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Genetics

Chromosome site for Epstein–Barr virus DNA in a Burkitt tumor cell line and in lymphocytes growth-transformed *in vitro*

(cytological hybridization/viral oncology)

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ABSTRACT **The Epstein–Barr virus (EBV) genome stably persists in latently infected Burkitt tumor cells and growth-transformed B lymphocytes. These cells usually contain multiple copies of episomal viral DNA. Cytological hybridization of recombinant viral DNA fragments to metaphase chromosomes of two latently infected cell lines demonstrates that viral DNA localizes to both chromatids of one homologue of chromosome 1 in Namalwa, a Burkitt tumor cell line, and to both chromatids of one homologue of chromosome 4 in IB4, a cell line with transformed growth properties *in vitro*. The site of chromosome association remains stable in a clone of IB4 cells. Probes from five separate regions of the EBV genome hybridize to the same chromosome regions. A previously undescribed achromatic site is identified within the region of EBV chromosome cytological hybridization. These observations suggest that most or all of the EBV genome is integrated into the chromosomal DNA of Namalwa and IB4 cells. Although the chromosomal sites of EBV DNA association are among those regions with homology to the EBV IR3 repeated DNA sequence, EBV IR3 did not mediate recombination between EBV and chromosomal DNA.**

The Epstein–Barr virus (EBV) genome is stably maintained and partially expressed in growth-transformed B lymphocytes and tumor cells of two human cancers, Burkitt lymphoma and nasopharyngeal carcinoma (1–10). Latently infected cells usually contain multiple episomal copies of nonintegrated viral DNA (11–18). The episomal DNA is formed by covalent joining of the direct repeats that are at both ends of the 175,000-base-pair linear virion EBV DNA (14).

Although episomal EBV DNA has been clearly demonstrated in latently infected cells, integrated EBV could also be present. The persistence of DNA in latently infected cells over years of active cell replication suggests the possibility of integration (15). Integration of EBV DNA not only could be important as a mechanism for persistence but also could result in alteration of virus or cell gene expression. A chromosome 8 to chromosome 2, 14, or 22 translocation is characteristic of malignant B lymphocytes, including those of African Burkitt lymphoma (16). One direct way in which EBV could promote Burkitt lymphoma is by integration at the site of the translocation.

Integration of EBV DNA has been suggested by the detection of viral DNA at a density intermediate between EBV DNA and cell DNA (17). However, covalent linkage of cell and viral DNA has not been established (18). Attempts to identify an association between EBV DNA and specific human chromosomes by somatic cell hybridization have yielded inconclusive and conflicting results, possibly due to the persistence of episomal copies of EBV DNA in the cell hybrids (19–22).

We report here the cytological hybridization of recombinant EBV DNA probes to metaphase chromosomes from two latently infected human cell lines, Namalwa and IB4. Namalwa is a culture of lymphocytes from an African Burkitt tumor biopsy sample (7, 23). IB4 is a line of latently infected neonatal lymphocytes established by growth transformation *in vitro* of cord blood lymphocytes with EBV (17). Viral DNA localizes to unique and nonidentical chromosomal sites in the two cell lines. These sites are among the chromosome sites that have DNA homology to the IR3 simple repeat array in EBV DNA (24, 25). Because virus–cell DNA homology could mediate integrative recombination at various chromosomal sites, we have investigated whether EBV IR3 recombined with homologous sequences of Namalwa or IB4 cells.

MATERIALS AND METHODS

Lymphocytes, Chromosomes, and Cytological Hybridization. IB4, IB4 clone D, and Namalwa cells were grown in RPMI 1640 medium supplemented with 10% newborn calf serum (8). The IB4 cell line was established by infecting cord blood lymphocytes with a 10^{-4} dilution of B95-8 culture supernatant containing EBV (8). At the 10^{-4} and 10^{-3} dilutions, only one of four infected cultures yielded continuous cell lines in the IB experiment, suggesting that the IB4 cell line was likely to result from a single infected cell. IB4 cells were maintained in culture for 1 year before cloning in soft agarose to yield IB4 clone D (8, 10). T cells for control hybridizations were prepared from the peripheral blood of human donors and stimulated with phytohemagglutinin (PHA) (25). Chromosomes were prepared from cells that were incubated in medium containing Colcemid (26). Chromosomes were Giemsa banded and photographed so that they could be identified after cytological hybridization and autoradiography (26). Slides were incubated for 2 hr at 68°C in 95% (vol/vol) formamide/0.15 M NaCl/0.15 mM sodium citrate to denature chromosomal DNA (26). EBV DNA *Eco*RI fragments from a cosmid EBV (W91 isolate except for the B95-8 *Eco*RI D–I, J het fragment) recombinant library (14, 27) were labeled by nick-translation (28) using ^{125}I-labeled dCTP (2,200 Ci/mmol; New England Nuclear; 1 Ci = 3.7×10^{10} Bq). The specific activity of each labeled DNA is given in Table 1. The iodinated DNA was denatured in 0.1 M NaOH, neutralized, and adjusted to a concentration of 150 ng/ml in a solution consisting of denatured *Escherichia coli* DNA at 30 μg/ml, 0.45 M NaCl, 45 mM sodium citrate, and 50% formamide. The DNA on each slide was hybridized for

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Abbreviations: EBV, Epstein–Barr virus; PHA, phytohemagglutinin; kb, kilobase(s).

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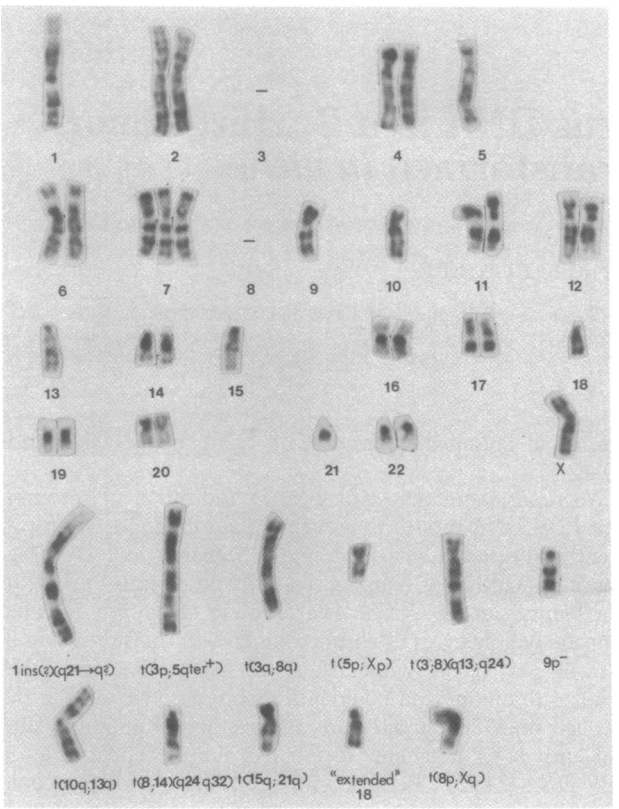

FIG. 1. Namalwa cell karyotype. Giemsa-banded normal human chromosomes are shown in the upper part of the figure. An achromatic site occurs in an otherwise normal chromosome 1 chromatid pair at 1p35. Abnormal chromosomes regularly found in Namalwa are identified in the lower part of the figure.

15 hr with 50 μl of labeled DNA at 42°C (26). Slides were washed at 25°C in hybridization buffer for 5 min, in 0.3 M NaCl/30 mM sodium citrate at 45°C for 10 min, and in 0.3 M NaCl/30 mM sodium citrate at 20°C for 5 hr. The slides were dehydrated in ethanol and exposed to NTB2 emulsion (Kodak). After development of the autoradiogram, previously photographed metaphase plates were relocated and again photographed. The grain distributions were determined over 90 regions of the chromosome complement, using the Giemsa-banded chromosomes as a guide.

Southern Blot Hybridizations. DNA was extracted from lymphocytes (24, 25) and digested with restriction endonucleases (Bethesda Research Laboratories). Lymphocyte DNA fragments were separated by electrophoresis in 0.3% agarose gels and transferred to nitrocellulose filters (29). Fragments of EBV *Bam*HI fragment K that map in U3, 0.1–0.5 kilobases (kb) to the left of IR3 and in U4, 1.5–2.2 kb to the right of IR3 (Fig. 2) were isolated from pDK225, an EBV *Bam*HI K–pBR322 recombinant plasmid (14, 24, 25, 30). The purified U3 and U4 subfragments were labeled by nick-translation with [^{32}P]dCTP (400 Ci/mmol; Amersham). Labeled DNA (10^8 cpm/μg) was hybridized to Southern blots of fragments of cell DNA in 0.9 M NaCl/90 mM sodium citrate at 72°C for 20 hr. Blots were washed and exposed to x-ray film with an intensifying screen.

RESULTS

Cytological Hybridizations. Cells were karyotyped by using Giemsa-banded metaphase plate preparations. The Namalwa karyotype has a trisomy 7 and several abnormal chromosomes with translocations or insertions (Fig. 1). There is a variant chromosome 8 to 14 translocation in which a portion of chromosome 3q is inserted at the chromosome 8 breakpoint. The 14q+ chromosome has the expected morphology. The IB4 karyotype has several deviations from the normal karyotype, including a 1q− and a 6p− chromosome. The IB4 clone D karyotype is normal except for a Robertsonian-like translocation between the long arm of chromosome 21 and an unidentifiable small fragment.

EBV *Eco*RI A, B, C + F, or D–I, J het fragment probes [Fig. 2; the C + F recombinant cosmid contains both the *Eco*RI C and the *Eco*RI F fragments of EBV DNA (27), the D–I, J het cosmid contains the terminal sequences of the linear genome in an *Eco*RI fragment from episomal viral DNA (14)] were individually hybridized to Namalwa, IB4, IB4 clone D, or PHA-stimulated lymphocyte chromosomes. After autoradiography, the distribution of grains per chromosome segment was determined. For this analysis, chromosome 1 was divided into seven segments, 2 into six segments, chromosomes 3, 4, and 5 into five segments, chromosomes 6–12 and X into four segments, chromosomes 13–20 into three segments, and chromosomes 21, 22, and Y into two segments. Grain counts over any segment were considered significant if the number of grains exceeded a random distribution indicated by a χ^2 value greater than 10 ($P < 0.01$). For Namalwa, the only specific grain density ($\chi^2 > 1{,}000$ in most experiments) was on the normal chromosome 1 at about 1p35 (Table 1 and Fig. 3). Each of the *Eco*RI fragment probes and probes made from equimolar mixtures of the recombinant *Eco*RI fragments hybridized to the chromosome 1p35 region. In IB4, the probe made from EBV *Eco*RI fragments C + F specifically hybridized only to chromosome 4 (Table 1). In order to determine whether the association of EBV DNA with IB4 chromosome 4 is stable, EBV DNA probes were hybridized to chromosomes of the D clone of IB4 (Table 1 and Fig. 4).

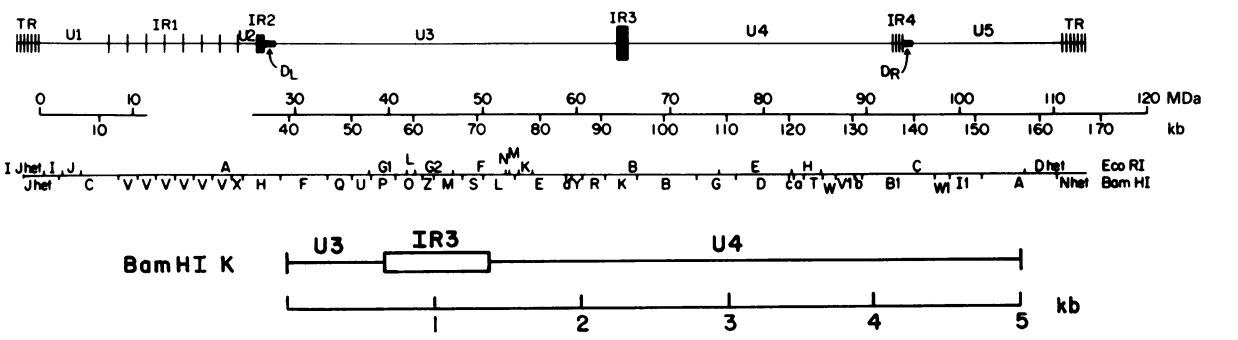

FIG. 2. Physical map of EBV DNA. Unique (U1, U2, U3, U4, and U5) and repeated (TR, IR1, IR2, IR3, and IR4) sequences and *Bam*HI and *Eco*RI restriction endonuclease sites are shown (14, 24, 25, 30). MDa, megadaltons.

Table 1. Hybridization of EBV DNA probes with specific sites on metaphase chromosomes

*Eco*RI fragment probes	Specific activity, dpm × $10^{-8}/\mu g$	Autoradiography, days	Metaphase plates analyzed	Total grains at specific site		Grains per day per chromatid at specific site (− background)*
				%	$\chi^{2\dagger}$	
Namalwa						
A, B, C + F, D–I, J het	6.5	7	23	3.5	140	0.17
A	8	10	15	10	>1,000	0.65
B	9	7	15	11	700	0.58
C + F	17	10	12	9.6	>1,000	0.18
D–I, J het	11	7	16	7.8	320	0.36
IB4						
C + F	15	13	18	11	>1,000	0.35
IB4 clone D						
A, B, C + F, D–I, J het	6.5	4	38	9	>1,000	0.37
A, B, C + F, D–I, J het	10	7	21	18	576	0.33
minus *Bam*HI K‡	10	20	14	16	>1,000	0.35
A	8	8.5	21	10	>1,000	0.60
B	9	5	46	12	>1,000	0.68
C + F	17	13	22	23	>1,000	0.38
D–I, J het	11	5	60	16	>1,000	0.62
	11	8	35	19	>1,000	0.50

* Background per chromosome region is determined by summation of all grains over all chromosome regions and division by 90, which is the number of regions into which the chromosome complement is divided (25, 26).

† χ^2 values were based on a comparison of the number of grains observed at the specific region in all metaphase blots with the number of grains expected from a random distribution of grains.

‡ Constructed to omit *Bam*HI fragment K, which includes IR3, which has a low level of homology to specific regions of all human chromosomes except for the Y chromosome. The *Eco*RI B fragment was replaced by recombinant clones of *Bam*HI E, Y, R, and B (14).

In IB4 clone D, the only specific grain density ($\chi^2 > 1{,}000$ in most experiments) was on chromosome 4q, at about 4q25 (Table 1 and Fig. 4). Each EBV DNA *Eco*RI fragment probe hybridized to this location. With the exception of one experiment, one or more grains were observed at the specific chromosome region in over 90% of the Namalwa and IB4 metaphase spreads analyzed for Table 1. The EBV probes did not yield any specific grain density ($\chi^2 < 10$ for each region) when

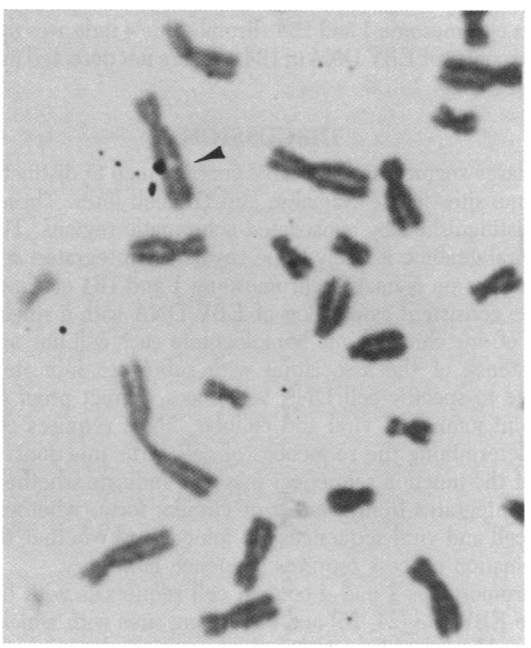

FIG. 3. Portion of Namalwa metaphase spread after hybridization, autoradiography, and Giemsa staining, showing (arrowhead) grains over one chromatid of chromosome 1 and an achromatic region at the same location on the other chromatid. The metaphase plate was hybridized to EBV *Eco*RI B fragment probe. Autoradiography was for 7 days. This chromosome 1 is atypical in not showing hybridization to both chromatids or to neither. It was selected because the unusual distribution of silver grains over only one chromatid allows the achromatic region to be seen on the other chromatid.

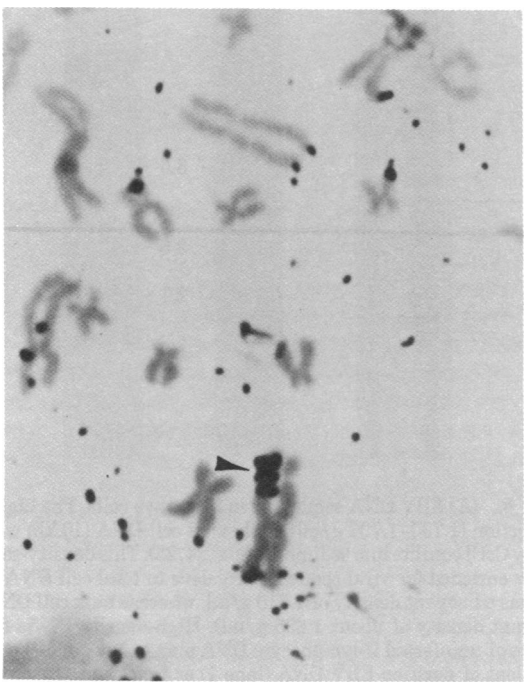

FIG. 4. Portion of IB4 metaphase spread after hybridization, autoradiography, and Giemsa staining, showing (arrowhead) grains over one chromatid of chromosome 4 and an achromatic region at the same location on the other chromatid. The metaphase plate DNA was hybridized to probe nick-translated from a mixture of EBV *Eco*RI A, B, C + F, and D–I, J het fragments. Autoradiography was for 5 days. This photograph was selected as described for Fig. 3.

hybridized in parallel to chromosomes of PHA-stimulated lymphocytes.

Most EBV *Eco*RI fragment probes yielded approximately the same number of grains per day in hybridization to specific IB4 clone D and Namalwa chromosome sites (Table 1). Reassociation kinetics (23) and comparative Southern blot hybridization with Namalwa DNA indicate that there is one copy of EBV DNA per Namalwa cell. Labeled Namalwa DNA hybridizes to all of the *Bam*HI fragments of EBV DNA on Southern blots, indicating that most or all of the viral genome is present in Namalwa cells (Fig. 5*A*). Because all of the EBV DNA probes hybridize to Namalwa chromosome 1, it is likely that the Namalwa 1p35 site contains most or all of the EBV genome. Further, because EBV DNA probes hybridize to IB4 chromosome 4 at 4q25 and Namalwa chromosome 1 at 1p35 with about the same level of efficiency and there is only one copy of EBV DNA in Namalwa cells, there is probably also only one EBV DNA molecule associated with the IB4 4q25 chromosome site.

Constricted achromatic regions are observed on both chromatids of one chromosome 1p35 in Namalwa and on both chromatids of one chromosome 4q25 in IB4 (Figs. 3 and 4).

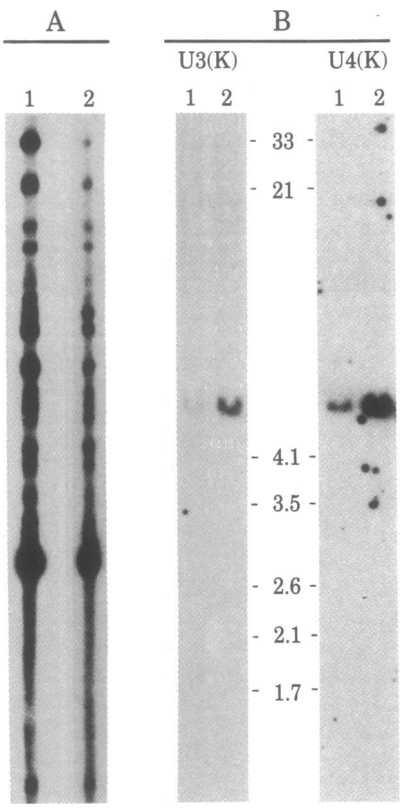

FIG. 5. (*A*) EBV DNA sequences in Namalwa cells. The high-density fraction (1.730–1.707 g/ml) of sheared cell DNA (10 kb) was isolated by CsCl equilibrium sedimentation (24, 25). This density-selected DNA is enriched for viral sequences relative to total cell DNA (EBV DNA has a buoyant density of 1.718 g/ml, whereas bulk cell DNA has a buoyant density of about 1.695 g/ml). High-density DNAs from a mixture of uninfected B-lymphocyte DNA plus 1 copy per cell genome equivalent of purified EBV DNA (lane 1) or from Namalwa lymphocytes (lane 2) were labeled by nick-translation and hybridized (2×10^8 cpm) to the separated *Bam*HI restriction enzyme fragments of purified EBV DNA in Southern blots. (*B*) Analysis to detect IR3-mediated integrative recombination. Sequences from the U3 and U4 domains of the *Bam*HI K fragment in recombinant pDK225 (Fig. 2) were labeled by nick-translation and hybridized to *Bam*HI-digested Namalwa (lane 1) and IB4 (lane 2) cell DNAs in identical Southern blots. Size markers (in kb) are intact adenovirus 2 DNA and *Eco*RI fragments of it.

These achromatic regions have not been observed previously and are found only on the chromatid pair to which EBV DNA hybridizes and not on the other chromosome homologue. The achromatic regions at 1p35 and 4q25 are presumed to be the direct result of viral DNA integration.

IR3-Mediated Recombination. Chromosome regions 1p35 and 4q25 contain DNA homologous to EBV IR3 (25). IR3-related DNA in human chromosomes could mediate recombination between cell and EBV DNA. Directed reciprocal recombination between viral IR3 and the related cell DNA could occur if EBV DNA circularizes prior to integration (31). If viral IR3 recombines with cell DNA, the U3 and U4 regions of EBV DNA (Fig. 2) would become separated. Parts of U3 and U4 as well as IR3 are in the same *Bam*HI fragment, K, in viral DNA (Fig. 2). EBV integration mediated by recombination at IR3 predicts that the *Bam*HI K fragment will be replaced by two *Bam*HI fragments that contain the U3 or U4 regions of *Bam*HI K, respectively, linked to cellular sequences. To test this prediction restriction endonuclease subfragments were isolated from the U3 and U4 domains of *Bam*HI K, labeled, and hybridized to *Bam*HI fragments of Namalwa or IB4 cell DNA in Southern blots (Fig. 5*B*). The U3 and U4 probes hybridize to a single *Bam*HI fragment in Namalwa or IB4 cells which is similar in size to the unrearranged viral *Bam*HI K fragment (24, 25). No new fragments are detected. Thus, the U3 and U4 regions of the integrated DNA have retained their previous linkage to each other, indicating that recombination at IR3 has not mediated EBV DNA integration in Namalwa or IB4 cell DNA. Hybridization of the probes to IB4 DNA is 5-fold more intense than the hybridization to Namalwa DNA, indicating that there are approximately five EBV DNA copies per IB4 cell (Fig. 5*B*).

IB4 cells contain approximately five copies of EBV DNA per cell (Fig. 5*B*; ref. 8) and most of the EBV DNA is episomal (8), whereas Namalwa cells contain only one copy per cell. The similar efficiency of cytological hybridization of EBV probes to Namalwa chromosome 1 and IB4 chromosome 4 indicates that episomal copies of EBV DNA in IB4 cells are not detected in these analyses.

DISCUSSION

Five large segments of the EBV genome map to distinct chromosome sites in the Namalwa and IB4 cell lines. These sites are distinguished by constricted achromatic regions. The entire viral genome is likely to be covalently integrated at these single sites on Namalwa chromosome 1 and IB4 chromosome 4. The consistent association of EBV DNA with a single segment of one chromosome homologue in each cell line and the persistence of the association with subclones indicate tight linkage to specific cell DNA sequences. Direct proof of the covalent joining of viral and cellular DNAs requires cloning and determining the sequences of cell–virus junctions. Analysis of the junction sequences may also indicate whether viral DNA integrates from a linear or circular form, whether specific cell and viral sequences are joined, and whether the recombination process requires sequence homology.

Chromosomes 1 and 4 contain cell sequences with homology to EBV IR3 (24, 25) and are among sites with which viral IR3 could recombine. Although our data indicate that EBV IR3 did not mediate integration of EBV DNA in Namalwa or IB4 cells, EBV DNA integration could still involve homologous recombination. Pentanucleotide homology appears to be sufficient to direct the integration of simian virus 40 DNA into cell DNA (32).

Other latently infected cell lines should be analyzed by cytological hybridization to determine whether EBV DNA can associate with chromosomes other than 1 and 4 and whether

integration is a constant feature of latent EBV infection. If integration is regularly observed in latent infection, it may be the principal mechanism of persistence of EBV DNA in progeny cells.

The finding that EBV DNA is not associated with chromosomes 8, 2, 14, or 22 in a growth-transformed cell line or in a Burkitt tumor cell line indicates that the reciprocal translocation involving chromosome 8 in Burkitt tumors is not a *cis* function of integrated EBV DNA. Thus, the simplest model to explain EBV association with Burkitt tumors is that EBV induces B-cell proliferation and thereby provides enhanced opportunity for chromosomal translocation and malignant degeneration.

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