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Methylation of the viral genome in an *in vitro* model of herpes simplex virus latency

(restriction endonucleases/5-methylcytosine/mitogens)

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ABSTRACT **An *in vitro* model of latency of herpes simplex virus type 1 (HSV-1) in a lymphoid cell line has been developed recently. CEM cells persistently infected with HSV-1 transiently ceased to produce virus for 24 days. This nonproductive state could either be reversed with phytohemagglutinin or maintained with concanavalin A. This system was used to study the relationship between DNA methylation and HSV-1 latency. DNA was probed for methylation by comparing the cleavage patterns generated by two pairs of restriction endonucleases (*Sma* I vs. *Xma* I and *Hpa* II vs. *Msp* I); these enzymes show differential activity reflecting methylation of the recognition sequences. Viral DNA in the concanavalin A-treated cells (not producing virus) was found to be extensively methylated. By contrast, no methylated copies were detected in viral DNA from producer cells. About 800 days after the initial infection, the productive culture once again became nonproductive. Viral sequences in the latter cells were also methylated. Reconstitution experiments revealed 1–2 copies of viral DNA in cells from the latent stages and 40–80 copies in cells from productive stages. Most (if not all) of the viral genome is present in cells from various productive and latent stages. No differences in sequence arrangement were detected (although a terminal fragment of intracellular HSV-1 DNA appeared to be under-represented in latent cells). These results suggest a role for DNA methylation in the mechanism of HSV-1 latency in this system.**

Herpes simplex virus type 1 (HSV-1) usually becomes latent in humans and animals after a primary infection. Although the virus or the viral DNA has been shown to reside latently in ganglion cells and in cells of the central nervous system (1), other potential reservoirs (e.g., lymphoid cells) have not been excluded. Understanding of the molecular events involved in latency has been hampered because existing *in vivo* models do not lend themselves readily to a biochemical approach and because, until recently, no *in vitro* models that unambiguously showed a reversible latency were available.

Hammer *et al.* (2) now have described a lymphoblastoid T-cell line, CEM, persistently infected with HSV-1. This line was usually productive—i.e., it demonstrated an average virus titer of 2×10^5 plaque-forming units/ml with a mean of 0.9% of the cells producing infectious virus and 10% or less positive for HSV-1 antigens by immunofluorescence. However, this line also demonstrated two nonproductive or latent stages, from day 83 to day 106 and from day 886 on. During both of these stages there was no evidence of virus production, and virus antigen expression diminished to undetectable levels. Furthermore, a single treatment of the nonproducing cells with concanavalin A resulted in permanent latency (Con A-cells)—i.e., no virion or viral antigen production. Virus production could be induced from cells of both spontaneous latent periods with a single treatment of phytohemagglutinin (ref. 2; unpublished data).

The ability to control virus expression with mitogens in this system provides an opportunity to study the molecular basis of latency. We have used this system to investigate the physical state of the viral genome in the latently and productively infected cells.

Vertebrate DNA methylation has been widely implicated recently in the regulation of gene expression (3–14). This methylation leads to the production of 5-methylcytosine predominantly at C-G dinucleotides (3). With the use of restriction endonucleases that detect methylated bases at such sequences (15, 16), we show here that the viral DNA is heavily methylated in the latently infected cells but it is not detectably methylated in cells from the productive phases of this infection nor in virions propagated on human embryonic lung cells. We also demonstrate the presence of most (if not all) of the viral genome in both producer and latent cells and the absence of major rearrangements of the intracellular viral DNA sequences compared to the virion DNA.

MATERIALS AND METHODS

Virus and Cells. Conditions for growth of the CEM cells and the propagation of the HSV-1 strain (a clinical isolate) used in this study have been described (2). At various points of this infection, aliquots of cells were frozen and stored at −70°C for later use.

DNA Isolation. HSV-1 DNA from virions was purified as described by Goldin *et al.* (17), except that $NaDodSO_4$ was omitted from the lysis buffer (to prevent precipitation in CsCl). Cellular DNA was obtained by lysis in 1% $NaDodSO_4$/10 mM Tris·HCl, pH 8.0/10 mM NaCl/10 mM EDTA, followed by extensive digestion with proteinase K (0.2 mg/ml). The lysate was extracted sequentially with phenol and chloroform and then dialyzed against 10 mM Tris·HCl, pH 8.0/5 mM EDTA. Further purification consisted of digestion with RNase (50 μg/ml) at 37°C for 1 hr, extraction with chloroform, precipitation with ethanol, and resuspension in 10 mM Tris·HCl, pH 8.0/0.1 mM EDTA.

Restriction Endonuclease Digestion. The restriction endonucleases were obtained from the following sources: *Sma* I, *Sal* I, *Bam*HI, *Pst* I, and *Hin*dIII from Biotec (Madison, WI); *Xma* I, *Hpa* II, and *Msp* I from New England BioLabs. The buffers used were those recommended by the manufacturers. At least a 5-fold excess of enzyme was used with each sample in a final volume of 200 μl. Reaction mixtures were incubated for 12 hr and monitored for completeness by the codigestion of λ DNA

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Abbreviations: Con A-cells, concanavalin A-treated latent cells; HSV-1, herpes simplex virus type 1; DBM-paper, diazobenzyloxymethyl-paper.

(New England BioLabs) in small aliquots of the reaction mixture.

Electrophoresis and Blot Hybridization. The digested DNA was fractionated on 0.8% agarose gels in 40 mM Tris·HCl, pH 7.8/10 mM sodium acetate/1 mM EDTA. The gel was stained in ethidium bromide (1 μg/ml), and the fluorescence pattern was photographed under short-wave ultraviolet light. DNA was then transferred to diazobenzyloxymethyl-paper (DBM-paper) (18) under partial vacuum; the paper was treated with prehybridization buffer (18) for 2 hr at 42°C and then hybridized with *in vitro* ^{32}P-labeled (19) HSV-1 DNA (1–2 $\times$ 10^8 cpm/μg) at 42°C for 12 hr (18). The papers were washed three times in 0.3 M NaCl/30 mM trisodium citrate/0.1% $NaDodSO_4$ at 42°C for 1 hr and once in 70% (vol/vol) formamide/0.15 M NaCl/15 mM trisodium citrate/0.1% $NaDodSO_4$ at 42°C for 2 hr. (These conditions selectively eluted cellular DNA hybrids while minimally disturbing the virus-specific hybridization, because of the high G+C content of HSV-1 DNA.) Partially dried papers were covered with Saran Wrap and exposed to Kodak XR2 x-ray film for various times at −70°C with Dupont Cronex intensifying screens.

RESULTS

Methylation of Intracellular HSV-1 DNA. The restriction endonuclease *Hpa* II has the same recognition sequence (C-C-G-G) as *Msp* I (isoschizomers) but, unlike *Msp* I, it cannot cleave if the internal cytosine of its recognition sequence is methylated (15). Similarly, although both *Sma* I and *Xma* I recognize the same sequence (C-C-C-G-G-G), only *Xma* I can cleave if the cytosine of the dinucleotide C-G is methylated (16). We have used these enzymes to probe for methylation of the intracellular viral sequences.

Fig. 1 shows the results of this analysis with DNA from (latent) Con A-cells at day 83 after Con A treatment. Cleavage of this DNA with *Xma* I or with *Msp* I revealed a pattern of DNA bands in the molecular weight range 2.2 $\times$ 10^6 to 0.3 $\times$ 10^6, which appeared to be indistinguishable from the pattern obtained with virion DNA. However, cleavage with the methylation-sensitive enzymes *Sma* I or *Hpa* II failed to reveal this pattern; instead, hybridization occurred predominantly in the region of DNA of molecular weight >30 $\times$ 10^6, indicating that intracellular viral sequences were resistant to cleavage by *Sma* I and *Hpa* II. These sequences were also resistant to cleavage by *Sal* I (data not shown), an analogous methylation-sensitive enzyme (12). Thus, the HSV-1 DNA sequences in these latently infected cells were heavily methylated.

The results obtained in the experiment shown in Fig. 1 were not due to differences in the cleavage of HSV-1 DNA by the two pairs of isoschizomers: the *Sma* I cleavage pattern of HSV-1 DNA from purified virions was identical to the pattern obtained with *Xma* I (Fig. 2). Thus, the virion DNA contained no detectable methylated C-C-C-G-G-G sequences.

The intracellular viral DNA at other stages of this model system was also analyzed by the same method. Fig. 2, lanes b and c, show the *Sma* I cleavage patterns of DNA from the producer cells at days 79 (P79) and 113 (P113), respectively (shortly before and shortly after the first latent phase). Both patterns appear to be identical to the *Sma* I and *Xma* I cleavage patterns of virion DNA as well as to their own respective *Xma* I patterns. Therefore, the intracellular HSV-1 DNA sequences of the producer cells show no detectable methylation.

In marked contrast to producer cells, the *Sma* I cleavage pattern of viral DNA sequences in L886 cells from the second latent phase (886 days after the initial infection) appears to be quite different from the corresponding *Xma* I pattern (Fig. 2). Virus-specific hybridization in the high molecular weight range

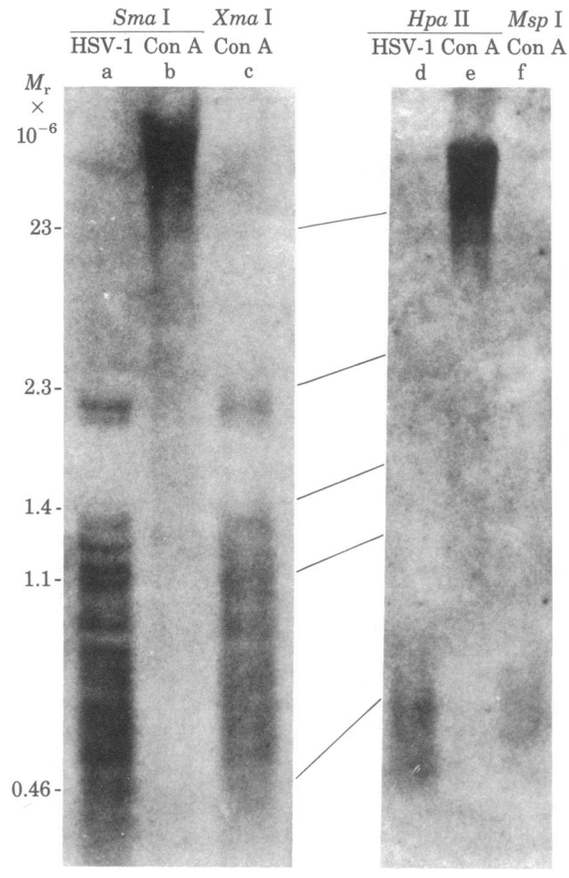

FIG. 1. Methylation pattern of intracellular HSV-1 DNA in Con A-cells. HSV-1 DNA (1.1 or 1.7 ng) was cleaved with *Sma* I (lane a) or *Hpa* II (lane d), respectively, and compared with DNA (20 μg) from Con A-cells (83 days after Con A treatment) cleaved with *Sma* I (lane b), *Xma* I (lane c), *Hpa* II (lane e), or *Msp* I (lane f). The digests were fractionated on a 0.8% agarose gel and transferred to DBM-paper under partial vacuum. The DBM-paper replica was then hybridized with ^{32}P-labeled HSV-1 DNA and exposed to x-ray film for 10 hr at −70°C. Adenovirus type 2 DNA cleaved with *Bam*HI, *Eco*RI, or *Sma* I provided the molecular weight calibrations.

of the *Sma* I-cleaved DNA is absent from the *Xma* I pattern of the same DNA. Instead, *Xma* I produced the characteristic pattern of *Sma* I- or *Xma* I-cleaved virion DNA. Hence, the intracellular viral DNA is also methylated in L886 cells.

The overall degree of methylation of total DNA of cells from different stages of this infection was estimated by cleaving cellular DNA from each of the following sources with *Hpa* II and *Msp* I: uninfected control cells; producer cells P79 and P113; and latent Con A-cells and L886 cells. Examination of the agarose gel after staining with ethidium bromide revealed no differences among the various cell lines in the extent of cleavage with *Hpa* II (data not shown). Therefore, no gross changes were detected in the degree of methylation of total cellular DNA from the different phases of this infection.

Physical State and Number of HSV-1 DNA Copies. An important question about latency is whether or not the complete viral genome is present during the latent state. This information cannot be obtained from the *Xma* I cleavage pattern because of the relatively poor resolution of the fragments. We therefore used another restriction endonuclease, *Hin*dIII, to cleave DNA from our various cell lines; this enzyme has fewer sites within the HSV-1 genome (20, 21) and is not affected by methylation (12). The number of HSV-1 copies per cell was also determined in the same experiment. In the infected CEM cells, no differ-

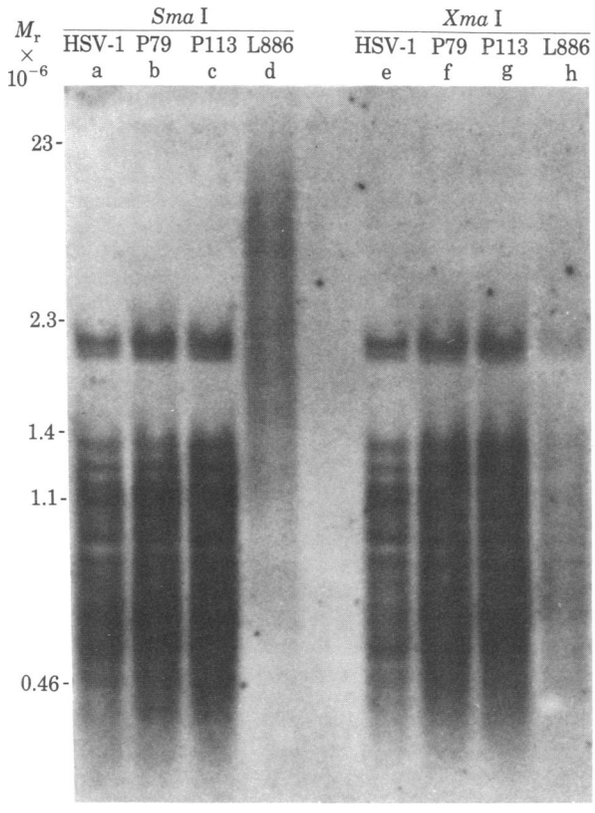

FIG. 2. Methylation patterns of DNA from producer cells of two different stages and from spontaneously latent cells. HSV-1 DNA (5.6 ng) was cleaved with *Sma* I (lane a) or *Xma* I (lane e) and compared with 2 μg of DNA from producer cells at day 79 (P79) and day 113 (P113) and 20 μg of DNA from spontaneously latent cells at day 886 (L886) cleaved with *Sma* I (lanes b–d) or *Xma* I (lanes f–h). Electrophoresis, transfer, and hybridization were as for Fig. 1.

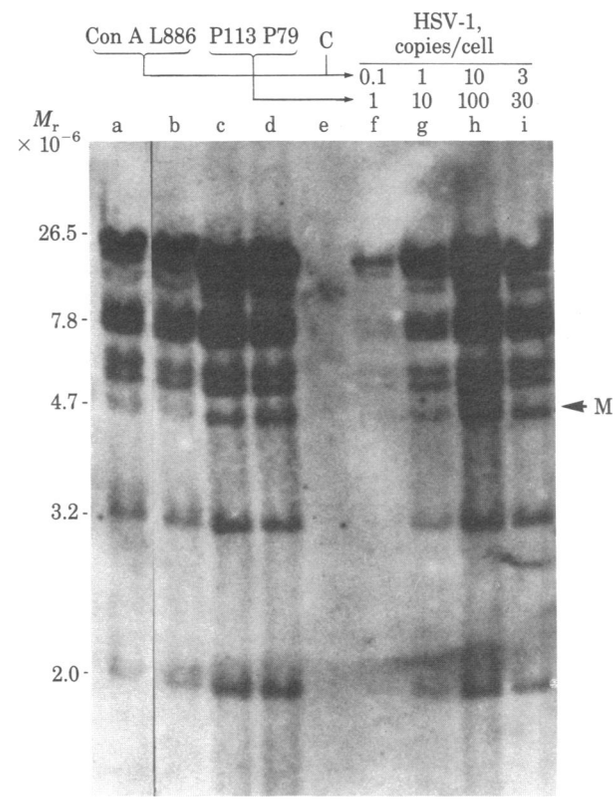

FIG. 3. Quantitation of the copy number of HSV-1 DNA in producer cells and latent cells by reconstitution. DNA (20 μg) from Con A-treated latent (Con A; 83 days after Con A treatment) (lane a), spontaneously latent (L886) (lane b), and uninfected control (C) (lane e) cells and DNA (2 μg) from producer cells of two different stages—P113 (lane c) and P79 (lane d)—were cleaved with *Hin*dIII, fractionated on a 0.8% agarose gel, transferred to DBM-paper, and hybridized with ^{32}P-labeled HSV-1 DNA. The copy numbers of intracellular HSV-1 DNA were estimated by comparison with the adjacent reconstitution lanes containing 10 μg of *Serratia marcescens* DNA (as carrier) plus varying amounts of *Hin*dIII cleaved HSV-1 DNA equivalent to the copies per cell indicated. Because 1/10th as much DNA was loaded in lanes c and d as in lanes a, b, and e, the calibrations for the former are 10-fold higher. Lanes f–h contained DNA (0.056, 0.56, and 5.6 ng, respectively) from the clinical isolate of HSV-1 used in this study; lane i contained 2 ng of HSV-1 DNA from strain Patton. The position of *Hin*dIII fragment M of HSV-1 is designated by an arrow.

ences were detected between the *Hin*dIII cleavage patterns of virus-specific DNA from producer or latent cells and the *Hin*dIII cleavage pattern of virion DNA (Fig. 3). Therefore, there is no detectable variation in the sequence arrangement of intracellular viral DNA and most, if not all of the viral genome is maintained (within the limits of sensitivity of restriction endonuclease analysis) in producer and latent cells. However, *Hin*dIII fragment M, a terminal fragment of the short region of HSV-1 DNA (refs. 20 and 21; Fig. 4), is distinctly under-represented in DNA from the latent cells but not in DNA from the producer cells (Fig. 3).

To estimate the copy number of intracellular viral DNA, small amounts of *Hin*dIII-cleaved HSV-1 DNA were mixed with bacterial DNA (as carrier) in reconstitution experiments. Because producer cells were anticipated to contain much more viral DNA than latent cells, we used less producer cell DNA (1/10th that of latent cell DNA) in this gel. Therefore, the amounts of viral DNA in the reconstitution lanes represent 0.1, 1, 10, or 3 copies of viral DNA per cell for the latent and the noninfected control cells but 1, 10, 100, or 30 copies for the producer cells. The intensity of hybridization, which was judged visually, was a function of the relative amounts of virus-specific DNA present in each lane. Our results indicate that producer cells P79 and P113 contain 40–80 copies of HSV-1 DNA per cell, whereas the latent Con A-cells and L886 cells contain about 1–2 copies of HSV-1 DNA per cell (Fig. 3). No virus-specific hybridization was detected in DNA from uninfected control cells.

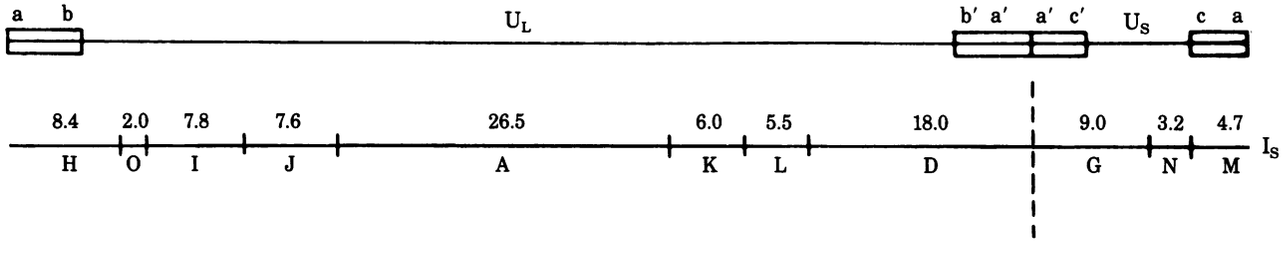

FIG. 4. *Hin*dIII cleavage map of HSV-1 DNA (strain Patton) in the I_S configuration (20, 21).

Fig. 3, lane i, shows the *Hin*dIII cleavage pattern of DNA from strain Patton, a laboratory strain of HSV-1. Although this appears to be identical to the *Hin*dIII pattern of the clinical isolate of HSV-1 used in this study, subtle differences were detected with two other restriction endonucleases, *Bam*HI and *Pst* I (data not shown). Therefore, the strain of HSV-1 used here is comparable to a well characterized, nondefective laboratory strain by this criterion.

At 600 days after the initial infection, we are able to detect viral sequences in Con A-cells at the level of 0.1 DNA copy per cell (data not shown).

DISCUSSION

To investigate the molecular mechanisms of latency of HSV-1, we have used an *in vitro* system in which the modulation of virus expression was achieved by mitogen treatment. A principal aim of this work was to investigate the relationship of DNA methylation to viral gene activity. Our results support the following conclusions.

(*i*) Cells in the two latent states examined harbor one or two copies of HSV-1 DNA per cell that is heavily methylated at C-C-G-G sites. By contrast, producer cells contain 40–80 copies of viral DNA which appears to be nonmethylated. However, no gross changes can be detected in the methylation of total cellular DNA between producer and nonproducer cells. The somewhat lower molecular weight of the *Sma* I-cleaved DNA of the L886 cells (Fig. 2, lane d), as compared to that of the Con A-cells (Fig. 1, lane b), may be due to a slight nonspecific degradation of the high molecular weight DNA in this digestion mixture, although the present experiments do not rule out a lower degree of methylation of the viral DNA.

(*ii*) Most (if not all) of the viral genome is present in latent cells. (This is expected because the virus can be reactivated from these cells.)

(*iii*) Although a terminal fragment of HSV-1 DNA in latent cells appears to have a lower molarity than in viral DNA from producer cells or virions, there is no detectable variation in the sequence arrangement of intracellular HSV-1 DNA.

The lowered representation of a terminal fragment of HSV-1 DNA within the latent cells may indicate a change in the configuration of the intracellular HSV-1 DNA. This could involve circularization of the intracellular DNA, which has been reported for two other herpesviruses, Epstein–Barr virus (22) and *Herpesvirus* saimiri (23) but not yet for HSV-1. Further possibilities include integration into host DNA, deletion of *Hin*dIII fragment M (or part of it) in most cells, or preferential retention of one or two of the four isomers of HSV-1 DNA in latent cells.

Our results are consistent with the evidence from a number of systems demonstrating an inverse correlation between DNA methylation and gene activity. Differences in methylation between active and inactive DNA have been observed in *Xenopus* ribosomal genes (4), viral genes (5, 7, 8, 11), chicken ovalbumin gene (9), chicken, human, and rabbit β-globin genes (10, 12, 14), mouse metallothionein-I gene (6), and the J region of the immunoglobin gene (13). To date, however, the cause–effect relationship of this phenomenon remains obscure. Similarly, in our system, it is premature to attribute the inactivity of HSV-1 DNA sequences to methylation. However, if latency can be divided into three stages—namely, establishment, maintenance, and reactivation—a role for DNA methylation in the maintenance of the latent state becomes an attractive possibility.

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1. Baringer, J. R. (1981) in *The Human Herpesviruses, an Interdisciplinary Perspective*, eds. Nahmias, A. J., Dowdle, W. R. & Schinazy, R. F. (Elsevier, New York), pp. 202–205.
2. Hammer, S. M., Richter, B. S. & Hirsch, M. S. (1981) *J. Immunol.* **127**, 144–148.
3. Ehrlich, M. & Wang, R. Y.-H. (1981) *Science* **212**, 1350–1357.
4. Bird, A. P. & Southern, E. M. (1978) *J. Mol. Biol.* **118**, 27–47.
5. Cohen, J. C. (1980) *Cell* **19**, 653–662.
6. Compere, S. J. & Palmiter, R. D. (1981) *Cell* **25**, 233–240.
7. Desrosiers, R. C., Mulder, C. & Fleckenstein, B. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3839–3843.
8. Groudine, M., Eisenman, R. & Weintraub, H. (1981) *Nature (London)* **292**, 311–317.
9. Mandel, J. L. & Chambon, P. (1979) *Nucleic Acids Res.* **7**, 2081–2103.
10. McGhee, J. D. & Ginder, G. D. (1979) *Nature (London)* **280**, 419–420.
11. Sutter, D. & Doerfler, W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 253–256.
12. van der Ploeg, L. H. T. & Flavell, R. A. (1980) *Cell* **19**, 947–958.
13. Yagi, M. & Koshland, M. E. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4907–4911.
14. Waalwijk, C. & Flavell, R. A. (1978) *Nucleic Acids Res.* **5**, 4631–4641.
15. Waalwijk, C. & Flavell, R. A. (1978) *Nucleic Acids Res.* **5**, 3231–3236.
16. Youssoufian, H. & Mulder, C. (1981) *J. Mol. Biol.* **150**, 133–136.
17. Goldin, A. L., Sandri-Goldin, R. M., Levine, M. & Glorioso, J. C. (1981) *J. Virol.* **38**, 50–58.
18. Wahl, G. M., Stern, M. & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3683–3687.
19. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113**, 237–251.
20. Skare, J. & Summers, W. C. (1977) *Virology* **76**, 581–595.
21. Spear, P. G. & Roizman, B. (1980) in *DNA Tumor Viruses*, ed. Tooze, J. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 615–745.
22. Lindahl, T., Adams, A., Bjursell, G. W., Kaschka-Dierich, C. & Tehn, U. (1976) *J. Mol. Biol.* **102**, 511–530.
23. Werner, F.-J., Bornkamm, G. W. & Fleckenstein, B. (1977) *J. Virol.* **22**, 794–803.