Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 847–850, February 1987
Medical Sciences

Expression of the hepatitis B virus genome in chronic hepatitis B carriers and patients with hepatocellular carcinoma

(gene expression/hepatitis B surface antigen/Southern blot analysis)

S. M. BOWYER*, G. M. DUSHEIKO*, B. D. SCHOUB†, AND M. C. KEW

*Department of Medicine, University of the Witwatersrand, Johannesburg 2193, South Africa; and †National Institute for Virology, Johannesburg 2131, South Africa

Communicated by J. H. S. Gear, October 3, 1986 (received for review July 3, 1986)

ABSTRACT **We examined the methylation status of CCGG sites in hepatitis B virus (HBV) DNA to determine whether methylation could be responsible for the selective expression of the HBV surface gene in chronic hepatitis B infection and hepatocellular carcinoma. Infected liver tissue from patients with low levels of viral replication was analyzed for HBV DNA copy number per haploid cell genome. Total cellular DNA, with sufficient HBV DNA, was digested with the restriction endonucleases *Msp* I and *Hpa* II, to determine whether the HBV DNA was methylated, or *Hin*dIII, to determine whether the HBV DNA was integrated or episomal. The cleavage fragments were analyzed by Southern blotting and hybridization to ^{32}P-labeled HBV DNA. In replicative chronic hepatitis B, hypomethylation of the HBV genome correlated with HBV expression in both virions and infected tissue. In carriers with nonreplicative infection, it was difficult to ascertain the role of methylation as copy number was low. HBV DNA copy number was also low in 17 out of 29 of the tumor tissues tested and as many as 14 out of 16 of the adjacent non-neoplastic tissues tested. Integrated sequences were hypermethylated in the PLC/PRF/5 cell line and in six of the tumor tissues suggesting that methylation plays a role in HBV gene repression. However, since DNA from five other tumors was hypomethylated, the belief that methylation *per se* is an absolute determinant of HBV core gene repression does not hold for human hepatocellular carcinoma tissue. Additional factors, such as gene rearrangements, therefore, must influence HBV expression in hepatocellular carcinoma.**

Chronic hepatitis B carriers may either have predominantly "replicative" or "nonreplicative" hepatitis B virus (HBV) infection (1). Chronically infected carriers with replicative infection evolve after several years from an early replicative phase to a later, predominantly nonreplicative phase in which HBV expression is diminished. In carriers with replicative infection, HBV DNA in liver exists as several episomal replicative intermediates. Both the core and surface genes are transcribed from episomal HBV DNA, intact virions are assembled and thus high levels of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) are present in serum. HBeAg is thought to be a breakdown product of the hepatitis B core antigen (2). The majority of long-standing HBV carriers and patients with HBV-related hepatocellular carcinoma (HCC) have low levels of HBV replication (3–5). Their serum is positive for HBsAg and antibody to HBeAg (anti-HBe) but negative for HBeAg, HBV DNA polymerase, and HBV DNA, indicating the absence of viral assembly; HBV DNA in liver is predominantly integrated (6). Transcription of integrated HBV sequences is assumed to be the source of HBsAg in these carriers (7). The human HCC cell line PLC/PRF/5 contains only integrated HBV DNA sequences and expresses only HBsAg (8).

Methylation of specific cytosine bases has been implicated in gene repression in viral infections (9–11). In particular, two studies (12, 13) have suggested that core antigen expression is regulated by methylation. Yoakum *et al.* (12) succeeded in expressing HBV core gene after 5′-azacytidine treatment of transfected human cell lines. This suggested that methylation was responsible for the repression since 5′-azacytidine inhibits methylation in two steps: it is incorporated into replicating DNA and inhibits methyltransferase (14). Miller and Robinson (13) concluded that methylation could be responsible for lack of expression of core antigen in the PLC/PRF/5 cells.

To determine whether these observations could explain the selective expression of HBsAg gene in carriers with nonreplicative infection or with HCC, we studied the state of methylation of CCGG sites in HBV DNA in liver tissue obtained from chronic HBV carriers and patients with HCC.

MATERIALS AND METHODS

Tissues from a total of 39 HBeAg-positive or -negative subjects were analyzed. Neoplastic tissue from 29 patients with histologically proven HCC was frozen immediately postmortem or after surgical removal and stored at −70°C. Sixteen adjacent non-neoplastic tissue specimens from patients with HCC were also studied. PLC/PRF/5 nude mouse tumor tissue was obtained from J. Alexander and S. Aspinall (Medunsa, South Africa). Seven percutaneous liver biopsy specimens from selected chronic carriers with replicative HBV infection were obtained. Two serum samples were also analyzed.

Tissue and Serum DNA Extraction. Liver biopsy specimens were cut into tiny fragments. Larger tissue samples (≈500 mg) were homogenized to a fine powder in liquid nitrogen. The tissue was then digested at 37°C overnight with proteinase K at 100 μg/ml (Boehringer Mannheim) in 2% (wt/vol) $NaDodSO_4$/0.05 M EDTA/0.2 M NaCl/0.1 M Tris·HCl, pH 8.2, followed by phenol/chloroform extraction. RNA was removed by digestion with pancreatic ribonuclease at 100 μg/ml (Boehringer Mannheim) for 1 hr at 37°C. Larger samples were purified by dialysis against TE buffer (10 mM Tris/1 mM EDTA, pH 8), while smaller samples were passed through an Elutip-d column (Schleicher & Schuell). This yielded from 20 μg (biopsy samples) to >600 μg of total cellular DNA.

Virion DNA was extracted from serum by proteinase K digestion and phenol/chloroform extraction, dialyzed, ethanol precipitated, and resuspended in TE buffer to an estimated concentration of 1 ng/μl. The single-stranded region of the HBV genome was made double stranded by the DNA polymerase reaction using DNA polymerase I (Klenow

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HCC, hepatocellular carcinoma; anti-HBe, antibody to hepatitis B e antigen.

fragment, Boehringer Mannheim) followed by a reverse transcriptase (Boehringer Mannheim) reaction using the protocol of Sattler and Robinson (15).

Restriction Endonuclease Mapping. Each DNA sample was digested with restriction endonucleases *Hpa* II (Boehringer Mannheim) and *Msp* I (New England Biolabs). Both enzymes cut the sequence CCGG, but methylation of the internal cytosine base rendered the sequence resistant to *Hpa* II endonuclease digestion but not to *Msp* I digestion (16). Since HBV DNA does not contain a *Hin*dIII site, integration of HBV DNA into total cellular DNA was examined by digestion of total cellular DNA with *Hin*dIII (Boehringer Mannheim). Completeness of digestion was monitored by adding 1 μg of ϕX174 DNA (New England Nuclear) to a 10-μl aliquot of each reaction mixture and verifying by electrophoresis that the appropriate phage cleavage products were present. After digestion, the DNA was loaded into the slots of a 1% agarose gel and electrophoresed at 30 V overnight. Two to 20 μg of total cellular DNA per gel lane was used, depending on the copy number of HBV DNA per genome. The DNA was transferred to a nitrocellulose filter by the method of Southern (17, 18). The filter was then hybridized to labeled, nick-translated pAM12 HBV DNA (19) for 24 hr at 37°C in 50% (vol/vol) formamide, 5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0.), 1× Denhardt's (0.02% each of Ficoll/polyvinyl pyrrolidone/bovine serum albumin in 3× SSC), 20 mM sodium phosphate buffer (pH 6.5), 10% (wt/vol) sodium dextran sulphate, and denatured carrier DNA at 100 μg/ml. Hepatitis B probe for these studies, pAM12 (a gift from the laboratory of J. Gerin, Rockville, MD), was purified by electroelution and labeled by nick-translation to high specific activity (1–2 × 10^8 dpm/μg) using [^{32}P]dCTP (Amersham).

The filter was washed five times at 65°C with 2× SSC in 0.1% $NaDodSO_4$, washed three times at 37°C with 0.1× SSC in 0.1% $NaDodSO_4$, and then autoradiographed for 2–7 days at −70°C using an intensifying screen.

Copy Number Screening. DNA from nonreplicative carriers was screened for HBV copy number per genome by spotting total cellular DNA onto a nitrocellulose filter in increasing concentrations (1–25 μg) together with purified HBV DNA (1–100 pg), as a calibrated standard. As a control, PLC/PRF/5 DNA was also included as it has a known copy number of four to six genomes per haploid mammalian cell DNA equivalent (8, 20). After hybridization, washing, and autoradiography, the HBV DNA copy number was measured by cutting out the circles on the filter, measuring the radioactivity by scintillation counting, and constructing a standard curve. The amount of HBV DNA in pg per mg of total cellular DNA was extrapolated from the standard curve and from this the copy number was calculated. Southern blots were performed whenever the copy number was found to be ≥0.2 so as not to exclude any specimens amenable to further study.

Table 1. Analysis of tissue and sera from chronic hepatitis B carriers

Carrier	HBsAg	HBeAg	Anti-HBe	Copy no.	HBV status	Meth. status	Diagnosis
C1	+	−	+	0.50	NA	NA	CPHB
C2	+	−	NT	0.30	NA	NA	CPHB
C3	+	−	+	0.57	I/E	H	CPHB
C4	+	+	NT	NT	E	H	CAHB
C5	+	+	NT	NT	E	H	CAHB
C6	+	+	+	NT	E	H	CAHB
C7	+	+	NT	NT	E	H	CAHB
S1*	+	+	−	NT	E	H	CAHB
S2*	+	+	−	NT	E	H	CAHB

NA, could not be analyzed; NT, not tested. +, Positive by RIA; −, negative by RIA. Meth., methylation. CPHB, chronic persistent hepatitis B. I, integrated; E, episomal; H, hypomethylated. CAHB, chronic active hepatitis B.
*Serum samples.

RESULTS

Two of the 39 subjects were HBsAg negative. One of these, 1T, was included in the study as copy number was sufficient to enable Southern blot analysis. Four of the carriers studied were HBeAg-positive and had a high copy number of HBV DNA. In only one anti-HBe-positive carrier did the HBV DNA copy number permit Southern blot analysis (Table 1). Seventy-nine percent (7/9) of the serum HBeAg-positive

Table 2. Analysis of tissue from patients with hepatocellular carcinoma

Patient	HBsAg	HBeAg	Anti-HBe	Copy no.	HBV status	Meth. status	Diagnosis
1L	−	−	−	0.5	NA	NA	N/N
1T*				0.68	I	Hypo	HCC
2L	+	−	+	0.04	NT	NT	N/N
2T*				0.04	NT	NT	HCC
3L	+	−	+	0.04	NT	NT	N/N
3T*				0.65	I	NA	HCC
4T*	+	−	−	0.27	NA	NA	HCC
5L	+	−	+	0.04	NT	NT	N/N
5T*				0.04	NT	NT	HCC
7L	+	−	+	0.04	NT	NT	N/N
7T*				0.04	NT	NT	HCC
8L	+	−	NT	0.04	NT	NT	N/N
8T*				0.60	I	NA	HCC
9L	+	−	NT	0.04	NT	NT	N/N
9T*				3.00	I	Hyper	HCC
10L	+	−	NT	0.72	I	Hypo	N/N
10T*				2.00	I	Hyper	HCC
K8T†	+	−	NT	2.98	I	Hypo	HCC
K9T†	+	−	NT	2.50	I	Hyper	HCC
K10T†	+	−	NT	6.95	E	Hypo	HCC
K11L	+	−	NT	0.19	NA	NA	N/N
K11T†				0.39	NA	NA	HCC
K12T†	+	−	NT	0.13	NT	NT	HCC
K13T†	+	−	NT	0.40	NA	NA	HCC
K14L	+	−	+	0.04	NT	NT	N/N
K14T†				0.30	NA	NA	HCC
K15L	+	−	+	0.10	NT	NT	N/N
K15T†				0.20	NA	NA	HCC
K16T†	+	−	−	2.00	E	Hypo	HCC
K17L	+	−	NT	0.23	NT	NT	N/N
K17T†				0.80	NT	NT	HCC
K18L	−	−	−	0.04	NT	NT	N/N
K18T†				0.30	NT	NT	HCC
6L	+	+	−	0.50	NA	NA	N/N
6T*				0.70	I	Hyper	HCC
K1L	+	+	NT	0.19	NA	NT	N/N
K1T†				1.06	NA	NT	HCC
K2T†	+	+	NT	1.90	E/I	Hyper	HCC
K3T†	+	+	NT	1.57	E/I	Hypo	HCC
K4T†	+	+	NT	0.25	NA	NA	HCC
K5T†	+	+	NT	5.0	NA	NA	HCC
K6T†	+	+	NT	6.00	I	Hyper	HCC
K7T†	+	+	NT	1.20	NA	NA	HCC
K19L	+	+	NT	3.50	NT	NT	N/N
K19T†				0.30	NT	NT	HCC
CL1	+	+	−	5.00	I	Hyper	PLC/PRF/5

NA, could not be analyzed; NT, not tested. I, integrated; E, episomal; hypo, hypomethylated; hyper, hypermethylated; N/N, non-neoplastic.
*HCC tissue from Taiwanese patients.
†HCC tissue from African patients.

HCC specimens and one-half (10/20) of the serum HBeAg-negative HCC tissues could be analyzed. The majority of non-neoplastic tissues from patients with HCC had a copy number <1, thus, only one gave a blot that could be analyzed (Table 2).

Total cellular DNA with sufficient HBV DNA was digested with *Hin*dIII to determine whether the HBV DNA was episomal or integrated. All the HBeAg-positive carriers studied had only episomal HBV DNA. The HBeAg-negative carrier studied had both integrated and episomal HBV DNA. Two serum HBeAg-negative HCC tissues had detectable episomal HBV DNA, but integrated copies were not visible in the Southern blot. In the remaining seven serum HBeAg-negative HCC tissues only integrated HBV DNA was detected. Two of the serum HBeAg-positive HCC tissues showed both episomal and integrated HBV DNA, while in the other two samples, only integrated HBV DNA was detected. Four tumors were omitted from the integration analysis as poor quality DNA obscured their integration status.

Methylation. The restriction patterns after digestion with *Hpa* II and *Msp* I were compared. Fewer bands and larger molecular weight fragments obtained after digestion with *Hpa* II indicated CCGG site methylation and hence resistance to *Hpa* II digestion. All the HBeAg-positive carriers had identical *Hpa* II and *Msp* I lanes, indicating that the viral DNA was not methylated (Fig. 1).

In long-standing, nonreplicative, chronic hepatitis B, viral DNA copy number was frequently low, and the role of methylation in influencing HBV gene expression was, therefore, difficult to ascertain (Table 1). For this reason the DNA from tissue of HBV-related HCC patients was studied in the expectation that clonal amplification of cells bearing HBV DNA would make analysis feasible. All HCC specimens were initially regarded as being representative of low viral expression since most patients with HCC have predominantly nonreplicative infection (3–5). Also, there is evidence to suggest a certain level of gene control in HCC (7).

Six tumor tissues showed hypermethylation of HBV DNA. Three of these were serum HBeAg-positive (Fig. 2), and one of these did show traces of episomal HBV DNA. Five of the tumor tissues showed a hypomethylated pattern. One of these, 1T, was the special case mentioned above: this patient was serum HBsAg-negative. Two of the others showed predominantly episomal DNA. Thus, if the restriction pattern seen is that of the episomal HBV DNA, one would expect these not to be methylated (Fig. 3). One, however, appeared to have only integrated sequences and these were hypomethylated. The fifth hypomethylated tumor was from an HBeAg-positive patient who had both episomal and integrated HBV DNA. Two tumors were excluded from the methylation study as their methylation status was not clear.

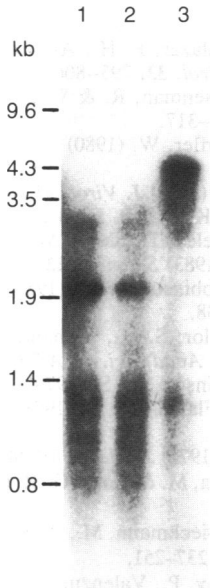

FIG. 1. Assessment of methylation of HBV DNA in liver tissue from a chronic carrier with replicative HBV infection (C4). DNA extracted from the tissue was electrophoresed undigested (lane 3) and following digestion with *Hpa* II (lane 1) or *Msp* I (lane 2).

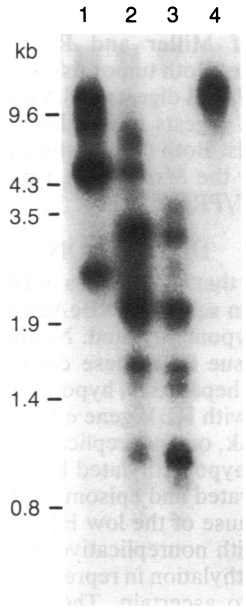

FIG. 2. Southern blot analysis of HBV DNA from a serum HBeAg-positive HCC patient (K6T). Total cellular DNA was digested with *Hin*dIII (lane 1), *Hpa* II (lane 2), or *Msp* I (lane 3) or was not digested (lane 4).

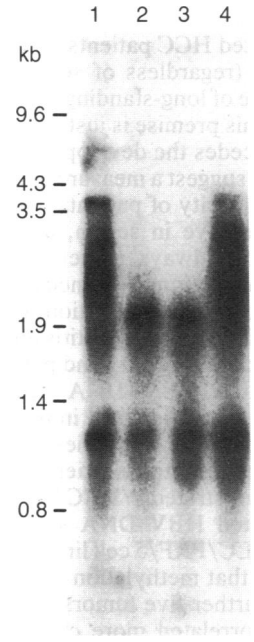

FIG. 3. Southern blot analysis of HBV-specific DNA from a serum HBeAg-negative HCC patient (K10T). Only episomal HBV DNA is evident from a comparison of the *Hin*dIII digest (lane 1) with the undigested DNA (lane 4). The HBV-specific DNA was hypomethylated as the restriction pattern of the *Hpa* II digest (lane 2) is identical to that obtained after digestion with *Msp* I (lane 3).

PLC/PRF/5 nude mice tumor restriction maps were compared to those of Miller and Robinson (13) for the PLC/PRF/5 cell line. Both tumor tissues have intense bands in both *Msp* I and *Hpa* II digests at 8.5 kilobases (kb) and 3.0 kb, and the *Hpa* II digests have additional bands between these common bands. Both tumors show a smaller band, less than 0.8 kb, in only the *Msp* I lane. Thus we could confirm methylation of PLC/PRF/5 cells.

DISCUSSION

Our results indicate that CCGG sites in HBV DNA extracted from HBV virions in serum of HBeAg carriers with replicative infection are hypomethylated. Neither are they methylated in infected tissue from these carriers. Thus, in early, replicative, chronic hepatitis B, hypomethylation of the HBV genome correlates with HBV gene expression. Although the signal was very weak, one nonreplicative carrier with a copy number of 0.6 had hypomethylated HBV DNA on Southern blot analysis; integrated and episomal HBV DNA was present. However, because of the low HBV DNA copy number found in carriers with nonreplicative infection, the possible converse role of methylation in repressing HBV gene expression was difficult to ascertain. The low HBV DNA copy number in long-standing, nonreplicative infection was confirmed when non-neoplastic liver tissues from HCC patients were studied: only 1 of the 16 adjacent non-neoplastic tissues from HCC patients had a copy number greater than one. In only one other case were interpretable Southern blots obtained from both tumor and adjacent non-neoplastic tissue from the same patient. The HBV DNA in this non-neoplastic tissue 10L was integrated but was hypomethylated. This result was interesting because DNA from the corresponding tumor tissue was hypermethylated, and the restriction maps obtained using tumor DNA were totally different than those obtained using DNA from the non-neoplastic tissue. The two tissues, although from the same patient, did not have common bands. There were also more bands in the Southern blot of the tumor tissue suggesting that either there had been rearrangements in the tumor clone or the tumor clone was the result of another integration. In an attempt to overcome the problem of low copy number in non-replicative infection, we included HBV-related HCC patients in the study and regarded all HCC tissue (regardless of serum HBeAg status) as being representative of long-standing infection with low-level viral replication. This premise is justified since long-standing HBV infection precedes the development of HCC and since there is evidence to suggest a measure of gene control in HCC (7). Also, only a minority of patients with HCC are HBeAg and HBV DNA positive in serum, and, where HBeAg is present, it is almost always present in low titer (3, 21). *Hin*dIII restriction digestion confirmed an inverse correlation between markers of HBV replication and integrated HBV DNA in patients with chronic hepatitis and HCC (5). Twelve of the 14 non-replicative carriers and patients with HCC had detectable integrated HBV DNA sequences. Although episomal HBV DNA was detected in five of these tissues, it was present in much lower quantities than was found in the specimens from chronic active carriers. Methylation of HBV DNA could be demonstrated in HCC but the results were not consistent. Integrated HBV DNA sequences were hypermethylated in the PLC/PRF/5 cell line and in six of the tumor tissues, suggesting that methylation may play a role in gene repression, but a further five tumors were hypomethylated. Viral expression correlated more closely with HBV DNA integration than HBV DNA methylation. All six hypermethylated tumors had integrated HBV DNA. Three were from serum HBeAg-negative patients, but the other three were serum HBeAg-positive. Low titers of HBeAg could be accounted for by coding from small quantities of episomal HBV DNA. However, this was only detected in one of the three specimens; this could reflect the degree of sensitivity of the methods employed.

HBeAg expression was detected in only one of the five hypomethylated HCC specimens. Expression of HBeAg in this HCC patient could not be attributed solely to the hypomethylated integrated sequences as episomal sequences were also present. The five hypomethylated HCC specimens included the special case that was serum HBsAg negative. A further two tumors were unusual in that they were HBeAg negative with only episomal HBV DNA detectable; no integrated sequences were visible. Thus, although the HBV DNA was hypomethylated in both of these patients, this would be expected if only the episomal sequences were visualized. The fifth hypomethylated tissue did not express the HBeAg, only integrated HBV DNA sequences were detected, and these were not methylated. Since all HCC with integrated HBV DNA did not have hypermethylated HBV DNA, our results suggest that methylation *per se* is not an absolute determinant of gene expression in HCC tissue. Thus, other factors, such as gene rearrangements, regulatory gene disruption or reading frame shifts must also be considered as possible influences of HBV expression in chronic hepatitis B and HCC.

This investigation was supported by a research grant from the Poliomyelitis Research Foundation and a grant from the Samuel and Sylvia Schnaid Foundation.

1. Brechot, C., Hadchouel, M., Scotto, J., Degos, F., Charnay, P., Trepo, C. & Tiollais, P. (1981) *Lancet* **ii**, 765–768.
2. Takahashi, K., Akahane, Y., Gotanda, T., Mishir, T., Imai, M., Miyakawa, Y. & Mayumi, M. (1979) *J. Immunol.* **122**, 275–279.
3. Kew, M. C., Desmyter, J., De Groote, G., Frösner, G., Roggendorf, M. & Deinhardt, F. (1981) *Prog. Med. Virol.* **27**, 41–48.
4. Hoofnagle, J. H. & Seeff, L. B. (1982) in *Progress in Liver Disease*, eds. Popper, H. & Schaffner, F. (Grune & Stratton, New York), Vol. 7, pp. 469–481.
5. Song, E., Dusheiko, G. M., Bowyer, S. & Kew, M. C. (1984) *Hepatology* **4**, 608–612.
6. Boninio, F., Hoyer, B., Nelson, J., Engle, R., Verme, G. & Gerin, J. (1981) *Hepatology* **1**, 386–391.
7. Dejean, A., Carloni, G., Brechot, C., Tiollais, P. & Wain-Hobson, S. (1982) *J. Cell. Biol.* **20**, 293–301.
8. Marion, P. L., Salazar, F. H., Alexander, J. J. & Robinson, W. S. (1980) *J. Virol.* **33**, 795–806.
9. Groudine, M., Eisenman, R. & Weintraub, H. (1981) *Nature (London)* **292**, 311–317.
10. Sutter, D. & Doerfler, W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 253–256.
11. Desrosiers, R. C. (1982) *J. Virol.* **43**, 427–435.
12. Yoakum, G. H., Korba, B. E., Lechner, J. F., Tokiwa, T., Gadzar, A. F., Seeley, T., Siegel, M., Leeman, L., Autrup, H. & Harris, C. C. (1983) *Science* **222**, 385–389.
13. Millar, R. H. & Robinson, W. S. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 2534–2538.
14. Jones, P. A., Taylor, S. M., Mohandas, T. & Shapiro, L. J. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1215–1219.
15. Sattler, F. & Robinson, W. S. (1979) *J. Virol.* **32**, 226–233.
16. Waalwijk, C. & Flavell, R. A. (1978) *Nucleic Acids Res.* **5**, 3231–3236.
17. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
18. Wahl, G. M., Stern, M. & Stark, G. R. (1979) *Biochemistry* **76**, 3683–3687.
19. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113**, 237–251.
20. Edman, J. C., Gray, P., Valenzuela, P., Rall, L. B. & Rutter, W. J. (1980) *Nature (London)* **286**, 535–538.
21. Tabor, E., Gerety, R. J., Vogel, C. L., Bayley, A., Anthony, P. & Barker, L. F. (1977) *J. Natl. Cancer Inst.* **58**, 1197–1200.