Biochimica et Biophysica Acta, 1048 (1990) 171–177
Elsevier

BBAEXP 92033

Differences in human cell lines to support stable replication of Epstein-Barr virus-based vectors

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(Received 17 July 1989)

Key words: Eukaryotic episomal vector; Epstein-Barr virus; (Human cell line)

Vectors carrying the origin of replication, ori-P, of the Epstein-Barr virus (EBV) are maintained extrachromosomally in human cells expressing the EBV nuclear antigen 1 (EBNA-1). We have studied the EBV vectors p201 and p292 in which both ori-P and EBNA-1 functions are present using the human cell lines A431 and HeLa. The two lines showed differences in their transfectability by the EBV vectors. Thousands of HeLa transfectants were obtained with either vector and these remained intact as episomes. A431 could only be efficiently transfected with p292 and a high ratio of chromosomal integrations and rearrangements were observed. The vector p292 expressed the EBNA-1 gene more efficiently than p201 and this was found to be associated with a harmful effect on the grown of both HeLa and A431 lines. These results indicate that EBV vectors behave differently, depending on the cell line and that over-expression of EBNA-1 from these vectors may be detrimental to the cells.

Introduction

Extrachromosomal vectors provide an efficient way to express cloned genes in tissue culture cells [1–3]. Moreover, they overcome the variability of expression seen when transfected genes integrate into the host genome, probably due to the effect of adjacent sequences. These vectors are maintained extrachromosomally as a consequence of their origin of replication (usually from viral replicons) and the presence of *trans*-acting factor(s) which act upon the replication origin. In SV40 and Polyoma-based vectors the large T-antigens bind to the origin of replication and initiate multiple round of replication leading to many thousands of copies per cell, providing efficient transient expression systems [2,3]. In contrast, vectors with origins of replication from viruses found as stable episomes in the nuclei of infected cells, i.e., bovine papilloma virus (BPV) or Epstein-Barr virus (EBV), maintain a constant number of copies per cell and are more appropriate for generating stable transfectants. BPV-based vectors are maintained as multi-copy plasmids in murine cells [4,5], while EBV-based vectors can be maintained in EBV-infected human cells. EBV-based vectors carry the origin of replication of EBV (termed ori-P) and the nuclear antigen encoded by the EBV gene EBNA-1 is the only virally encoded *trans*-acting factor needed for replication [6]. However, EBV vectors can also be maintained extrachromosomally in normal human and other mammalian cell lines if the EBNA-1 function is provided *in trans* [6]. In this paper we describe the study of EBV-based shuttle vectors in two human cell lines, A431 and HeLa. We find that the efficiency of transfection and stability of the plasmids is cell line-dependent, and that vectors designated to increase EBNA-1 expression are detrimental to the cells.

Materials and Methods

Plasmids and cells

The EBV plasmids p201 and p291 (p291 which was modified to give p292), were obtained from B. Sugden (Madison, WI, Ref. 6) through D. Kioussis. p292-H2K was obtained by subcloning the H2-K^b gene [8] under the control of the Rous Sarcoma virus LTR [9] into the SalI site of p292. p228 was obtained from pHeBo [7] by D. Kioussis. A431 cells (obtained from Oncogene Sci-

Abbreviations: EBV, Epstein-Barr virus; EBNA-1, EBV nuclear antigen 1; BPV, bovine papilloma virus;

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ence Inc., New York) and HeLa cells were cultured in Dulbecco modified Eagle's medium (Gibco Laboratories) supplemented with 100 U of penicillin per ml, 100 mg of streptomycin per ml, and 10% foetal calf serum (Gibco Laboratories). BW14 is an EBV-infected lymphoblastoid cell line obtained from M. Gallem (U.C., London).

DNA transfections

Plasmids were introduced into cells by the calcium phosphate co-precipitation method [10]. Cells ($(5–8) \cdot 10^5$ plated the previous day) were treated with 1 ml of a calcium phosphate DNA coprecipitate containing 10 μg of DNA for 20 min at 37 °C, 9 ml of complete medium was added, and the cells were incubated for 4–6 h at 37 °C and then exposed to 20% glycerol in phosphate-buffered saline for 2 min. 48 h after transfection, cells were trypsinized and plated at 1 : 10, 1 : 20 and 1 : 40 dilutions. The next day, the cells were fed with complete medium containing hygromycin B (Calbiochem) at 200 μg per ml. Hygromycin resistant colonies were picked at about 3–4 weeks after transfection. The resulting cell lines were maintained in medium containing 200 μg of hygromycin per ml.

DNA analysis

Low molecular weight DNA was prepared from $(1–2) \cdot 10^7$ cells as described [11]. To check for losses during isolation a known amount of a purified recombinant plasmid DNA (M13 phage, replicative form, containing a human γ-globin gene) was added prior to the precipitation of proteins and high molecular weight DNA. For plasmid rescue experiments DNA from $(2–4) \cdot 10^6$ cells was used to transform *Escherichia coli* HB101 as described [12].

For Southern blot analysis digested DNA was electrophoresed on 0.8% agarose gels and blotted onto nitrocellulose filters. Probes were radiolabelled by the random oligonucleotide priming method [13]. Hybridizations were done at 65% in 3 × SSC, 10 × Denhardt solution, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA and 10% dextran sulphate (1 × SSC is 0.1 M NaCl, 0.015 M sodium citrate; 1 × Denhardt solution is 0.02% Ficoll 400, 0.02% polyvinylpirrolydone, 0.02% bovine serum albumin). Filters were washed with 0.1 × SSC, 0.1% SDS at 65 °C and autoradiographed.

S1 protection analysis

Total RNA was prepared by the lithium chloride method [14]. Levels of EBNA-1 RNA were measured by S1 analysis [15]. To prepare a probe the *Hin*dIII fragment of p292 containing the EBNA-1 gene was subcloned into pGEM4 (Promega). An *Nae*I-*Nco*I fragment of that plasmid was labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase and hybridized to 20 μg of total RNA at 54 °C for 18 h. After S1 digestion, products were analysed by electrophoresis on 8% acrylamide-urea gels and autoradiography.

Results

Transfection of A431 and HeLa cells

We first transfected A431 with the EBV vector p201 (Fig. 1) which carries both ori-P and EBNA-1 functions, as well as the *hph* gene (encoding a hygromycin B phosphotransferase under an HSV thymidine kinase promoter) as a selectable marker [16]. Very few colonies grew in selective media (2–4 colonies/10 μg DNA per $8 \cdot 10^5$ cells), which indicates a transfection efficiency several hundred-fold lower than previously that reported for this vector [6]. When the same DNA preparation of p201 was used to transfect HeLa cells, many resistant colonies were obtained ($(2–3) \cdot 10^3$/10 μg DNA per $8 \cdot 10^5$ cells). This is probably not due to differences in DNA uptake because A431 cells are readily transfectable as assayed by transient expression assays (data not shown), indicating that differences in the numbers of stable transfected colonies are cell line-dependent. As the ability of an EBV-based vector to replicate is dependent on EBNA-1 *in trans*, a plasmid containing EBNA-1 under the control of the SV40 early promoter (p292) was used to increase EBNA-1 expression. (In p201 EBNA-1 is apparently transcribed from a fortuitous promoter in pBR322). This plasmid also contains the mouse H2-K^b gene (p292-H2K, Fig. 1) which allows the study of heterologous gene expression. After transfection into A431 cells colonies were observed at high frequency ($(1–3) \cdot 10^3$/10 μg DNA per $8 \cdot 10^5$ cells), which was 500–1000-times greater than that observed with p201, and is similar to the frequency observed with p201 or p292 vectors in HeLa cells. This suggests that an insufficient level of expression of EBNA-1 was re-

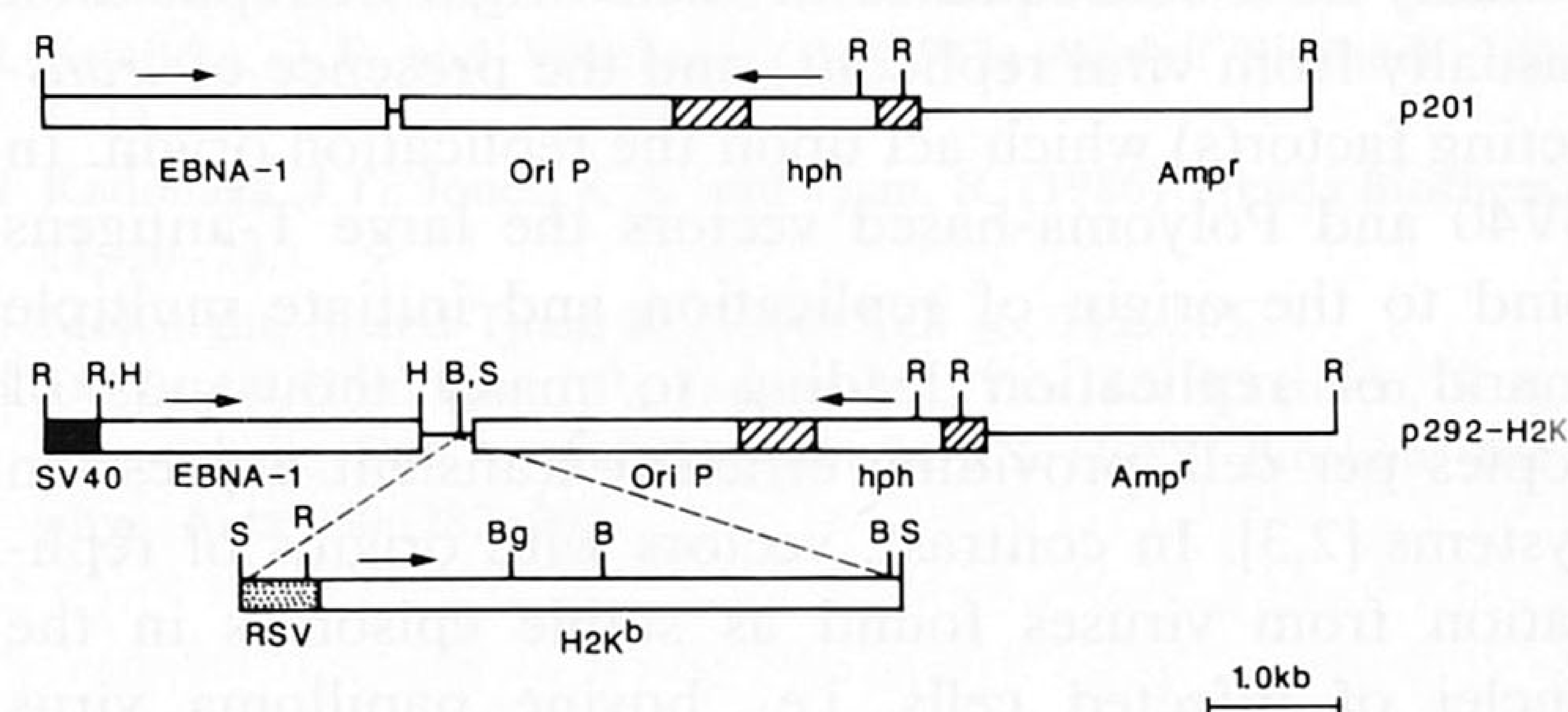

Fig. 1. Maps of EBV plasmids p201 and p292 [6]. The *hph* gene encoding hygromycin resistance uses the herpes virus thymidine kinase (TK) promoter and poly(A) addition sequences (hatched boxes). Ori-P lies on a *Sph*I-*Sac*II fragment from EBV (nucleotides 7358–9517 in the EBV genome [24]). EBNA-1 is encoded on a *Bam*HI-*Pvu*II fragment from EBV (nucleotides 107566–110177) in p201. In p292 it is a *Sau*3AI-*Hin*dIII fragment (nucleotides 107930–110493) and is transcribed from the SV40 early region. The H2-K^b gene subcloned in p292 is transcribed from the LTR of RSV. Other sequences, including the β-lactamase gene, are from pBR322. Relevant restriction sites are shown: *Eco*RI (RI), *Bam*HI (B), *Sal*I (S), *Hin*dIII (H), *Bgl*II (Bg).

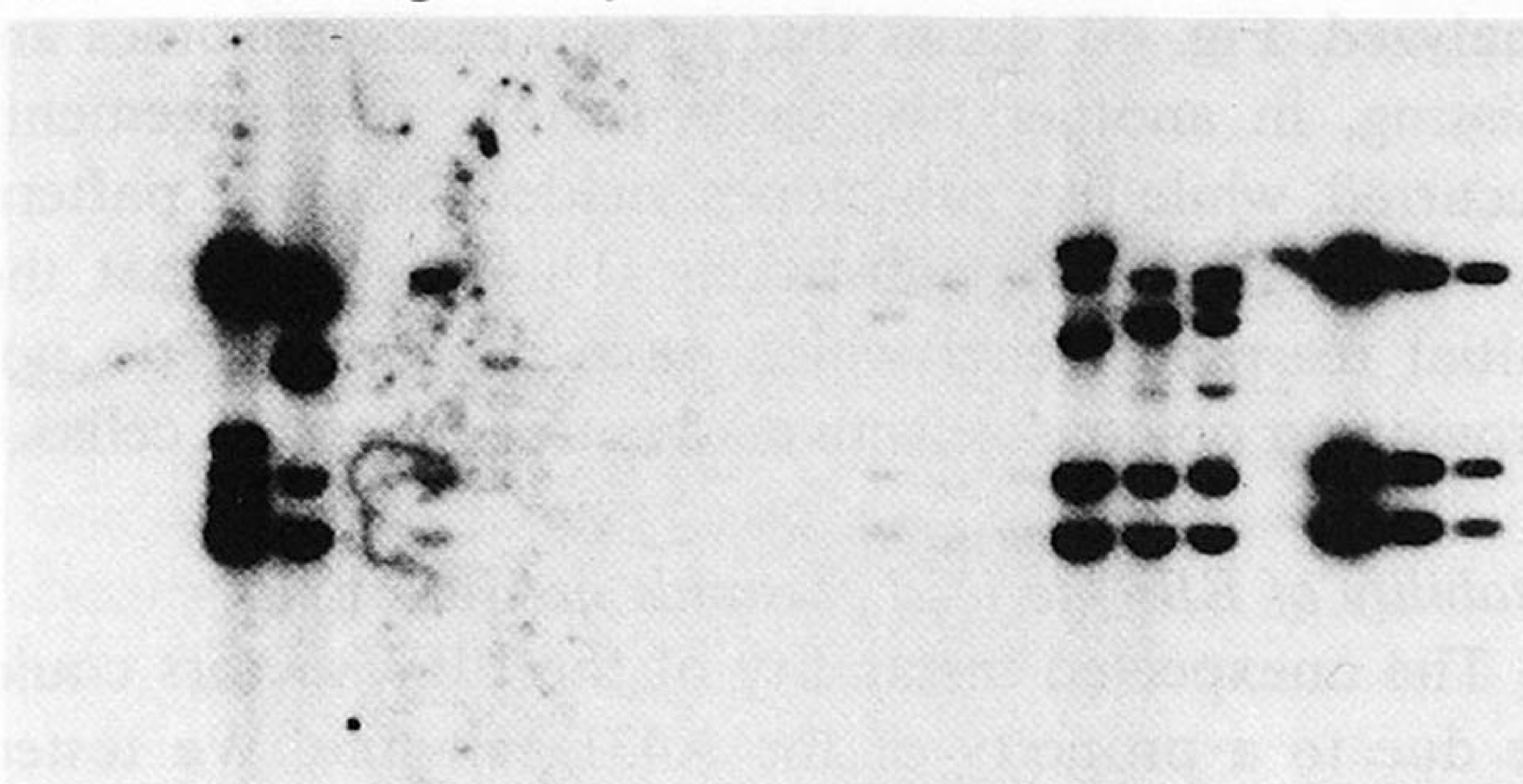

Fig. 2. Southern blot analysis of low molecular weight DNA isolated from A431 clones transfected with p292-H2K. DNA from approx. $2 \cdot 10^6$ cells, from nineteen independent A431 transfectants (lanes a through s) was digested with *Bam*HI and probed with p292-H2K. Lanes 1 through 3 are markers containing reconstructions of 10, 3 and 1 plasmid copy per cell, respectively, obtained by mixing 300, 100 and 30 pg, respectively, of p292-H2K plasmid DNA with DNA from untransfected A431 cells.

sponsible for the poor transfection efficiency of A431 cells with p201. Similar observations were made with the p292 vector alone, showing that the H2-K^b gene was not responsible for the effect.

Stability of EBV vectors in A431 cells

The presence of exogenously introduced DNA was analyzed by Southern blot and plasmid rescue of low molecular weight DNA preparations. Analysis of two A431 clones transfected with p201 failed to show any extrachromosomal copies of p201 (data not shown), suggesting that the vector had integrated into the A431 genome. A constrasting result was obtained with p292-H2K. Fig. 2 shows an autoradiograph of *Bam*HI digested DNA from p292-H2K transfected A431 clones probed with the total p292-H2K DNA. Episomes were detected in 11 of the 19 clones analyzed, ranging from < 1–10 copies per cell. However, episomes from 7 of the 11 clones had undergone rearrangements as shown by hybridization of the probe to extra bands when compared with the input plasmid. The inability to detect episomes in 8 of the 19 clones was not due to losses during isolation because hybridization to an added external standard DNA (see Materials and Methods) was positive (data not shown).

Plasmid rescue experiments (Table I) gave results consistent with the hybridization data. Only DNA extracted from A431 clones carrying episomes gave rise to ampicillin resistant colonies when transfected into *E. coli*. However, the plasmids produced aberrant restriction digestion patterns as seen in Fig. 3. It is interesting to note that more than one molecular species of episome was recovered from single A431 clones. Whether this is due to a mixture of different species co-existing in the same cell, or a mixture of cells containing different episomes is not clear.

TABLE I

Analysis of episomes recovered from cells transfected with p292-H2K

Low molecular weight DNA was prepared from clones grown for 8–9 weeks. (*, denotes 12 weeks) and used to transform *E. coli* to ampicillin resistance. Transformation efficiency was in the range $0.9\text{–}4 \cdot 10^7$ transformants per μg pBR322. Structure of plasmid was checked by restriction analysis.

A431 Clone	amp^r colonies	Plasmid DNA	
		unrearranged	rearranged
FP2	34	0	4 (2 species)
FC9	76/0 *	1	5 (1 species)
FC3	36/40 *	2	4 (1 species)
AP5b	0	–	–
A431	0	–	–
HeLa BH22.1	198	All (6)	
BH21.2	453	All (6)	
BH21.3	191	All (6)	
BH21.4	0		
HeLa	0	–	

Fig. 3 shows the restriction pattern corresponding to recovered episomes from three independent A431 clones and the non-transfected p292-H2K plasmid as a control. In two cases (p2.4 and p3.1) rearrangements resulted in episomes larger than the the introduced plasmid, as shown by the extra bands obtained with *Bgl*II, which recognizes only a single site (Fig. 1) in the control plasmid. In all cases, the H-2K^b gene seems to be intact

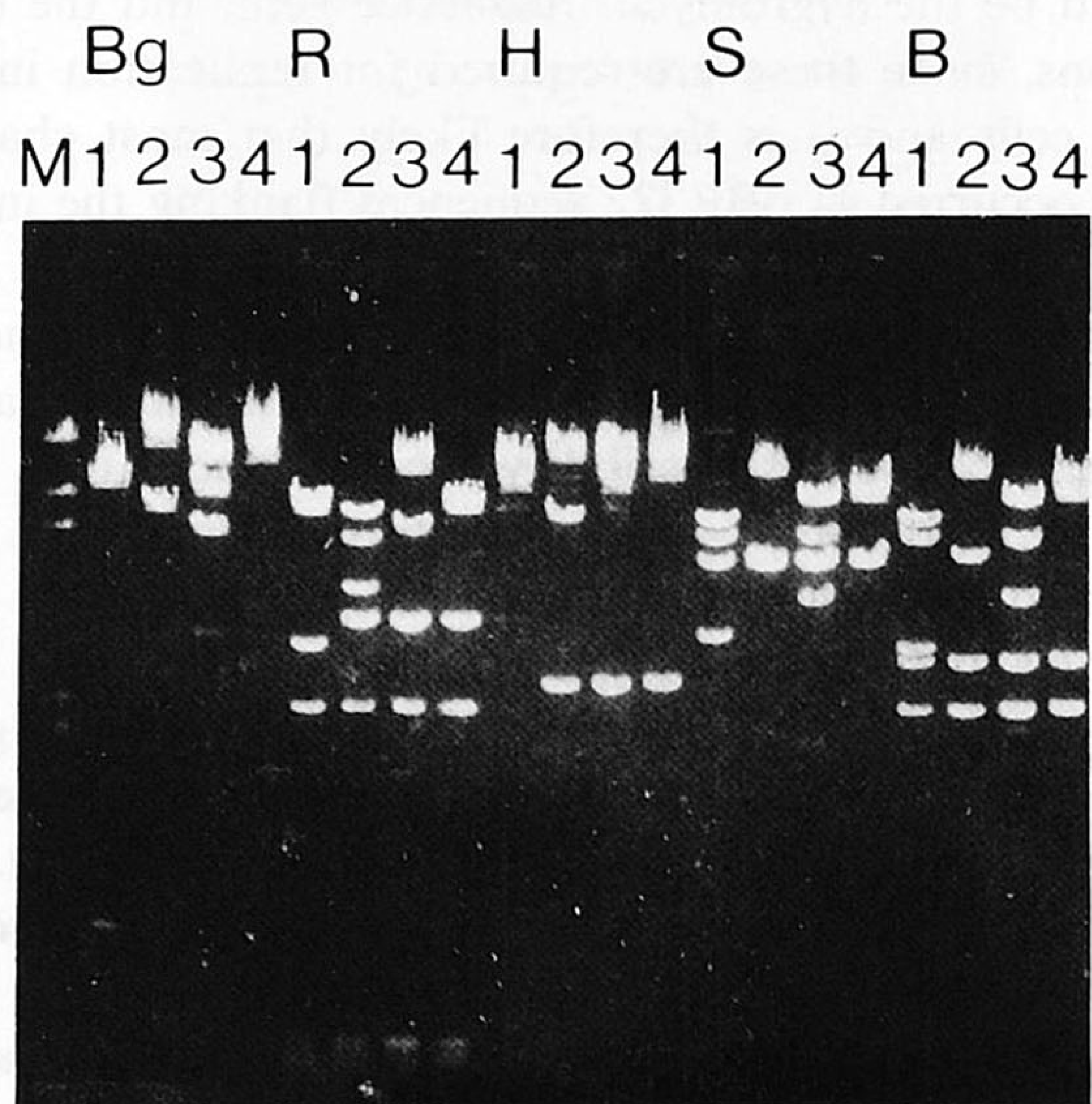

Fig. 3. Restriction analysis of episomes rescued from A431 clones transfected with p292-H2K. Low molecular weight DNA from A431 clones was used to transform *E. coli* HB101. Plasmid DNA from amphicillin resistant colonies obtained with DNA from three independent A431 clones was digested with the following restriction enzymes: *Bgl*II (Bg), *Eco*RI (R), *Hin*dIII (H), *Sal*I (S) and *Bam*HI (B). Lanes 1-3 contain plasmids p1.1, p2.4 and p3.1 (respectively). p292-H2K plasmid DNA (lane 4) was included as a control. M, are *Hin*dIII fragments of λDNA used as size markers. The lanes are overloaded intentionally to enable detection of small fragments.

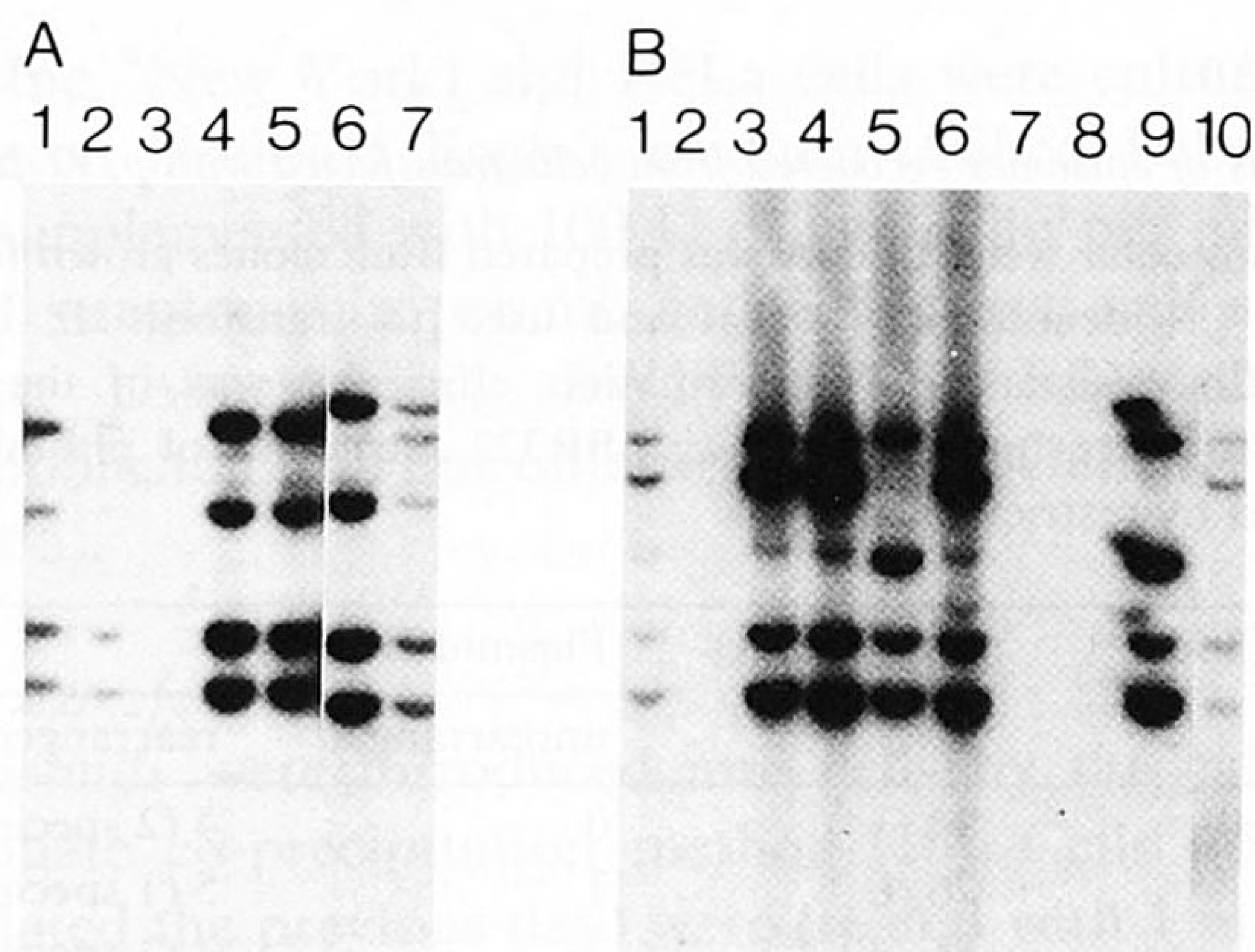

Fig. 4. Southern blot analysis of low molecular weight DNA isolated from A431 clones harboring rearrangement forms of p292-H2K. DNA from approx. $2 \cdot 10^6$ cells was digested with *Bam*HI and probed with p292-H2K. (A) Clones obtained by transfection of A431 cells with rescued p2.4; five independent clones (lanes 3 through 7) as well as a population of p2.4 transfectants (lane 2) are shown. p2.4 plasmid DNA was included as a control (lane 1). (B) Subclones of the A431 cell line which gave rise to p3.1 (lane 2 through 10). p3.1 plasmid DNA was included as a control (lane 1).

as judged by the *Bam*HI digestion pattern. The EBNA-1 gene, which is contained on a *Hin*dIII fragment, is intact in two cases (p2.4 and p3.1). All the episomes have the correct 2.2 kb *Eco*RI fragment encompassing most of the pBR322 sequences. The latter is perhaps not surprising as it contains the β-lactamase gene and the origin of replication which were selected for during the rescue procedure. The other bias in the rescue analysis would be the hygromycin resistance gene and the ori-P regions, since these are required for replication in human cells and it is therefore likely that most changes have occurred in pBR322 sequences flanking the insert.

It is possible that the rearranged EBV plasmids have a selective advantage when grown in A431 cells and do not undergo further changes after the initial rearrangements. To test this possibility, we introduced pl.1, p2.4 and p3.1 into A431 cells. Transfection with p1.1. was 500-fold less efficient than p2.4 and p3.1, the latter gave colonies ($(0.5–1.0) \cdot 10^3$ colonies/10 μg DNA per $8 \cdot 10^5$ cells) at a frequency similar to that of the original p292-H2K plasmid. However, only the p2.4 transfected clones survived in culture prior to analysis. Fig. 4A shows the Southern analysis of low molecular weight DNA from A431 clones transfected with p2.4. One clone has an additional band and another clone has no episomes; the other three, as well as a population of p2.4 transfected cells (about 100 clones), gave an unchanged pattern. The A431 clone from which p2.4 was obtained, however, lost its episomes after 12 weeks in culture.

An additional experiment on the stability of these rearranged EBV plasmids was carried out with the parental line which produced p3.1. The line was recloned after 8 weeks in culture by limiting dilution and low molecular weight DNA from nine subclones was analyzed. Fig. 4B shows that in two cases episomes are missing, in another two cases further rearrangements occurred, while five sub-clones yielded the same pattern as the original 3.1 parent cells. This suggests that the initial rearrangements which gave rise to p3.1 do not provide an obvious selective advantage in A431 cells.

Stability of EBV-derived plasmids in HeLa cells

The unexpected instability of the EBV vectors could be due to a property of the A431 cell line. We tested this possibility by analyzing HeLa clones transfected with p292-H2K. Fig. 5 shows an autoradiograph of *Bam*HI digests of low molecular weight DNA from ten independent clones hybridized with p292-H2K. All but one had a relatively high copy number (30 copies per cell) of episomes and only one showed an extra band compared to p292-H2K plasmid DNA control.

Similar observations were made with plasmid obtained from bacterial colonies resulting from transformation with low molecular weight DNA extracted from HeLa cells. In all cases, a restriction pattern identical to p292-H2K was observed (Table I). Furthermore, the cloned H-$2K^b$ gene was efficiently expressed in HeLa and H-$2K^b$ specific mRNA levels were found to be related to the copy number of episomes (data not shown). The same conclusion about stable extrachromosomal replication of EBV vectors in HeLa cells was obtained from the analysis of HeLa clones transfected with p201. Fig. 6 shows a Southern blot of total DNA probe with p228 (a plasmid derived from pHeBo (7) which contains ori-P, the *hph* gene and pBR322 sequences). This was used to avoid possible cross-hybridization with endogenous sequences homologous to H-2 and EBNA-1 genes [17]. Enzymes which do not cut

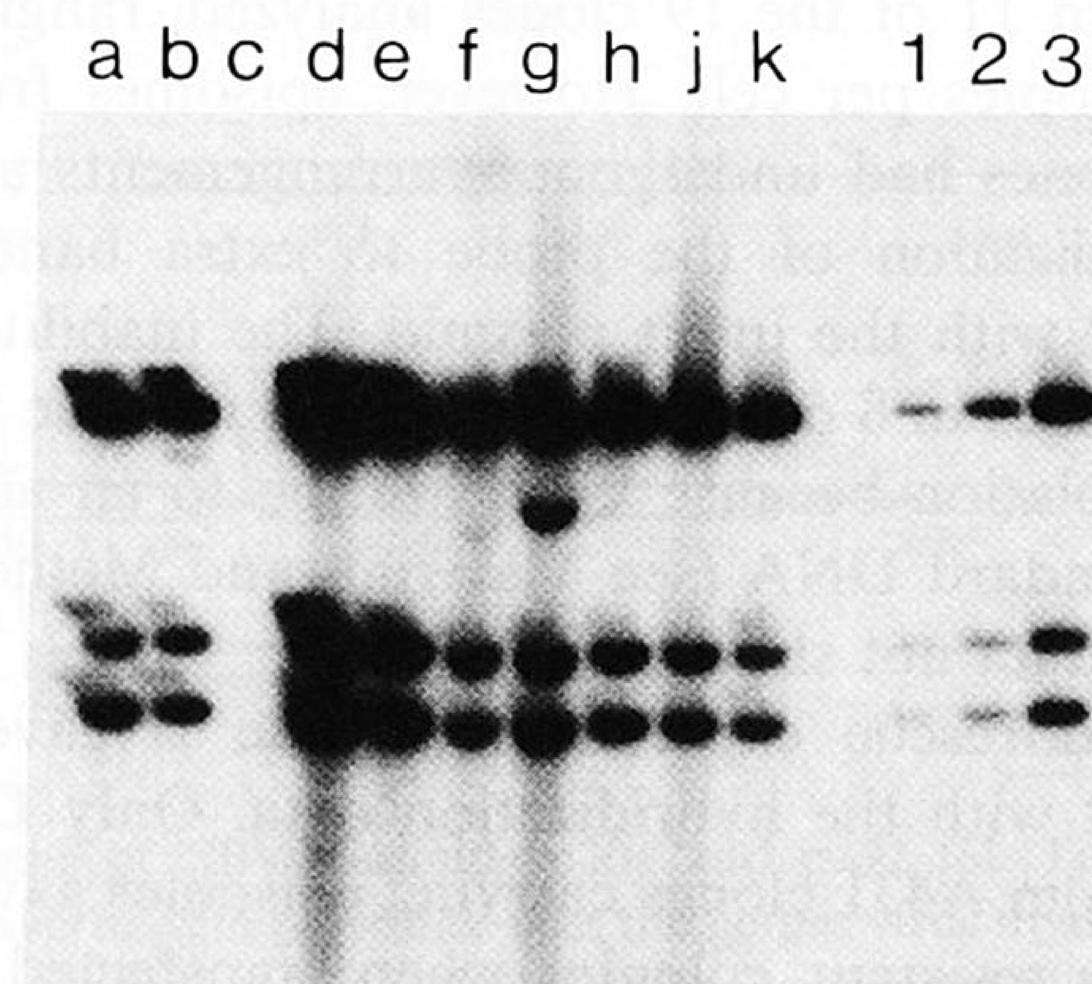

Fig. 5. Southern blot analysis of low molecular weight episomal DNA isolation from HeLa clones transfected with p292-H2K. DNA from approx. $2 \cdot 10^6$ cells from ten independent HeLa transfectants (lanes a through k) was digested with *Bam*HI and probed with p292-H2K. Lanes 1 to 3 are markers containing reconstructions of 1, 3 and 10 plasmid per cell, respectively, obtained by mixing 30, 100 and 300 pg of p292-H2K plasmid DNA with DNA from untransfected HeLa cells.

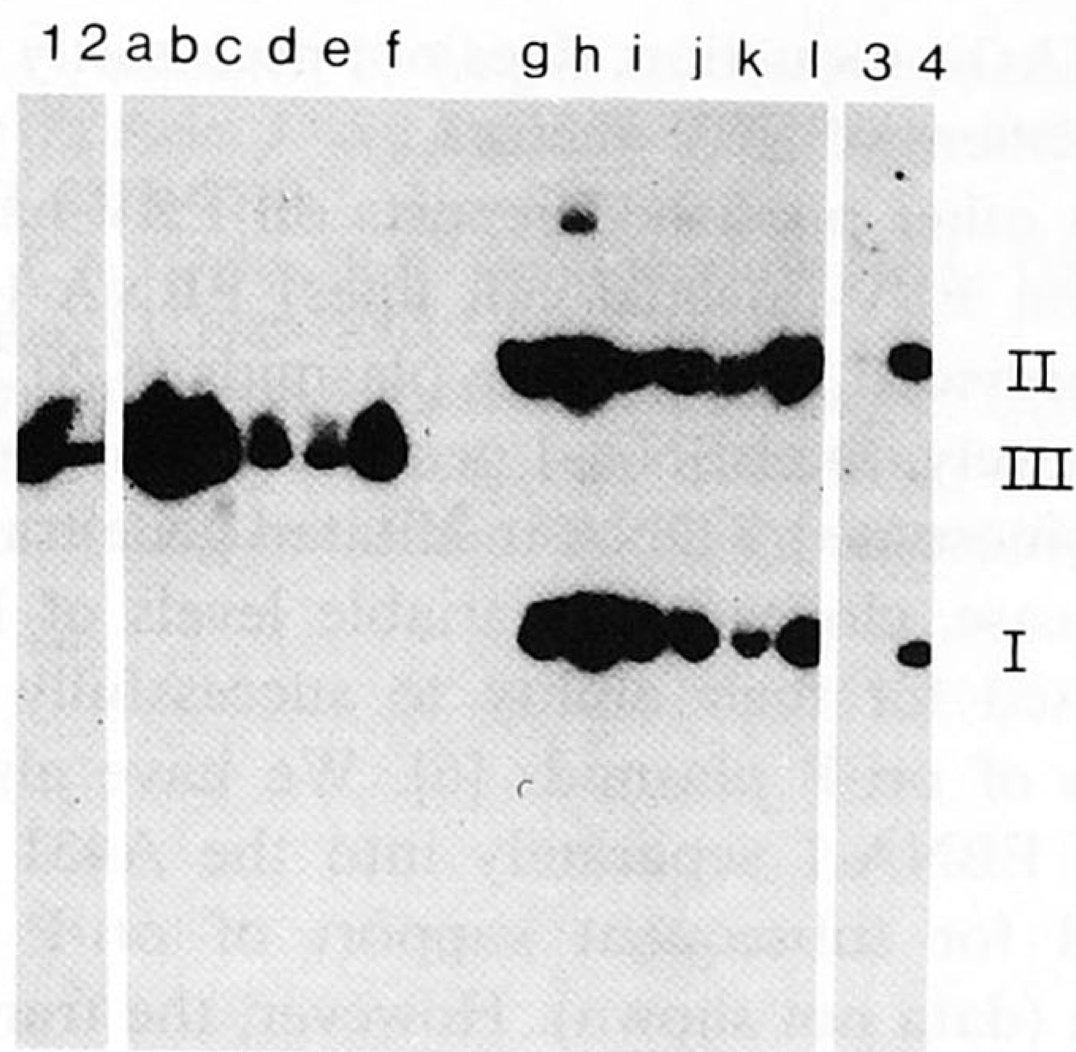

Fig. 6. Southern blot analysis of total DNA isolated from HeLa clones transfected with p201. Six clones, representative of a total of ten independent clones, are shown. Lanes a through f are DNA samples digested with *Bst*EII (which linearizes p201). Lane g through l are DNA samples p201. p201 plasmid DNA was mixed with toal DNA from untransfected cells to provide markers for 1 and 10 copies of plasmid per cell. Migration of form III is indicated by *Bst*EII digested p201 (lanes 1 and 2, corresponding to 10 and 1 plasmid copy per cell, respectively). Migration of forms I and II is indicated by *Bgl*II-treated p201 (lanes 3 and 4, corresponding to 1 and 10 plasmid copy per cell, respectively).

(*Bgl*II) or cut p201 once (*Bst*EII) were chosen. This analysis showed that these clones have intact extrachromosomal copies of p201 (in nine out of ten clones analyzed), from 3–30 copies per cell, and that no rearrangement had occurred.

Growth of A431 and HeLa cells transfected with p201 and p292-H2K vectors

Although the plasmid p292-H2K is more stable in HeLa cells than in A431, we observed in both A431 and in HeLa cells that after 8–9 weeks in culture the cells underwent a crisis during which the growth rate was decreased and many dead cells accompanied every passage. Interestingly, HeLa cells transfected with p201 and the rare A431 transfectants obtained with p201 did not show any crisis and grew faster than their counterparts obtained with p292, with or without the cloned H-$2K^b$ gene.

A possible explanation for the occurrence of this crisis is that the loss of the dominant selectable marker (due to inactivation, or lack of replication of the vector) could give rise to cells unable to survive selection. However, the change occurred rapidly and, in the case of already characterized clones, it was known that they contained 10–30 intact episomes per cell, which makes the above explanation unlikely. After continued growth, some clones survived the crisis, but their growth rate was slower than that of the parental line and they showed differences in morphology. Nevertheless, intact episomes were maintained in HeLa cells, as shown by Southern hybridization of three of the HeLa clones in Fig. 5 (lanes a, b and d).

Expression of the EBNA-1 gene in p201 and p292 based vectors

Because the p201 and p292 plasmids have a different promoter on the EBNA-1 gene, the different growth properties of the cells transfected with p201 and p292 based vectors could be caused by differential expression of the EBNA-1 gene in the two vectors. We therefore analyzed RNA from HeLa cells transfected with p201 and p292-H2K vectors for the steady-state level of EBNA-1 mRNA. Fig. 7 shows S1 analysis of total RNA from four independent HeLa clones as well as a population of transfectants obtained with p201 or p292.

Clones carrying p292 have 5 to 10-fold higher levels of steady-state EBNA-1 mRNA than clones with p201. These levels were even higher than in an EBV infected lymphoblastoma line used as a control. It therefore seems likely that the level of EBNA-1 mRNA may determine the physiological changes leading to the differences observed between cells transfected with EBV vectors.

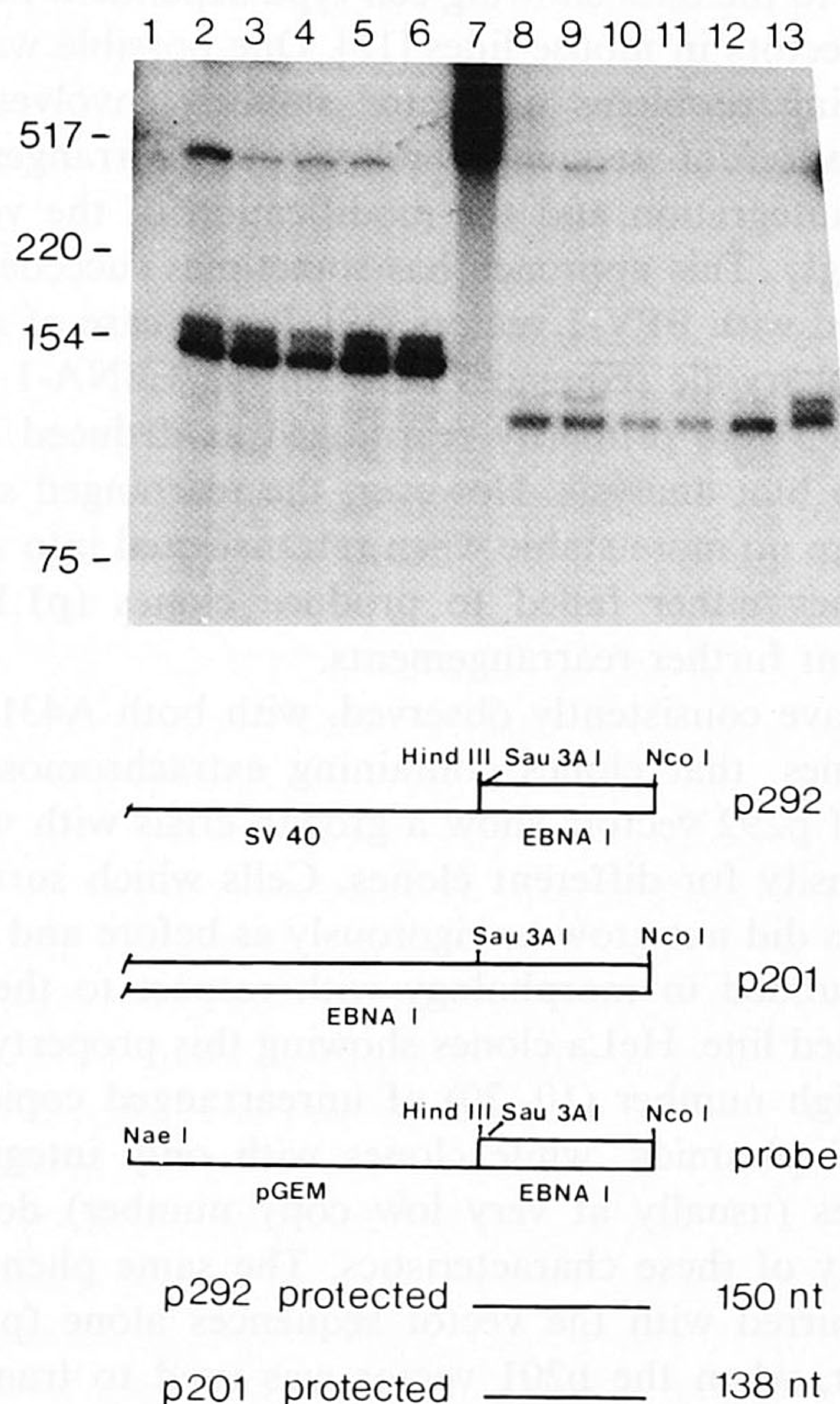

Fig. 7. S1 analysis of EBNA-1 transcripts in HeLa clones transfected with p292 and p201. Lanes 3 through 7 are individual clones and lane 2 is a population of cells transfected with p292. Lanes 9 through 12 are individual clones and lane 8 is a population of p201 transfectants. Lane 1 (untransfected cells) and lane 13 (EBV-infected lymphoblastoid BW14 cell line) are the negative and positive controls respectively. Size markers shown at the left are those of *Hin*fI digested pBR322.

Discussion

Both A431 and HeLa cells are EBV negative and therefore plasmids containing both the EBNA-1 gene and EBV ori-P origin of replication are needed for extrachromosomal persistence of EBV vectors [6]. Rare transfectants of A431 were obtained with the vector p201, but they lacked extrachromosomal sequences. In contrast, p201 produced thousands of transfectants in HeLa cells, in which it was found predominantly in an intact extrachromosomal form. When p292-H2K was used, high transfectant numbers were obtained with both A431 and HeLa cells. However, whereas most HeLa clones had a steady level of extrachromosomal intact vector molecules (about 30 copies per cell) only a fraction of A431 transfectants had episomes. They were very often in a rearranged form and were present at low copy numbers < 1–10 copies per cell.

These observations suggest that cellular properties rather than specific vector sequences are responsible for the different behaviour of EBV vectors in human cell lines. Consequently, not all human lines may be suitable hosts for a given EBV vector. This observation has some parallels to the data showing cell type dependent fate of BPV-1 vectors in mouse lines [18]. One possible way of overcoming problems of vector stability involves the identification of sequences related to rearrangement and/or integration and the modification of the vector accordingly. This approach has sometimes succeded, as illustrated with BPV-1 vectors [19]. In the case of A431 cells, prokaryotic sequences between the EBNA-1 gene and ori-P, were primarily rearranged as deduced from Southern blot analysis. However, the rearranged structures were no more stable when retransfected into A431 cells. They either failed to produce clones (p1.1) or underwent further rearrangements.

We have consistently observed, with both A431 and HeLa lines, that clones containing extrachromosomal copies of p292 vectors show a growth crisis with variable intensity for different clones. Cells which survived this crisis did not grow as vigorously as before and were more rounded in morphology with respect to the untransfected line. HeLa clones showing this property still had a high number (10–30) of unrearranged copies of the EBV plasmids, while clones with only integrated sequences (usually at very low copy number) do not show any of these characteristics. The same phenomenon occurred with the vector sequences alone (p292). However, when the p201 vector was used to transfect HeLa cells, episome-containing clones looked and grew normally. The significant difference between these vectors is the SV40 promoter driving the EBNA-1 gene in the p292 type plasmids and resulting in 10-fold higher EBNA-1 transcript levels in (p292) 'abnormally' growing clones than in (p201) 'normal' clones. This shows that the addition of a strong constitutive promoter to drive EBNA-1 production, does not necessarily result in an improvement of EBV vectors.

In most other published reports on EBV based vectors (in non EBV-infected cell lines) EBNA-1 expression was provided by p201-like plasmids [6,20,21,22,23] or, alternatively, the EBNA-1 gene had been introduced in the chromosome by DNA meditated gene transfer. In the latter case, clones with variable levels of EBNA-1 were selected for their ability to successfully support replication of ori-P plasmids [6]. We have also introduced the EBNA-1 separately into the A431 genome and tested for subsequent support of ori-P plasmid replication (data not shown). However, the transfection efficiency with ori-P plasmids was extremely low and very variable levels of expression were found. It is therefore possible that cell lines differ in the level of their requirement for EBNA-1 to support replication of ori-P containing plasmids. Furthermore, it seems that the expression of EBNA-1 should be balanced between levels too low to support replication of plasmids and levels too high, where cell physiology is impaired.

Acknowledgements

We wish to thank D. Kioussis for providing reagents, as well as for helpful discussions. This work was supported by Oncogene Science Inc., New York and by the MRC (U.K.).

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