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Epstein–Barr virus and human chromosomes: Close association of the resident viral genome and the expression of the virus-determined nuclear antigen (EBNA) with the presence of chromosome 14 in human–mouse hybrid cells

(anticomplement immunofluorescence/nucleic acid hybridization/quinacrine mustard–33258 Hoechst staining/transformation/oncogenesis)

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ABSTRACT **Fourteen hybrid clones derived from the fused cultures of human lymphoblastoid FV5 cells and 5-bromodeoxyuridine-resistant mouse fibroblastic MCB2 cells grown in hypoxanthine/aminopterin/thymidine selective medium were examined for the presence of Epstein–Barr virus (EBV) DNA, the expression of the virus-determined nuclear antigen (EBNA), and the presence of human chromosomes, in the course of serial passage *in vitro*. Among the hybrid clones tested, 3 were positive for EBV DNA and EBNA, whereas the remaining 11 were totally negative. The chromosome investigations showed that human chromosome 14 was consistently involved in all three EBV genome-positive and EBNA-positive hybrid clones, but not in any negative clones. In 10 subclones isolated from 1 of the 3 positive clones, all of which contained only chromosome 14 of the human chromosomes, a concordant segregation of EBNA, EBV DNA, and chromosome 14 was evident. These findings suggest that the resident EBV genome is closely associated with chromosome 14 and the presence of this particular chromosome alone is sufficient for the maintenance and the expression of EBV genetic information in human lymphoblastoid cells.**

Epstein–Barr virus (EBV) (1) characteristics, such as the viral DNA and the virus-determined nuclear antigen (EBNA) (2), are involved with high frequency in human lymphocytes transformed by EBV *in vitro*, lymphoblastoid cell lines with B cell markers, and malignant tumors such as Burkitt lymphoma and nasopharyngeal carcinoma suspected of being caused by the virus (3). It appears to be important to determine with which human chromosome(s) the maintenance and expression of such viral genetic information is closely associated in EBV-induced oncogenesis (4–6, §). The investigations described here have been carried out by using somatic hybrids between human and mouse cells and taking advantage of the preferential loss of human chromosomes in the hybrid cells (7), on the basis of the successful assignment of simian virus 40 T antigen gene to specific human chromosomes (8, 9).

We report here that the resident EBV genome and the expression of EBNA can be closely associated with human chromosome 14. The results were obtained by the analysis of somatic cell hybrids between mouse fibroblastic cells and EBNA-positive human lymphoblastoid cells that contained only a small number of EBV genomes.

MATERIALS AND METHODS

Cells. FV5 and MCB2 cells were used for somatic cell hybridization. The FV5 cells are cloned cells of the nonproducer human lymphoblastoid FVNC cell line previously established in our laboratory (10), grown in suspension in Eagle's minimum essential medium supplemented with 10% fetal calf serum and 10% tryptose phosphate broth. They are intensely positive for EBNA immunofluorescence, but contain only one to two EBV genome equivalents per cell (11). The monolayer MCB2 cells are cloned cells of a 5-bromodeoxyuridine (BrdUrd)-resistant MCB fibroblastic cell line derived from a methylcholanthrene-induced DDD mouse tumor in our laboratory (unpublished data). After several passages *in vitro* of the tumor cells, they were incubated in growth medium containing BrdUrd at 10–100 μg/ml to select the drug-resistant MCB cells. The clone MCB2 is completely resistant to BrdUrd at 100 μg/ml and is totally negative for EBV genome and EBNA immunofluorescence.

Fusion and Selection of Hybrid Cells. Cell hybridization was carried out as described elsewhere (12). FV5 cells (3×10^6) were mixed with 1×10^6 trypsinized MCB2 cells, together with ultraviolet-inactivated Sendai virus (400–1000 hemagglutinating units per ml). These mixtures were kept at 4°C for 15 min and then incubated at 37°C for 20 min with frequent shaking. After washing, they were grown in minimum essential medium containing 10% fetal calf serum and 10% tryptose phosphate broth for 24 hr and further incubated in growth medium supplemented with 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (HAT selective medium). Hybrid cell colonies were obtained in 2–3 weeks and then seeded into semisolid agar medium (13). A number of hybrid clones and subclones were isolated from several different colonies.

Detection of EBNA. The anticomplement immunofluorescence test was carried out for the detection of EBNA (2). Cells grown on coverslips were dried at room temperature and fixed in acetone/methanol (1:1, vol/vol) at −20°C. The coverslips were treated with heat-inactivated standard EBV-seropositive serum from a normal person (anti-EBNA of 1:640), in parallel with inactivated seronegative serum (anti-EBNA of <1:5), containing complement (seronegative serum with anti-EBNA of <1:5) at 37°C for 50 min. They were washed four times with balanced salt solution, stained with fluorescein

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Abbreviations: EBV, Epstein–Barr virus; EBNA, EBV-determined nuclear antigen; BrdUrd, 5-bromodeoxyuridine.

§ Glaser, R., Croce, C. & Nonoyama, M. (1977) in *Third International Symposium on Oncogenesis and Herpesviruses, Abstracts of Papers*, p. 83.

isothiocyanate-conjugated antihuman β1C/β1A goat serum (Hyland Laboratories, Costa Mesa, CA) at 37°C for 50 min, washed again four times, and mounted in 50% (vol/vol) balanced salt solution/glycerol. All the stained preparations were examined under a Leitz Orthoplan fluorescence microscope.

Detection of EBV Genome. Extraction of cell DNA and hybridization with EBV-specific cRNA have been described previously (14). Cells were treated with Pronase (1 mg/ml) and 1% sodium dodecyl sulfate, followed by extraction of DNA with phenol. The DNA was denatured with alkali and fixed onto nitrocellulose filters, which were then baked at 80°C under reduced pressure, and incubated with EBV-specific radioactive cRNA (1.5×10^5 cpm per filter) in 0.90 M NaCl/0.09 M Na citrate at 66°C for 20 hr. The filters were washed with 0.3 M NaCl/0.03 M Na citrate, treated with bovine pancreatic RNase (20 mg/ml) and washed again with the same buffer. The hybridized radioactive material was measured in a scintillation counter and the DNA on the filters was measured by the diphenylamine test. The counting data were normalized to 50 μg of DNA, and the number of EBV genome equivalents per cell was calculated from the value of 50 genome equivalents per cell for nonproducer Raji cells. Nonspecific background hybridization to Simpson cell DNA was subtracted before the calculation was made.

Chromosome Analysis. Parental chromosomes in interspecies cell hybrids were differentially stained as described elsewhere (15). Hybrid cells between human FV5 and mouse MCB2 were exposed to colchicine at a final concentration of 0.05 μg/ml for 30 min. After trypsinization, they were treated with 0.56% KCl solution at 37°C for 10 min, fixed by three changes of methanol/acetic acid (3:1) for 30 min, and then spread on slides and air-dried. The slides were stained with a mixed solution of quinacrine mustard (50 μg/ml) and 33258 Hoechst (0.5 μg/ml) dissolved in McIlvaine's buffer, pH 7.0, for 10–15 min, rinsed, and mounted in McIlvaine's buffer, pH 4.5. Well-spread metaphase plates were photographed with an Olympus fluorescence microscope, and the chromosome analysis was made with 50 metaphases per sample, on the basis of the selective 33258 Hoechst affinity for mouse centromeres and the specific quinacrine banding patterns of human and mouse chromosomes.

Enzyme Analysis. Hybrid cells were examined for the presence of human nucleoside phosphorylase, known to be linked to chromosome 14 (16), by cellulose acetate gel electrophoresis (17). After 1×10^8 cells were sonicated and centrifuged at $10,000 \times g$ for 60 min, the resulting extract was applied to a cellulose acetate gel and electrophoresed in 0.02 M sodium phosphate buffer, pH 7.0, at 4°C at 200 V for 90 min. The gel was then incubated in the reaction mixture [0.2 ml of inosine at 5 mg/ml; 10 μl of xanthine oxidase (Boehringer) at 4 units/ml; 0.2 ml of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) at 2 mg/ml; 0.2 ml of phenazine methosulfate at 0.4 mg/ml; 1.4 ml of Tris/$MgSO_4$ buffer, pH 7.4] at 37°C for 15 min.

RESULTS

Isolation of Somatic Hybrids between Human Lymphoblastoid FV5 and Mouse Fibroblastic MCB2 Cells. When human lymphoblastoid FV5 cells were fused with BrdUrd-resistant mouse MCB2 fibroblasts in the presence of Sendai virus and then incubated in hypoxanthine/aminopterin/thymidine selective medium, discrete cell colonies became evident in 2–3 weeks. As shown in Fig. 1, the cells grew in an attached form on culture flasks and were polygonal, in contrast to their parental round FV5 and spindle-shaped MCB2 cells. These polygonal cells contained both human and mouse chromosomes, which were distinguished by the selective affinity of 33258 Hoechst for mouse centromeres and the differences of the quinacrine banding patterns. Many clones and subclones were then isolated from several hybrid colonies in semisolid agar medium.

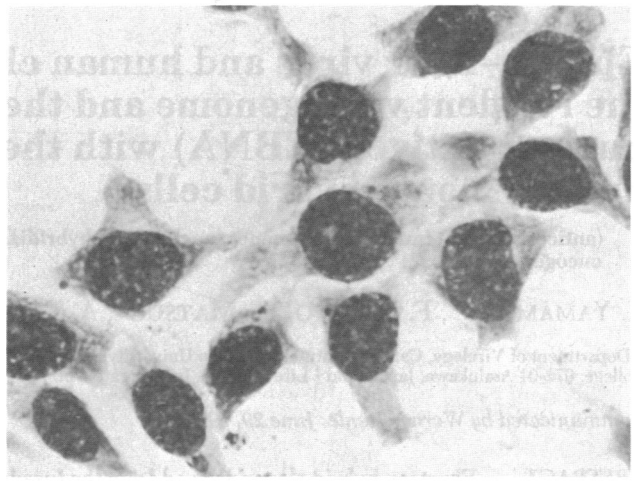

FIG. 1. Interspecific hybrid cells between human lymphoblastoid FV5 cells and mouse fibroblastic MCB2 cells. Note polygonal morphology. (Giemsa stain; ×490.)

Expression of EBNA in FV5/MCB2 Hybrid Clones and Subclones. Fourteen FV5/MCB2 hybrid clones were first examined for the expression of EBNA in the course of serial passage *in vitro*, together with their human parental FV5 and mouse parental MCB2 lines. All FV5 cells were intensely positive for the immunofluorescence, while MCB2 cells were totally negative. When the hybrid clones were tested at 3 months after their isolation, 11 of 14 clones were EBNA-positive, as illustrated in Fig. 2. They were clones 3, 5, 7, 8, 10, 13, 14, 16, 17, 18, and 19. The percentages of the positive cells ranged from 30 to 100. The remaining three, clones 1, 12, and 24, were totally negative. These 14 hybrid clones were further examined 18 months after their isolation (Table 1). Most of the positive clones at 3 months, however, were completely negative. Only clones 17, 18, and 19 were still positive in all cells. Clone 19 was again seeded in semisolid agar medium, and the resulting 10 subclones were also positive for EBNA in all cells (Table 2). Clones 17, 18, and 19 were eventually EBNA-negative, when

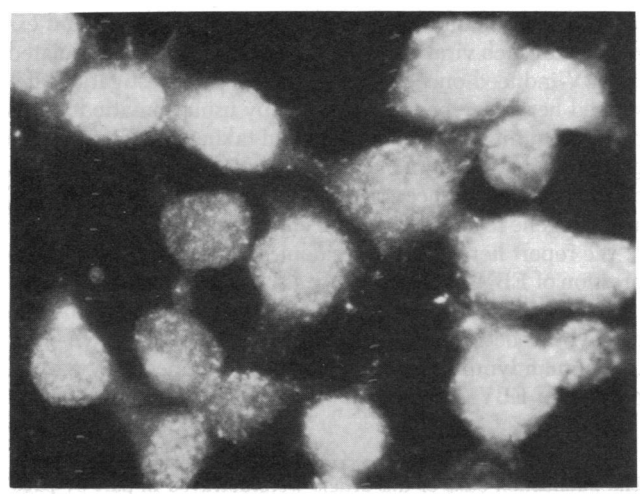

FIG. 2. Intense EBNA immunofluorescence in all nuclei in FV5/MCB2 clone 19. (Stained with EBV-seropositive human serum, followed by fluorescein isothiocyanate-conjugated antihuman β1C/β1A goat serum; ×490.)

Table 1. Relation between the presence of EBV genome, the expression of EBNA, and the presence of chromosome 14 in FV5/MCB2 hybrid clones*

FV5/MCB2 clone	EBNA-positive, %	EBV genome equiv. per cell	Human chromosomes in FV5/MCB2 clones: A			B		C							D			E			F		G			
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
1	0	<1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	0	ND	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5	0	ND	−	−	−	−	−	−	−	−	−	−	−		−	−	−	−	−	−	−	−	−	−	−	−
7	0	<1	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
8	0	ND	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−
10	0	<1	−	−	+	+	+	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
12	0	<1	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	+	−	−
13	0	<1	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
14	0	ND	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
16	0	<1	+	+	+	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	+	+	−	−	−	−
17	95–100	1–2	−	+	+	−	−	−	+	−	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−
18	95–100	1–2	+	+	+	−	−	−	+	−	−	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−
19	95–100	1–2	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
24	0	ND	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

ND, not determined.
* Results obtained 18 months after isolation of clones.

tested after 3 years. One of the 10 subclones of clone 19 became negative 3 months after the isolation; two additional subclones were negative at 6 months; and all remaining positive subclones were also converted into totally negative after 10 months (Table 2).

Presence of EBV Genome in FV5/MCB2 Hybrid Clones and Subclones. In addition to EBNA test, the FV5/MCB2 hybrid clones and subclones were also examined for the presence of EBV genome by cRNA·DNA hybridization, at 18 months after isolation of clones and throughout the incubation period of subclones. The positive control Raji cell line contained 50 EBV genome equivalents per cell, whereas the FV5 human parental lymphoblastoid cells contained only 1–2 EBV genome equivalents per cell. No detectable EBV DNA was present in either the negative control Molt-4 cells or the MCB2 mouse parental fibroblasts. When the hybrid cells were examined, EBNA-positive clones and subclones, all of which contained 100% immunofluorescent nuclei, were also positive for only 1–2 genome equivalents per cell. No detectable EBV genome was involved in any EBNA-negative clones tested. Also, the loss of EBNA was accompanied by the loss of EBV DNA. These results are summarized in Tables 1 and 2.

Chromosome Analysis of FV5/MCB2 Hybrid Clones and Subclones. The chromosome investigations of the FV5/MCB2 hybrid cells were carried out in parallel with the immunofluorescence and the nucleic acid hybridization at the same passage levels, by the quinacrine mustard/33258 Hoechst double staining. The modal numbers of chromosomes of the human parental FV5 cells and the mouse parental MCB2 cells were 48 and 58, respectively. In all the 14 hybrid clones examined at 3 months of their isolation, the number of human chromosomes was small, ranging from 3 to 13, though mouse chromosomes were well retained. Detailed analyses were carried out at 18 months, and the results are summarized in Table 1. Among the three EBV genome-positive and EBNA-positive clones, clone 17 contained chromosomes 2, 3, 7, 14, 15, and 17, and clone 18 contained chromosomes 1, 2, 3, 7, 14, 15, 17, and 21. On the other hand, in clone 19, chromosome 14 alone was present (Fig. 3). The human chromosome that was common to these three positive clones was therefore chromosome 14. Fig. 4 shows partial karyotypes of chromosomes 14 from four cells of the parental FV5, clone 17, clone 18, and clone 19. In contrast, human chromosomes involved in the 11 negative clones were nos. 1, 2, 3, 4, 5, 6, 7, 9, 11, 13, 15, 17, 18, 19, 20, 21, and 22. Among these, nos. 1, 2, 3, 7, 15, 17, and 21 were also noted in the positive clones. Chromosome 14 was not involved in any negative clones tested. The chromosome investigations were further carried out with the 10 subclones of clone 19, as summarized in Table 2. When examined 1 month after their isolation, these subclones were EBNA-positive and EBV genome-positive in consistent association with chromosome 14 alone. At 3 months, however, one of the 10, subclone 19-1, was

Table 2. Concordant segregation of EBNA, EBV genome, and chromosome 14 in FV5/MCB2 hybrid subclones

FV5/MCB2 subclone	EBNA, EBV genome, and chromosome 14 in FV5/MCB2 subclones: 1 mo	3 mo	6 mo	10 mo
19-1	+/+/+	−/−/−	−/−/−	−/−/−
19-2	+/+/+	+/+/+	−/−/−	−/−/−
19-5	+/+/ND	+/+/ND	+/+/ND	−/−/ND
19-8	+/+/ND	+/+/ND	+/+/ND	−/−/ND
19-13	+/+/ND	+/+/ND	+/+/ND	−/−/ND
19-15	+/+/+	+/+/+	−/−/−	−/−/−
19-18	+/+/+	+/+/+	+/+/+	−/−/−
19-19	+/+/ND	+/+/ND	+/+/ND	−/−/ND
19-22	+/+/ND	+/+/ND	+/+/ND	−/−/ND
19-25	+/+/+	+/+/+	+/+/+	−/−/−

Months after isolation of subclones are given in the headings. Results are expressed as presence or absence of: EBNA/chromosome 14/EBV DNA. ND indicates the presence of EBV DNA was not determined.

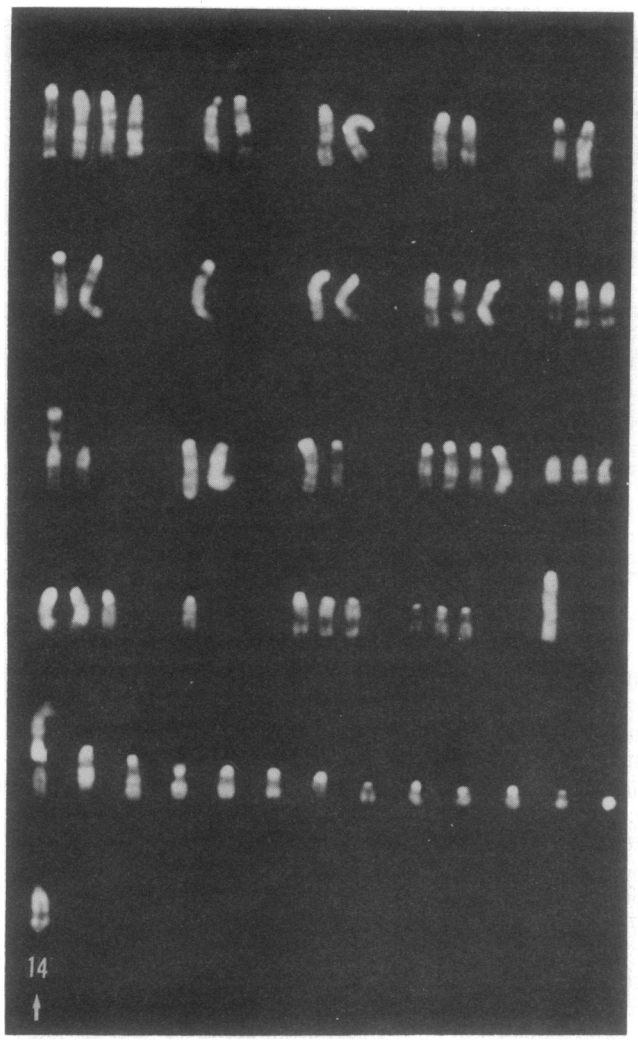

FIG. 3. Karyotype of FV5/MCB2 clone 19. Arrow indicates human chromosome 14. All other chromosomes originate from the mouse MCB2. (Stained with a mixed solution of quinacrine mustard and 33258 Hoechst; × 1000.)

converted into totally EBNA-negative and EBV genome-negative with the concomitant loss of chromosome 14. Subsequently, at 6 months two additional subclones, 19-2 and 19-15, became negative for immunofluorescence, EBV DNA, and chromosome 14. The remaining 7 subclones were eventually negative not only for EBV markers but also for this particular chromosome, when examined after 10 months of their isolation.

Enzyme Analysis of FV5/MCB2 Hybrid Clones and Subclones. To confirm the results obtained by the quinacrine banding procedure in combination with 33258 Hoechst staining, the enzyme analysis of the FV5/MCB2 hybrid cells was carried out. As illustrated in Fig. 5, EBV genome-positive and EBNA-positive clones and subclones were also positive for the human nucleoside phosphorylase, known to be specifically linked to chromosome 14, and the heteropolymer between the human and the mouse enzyme. No significant expression of this particular human enzyme was evident in any negative clones tested.

DISCUSSION

Several reports (4–6, §) have recently described the relation between the presence of EBV genome, the expression of EBNA, and the presence of human chromosomes in somatic hybrids

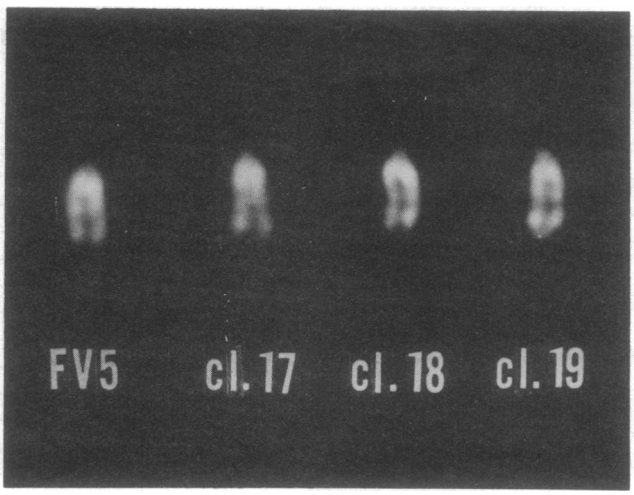

FIG. 4. Comparison between partial quinacrine-banded karyotypes of chromosomes 14 from FV5, and FV5/MCB2 clone 17, clone 18, and clone 19.

between human lymphoblastoid cells and mouse cells, taking advantage of the preferential loss of human chromosomes in such hybrid cells (7). This particular approach has been used to identify specific human chromosome(s) to which the EBNA antigen gene is assigned, along the line of simian virus 40 work (8, 9).

Klein *et al.* (4) previously suggested that the EBV genome may be associated with a small number of human chromosomes, perhaps only one, because their hybrids that had lost detectable viral genomes and EBNA still contained a considerable number of human chromosomes. They have, however, observed recently that the persistence of the genetic information of EBV does not require the presence of specific human chromosomes

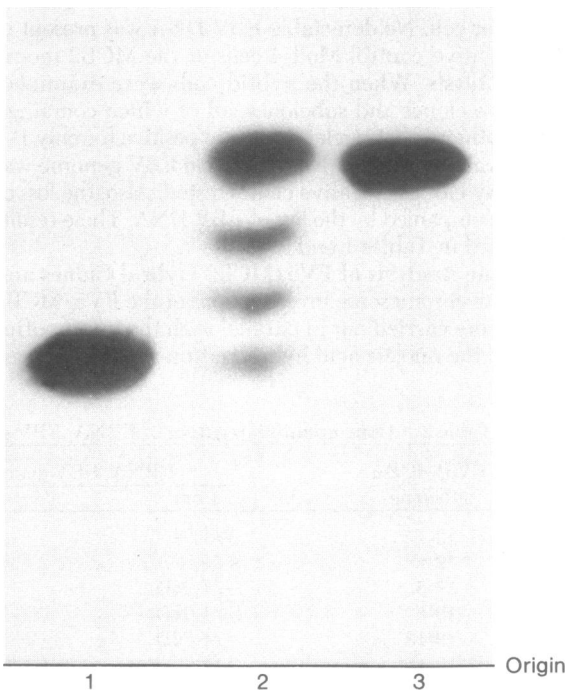

FIG. 5. Zymogram of nucleoside phosphorylase in cellulose acetate gel. Lane 1 represents the pattern of the human parental FV5; lane 2 represents the expression of the human enzyme and of the heteropolymer between the human and the mouse enzyme in FV5/MCB2 clone 19; lane 3 represents the pattern of the mouse parental MCB2.

(6). On the other hand, Glaser *et al.* (5) also suggested that EBV genome and the expression of EBNA were not associated with all or most human chromosomes, but may be associated with at least one of chromosomes 3, 4, 5, 6, or 10, on the basis of analysis of EBV genome-negative and EBNA-negative hybrid cells. More recently, they have reported, on the basis of investigations of their positive clone, that the expression of EBNA might be associated with the presence of chromosomes 7 and 17 (§).

We have also been trying for several years to determine with which human chromosome the resident EBV genome and the expression of EBNA are specifically associated, through somatic hybrids between human and mouse cells. In order to achieve clear-cut results, EBNA-positive lymphoblastoid cells that contained only one to two EBV genome equivalents per cell were used. In these particular cells, the expression of EBNA was considered to be attributed to the function of this small number of the resident EBV genomes.

Of 14 hybrid clones tested, human chromosome 14, as identified by both the banding procedure and the electrophoretic enzyme assay, was consistently involved in all three EBV genome-positive and EBNA-positive clones, but not in the remaining 11 negative clones. The presence of any other human chromosomes was not correlated with either the presence of EBV genome or the expression of EBNA. Furthermore, a concordant segregation of EBV genome, EBNA, and chromosome 14 was obviously evident in positive subclones, which had contained chromosome 14 alone as human chromosomes.

These findings strongly suggest that the resident EBV genome can be closely associated with human chromosome 14, and the presence of this particular chromosome alone seems sufficient for the maintenance and the expression of the genetic information of EBV.

It has recently been reported that a translocation from chromosome 8 to the long arm of chromosome 14 is observed in lymphomas, including Burkitt tumors (18–20). Whether or not the close association of EBV genome and EBNA with chromosome 14 is related to the mechanism of EBV-induced oncogenesis is deemed worthy of investigation.

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