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The Level of c-*fgr* RNA Is Increased by EBNA-2, an Epstein-Barr Virus Gene Required for B-Cell Immortalization

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The efficient immortalization of primary resting human B lymphocytes by Epstein-Barr virus (EBV) requires several viral genes and presumably the altered expression of an unknown number of cellular genes as well. In this paper, I show that infection of primary human B cells with EBV increased the transcript level of the proto-oncogene, c-*fgr*, 10-fold. This effect on the level of c-*fgr* transcripts in B cells was not secondary to blast formation, because levels of c-*fgr* RNA were also increased 10-fold in two proliferating EBV-negative Burkitt's lymphoma-derived cell lines, Ramos and BJAB, 2 days after infection with EBV. Two lines of evidence indicated that EBV nuclear antigen 2 (EBNA-2) mediates this increase in c-*fgr* RNA levels: acute infection of BJAB and Ramos cells by a mutant strain of EBV that lacked the EBNA-2 open reading frame, P3HR1, did not affect c-*fgr* RNA levels; and cell lines constitutively expressing only the EBNA-2 gene of EBV had increased levels of c-*fgr* RNA relative to those in the parental cell lines. Since P3HR1, a nonimmortalizing strain of EBV, failed to affect c-*fgr* RNA levels and since a viral gene required for B-cell immortalization was responsible for the induction of c-*fgr*, the data indicate a possible role of c-*fgr* expression in B-lymphocyte immortalization by EBV and a mechanism by which EBNA-2 contributes to the immortalizing activity of EBV.

Epstein-Barr virus (EBV) is a human herpesvirus that causes infectious mononucleosis and is associated with two human cancers, Burkitt's lymphoma and nasopharyngeal carcinoma (29). In vitro EBV infects primary human B lymphocytes and efficiently transforms them into blasts that can proliferate indefinitely in culture. Except for infection by EBV, no conditions are available for inducing and maintaining proliferation of primary human B-cells in culture. In these immortalized cells, the virus establishes a latent state, with multiple copies of the viral genome (172 kilobases) maintained as plasmids. At least eight viral genes are expressed in EBV-immortalized lymphoblastoid cell lines (22), and at least three of them, EBV nuclear antigen 1 (EBNA-1), EBNA-2, and latent membrane protein (LMP), may be required for B-cell immortalization (3, 10, 56, 59). Genetic studies have identified one viral gene, EBNA-2, as essential but probably not sufficient for immortalization (9, 14, 33). The cellular genes required for immortalization are unknown.

The cellular gene c-*fgr* encodes a protein tyrosine kinase and is a member of the *src* gene family. The gene *fgr* was first identified in an altered form, v-*fgr*, in the oncogenic retrovirus Gardner-Rasheed feline sarcoma virus (35) and has recently been identified in a second viral isolate, the Theilen-Pedersen I feline sarcoma virus (16). The genes of the *src* family—*src*, *fgr*, *yes*, *syn*, *lyn*, *lck*, and *hck*—possess a conserved exon-intron structure and encode proteins of similar size that have a strong similarity in the amino acid sequences of the tyrosine kinase domains. Two members of this family besides *fgr* have been found in oncogenic retroviruses: *src* (15) and *yes* (18). The association of tyrosine kinase activity with retroviral transforming viruses and with growth factor receptors has led to the notion that the products of the genes of this family function in growth regulation.

Transcripts of c-*fgr* have been detected in both normal and neoplastic cells, but from a limited number of tissues. Tumors of human lymphoid and myeloid cell lineages express c-*fgr* RNA, as do human and murine primary myeloid cells (19, 24, 57). The c-*fgr* gene product, therefore, like the *src* gene product, may function in terminally differentiated cells and in tumor formation.

In screening a variety of cell lines derived from human tumors, Cheah et al. (6) detected c-*fgr* RNA only in cell lines derived from certain lymphomas. The expression of c-*fgr* RNA correlated with the presence of EBV in these cell lines. This correlation held for cell lines containing endogenous EBV, e.g., Raji or Namalwa, as well as for EBV-negative Burkitt's lymphoma cells that had been infected with EBV in vitro and then selected to stably maintain the viral genome. No correlation with EBV was observed for the expression of two other protein tyrosine kinase genes, *fms* and *fes*. The question remained whether EBV infection, i.e., the virus itself and not a selection process, affected c-*fgr* RNA levels, and if so, which viral gene(s) was responsible.

In this manuscript, I report the possible participation of c-*fgr* expression in B-cell immortalization. I demonstrate that soon after infection of primary human B lymphocytes and of two EBV-negative Burkitt's lymphoma-derived cell lines by EBV, the levels of c-*fgr* transcripts increased. EBNA-2, an EBV gene known to be required for B-lymphocyte immortalization, was identified as the viral gene that induced the accumulation of c-*fgr* RNA by two lines of evidence: (i) infection of EBV-negative Burkitt's lymphoma-derived cell lines with a nonimmortalizing, mutant strain of virus that lacked the EBNA-2 gene failed to increase the level of c-*fgr* and RNA; (ii) cell lines that were derived from an EBV-negative Burkitt's lymphoma and that constitutively expressed only the EBNA-2 gene of EBV had increased levels of c-*fgr* RNA.

MATERIALS AND METHODS

Cells and viruses. All cell lines were grown in RPMI 1640 medium with the following serum additives: 10% fetal bovine serum for cell lines derived from EBV-negative Burkitt's

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lymphoma biopsies, BJAB (21) and Ramos (20), and all recently transformed lymphoblastoid cell lines, 11/17-3, 11/17-4, 11/17-5, THLB1, and THLB 5 (47); 5% calf serum for a clone of the B95-8 marmoset cell line (30); and 10% calf serum for a clone of P3HR1, HH514 clone 16, that lacks *het* DNA (34).

Primary human B lymphocytes were isolated from peripheral blood donated by the American Red Cross. The cells were obtained from the cell fraction left after aphoresis. The purification of B cells included the following: isolation of cells by centrifugation over Renografin-Ficoll, incubation for 1 h in complete medium to allow monocytes to attach, removal of nonadherent cells to plates coated with rabbit anti-human immunoglobulin, removal and discarding of nonadherent primary T cells after 2 h, and incubation with medium or virus stock (45). These B-cell populations may contain other cell types, because not every cell stained for cell surface immunoglobulin by immunofluorescence.

Viral stocks were obtained from the supernatants of cultures of B95-8 or P3HR1 cells incubated with 20 ng of 12-*O*-tetradecanoylphorbol-13-acetate and 3 mM sodium butyrate for 5 days.

Recombinant plasmids. The plasmid pCMV-BZLF-1 contains the EBV gene BZLF-1 driven by the cytomegalovirus immediate-early promoter and enhancer (13). Plasmid p220, constructed by N. Warren and B. Sugden (this laboratory), was derived from p201 (59) by insertion of a polylinker at the *Nar*I site and expresses EBNA-1. Plasmids p554, p633, and p615, constructed by W. Hammerschmidt, have been described previously (14) and are shown in Fig. 3. Plasmid pZip-E2 (56) was obtained from F. Wang and E. Kieff (Harvard University) and contains the EBNA-2 open reading frame driven from the murine leukemia virus long terminal repeat. The antisense *fgr* probe was derived from pv-*fgr*1 (49), which was obtained from K. Robbins (National Cancer Institute), by digestion with *Sma*I and *Bam*HI and insertion of this fragment into M13mp19. Single-stranded virion DNA was then isolated by standard procedures (instruction manual from Bethesda Research Laboratories, Inc.). The clone TDp326 contains a 0.62-kilobase *Pvu*II fragment from the human c-*fgr* gene that includes exon 7 and part of exon 8 subcloned into T3T718 and was obtained from T. Ley (24).

Infection. The EBV-negative Burkitt's lymphoma-derived cell lines were incubated at 2×10^5 cells per ml with the virus stock for 2 h. The cells were collected by centrifugation and then suspended in fresh medium for 48 h. Cells were harvested, washed, pelleted, and stored at −70°C for RNA isolation. For primary B lymphocytes, 5×10^7 cells per 100-mm plate were incubated for 2 h with 8 ml of medium or virus stock while the B cells were still bound to the plate. The virus stock was replaced with fresh medium, and the cells were incubated overnight. Cells were removed from the plate by pipetting. Uninfected cells were harvested for RNA isolation, and infected cells were cultured for 5 days and then harvested.

The percentage of cells infected with EBV was determined by EBNA staining by anticomplement immunofluorescence with human serum, as described by Reedman and Klein (36).

Electroporation. Ramos cells (4×10^6) were electroporated with the UW electroporator described previously (23). The conditions used were as follows: peak voltage, 680 V; fall time, 40 ms. Cells were counted after 2 to 4 days and plated at 10^6 and 10^5 cells per plate with the drug used for selection, either G418 (2 mg/ml) or hygromycin (300 μg/ml). For cells electroporated with pZip-E2 (10 μg), five of six plates originally containing 10^5 cells had resistant cells after 3.5 weeks. Two of these were positive for EBNA-2 expression. For cells electroporated with p220 (10 μg) one plate of 10^6 cells had cells that were resistant to hygromycin and expressed EBNA-1.

Cells containing plasmid-derived virus. P3HR1 cells were electroporated with pCMV-BZLF-1 and p554, p633, and p615 as described previously (14) and briefly below. The supernatant from these cultures contained plasmid-derived virus. Ramos cells containing p220 (Ramos+p220) (4×10^6) were incubated overnight with supernatant (5 ml). The next day the cells were seeded at 10^6 or 10^5 cells per plate with 2.0 mg of G418 per ml. Cells populations resistant to G418 arose from cultures incubated with the indicated supernatants with the following frequencies: p554, one of three cultures originally plated at 10^6 cells per plate; p633, five of six cultures originally plated at 10^5 cells per plate; and p615, three of six cultures originally plated at 10^5 cells per plate. All selected populations tested by Western analysis expressed the appropriately sized EBNA-2 protein, either full length from p554 and p633 or truncated from p615.

Northern analysis. RNA was isolated with either urea lysis buffer and phenol-$CHCl_3$ (41) or guanidinium isothiocyanate (7). Total RNA was quantitated by optical density at 260 nm. RNA was separated in a formaldehyde–1% agarose gel, transferred to a nylon membrane (Zeta probe), and cross-linked by UV irradiation. To obtain increased sensitivity, I probed the blot in two steps. First, antisense v-*fgr* in a single-stranded M13 vector (2 μg) was hybridized in 10 ml hybridization buffer (8) for about 24 h at 68°C. The blot was washed three times and then hybridized overnight with a ^{32}P-radiolabeled probe of single-stranded M13mp18 without an insert. The probe was made by the oligonucleotide random primer method (12). The blots were exposed to preflashed film, and autoradiogram exposures in the linear range were quantitated with a laser densitometer.

Western immunoblot analysis. Rabbit antiserum to an EBNA-2–trypE fusion protein was kindly provided by Oleg Pavlish and Georg Bornkamm (Munich). Cell lysates were separated on a 7.5% acrylamide gel, electrophoretically transferred to nitrocellulose, and developed with the EBNA-2 antiserum and goat anti-rabbit antibody conjugated with alkaline phosphatase. Affinity-purified rabbit antisera was used for detection of LMP (2) and EBNA-1 (L. Sternas, T. Middleton, and B. Sugden, submitted for publication).

S1 nuclease analysis. The clone TDp326 was digested with *Xmn*I; the 2.36-kilobase fragment was isolated and 5′ end labeled with [γ-^{32}P]ATP (25). This probe (75,000 cpm) was hybridized with total cellular RNA in 15 μl at 53°C overnight. Hybridization and S1 nuclease protection analyses were performed as described previously (11). The blots were exposed to preflashed film, and autoradiogram exposures in the linear range were quantitated with a laser densitometer.

In vitro transcription of c-*fgr* RNA. In the plasmid TDp326, the genomic fragment of c-*fgr* is positioned downstream from the T7 promoter. This plasmid was transcribed with T7 polymerase (Stratagene) and digested with DNase. The RNA was extracted, precipitated, suspended in buffer, and quantitated by A_{260}. A portion of the RNA separated on a 4% acrylamide–urea gel indicated that the appropriate-sized RNA had been made.

RESULTS

Primary human B lymphocytes infected with B95-8 virus had increased levels of c-*fgr* transcripts. If increased expres-

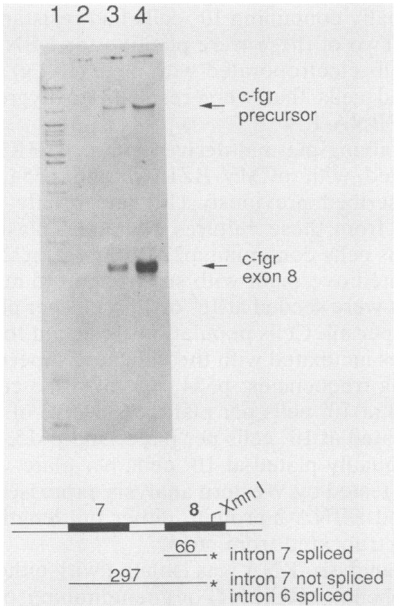

FIG. 1. Increased expression of c-*fgr* RNA in uninfected or B95-8-infected primary human B lymphocytes. Primary human B lymphocytes were isolated from peripheral blood and infected with B95-8 or left uninfected. Uninfected and infected cells were obtained from the same preparation. Indirect immunofluorescence staining indicated that 69% of the infected B-cell population was EBNA positive. Total cellular RNA was isolated from uninfected cells after 1 day in culture and from infected cells after 5 days. The RNA (5 μg) was hybridized with 75,000 cpm of radiolabeled probe consisting of a fragment of the c-*fgr* gene that includes exon 7 and part of exon 8. The diagram at the bottom indicates the S1 nuclease protected fragment sizes arising with different extents of processing. The 66-nucleotide protected band was produced with RNA lacking both introns 6 and 7 and was designated c-*fgr* exon 8; the 297-nucleotide band was produced with RNA in which intron 7 has not been removed and was denoted c-*fgr* precursor. Lanes: 1, DNA markers, pBR322 digested with *Msp*I; 2, tRNA control; 3, 5 μg of total RNA from uninfected B cells; 4, 5 μg of total RNA from infected B cells.

sion of c-*fgr* played a role in B-cell immortalization, the levels of c-*fgr* RNA should increase in primary human B lymphocytes after infection with B95-8, the prototype immortalizing strain of EBV. The levels of c-*fgr* transcripts were analyzed by S1 nuclease protection assays in B cells that were uninfected or infected for 5 days with the B95-8 strain of EBV (Fig. 1). EBV infection produced at least a 10-fold increase in c-*fgr* transcripts. Similar results were also observed with different RNA preparations analyzed by Northern (RNA) blotting (data not shown).

To determine whether the elevated levels of c-*fgr* RNA were maintained, the level of c-*fgr* transcripts in immortalized lymphoblastoid cell lines that were in culture for less than 1 year (47) was compared by S1 nuclease analysis with the level found in Ramos cells, which are derived from an EBV-negative Burkitt's lymphoma. Although all of the EBV-positive lymphoblastoid cell lines had higher levels of c-*fgr* transcripts than did the EBV-negative Ramos cells, the level of c-*fgr* transcripts clearly varied, with cell lines 11/17-3, 11/17-4, 11/17-5, THLB-1, and THLB-5 cells having 12-, 1.5-, 5-, 20-, and 10-fold increases, respectively, above levels in Ramos cells.

Infection with B95-8 virus but not P3HR1 virus increased the levels of c-*fgr* RNA. B95-8 is an immortalizing virus, and

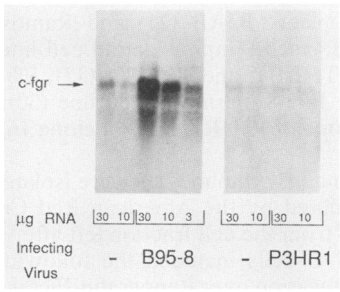

FIG. 2. c-*fgr* RNA levels in BJAB cells infected with EBV. BJAB cells were infected with EBV strain B95-8, an immortalizing strain, or P3HR1, a nonimmortalizing, mutant strain. The percentage of cells infected was the same for both strains (83 and 84%, respectively). Total cellular RNA was isolated 48 h after infection, separated in a 1% agarose–formaldehyde gel, transferred to Zeta probe, and hybridized with a probe of antisense v-*fgr* in an M13 vector.

P3HR1 is a mutant EBV strain that cannot immortalize human B lymphocytes (34). A deletion in P3HR1, which encompasses nucleotides 45,644 to 52,450 of B95-8 (1), affects two viral genes, EBNA-LP and EBNA-2, that are expressed from B95-8 virus in latently infected cells (9, 42, 44). P3HR1 lacks the last two exons of EBNA-LP and the entire open reading frame for EBNA-2.

Two EBV-negative cell lines derived from Burkitt's lymphomas, BJAB and Ramos, were infected with B95-8 virus. Total cellular RNA was extracted from infected and uninfected cells, and the levels of c-*fgr* RNA were measured by Northern analysis with v-*fgr* sequences as a probe. The data obtained with BJAB cells are shown in Fig. 2. B95-8 infection of BJAB and Ramos cells increased c-*fgr* RNA approximately 10-fold after 48 h (Fig. 2 and data not shown). With RNA from both BJAB and Ramos cells, the v-*fgr* probe hybridized to a 2.6-kilobase RNA that comigrated with an RNA from Raji cells, an EBV-positive Burkitt's lymphoma-derived cell line that expresses high levels of c-*fgr* RNA (6, 31). Subsequent analysis by S1 nuclease protection indicated that the 2.6-kilobase RNA was c-*fgr* RNA, and not the RNA of another tyrosine kinase that cross-reacts with the v-*fgr* probe (data not shown).

BJAB and Ramos cells were also infected with the nonimmortalizing strain of EBV, P3HR1. Infection with this viral strain did not affect the level of c-*fgr* RNA in either cell line (Fig. 2 and data not shown). The lack of effect of P3HR1 infection was not a result of infection efficiency, since the percentage of cells infected by each viral strain was similar. P3HR1 virus infected 84% of BJAB cells and B95-8 infected 83% of BJAB cells, as monitored by immunofluorescence staining for viral nuclear antigens. The level of c-*fgr* RNA in primary human B lymphocytes was also not affected by infection with P3HR1 virus as monitored by Northern analysis (data not shown). The infection efficiency of primary human B lymphocytes by P3HR1 virus cannot be quantitated by immunofluorescence staining for viral nuclear antigens (40); however, the same P3HR1 stock was capable of infecting 90% of BJAB cells.

To determine the approximate copy number of c-*fgr* transcripts, the amount of hybridizing RNA from 30 μg of Ramos total cellular RNA or 1 μg of Raji cellular RNA was compared by S1 nuclease protection analysis with the amount hybridizing with known concentrations of *fgr* RNA sequences transcribed in vitro (data not shown). Assuming 10 pg of total RNA per Ramos cell, based on RNA recover-

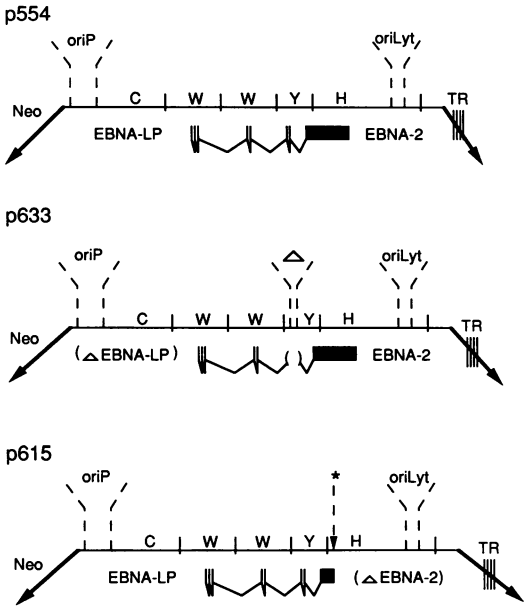

FIG. 3. Diagrams of the plasmids p554, p633, and p615. The thin horizontal lines represent contiguous sequences derived from EBV, and the vertical lines labeled TR denote the terminal repeats of EBV. The solid heavy lines represent sequences derived from the vector pKan 2. The vertical lines above the EBV sequences denote sites of cleavage with *Bam*HI, and the letters between these lines indicate the different BamHI cleavage products of EBV. oriP and oriLyt represent respectively the origin of plasmid and lytic replication of EBV. oriP permits the plasmid to replicate in cells that express EBNA-1; oriLyt, together with the terminal repeats, permits the plasmid to replicate and be packaged into virions during the viral lytic cycle. Neo represents the gene that confers resistance to the drug G418. The bicistronic transcript of EBNA-LP and EBNA-2 is depicted below each plasmid with the vertical lines (EBNA-LP) and the solid box (EBNA-2) denoting its exons. Plasmids p633 and p615 are derived from p554. Plasmid p633 contains a deletion that removes the last two exons of the EBNA-LP open reading frame (△) but leaves that of EBNA-2 intact. Plasmid p615 contains termination codons in all three reading frames at a site one-third of the distance into the EBNA-2 open reading frame, denoted by an asterisk.

ies, there were approximately 10 molecules of c-*fgr* RNA per uninfected Ramos cell and 100 copies per infected cell. Similar copy numbers were found in uninfected and infected BJAB cells. Raji, an EBV-positive, Burkitt's lymphoma-derived cell line that contains about 25 pg of total RNA per cell, had about 750 molecules of c-*fgr* RNA per cell. Even after infection of Ramos cells with EBV, the level of c-*fgr* RNA was less than that in Raji cells.

At least eight gene products are expressed in lymphoblastoid cell lines latently infected with EBV. Three of these proteins can affect the expression of other genes—EBNA-1 (38, 48), LMP (55), and EBNA-2 (56). To investigate whether any of these genes might be responsible for affecting c-*fgr* RNA levels, I compared by Western blots the expression of these viral genes in EBV-negative, Burkitt's lymphoma-derived cells infected with B95-8 or P3HR1 for 48 h (data not shown). Similar levels of EBNA-1 and LMP were observed in BJAB cells infected with B95-8 or P3HR1 for 48 h. As expected, since the P3HR1 virus lacked the EBNA-2 open reading frame, EBNA-2 protein was detected only in cells infected with B95-8 virus. Since c-*fgr* RNA expression was not affected by infection with P3HR1 virus, it seemed likely that EBNA-2 and/or EBNA-LP, the two genes affected by the deletion in the P3HR1 genome, was responsible for the induction of c-*fgr* RNA either alone or with other viral gene(s).

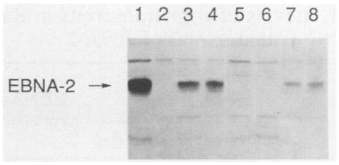

FIG. 4. Expression of EBNA-2 in Ramos cells containing various constructs. Cell extracts from 10^6 cells were separated in a 7.5% acrylamide gel, electrophoretically transferred to nitrocellulose, and probed with a rabbit antiserum to EBNA-2. Lanes: 1, 721, a latently infected EBV-positive lymphoblastoid cell line; 2 through 4, Ramos+p220 cells carrying defective virus genomes derived from plasmid p615 (lane 2), p633 (lane 3), or p554 (lane 4); 5, Ramos+p220 cells; 6, Ramos cells; 7 and 8, two populations (i and ii) of Ramos cells electroporated with pZip-E2.

EBNA-2 gene product is sufficient to produce increased levels of c-*fgr* RNA. To determine whether EBNA-2, EBNA-LP, or both were responsible for increases in c-*fgr* RNA levels, vectors were used to introduce these genes singly or together into Ramos cells.

EBV particles can be produced in which the viral genome is replaced by concatamers of certain plasmids (14). The plasmids shown in Fig. 3 contained all of the elements to permit (i) replication and packaging during the viral lytic cycle, (ii) nonlytic replication in cells that expressed EBNA-1, (iii) drug selection, and (iv) expression of part of the EBV genome. To produce these viral particles, a plasmid was electroporated into the P3HR1 virus producer cell line, also called P3HR1, together with an inducer of the viral lytic cycle, pCMV-BZLF-1. The supernatant from this culture contained parental P3HR1 virus, virus particles with genomes that were concatamers of the plasmid, and virus in which the genome was a recombinant between the parental virus and the plasmid.

Ramos+p220 cells, which were EBV negative and expressed EBNA-1, were infected with culture supernatant made with plasmid p554, p633, or p615. Cell lines resistant to G418 were selected and analyzed for the expression of EBNA-2 (Fig. 4). Resistant cell lines infected with vp554 or vp633 expressed full-length EBNA-2 (Fig. 4), whereas those infected with vp615 expressed a truncated form (detected with human serum; data not shown). Cell lines expressing full-length EBNA-2 had increased levels of c-*fgr* RNA relative to those in parental cells (Table 1). The same results were observed with two, three, and four resistant cell lines obtained after infection with vp554, vp633, and vp615, respectively. These resistant cell lines were derived from two different recipient Ramos cell lines that express EBNA-1 (data not shown). Hammerschmidt and Sugden (14) determined that virus particles derived from p554 and p633, but not p615, complement P3HR1 virus to immortalize B cells, thus demonstrating that EBNA-2 is required for immortalization. Virus particles derived from the same plasmids also induced increased levels of c-*fgr* RNA when the genomes were maintained in Ramos cells (Table 1).

Since the culture supernatants contained not only the virus particles derived from a plasmid but also parental and recombinant viruses, cells may have been infected with more than one virus particle. Cell lines resistant to G418, therefore, were analyzed for the presence of parental virus. Southern blots of total cellular DNA (12 μg) probed with the sequences that encode LMP, a viral gene present in the

TABLE 1. Levels of c-*fgr* transcripts in Ramos cells that express EBNA-2

Cell line[a]	Intact open reading frame		EBNA-2 expression[b]	Level of c-*fgr* transcripts[c]
	EBNA-LP	EBNA-2		
Ramos			−	1
Ramos+p220			−	1
Ramos+p220+vp554	+	+	+	5–10
Ramos+p220+vp633	−	+	+	5–10
Ramos+p220+vp615	+	−	−	1

[a] EBV-negative Burkitt's lymphoma-derived Ramos cells were electroporated with and selected for cells that carry p220 and that express EBNA-1. Ramos+p220 cells were infected with culture supernatant that contained virus derived from plasmids p554, p633, or p615 (Fig. 3) and selected with G418. The plasmid-derived virus genomes are designated vp554, vp633, and vp615, respectively. Generation of these viruses was as described previously (14) and briefly in the text.

[b] EBNA-2 expression (Fig. 4) was monitored by Western analysis with a monospecific rabbit antiserum.

[c] The level of *fgr* transcripts was determined by Northern analysis and S1 nuclease protection analysis. The levels have been normalized to that found in the parental Ramos cell line, which contains approximately 10 molecules of *fgr* RNA per cell.

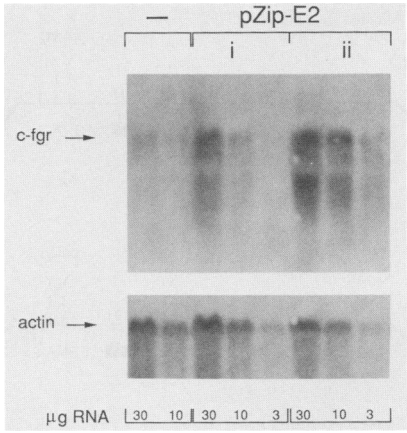

FIG. 5. Expression of c-*fgr* RNA in Ramos cells and Ramos cells transfected with an EBNA-2 expression vector, pZip-E2. Total cellular RNAs from Ramos cells and two Ramos cell lines (i and ii) that carry pZip-E2 and that express EBNA-2 were analyzed for c-*fgr* RNA by Northern blotting as described in the legend to Fig. 1. The probe hybridizing to the blot in the top panel was antisense v-*fgr* in an M13 vector. The blot was reprobed with actin sequences (bottom panel) to confirm the amount of RNA applied.

parent virus but not the plasmid-derived virus, indicated that the LMP sequence was not present in the selected cell lines at a level of one molecule per cell (data not shown). Therefore, the EBNA-2 expression sequences, but not the parental P3HR1 viral genome, were maintained in the cell lines analyzed for c-*fgr* expression.

EBNA-2 was strongly implicated as the viral gene responsible for affecting c-*fgr* expression by the results with acute infection with P3HR1 and B95-8 virus and with cell lines constitutively expressing EBNA-2 or EBNA-LP. To eliminate the possibility that other EBV sequences contained in the selected cells contributed to the effect on c-*fgr* RNA, I selected cell lines in which the EBNA-2 open reading frame was the only EBV sequence present. Plasmid pZip-E2, which expresses the EBNA-2 open reading frame from the Moloney murine leukemia virus long terminal repeat, was electroporated into Ramos cells, and cells resistant to G418 were selected. The two cell lines (i and ii) that expressed EBNA-2 by Western analysis (Fig. 4) had increased levels of c-*fgr* RNA (Fig. 5). S1 nuclease protection analyses of RNAs from these cell populations indicated 3- and 10-fold increases in c-*fgr* transcripts in cell lines i and ii, respectively. The amount of RNA applied to the gel was confirmed by reprobing the blot with actin sequences (Fig. 5).

DISCUSSION

The transformation of resting human B lymphocytes into proliferating blasts by EBV was accompanied by at least a 10-fold increase in the level of c-*fgr* transcripts. This increase was not simply a result of blast formation, because EBV also induced elevated levels of c-*fgr* RNA in proliferating Ramos and BJAB cells. Two lines of evidence indicate that EBNA-2 mediates the increased levels of c-*fgr* transcripts: first, a mutant strain of EBV that lacked the EBNA-2 open reading frame failed to affect c-*fgr* RNA levels; second, cell lines constitutively expressing EBNA-2 possessed higher levels of c-*fgr* transcripts than the parental lines. These results support the ideas that (i) a function of EBNA-2 is the regulation of cellular gene expression and (ii) induction of c-*fgr* RNA may play a role in B-cell immortalization.

The EBNA-2 gene product is required for B-cell immortalization (14); however, the biochemical function of this protein has not been defined. The experiments described in this paper demonstrate that EBNA-2 affects the RNA levels of the cellular gene c-*fgr*. The mechanism by which the EBNA-2 protein induces the expression of this cellular gene—transcription, RNA stability, RNA processing, etc.—is not known. EBNA-2 also increases the level of the latent viral protein, LMP (unpublished data). LMP is an oncogene, since expression of LMP in rodent fibroblasts confers anchorage-independent growth on these cells (3, 54). EBNA-2, therefore, affects the expression of both a cellular protooncogene and a viral oncogene. Increased expression of the cellular gene CD23 was reported in one EBV-negative, lymphoma-derived cell line, Loukes, that constitutively expressed EBNA-2 (56). However, this effect may not be general for all cells. Binding of radiolabeled anti-CD23 antibody by the method of Sugden and Metzenberg (46) indicated that EBNA-2 expressed from the p554-derived virus did not increase the level of CD23 protein in Ramos+p220 cells (unpublished data).

Cheah et al. (6) reported detectable levels of c-*fgr* mRNA in BJAB cells that stably maintain the B95-8 genome and in Ramos cells that stably maintain the P3HR1 genome, whereas they detected no c-*fgr* mRNA in BJAB or Ramos cells. These virally infected cells are very different from the recently infected cell lines described in this paper. Unlike the infection of primary B-lymphocytes, when EBV-negative, Burkitt's lymphoma-derived cell lines such as Ramos cells are infected with EBV, the virus is lost in the population within a few weeks (37; unpublished data). A rare cell, however, can be selected that maintains the virus (5). It is possible that a cell that expressed elevated levels of c-*fgr*, independent of the virus, was selected while the Ramos-P3HR1 cell line used by Cheah and co-workers was being established.

Of the seven *src*-like genes identified, the *src* (39), *syn-slk-fyn* (17, 43), *lyn* (58), and *yes* genes have been found to be expressed in many tissue types. The others, (*fgr* and *lck* [28] and *hck* [32, 60]), have been detected in fewer tissues, principally in hematopoietic cells. Several observations have associated the expression of c-*fgr* with alterations in cell growth or differentiation of hematopoietic cells. Increased

levels of transcripts of c-*fgr* have been detected in primary monocytic cells stimulated to proliferate with colony-stimulating factor 1 (57). The addition of 12-*O*-tetradecanoylphorbol-13-acetate to the promonocytic cells U937 induced an increased accumulation of *fgr* transcripts and a differentiated phenotype (24). The association of increased c-*fgr* expression with immortalization of B cells by EBV is another example of a possible function of c-*fgr* in hematopoietic cells.

The notion that increased levels of an *src*-like cellular gene product may play a role in transformation was first proposed for the *lck* gene (28). High levels of *lck* cellular tyrosine kinase activity were first found in the cell line LSTRA, which is derived from a Moloney murine leukemia virus-induced T-cell lymphoma. In these cells the virus affects neither the coding sequences of the *lck* gene nor the activity of the enzyme it encodes, but rather increases the levels of the protein by insertion within the transcribed region of the *lck* gene. This insertion increases both the rate of transcription by the use of the promoter in the Moloney murine leukemia virus long terminal repeat (53) and the efficiency of translation by eliminating several upstream potential AUG start sites (27). Whether this increase in enzyme level is sufficient for transformation of T cells is unknown. The modulation of *lck* levels during the activation of primary murine T cells further supports a role for *lck* expression in the growth control of T cells (26, 52).

A possible function for *fgr* protein is also suggested by recent findings with the *lck* gene product, $p56^{lck}$. The *lck* protein can be coimmunoprecipitated with the T-cell surface antigens CD4 and CD8 from murine T-cell lines (50). Furthermore, cross-linking of the cell surface CD4 molecules with anti-CD4 antibodies increases the tyrosine kinase activity of the immunoprecipitated CD4 complex (4, 51). $p56^{lck}$, therefore, may be involved in signal transduction mediated through the CD4 receptor during T-cell activation. By analogy, I speculate that the increased level of c-*fgr* in B cells functions in the transduction of growth signals from the cell surface. This scenario is especially intriguing because the viral gene EBNA-2 increases the levels of both c-*fgr* and LMP, a viral oncogene located on the cell surface.

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