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fgr proto-oncogene mRNA induced in B lymphocytes by Epstein–Barr virus infection

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Several acute transforming retroviruses encode tyrosine-specific protein kinases which possess structural and functional relationships to cell-surface receptors for certain growth factors[1,2]. One such tyrosine kinase is encoded by the *onc* gene, v-*fgr*, of Gardner–Rasheed feline sarcoma virus (GR-FeSV)[3–6]. Recently, we have isolated and characterized the human gene, c-*fgr*, corresponding to the viral *onc* sequence and have shown that c-*fgr* is a unique gene located on the short arm of chromosome 1 (ref. 7). Here we report that certain lymphomas (but not sarcomas or carcinomas) express *fgr*-related messenger RNA. This transcript is detected in Burkitt's lymphoma cell lines naturally infected with Epstein–Barr virus (EBV), but not in EBV-negative Burkitt's lymphoma cells. Normal umbilical cord or peripheral blood lymphocyte lines established *in vitro* by EBV infection also contain detectable c-*fgr* mRNA. Moreover, a 50-fold increase of the steady-state c-*fgr* mRNA concentration is observed when uninfected Burkitt's lymphoma cell lines are deliberately infected with EBV. These findings demonstrate for the first time the induction of a proto-oncogene in response to infection by a DNA tumour virus.

To investigate whether the *fgr* proto-oncogene was expressed in human tumour cells, RNAs were prepared from selected cell lines representing a diverse spectrum of human cancers. After selection on oligo(dT) columns, poly(A)$^+$ RNAs were examined for the presence of v-*fgr*-related mRNA by Northern blotting. c-*fgr* transcripts were detected in 6 of 11 cell lines derived from myelo- or lymphoproliferative disorders, but not in any of the 9 carcinoma or 6 sarcoma cell lines examined. In a representative experiment (Fig. 1) the *fgr*-related mRNA detected was 3 kilobases (kb) long, which is in close agreement with the length of the transcript detected in normal human lung tissue[7].

Because our initial survey had indicated the presence of the c-*fgr* transcript in cell lines derived from lymphoproliferative disorders, our analysis was extended to include various B-cell lines derived from African and American undifferentiated lymphomas of the Burkitt and non-Burkitt types. As summarized in Table 1, the c-*fgr* transcript was detected in approximately half of the lymphoma cell lines tested. All cell lines infected with EBV expressed detectable *fgr* mRNA. One non-Burkitt's

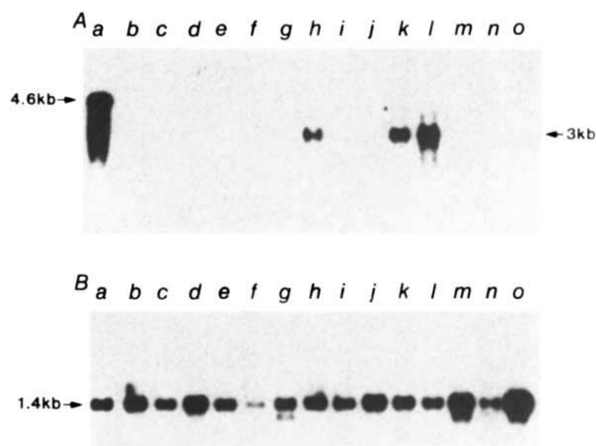

Fig. 1 *A*, Survey of human tumour cell lines for expression of c-*fgr*-related RNA. Poly(A)$^+$-containing RNA was prepared, as described elsewhere[22], from human tumour cell lines and examined by the Northern technique using a v-*fgr*-specific DNA fragment as probe[7]. Carcinoma-derived cell lines A2054, A549, A2182, A431, A1847 and A2199 (ref. 23 and S. A. Aaronson and N. Ellmore, unpublished) (lanes *b–g*, respectively), lymphoma-derived cell lines JI, MC116, JD38, Raji and Namalwa (lanes *h–l*, respectively), as well as sarcoma-derived cell lines A375, A1632 and A2984 (ref. 23 and S. A. Aaronson and N. Ellmore, unpublished) (lanes *m–o*, respectively), were examined. RNA from GR-FeSV-infected mink cells (lane *a*) was used as a control. The relative amounts of hybridizable RNA transferred to nitrocellulose filters were shown to be similar by rehybridization with a GR-FeSV-derived, actin-specific probe (*B*).

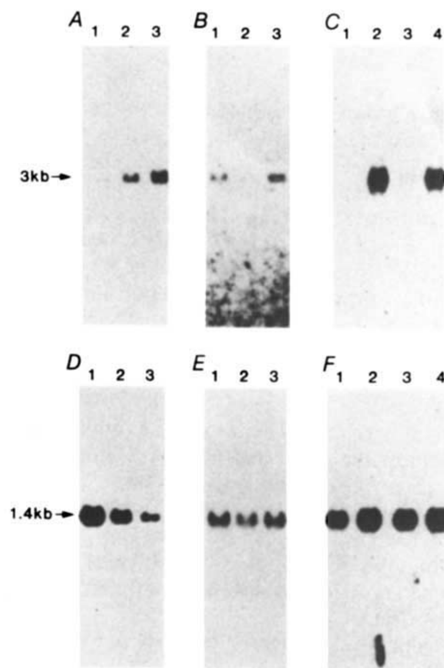

Fig. 2 Expression of *fgr*-related mRNA in cells infected with EBV *in vitro*. Poly(A)$^+$-containing RNAs prepared from EBV-infected normal peripheral (*A*) or umbilical cord (*B*) blood lymphocytes, as well as uninfected (*C*, lanes 1, 3) or EBV-infected (*C*, lanes 2, 4) BJAB (lanes 1, 2) or Ramos (lanes 3, 4) Burkitt's lymphoma cell lines[24,25], were analysed as described in Fig. 1 legend. Relative amounts of hybridizable RNA transferred to nitrocellulose filters were shown to be similar by rehybridization with a GR-FeSV-derived actin-specific probe (*D–F*).

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lymphoma cell line that expressed detectable c-*fgr* mRNA was not nuclear antigen(EBNA)-positive. These results revealed an association of *fgr* proto-oncogene mRNA with Burkitt's lymphoma cells which had been naturally infected with EBV.

To further assess the association between expression of the *fgr* proto-oncogene and EBV infection, we examined RNAs prepared from normal umbilical cord or peripheral blood lymphocyte cell lines established as a result of EBV infection. Figure 2 shows that the *fgr* proto-oncogene transcript was expressed at detectable levels in each of these cell lines, providing further evidence that EBV infection was responsible for the increased concentration of *fgr* proto-oncogene transcripts detected in the lymphoid cells. Preliminary efforts to determine whether normal B lymphocytes express c-*fgr* mRNA have revealed that normal peripheral blood mononuclear cells contain this transcript. However, such preparations consist of a variety of cells, of which only about 10% are B lymphocytes. Thus, further studies will be required to identify the source of c-*fgr* mRNA in peripheral blood (see note added in proof). In any case, the fact that normal lymphocytes become immortalized as stable cell lines in response to EBV infection, but do not acquire fully neoplastic properties, suggests that expression of c-*fgr* RNA alone is not sufficient to induce the neoplastic state.

We extended our analysis to EBV-negative Burkitt's lymphoma cells which are susceptible to EBV infection. BJAB cells infected with a prototype EBV, B95-8, which is capable of immortalizing normal B lymphocytes[8,9], or Ramos cells infected with a nontransforming derivative, P3HR-1 (ref. 10), were examined for the presence of *fgr* proto-oncogene transcripts. These converted cell lines are known to closely resemble their parental lines in morphology and with regard to karyotype and HLA- and B-antigen typing[11]. As shown in Fig. 2*C*, uninfected BJAB or Ramos cell lines expressed little or no detectable c-*fgr* mRNA, whereas cells infected with either of the two EBV strains expressed the c-*fgr* transcript. We conclude from these results that the increase in c-*fgr* RNA concentration was a specific event associated with EBV infection. Moreover, as infection with either an immortalizing or a non-immortalizing EBV strain led to an increase in the steady-state concentration of *fgr* mRNA, we suggest that altered c-*fgr* expression alone is insufficient for lymphocyte immortalization.

To assess the level of c-*fgr* transcript induction in response to EBV infection, DNA fragments containing a dihydrofolate reductase exon (*dhfr*)[12] or human c-*fgr* sequences[7] were used as probes in quantitative S_1 nuclease protection experiments. As a positive control for each probe, RNA prepared from normal human peripheral blood mononuclear cells was examined (Fig. 3). As expected, a major band of 144 nucleotides was protected with the *dhfr* probe in the cell lines that were in cycle[12]. The c-*fgr* DNA probe detected sequences of 165 and 120 nucleotides, sizes which were consistent with those of v-*fgr*-related stretches present in the DNA fragment used as a probe (data not shown). The intensity of bands detected with each DNA probe increased as a function of RNA concentration, making it possible to quantitate the level of c-*fgr* mRNA induction. RNAs from uninfected or EBV-infected cell lines contained similar amounts of *dhfr* mRNA, as indicated by the intensity of the 144-nucleotide *dhfr* probe fragment (Fig. 3*B*). In contrast, c-*fgr* sequences of 165 and 120 nucleotides were protected by RNA from EBV-infected but not uninfected BJAB or Ramos cells. By varying the exposure times, we calculated that the steady-state concentration of c-*fgr* mRNA increased by at least 50-fold in response to EBV infection. Based on other data obtained with probes of similar specific activity[13], we estimate that the EBV-infected lines contain <20 copies of c-*fgr* mRNA per cell.

The correlation of increased *fgr* proto-oncogene expression with EBV infection of transformed lymphocytes was nearly absolute. Increased concentrations of c-*fgr* mRNA were detected in Burkitt's lymphoma cells whether infected naturally or deliberately in tissue culture. In addition, each of the umbilical cord or peripheral blood lymphocyte cell lines established by EBV infection expressed c-*fgr* mRNA. Increases in the levels of transcripts related to other mammalian tyrosine kinase-coding *onc* genes (v-*fms* and v-*fes*) were also detected in some EBV-infected Burkitt's lymphoma cell lines (data not shown), but these RNAs were not consistently detected in EBV-infected lines and were, in some cases, detectable in uninfected cell lines. Taken together, these results establish a specific relationship between EBV infection and expression of the *fgr* proto-oncogene, which suggests that EBV infection results in transcriptional activation of c-*fgr*. Further studies will be required to show whether increased transcription rates alone are responsible for the increase in steady-state concentration c-*fgr* mRNA.

Integration of EBV into host chromosomal DNA of Namalwa and IB4 cell lines has been described elsewhere[14]. In Namalwa cells, the site of integration is close to the *fgr* proto-oncogene locus on chromosome 1 (refs 7, 14), whereas in IB4 cells the

Table 1 Detection of *fgr* proto-oncogene mRNA in cell lines derived from Burkitt's and non-Burkitt's undifferentiated lymphomas

Histopathology	Cell line	EBNA status	Chromosome translocation	Geographical origin	Expression of v-*fgr*-related transcript*
Burkitt's lymphoma	Namalwa	+	8; 14	Africa	+++
	Keeper	+	8; 14	S. America	+++
	AG876	+	8; 14	Africa	++
	JI	+	2; 8	Europe	++
	Raji	+	8; 14	Africa	++
	LY47	+	8; 22	Africa	+
	BL2	−	8; 22	Europe	−
	ST486	−	8; 14	USA	−
	MC116	−	8; 14	USA	−
	CA46	−	8; 14	S. America	−
Non-Burkitt's lymphoma	EW36	−	8; 14	USA	−
	DS179	−	8; 14	USA	+
	JD38	−	8; 14	USA	−
	JLPC119	−	8; 14	USA	−

The characteristics of the cell lines examined have been described previously[19,20]. The concentration of 3-kb *fgr*-related mRNA was determined by analysing 10 μg of poly(A)$^+$ RNA in formamide-formaldehyde agarose gels as described[21]. The relative amounts of hybridizable RNA transferred to nitrocellulose filters were determined by rehybridization with a GR-FeSV-derived actin-specific probe.

* Detectable levels were graded by exposing hybridized nitrocellulose filters for various times as follows: +++, detected after overnight exposure; ++, detected after 2 days of exposure; +, detected after 5 days of exposure; −, not detectable after 5 days of exposure.

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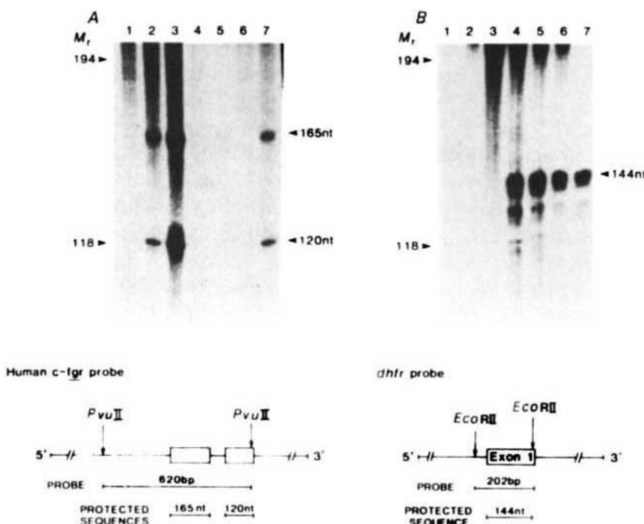

Fig. 3 Quantitation of *fgr* proto-oncogene mRNA induction in response to EBV infection. DNA fragments derived from the region 6.0–6.62 kb of the human *fgr* proto-oncogene[7] (*A*) or the 5′ end of the dihydrofolate reductase gene[12] (*B*), were cloned into M13 phage vectors and used as templates for the synthesis of uniformly labelled anti-sense DNA probes. Conditions for hybridization of total cellular RNA with the DNA probe, S_1 nuclease digestion and polyacrylamide gel electrophoretic analysis of protected sequences were as described elsewhere[26,27]. Samples tested included 5 μg (lanes 2) or 20 μg (lanes 3) total cellular RNA from normal human peripheral blood mononuclear cells, 20 μg RNA from uninfected Ramos (lanes 4) or BJAB (lanes 6) cells, as well as P3HR1-infected Ramos (lanes 5) or B95-8-infected BJAB (lanes 7) cell lines. Negative controls consisted of labelled probe hybridized in the absence of RNA and treated with S_1 nuclease (lanes 1). As expected, no *dhfr* mRNA was detected in peripheral blood mononuclear cells as these cells were not in cycle. The diagrams at the bottom of the figure show human c-*fgr* and *dhfr* exons and their protected sequences. The extent and locations of the human c-*fgr* exons shown were confirmed by nucleotide sequence analysis.

EBV genome integrates into chromosome 4. In the case of the Namalwa cell line, EBV may have a *cis*-acting effect on the expression of the *fgr* proto-oncogene. However, in most cases the influence of EBV on c-*fgr* is more likely to be *trans* as the EBV genome typically exists in the host as an episome. Thus, the isolation of EBV genes having specific functions may make it possible to identify the region of the EBV genome responsible for the increase in *fgr* proto-oncogene mRNA.

Several lines of evidence suggest that Burkitt's lymphoma results from a multi-step process involving the alteration of several genetic elements[15]. Epidemiological data suggest that EBV has an important role in the aetiology of African Burkitt's lymphoma[16]. Molecular studies have strongly implicated alterations of *myc* proto-oncogene expression in the process leading to American as well as African Burkitt's malignancies[17,18]. However, there is no evidence to indicate that such genetic alterations are the only changes required to achieve lymphoid cell transformation. From the results of the present study we conclude that increased concentrations of c-*fgr* mRNA alone are not sufficient to induce malignant transformation of normal lymphocytes or even to immortalize them as continuous cell lines. Moreover, we have no direct evidence that EBV-induced immortalization involves c-*fgr*. Nevertheless, our results are consistent with the possibility that one step in EBV-induced B-cell immortalization involves transcriptional activation of the *fgr* proto-oncogene in response to an EBV-encoded function.

We thank N. Ellmore, L. Mickley and C. Lengel for technical assistance; N. Raab-Traub, I. T. Magrath and D. Ablashi for providing cell lines; T. Kawakami for making unpublished nucleotide sequence data available to us; T. Shimada and A. Nienhuis for the *dhfr* probe; and S. Aaronson for helpful discussions.

Note added in proof: Recent experiments have revealed that c-*fgr* mRNA is not expressed in normal resting or activated B lymphocytes.

Received 8 July; accepted 24 October 1985.

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Expression of cauliflower mosaic virus reverse transcriptase in yeast

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Cauliflower mosaic virus (CaMV) is a double-stranded DNA plant virus[1–3] for which evidence has been presented that its replicative cycle involves a reverse transcription step[4–14]. Until now, however, there has been no direct evidence for such a step. The sequence of the CaMV genome contains a number of open reading frames, one of which (ORF V) encodes a protein of predicted sequence homologous to retroviral reverse transcriptases[10]. We have cloned this gene, and expressed it in the yeast *Saccharomyces cerevisiae*. We report here that yeast expressing this gene accumulate significant levels of reverse transcriptase activity. This provides strong evidence that CaMV really does replicate through an RNA intermediate, converted to DNA by reverse transcriptase.

To obtain the direct expression of the ORF V gene in yeast cells, we constructed the plasmid pAM.ORFV in which the entire ORF V gene is inserted immediately downstream from the acid phosphatase (APase) promoter of the yeast expression vector pAM82 (ref. 15) (Fig. 1*b*). The resulting Leu^+ yeast transformant AH22/pAM.ORFV was obtained. The integrity of the ORF V gene in pAM.ORFV was confirmed by fine restriction mapping of a rescued pAM.ORFV from AH22/pAM.ORFV cells (Fig. 1*b*).

AH22/pAM.ORFV and AH22/pAM82 cells were grown in a liquid medium and then induced in phosphate-deprived medium. The extract from AH22/pAM.ORFV cells, but not

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