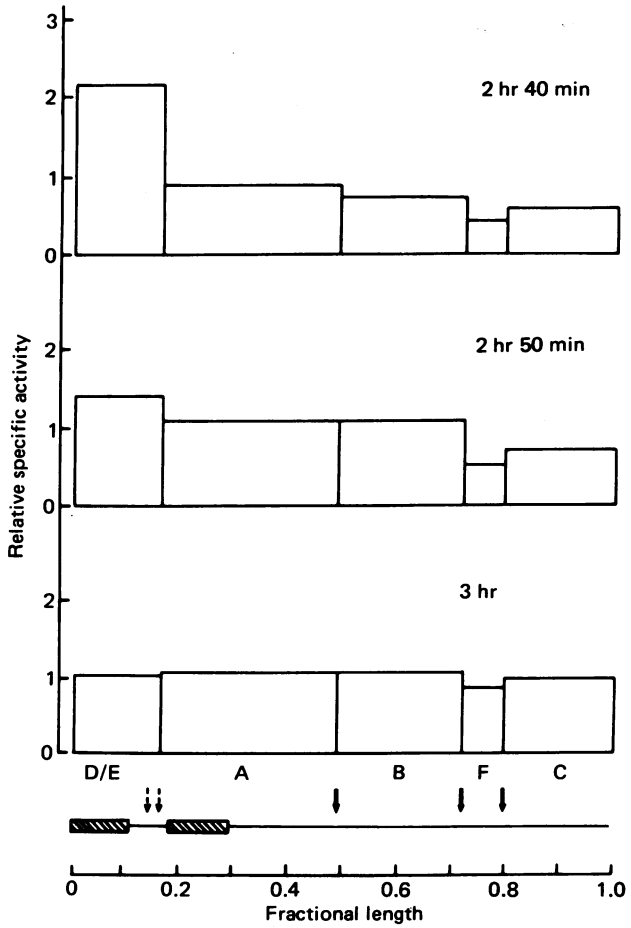

FIG. 1. Hybridization of Pr viral DNA synthesized during the first round of replication to separated fragments of *Bgl* II-digested mature Pr viral DNA. Cells were incubated for 24 hr in EDS − PO_4 + FUra to inhibit cellular DNA synthesis (14) and then further incubated in the same medium containing $^{32}PO_4$ (100 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) to prelabel the nucleotide pools. To synchronize partially the onset of viral DNA replication, cells were infected (20 plaque-forming units per cell) in the presence of fluorodeoxyuridine (2 μg/ml), an inhibitor of DNA synthesis. The inhibitory action of the drug was reversed by the addition of thymidine at 2 hr and 30 min after infection, and the cells were harvested at various times thereafter. Viral DNA was purified and annealed with filter strips to which separated fragments (A–F) of *Bgl* II-digested mature viral DNA had been fixed. After appropriate exposure times, autoradiograms of the strips were scanned. The percentage of the total ^{32}P-labeled DNA bound to DNA in each band on the filter strips was divided by the values obtained when mature ^{32}P-labeled DNA was similarly hybridized to DNA on a filter strip. The hatched bars on the map at the bottom indicate the repeats. Arrows indicate nuclease cleavage sites. Because all the sequences present in one repeat will hybridize to both repeats, the specific activities of both are the same, even though the site of initiation might be present on only one. The specific activity of fragment A is relatively low, even though it contains some of the same sequences as fragments D/E, but evidently the other sequences in fragment A have a lower specific activity.

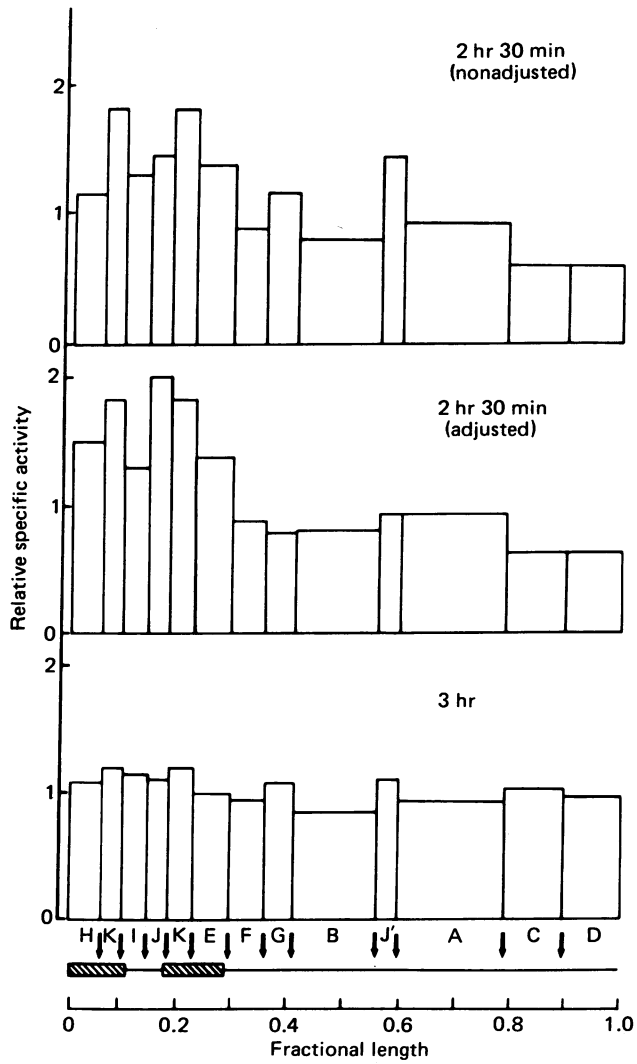

FIG. 2. Hybridization of Pr viral DNA synthesized during the first round of replication to separated fragments of *Kpn* I-digested mature Pr viral DNA. Cells were treated and infected as described in the legend to Fig. 1, except that the infected cells were not treated with fluorodeoxyuridine; i.e., no attempts were made to synchronize the initiation of viral DNA replication. The DNA was purified and annealed with the filter strips as described in the legend to Fig. 1. The data were also expressed the same way. Some ambiguity exists (nonadjusted data) whether the origin of replication is near or at the end of the molecule, because *Kpn* I end fragment H migrates with fragment G, which is part of the long unique sequence. Because it is likely that fragment G had a specific activity similar to that of adjoining fragments, the data have been modified (adjusted) to reflect this possibility. Similarly, although fragments J and J′ comigrate and, therefore, in the nonadjusted data have similar specific activities, it is likely that the specific activity of J′ is similar to that of the adjoining fragments and that fragment J has a correspondingly higher specific activity. The results are expressed in this manner in the adjusted data.

(*i*) The results obtained with different concentrations of labeled viral DNA were compared to ensure that all DNA fragments were present in excess on the filter strips. (*ii*) Different exposure times of the autoradiogram were scanned to ensure that blackening of the film over all the DNA bands was linear. (*iii*) To compensate for possible lack of quantitative transfer of all the DNA fragments to the filter, as well as for possible differences in the efficiency of hybridization of the different DNA fragments, the results were expressed as follows: the percent of the total radioactive DNA in the experimental sample that had associated with a given DNA band on the filter strips was divided by the percent of the total radioactive DNA in a mature viral DNA sample that had associated with the same DNA band on the filter strips (for greater detail, see legend to Fig. 1).

The data in Figs. 1 and 2 show that a gradient of specific activity along the genome was observed during the earliest stage of DNA replication when either *Kpn* I or *Bgl* II DNA fragments were tested. Ten minutes after DNA synthesis was allowed to start by addition of thymidine (Fig. 1), the regions of greatest specific activity were near the end of the molecule bearing the

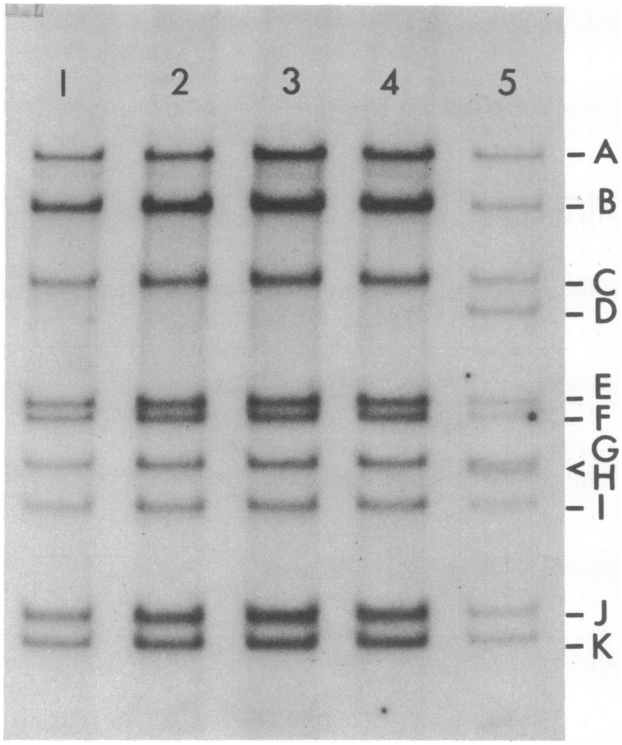

FIG. 3. Autoradiogram of *Kpn* I-digested Pr viral DNA labeled during the first round of replication. Viral DNA was labeled and purified as in the experiment described in the legend to Fig. 2. The DNA was digested with *Kpn* I restriction enzyme and the fragments were separated on agarose gels. Tracks 1, 2, 3, and 4 contain *Kpn* I-digested viral DNA labeled up to 2 hr and 30 min, 2 hr and 40 min, 2 hr and 50 min, and 3 hr, respectively. Track 5 contains *Kpn* I-digested mature viral DNA.

inverted repeat, indicating that the main origin of replication is near (or at) that end of the molecule. Similar types of results, although less pronounced, were also obtained when no attempts were made to synchronize the initiation of DNA replication (Fig. 2). As synthesis continued, the specific activities of the fragments, as expected, equalized, and the gradient of specific activity moved from the end of the molecule bearing the inverted repeat to the other end. It is unlikely that an appreciable amount of radioactivity was incorporated into the viral DNA by repair synthesis, because most of the labeled DNA acquired a higher buoyant density when the infected cultures were incubated in the presence of bromodeoxyuridine. Thus, it appears that replication is initiated mainly near (or at) the end of the molecule bearing the inverted repeats and proceeds unidirectionally to the other end. That the origin of replication of herpes simplex virus DNA might be in the region of the repeats has been previously reported (15).

We had concluded, on the basis of electron microscope examinations, that there are two origins of replication on Pr DNA—one near or at the end of the molecule, the other located approximately 20 μm from one of the ends, from which replication proceeds bidirectionally. This second internal origin of replication is, however, just barely detectable by gel transfer hybridization (see Figs. 1 and 2). Therefore, it is possible that DNA synthesis is initiated at that position on only relatively few molecules. This possibility is reinforced by the finding that while most of the viral DNA replicates during the first round of replication at a rate of approximately 1 μm/min (13) a small amount of the DNA replicates at approximately twice that rate. It seems likely that these results reflect unidirectional replication of the bulk of viral DNA and bidirectional replication of some

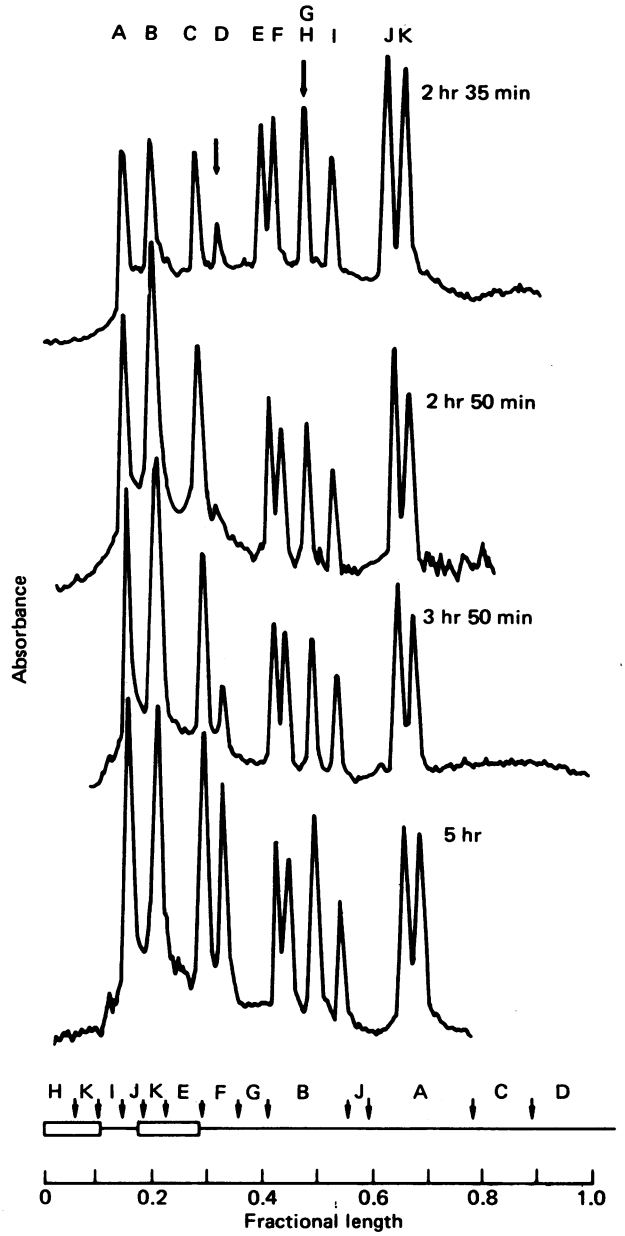

FIG. 4. Microdensitometer tracing of autoradiograms of *Kpn* I-digested Pr viral DNA labeled for various times. This experiment was performed in a similar manner to the one described in the legend to Fig. 3. The results obtained were slightly different in that some labeled end fragments were detected in this experiment during the first round of viral DNA replication.

of the DNA, which would be consistent with only relatively rare initiation at the internal origin of replication.

Analysis by direct digestion with restriction enzymes of the structure of replicating DNA during the first round of replication

Fig. 3 shows an autoradiogram of a typical *Kpn* I digest of Pr DNA labeled for various times during the first round of replication. Fig. 4 shows the scan of autoradiograms of restriction digests of another preparation of viral DNA similarly labeled. Two points emerge from these data:

(*i*) In general, the amount of radioactivity associated with different DNA fragments confirms the data obtained by the Southern procedure (Figs. 1 and 2). Quantitative analysis of the scans of the autoradiograms (data not shown) indicated that the

fragments originating from the end of the molecules bearing the inverted repeat had the highest specific activities during the earliest stages of replication. As replication continued, the specific activities of the fragments equalized.

(*ii*) Most of the replicating molecules are "endless"; i.e., the normal end fragments of the molecules were underrepresented and, instead, a new fragment composed of the two end fragments appeared. In the experiment illustrated in Fig. 4, some labeled *Kpn* I end fragments (H and D) were detectable; they were, however, underrepresented. This was even more pronounced in the experiment illustrated in Fig. 3, in which no end fragments were detected. On the other hand, band B, which has the molecular weight expected of the joined H and D fragments, is overrepresented. That band B, indeed contains the joined fragments H and D was shown by excising the DNA in band B from the gel and annealing it with filter strips to which the separated *Kpn* I fragments of Pr DNA had been fixed. Band B purified from restriction enzyme-digested mature viral DNA hybridized only to fragment B; band B purified from *Kpn* I-digested DNA synthesized during the first round of replication hybridized to fragments B, D, E, and H. (Fragments H and E contain similar sequences; see map at the bottom of Fig. 2.)

The fact that Pr DNA is "endless" during its first round of replication is also illustrated by the results shown in Fig. 5,

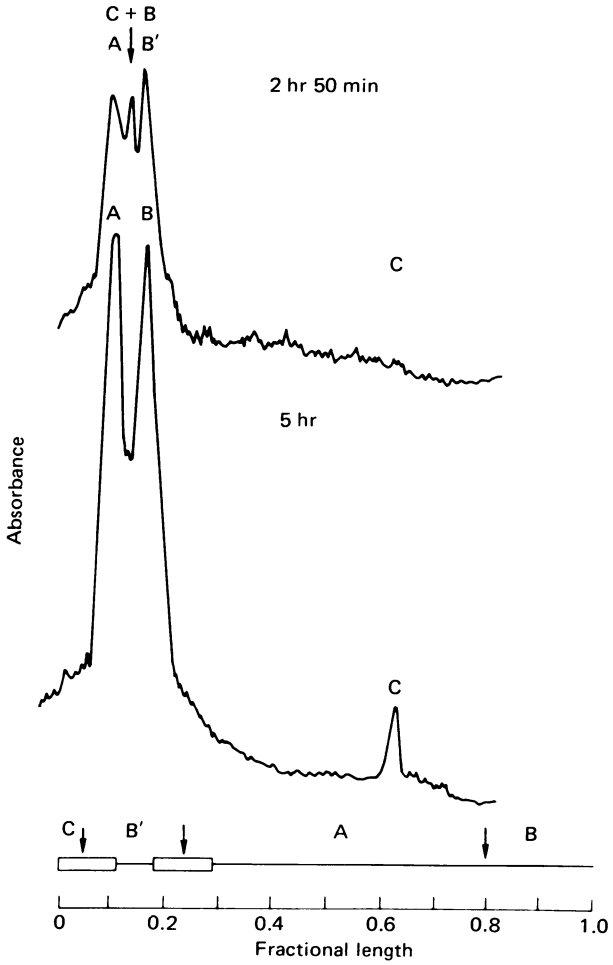

FIG. 5. Microdensitometer tracing of an autoradiogram of *Hin*dIII-digested Pr viral DNA labeled for various times. The experiment was performed as described in the legend to Fig. 3.

which shows a *Hin*dIII digest of newly synthesized DNA that had accumulated in the infected cells during the first round of DNA replication. Of interest is the fact that fragment C (M_r 4.5×10^6), which is the end fragment containing the main origin of replication (see bottom of Fig. 5 and Figs. 1 and 2), is virtually absent and that a new fragment appears with a molecular weight of 23×10^6; i.e., a molecular weight equal to that of the two end fragments B (M_r 18.5×10^6) and C (M_r, 4.5×10^6) joined together. By 5 hr after infection (bottom panel, Fig. 5), most of the DNA that had accumulated up to that time in the infected cells had matured; fragment C was present and the fragment consisting of the two joined end fragments was no longer detectable. The fact that most of the viral DNA that had accumulated in the infected cells up to 5 hr after infection had matured is also illustrated in Fig. 4, which represents a similar analysis after digestion with *Kpn* I.

Thus, Pr DNA during its first round of replication is mainly endless. Instead of the free ends present in mature DNA, a new fragment consisting of the two ends joined together is found. This indicates that the DNA is in the form of either circles or large concatemers. However, because most of the DNA synthesized during the first round of DNA replication sediments with an S value approximately twice that of mature DNA (4), which is inconsistent with very large concatemeric structures, we conclude that most of the DNA replicates in the form of circles. The presence in some of the experiments of some free labeled ends indicates that replication can, however, be also initiated on linear molecules, either unit length or small concatemers.

Some variation between experiments was found with respect to the relative amount of DNA that is endless during the first round of replication. Whereas Fig. 4 shows that approximately 75% of the replicating DNA is endless, in other, similar experiments (for example, see Figs. 3 and 5) virtually all the replicating DNA was endless. We do not know the reason for this variation.

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1. Honess, R. W. & Watson, D. H. (1977) *J. Gen. Virol.* **37**, 15–37.
2. Stevely, W. S. (1977) *J. Virol.* **22**, 232–234.
3. Ben-Porat, T., Rixon, F. J. & Blankenship, M. L. (1979) *Virology* **95**, 285–294.
4. Ben-Porat, T. & Tokazewski, S. A. (1977) *Virology* **79**, 292–301.
5. Ben-Porat, T., Kaplan, A. S., Stehn, B. & Rubenstein, A. S. (1976) *Virology* **69**, 547–560.
6. Ben-Porat, T. & Rixon, F. J. (1979) *Virology* **94**, 61–70.
7. Jacob, R. J., Morse, L. S. & Roizman, B. (1979) *J. Virol.* **29**, 448–457.
8. Jean, J.-H., Blankenship, M. L. & Ben-Porat, T. (1977) *Virology* **79**, 281–291.
9. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–518.
10. Kaplan, A. S. (1969) in *Virology Monographs*, eds. Gard, S., Hallauer, C. & Meyer, K. F. (Springer, Berlin), Vol. 5, pp. 1–115.
11. Eagle, H. (1959) *Science* **130**, 432–437.
12. Warner, J. R., Knopf, P. M. & Rich, A. (1963) *Proc. Natl. Acad. Sci. USA* **49**, 122–129.
13. Ben-Porat, T., Blankenship, M. L., DeMarchi, J. M. & Kaplan, A. S. (1977) *J. Virol.* **22**, 734–741.
14. Kaplan, A. S. & Ben-Porat, T. (1961) *Virology* **13**, 78–92.
15. Hirsch, I., Cabral, G., Patterson, H. & Biswal, N. (1977) *Virology* **81**, 48–61.