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Expression of Epstein-Barr Viral Early Antigen in Monolayer Tissue Cultures After Transfection with Viral DNA and DNA Fragments

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Antigens associated with the Epstein-Barr virus (EBV) replicative cycle were found in the nucleus and cytoplasm of human placental, Vero, BSC-1, and owl monkey kidney cells transfected with EBV DNA prepared from several different strains of virus. The number of antigen-positive nuclei increased when transfection was followed by cell fusion induced by inactivated Sendai virus. About 1,200 antigen-positive foci were induced per μg of EBV DNA. On the basis of their reactivity with various well-characterized human sera, it appears that the antigens are part of the early antigen complex. None of the four restriction endonucleases, *Eco*RI, *Hin*dIII, *Sal*I, and *Bam*HI, destroyed the ability of EBV DNA to induce early antigen. However, only *Sal*I seemed to leave intact the full spectrum of antigen expression by the HR-1 and FF41 strains of EBV DNA. By means of transfection with recombinant DNA plasmids containing different EBV (FF41) DNA fragments generated by *Eco*RI, we showed that the coding region for early antigen was at least partially contained on the 17.2-megadalton *Eco*RI B fragment.

The DNA of Epstein-Barr virus (EBV) is infectious by the calcium technique of transfection (17). When DNA prepared from virions of two transforming EBV strains is added to human placental cells which are then co-cultivated with lymphocytes obtained from umbilical cord blood, continuous B lymphoblastoid cell lines form. These lines contain EBV DNA and express EBV nuclear antigen. The exact mechanism by which lymphocytes are immortalized after co-cultivation with placental cells transfected with EBV DNA is not yet known. One possible mechanism is that virus is replicated in the placental cells and then passed on to the lymphocytes. Evidence in favor of this idea was that antigens were expressed in the fibroblastic cells transfected with EBV DNA. These antigens were detected with certain EBV antibody-positive human sera and not with human sera which lacked EBV antibody. No antigens were found in cells which received only carrier calf thymus DNA.

The present experiments were undertaken in response to several questions about antigen expression in the transfected placental cells. Was the particular strain of placental cells we used unique in its susceptibility to transfection by EBV DNA? What was the cellular location of the induced antigens? Did the antigen correspond to any known EBV antigen which had been identified by immunofluorescence in EBV-infected lymphocytes? Several experiments were directed at the goals of defining the polypeptide composition of the antigens induced by transfection and of pinpointing the coding region for the antigen on the large EBV genome. Such experiments included efforts to enhance antigen expression after transfection, to determine whether antigen induction after transfection was affected by predigestion of viral DNA with restriction endonucleases, and to learn whether antigen would be expressed after transfection with individual cloned DNA fragments.

MATERIALS AND METHODS

Viral DNA. DNA was prepared from virions of the FF41 strain by concentration of cell culture fluid with polyethylene glycol, DNase treatment of partially purified virus, extraction with Sarkosyl and pronase, and CsCl equilibrium centrifugation of the DNA as described previously (4). DNA from the virions of the EBV B95-8 and HR-1 strains was provided by M. Nonoyama of the Life Sciences Co. through the office of Biologic Resources, National Cancer Institute.

Transfection and cell fusion. Cell monolayers of human placental (10), Vero, BSC-1, and owl monkey kidney cells were 2 to 9 days old before transfection. Cells were cultivated in minimal essential medium

(MEME) plus 10% fetal calf serum and antibiotics in 5% CO_2. The cells were seeded in 60-mm plastic petri dishes containing four to six glass cover slips measuring 10.5 by 22 mm. At 1 to 4 h before addition of DNA, the spent medium was removed, and 5 ml of fresh medium was added. $CaPO_4$ precipitates of viral DNA were formed as described by Graham and van der Eb (9). Calf thymus DNA was used at a concentration of 10 μg/ml as a carrier. The precipitates were allowed to form at room temperature for 20 to 30 min, and then 0.5 ml was added to the cultures. After 4 h of incubation at 37°C, the medium containing DNA was removed, and the cultures were washed once with MEME and incubated at room temperature in 25% glucose in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-buffered saline (HeBS) for 4 min, a modification of the method described by Stow and Wilkie (19). Cells were washed once with HeBS and once with MEME; fresh medium with 10% fetal calf serum was then added. Sendai virus fusion factor was prepared from virions propagated in embryonated chicken eggs. The Sendai virus was inactivated with beta-propriolactone as described by Neff and Enders (18). A 0.5-ml amount of a 1:2 dilution of fusion factor was added per dish.

Immunofluorescence. Three to four days after transfection, cover slips were removed, washed once with Hanks balanced salt solution, and routinely fixed at −20°C for 5 min in a mixture containing 66% acetone and 33% methanol. In later experiments, some cover slips were fixed only in acetone. Fixed cover slips were stored at −70°C until they were processed by indirect immunofluorescence. We employed fluorescein-conjugated anti-human immunoglobulin G (IgG) purchased from Wellcome Reagents Division, Burroughs Wellcome Co., at a dilution of 1:30. The usual source of antibody to detect the antigens in transfected cells was serum from an American patient with nasopharyngeal carcinoma. This serum was routinely used at a dilution of 1:40. The serum contained antibody to various EBV-associated antigens at the following titers: IgG antibody to D component of early antigen, 1:640, to viral capsid antigen, 1:2,560, and to EBV nuclear antigen, 1:640; IgA antibody to D component of early antigen, 1:40, and to viral capsid antigen, 1:80.

Restriction enzyme digestion. The enzymes *Sal*I, *Eco*RI, *Bam*HI and *Hin*dIII were purchased from New England Biolabs. Each digestion reaction contained 500 ng of EBV strain FF41 or HR-1 DNA and about 4 U of enzyme. The digestions were continued for 2 h at 37°C in the appropriate buffers. A sample of the digestion mixture containing about 50 ng of DNA was electrophoresed in a 0.5% agarose gel for 18 h at 40 V. The DNA was transferred to nitrocellulose and hybridized with a nick-translated ^{32}P-labeled probe of intact EBV DNA. The remainder of the digested DNA was used for transfection.

Cloning of *Eco*RI fragments of EBV DNA. *Eco*RI fragments of EBV (FF41) DNA were inserted into the unique *Eco*RI site of the plasmid pACYC184 (2). The methods of ligation, transfection, and screening for recombinant plasmids have been described in detail previously (4). In this instance, however, the recipient *Escherichia coli* strain was HB101, and bacterial colonies which had acquired a chimeric plasmid were resistant to tetracycline (15 μg/ml) and sensitive to chloramphenicol (20 μg/ml). A preliminary identification of the cloned fragments was made by comigration of *Eco*RI-digested plasmid DNA fragments with fragments of virion DNA. A more definitive identity of the cloned *Eco*RI fragment was established by hybridization of ^{32}P-labeled cloned DNA to Southern blots containing *Bam*HI fragments of virion DNA.

Purification of recombinant plasmids. For transfection experiments, we used recombinant plasmid DNA which had been purified on sucrose velocity gradients. The bacteria were grown shaking at 37°C in 1 liter of Luria broth containing 12.5 μg of tetracycline per ml. At an optical density (640 nm) of 0.75, 25 μg of chloramphenicol per ml was added, and incubation was continued overnight. The bacteria were collected by centrifugation and suspended in 20 ml of a solution of 0.1 M Tris-hydrochloride, pH 8.5, 0.01 M EDTA, 10% sucrose, and 100 μg of lysozyme per ml (Worthington). After the suspension was placed for 10 min on ice, Triton X-100 was added to a final concentration of 1%. The lysate was centrifuged for 1 h at 4°C in an SW27 rotor at 25,000 rpm. The supernatant was extracted twice with phenol, and the aqueous phase was made 0.3 M sodium acetate and precipitated with alcohol. The precipitate was suspended in 1 ml of a solution of 0.01 M Tris-hydrochloride, pH 7.4, and 0.001 M EDTA (TE); RNase was added to a concentration of 50 μg/ml, and the sample was incubated for 10 min at room temperature. The material was centrifuged through continuous 5 to 20% sucrose gradients in 0.1 M NaCl, 0.05 M Tris-hydrochloride, pH 7.4, 0.005 M EDTA for 3 to 15 h at 4°C in a SW41 rotor at 25,000 to 35,000 rpm. The time and speed of centrifugation were adjusted to the size of the plasmid. Fractions were collected through a Uvicord III spectrophotometer; those fractions containing plasmid DNA were identified by optical density and by electrophoresis in 0.5% agarose gels containing ethidium bromide. Fractions containing plasmid DNA were pooled, precipitated with ethanol, and suspended in 1 ml of TE. The identity of the purified EBV *Eco*RI fragment was verified by digestion with *Bam*HI followed by electrophoresis alongside *Bam*HI fragments known to be contained in the *Eco*RI fragment.

RESULTS

Cellular location of antigen induced by transfection. In our original experiments, monolayer cultures of human placental cells were transfected with viral DNA; 3 to 4 days later, the monolayers were dispersed with trypsin-EDTA, and cell spreads were prepared for immunofluorescence with a cytocentrifuge. Although in these early experiments we clearly identified cells with antigens detectable by EBV antibody-positive human sera, we were unable to define the location of the antigen. Therefore, in the present experiments, we modified the method by transfecting human placental cell monolayers which were grown on glass cover slips (Fig. 1 and 2). We observed cells with two generally different types of antigen expression.

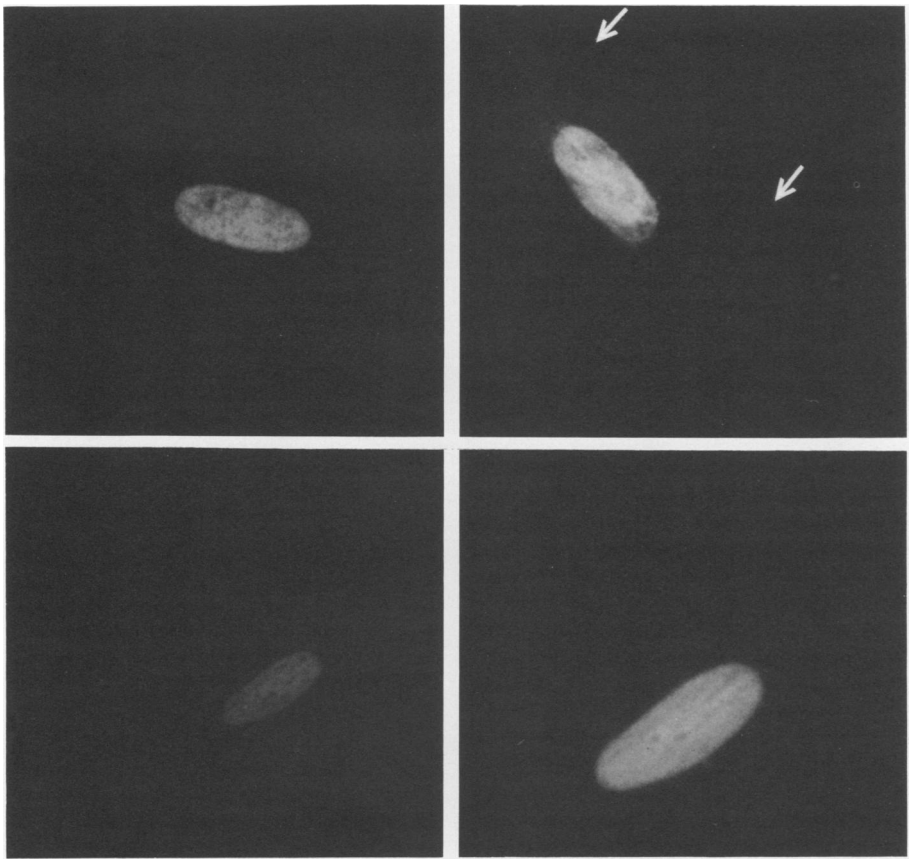

FIG. 1. *Nuclear antigen in human placental cells transfected with EBV DNA. The arrows indicate the presence of some antigen in the cytoplasm.*

In one type, the antigen was confined to the nucleus (Fig. 1). In other cells there was, in addition, antigen found in the cytoplasm (Fig. 2). This cytoplasmic antigen took several forms. In some cells, antigen granules seemed to burst through the nuclear membrane; in others, it was present in coarse clumps in the cytoplasm. Some cells contained antigen diffusely through the cytoplasm; in others, it seemed to be concentrated at the nuclear membrane.

Enhanced antigen expression after cell fusion. We were able to increase the number of nuclei with viral antigen by fusing the cells with inactivated Sendai virus shortly after transfection (Fig. 3). Under these conditions, foci with 2 to 10 or more antigen-positive nuclei could be observed. In these foci, the antigen was usually most pronounced in the nucleus and rarely burst into the cytoplasm. The absence of antigen in the nucleolus is evident in Fig. 3.

Cell fusion did not seem to increase the sensitivity of the placental cell monolayers to transfection, as measured either by a decrease in the minimum amount of DNA which would initiate infection or by an increase in the number of antigen-positive foci per dose of DNA (Table 1). The minimal infectivity of EBV DNA, as assessed by its ability to induce antigen in placental cells, can be calculated from the data in Table 1. There were four cover slips per dish; assuming all of the DNA reached cells on the cover slip, the infectivity was about 1,200 to 1,500 antigen-inducing units per μg. The infectivity was similar with and without Sendai virus. However, cell fusion made the assay for transfection easier to score, since there were more positive nuclei in each focus; therefore, fusion was used in subsequent experiments.

Antigens induced by transfection with three different EBV strains in several different monolayer cultures. The original experiments were carried out with a single strain of human placental fibroblasts, designated in our laboratory as FF260. We subsequently found that another placental cell strain (FF498) and our laboratory's strain of human foreskin fibro-

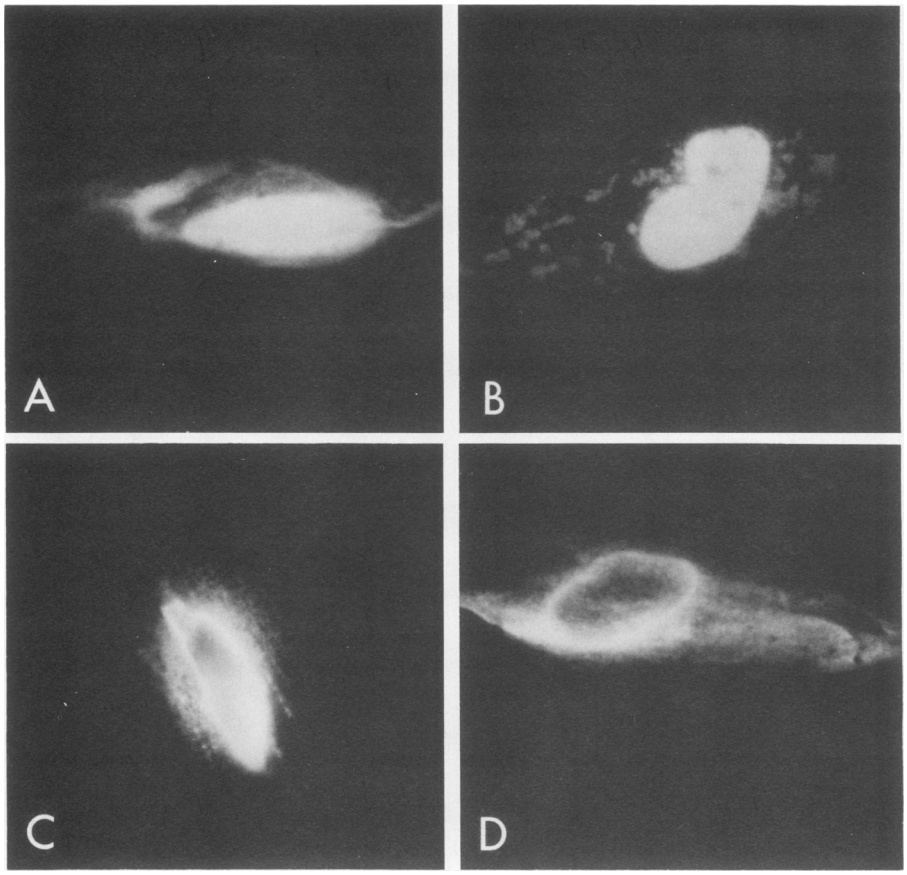

FIG. 2. *Cytoplasmic antigen in human placental cells transfected with EBV DNA. The cells in C and D have antigen concentrated at the nuclear membrane. The cell shown in C has a characteristic burst of antigen in the cytoplasm. The cell shown in B has cytoplasmic antigen clumps.*

blasts ("Taylor") also displayed antigens after transfection with EBV DNA (data not shown). In the FF260 cells, antigens similar to those shown in Fig. 1 to 3 were found after transfection with DNA prepared from three EBV strains; there did not appear to be significant variation either in the morphological appearance of the antigens or in the infectivity of the DNA from the three different strains (Table 2).

Antigens generally similar to those found in the placental cells were also found after transfection of three continuous lines of monkey kidney cells, the Vero and BSC-1 lines of African green monkey kidney cells, and the 637/10 line of owl monkey kidney cells (3) (Table 2).

Reactivity of different human sera with the antigen induced by transfection. In our early attempts to identify viral antigens in monolayer tissue cultures transfected with EBV DNA, we used sera with a broad range of reactivity to the full spectrum of EBV antigens detectable by immunofluorescence in lymphoid cells. By screening, we found reactive sera from patients with American Burkitt lymphoma, chronic lymphocytic leukemia, and infectious mononucleosis. We tested one serum pair taken before and after the onset of infectious mononucleosis. The pre-illness serum lacked antibody against the nuclear antigen in transfected cells, and the convalescent serum contained antibody to the antigen. Positive sera always failed to react with monolayer cultures which had received only carrier DNA. These results indicated that the antigens were specific for EBV.

At this juncture, we wished to learn whether the antigens found in the nuclei and cytoplasms of transfected cells corresponded to any of the known EBV antigens characterized in EBV-infected lymphocytes. We tested a panel of 18 EBV-positive sera whose reactivity to viral capsid, early, and nuclear antigens were known (Table 3). In the initial series of tests, the antigen consisted of human placental cells transfected with EBV (B95-8) DNA and then fixed in a

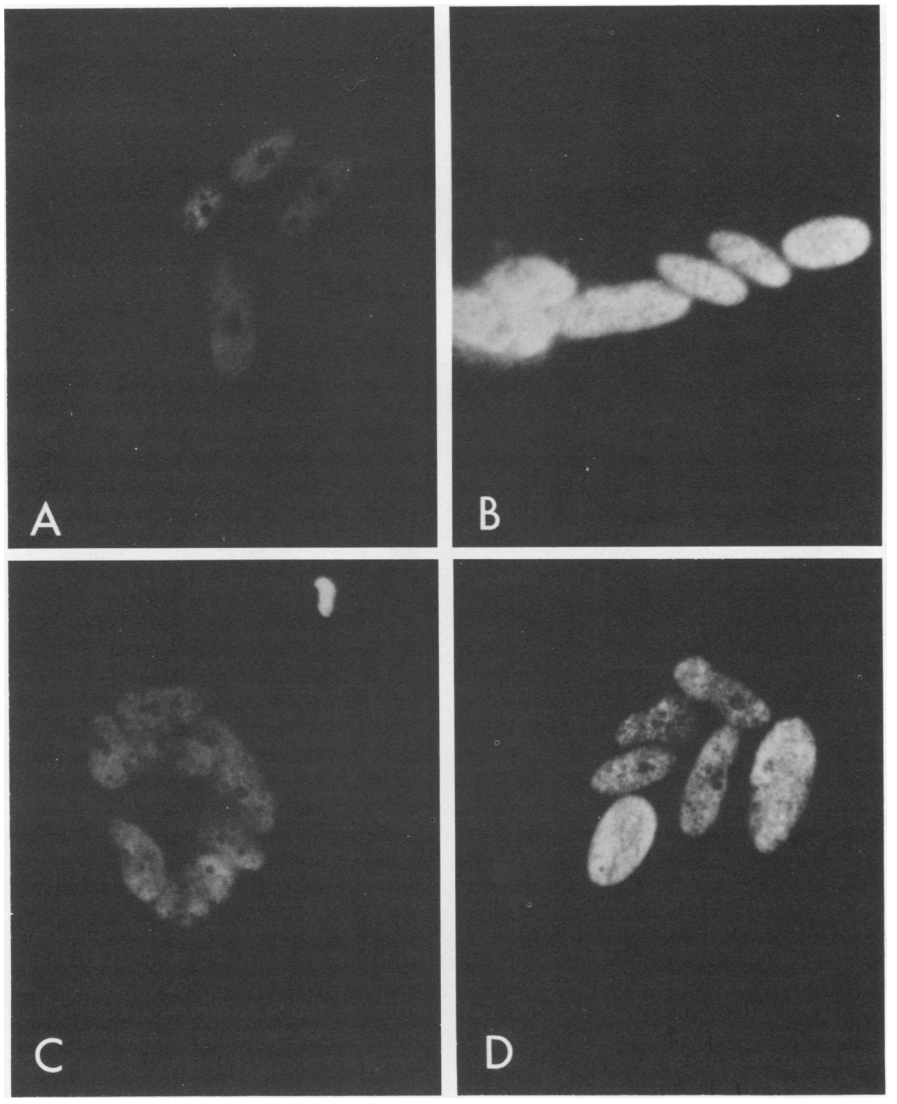

FIG. 3. *Antigen expression in human placental cells which have been transfected with EBV DNA and then fused with Sendai virus. The polykaryon in A has faint immunofluorescence of the cytoplasm. In A, C, and D it is evident that the antigen is not present in the nucleolus.*

mixture of acetone and methanol. Five sera with high titers of antibody to viral capsid antigen, but lacking in antibody to early antigen, produced no reaction; this group included sera which were anti-EBV nuclear antigen positive (346, 350, and 353) and negative (344 and 354). Negative reactions were also seen with two sera (342 and 343) with low titers of antibody to the D component of early antigen (1:10 to 1:40). However, a positive reaction was obtained with four sera (345, 361, 362, and standard) with anti-D titers of $\geq$1:80. These four sera were obtained from patients with lymphoma, nasopharyngeal carcinoma, infectious mononucleosis and "chronic mononucleosis." When the antigen consisted of transfected placental cells fixed only in acetone, the four sera with high anti-D titers were positive, as were four sera (25, 43, 48, and 58) with high antibody to R, from African patients with Burkitt lymphoma. Three sera with moderate (1:10 to 1:40) anti-R levels (341, 356, and 357) failed to detect the antigen. The R antigen appeared to consist mainly of clumped cytoplasmic granules. These results suggested that placental cells transfected with EBV DNA produced mainly EBV early antigens, both the

D and R components (11).

Effect of restriction endonuclease digestion of EBV DNA. To learn whether the antigens could be induced by EBV DNA fragments, aliquots of 500 ng of EBV strain HR-1 and FF41 DNA were treated individually with four different restriction endonucleases, and the digested DNA was tested for residual ability to induce the antigens (Table 4). A 50-ng sample of the digested DNA was electrophoresed through a 0.5% agarose gel and analyzed by Southern blot hybridization to ensure that the digestions had been complete. This gel revealed no DNA of the size of complete EBV DNA or fragments which represented partial digestion products (data not

TABLE 1. *Titration of EBV (HR-1) DNA by antigen induction on human placental cells with and without cell fusion*

Amt of DNA (ng)	With Sendai virus		Without Sendai virus	
	No. of foci[a]	Avg	No. of foci[a]	Avg
500	67, 83, 30	60	90, 118	104
100	35, 46, 15	32	26, 28, 15	23
10	2, 3, 4	3	3, 4, 4	3.7
None[b]	0, 0	0	0, 0	0

[a] Per approximately 200,000 cells on a cover slip. Standard antiserum used at 1:40. Cells were tested for antigen 4 days after transfection.

[b] Carrier calf thymus DNA present.

TABLE 2. *Detection of antigen induced by different strains of EBV DNA in various monolayer cell cultures*

Cells	DNA		No. of foci per cover slip[a]	Avg
	Strain	Amt (ng)		
Human placental	B95	500	43, 19, 29, 21	28
Human placental	FF41	1 μg	65, 25, 53	48
Human placental	HR-1	100	6, 20	13
Vero	HR-1	500	50, 32	41
BSC-1	HR-1	500	13, 9	11
Owl monkey kidney	HR-1	500	25, 29	27

[a] Standard serum 1:40 used as source of antibody in all experiments.

TABLE 3. *Correlation between antibody to EBV early antigen and antibody to the antigen induced by transfection*

Serum no.	Titer				No. of foci per cover slip
	Anti-VCA[a]	Anti-D[a]	Anti-R[a]	Anti-EBNA[b]	
341[c]	1,280	<10	10	<2	0
342	1,280	10		40	0
343[d]	1,280	40	?	10	0
344	1,280	<10	<10	<2	0
345	1,280	80	?	<2	14
346	1,280	<10	<10	80	0
350	640	<10	<10	≥80	0
353	1,280	<10	<10	≥160	0
354	1,280	<10	<10	<2	0
356[d]	1,280	<10	40	10	0
357[d]	1,280	<10	40	<2	0
361	5,120	1,280	?	≥160	27
362	2,560	320	?	10	42
Standard	2,560	640	?	640	76
25[e]	1,280	80	1,280	320	18
43	1,280	<10	≥320	80	19
48	5,120	<10	320	80	15
58	1,280	<10	320	40	6
Standard	2,560	640	?	640	19

[a] IgG antibody. The D component is located throughout the cell and is stable to methanol. The R component is mainly cytoplasmic and is destroyed by methanol (11). VCA, Viral capsid antigen.

[b] EBNA, EBV nuclear antigen.

[c] Sera 341 to 362 were tested on human placental cell cover slips transfected with 500 ng of EBV (B95-8) DNA and fixed with acetone-methanol, 2:1.

[d] These sera were also negative on cover slips fixed in acetone alone.

[e] Sera 25 to 58 were from African patients with Burkitt lymphoma and were tested on human placental cell cover slips transfected with 200 ng of EBV (B95-8) DNA and fixed in acetone.

TABLE 4. *Effect of restriction endonuclease digestion on the ability of EBV DNA to induce antigen in human placental cells*

Enzyme	EBV (HR-1) DNA[a]			EBV (FF41) DNA[a]		
	No. of foci per cover slip	Avg	Cytoplasmic antigen	No. of foci per cover slip	Avg	Cytoplasmic antigen
*Eco*RI	8, 8, 4, 8	7	−	7, 6, 0, 1, 15, 4	5.5	+
*Hin*dIII	4, 5, 4	4.3	−	10, 13, 19, 18, 16, 2	13.0	−
*Sal*I	8, 30, 22, 18	19.5	+	8, 5, 11, 15, 7	9.2	+
*Bam*HI	0, 5, 4, 3	3.0	−	7, 6, 0, 6, 9, 6	5.6	−
None	46, 40, 12, 19	29.3	+	10, 4, 24, 47, 8	18.6	+

[a] A 500-ng amount of DNA per dish.

shown). Similar results were obtained with both strains of viral DNA. Treatment with all of the restriction enzymes lowered the ability of the DNA to induce the antigens in comparison with undigested DNA, but no enzyme completely eliminated antigen induction. However, there were qualitative differences in antigen expression by cells transfected with EBV DNA digested with different enzymes. Antigen produced by the complete mixture of *Bam*HI fragments was always faint and limited to the nucleus. *Sal*I-digested DNA, in contrast, induced antigens with "bursts" and cytoplasmic granules similar to those caused by intact DNA.

Antigen induction by cloned EBV DNA fragments. The foregoing results suggested that individual fragments of EBV DNA might be able to induce the antigen. A series of bacterial clones containing EBV *Eco*RI fragments was prepared, and the identity of the chimeric plasmids was confirmed by hybridization of plasmid DNA to Southern blots of *Bam*HI fragments of virion DNA (Table 5). Both placental cells and Vero cells were transfected with plasmids containing different *Eco*RI fragments. We also tested *Bam*HI-W, the internally reiterated fragment. Together, the fragments tested represent a total molecular weight of approximately 73 × 10^6 or about 70% of the EBV genome. Antigen was regularly detected with the standard human antiserum in both Vero and human placental cells transfected with the *Eco*RI fragment B of EBV (FF41) DNA. Antigen was not observed when transfection was carried out with any of the other *Eco*RI fragments listed in Table 5 or with the reiterated *Bam*HI fragment W.

TABLE 5. *Expression of EBV early antigen in Vero cells transfected with chimeric plasmids containing different EBV EcoRI fragments*

Laboratory designation of clone	*Eco*RI fragment[a]	Hybridization to *Bam*HI fragments[a] of B95-8 DNA	No. of foci per cover slip[b]
pHH11-29	B	B, E, G, K, R, Z, d	11, 6, 21, 26
pHH11-14	C	A, I, V, X, c	0, 0, 0, 0
pHH11-13	E	D, G, b'[c]	0, 0
pHH11-24	F	L, M, S	0, 0
pHH11-18	G_1	O, P, U	0, 0
pHH11-22	H	T, b, b'[c]	0, 0
pHH11-11	I	ND[d]	0, 0
pHH11-30	J	C	0, 0

[a] For the location of these fragments on the physical map of EBV (FF41) DNA, see reference (4).

[b] After transfection with approximately 3 μg of purified chimeric plasmid DNA which had been digested with *Eco*RI.

[c] A previously unidentified fragment which is located in *Eco*RI-E and *Eco*RI-H of FF41 DNA.

[d] ND, Not done. The identity of the cloned fragment was made by co-migration with viral DNA.

DISCUSSION

EBV genes can be expressed in nonlymphoid cell types. In vivo EBV DNA and antigens have been found in epithelial cells in biopsies of nasopharyngeal cancer. EBV DNA has been shown by in situ hybridization in epithelial cells found in saliva and in ductal cells of the parotid gland (15, 22; H. Wolf, personal communication). However nonlymphoid cells are resistant to infection with virus in vitro presumably because cells containing virus receptors cannot be cultured. The single possible exception is certain explants of nasopharyngeal carcinoma biopsies which have been shown to synthesize early antigens in response to viral infection in vitro (6). Otherwise, some alternative method must be used to bypass the barrier to virus entry presented by the absence of a receptor. So far, several techniques have proved successful. Somatic cell hybrids formed between EBV genome-carrying lymphoid cells and epithelial cell lines will express viral antigens either upon induction or spontaneously (7). Similarly, antigens associated with the viral replicative cycle are found in continuous human amnion cells within several hours after they are fused with lymphoblastoid cells carrying the EBV genome (16). Microinjection of intact EBV DNA into human fibroblasts has led to the expression of viral antigens (8). Introduction of Raji cell membranes which contain EBV receptors into other cells has permitted cells which lack receptors to be infected with virus (21). Transfection with pure viral DNA has led to the synthesis of transforming activity and to viral antigen expression (17).

Because of the relative ease of the latter technique it should prove useful in experiments designed to define the biological function of different regions of the EBV genome. The present experiments represent a useful step in that direction. We have defined an antigen system which is regularly induced by transfection with intact EBV DNA and a mixture of DNA fragments produced by restriction endonuclease digestion. Furthermore, we locate in a general way the region encoding the antigen on the EBV map by transfecting with individual cloned EBV DNA fragments. We find that early antigen is induced by a 17.2-megadalton fragment located in the long, unique region of the DNA. This genomic region is one of several which King et al. found are newly transcribed when Raji cells are induced to express early antigen after treatment with iododeoxyuridine (13).

Evidence indicates that the antigens synthesized are mainly the D and R components of the early antigen complex. In a manner similar to that of the antigen we observed in transfected placental cells, the D antigen in lymphoid cells arises first in the nucleus and then spills out into the cytoplasm (11). This would correspond to the bursts which we observed. The R antigen in lymphocytes forms cytoplasmic aggregates, corresponding to the granules found in the fibroblastic cells. Although the antigen is initially in the nucleus, it does not correspond to the EBV nuclear antigen on several grounds. Thus, it can be detected by indirect anti-immunoglobulin immunofluorescence and does not require complement for detection. Until now, the demonstration of EBV nuclear antigen has required complement fixation (14). Furthermore, antigen expression is not limited to the nucleus, but extends into the cytoplasm (Fig. 2) and is associated with cytopathic effects. EBV nuclear antigen expression is limited to nuclei and is noncytopathic. Finally, sera with high anti-EBV nuclear antigen titers, but lacking in antibody to early antigen, do not seem to identify the antigen (Table 3). Thus far, our attempts to demonstrate EBV nuclear antigen in the transfected fibroblasts have been confounded by nonspecific immunofluorescence of cytoskeletal components. Similarly, the antigen is apparently not the rheumatoid arthritis-associated nuclear antigen which is destroyed by acetone fixation (1) or the transiently induced nuclear antigen which is induced in cells which already contain an EBV genome (5). Nonetheless, the identification of the antigen as early antigen must be tempered with the caveats that few monospecific antibodies are yet available in the EBV system, and that precise definition of the antigen will require that its polypeptide composition be known. The only monoclonal antibodies which are available recognize the membrane antigen on the surface of virions and on the cytoplasmic membrane of cells producing virus (12, 20). Thus far, we have not been able to show that they react with the intracellular antigens induced by transfection (unpublished data).

Enhanced expression by cell fusion. When Sendai virus fusion factor was added after transfection with viral DNA, we observed polykaryons with the nuclear antigen. Polykaryon formation per se was not responsible for antigen expression since no antigen was present in cells transfected with carrier DNA and then fused. Several hypotheses might account for the increased number of antigen-positive nuclei after cell fusion. Sendai virus did not seem to enhance the uptake of the $CaPO_4$ precipitate; if uptake were facilitated by fusion factor, there would have been a greater number of antigen-positive foci when the factor was present, whereas the data in Table 1 show that the number of foci was the same with and without Sendai virus. Another possibility is that fusion somehow alters the competence of the cells to express viral DNA. If fusion per se affected competence, more foci would be seen with fusion factor than without it. However, fusion may permit competence which is being expressed in one cell to be transferred to another. By this model, all of the cells would contain viral DNA, but only a few would be competent to express it. Thus, if a cell which was competent to express DNA was introduced into a polykaryon, it would influence the competence of other cells in the polykaryon. The most likely explanation is that cell fusion promotes antigen expression by allowing antigen which is produced in the cytoplasm of the polykaryon to be transferred back to all of the nuclei in the polykaryon. The nuclear location of the antigen might indicate that the antigen has some DNA-binding properties.

Nature of EBV infection in the transfected cells. If cover slips are transfected with EBV DNA in the absence of added fusion factor and examined for antigens by immunofluorescence, scattered individual antigen-positive cells are seen, but no foci consisting of multiple antigen-positive cells are found. This finding allows two interpretations. If complete virions are replicated in the cells expressing antigen, then virus is unable to pass to adjacent cells which lack receptors for EBV. Alternatively, cells which are transfected with EBV DNA may undergo principally an abortive infection with synthesis only of early viral antigens and not infectious virus.

We obtained evidence which supports both alternatives. The placental cells do not seem to have virus receptors, since they cannot be infected with concentrated intact EBV (unpublished data). When the placental cells are transfected with EBV DNA and co-cultivated with lymphocytes, the lymphocytes transform. In some but not all experiments, transformation can also be achieved by adding supernatant fluids from transfected placental cells to lymphocytes, and in one (unpublished) experiment, the transforming activity was filterable through a 0.45 μm filter. Furthermore, the transformed lymphocytes are B cells, which mirror the tropism of intact virus. Recently, we neutralized the transforming activity (unpublished data). These findings support the idea that some complete virus is being made in the placental cells, but the data do not allow definitive conclusions on this point. We have not yet demonstrated morphologically recognizable virions in the fluids or cells of transfected monolayers. It is

also possible that no complete virus is made in placental cells and the transforming activity represents transfection of lymphocytes by DNA which has somehow been altered by its contact with the placental cells, perhaps by proteination. DNA either naked or altered may be transferred from the placental cells to lymphocytes by cell fusion.

The data we present in this report indicate that most of the viral replicative cycle in the placental cells is abortive and accompanied only by synthesis of early antigens. If a significant number of cells produced virions, we would have expected to see expression of viral capsid antigen, but this was not the case. It is possible that only a few cells produced the viral capsid antigen, or that its level was too low to be detected by our method.

The DNA of both transforming and nontransforming strains synthesize early antigen upon introduction into placental cells, and neither appear to cause changes in cell growth which we associate with morphological transformation. This was a surprising finding especially for the strains B95-8 and FF41, which are potent in their ability to immortalize lymphocytes. It suggests that the direction of genome expression either toward cell-transforming functions or toward viral replicative and cell lytic functions is modified by the host cell.

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