Human Neonatal Lymphocytes Immortalized after Microinjection of Epstein-Barr Virus DNA

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Epstein-Barr virus (EBV) is a highly efficient acute transforming agent in human cells, provided that the intact virus is used. To investigate the ability of viral DNA alone to transform cells, we introduced the EBV genome into human lymphocytes. After microinjection of EBV DNA into neonatal B lymphocytes, we established a cell line that in early passages contained multiple viral fragments. This cell line retained sequences from the short, unique (U_s) region of the EBV genome and sequences from *EcoRI*-E. The viral sequences were not expressed; however, the cells expressed a 2.3-kilobase polyadenylated message homologous to the *c-fgr* oncogene, a cellular locus believed to be activated by EBV infection [M. S. C. Cheah, T. J. Ley, S. R. Tronick, and K. C. Robbins, *Nature (London)* 319:238-240.]. The cell line was monoclonal with rearrangement at the immunoglobulin locus and had a reciprocal translocation t(1;7)(p34;q34) and a deletion of sequences within the locus for the beta chain of the T-cell receptor. The close proximity of the translocation to the chromosomal loci for *c-fgr* on chromosome 1 and the T-cell receptor beta chain on chromosome 7 suggests that structural alteration of these genes was critical to this transformation event.

Although infection of B lymphocytes with Epstein-Barr virus (EBV) routinely results in transformation (37), identification of transforming functions of EBV by gene transfer has been an elusive objective. One impediment has been the difficulty in delivering viral DNA into these cells. Lymphocytes are nuclease rich and relatively refractory to transfection. Microinjection has been used to introduce EBV DNA into human and rat fibroblasts and African green monkey kidney cells (13); early antigen expression was induced in the recipient cells. EBV DNA has been successfully transfected into human placental, Vero, BSC-1, and owl monkey kidney cells (15). These cells did not express EBV nuclear antigen but did express antigens associated with a productive viral cycle. However, synthesis of EBNA has been induced in lymphocytes after Sendai virus envelope-mediated gene transfer of the entire EBV genome or cloned fragments of EBV DNA (49). These studies indicated that induction of EBNA and transient growth stimulation could be induced in lymphoid cells, but the recipient cells were not permanently transformed. This inability to transform perhaps occurred because the viral DNA was damaged during transfer through the cytoplasm and was not stably maintained.

Studies of the sensitivity of transformation to inactivation (17, 30) and of viral transcription in latently infected growth-transformed cells (26) have indicated that the transformation process is complex and involves much of the genome. To determine whether EBV-induced growth transformation of lymphocytes could occur in the absence of infection by whole virus, we microinjected EBV (B95-8) DNA purified from virions into the nuclei of mononuclear cells obtained from human umbilical cord blood. Injection of DNA directly into nuclei may prevent DNA degradation which may occur in the cytoplasm after transfection, electroporation, or vesicle fusion. Neonatal lymphocytes are ideal for such studies as they do not spontaneously transform, nor do they exhibit chromosomal abnormalities in culture. In contrast to the uninjected cord lymphocytes, injection of cells with B95-8

viral DNA consistently resulted in transient growth stimulation for several months in culture. Only one of these abortive transformations resulted in establishment of a permanent cell line. This paper describes an analysis of the EBV DNA content of this cell line and the chromosomal abnormalities which may have contributed to the transformation event.

MATERIALS AND METHODS

Isolation of B lymphocytes. Mononuclear leukocytes were obtained by centrifugation of fresh human cord blood on Ficoll (LSM; Litton Bionetics). The leukocyte layer was depleted of monocytes by incubation in plastic tissue culture ware which had been pretreated with fetal calf serum. Nonadherent cells were washed and suspended in RPMI-20% fetal calf serum until use. Just before microinjection, monolayers were prepared with anti-human F(ab')₂-treated polystyrene petri dishes. Petri dishes were incubated for 30 min at 37°C with affinity-purified goat anti-human F(ab')₂ (Atlantic Antibodies). The monocyte-depleted cord blood lymphocytes were allowed to adhere to the F(ab')₂ antibody for 30 min at 37°C. Nonadherent T cells were washed from the petri dishes, providing a B-cell-enriched monolayer target for microinjection.

Preparation of EBV DNA. EBV was obtained from the supernatant fluids of 12-*O*-tetradecanoylphorbol acetate-induced B95-8 cells by centrifugation and layered onto a 15 to 30% sucrose gradient. Virions thus obtained were treated with proteinase K and sodium dodecyl sulfate. Viral DNA was isolated by CsCl density gradient centrifugation. After ethanol precipitation, the viral DNA was suspended in microinjection buffer (114 mM KCl, 20 mM NaCl, 3 mM NaH₂PO₄ · H₂O, 3 mM MgCl₂ · 6H₂O, pH 7.4).

Microinjection. Recipient cells were microinjected with glass microcapillaries drawn with a de Fonbrune microforge (Curtin Matheson Scientific, Inc.). Injections were carried out on a Zeiss IM inverted phase-contrast microscope. Micromanipulators provided control of the microcapillary needle, and a Hamilton syringe allowed maintenance of continuous pressure on the DNA solution. Microinjection

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FIG. 1. Linkage map of *Bam*HI and *Eco*RI restriction endonuclease fragments of the EBV genome. Below is a schematic map of the arrangement of unique (U) and internal repeat (IR) sequences of virion DNA and of the terminal repeats (TR), which vary in number, at each end of the molecule. d, Dalton.

was controlled by determining visually by cellular refractile changes that solution had been delivered. Approximately 200 cells were injected per assay. DNA concentration was adjusted to deliver five copies of EBV DNA per 10-fl injection.

Nucleic acid preparation and hybridization conditions. For isolation of nucleic acids for hybridization, cells were lysed in 4 M guanidine isothiocyanate–0.5% sarcosyl solution, and the lysate was centrifuged in a CsCl step gradient. Poly(A)⁺ RNA was obtained by oligo(dT)–cellulose chromatography. The DNA was dialyzed against 10 mM Tris hydrochloride–1 mM EDTA, pH 8.0, ethanol precipitated, and digested with *Eco*RI. After electrophoresis and transfer to nitrocellulose, the DNA was probed with *Bam*HI or *Eco*RI fragments of EBV DNA. These isolation procedures were carried out at periodic intervals after cell outgrowth.

Hybridization with ³²P-labeled recombinant EBV DNA probes was done in buffer consisting of 10× Denhardt reagent, 100 µg of single-stranded calf thymus DNA per ml, 100 µg of poly(A) per ml, 0.2% sodium dodecyl sulfate, and 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The labeled probe was hybridized to pretreated blots at 72°C for 18 h. The blots were washed at 72°C in 0.2% sodium dodecyl sulfate in decreasing SSC concentration to 0.1× SSC. Hybridizations to pHJ and T-beta probes (see below) were done at 68°C in buffer as stated above; the blots were washed at 68°C.

The EBV *Bam*HI C fragment was cloned into the pGEM-2 vector to allow generation of SP6 transcripts for Southern and Northern blot hybridizations. SP6 *Bam*HI-C labeled with ³²P was hybridized in buffer containing 50% formamide at 50°C. Washes were done at 75°C in the aqueous buffer described above.

A 0.841-kilobase *Ava*I-*Bam*HI fragment of *v-fgr* which contains only the tyrosine kinase sequences of the oncogene was subcloned into the pGEM1 vector. Transcripts from 2 µg of linearized DNA template were labeled with [³²P]GTP in a 50-µl reaction mixture consisting of 40 mM Tris hydrochloride (pH 7.5), 6 mM MgCl₂, 2 mM spermidine, 100 µg of bovine serum albumin per ml, 10 mM dithiothreitol, and 20 U of SP6 polymerase with a 0.5 mM concentration of each unlabeled nucleotide. Northern blots were hybridized in 50% formamide at 44°C for 18 h. The blots were washed at 65°C in 0.2% sodium dodecyl sulfate–0.1% sodium pyrophosphate in SSC in decreasing concentrations to 0.1× SSC. RNase digestion at 37°C was included in the wash protocol.

Probes. Cloned *Bam*HI and *Eco*RI EBV (W91) fragments were used to detect EBV DNA in *Eco*RI-digested DNA from the microinjected cells (39). *Bam*HI-C, subcloned into the pGEM-2 vector, was used in later hybridizations to both Southern and Northern blots of poly(A)⁺ RNA. pHJ (10), a probe specific for the joining region of the human immunoglobulin heavy chain, was used to probe *Hind*III-digested

cellular DNAs. A cDNA clone, Jurkat-2 (52), specific for the beta chain of the T-cell receptor was used to probe *Eco*RI-digested cellular DNAs. A 0.841-kb *Ava*I-*Bam*HI fragment of *v-fgr* ligated into pGEM-1 was used to probe Northern blots of poly(A)⁺ RNA from the microinjected cell line.

RESULTS

Establishment of the MIB cell line. Transformation of cord blood lymphocytes by microinjection of EBV DNA was a rare event. In 23 attempts to transform cells with B95-8 DNA, only one permanent line was established. Microinjected and uninjected cells from the same cord blood preparation were allowed to elute from (Fab')₂-treated petri dishes, and these cells were cultured together. The majority of cultures could not be expanded, and the cells died within 2 months. In six of the assays, cells remained viable for 3 to 5 months; in that time, culture volumes were expanded as cell numbers increased. The usual circumstance was eventual decline in cell numbers and death of the culture. Only in the case of MIB could expansion of the cell culture continue as a permanent line was established.

EBV DNA content. EBV DNA within the virion is a 172-kb-pair linear genome containing multiple random nicks (38). Although the standard EBV-transformed lymphoblastoid cell lines contain the intact EBV genome, usually episomal (1, 16, 32, 34), some fragmentation of the genome could occur during viral DNA isolation or during the microinjection process itself. It was therefore anticipated that not all viral sequences would necessarily be retained in this transformed cell line. To identify the viral sequences retained, high-molecular-weight DNA was extracted from the MIB cell line, digested with *Eco*RI, transferred to nitrocellulose, and hybridized to ³²P-labeled *Eco*RI or *Bam*HI EBV (W91) recombinant fragments (Fig. 1). DNA preparations from the early outgrowth of MIB contained fragments homologous to the EBV fragments listed in Table 1. The majority of these fragments were not detectable in preparations of DNA from later passages. Loss of viral sequences during passage could be due to unstable integrative events in contrast to the regulated replication and expression of the intact EBV plasmid.

Table 1 shows the hybridization results obtained with duplicate Southern blots of *Eco*RI-digested cellular DNA. In intact viral DNA, *Eco*RI-A is a 30- to 45-kb fragment, depending on the number of IR1 sequences it includes. However, hybridization with *Eco*RI-A to the *Eco*RI-digested MIB DNA identified four fragments with molecular weights of 16.5, 6.6, 4.2, and 2.0 kb. The 6.6- and 4.2-kb fragments were also detected with *Bam*HI-C, and the 2.0-kb fragment was detected by *Bam*HI-V; both sequences map within *Eco*RI-A. The multiple *Eco*RI fragments indicate that the entire *Eco*RI-A sequence was not retained and that small

fragments of this sequence integrated into cell DNA. Multiple *EcoRI* fragments were also detected with *EcoRI*-E (5.2, 4.5, and 2.8 kb) and *EcoRI*-Dhet (6.6, 3.8, and 3.2 kb). In the intact genome, *EcoRI*-E is 11.3 kb in size, *EcoRI*-Dhet varies with a range of 11.1 to 13.8 kb. The sequences detected in MIB represent portions of these *EcoRI* fragments. In the hybridizations to DNA derived from early cell passages, only *EcoRI*-F detected a fragment of the expected size, 8.1 kb. Hybridization with the *EcoRI*-B sequence detects 18-, 10.5-, and 3.6-kb fragments in the cellular DNA. *BamHI*-B, which maps within the *EcoRI*-B sequence, hybridized to 18-, 12.3-, 6.9-, and 3.6-kb fragments in MIB DNA. These data indicated that, at early times after cell outgrowth, not all viral sequences were retained. The multiple *EcoRI* fragments detected by several of the probes indicated that multiple rearrangements or integrative events occurred. Further analysis of the DNA from the transformed line was carried out at later passages to determine whether the loss of viral sequences was a continuing process.

Hybridization to DNA from later passages was compared with hybridizations to reconstructions of 1, 10, or 100 copies of the recombinant fragment or dilutions of cellular DNA from the latently infected Raji cell line which contains approximately 60 copies of the EBV genome. Hybridization with *BamHI*-V, -X, -H, and -F within *EcoRI*-A, *BamHI*-K within *EcoRI*-B, *BamHI*-E, or *EcoRI* fragments DIJhet, F, and G1, which detected one copy in reconstruction hybridizations, detected no homologous fragments in MIB DNA.

TABLE 1. Sequences detected by hybridization with recombinant EBV nick-translated probes or SP6-generated riboprobes against Southern blots of DNA extracted from transformed MIB cells 4 months or 1 year after the start of cell outgrowth^a

Probe	<i>EcoRI</i> restriction fragment(s) (kb) detected after:	
	4 Mo	1 Yr
<i>EcoRI</i> -A	16.5	
	6.6	
	4.2	
	2.0	
<i>BamHI</i> -C	6.6	
	4.2	
	3.0	
		3.0
<i>BamHI</i> -V	3.6	
	2.0	
<i>EcoRI</i> -F	8.1	
<i>EcoRI</i> -B	18.0	
	10.5	
	3.6	
<i>BamHI</i> -B	18.0	
	12.3	
	6.9	
	3.6	
<i>EcoRI</i> -E	5.2	
	4.5	
	2.8	2.8
<i>EcoRI</i> -Dhet	6.6	
	3.8	
	3.2	

^a Cellular DNA was digested with *EcoRI*.

FIG. 2. Hybridization with SP6 *BamHI*-C, a riboprobe specific for the *BamHI*-C region of EBV. *BamHI*-C was cloned into the pGEM-2 vector, and RNA was synthesized with SP6 polymerase; 2×10^8 cpm of labeled RNA was hybridized to Southern blots of *EcoRI*-digested DNA from the microinjected cell line (MI), H9 (a T-cell leukemia), Bjab (an EBV-negative BL line), and Raji (an EBV-positive BL line). Raji DNA was diluted 1/50. Hybridization was carried out in 50% formamide at 55°C. Blots were washed at 75°C, and a ribonuclease A treatment at 37°C was included in the wash protocol. A 3.0-kb band was visible in the MIB line (lane 1) and in Raji DNA (lane 4) after hybridization with SP6 *BamHI*-C RNA. This represents the *EcoRI* J fragment of EBV, which maps within the *BamHI* C fragment. Also present in lane 4 is a 3.9-kb band representing *EcoRI*-I sequences in Raji DNA. The probe did not detect sequences in either Bjab or H9 DNA.

Hybridizations with *BamHI*-B, -Y, and -R within *EcoRI*-B, *EcoRI*-C, and *EcoRI*-H detected 7.5-kb fragments. An identically sized fragment was detected with a clone representing ribosomal DNA. Homology of EBV and herpes simplex virus DNAs with ribosomal sequences has been previously described (24, 51). Hybridization with *EcoRI*-E faintly detected a 2.8-kb fragment within the MIB cell line but did not detect sequences in DNA prepared from the EBV-negative H9 or Bjab cell line. In early passages, hybridization with the *EcoRI* E fragment had also detected a 2.8-kb fragment in MIB DNA. Hybridization with a ^{32}P -labeled SP6 riboprobe of *BamHI*-C, synthesized from right to left on the EBV genome, detected an intact *EcoRI* J fragment in the MIB cell line and in a 1:50 dilution of Raji DNA (Fig. 2). Although the EBV copy number in Raji may vary from 50 to 100 copies, the lower intensity of hybridization in the MIB cell line may indicate that viral DNA is present at less than one copy per cell. To summarize, the only viral DNA sequences that were retained in later cell passages of the MIB cell line included an intact *EcoRI* J fragment and a small sequence from *EcoRI*-E.

We cannot exclude the possibility that additional frag-

FIG. 3. (A) Cellular DNAs digested with *Hind*III and probed with pHJ, a probe specific for the joining region of the human immunoglobulin heavy chain. The pHJ probe was labeled by nick translation with [α - 32 P]dCTP and hybridized to Southern blots of cellular DNA digested with *Hind*III. Germ line configuration is present in a T-cell leukemia line, H9 (lane 3), and in nasopharyngeal carcinoma (NPC) tissue (lane 5). The germ line band is 10.2 kb. An identical rearranged band at 8.7 kb is visible in the MIB line (lane 1) and in DNA of Bjab, an EBV-negative B-lymphoblastoid line (lane 4). The B-cell leukemic DNA has rearranged bands at 5.7 and 3.9 kb (lane 2). The intensity of hybridization in lane 2 is due to unequal loading of DNA. (B) *Eco*RI-digested cellular DNA probed with the cDNA clone Jurkat-2, which is specific for the beta chain of the T-cell receptor. The T-beta probe, isolated as insert DNA, was labeled by nick translation and hybridized to Southern blots of cellular DNAs. Lanes 3 to 5, containing DNAs from an NPC biopsy, Bjab, and Raji, respectively, represent the germ line configuration of T-beta sequences. The two constant (C) regions (C β 1 and C β 2) and the variable (V)-region genes are labeled at the right. H9, a T-cell leukemic line (lane 2), and MIB (lane 1) exhibit rearranged C β 1 bands at 9.0, 16.0, and 27.0 kb. Arrows at the left mark these rearranged fragments.

ments of EBV DNA have been retained in MIB cells. Probes which detect homologous sequences in one-copy reconstructions may not have detected those sequences in MIB. The *Eco*RI-E sequences appeared to be present at much less than one copy per cell; this retention at low level may be true of other EBV sequences as well.

Lack of transcription from retained sequences. At later cell passages, when it had been determined that sequences from *Eco*RI-J and -E were still retained, possible transcription from these sequences was investigated. The *Eco*RI-J sequence within *Bam*HI-C hybridizes to a number of early and late EBV mRNAs (20, 21). A 2.3-kb mRNA and a 2.0-kb mRNA are known to be partly transcribed from this region in latently infected cells. Throughout EBV infection, two small poly(A)⁻ transcripts (the EBV-encoded RNAs [EBERS]) are present in abundance. The coding sequences for the EBERS are wholly within the *Eco*RI J fragment of EBV (4, 43). To determine whether the *Eco*RI-J sequences were expressed in the MIB cells, Northern blots were prepared from poly(A)⁻ and poly(A)⁺ RNAs. These were probed with a *Bam*HI-C SP6 transcript. There was no evidence of expression of *Eco*RI-J in the MIB cell line although the probe detected abundant transcription from the *Eco*RI-J sequence in B95-8 cells; transcription of the 4.75-kb late transcript and the EBERS was seen with this probe in these productively infected cells.

Although fragments of 5.2, 4.5, and 2.8 kb homologous to *Eco*RI-E were found in early passages of MIB, only the 2.8-kb sequence was retained in late passages of the line and was present at less than one copy per cell. A nick-translated *Eco*RI-E probe detected no transcription from the region

when Northern blots of poly(A)⁺ RNA from MIB were probed. Griffin and Karran (14) obtained immortalized African green monkey kidney cell lines after transfection with cosmid clones containing *Eco*RI-E sequences. The retention of *Eco*RI-E sequences in transformation events in both epithelial cells and lymphocytes is interesting.

Determination of clonality of the cell line. The detection of multiple fragments of *Eco*RI-digested MIB DNA homologous to *Bam*HI-C, *Bam*HI-V, *Eco*RI-E, *Eco*RI-Dhet, and *Eco*RI-B suggested that the cell line was polyclonal and that the hybridization data reflected different integration events within several microinjected cells. The clonality of cells of lymphocytic origin can be assessed by determining the pattern of rearrangement of immunoglobulin or T-cell receptor genes (2, 3, 50).

To assess the clonality of the MIB cell line, immunoglobulin gene rearrangement was analyzed by using the pHJ probe which contains the joining-region sequences of the immunoglobulin heavy-chain locus (10). MIB DNA was compared with DNAs from a well-differentiated EBV-positive B-cell leukemia, Leu, and Bjab, an immunoglobulin-producing EBV-negative B-cell line (Fig. 3A). The unrearranged genomic fragment of 10.2 kb was detected in nasopharyngeal carcinoma and T-cell lymphoma (H9) DNAs. *Hind*III digestion revealed clear rearrangement in the leukemia DNA with bands of 5.7 and 3.9 kb and rearrangement in the MIB cell line with an additional band at 8.7 kb. A band of similar size was detected in the Bjab cell line. Restriction with *Eco*RI and *Bam*HI also identified similar gene rearrangement in the Bjab and MIB cell lines (data not shown) although the two cell lines have clearly

distinct karyotype and surface markers (27). The pattern of rearrangement of MIB DNA at the immunoglobulin-joining region suggests that these cells are a clonal population of B-cell lineage. Rearrangement at this locus occurs early in B-cell development and is not seen in T-cell neoplasia (35).

Karyotype analysis provided additional evidence for the monoclonal nature of the MIB cell line. Quinacrine fluorescence banding revealed that all cells were XO with a reciprocal translocation, t(1;7)(p34;q34). The breakpoint on chromosome 7 has been identified as a fragile site involved in chromosomal translocations in cultured human T lymphocytes (53) and is close to the gene which encodes the beta subunit of the T-cell receptor (22). Translocations at the region p34-p36 of chromosome 1 occur in 5% of acute nonlymphocytic leukemias and in 10% of non-Hodgkin lymphomas (28).

Gene rearrangement at the T-beta locus. Karyotypic analysis of MIB cells disclosed a reciprocal translocation involving chromosomes 1 and 7. Because of the proximity of the chromosomal breakpoint to the coding region of the beta chain of the T-cell receptor (22), we determined the arrangement of the T-beta sequences in MIB. The DNA arrangement of this gene was analyzed by using the cDNA clone Jurkat-2, which is specific for the beta chain of the T-cell receptor. Hybridization with the Jurkat-2 clone to *Eco*RI-digested MIB DNA was compared with hybridizations to H9 (a T-cell leukemia), CH5 (a biopsy of nasopharyngeal carcinoma), Raji, and Bjab cells. Based on published hybridizations of the Jurkat-2 clone to leukemic human T-cell CEM DNA (52), we can identify the fragments containing the two constant-region genes and the variable-region genes of the T-beta receptor. The constant-region sequences were present in 12.0- and 4.0-kb fragments in *Eco*RI-digested DNAs from CH5, Raji, and Bjab cells (Fig. 3B, lanes 3 to 5, respectively). Variable-region T-beta sequences migrated as 9.0-, 6.0-, 3.6-, and 2.4-kb fragments. H9 DNA (lane 2) contained the C β 2 4.0-kb fragment and a 9.0-kb rearranged C β 1 fragment. The other C β 1 allele appeared to be deleted in H9. MIB DNA (lane 1) showed strong hybridization to three fragments of 27.0, 16.0, and 4.0 kb. The larger bands were rearrangements of the C β 1 loci of both alleles; the smallest band represented C β 2. Variable sequences could be seen at 6.0 and 2.4 kb. The 9.0- and 3.6-kb fragments containing variable-region sequences were not present in MIB DNA. In summary, the MIB cell line is rearranged at both C β 1 loci of the T-beta receptor and is deleted for DNA fragments of 9.0 and 3.6 kb which contain variable-region sequences. These rearrangements and deletions may be the result of gene rearrangement at the T-beta locus or may reflect the close proximity of the chromosomal breakpoint.

Expression of the *c-fgr* locus in MIB cells. It is likely that the chromosomal translocation was a contributing factor in this transformation event. The breakpoint involved in this translocation occurs in hematological neoplasia (2, 28). Known cellular proto-oncogenes which have been mapped to chromosome 1 include *N-ras* at 1p13 (8), *B-lym* at 1p32 (33), *lck* at 1p32-35 (31), and *c-fgr* at 1p34-36 (28, 36, 46). The close proximity of *c-fgr* to the MIB translocation is of particular interest in that a recent report indicates that expression of the *fgr* oncogene is induced by EBV infection (5). To determine whether *c-fgr* was expressed in MIB cells, poly(A)⁺ RNA from the MIB cell line and the EBV-infected Raji cell line was hybridized to a ³²P-labeled SP6 RNA probe synthesized from an 841-base-pair *Ava*I-*Bam*HI fragment representing only *v-fgr* oncogene sequences (Fig. 4). The MIB line expressed a 2.3-kb message homologous to *v-fgr* in

contrast to the abundant 2.8-kb message detected in Raji cells. The mRNA homologous to *v-fgr* was not present at an elevated level. The altered size of the mRNA homologous to *v-fgr* in MIB may reflect the close proximity of the chromosomal translocation or perhaps expression of a related cellular locus. A recent report (31) describes a cellular gene, *lck*, which is less than 70% homologous to *c-fgr*, maps at 1p32-36 adjacent to the *fgr* locus on chromosome 1, and encodes a 2.3-kb mRNA.

DISCUSSION

In this work, neonatal cord lymphocytes immortalized after microinjection of EBV DNA did not retain the intact viral genome. The majority of viral sequences were lost from this line, and the EBV sequences were not expressed. This is in marked contrast to cell lines established by infection with EBV which retain multiple episomal copies of the intact viral genome and express several mRNAs (48). The result that we achieved is clearly different from the normal interaction of the virus with a recipient host cell.

It is possible that regulated expression of several gene functions is required for transformation by EBV. Fragmentation of the genome, likely to occur in delivery of DNA by microinjection, would interfere with this ordered process. The presence and eventual loss of viral sequences in MIB suggests that alterations in the recipient cells, ending in their

FIG. 4. Total RNAs were prepared from Raji and MIB cells by centrifugation of cell lysates through a cesium chloride step gradient. Poly(A)⁺ RNA was obtained by passage of total RNA through oligo(dT) columns. Raji (lane 1) and MIB (lane 2) poly(A)⁺ RNAs (5 μ g each) were electrophoresed in a formaldehyde-agarose gel and blotted onto nitrocellulose. A 0.841-kb *Ava*I-*Bam*HI fragment of *v-fgr*, ligated into pGEM1, was labeled with SP6 polymerase and [³²P]CTP and hybridized to the Northern blot at 44°C in 50% formamide. The blot was washed at 65°C. A band of 2.8 kb representing *c-fgr* mRNA is present in the Raji RNA; a 2.3-kb mRNA is present in the MIB RNA fractions. The markers at the left denote 5.2- and 2.0-kb rRNAs.

transformation, may have involved only transient viral expression.

Variation from the usual pattern of viral expression occurs in Burkitt's lymphoma (BL). Three nuclear antigens and a cytotoxic T-cell target (LYDMA) present at the surfaces of EBV-infected cells are known to be expressed in latent EBV infection (12, 18, 19, 26, 47). However, the latent protein LYDMA appears not to be expressed in all BL cell lines; several EBV-positive BL lines are not susceptible to HLA-matched LYDMA-specific killing (42). Another of the latent viral functions, EBNA-2, is apparently essential for transformation in that P3HR-1, a nontransforming subclone of the transforming BL Jijoye strain, is deleted for the sequences which encode EBNA-2 (25, 40, 41). Superinfection of the Raji cell line with HR-1 virus generates transformation-competent recombinants which have regained the EBNA2 sequences (44). Daudi cells, derived from a BL cell, are also deleted for EBNA-2 sequences (23). This implies that the viral sequences which may be necessary for initiation of transformation are no longer required in BL cells. This may be due to changes effected in the cells by viral expression.

The permanent changes which result from chromosomal translocation of cellular sequences in MIB may be sufficient for transformation in the absence of continued viral expression. Genesis of a nonlethal growth-inducing translocation would presumably be a rare event probably not fundamental to the relatively efficient mechanism of EBV transformation but intrinsic to the multistep progression to malignancy. It is striking that the MIB chromosomal translocation which involves DNA sequences having the inherent potential to rearrange, and which may alter a cellular oncogene, so parallels the translocations involving the immunoglobulin loci in BL cells. This malignancy is characterized by distinctive chromosomal translocations, t(8;14), t(2;8), and t(8;22), which involve rearrangement of the immunoglobulin heavy- or light-chain loci (6, 7, 10, 11, 29, 45). These translocations are apparently due to aberrant somatic recombination resulting in translocation of the *c-myc* oncogene. Translocation of *c-myc* seems to result in deregulation of expression of that gene (9). The deregulation must be a significant contribution to the malignant course of cell growth.

Similarly, the translocation in the MIB cell line may have resulted in deregulation of a cellular oncogene. A 2.3-kb mRNA homologous to *c-fgr* is expressed in the MIB cell line. The locus of this oncogene is in close proximity to the chromosomal breakpoint in the microinjected line (46). This site is also near the location of the *lck* gene and is a region of chromosomal anomalies in human lymphomas (28, 31). Both *fgr* and *lck* encode protein tyrosine kinases belonging to the *src* family of proteins believed to play a role in neoplastic transformation. It has been shown that a 3.0-kb message homologous to *c-fgr* is found in EBV-transformed peripheral blood cells and EBV-positive BL cell lines (5). Both transforming and nontransforming EBV isolates induce *c-fgr* expression; therefore, induction of that proto-oncogene is not alone sufficient for transformation. Further study is necessary to determine whether the transformation event which occurred in MIB involved alteration of the *c-fgr* or *lck* locus or perhaps alteration of a related locus mapping to the breakpoint region.

EBV expression, which initially produces polyclonal-cell proliferation, may potentiate cellular rearrangement in multiple stages of progression in the malignant process. The multiple DNA rearrangements and chromosomal translocation of the MIB cell line may reflect aberrant activation of

somatic DNA-rearranging mechanisms and indicate possible viral involvement in this process.

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