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Expression of Hepatitis B Surface Antigen in Human Cells by a Recombinant BK Virus DNA Vector

By A. CAPUTO,[1]* G. BARBANTI-BRODANO,[1] P. RESCHIGLIAN,[1] M. GIANNI,[2] M. MOTTES,[2] P. MIRANDA,[2] G. MILANESI,[2] B. B. KNOWLES[3] AND R. P. RICCIARDI[3]

[1]*Institute of Microbiology, University of Ferrara, Via L. Borsari 46, I-44100 Ferrara,* [2]*Institute of Biochemical and Evolutionary Genetics, National Research Council, Via Abbiategrasso 187, I-27100 Pavia, Italy and* [3]*The Wistar Institute of Anatomy and Biology, 3601 Spruce Street, Philadelphia, Pennsylvania 19104, U.S.A.*

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SUMMARY

The construction of the first stable human cell lines that express and secrete authentic hepatitis B virus surface antigen (HBsAg), using a BK virus (BKV) episomal plasmid vector, is described. The amount of HBsAg produced by BKV vectors (up to 600 ng/10^7 cells) was comparable to other eukaryotic vector systems. The level of HBsAg expression remained the same regardless of the orientation of the HBsAg gene, substitution of the HBsAg gene promoter with the mouse metallothionein I gene promoter or the tissue origin of the human cell lines used to establish stable cellular transformants. Northern blot analysis also indicated synthesis of normal HBsAg transcripts. Surprisingly, however, the vectors were maintained at far lower than expected copy number (one to five copies/cell). Reasons for this are discussed.

Hepatitis B virus (HBV) causes acute and chronic liver disease and has been implicated in hepatocellular carcinoma. The HBV surface antigen (HBsAg), present on the viral envelope, induces the immune response during infection (Tiollais *et al.*, 1985). Due to the difficulty in propagating HBV in tissue culture, several eukaryotic vector systems have been used to study the transcription and expression of the HBV genome (Feitelson, 1985). These vector systems are also useful in obtaining purified HBV antigens for viral vaccines. However, in no case have stable episomal vector-transformed human cell lines been described which synthesize the HBsAg. Generation of human viral vaccine in a human cell line is preferable to using cell lines from other species since potential contamination by foreign cellular proteins in the purification is obviated. In this study the BK virus (BKV) plasmid vector pBK, which has been shown to reside as a stable episome in human cells (Milanesi *et al.*, 1984), was used to obtain human cell lines that express HBsAg.

Vectors were constructed as described in the legend to Fig. 1 using standard cloning procedures (Maniatis *et al.*, 1982). Vectors pBK-H1, pBK-H2 and pBK-TK-H2 were derived from pBK or pBK-TK (Milanesi *et al.*, 1984). In vectors pBK-MT-H3 and pBK-TK-MT-H4 the endogenous promoter of HBsAg gene was substituted by the mouse metallothionein I (MT-I) gene promoter. All cell lines were of human origin, namely 143B thymidine kinase-deficient (TK^-) derived from an osteosarcoma (Croce *et al.*, 1979), HeLa TK^- derived from a cervical carcinoma, 293 originated from embryonic kidney cells transformed with adenovirus 5 (Graham *et al.*, 1977) and PLC-PRC-5 hepatoma cells (Alexander *et al.*, 1976), and were maintained in culture as described in the specific references. Cell monolayers (about 10^7 cells) were transfected (Wigler *et al.*, 1979) with 10 μg of each BKV-HBsAg plasmid. To select for TK^+ transformants, 143B and HeLa TK^- cells were transfected with plasmids containing the herpes simplex virus type 1 (HSV-1) TK gene and maintained in HAT medium (Littlefield, 1965). To select for

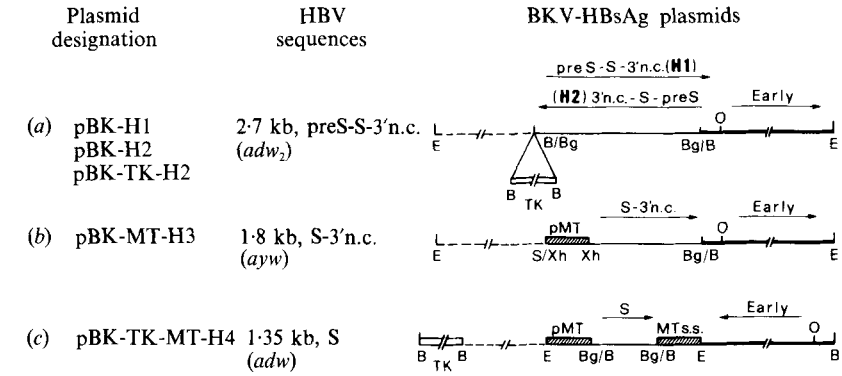

Fig. 1. Construction of five BKV-HBsAg plasmids. (*a*) The vectors pBK-H1 and pBK-H2 were constructed by inserting the 2·7 kb *Bgl*II fragment of the HBV genome (subtype adw_2 from pHBs3; Ono *et al.*, 1983) into the unique *Bam*HI site of the pBK vector DNA. The *Bgl*II fragment contains the 5′ pre-S1 and -S2, the S coding region and the 3′ non-coding region of the S gene (Tiollais *et al.*, 1985). Plasmids pBK-H1 and pBK-H2 have opposite orientations of the HBsAg gene. Plasmid pBK-TK-H2 was prepared by ligating the 2·7 kb *Bgl*II fragment of HBV to pBK-TK DNA, partially digested with *Bam*HI; only constructs in which the direction of transcription of the HBsAg gene was opposite to that of the BKV early region were obtained. (*b*) For construction of pBK-MT-H3, plasmid pBK-MT-gB, containing the HSV-1 glycoprotein B (gB) gene, was the source of the MT-I gene sequences (R. Manservigi *et al.*, unpublished results). The *Xho*I–*Bgl*II fragment (1·8 kb) of the HBV genome (subtype *ayw* from pCP9; Galibert *et al.*, 1979; Tiollais *et al.*, 1981), containing the S coding and 3′ non-coding sequences, was substituted for the gB gene (*Xho*I–*Bam*HI fragment) in pBK-MT-gB. In pBK-MT-H3, the *Xho*I site is immediately upstream of the AUG translational starting codon of the S gene. Therefore, in this construct, transcription of the S gene is directed by the MT-I gene promoter. (*c*) The pBK-TK-MT-H4 plasmid was obtained by inserting the 5·3 kb *Eco*RI fragment, derived from pJYMMT(E)HBV (Hsiung *et al.*, 1984), into the unique *Eco*RI site of the vector pBK-TK. This *Eco*RI fragment contains the 1·35 kb S gene of HBV (subtype *adw*) as well as the promoter and 3′ signal sequences of the MT-I gene. Dashed lines indicate pML DNA sequences; open boxes indicate the HSV-1 TK gene; thin lines indicate HBV DNA sequences; thick lines indicate BKV DNA; dashed boxes represent the metallothionein promoter (pMT) and the 3′ signal sequences of the MT-I gene (MT s.s.), respectively. The arrows indicate the direction of transcription from the promoters of the HBV pre-S region, MT-I gene and BKV early region. E (*Eco*RI), B (*Bam*HI), Xh (*Xho*I), Bg (*Bgl*II), S (*Sal*I); O, BKV origin of replication; preS, 5′ pre-S1 and -S2 coding and regulatory sequences; S, S gene; 3′ n.c., 3′ non-coding signal sequences for the pre-S and S genes.

resistance to the antibiotic G418, cells were cotransfected with one of the BKV-HBsAg plasmids and pSV2neo DNA (Southern & Berg, 1982), at a ratio of 10 : 1, and maintained in the presence of G418 (Sigma; 400 μg/ml). Resulting colonies were individually cloned and expanded into cell lines. HBsAg was detected in the culture supernatant fluid and cell extracts by radioimmunoassay (RIA) or ELISA (Ausria II or Auszime Monoclonal; Abbott Laboratories, North Chicago, Ill., U.S.A.); the clones positive for HBsAg were maintained in culture and examined several times for HBsAg expression and state of plasmid DNA over a period of at least 6 months. For quantification of secreted HBsAg, cells were split into new cultures, incubated for 6 days in growth medium and, at the sixth day, 200 μl of medium or cell lysate was assayed for HBsAg and the cells were counted. The amounts of HBsAg for the different cell lines correspond to ranges from three to eight clones. Density determination of HBsAg was by CsCl gradient analysis (Shih *et al.*, 1984). Aliquots (200 μl) of fractions (500 μl) were assayed for HBsAg and CsCl density was determined by refractometry. For immunoprecipitation, cell monolayers were labelled overnight with 500 μCi/12 × 10^6 cells of [^{35}S]methionine (Amersham; 400 Ci/mmol), the medium was concentrated by ultrafiltration (Minicon-B15, Amicon), reacted with a mouse IgG monoclonal antibody to HBsAg and analysed on an SDS–polyacrylamide gel (Laemmli, 1970; Bonner & Laskey, 1974). The MT promoter was induced with $CdSO_4$ according to Mayo *et al.* (1982). Total cellular and low mol. wt. DNA extraction and Southern blotting were carried out as described (Milanesi *et al.*, 1984). For Northern blot analysis, total and poly(A)$^+$ RNA were

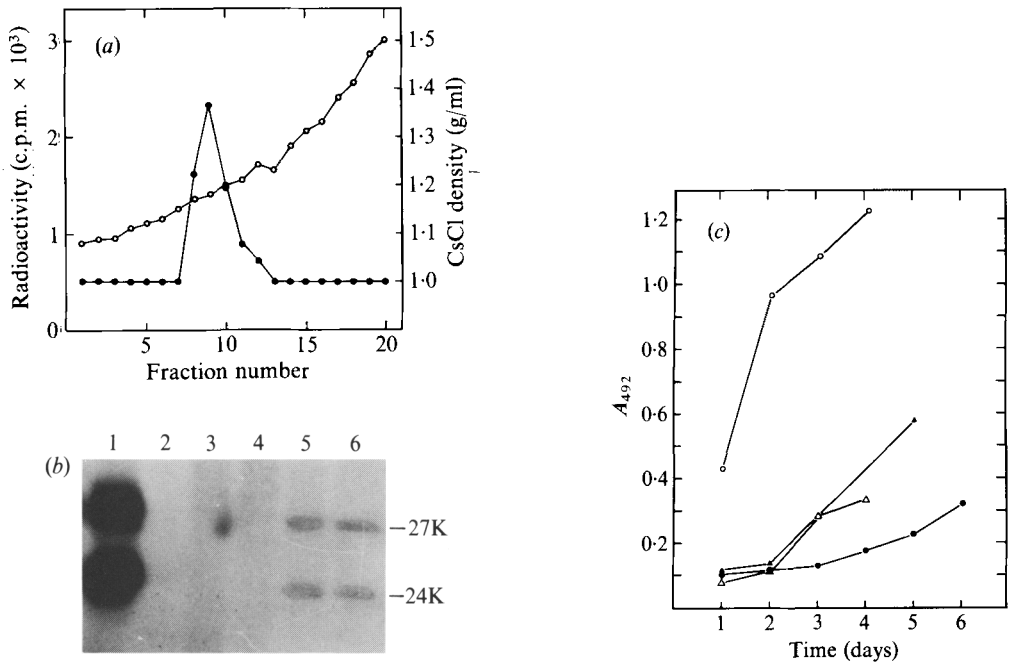

Fig. 2. Characterization of HBsAg. (*a*) Buoyant density of HBsAg purified in CsCl gradients from growth medium of 143B cell clone 1, transformed by pBK-TK-H2 (fractions were collected from the top of the gradients); ●, radioactivity; ○, CsCl density. (*b*) Immunopreciptiation of HBsAg labelled *in vivo* with [^{35}S]methionine, from 143B stable clones and PLC-PRC-5 human hepatoma cells (control cell line). Lanes 1, 5 and 6, PLC-PRC-5 and 143B (pBK-TK-H2) clones 2 and 3, respectively; cell lysate immunoprecipitated with anti-HBsAg monoclonal antibody and with P3 non-specific monoclonal antibody (lanes 2, 3 and 4). The molecular weight markers on the right indicate the two HBsAg-specific bands. (*c*) HBsAg production in 143B and 293 stable cell clones transformed with pBK-TK-H2 and pBK-H2, respectively. Cells were split and cultured in growth medium for 4 or 6 days. Aliquots (200 µl) of medium were harvested at each day and assayed for HBsAg by ELISA. ●, 143B (pBK-TK-H2) clone 2; △, 143B (pBK-TK-H2) clone 3; ▲, 293 (pBK-H2) clone 8; ○, 293 (pBK-H2) clone 13.

separated on 1% agarose–6% formaldehyde gels and hybridized as described (Vasavada *et al.*, 1986). RNA dot blot analysis was performed as described (White & Bancroft, 1982).

Human TK$^-$ 143B osteosarcoma cells were transfected with pBK-TK-H2 DNA (Fig. 1*a*) and selected in HAT medium. Out of 22 TK$^+$ stable derived clones, 14 secreted HBsAg into the culture medium, as determined by RIA; five of these positive clones were further assayed. The amount of HBsAg secreted into the medium in 6 days ranged between 100 and 600 ng per 10^7 cells. Negligible amounts of HBsAg were detected in the cell lysate. Production of authentic HBsAg was verified by the observations that (i) HBsAg from the culture supernatants banded in isopycnic CsCl density gradients at a density of 1·2 g/ml (Fig. 2*a*) and (ii) immunoprecipitation of concentrated culture medium with an HBsAg-specific monoclonal antibody revealed the 24K and 27K proteins, encoded by the S gene (Fig. 2*b*), but none of the higher mol. wt. products coded by the pre-S1 and -S2 sequences. Moreover, electron microscope analysis of the banded material indicated the presence of particles 20 to 25 nm in diameter characteristic of human infected serum (not shown). The time course of accumulation of HBsAg is shown for several transformants in Fig. 2(*c*). The amount of HBsAg detected in the culture medium of 143B stable cell lines remained the same regardless of whether these cells were grown in the absence of HAT (data not shown), or whether stable cell lines were derived from cotransfections with pBK-H2 (Fig. 1*a*) and pSV2neo DNA.

No consistent increase in HBsAg production was obtained with different cell types stably transformed after transfection by plasmids pBK-TK-H2 and pBK-H2. These host cell lines

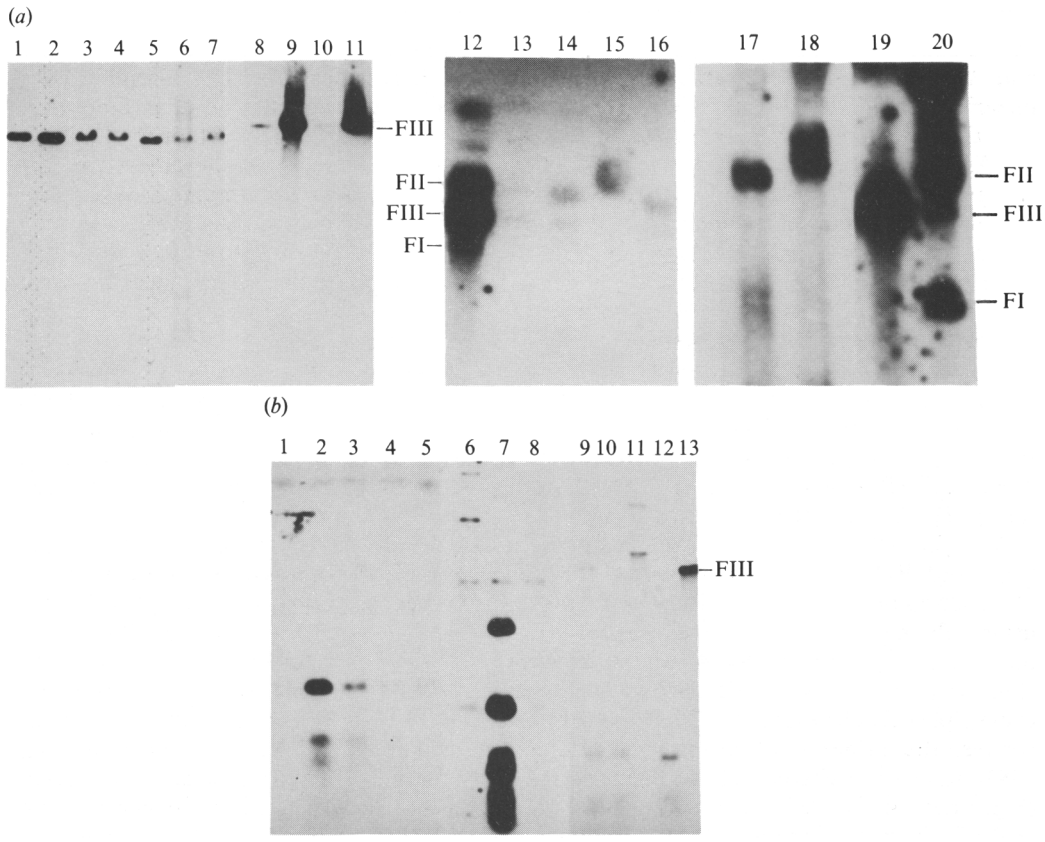

Fig. 3. Southern blot analysis of recombinant DNAs extracted from 143B, 293 and HeLa cell clones transformed with pBK-TK-H2 or pBK-H2 DNAs. Hirt supernatant DNA from 5×10^6 cells or 5 μg of total cellular DNA were analysed. (*a*) *Sal*I digestion. Lanes 1 and 2, control pBK-TK-H2 DNA linear form III (FIII), 50 and 100 pg, respectively; lanes 3 to 7, Hirt supernatant DNAs of 143B (pBK-TK-H2) clones 1 to 5. In lanes 5 and 6, the band migrating more slowly than FIII corresponds to the circular form (FII) of pBK-TK-H2 DNA. In lanes 3 and 6, the low mol. wt. bands may represent deleted plasmid molecules. Lanes 8 to 10, Hirt supernatant DNAs of 143B (pBK-H2) clones 2, 35 and 90; lane 11, pBK-H2 FIII DNA, 50 pg; lane 12, circular, linear and supercoiled (FI) forms of pBK-TK-H2 DNA, 200 g.e.; lanes 13 to 16, total cellular DNA of HeLa (pBK-TK-H2) clones 8, 6, 3 and 5; lanes 17 and 18, total cellular DNA of 293 (pBK-H2) clones 8 and 13; lane 19, pBK-H2 FII DNA, 10 g.e.; lane 20, pBK-H2 uncut DNA, 10 g.e. Due to *Sal*I partial digestion, pBK-H2 DNA is present as FII and FIII in lanes 13 to 16. Similarly, the major bands comigrate with FII of the pBK-H2 control DNA in lanes 17 and 18; a faint band comigrating with FI of pBK-H2 DNA is present in lane 17. (*b*) *Mbo*I digestion. Lanes 1 to 5, Hirt supernatant DNAs of 143B (pBK-TK-H2) clones 1 to 5; lanes 6 to 8, Hirt supernatant DNAs of 143B (pBK-H2) clones 2, 35 and 90; lanes 9 and 10, total cellular DNAs of 293 (pBK-H2) clones 8 and 13; (lane 11, pBK-H2 uncut DNA, 5 g.e.; lane 12, pBK-H2 DNA digested with *Dpn*I, 5 g.e.; lane 13, pBK-H2 DNA linearized with *Sal*I, 5 g.e. In lanes 6 to 9, high mol. wt. bands correspond to partially digested forms of the plasmid DNA.

included the HeLa TK^- cervical carcinoma cells and the human embryonic kidney cell line 293, whose derived clones are represented in Fig. 2(*c*). The orientation of the HBsAg gene in the pBK-H2 construct did not seem to influence expression of HBsAg; stable clones of 293 cells transformed by pBK-H1 and pBK-H2 (Fig. 1*a*) secreted HBsAg at comparable levels (20 to 120 ng/10^7 cells and 30 to 200 ng/10^7 cells, respectively). Finally, the substitution of the HBV promoter with the MT-I promoter (pBK-MT-H3 and pBK-TK-MT-H4, Fig. 1*b* and *c*) did not augment the expression of HBsAg in any of the tested stable cellular clones containing these constructs (10 to 70 ng/10^7 cells). A 1·5-fold increase in HBsAg production was obtained from HeLa cell clones transformed by pBK-TK-MT-H4 upon induction of the MT-I promoter by 40 μM-$CdSO_4$ (not shown).

The state of the recombinant DNA molecules in stable clones was analysed by Southern blot hybridization using pBK-H2 as the probe. Fig. 3 shows the results for 14 clones of 143B, HeLa and 293 cells, transfected with pBK-TK-H2 and pBK-H2 plasmids. The plasmids were linearized by *Sal*I digestion (Fig. 3*a*, lanes 1, 2, 11 and 19). Similar length bands that comigrated with the control plasmids were produced by *Sal*I digestion of the low mol. wt. or total cellular DNAs (Fig. 3*a*, lanes 3 to 10, 13 to 18). These results, together with the analysis of the undigested DNAs (not shown), indicate that the transfected plasmids are maintained in a free state and not rearranged in human cells. Densitometric analysis of these DNAs in comparison to known quantities of plasmid DNA showed that one to five genome equivalents (g.e.) of episomal recombinant plasmid were present per cell. The recombinant DNA vectors were sensitive to digestion with *Mbo*I (Fig. 3*b*, lanes 1 to 10), which specifically cleaves DNA that has replicated in eukaryotic cells, whereas the same DNAs were resistant to digestion with the isoschizomer *Dpn*I (not shown), demonstrating that the episomal recombinant molecules replicate in human cells. Furthermore, Hirt supernatant DNA from HeLa cell clones transformed by pBK-TK-H2 was able to transform *Escherichia coli* C600 to the ampicillin-resistant phenotype, confirming that BKV-HBsAg plasmid DNA persists as a stable episome in human cells. Restriction endonuclease analysis of DNA from several bacterial colonies showed that pBK-TK-H2 DNA was not rearranged (not shown).

A major HBV-specific transcript of approximately 2·3 kb, corresponding to the reported length of HBsAg mRNA (Pourcel *et al.*, 1982; Tiollais *et al.*, 1985), was observed by Northern blot analysis of RNA from 143B, 293 and HeLa cells transfected with pBK-H2 or pBK-TK-H2 using ^{32}P-labelled HBsAg DNA as the probe (Fig. 4*a*, lanes 2, 3, 5, 6, 8 and 9). In addition, three minor transcripts of about 6·4, 4·7 and 0·7 kb were detected in three different clones of 143B cells (Fig. 4*a*, lanes 2, 3 and 9). Although the origin of these minor transcripts is not clear, the 0·7 kb transcript could correspond to the RNA, synthesized *in vitro* by RNA polymerase III, that maps between the surface and core antigen coding sequences on the HBV genome (Standring *et al.*, 1983). In non-transfected cells, no bands corresponding to the HBsAg transcript were detected (Fig. 4*a*, lanes 1, 4 and 7). In order to compare the amount of HBsAg transcripts to the transcript of the housekeeping cellular dihydrofolate reductase (DHFR) gene, RNA from a HeLa pBK-TK-H2 cell transformant and from normal HeLa cells was hybridized to HBsAg DNA (Fig. 4*b*, columns 1 and 2) and DHFR DNA probes (Fig. 4*b*, columns 3 and 4). Also shown is hybridization to HSV-1 TK DNA (Fig. 4*b*, columns 5 and 6) as well as to BKV DNA probes (Fig. 4*b*, columns 7 and 8). An equivalent amount of endogenous DHFR RNA was detectable in transformed and untransformed cells (Fig. 4*b*, columns 3 and 4), and the amount of HBsAg, DHFR, TK and BKV transcripts in transformed cells was similar (Fig. 4*b*, columns 1, 3, 5 and 7). Since the steady-state level of DHFR transcripts is about 100 molecules per cell (Morandi *et al.*, 1982; Riva *et al.*, 1986), we conclude that HBsAg transcripts are maintained at a comparable level by pBK-TK-H2 in HeLa cells.

We describe here the construction of the first stable human cell lines that express and secrete authentic HBsAg, using a BKV episomal plasmid vector. The amount of HBsAg produced by BKV vectors is comparable to other eukaryotic vectors systems, e.g. bovine papillomavirus (BPV), murine sarcoma virus and simian virus 40 (SV40) (Stratowa *et al.*, 1982; Denniston *et al.*, 1984; Will *et al.*, 1984). The orientation of the HBsAg gene relative to the BKV sequences, the type of HBsAg gene promoter, the HBV subtype as well as the tissue origin of the human cell clones did not determine major differences in copy number or level of expression of the HBsAg gene using different BKV plasmid constructs.

The low amplification of the pBK-HBsAg plasmids in human cells was unexpected since the progenitor pBK vector was shown to occur at a frequency of 40 to 100 copies per cell in HeLa and 143B cells (Milanesi *et al.*, 1984). Moreover, pBK vectors containing the HSV-1 glycoprotein B or β-interferon genes amplify as episomal molecules at 40 to 1000 copies per cell in 293 and HeLa cells (R. Manservigi *et al.*, unpublished results; G. Milanesi, unpublished results). One possible explanation for the low copy number of the pBK-HBsAg plasmids is the small amount of BKV T antigen, a viral protein necessary for DNA replication (Tegtmeyer, 1980), synthesized in transformed cell clones; T antigen was detected by ELISA but not by

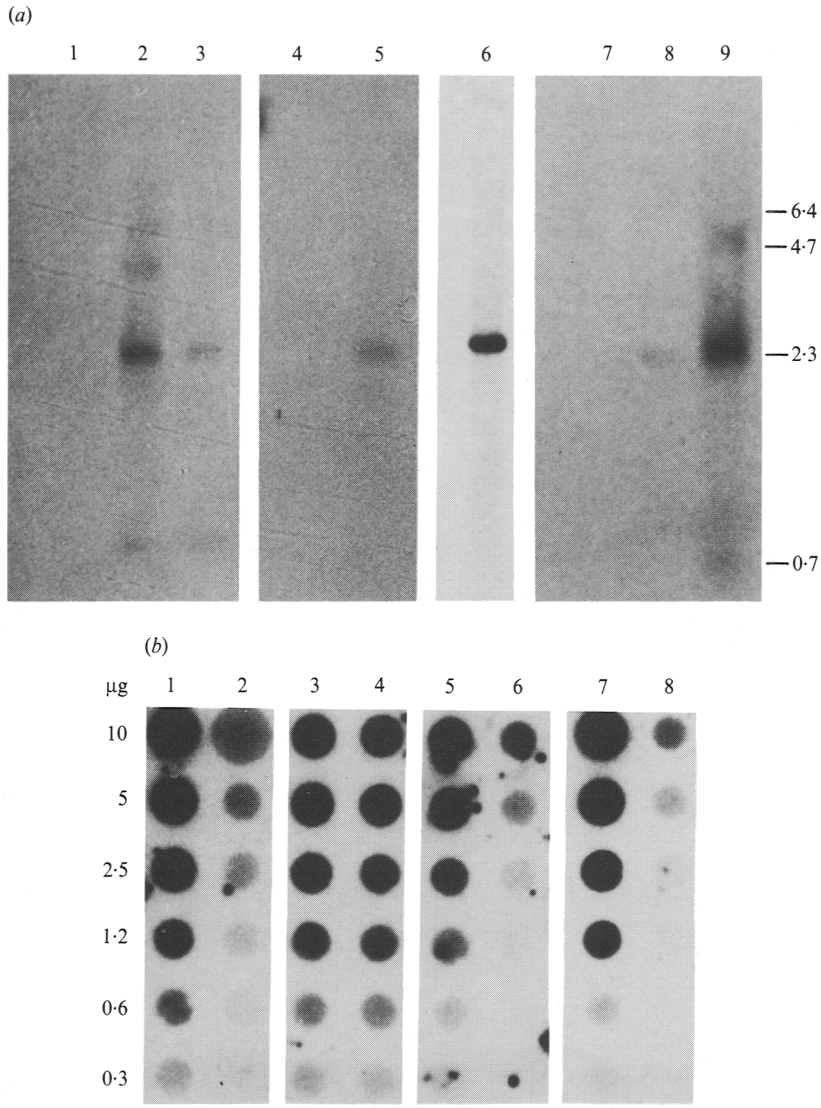

Fig. 4. (*a*) Northern blot analysis of total (30 μg) of poly(A)$^+$ (4 μg) RNAs from 143B, 293 and HeLa stable clones transformed with pBK-TK-H2 or pBK-H2 DNA and hybridized to a probe of HBsAg DNA (*Xho*I–*Bgl*II 1·8 kb fragment). Lanes 1 to 6, poly(A)$^+$ RNAs; lane 1, non-transfected 143B cells; lanes 2 and 3, 143B (pBK-H2) clones 2 and 35; lane 4, non-transfected 293 cells; lane 5, 293 (pBK-H2) clone 13; lane 6, HeLa (pBK-TK-H2) clone 5. Lanes 7 to 9, total cytoplasmic RNAs; lane 7, non-transfected 143B; lanes 8 and 9, 143B (pBK-TK-H2) clones 2 and 5. Molecular size markers are shown on the right in kb. (*b*) Dot blot analysis of cytoplasmic RNA from one clone of HeLa cells transformed with pBK-TK-H2. Decreasing amounts of RNA (μg) were loaded. Columns 1, 3, 5 and 7, Hela (pBK-TK-H2) clone 5; columns 2, 4, 6 and 8, non-transfected HeLa cells. Columns 1 and 2 were hybridized to a probe of HbsAg DNA (2·7 kb *Bgl*II fragment), columns 3 and 4 to the human DHFR 0·82 kb cDNA probe (Morandi *et al.*, 1982), columns 5 and 6 to HSV-1 TK gene, and columns 7 and 8 to BKV DNA. All probes had the same specific activity and equal amounts of radioactivity were used for hybridization.

immunofluorescence. Alternatively, sequences in the HBsAg gene might inhibit the amplification of pBK plasmid DNA. Recently, Roberts & Weintraub (1986) showed that specific sequences of BPV DNA repress replication of a composite BPV–SV40 plasmid in COS cells and this negative control on replication was due to both *cis*- and *trans*-acting sequences from BPV. Similarly, sequences in the HBsAg gene might reduce plasmid amplification when linked to BKV sequences. In that case, S gene-coding sequences are probably responsible for the effect, since pBK-MT-H3 does not contain the pre-S1 and -S2 regions (Fig. 1*b*), and pBK-TK-MT-H4 contains neither the pre-S1 and -S2 nor the 3′ non-coding regions (Fig. 1*c*). Analysis of amplification of S gene deletion mutants cloned in the pBK vector would address this possibility.

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