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A transforming function of the BARF1 gene encoded by Epstein - Barr virus

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We report a new rodent cell-transforming gene, presumably involved in viral replication, encoded by Epstein-Barr virus. We previously showed that the corresponding open reading frame BARF1 is transcribed before the onset of viral DNA synthesis, and translated into a 33 kd early polypeptide (p33). Here we show that recombinant plasmids containing the BARF1 induce morphological change, anchorage-independent growth and tumorigenic transformation of established mouse fibroblast lines. The BARF1-transformed cells and the tumour tissues isolated from new-born rats after injection of such transformed cell both express p33. Transforming activity was obtained from either the genomic fragment or the cDNA sequence.

Key words: Early gene/EBV/Viral transforming gene

Introduction

The Epstein-Barr virus (EBV), a ubiquitous human herpes virus and a causative agent of infectious mononucleosis, is closely associated with two human cancers: Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC) (Epstein, 1974; Klein, 1973; de Thé, 1980; Ooka, 1985). This virus is also associated with the development of lymphoma induced in acquired immune deficiency syndrome (AIDS) patients (Birx *et al.*, 1986). The function of EBV in these human cancers is not yet known. An EBV gene, coding for a latent membrane protein (LMP) has been reported to transform Rat-1 cells to malignancy (Wang *et al.*, 1985) and, with lower efficiency, Balb/c3T3 cells (Baichwal and Sugden, 1988). It is incapable of transforming NIH3T3 cells (Wang *et al.*, 1985). A 40 kb fragment of the right-hand part of the EBV genome can immortalize primary monkey kidney epithelial cells (Griffin and Karran, 1984).

The appearance of antibodies against the viral early antigens (EA) is an important marker for the diagnosis of EBV-related diseases (de Thé, 1982), indicating an important role of EA in the development of cancer. The EBV-encoded early proteins, involved in the viral DNA synthesis, are the presumed constituents of EA complex since the synthesis of EA and early proteins are inhibited by the inhibitors of DNA synthesis like arabinofuranosylthymine (Ooka and Calander, 1980) or phosphonoacetic acid (Yajima *et al.*, 1976). EBV DNA polymerase and DNase activities belonging to early proteins are specifically neutralized by antibodies against EA (Ooka *et al.*, 1979, 1984; Zhang *et al.*, 1988). Also an ICP8-like early 135 kd protein encoded by the BALF-2 open reading frame (ORF) was recognized by only EA antibody (Zhang *et al.*, 1988). The EBV replication event in nasopharyngeal epithelial cells seems to be an essential step for the development of EBV-related cancers (Allday and Crawford, 1988). Taking all these facts into account, the genes expressed during early stages of the viral life cycle may have an essential role in viral replication and/or oncogenic activity. We therefore decided to determine the possible function of viral genes transcribed during the early stage of the viral cycle.

In initial experiments, we suggested that a gene corresponding to the BARF1 ORF which was not transcribed in EBV-carrying Raji cells incapable of synthesizing viral DNA, might be involved in EBV replication. The BARF1 ORF gene is transcribed just before the onset of EBV DNA synthesis in EBV producer cells and translated into a 33 kd early protein (p33) which is recognized by high EA titre NPC sera (Zhang *et al.*, 1988). In this study we show that recombinant plasmids containing genomic or cDNA sequences corresponding to the BARF1 ORF can induce morphological change, anchorage-independent growth and tumorigenic transformation of the established mouse fibroblast lines, Balb/c3T3 and NIH3T3. Transformed cells and also tumour tissues isolated from new-born rats after injection of BARF1-transformed cells both express p33.

Results

Expression of p33 in NIH3T3 or Balb/c3T3 cells

In order to characterize the BARF1 gene, we expressed it in eukaryotic cells. Two recombinant plasmids were constructed, one that contained the 1.1 kb *Sma*I genomic fragment covering the whole of the BARF1 ORF (Baer *et al.*, 1984) and a second from a 0.74 kb cDNA containing the total BARF1 ORF plus 41 bp at the 5′ end, as well as a polyadenylation site sequence (Zhang *et al.*, 1988) (Figure 1). Both DNAs were inserted into pZip-Neo-SV(X)1, a vector that provides the M-MuLV long terminal repeat and neomycin selection gene sequence (Cepko *et al.*, 1984; Gong *et al.*, 1987). The recombinant plasmid with *Sma*I genomic fragment (pZAR1) was transfected into NIH3T3 cells using a transfection technique that consists of calcium phosphate co-precipitation in suspension followed by polyethylene glycol-sucrose shock (Shen *et al.*, 1982). The cultures were trypsinized 3 days later and seeded onto 10 cm plastic plates. After 2 weeks, several dozen foci were evident in each plate. A number of morphological forms, such as elongated and dense clumped cells were observed (Figure 2A, b and c). Neomycin (neo) was added (350 μg/ml) to test their resistance. After 2 weeks, 80% of foci were resistant to this drug. We then selected neo-resistant clones and analysed them on an immunoblot using NPC sera in order to know whether the p33 protein was expressed. Only 30% of selected clones expressed p33

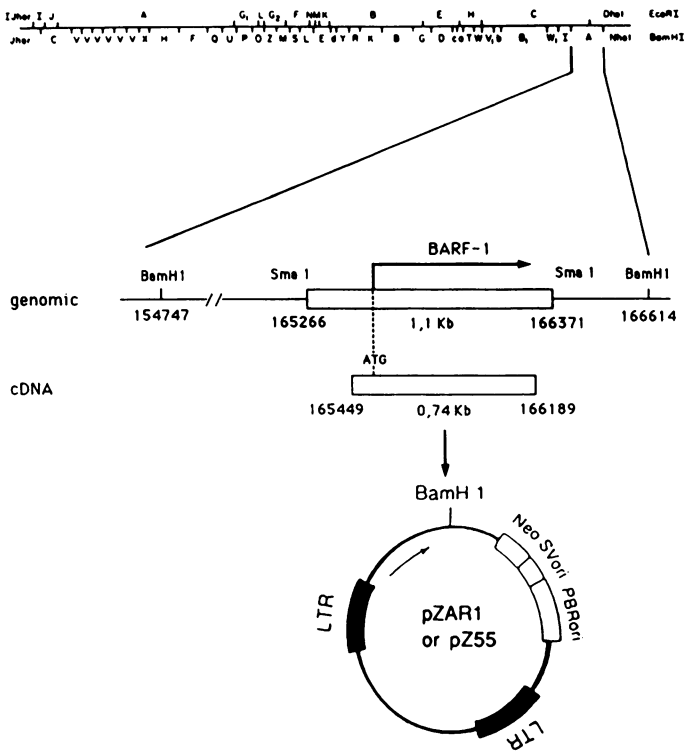

Fig. 1. Construction of eukaryotic recombinant expression plasmids. The endonuclease restriction map of EBV genome and the position of *Bam*HI A fragment are shown at the top of the figure. The isolation of the BARF1 genomic fragment (1.1 kb) and the corresponding cDNA sequence (0.74 kb) was described in Materials and methods and their exact positions on the map of the genome are shown in the middle of the figure. The expression vector pZip-Neo-SV(X)1 is shown at the bottom of the figure. It contains two LTRs of the Moloney murine leukaemia virus, neomycin-phosphotransferase gene (neo), and the origins of replication of simian virus 40 (SVori) and pBR322 (pBRori). The cloning strategy was described in detail in Materials and methods. The arrows indicate the direction of transcription and translation.

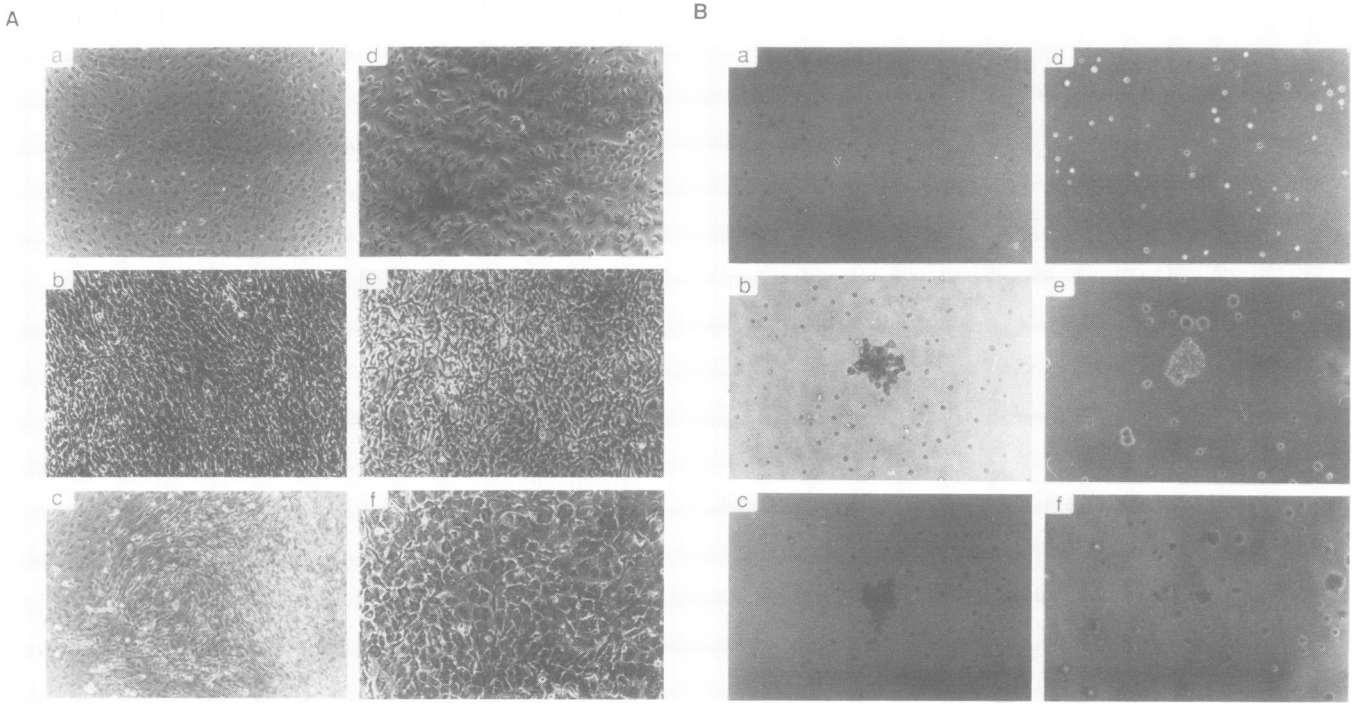

Fig. 2. Morphology of a transformed focus induced in NIH3T3 and Balb/c3T3 cells by transfection of BARF1 contained pZip vectors. (**A**) negative control NIH3T3 cells; **b** and **c**, pZAR1-transfected NIH3T3 cells; **d**, negative control Balb/c3T3 cells; **e** and **f** pZAR1-transfected Balb/c3T3 cells, and growth in soft agar of Balb/c3T3 cells seeded directly after transfection. (**B**) **a**, negative control pZip cells; **b** and **c**, pZAR1-transfected cells or neomycin (Gibco) selected p33 positive clones; **d**, pZip-transfected clone as control (Bpzip-10); **e** and **f**, BZAR1-33 and BZAR1-16 (p33 protein expressing clones). × 100.

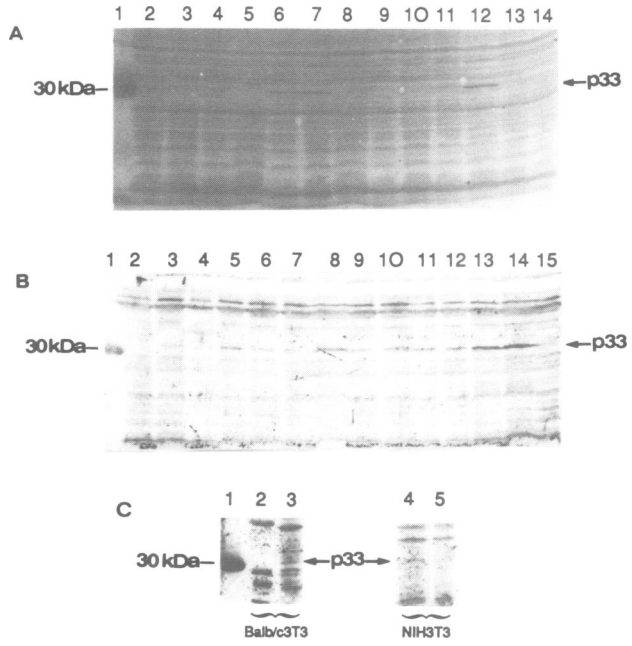

Fig. 3. Expression of BARF1 gene products in rodent cells and in tumour tissues detected by immunoblots. NIH3T3 (**A**) or Balb/c3T3 (**B**) cells were transfected with pZip-Neo-SV(X)1 as negative control (**A, lane 14** for NIH3T3 cells and **B, lane 2** for Balb/c3T3 cells) or, with pZAR1 (**A, lanes 2–13** for NIH3T3 cells and **B, lanes 8–14** for Balb/c3T3 cells) or pZ55 (**B, lanes 3–7** for Balb/c3T3 cells) which contain genomic or cDNA sequences corresponding to the BARF1 open reading frame respectively. (**A Lane 1**, mol. wt markers; **lanes 2–13**, pZAR1-transfected neo-resistant NIH3T3 cells; **lane 14**, pZip-transfected NIH3T3 cells. (**B**) **Lane 1**, mol. wt markers; **lane 2**, BZip-10; **lane 3**, BZ55-8; **lane 4**, BZ55-10; **lane 5**, BZ55-12; **lane 6**, BZ55-15; **lane 7**, BZ55-13; **lane 8**, BZAR1-2; **lane 9**, BZAR1-49; **lane 10**, BZAR1-41, **lane 11**, BZAR1-16; **lane 12**, BZAR1-27; **lane 13**, BZAR1-33; **lane 14**, BZAR1-84; and **lane 15**, negative control Balb/c3T3 cells. (**C**) p33 expression in tumour tissues isolated from new-born rat after injection of BZAR1-27 clone or neo-resistant p33 expressing NIH3T3 clone. **Lane 1**, mol. wt markers; **lanes 2** and **5**, negative controls; **lane 3**, BZAR1-27 clone; **lane 4** neo-resistant p33 expressing NIH3T3 clone.

Table I. Anchorage-independent growth of Balb/c3T3 cells transfected with recombinant plasmids

Transfection	Foci/μg of DNA/1 $\times$ 10^4 cells
Control (Balb/c3T3 cells)	0
pZIP-neo-SV(X)1	0
pZ55	0.014
pZAR1	0.018

The cells were put in agarose 3 days after the transfection and the number of colonies was counted 3 weeks after plating the cells in agarose.

(Figure 3A). In control NIH3T3 cells or pZip-transfected NIH3T3 cells, 4–6 foci/plate were observed in both cases. With the exception of three foci with pZip-transfected NIH3T3 cells, these foci were not resistant to neomycin. Southern blot analysis confirmed that p33 was expressed only in clones possessing the BARF1 sequence (results not shown).

This low rate of p33-positive foci might come from high spontaneous transformants which are resistant to 350 μg/ml neomycin. To characterize further the transforming activity

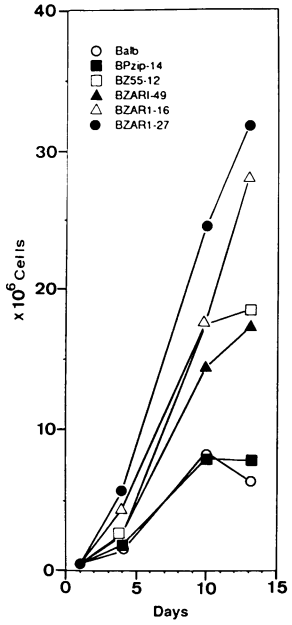

Fig. 4. Kinetics of cell growth. 0.5 $\times$ 10^6 cells from Balb/c3T3 and Bpzip-14 clone as controls and from p33-expressing BZ55-12, BZAR1-49, BZAR1-16 and BZAR1-27 clones were seeded into multiple 10 cm plates. At the times indicated, cells from double plates were harvested by trypsinization and cell number determined using a haemocytometer.

of the BARF1 gene, we tested another rodent cell line, Balb/c3T3. Three days after transfection with pZAR1, Balb/c3T3 (5 $\times$ 10^5) were either plated onto plastic dishes containing 500 μg/ml of neomycin or directly seeded in soft agar (0.3%). After 21 days, we observed 11 colonies/plate which were neo-resistant. These colonies were epitheloid (Figure 2A, f) or spindle-shaped cells growing in a disordered way (Figure 2B, e). Ninety per cent of these clones expressed p33, but the quantity of protein varied with the clones (Figure 3B). Among them four clones (BZAR1-16, 27, 33 and 41) expressed large amounts of p33 (Figure 3B, lanes 10–14). All p33-positive clones grew in soft agar as progressively enlarging colonies within 2 weeks of seeding (Figure 2B, e and f). These colonies were microscopically visible by 7–14 days. In contrast, no colonies were observed in normal Balb/c3T3 cells or pZip-transfected Balb/c3T3 cells clones (Figure 2B, d) over 1 month of cultivation. The pZAR1-transfected cells (5 $\times$ 10^5 cells/plate) directly seeded in soft agar gave an average of nine colonies/plate consisting of 10–100 cell heaps (Figure 2B, b and c). The number of p33 expressing neo-resistant foci per plate corresponds to the number of colonies observed in soft agar when cells were directly seeded after transfection without previous neo-selection. Cloning efficiency (number of colonies per 1 μg DNA per 10^4 cells) was 0.018 in both cases (Table I).

p33 expressing cell growth

Cell growth was analysed on three selected clones—BZAR1-16, 27 and 33—which express p33 protein and have gross morphological changes. After seeding (5 $\times$ 10^5 cells/plate) the culture medium was changed at 3- or 4-day intervals. The three clones multiplied much faster than

Table II. Characteristics of Balb/c3T3 cell clones

Cell lines and clones	Morphologic[a] transformation	Growth in[b] 0.3% agar	33-kd protein[c] expression	Tumour	Tumorigenicity[d] (tumour size in cm)				
					7 days	9 days	14 days	21 days	28 days
DMEM[e]				No	No	No	No	No	No
HeLa[f]				Yes	0.7 × 0.9	0.8 × 1.1	1.2 × 1.8	1.8 × 2.7	2.4 × 3.3
Balb/c3T3	−	−	−	No	DT[g]	No	No	No	No
Bpzip-2	−	−	−	No	DT	DT	No	No	No
Bpzip-10	−	−	−	No	DT	No	No	No	No
Bpzip-14	−	−	−	No	DT	No	No	No	No
BZ55-6	±	±	−	No	No	No	No	DT	DT
BZ55-7	+	+	+	Yes	DT	DT	0.4 × 0.4	2.2 × 3.0[i]	−
BZ55-8	+	+	++	Yes	0.5 × 0.6	0.7 × 1.0	2.1 × 2.8	2.6 × 3.5[h]	−
BZ55-9	−	±	+	Yes	DT	DT	0.4 × 0.4	2.5 × 2.9[i]	−
BZ55-10	±	+	±	Yes	DT	DT	DT	1.0 × 1.2	1.4 × 1.5
BZ55-11	+	++	+	Yes	0.7 × 0.7	0.6 × 0.8	0.5 × 0.5	0.8 × 0.9	0.6 × 0.9
BZ55-12	+	++	+	Yes	0.5 × 0.6	DT	1.3 × 1.8	2.1 × 2.6[h]	−
BZ55-14	+	+	+	Yes	0.5 × 0.6	0.7 × 0.8	1.4 × 1.9	2.1 × 2.6[h]	−
BZ55-15	−	±	±	No	DT	No	No	DT	No
BZAR1-2	+	++	++	Yes	DT	DT	DT	0.4 × 0.4	0.5 × 0.5
BZAR1-14	+	++	+	Yes	0.4 × 0.4	0.4 × 0.5	1.0 × 1.4	1.6 × 2.4[h]	−
BZAR1-16	+	++	++	Yes	DT	DT	0.6 × 0.8	2.0 × 2.9[i]	−
BZAR1-27	+	+++	++	Yes	DT	0.8 × 1.0	2.0 × 2.6	3.1 × 3.8[i]	−
BZAR1-33	+	++	+++	Yes	DT	0.5 × 0.5	0.6 × 0.7	1.7 × 2.2	2.4 × 3.4
BZAR1-41	+	+	++	Yes	DT	DT	0.7 × 1.0	1.7 × 2.7	2.3 × 3.4
BZAR1-49	+	+	+	Yes	DT	DT	DT	1.1 × 1.3	2.8 × 3.6
BZAR1-51	±	+	+	Yes	DT	DT	DT	1.7 × 2.4	2.4 × 3.3
BZAR1-63	−	−	−	No	DT	DT	No	No	No
BZAR1-84	+	+	++	Yes	DT	DT	0.4 × 0.4	2.0 × 2.4	1.8 × 2.6

All the clones (Bpzip, BZ55 and BZAR1 clones) were neo-resistant.
[a]−, no morphological transformation, ±, dense growth, +, heaping of cells.
[b]5×10^5 cell suspension were plated in 0.3% soft agar medium containing 10% FCS. Visible colonies comprising 10−100 cells were scored on the 21st day. −, no growth in 0.3% soft agar; ±, <10%; +, 10−20%; ++, 20−50%; +++, >50% cell colony with 10−100 cells.
[c]Intensity of p33 band on immunoblot after electrophoresis of 5×10^6 cells; ±, positive, but very low quantity.
[d]Tumour formation in new-born rats: new-born rats were inoculated s.c. with 1×10^7 cells/animal for each cell line. Tumour was monitored for the indicated time (days). The size of tumour is indicated in cm.
[e]100 μl of DMEM culture medium without serum was injected per animal.
[f]1×10^7 HeLa cells per animal as positive control.
[g]DT, diffused trace: <0.2 × 0.2 cm.
[h]16th day value; the animals were killed to isolate the tumour tissue sample.
[i]The animals developed large tumours and were killed to isolate tumour tissue.

control Balb/c3T3 cells (with or without pZip vector). Three days after seeding, these cell clones grew 2- to 4-fold more than controls (Figure 4) and cell number increased progressively with time. After 2 weeks of culture, the BARF1-transformed cell populations reached 3.3×10^7 (5×10^5 at seeding time) and continued to increase later. However, control cultures stopped multiplying at confluence ($\sim 7 \times 10^6$). The culture medium from these clones after 4 days contained some heterogeneous extracellular particles. This medium was then precipitated by 50% ammonium sulphate and analysed by immunoblots. The p33 was found among some other polypeptides, whereas no such polypeptides were observed in the control culture medium (non-transformed Balb/c3T3 cells). We do not know if these proteins were secreted from alive cells or resulted from dead cells (results not shown).

0.74 kb cDNA transform Balb/c3T3 cells

We next investigated whether the 0.74 kb cDNA sequence corresponding to BARF1 ORF would transform Balb/c3T3. The recombinant cDNA sequence inserted into pZip vector (pZ55) (Figure 1) was used for this experiment. This cDNA previously constructed from mRNA of EA expressing p3HR1 cells was completely sequenced and its 5′ end localized at genomic position 165 449 and its 3′ end position at 166 189 with no splicing and nucleotide change (Zhang *et al.*, 1988). The recombinant pZ55 gave similar results to pZAR1. The cloning efficiency was 0.014 (Table I) for colonies formed in soft agar and for neo-resistant transformed foci. The transformed BZ55 clones were morphologically similar to the BZAR1 clones and 80% expressed p33. However, the quantity of p33 expressed by BZ55 clones (Figure 3B, lanes 3−7), as determined by immunoblot, was lower than that of BZAR1 clones (Figure 3B, lanes 8−14). This might be due to the lack of the 183 bp sequence upstream of cDNA that is possibly necessary for the efficient expression of this gene. We conclude that a cDNA representative of a single messenger could have a transforming activity equivalent to that of the genomic sequence.

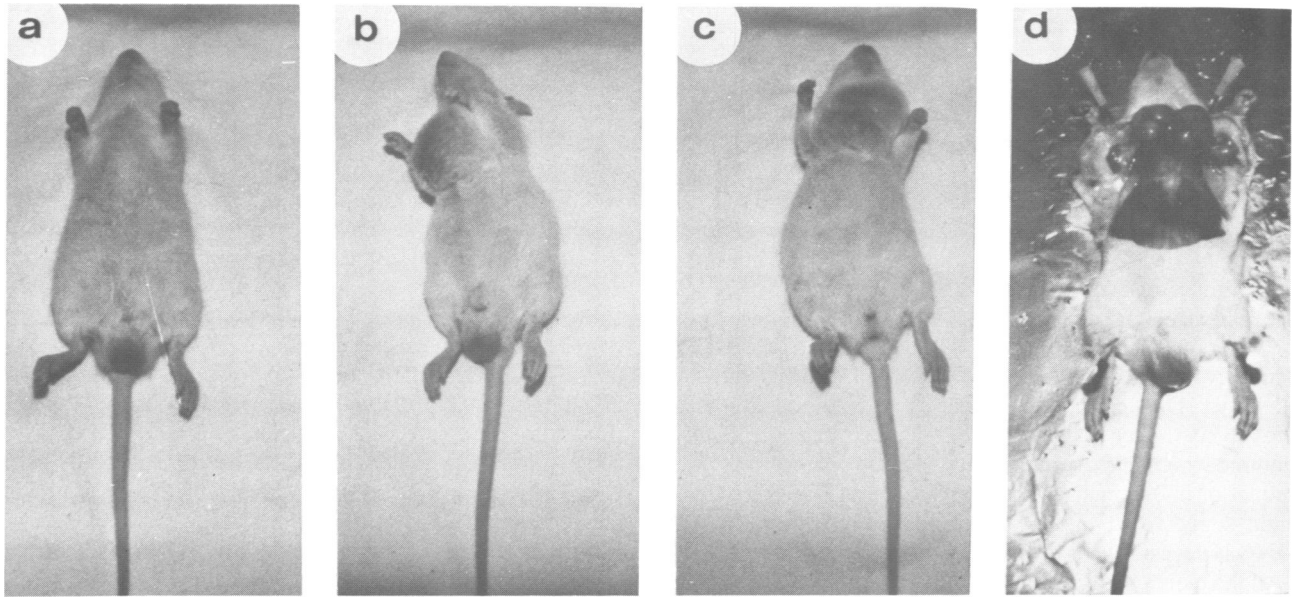

Fig. 5. New-born rats previously treated with anti-thymocyte sera injected with control cell clones and with p33-expressing clones. (**a**) Negative controls, the rat injected with 1×10^7 cells of Balb/c3T3 or Bpzip clones and the culture medium without serum. (**b**) A rat injected s.c. with 1×10^7 cells of BZAR1-33 clone, the animal was killed on the 28th day and the photograph taken. (**c**) A rat injected s.c. with 1×10^7 cells of BZ55-12 clone, the photograph was taken on the 21st day, when the animal was killed. (**d**) The naked tumour as shown in (b) and (c).

Tumour formation in new-born rats

To investigate whether the BARF1-transformed Balb/c3T3 clones could grow as tumours in animals, we used a new-born rat treated with anti-thymocyte sera since these animals can develop metastasis during tumour formation (WHO, 1987). Tumorigenicity of the p33 expressing BZAR1- and BZ55-transformed cell clones was initially assayed by injection of 1×10^7 cells/animal. Culture medium without serum, Balb/c3T3 cells and pZip carrying Balb/3T3 cells were used as negative controls and HeLa cells (1×10^7) as a positive control. Table II shows that no tumour formation was observed in any negative controls. However, the rats injected with HeLa cells or BARF1 containing clones (BZAR1 and BZ55) formed progressively enlarging tumours at the site of inoculation (Figure 5); 16 out of 19 clones tested developed tumours (Table II). Three clones did not give rise to tumours, one cDNA clone BZ55-6 which did not express p33 and another cDNA, BZ55-15, which initially exhibited no morphological changes, formed colonies at a lower rate in soft agar and expressed a very low level of p33 but did not give rise to tumours. The genomic clone BZAR1-63, which was neo-resistant, did not express p33 and did not give rise to tumours (Table II). Clones expressing a high level of p33 formed tumours at early times (7th day) and yielded at $1.0 \times 1.2-3.1 \times 3.8$ cm tumour by the 21st−28th day. All these clones grew well in soft agar. The tumour size with BZAR1 cells was superior to that of HeLa cells as in the case of BZAR1-27, BZAR1-33, BZAR1-41 and BZAR1-49 (Table II).

To examine if the tumour tissue expressed p33, protein extracts from BZAR1-33 (which highly expressed p33) biopsies were analysed by immunoblots. We found p33 in the tumour tissues (Figure 3C, lane 3), whereas control biopsies removed from subcutaneous tissue of the control animals were negative (Figure 3C, lanes 2 and 5). In order to examine if the tumours induced by the other p33-expressing clones express p33, we analysed five cDNA clones (BZ55-7, 9, 10, 11, 12) and five genomic clones (BZAR1-14, 16, 27, 41, 49) by immunoblots. p33 expression was found in all the tumours (data not shown). p33 expression was also obtained in tumours produced after injection of the pZAR1-transfected NIH3T3 clone (Figure 3C, lane 4). Histological examination of the tumours revealed a fibrosarcoma-like tumour with cells having heterogeneous nuclei and metaphasic chromosomes. The data indicate that BARF1 gene product can induce tumorigenic transformation in the established rodent cell lines Balb/c3T3 and NIH3T3.

Discussion

The BARF1-transformed cells show a loss of contact inhibition, increased multiplication, morphological changes, anchorage-independent growth and tumorigenicity in new-born rats. This property resembles that of LMP encoded by BNLF1 gene (Wang *et al.*, 1985; Baichwal and Sugden, 1988). There is however, no significant amino acid sequence homology between BARF1 and BNLF1 ORFs. We have never observed the growth of neo-resistant pZip carrying Balb/c3T3 cell clones (without BARF1 sequence) or control Balb/c3T3 cells in soft agar (Figure 2B, a and d) over a 1-month period of culture. This contrasts with results obtained from pSV2Neo-transfected Balb/c3T3 cells (Baichwal and Sugden, 1988). Sixteen out of 19 anchorage-independent and p33-expressing Balb/c3T3 clones developed tumours. According to our results, all the p33-expressing clones except for the cDNA clone BZ55-15 gave rise to tumours in new-born rats. By contrast, all the p33-negative clones (Balb/c3T3 cells and Bpzip clones as negative controls, a cDNA clone BZ55-6 and a genomic clone BZAR1-63) did not give any tumours. Moreover, all the tumour biopsies tested expressed p33. This strongly indicates that the 33 kd protein intervenes in the tumour formation in new-born rats. This high level of tumorigenic growth

(85%) differs from that reported with LMP-expressing Balb/c3T3 clones (Baichwal and Sugden, 1988). In the latter case, only two out of five LMP-expressing Balb/c3T3 clones gave tumours (Baichwal and Sugden, 1988) and no tumour was observed with LMP-expressing NIH3T3 cells (Wang *et al.*, 1985), except in the case of Rat-1 cells. The lower transforming activity of LMP in Balb/c3T3 cells could reflect the very different growth factor requirements of this cell line (Rozengurt, 1986). In this regard, the 33 kd protein appears to be a more efficient transforming agent than LMP. The potential oncogenic properties of BARF1 gene are, therefore like other oncogenes (Bishop, 1987), although no detectable homology of amino acid sequence was found with any known oncogenes. Our results showed that the cDNA sequence had a relatively lower transforming efficiency than the *Sma*I genomic fragment as judged by cloning efficiency, the amount of p33 expression and certain tumorigenicity. This might suggest that the 183 bp sequence upstream of the cDNA is necessary for the efficient expression of this gene.

All the clones growing in 0.3% agar gave rise to tumours. The anchorage-independent growth is therefore a good criterion for the transformation of the cells. From the test of tumorigenicity, the clones expressing a high level of p33 generally gave relatively big tumours. However, the cDNA clone BZ55-11, which grew well in soft agar, expressed p33-induced tumours early (7th day) without further evolution of tumour size over 1 month of inoculation (Table II). The same phenomenon was also observed for the genomic clone BZAR1-2 forming very small tumours only at the 21st day. It therefore seems that the tumour size is not strongly related to the anchorage-independent growth or the level of p33 expression. This also indicates that the observation of *in vitro* transforming activity (both anchorage-independent growth and p33 expression level) did not correspond exactly to the results obtained *in vivo* (the tumorigenicity assay).

We have recently observed large amounts of transcripts of this gene in tumour biopsies from NPC patients. Since EBV replication in pharyngeal epithelial cells is considered as an important key for understanding the development of NPC and BL tumours (Allday and Crawford, 1988), the early BARF1 gene, which is probably involved in the viral DNA replication, could also be invovled in EBV-related tumour development. It is interesting to note that several regions encoding early genes involved in viral replication (E1A/E1B of adenovirus or E6/E7 region of human papilloma virus type 18) possess a transforming activity (Gallimore *et al.*, 1984; Bedell *et al.*, 1987). Also the HSV replication enzyme, ribonucleotide reductase, was reported to be a potential pathogene (Cameron *et al.*, 1988). These observations tend to indicate that some early genes involved in viral replication may also be important in transformation events. Since the cell lines used in this experiment are already immortalized, it is important to know whether the BARF1 gene has only a transforming activity like adenovirus E1B (Gallimore *et al.*, 1984), polyoma virus middle T antigen or the *ras* gene (Barbacid, 1987), or possesses both immortalizing and transforming activities like the large T antigen of SV40 (Tooze, 1981). In future it will be relatively straightforward to ask whether this gene can transform primary cells. Failure of BARF1 to immortalize would suggest the presence on the EBV genome of another activity, e.g. EBNA2 gene—one of candidates of immortalizing genes (Miller *et al.*, 1974) that possibly co-operated with BARF1. We can ask also whether it needs the activity of other co-operating cellular oncogenes, e.g. c-*myc* oncogene (Land *et al.*, 1983). Further studies will address this question.

In this regard, specific antibodies to p33 in pre-NPC sera might be a more specific marker for immediate risk patients, with regard to developing clinical NPC, than the IgA class antibodies directed to EA which today represents the best epidemiological and clinical marker for NPC (de Thé and Zeng, 1986).

Interestingly, a large EBV fragment that includes the BARF1 ORF immortalizes primary monkey kidney epithelial cells. These immortalized cells expressed an EBV-specific 30 kd protein (Griffin and Karran, 1984). We do not know if both proteins were translated from the same ORF. In this context, it is interesting to ask whether only the BARF1 gene is capable of immortalizing the same cells. Anyway, it will be important to determine whether the viral gene capable of transforming rodent cells is really involved in the transformation of 'human' lymphoid or epithelial cells and consequently in the development of EBV-associated cancers.

Materials and methods

Vector construction

The EBV DNA *Bam*HI fragment is a segment of episomal EBV DNA which extends from the last *Bam*HI site at the right end of the linear EBV genome to the first *Bam*HI site at the left end (see on the map of the EBV genome shown on the top of Figure 1). The BARF1 genomic fragment (1.1 kb) was isolated from the *Bam*HI A fragment of the B95-8 viral genome by digestion with restriction enzyme *Sma*I. The cDNA sequence (0.74 kb) corresponding to BARF1 ORF was isolated from pGEM plasmid containing the BARF1-corresponding cDNA sequence (Zhang *et al.*, 1988) by digestion with restriction enzymes *Hin*dIII and *Eco*RI. They were both treated with T4 DNA polymerase to form the blunt ends and then ligated into pZip-Neo-SV(X)1 vector (Cepko *et al.*, 1984) after *Bam*HI digestion and T4 DNA polymerase and calf alkaline phosphatase to form, respectively, pZAR1 (with genomic sequence) and pZ55 (with cDNA sequence) (see Figure 1). pZip-Neo-SV(X)1 encodes the neomycin phosphotransferase enzyme, which confers resistance to G418 in mammalian cells (Southern and Berg, 1981). Plasmids were transformed into *Escherichia coli* C600R5 and identified by colony hybridizaiton and mini-preparation to have each of the restriction endonuclease sites and orientations shown in Figure 1. Plasmid DNAs were then prepared in bulk for transformations.

Cell culture and transfection

Balb/c3T3 (CIRC, Lyon, France) and NIH3T3 cells (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin and streptomycin in a 5% CO_2, 37°C incubator. Cells at 70–80% confluence were transfected with 50 μg of plasmid DNA by the calcium phosphate technique (Graham and Van der Eb, 1973; Wigler *et al.*, 1979) with some modifications as described (Shen *et al.*, 1982). Briefly, cells at 70–80% confluence were trypsinized and counted. 1.5×10^6 cells were suspended in 1 ml of complete medium and contacted with plasmid DNA for 20 min at room temperature and then incubated at 37°C for 3–4 h in 10 ml of plating medium [20 ml of conditioned medium from the original cell culture to be transfected plus 17.5 ml of fresh medium 2.0 ml of 2 × HeBS (Hepes, 10 g/l; NaCl, 16 g/l; KCl, 0.74 g/l; $Na_2HPO_4 \cdot 7H_2O$, 0.375 g/l; dextrose, 2 g/l), and 0.25 ml of 2 M $CaCl_2$]. At 3–4 h after plating, the cells were shocked with 40% of polyethylene glycol (PEG) (mol. wt 6000, Merck) in 7% (w/v) sucrose/DMEM/10 mM $MgCl_2$ for 2–3 min. Finally, the cells were washed with medium without serum and refed. This technique affects considerably the efficiency of transfection since the cells were transfected in suspension with plasmid DNA. At 72 h post-transfection, 350 μg/ml G418 (Gibco) of NIH3T3 cells and 500 μg/ml G418 were added to the medium for selection of drug-resistant cells. Selective media were changed every 5 days; after 2–3 weeks, colonies of G418-resistant cells were visible. Isolated G418-resistant colonies from the pZAR1 and pZ55 transfections were recovered with stainless steel cloning cylinders (Bellco Glass, Inc. Vineland NJ) and continued to be cultured.

Soft agar assay

Anchorage-independence cell growth was assayed in 0.3% agarose (Seaplaque, FMC) in medium with 10% FCS essentially as described (Wang *et al.*, 1985). The medium was changed 24 h before trypsinization and the cells were then trypsinized and counted. First, 10 ml of 0.6% agarose in phosphate-buffered saline (PBS) × 1 was layered onto the 10-cm plate. 0.5×10^6 cells in 3 ml of complete medium were mixed with 0.6% agarose medium and layered over 0.6% agarose. Cultures were fed regularly with some complete medium. The colonies are visible microscopically by 1 week and were photographed with a Nikon phase-contrast microscope equipped with a Polaroid camera.

Cell growth assay

On day 0, 0.5×10^6 cells were added to 10 cm dishes. At the times indicated, double dishes were trypsinized, the cells from each dish collected in complete medium, and the cell number determined with a haemocytometer. The cell number from double plates was averaged to give the final number.

Immunoblot analysis

The cultured cells were trypsinized and washed with PBS × 1. For tumour tissue, 1 g of tissue was homogenized in 1 ml of TE buffer (10 mM Tris−HCl, pH 7.5 and 1 mM EDTA). 5×10^6 cells of each clone in 25 μl of TE buffer or 5 μl of homogenized tumour tissue were heated in 1 vol of denatured buffer × 2 (62.5 mM Tris−HCl, pH 6.8, 4% SDS, 0.1 g/ml β-mercaptoethanol, 1 g/ml glycerol, 0.02 mg/ml bromophenol blue) in a boiling water bath for 3 min and sonicated. The aliquots were electrophoresed on a 12% polyacrylamide gel and transferred onto nitrocellulose by Sartoblot II (Sartorius, France). The immunoblots were treated with TU132 NPC serum (VCA titre = 1280, EA titre = 1280, EBNA titre = 1280) and the p33 protein was visualized by antiserum against anti-human IgG coupled with peroxidase.

Tumorigenicity analysis

Balb/c3T3 cells, transfected with pZip-Neo-SV(X)1 vector or transfected with pZAR1 and pZ55, were just grown to confluence. Cells were freshly trypsinized and washed with medium without serum. Cell number was determined using a haemocytometer, and ajusted to 1×10^7 cells/100 μl of medium without serum, which was then injected s.c. into new-born rats. Three of four animals were injected per condition. Animals were examined weekly for tumour development.

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