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## Expression of Epstein-Barr virus (EBV) DNA and cloned DNA fragments in human lymphocytes following Sendai virus envelope-mediated gene transfer\*

(Epstein-Barr virus/lymphocyte transformation/gene transfer)

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**ABSTRACT** Purified EBV DNA and cloned DNA fragments were trapped in Sendai virus (SV) envelopes during envelope reconstitution. The DNA-loaded reconstituted envelopes (RSVE/DNA) served as gene-transfer vehicles using the capability of RSVE to fuse with normal and tumor cells. The efficiency of RSVE-mediated EBV DNA transfer into lymphoid tumor cells and fresh human lymphocytes was 5–10% of the enveloped <sup>3</sup>H-labeled *Eco*RI fragment B of EBV DNA. Purified intracellular EBV (B95-8 strain) DNA induced EBV nuclear antigen (EBNA) in 0.2–1% of human lymphocytes, transiently stimulated cellular DNA synthesis, but did not fully transform cells. Cloned *Sal* I F1 fragment [≈9 kilobase pairs (kbp)] and a smaller *Bam*HI K (5.2 kbp) fragment from the same region of B95-8 EBV DNA induced EBNA in 2–4% of human lymphocytes but did not stimulate DNA synthesis nor transform cells. Cloned *Bam*HI D1 fragment (≈9 kbp) from AG-876 virus DNA, or a combination of cloned *Bam*HI X and H fragments (≈2 and 7 kbp, respectively) from the similar region of B95-8 virus DNA, significantly stimulated lymphocyte DNA synthesis, but EBNA could not be detected and transformation was not achieved. Early antigen and viral capsid antigen were not observed with any of the fragments tested. Our results suggest that the induction of EBNA and stimulation of lymphocyte proliferation are not controlled by the same region of EBV DNA.

Epstein-Barr virus (EBV) is a lymphotropic transforming herpesvirus that causes infectious mononucleosis and is associated with Burkitt lymphoma, nasopharyngeal carcinoma, and several acute lymphoproliferative diseases (reviewed in refs. 1–3). Classical genetic mapping of viral functions is hampered by the host range restriction to B lymphocytes and restricted permissivity of B cells for virus replication (1, 3). Attempts to extend the host range of the virus by DNA transfection, microinjection, or virus-receptor transplantation resulted in virus replication (refs. 4–6; reviewed in ref. 7). Many viral proteins have been mapped to fragments of EBV DNA by using cloned fragments of EBV DNA and hybrid selection, *in vitro* translation, and immunoprecipitation (8, 9). Some viral proteins, including EBV nuclear antigen (EBNA), have been mapped by using cloned EBV DNA fragments in prokaryotic or eukaryotic expression systems (10, 11).

Although a great deal is known about viral gene expression in latently infected growth-transformed cells, the cell-transforming functions of EBV have not been distinguished from viral genes whose function may be solely related to maintenance of viral latency. Some transfection and micro-

injection experiments employing recombinant EBV DNA have been performed with already transformed or permanently growing cells (4, 10–12). Introduction of EBV DNA into normal human T lymphocytes (7), human placental cells (6), or rodent splenocytes (5) resulted in virus replication rather than *in vitro* transformation. Possibly, the cell-transforming activity of EBV is limited to natural host cells of the virus—i.e., the mature human B lymphocytes and epithelial cells (1, 3, 5).

The purpose of these experiments is to begin to assay the function of parts of the EBV genome in normal B cells. Since DNA transfer into lymphocytes by calcium phosphate, DEAE-dextran, and protoplast fusion are inefficient processes, we have tried a different approach—i.e., use of DNA-loaded reconstituted Sendai virus (SV) envelopes as gene-transfer vehicles (13–15). SV envelopes (SVE) can be isolated, solubilized, and reconstituted (RSVE), retaining the ability to fuse with target cell membranes (16). EBV nucleocapsids can be enveloped by RSVE (14). The fusible RSVE/EBV vesicles have been used to transfer EBV genome into an extended host range (14). The internalized viral DNA was expressed in up to 1% of the target cells (14). These experiments suggested the possibility that EBV DNA present during reconstitution could be enveloped and transferred into living eukaryotic cells.

In the present work, we present data on the application of the RSVE system for transferring purified EBV DNA and cloned DNA fragments into fresh human lymphocytes. The biological activity of EBV DNA and DNA fragments in human B cells was monitored by following stimulation of cellular DNA synthesis, cell immortalization, and expression of EBV-determined antigens. Our results suggest that cell transformation by EBV may require collaboration between several viral and cellular genes.

### MATERIALS AND METHODS

**Cells.** Human cord blood lymphocytes or cells from EBV-seronegative donors were separated on Ficoll-Isopaque (Pharmacia). B cell-enriched fractions were obtained by separating human B and T cells on nylon wool columns. Tumor cells utilized in this study were Raji (EBV-positive BL; ref. 17), Ramos (EBV-negative American BL; ref. 18), and B95-8 (EBV-transformed marmoset lymphocytes; ref. 19). All cells

Abbreviations: EBV, Epstein-Barr virus; EBNA, EBV nuclear antigen; EA, early antigen; VCA, viral capsid antigen; SV, Sendai virus; SVE, SV envelopes; RSVE, reconstituted SVE; RSVE/DNA, DNA-loaded RSVE; ACIF, anti-complement immunofluorescence.

\*Preliminary results of this study have been reported during the University of California, Los Angeles, Symposium on Normal and Neoplastic Hematopoiesis, March 27–April 1, 1983, Steamboat Springs, CO (13).

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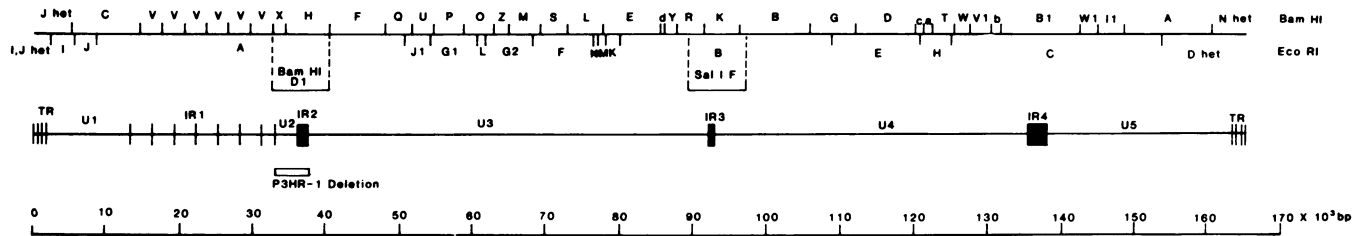


FIG. 1. Restriction endonuclease map of prototypic EBV isolate (22), indicating the position of the cloned EBV (AG-876) *Bam*HI D1 fragment and EBV (B95-8) *Sal*I F1 fragment below the linkage map of *Bam*HI and *Eco*RI fragments for a typical EBV isolate. The middle line indicates the location of direct terminal repeat sequences, TR, and discrete direct internal repeat sequences, IR1-4, separated by unique sequence regions, U1-5 (24). Also shown is the region of DNA deleted from the nontransforming EBV (P3HR-1) isolate (23, 25).

were maintained as stationary suspension cultures in RPMI 1640 (GIBCO) medium supplemented with 10% fetal bovine serum, penicillin (100  $\mu$ g/ml), and streptomycin (100  $\mu$ g/ml).

**Viruses.** SV was propagated and its hemagglutinating titer was determined as described (5, 16). One milligram of SV was approximately equivalent to 15,000 hemagglutination units. The transforming B95-8 virus was harvested from supernatants of starving B95-8 cells as described (5) and concentrated to 0.2% of initial volume. The virus was capable of inducing EBNA in 10% of Ramos cells 2 days after infection at a 1:200 dilution and was used at a 1:100 dilution.

**EBV DNA and Cloned DNA Fragments.** The procedure for isolation of high molecular weight intracellular EBV DNA by repetitive CsCl equilibrium centrifugation has been described (20). The cloned *Bam*HI X, H, and K fragments and *Sal*I F fragment from B95-8 EBV DNA and the cloned *Eco*RI fragments from W91 EBV DNA were isolated, propagated, and characterized as described (21, 22). The cloned *Bam*HI D1 fragment from AG-876 EBV DNA occupies the same map location as the B95-8 EBV *Bam*HI X and H DNA fragments (refs. 17 and 23; see also the restriction endonuclease map in Fig. 1).

**Preparation of DNA-Loaded SV Vesicles (RSVE/DNA).** The four principal steps of the procedure and its efficiency are summarized in Table 1. SV particles (5 mg of viral protein) in 100 mM NaCl/50 mM Tris-HCl, pH 7.4, were extracted by Triton X-100 for 1 hr at room temperature. The detergent/SV protein ratio was 2:1 (wt/wt). After centrifugation at 100,000  $\times$  g for 1 hr, the supernatant containing  $\approx$ 1 mg of SVE protein in the detergent was collected. Triton-solubilized SVE (1 mg) and EBV DNA (50  $\mu$ g) or cloned EBV DNA fragments (50  $\mu$ g) were mixed (step 1 in Table 1) and dialyzed in Spectrapor 2 dialysis bags at 4°C for 48 hr against 2 mM CaCl<sub>2</sub>/2 mM MgCl<sub>2</sub>/10 mM Tris-HCl, pH 7.4/2 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>/0.1 mM phenylmethylsulfonyl fluoride (steps 1 and 2 in Table 1). The RSVE/+DNA step 2 vesicles were then digested with a DNase (Sigma; 50  $\mu$ g/ml) for 1 hr

Table 1. Procedure of EBV DNA entrapment in RSVE and its efficiency

Step	Radioactivity, cpm $\times 10^{-6}$	Amount of DNA, $\mu$ g	% of starting material radioactivity
(1) SVE + <i>Eco</i> RI [ <sup>3</sup> H]B	1.1	50	100
(2) RSVE/+ <i>Eco</i> RI [ <sup>3</sup> H]B	0.7	32	64
(3) RSVE/ <i>Eco</i> RI [ <sup>3</sup> H]B	0.055	2.5	5.0

Triton X-100-extracted SVE are mixed with EBV DNA and a probe (*Eco*RI [<sup>3</sup>H]B) (step 1). Removal of the detergent by dialysis results in envelope reconstitution (RSVE). Some of the DNA probe is trapped inside the vesicles, but most is adsorbed on the surface (RSVE/+ *Eco*RI [<sup>3</sup>H]B) (step 2). DNase treatment of the RSVE/+ *Eco*RI [<sup>3</sup>H]B vesicles and ultracentrifugation remove the adsorbed DNA (RSVE/*Eco*RI [<sup>3</sup>H]B) (step 3). For further details, see *Materials and Methods*.

at 37°C to remove any DNA not entrapped inside the vesicles. The vesicles then were sedimented at 100,000  $\times$  g for 1 hr at 4°C to remove nonreconstituted elements and DNase and were resuspended in 0.25 ml (step 3, final preparation of RSVE/DNA vesicles, in Table 1).

**RSVE-Mediated DNA Transfer.** RSVE/EBV DNA vesicles were absorbed at 4°C to target cells suspended in a fusion buffer (160 mM NaCl/20 mM Tricine-NaOH, pH 7.5). After 10 min at 4°C, 5 mM CaCl<sub>2</sub> was added, followed by a 20- to 30-min incubation at 37°C. The cells were then washed twice in RPMI 1640 medium, resuspended at 2  $\times$  10<sup>6</sup> cells per ml, and cultivated under standard conditions.

**Cellular DNA Synthesis-Stimulation Assay.** Cellular DNA synthesis was followed by measuring incorporation of [<sup>3</sup>H]thymidine (2.0 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq). Triplicate cultures of cells (2  $\times$  10<sup>5</sup> cells per 0.1 ml per well) were kept in Falcon 96-well microtiter plates. At different times (7, 14, and 21 days) after EBV DNA transfer or EBV infection, 0.8  $\mu$ Ci of [<sup>3</sup>H]thymidine was added to each well and incubated at 37°C for 16-20 hr. The cells were collected on glass fiber filters by a Titertek cell harvester and counted by liquid scintillation using Insta-Gel (Packard).

**Immunofluorescence Staining.** EBNA was determined by anti-complement immunofluorescence (ACIF) assay (26) using smears of 2:1 (vol/vol) acetone/methanol-fixed cells 2 or 7 days after the DNA transfer or EBV infection. EBV-positive and EBV-negative sera were used at 1:10 dilution. Early antigen (EA) and viral capsid antigen (VCA) were determined by indirect immunofluorescence (27) using mouse monoclonal anti-EA (D and R) and anti-VCA antibodies (Biotech, Rockville, MD) and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Cappel Laboratories, Cochranville, PA) as a second antibody.

## RESULTS

**Trapping of EBV DNA in RSVE and Transfer into Lymphocytes.** The efficiency of EBV DNA entrapment in RSVE was determined by using <sup>3</sup>H-labeled nick-translated *Eco*RI fragment B probe (*Eco*RI [<sup>3</sup>H]B) (Table 1). Although more than 50% of the DNA was associated with the reconstituted vesicles (step 2), only 5% of it was trapped inside the vesicles, as judged by the resistance to DNase digestion (step 3). Titration experiments with various amounts of DNA added to the co-reconstitution mixture revealed that maximal entrapment could be achieved with 40-50  $\mu$ g of EBV DNA per 1 ml of solubilized SVE proteins (unpublished data). Up to 2.5  $\mu$ g of DNA could be loaded into the reconstituted vesicles (2 mg of envelope protein) under these conditions.

Previously, we have demonstrated that RSVE retained the cell-binding and -fusing activities of intact virions (5, 14, 16). As expected, loading with DNA did not impair the cell-recognition properties of the RSVE (Fig. 2). A typical titration experiment of RSVE/[<sup>3</sup>H]DNA vesicles against target cells, in this case Raji, is shown in Fig. 2. The optimal amount of RSVE that could be added without causing a significant cell

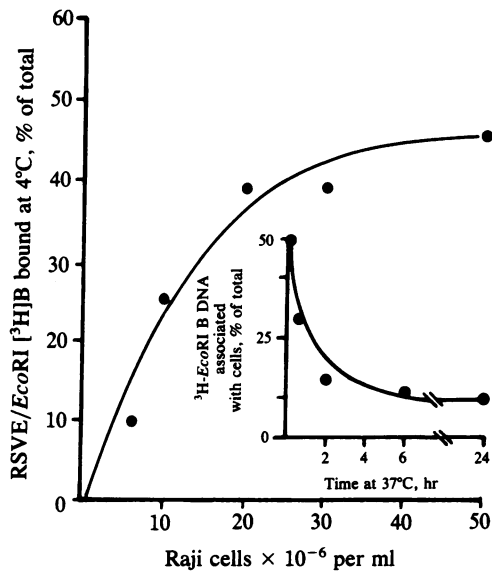


FIG. 2. Binding of RSVE/EcoRI [<sup>3</sup>H]B vesicles to Raji cells at 4°C and 37°C (*Inset*). RSVE/EcoRI [<sup>3</sup>H]B vesicles (25  $\mu$ l; 5500 cpm) were adsorbed to Raji cells for 10 min at 4°C. The cells were then washed and analyzed for residual radioactivity. (*Inset*) RSVE/EcoRI [<sup>3</sup>H]B vesicles (125  $\mu$ l; 27,000 cpm) were adsorbed to  $150 \times 10^6$  Raji cells and incubated as described above. After taking the 4°C sample, the cells were supplemented with CaCl<sub>2</sub> (5 mM), transferred to 37°C for 30 min, washed twice in RPMI containing 10% fetal calf serum, and cultured under standard conditions at  $0.5 \times 10^6$  cells per ml. Samples were taken at the times indicated in the figure and analyzed for the residual radioactivity. The 24-hr sample was first treated with 0.125% trypsin (10 min at 20°C) to remove any vesicles that could still be adsorbed on the cell surface.

lysis was 2–5  $\mu$ g of envelope protein per  $1 \times 10^6$  cells.

RSVE-mediated DNA transfer was performed by interacting RSVE/DNA vesicles with target cells at 37°C. The main steps were: (i) adsorption of the vesicles to cells at 4°C, (ii) vesicle–cell fusion at 37°C (20–30 min), and (iii) cell culture and analysis. Fig. 2 *Inset* shows a quantitative analysis of the RSVE-mediated gene transfer into Raji cells, with RSVE containing EcoRI [<sup>3</sup>H]B probes. About 50% of the added DNA remained associated with cells at 4°C. A subsequent incubation at 37°C for 30 min resulted in decrease of the label, likely because of detachment of unfused DNA-loaded

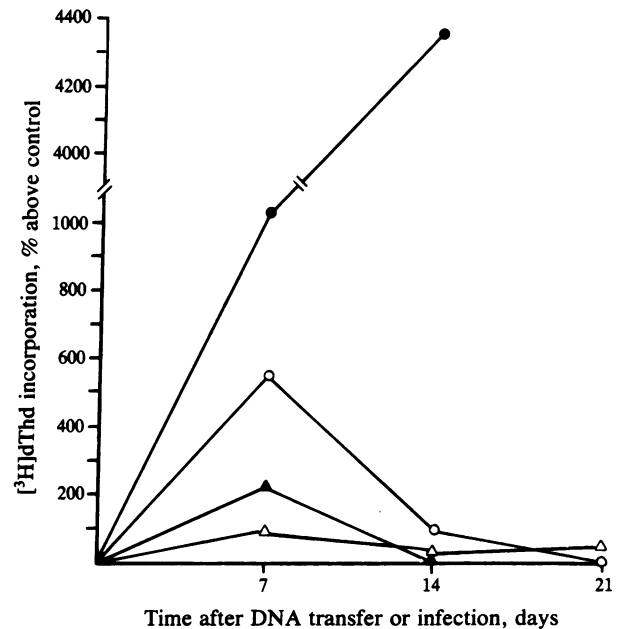


FIG. 3. Kinetics of cellular DNA synthesis in fresh human lymphocytes after the RSVE-mediated EBV DNA and DNA fragment transfer or after infection with intact virus. Fresh lymphocytes from the EBV-seronegative donor were isolated, resuspended in fusion buffer, and fused with RSVE/DNA as described. After fusion, the cells were washed in RPMI containing 10% fetal calf serum and cultured under standard conditions. Cellular DNA synthesis was assayed at indicated time intervals as described. Stimulation is expressed as percentage of mean cpm (incorporated [<sup>3</sup>H]thymidine) for each system above the cpm of the control—i.e., cells treated with empty RSVE. ●, Intact B95-8 EBV added to untreated cells; ○, cells exposed to RSVE/DNA; ▲, cells exposed to RSVE/BamHI D1 fragment; △, cells exposed to RSVE/Sal I F1 fragment.

vesicles by viral neuraminidase (16). To reveal the actual amount of EBV DNA transferred into the cell interior, cells were cultured for 24 hr after the fusion, washed, treated with trypsin (0.125%) to remove any nonfused vesicles, and analyzed for residual radioactivity. About 10% of the added probe remained associated with the Raji cells, indicating that the amount of internalized EBV DNA was about 100 molecules per cell (24-hr point in Fig. 2 *Inset*). The pattern of RSVE/EcoRI [<sup>3</sup>H]B vesicle interaction with human B lym-

Table 2. Induction of cell DNA synthesis and EBNA in human lymphocytes after RSVE-mediated DNA transfer

System	Stimulation of cellular DNA synthesis, SI weeks after infection			% EBNA-positive cells	Cell transformation
	1	2	3		
RSVE	1	1	1	0	—
RSVE/EBV DNA	3.1; 1.6; 6.4	2.7; 5.4; 2	1.5; 1	1; 0.2	—
RSVE/Sal I F1	0.6; 1.3; 0.5	0.97; 0.74	0.3; 2	4; 2; 0.2	—
RSVE/BamHI K	1	0.9; 0.2	0.5	0.5	—
RSVE/BamHI D1	3.1; 2.1; 1.5	1.5; 1.7; 2.2	0.9	0; 0	—
RSVE/BamHI H-X	2.3; 1.8	1		0; 0	—
RSVE/BamHI D1/Sal I F1	1.1	1.3		NT	—
RSVE/pBR322	1.1	0.9		0	—
EBV	2; 8	7.7; 38	30; 40	2; 5	+

Separation of human lymphocytes, reconstitution of EBV DNA or cloned restriction endonuclease fragments with SVE, and gene transfer were as described. After the transfer, the cells were resuspended in RPMI 1640 with 10% fetal calf serum and cultured under standard conditions at  $2 \times 10^6$  cells per ml. EBNA was determined by ACIF assay (26) 2 days after infection. EA and VCA were determined by direct immunofluorescence. Cellular DNA synthesis was determined by measuring incorporation of [<sup>3</sup>H]thymidine at the indicated time after infection. The results are expressed as a relative stimulation (stimulation index, SI)—i.e., increase over the [<sup>3</sup>H]thymidine incorporation in controls (cells treated with empty RSVE). Repetitive experiments are listed from left to right in each column. [<sup>3</sup>H]Thymidine incorporation in controls (empty RSVE) for the three experiments listed in column 1 (1 week) were (in cpm):  $2301 \pm 213$ ,  $707 \pm 102$ , and  $230 \pm 121$ , respectively. NT, not tested.

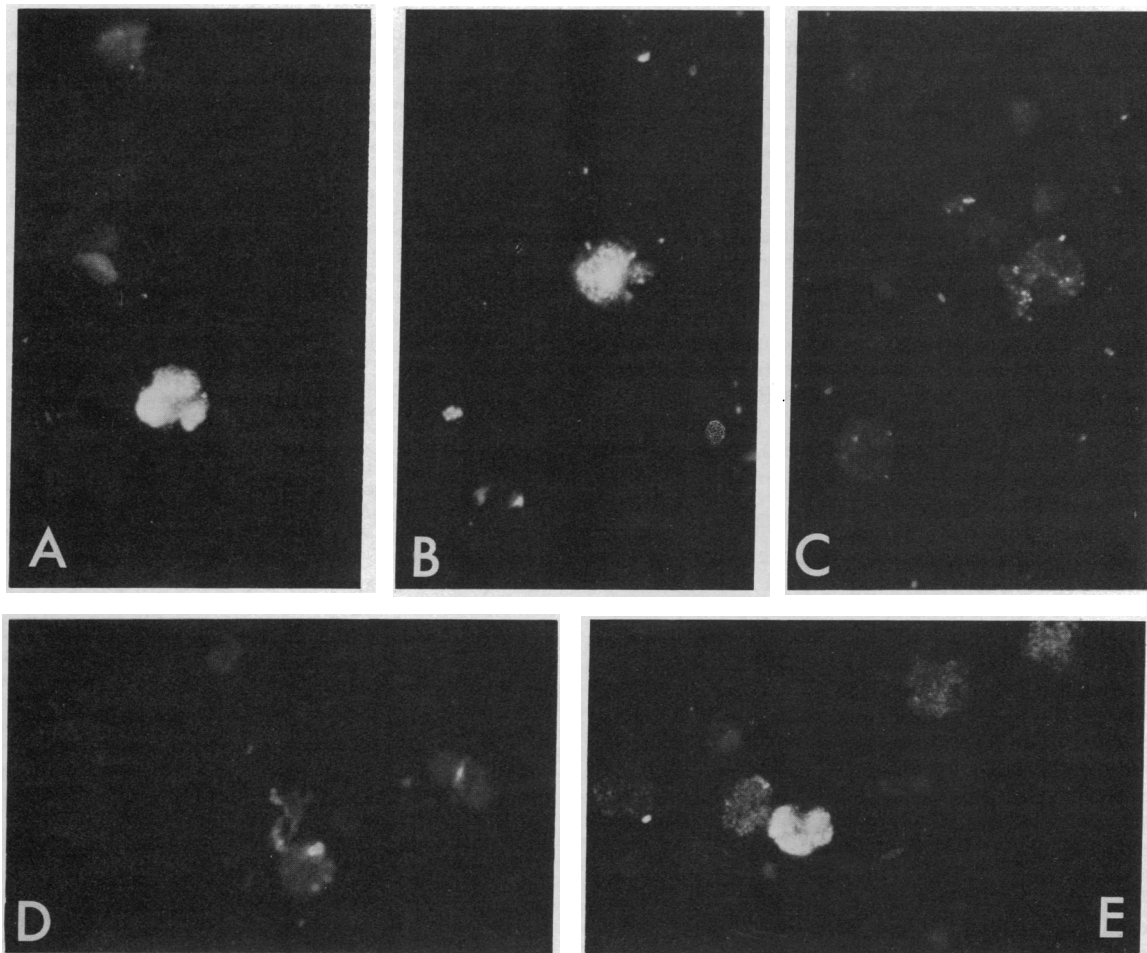
phocytes was similar to that of Raji cells (not shown). The amount of internalized DNA was usually lower, 4–5%.

**Expression of the RSVE-Transferred EBV DNA and Cloned DNA Fragments in Human B Lymphocytes.** Transformation of B cells by EBV involves stimulation of cellular DNA synthesis, induction of EBNA, cell division, and, finally, self-sustained outgrowth of EBV-infected B-cell lines (1, 3). We followed all these parameters in B cells containing RSVE-transferred EBV DNA and fragments and compared them with the cells infected with intact virus. The results are shown in Table 2 and Figs. 3 and 4. EBV DNA induced a significant increase in cellular DNA synthesis—1.6–6.4 times the control (RSVE alone) levels. The extent of the stimulation was lower than after exposure to intact EBV. As shown in Fig. 3, stimulation was transient, decreasing to control levels after 3 weeks in culture. RSVE-transferred EBV DNA also induced ACIF-detectable EBNA in 0.2–1% of the cells 48–72 hr after infection (Table 2). The proportion of EBNA-positive cells decreased, and no cell transformation was observed.

Cloned regions of EBV DNA tested for the virus-related functions included *Bam*HI restriction endonuclease fragments D1, H, X, and K and *Sal* I F1 fragment. It has been reported recently that the *Sal* I F1 fragment [9 kilobase pairs (kbp)] or part of it obtained after digestion with *Bam*HI endonuclease (*Bam*HI K fragment, 5.2 kbp) contains gene(s) coding for an EBNA-like antigen in mouse LTK<sup>-</sup> cells (10).

Since EBNA has been postulated as a transforming antigen of EBV (28, 29), we tested whether the EBV DNA region containing EBNA-encoding gene(s) might act as an autonomous transforming unit. Neither *Sal* I F fragment nor *Bam*HI K fragment stimulated any significant cellular DNA synthesis or caused cell transformation (Table 2 and Fig. 3). The fragments induced EBNA in 2–4% of human lymphocytes. Fig. 4 A and B shows EBNA-expressing human B cells detected by ACIF test 48 hr after *Sal* I F fragment transfer, in comparison to the EBNA induction by intact virus (Fig. 4E).

We next tested fragments from the EBV DNA region that is deleted in the nontransforming (but EBNA-inducing) P3HR-1 virus (23, 25, 30). This region is encompassed within the *Bam*HI D1 fragment of the AG-876 EBV DNA or the combined *Bam*HI H–X fragments of B95-8 virus DNA (refs. 20–22; see also Fig. 1). The *Bam*HI D1 or H–X fragments transiently stimulated lymphocyte DNA synthesis. The stimulation was lower than after exposure to intact virion. In contrast to EBV DNA, or *Sal* I F and *Bam*HI K fragments, *Bam*HI D1 or H–X did not induce any ACIF-detectable EBNA in human B cells. When both the *Sal* I F1 and *Bam*HI D1 fragments were introduced into the cells, the stimulation of DNA synthesis by *Bam*HI D1 alone was reduced, and no cell transformation was achieved (Table 2). Plasmid DNA or control empty RSVE did not have any effect on human lymphocyte DNA synthesis or EBNA expression.



**FIG. 4.** Expression of EBNA in lymphocytes after RSVE-mediated EBV DNA transfer: detection by immunofluorescence staining. Two days after RSVE-mediated EBV DNA transfer, lymphocytes were fixed in acetone/methanol on glass slides and assayed by the ACIF technique as described (26). (A and B) Cells transferred with *Sal* I F1 fragment and stained with anti-EBNA-positive human serum (M.H.; anti-EBNA titer 1:160, 1:10 dilution) as the first antibody. (C) Cells from the same system but stained with EBV-negative serum (C.K.; 1:10 dilution) as the first antibody. (D) Cells transferred with *Bam*HI D1 fragment and stained with the anti-EBNA-positive M.H. serum. (E) Untreated cells exposed to intact EBV and stained with the M.H. serum. ( $\times 500$ .)

Neither intact virus nor RSVE-transferred DNA or DNA fragments induced EBV-determined antigens associated with the lytic cycle of the virus, EA, and VCA (Table 2).

### DISCUSSION

The present work describes an efficient way of transferring genetic information into fresh human lymphocytes. The procedure consists of two steps: (i) loading isolated SVE with DNA and (ii) transferring DNA by following a spontaneous fusion of hybrid RSVE/DNA vesicles with the target cells. The natural process of envelope-cell fusion contributes to the high transfer efficiency and limits cell damage. Since the technique differs in principle from the other methods of gene transfer, such as the DNA transfection or microinjection by microcapillaries, we refer to it by the term RSVE-mediated gene (DNA) transfer (13).

The RSVE-mediated gene transfer has been used in the present work for studies on the expression of purified EBV genome and cloned EBV DNA fragments in the natural target cells of the virus, the human B lymphocytes. We have been interested in particular in evaluating the effect of DNA fragments on cell proliferation and transformation and the relationship of this phenomena to the induction of EBNA. The following results are indicated. (i) *Sal* I F1 fragment and *Bam*HI K fragment of EBV DNA are capable of inducing EBNA reactivity in normal human lymphocytes, confirming the results previously obtained in tumor cells (10, 11). (ii) The EBNA-inducing *Sal* I F1 and *Bam*HI K fragments neither stimulated lymphocyte DNA synthesis nor transformed the cells (Table 2), indicating that induction of EBNA reactivity by itself is not sufficient to achieve cell proliferation and transformation. This finding is in contrast to previous postulates suggesting that EBNA plays a direct role in stimulating and maintaining cell proliferation (28, 29). The discrepancy may be due to different experimental approaches. In the experiments described by Klein *et al.* (28), purified EBNA subcomponent (48 kDa) was microinjected into permanently growing mouse fibroblasts. Possibly, the fragment further activated the existing growth-stimulatory mechanism of the transformed cells. In our experiments, EBNA-encoding DNA fragments were introduced into resting normal lymphocytes. The observed lack of lymphocyte stimulation, combined with the efficient expression of EBNA, makes it unlikely that *Sal* I F1 or *Bam*HI K fragments contain an autonomous lymphocyte-transforming gene. (iii) Transient cellular DNA synthesis-stimulatory activity was detected after transfer of intact EBV DNA, the *Bam*HI D1 region of AG-876 virus DNA, and the combination of *Bam*HI H and X fragments of B95-8 DNA. It recently has been reported by Glaser and colleagues that transfer of cloned *Bam*HI X, H, and F fragments (from B95-8 virus DNA) into a virus-non-producer P3HR-1/epithelial hybrid tumor cell line resulted in rescue of transforming virus (31), possibly as a result of recombination between the fragments and the endogenous P3HR-1 virus genome (32). As shown in our experiments, these fragments alone are not sufficient for inducing B-cell transformation. A mixture of *Bam*HI D1 and *Sal* I F1 fragments was not capable of transforming the cells either, suggesting that still another region of the EBV DNA is necessary for stabilizing the DNA synthesis-stimulating and EBNA-inducing activities of *Bam*HI D1 (or *Bam*HI H-X) and *Sal* I F1 (*Bam*HI K) fragments, respectively. This could be, for example, the *Eco*RI joining (*J*) region coding for the most abundant mRNA detected during lymphocyte transformation by an intact virus (33).

Our results suggest that transformation of human lymphocytes by EBV may require functional collaboration between several viral and perhaps cellular genes. The approach described in this work may permit identification of these genes.

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