Characterization of the Epstein-Barr Virus-Inducible Gene Encoding the Human Leukocyte Adhesion and Activation Antigen BLAST-1 (CD48)

ROBERT C. FISHER AND DAVID A. THORLEY-LAWSON*

*Department of Pathology, Tufts University School of Medicine,
136 Harrison Avenue, Boston, Massachusetts 02111*

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BLAST-1 (CD48) (previously referred to as BCM-1 by the Human Gene Nomenclature Committee) is an early-activation-associated membrane glycoprotein expressed on the surface of human leukocytes and induced to a high level following infection of B cells by the Epstein-Barr virus. It is a member of the immunoglobulin superfamily, mediates cell adhesion, and has significant sequence homology to two other adhesion molecules, CD2 and LFA3. Here we report the isolation and characterization of the BLAST-1 gene. The gene is at least 28.6 kb in length, is split into 4 exons, and contains a restriction fragment-length polymorphism. The overall genomic organization is consistent with other members of the immunoglobulin superfamily, in which extracellular immunoglobulinlike domains are encoded by discrete exons. Transcription is initiated at a series of major and minor sites in both normal and tumor-derived lymphoid cells. Appropriately located TATA and CCAAT box sequences were not detected. These characteristics have also been demonstrated for the recently described B-cell-specific genes B29 and CD20. The expression of these genes in B cells may involve the use of multiple promoters and novel transcription initiator-binding proteins. A 1.58-kb genomic DNA fragment, consisting of the 5'-flanking region located immediately upstream of the ATG initiation codon, was able to drive the expression of a reporter gene in an orientation-dependent and tissue-restricted manner.

Recognition of antigen by resting lymphocytes initiates a complex set of intracellular signals that result in a coordinated cascade of transient gene activation steps, leading to the synthesis of gene products that are necessary for cellular activation and proliferation. This process of tightly controlled gene expression can be disrupted upon viral infection and lead to deregulation of normal cellular gene control. Infection of resting human B cells in vitro by Epstein-Barr virus (EBV) exemplifies this scenario. The virus is able in vitro to activate and drive the cells into the lymphoblastoid stage of differentiation, in the absence of accessory cells, by a process referred to as immortalization or growth transformation (1, 19). However, unlike normally activated B cells, which proceed to terminal differentiation, EBV-infected lymphoblastoid cells are fixed at this stage of differentiation and will form continuously proliferating EBV-positive lymphoblastoid cell lines (LCL) in culture. The virus persists latently in the infected cell and expresses a limited number of genes, whose products are referred to as latent gene products (reviewed in reference 30).

LCLs may represent an in vitro equivalent to the premalignant stage in B-cell tumorigenesis. This conclusion is based in part on the observation that immunosuppressed transplant patients often possess polyclonal B-cell lymphoproliferative lesions (17) that have the potential to switch to fatal monoclonal EBV-positive B-cell lymphomas (16). Dissecting the molecular mechanism that explains the immortalization process should allow the identification of genes that are crucial for regulating normal lymphocyte activation and growth. In addition, this will increase our understanding of the contribution of these genes to B-cell neoplasia.

Two likely targets of the deregulating process are the

interaction of growth factors with their cognate receptors and adhesion molecules involved in mediating cellular interactions. EBV-transformed LCLs have been shown to produce autocrine growth factors (4, 12) and form multicellular clumps that characterize the LCL-like phenotype (43). Cell clumping may promote autocrine-induced growth by bringing cells into closer proximity, potentially allowing cross-feeding and the transmission of growth signals through receptor-ligand interactions.

At present, it is unclear which latent gene products are responsible for immortalization. The Epstein-Barr nuclear antigen 2 has a critical role, since nontransforming viral strains, lacking the Epstein-Barr nuclear antigen 2-coding region, can be genetically rescued by complementation with the wild-type Epstein-Barr nuclear antigen 2 gene (15). In addition, the latent membrane protein has been shown to induce surface molecules that are characteristic of the immortalized cell phenotype, such as the proteins LFA-1, LFA-3, and ICAM-1 (56).

We have previously shown that another surface molecule, BLAST-1, is upregulated by EBV (55). BLAST-1 (previously referred to as BCM-1 by the Human Gene Nomenclature Committee) is a 45-kDa early-activation-associated glycoprotein anchored to the membrane via a glycosyl-phosphatidylinositol (GPI)-containing lipid (49). It is also expressed by activated T lymphocytes and monocytes and is the molecule recognized by the CD48 antibody cluster (59). BLAST-1 is a member of an immunoglobulin superfamily (IgSF) subgroup that includes CD2, LFA-3, and CEA (29, 50). Like the other members of this subgroup, BLAST-1 also functions as an adhesion molecule; however, unlike the other members it has the unusual property of binding to itself (48a).

We have chosen BLAST-1 as a model for studying the mechanism by which EBV deregulates the expression of cell surface activation molecules. By using a previously de-

* Corresponding author.

scribed BLAST-1 cDNA clone isolated by our laboratory, genomic clones were identified and used to determine the genomic organization of the BLAST-1 gene and to define the promoter region at both the structural and functional levels.

MATERIALS AND METHODS

Cell lines and purified lymphocytes. Erythroleukemia cell line K562, helper T-cell line Jurkat, and EBV-positive Burkitt's lymphoma cell line Namalwa were all obtained from the American Type Culture Collection, Rockville, Md. ER-LCL was established as previously described (50), while a primary fibroblast cell line, ER-Fib, was established from the same individual by culturing a skin punch biopsy. IB4 LCL was a gift from Elliot Kieff (Harvard University Medical School, Boston, Mass.). All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U of penicillin per ml, and 50 µg of streptomycin per ml.

B and T cells were purified from leukocyte by-products of platelet pheresis, obtained from the Blood Bank of the Dana Farber Cancer Institute (Boston, Mass.), as described elsewhere (23, 50). Generally, the protocols yielded B-cell preparations with ≥90% B cells and T-cell preparations with ≥98% T cells. B cells were activated with *Staphylococcus aureus* Cowans strain I (0.0075% [vol/vol]; SAC/Pansorbin; Calbiochem, La Jolla, Calif.) plus human recombinant interleukin-2 (10 U/ml; gift of James Mier, New England Medical Center, Boston, Mass.), and T cells were activated with phytohemagglutinin (1 mg/ml; Burroughs-Wellcome Co., Greenville, N.C.), for 12 h in both cases. The activation status of the cells was confirmed by measuring the incorporation of [³H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, Mass.) after 3 days of stimulation *in vitro*.

Extraction of RNA and genomic DNA. Total RNA isolation and purification of poly(A)⁺ RNA by oligo(dT) chromatography were performed as described elsewhere (50). High-molecular-weight DNA was isolated as described previously (22).

Genomic Southern blot hybridization analysis. Southern blot analysis of genomic DNA was performed by digesting 10 µg of DNA with *Eco*RI, *Hind*III, or *Bam*HI according to the manufacturer's specifications (BRL, Gaithersburg, Md.). The resulting digests were electrophoresed through a 0.8% agarose gel containing no ethidium bromide at 40 V for approximately 18 h and subsequently transferred to a Zeta-Probe nylon membrane (Bio-Rad, Richmond, Calif.) by capillary blot transfer (47). The membrane was prehybridized and hybridized in 5× SET (0.75 M NaCl, 5 mM EDTA, pH 8.0, 0.1 M Tris-HCl, pH 7.8)–50 mM Na₂HPO₄–0.16 µg of heparin per ml–1% sodium dodecyl sulfate (SDS)–100 µg of denatured salmon sperm DNA per ml at 68°C for 14 h. The membrane was hybridized to 10⁷ cpm of ³²P-labeled BLAST-1 cDNA 2A1 fragment (50) per ml that was labeled by the random primer method (10) for approximately 24 h at 68°C. The membrane was washed successively with 2× SET–0.5% SDS, 1× SET–0.25% SDS, 0.5× SET–0.125% SDS, and 0.1× SET–0.025% SDS at 68°C and exposed to X-ray film at –70°C for 4 days with an intensifying screen.

Isolation and characterization of genomic clones. Genomic DNA clones were isolated from a λ Charon 4A human fetal liver genomic library (33) containing 15- to 20-kb inserts with *Eco*RI linkers. The library (a generous gift of Edward Fritsch, Genetics Institute, Boston, Mass.) had undergone only two amplifications and should be representative of the human genome. Screening was performed as described

previously (3) utilizing the BLAST-1 cDNA 2A1 (50), which contains the entire protein coding region along with 5' and 3' untranslated (UT) regions. Genomic clones positive after three rounds of plating and screening were grown up by a rapid phage lysate plate method (35). Phage DNA was characterized by restriction endonuclease mapping and Southern blot analysis using 2A1 cDNA fragments. Further restriction mapping and DNA sequencing was performed following subcloning into the Bluescript KS(+) plasmid (Stratagene, La Jolla, Calif.). DNA sequencing was performed by the dideoxynucleotide chain termination method (44) using denatured double-stranded templates, universal primers, and the Sequenase T7 DNA polymerase enzyme (United States Biochemical, Cleveland, Ohio). If necessary, restriction fragments were further subcloned or unidirectional overlapping deletions (18) were generated by the *Exo*III-mung bean nuclease deletion system (Stratagene). Exon-intron boundaries were localized by comparing genomic and cDNA sequences by the alignment program of the Microgenie sequence analysis program (Beckman, Irvine, Calif.).

RNase protection analysis. A 319-bp *Rsa*I genomic fragment containing the translated portion of exon 1 and 235 bp upstream of the ATG start site was isolated, *Hind*III linkers (New England Biolabs, Beverly, Mass.) were added, and the fragment was subcloned into the *Hind*III site of the Bluescript KS(+) plasmid. The template was prepared, and antisense riboprobes were generated with T7 RNA polymerase (Stratagene) in the presence of [α-³²P]UTP (800 Ci/mmol; New England Nuclear) according to the specifications of the manufacturer. The 459-bp RNA runoff products were gel purified with a 4% acrylamide–8.3 M urea denaturing gel, localized by autoradiography, and eluted into 10 mM sodium acetate (pH 6.0)–1 mM EDTA–0.5% SDS–10 µg of yeast tRNA per ml by shaking vigorously at 37°C. Total RNA (40 µg) was mixed with 2 × 10⁶ cpm of riboprobe in the presence of 80% formamide, 40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 7.0, 400 mM NaCl, 1 mM EDTA, and 10 µg of yeast tRNA; denatured at 90°C for 10 min; and hybridized for 14 h at 40°C. Then 350 µl of 500 mM NaCl–10 mM Tris-HCl, pH 7.5, containing 40 µg of RNase A (Sigma Chemical Co., St. Louis, Mo.) per ml and 2 µg of RNase T₁ (Sigma Chemical Co.) per ml, was added to each sample and incubated for 15 min at 37°C. This was followed by the addition of 10 µl of 10% SDS and 2.5 µl of 20-mg/ml proteinase K for an additional 15 min at 37°C. The digested mixtures were extracted with phenol-chloroform and ethanol precipitated in the presence of 10 µg of yeast tRNA. The resulting protected fragments were resuspended in sample buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF) and electrophoresed through a 6% acrylamide–8.3 M urea denaturing sequencing gel alongside ³²P-labeled *Msp*I-digested pBR322 DNA. The resulting gel was fixed in 10% acetic acid, dried, and exposed to X-ray film at –70°C for 4 days without an intensifying screen.

Primer extension analysis. A 41-nucleotide oligonucleotide complementary to the 5'-end region of cDNA clone 2A1 (5' GCA GCA GTA GCA ATT CCA GAG CCA GAC ACG AAT CCC AAC CT) was synthesized at the Center for Blood Research, Boston, Mass. The oligonucleotide was purified by electrophoresis through a 10% acrylamide gel, eluted, and 5' end labeled with [γ-³²P]ATP with polynucleotide kinase (Promega, Madison, Wis.) as described elsewhere (35). The labeled oligonucleotide was separated from unincorporated ATP by a P6 biospin column (Bio-Rad)

chromatographic step with a resulting specific activity of 4.4×10^7 cpm/pmol. Poly(A)⁺ RNA (5 µg) was added to a 10-µl volume containing 4×10^6 cpm of end-labeled primer, 0.1 M NaCl, 16.7 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.5, and 0.03 mM EDTA; heated to 80°C for 4 min; and then incubated at 50°C for 3 h. The hybridized primer-RNA complex was precipitated and resuspended in 50 µl containing 0.8 mM deoxynucleoside triphosphates, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, and 10 mM dithiothreitol; 1,000 U of Moloney murine leukemia virus reverse transcriptase (BRL) was added, and the mixture was incubated for 1 h at 42°C. The reaction was terminated by the addition of 2 µl of 0.25 mM EDTA, pH 8.0, followed by an additional 30-min incubation step at 37°C in the presence of 1 µg of RNase A. The digestion mixtures were phenol-chloroform extracted and ethanol precipitated in the presence of 10 µg of yeast tRNA. The precipitated extension products were resuspended in sample buffer and electrophoresed through an 8% acrylamide-7.2 M urea sequencing gel alongside a sequencing ladder generated with the identical primer used for the extension reaction and a subcloned genomic fragment containing exon 1 and the 5'-flanking region. The gel was fixed in 10% acetic acid, dried, and exposed to X-ray film at -70°C for 4 days without an intensifying screen.

Functional identification of the promoter region. A 1,576-bp *Hind*III-*Nsp*I genomic DNA fragment was isolated and blunt ended at the *Nsp*I site with T4 DNA polymerase (New England Biolabs), and a *Hind*III linker was added and cloned in both orientations into the *Hind*III site of SL1CAT (gift of Stephanie Lee, New England Medical Center). This plasmid contains the structural gene for the bacterial enzyme chloramphenicol acetyl transferase (CAT) in addition to the simian virus 40 early splicing and polyadenylation signals cloned into the polylinker region of the Bluescript KS(+) plasmid. The orientation of the insert was determined by DNA sequencing. Plasmid DNA used for transfections was purified with Qiagen maxicolumns (Qiagen, Studio City, Calif.). Transfections were performed by electroporation with a Cell Zap II electroporation apparatus (Anderson Electronics, Cambridge, Mass.) and Gene Pulser cuvettes (Bio-Rad) at 250V/1,000 µF (625 V/cm) as described elsewhere (7), except that interleukin-2 was left out of the growth medium and 10^7 cells in a volume of 250 µl were electroporated. Cell lysates were prepared from cells 24 h posttransfection by three cycles of freezing and thawing. For measuring CAT activity, 10 µg of cell lysate, as determined by the Bradford dye-binding assay (Bio-Rad), was incubated in a total volume of 170 µl containing 0.25 M Tris-HCl, pH 7.5, 68 µM acetyl coenzyme A (Sigma Chemical Co.), and 0.05 µCi of [¹⁴C]chloramphenicol (57 mCi/mmol; Amersham, Arlington Heights, Ill.) for 2 h at 37°C. Reaction products were recovered in ethyl acetate, separated by thin-layer chromatography, and quantitated by liquid scintillation counting.

RESULTS

Southern blot hybridization analysis of the BLAST-1 gene. Genomic DNA isolated from an LCL and a primary fibroblast line originating from the same individual (ER-LCL and ER-Fib) was digested with *Eco*RI, *Hind*III, or *Bam*HI; size fractionated; and transferred to nylon membrane. The resulting blot was hybridized to the full-length BLAST-1 cDNA, 2A1 (50). The hybridization pattern (Fig. 1) is consistent with BLAST-1 being a single-copy gene that possesses a simple

FIG. 1. Southern blot analysis of the BLAST-1 gene. DNA from ER-LCL and ER-Fib was digested with *Eco*RI, *Hind*III, or *Bam*HI, while DNA from IB4 LCL and Namalwa EBV-positive Burkitt's lymphoma was digested with *Bam*HI. The hybridizing probe was the cDNA 2A1. *Hind*III-digested DNA size markers (in kilobases) are shown by arrows to the left of the autoradiogram.

organization with a minimum of four exons, since the enzymes used do not digest the cDNA. In addition, there are no obvious structural rearrangements. *Bam*HI digests of DNA from two additional B-cell lines, IB4 and Namalwa, revealed the presence of a restriction fragment-length polymorphism (RFLP), as indicated by the additional fragment of 1.85 kb (Fig. 1).

Isolation of BLAST-1 genomic clones. Approximately 4.7×10^5 plaques from a λ Charon 4A human fetal liver genomic library were screened with the full-length BLAST-1 cDNA 2A1 (50), yielding 10 genomic clones that were positive after three rounds of plating and screening. The entire BLAST-1 cDNA is spanned by four contiguous fragments of 408 bp (most 5'; *Eco*RI-*Alu*I), 154 bp (*Alu*I-*Sac*I), 212 bp (*Sac*I-*Sac*I), and 391 bp (most 3'; *Sac*I-*Eco*RI). DNA from the λ clones digested with *Eco*RI was analyzed by using these fragments as probes. Hybridization results for clones 2C, 5C, and 8D suggested that they contained unique inserts covering the entire coding region of the gene.

Exon-intron structure. Further characterization of genomic clones 2C, 5C, and 8D involved restriction enzyme mapping with one or more enzymes followed by Southern

TABLE 1. Exon-intron organization of BLAST-1

Domain ^a	Exon no.	Intron no.	Size (kb)	Sequence of exon/intron boundary ^b
5' UT and LP	1	1	0.168 ^c	ATT CAA G/gtactt
EXD1	2	2	0.303	tggcag/GT CAG TTG
EXD2	3	3	0.265	GTG CTT G/gtgagt
GPI and 3' UT	4	3	0.383	tttcag/AC CCT GTA
				ACC CTG G/gtaaga
				cttcag/CC CGG TCC

^a LP, Leader peptide; EXD1 and EXD2, extracellular Ig-like domains 1 and 2.

^b Uppercase, Exon sequences; lowercase, intron sequences.

^c This exon size is based on the most 5' transcriptional initiation site detected by RNase protection analysis.

blot hybridization with 2A1 cDNA subfragments. Genomic clones 5C and 8D were found to overlap, while clone 2C, which contains unique restriction fragments hybridizing only to a 5' cDNA probe, does not overlap with either 5C or 8D. 8D hybridizes to both 5' and 3' cDNA probes, whereas 5C hybridizes only to 3' probes. These results, in the context of the information obtained from the genomic Southern blot (Fig. 1), strongly suggest that the 5' end of the gene is separated from the rest of the gene by a large intron. Given the size of the first intron, it is not surprising that overlapping clones containing exons 1 and 2 were not obtained, since the library was screened only with the cDNA. Inserts from genomic clones 2C, 5C, and 8D were subcloned into the Bluescript KS(+) plasmid for detailed restriction mapping and DNA sequencing. Subcloned restriction fragments that hybridized to 2A1 cDNA fragments were analyzed by DNA sequencing to define the exon-intron boundaries. The genomic sequence was compared with the cDNA sequence by

the alignment program contained in the Microgenie DNA sequence analysis program.

The completed exon-intron organization is summarized in Table 1 and depicted in Fig. 2. The genomic organization accounts for the entire 2A1 cDNA sequence, which is encoded by a gene of at least 28.6 kb. The map is also consistent with the genomic Southern blot results with the 2A1 cDNA probe. The only exception is a weakly hybridizing 3.4-kb *Eco*RI fragment. This may be related to a minor 4-kb mRNA of unknown origin that has been detected by Northern (RNA) blot analysis using the cDNA as a probe (50). Infrequently, weakly hybridizing fragments are also detected for *Hind*III- or *Bam*HI-digested genomic DNA.

The gene consists of four exons 121, 303, 265, and 388 bp in length. The exons are separated by introns that vary in size from 1.95 to ≥ 22 kb in length. The first exon encodes the 5' UT region and leader peptide. In the nomenclature established for describing IgSF domains (57), exon 2 encodes a V-like Ig domain lacking the characteristic disulfide bond, while exon 3 encodes the C2-SET-containing Ig domain. Exon 4 encodes both the portion of the protein containing the predicted serine attachment site for the GPI anchor and the 3' UT region (29, 49). There is a single polyadenylation signal (AATAAA) within the 3' UT region, which is consistent with the observation of a single major mRNA species by Northern blot analysis.

The sequences of the exon-intron boundaries conform to the consensus sequences for splice donor and acceptor sites (24), except for the exon 3 splice donor site, which reads exon-GTAAGA instead of GTAAGT. The genomic sequence agrees with the previously published cDNA sequence for clone 2A1 (50), except for the following differences: a T in place of an A at position 232 of the cDNA, resulting in a nonconservative change of an asparagine to an isoleucine, and the addition of an AGG at position 32 of the cDNA. These differences may be the result of DNA polymorphisms, since the cDNA and genomic clones were isolated

FIG. 2. Genomic structure of the BLAST-1 gene. (A) Genomic clones 2C, 5C, and 8D, which contain the complete coding region of the BLAST-1 gene. The undefined 3' end of phage clone 2C is indicated by a broken line. (B) Partial restriction endonuclease map and schematic representation of the exon-intron structure. Restriction enzyme sites: R1, *Eco*RI; H3, *Hind*III; B, *Bam*HI; B*, *Bam*HI RFLP. Detailed regions of the restriction map are indicated by a thick line, while partially mapped regions are indicated by a thin line. The locations of exons are denoted by boxes. (C) The structure of the cDNA 2A1 is shown, with positions for the translational initiation codon (ATG), predicted GPI attachment site (49), stop codon (TGA), polyadenylation signal (AATAAA), and poly(A) tail [(AAA)_n] indicated. The predicted structural regions are labeled 5' UT, LP (leader peptide), EXD1 and EXD2 (extracellular Ig-like domains 1 and 2), and 3' UT.

from recombinant libraries prepared with genetic material from different individuals.

During restriction enzyme mapping of overlapping genomic clones 5C and 8D, an additional *Bam*HI restriction site mapping to the third intron was detected in 5C. The lack of a *Bam*HI site at this position would increase the size of the exon 4-containing *Bam*HI fragment by an additional 450 bp, thereby explaining the *Bam*HI RFLP that had been seen in genomic Southern blots using cDNA 2A1 as a probe (Fig. 1). Furthermore, additional Southern blot analysis using genomic fragments originating from intron 3 as hybridization probes suggests that the *Bam*HI RFLP is due to a point mutation or small deletion, rather than a large deletion, resulting in the loss of the *Bam*HI restriction site (data not shown).

Mapping transcriptional initiation sites. To define the 5' end of the BLAST-1 gene, the transcriptional initiation site(s) was defined initially by RNase protection analysis. A 319-bp *Rsa*I genomic DNA fragment containing the translated portion of exon 1 and 196 bp of 5'-flanking sequence was subcloned into the Bluescript KS(+) plasmid, and uniformly labeled antisense RNA transcripts were prepared. Total RNAs isolated from the BLAST-1-positive ER-LCL, Jurkat helper T-cell line, phytohemagglutinin-activated normal peripheral blood T cells, and SAC plus recombinant-interleukin-2-stimulated normal peripheral blood B cells, along with total RNA from the BLAST-1-negative cell line K562, were analyzed. Between six and eight protected fragments were detected in all of the positive samples (Fig. 3A). For simplicity, the sites are referred to as B1 to B8, with B8 representing the 5'-most initiation site.

Overall, the sites were similar for the different cell types, with the exception that the Jurkat line did not use sites B1 and B2 detectably. The absence of these sites, as compared with phytohemagglutinin-activated normal T cells, may be an artifact of this particular cell line or reflect heterogeneity among different T-cell populations or be a property of certain T-cell neoplasias.

To confirm the RNase protection results and more accurately map the initiation sites, primer extension analysis was performed using a 41-mer oligonucleotide complementary to positions 48 to 88 of the BLAST-1 cDNA. This region is immediately downstream of the translational initiation codon and includes a portion of the leader peptide. Six extension products clustered over a distance of approximately 40 bp were detected with mRNA from the B-cell line ER-LCL. These extension products were not present when RNA from the negative-control cell line K562 was used (Fig. 3B). Four of the extension products matched up with initiation sites B1, B3, B4, and B5. Unexpectedly, two additional initiation sites were detected approximately 250 bp upstream of B8. The two extension products were not detected by the RNase protection analysis, since they lie 5' of the subcloned genomic DNA fragment used to generate the riboprobe. Assignment of these two sites is therefore tentative, since they have not been confirmed by another method.

The inability to confirm initiation sites B2, B6, B7, and B8 may be the result of the greater sensitivity of the RNase protection method and/or incomplete primer extension due to the inducing of the reverse transcriptase by the mRNA secondary structure to pause or terminate prematurely. Efforts to decrease mRNA secondary structure by increasing the temperature during hybridization or altering the kinetics of hybridization by decreasing the salt concentration in the hybridization buffer resulted in a dramatic increase in the background without significantly increasing the lengths

or pattern of the extension products (data not shown). For the reasons stated above, the RNase protection assay appears to be the technique that is least susceptible to artifacts, especially for genes such as BLAST-1 which possess multiple initiation sites. As a result, we are assigning initiation site B8 to represent the 5' end of the gene. B8 is 86 bp upstream of the ATG initiator codon, thus giving exon 1 a final length of 168 bp. These findings indicate considerable heterogeneity in the 5' ends of BLAST-1 mRNA. In addition, RNase protection analyses of mRNA isolated from activated normal B and T cells indicate that the same initiation sites used by the tumor-derived cell lines are also used by normal lymphocytes during the activation process. The similarities in the pattern of initiation sites suggest that they are not preferentially used in a tissue-specific manner.

Structure of the 5' end of the gene. A 2,144-bp *Hind*III genomic DNA fragment containing the 5' portion of intron 1, exon 1, and the adjacent 5'-flanking region was subcloned. Overlapping unidirectional deletions of this fragment were generated and analyzed by DNA sequencing in order to identify distinctive structural elements or putative regulatory regions (Fig. 4). The initial comparison of the sequence with those in the primate DNA data banks (GenBank, Microgenie release 61) revealed a 282-bp *Alu* repeat sequence at positions -1000 to -718 showing approximately 85% homology to a consensus *Alu* repeat sequence (28). This *Alu* repeat region belongs to subfamily S and the B subclass, on the basis of identity at a number of diagnostic positions for the consensus *Alu* sequence (28). At the 3' end is a 31-bp A-rich region from -718 to -687 that is commonly found at the 3' end of *Alu* repeats (45). Spanning the region from -1031 to -1001 just 5' of the *Alu* repeat and -683 to -614 3' of the repeat are stretches of DNA sequence that have significant identity to several repeat families, including the O (52), the THE (41), and the *Msl*II (21) repeats. Additional searches of other primate data banks (EMBL, GCG version 6.0) revealed no additional sequence similarities.

A data base was constructed containing DNA-binding motifs for a number of ubiquitous and tissue-specific transcriptional factors that are involved in controlling gene expression in leukocytes and viruses (26, 27). The BLAST-1 sequence upstream of the translational initiator codon was analyzed by homology comparison at the zero- or single-mismatch level. Figure 4 shows a summary of the results, in which the sequence upstream of the transcriptional initiation sites does not contain the TATA and CCAAT box sequences commonly present in the promoter region of eukaryotic genes (36). A number of sites resembling the consensus sequence for ubiquitous transcription factors AP1 (TG/TAGTCA [34]), AP2 (CCCCANC/GC/GC/G [25,40]) and AP3 (TGTGGA/TA/TA/T [38]) were located. In addition, two binding sites for the Ig enhancer-binding factor KE2 (GGCAGGTGG [46]) were located. The relevance of these binding sites can be determined only by fine mapping of the promoter region.

Functional identification of the promoter region. A genomic DNA fragment extending from -1577 to -2 was isolated and inserted in both orientations upstream of the bacterial enzyme CAT to test for promoter activity following transfection into the EBV-positive B-cell line IB4 and the erythroleukemia cell line K562. In addition, RSV-CAT containing the Rous sarcoma long terminal repeat (13) and SL1CAT, the parent plasmid without an insert, were used as positive and negative controls, respectively. The cloned insert was active only in the sense orientation and only when transfected into IB4 (Fig. 5). The positive control RSV-CAT was

FIG. 3. Mapping transcription initiation sites by RNase protection and primer extension analyses. (A) RNase protection analysis was performed by hybridizing a 5'-flanking region-exon 1 riboprobe to total RNA from the indicated samples, digesting them with RNase A and RNase T₁, and resolving protected fragments on a denaturing acrylamide-urea sequencing gel. Additional samples include undigested and digested probe controls, which contain no unknown RNA sample but were digested in the absence or presence of RNase A and RNase T₁. Size markers (in base pairs) are *Msp*I-digested pBR322 DNA and are indicated to the right of the gel. Arrows labeled B1 to B8 denote major sites of transcriptional initiation. (B) Primer extension analysis was performed by hybridization of poly(A)⁺ RNA from the ER-LCL and K562 erythroleukemia cell lines to an end-labeled primer, corresponding to the 5' end of cDNA 2A1, and extension with Moloney murine leukemia virus reverse transcriptase. As a mock control, the extension reaction was performed in the absence of RNA. All extension reactions were run through a denaturing acrylamide-urea sequencing gel alongside a sequencing ladder generated with the primer and the subcloned 5' end of the gene. Major transcriptional initiation sites are denoted by arrows and the notation described above if a previously mapped site was confirmed. For both analyses, the ATG translational initiator codon is labeled.

active in both cell lines, while the negative control SL1CAT was not. These results demonstrate that the subcloned genomic DNA fragment contains a promoter region(s) and is regulated in a tissue-specific manner, as observed during endogenous BLAST-1 transcription.

DISCUSSION

After infection of resting B cells with EBV, there is an increased expression of a number of cell surface molecules along with the appearance of newly synthesized surface

FIG. 4. Nucleotide sequence of the 2,144-bp *Hind*III DNA fragment containing the 5' region of intron 1, exon 1, and the 5'-flanking region. The transcription initiation sites determined by RNase protection (○) and primer extension (●) analyses are indicated. The 5' end of cDNA 2A1 is denoted (★), and the translated portion of exon 1 and the corresponding amino acid sequence are denoted in italics. The location of the 41-mer oligonucleotide used for the primer extension analysis is underlined. Putative regulatory control elements are boxed and appropriately labeled. The numbering of the sequence is based on ATG at +1.

FIG. 5. Identification of the promoter region in the 5'-flanking region of the BLAST-1 gene. A *HindIII*-*NspI* fragment (-1577 to -2) from the 5' end of the BLAST-1 gene was ligated into the *HindIII* site of the SL1CAT plasmid in both orientations with respect to the bacterial CAT gene. IB4 and K562 cell lines were transfected with 50 μ g of sense BLAST-1-CAT, anti-sense BLAST-1-CAT, or SL1CAT negative-control or 25 μ g of RSV CAT positive-control plasmids. Transfections were performed in triplicate, with a representative sample shown in the figure. Acetylated (3AC-CM and 1AC-CM) and unacetylated (CM) forms of [14 C]chloramphenicol are indicated by arrows to the right of the autoradiograph. The percent conversion of chloramphenicol to acetylated forms for each transfection represents the average from three transfections.

molecules (6), both of which play an important role during normal lymphocyte activation and proliferation. One of these molecules is referred to as BLAST-1. At present, the transcriptional regulation of only a limited number of B-cell surface molecules, including major histocompatibility complex class I and II genes (11; reviewed in reference 51), Ig genes (reviewed in reference 5), and the interleukin-2 receptor α gene (reviewed in reference 14), has been studied in detail. However, studies of regulatory control regions mediating expression of other cell surface molecules present on B cells are just beginning (8, 53), and to date no studies on the transcriptional regulation of activation molecules by EBV have been published. Our overall goal is to use BLAST-1 as a model gene to understand the mechanism by which EBV deregulates expression of cell surface activation molecules. In order to address the question, the gene encoding BLAST-1 was isolated and characterized. Its exon-intron organization was determined, and the promoter region was analyzed both structurally and functionally.

The BLAST-1 gene isolated from three genomic clones is at least 26.8 kb in size and consists of four exons. The promoter region contains many major and minor initiation sites for BLAST-1 mRNA transcription. This is expected, since the TATA and CCAAT sequences, often associated with only one or two sites of initiation, are absent. The presence of eight initiation sites over a distance of approximately 60 bp strongly suggests the existence of multiple promoter regions for controlling BLAST-1 gene expression.

Sequence analysis of the promoter and upstream region revealed very little homology to other known promoter and enhancer elements present in leukocytes and viruses. The 5'-flanking region was analyzed for the presence of binding

sites for known transcription factors, and a number of sites, including AP1- and AP2-binding sites, which are known to mediate the TPA-induced responses (2, 25) were detected. These sites may be physiologically important, since BLAST-1 is phorbol myristate inducible (59).

The BLAST-1 promoter possesses a major characteristic of an inducible gene by being expressed at a high level in a limited tissue setting after stimulation. In addition, it possesses the attributes of a housekeeping gene by having transcriptional initiation sites spread over a large distance and lacking a TATA box. The presence of a large number of initiation sites and the lack of a TATA box are also characteristics found in two other genes expressed by B cells, CD20 (54) and B29 (20). This suggests that the transcriptional initiation complex that has been characterized thus far for TATA box-containing promoters (39) may be modified for this group of genes. This would be needed in order to accommodate the spatial arrangement of initiation sites and the lack of a TATA box.

A 1.58-kb genomic DNA fragment cloned upstream of the reporter gene CAT was able to drive CAT expression only in the sense orientation and appropriate cell type (B lymphoblastoid), thereby functioning similarly to the endogenous BLAST-1 promoter. When approximately 1,300 bp are removed from the 5' end of the fragment, the resulting chimeric CAT construct is also active in the negative-control cell line, which suggests that a putative silencer or negative-control region involved in tissue-specific expression has been removed (10a). The deleted sequence contains an *Alu* repeat sequence, which may have a role in controlling tissue specificity, since a repetitive element has previously been shown to function as a negative regulatory element in the case of the rat insulin gene (31).

The overall organization of the BLAST-1 gene is reminiscent of Ig genes and other members of the IgSF, in which the structural or functional domains are encoded by discrete exons (57). Unlike most IgSF members, exon 4 does not encode a transmembrane domain; rather, it encodes the portion of the protein that becomes modified through the addition of the GPI anchor. A significant portion of BLAST-1 on LCLs is resistant to digestion with phosphatidylinositol-specific phospholipase C (49). However, RNase protection analysis of RNA from such cells failed to identify an alternately spliced form of the BLAST-1 RNA that could encode a transmembrane form (48, 57). Thus, unlike LFA-3, there is no evidence at present for a transmembrane form of BLAST-1. On the basis of DNA sequence similarity, a subset of the Ig superfamily was proposed and includes BLAST-1, CD2, LFA-3, MRC OX45, NCA, and CEA (29), with BLAST-1 being most highly related to CD2, LFA-3, and MRC OX45 (29). This similarity now extends to include the function of cell adhesion molecules. At present, the gene structure has been elucidated only for CD2 (9, 32), and comparison with BLAST-1 indicates that they both have similarly sized exons encoding the Ig-like domains. In addition, two of the introns that flank these two exons are also of similar sizes. The major difference between the two gene structures, apart from CD2's possession of an additional exon encoding the cytoplasmic tail and 3' UT region, is the size of the first intron. In the case of CD2, the intron is 0.77 kb, while for BLAST-1 it is ≥ 22 kb. It may be worthy to note that CD4, another related member of the IgSF, also contains a large intron of >22 kb (34a). It will be of particular interest to know the gene structure of LFA-3, since it is most closely related to BLAST-1.

Previous analysis has shown that the interaction between

LFA-3 and its receptor CD2 mediates thymocyte-thymic epithelium adhesion and cytolytic conjugate formation (reviewed in reference 48). LFA-3 has been shown to interact with the V-like Ig domain of CD2 (42), demonstrating that Ig-like structural domains possessing significant homology are capable of interacting with each other and mediating heterotypic cellular interactions. Recent evidence from our laboratory demonstrates that BLAST-1 mediates homotypic and heterotypic adhesion, acting as its own receptor ligand. In addition, binding to T cells is greatly increased by preactivation with certain anti-CD2 monoclonal antibodies (48a). Recent genetic linkage studies and pulse-field analysis indicate that the genes for CD2 and LFA-3 are closely linked and that in the human and mouse this linkage region is similar to that around the location of the BLAST-1 gene. This has fueled speculation that these genes arose from a common ancestor and that there may exist another CD2-like gene, near the BLAST-1 locus, that encodes a BLAST-1 recognition partner (58). In light of our new data that BLAST-1 is its own receptor ligand, this speculation must be reconsidered. One possibility is that BLAST-1 is the primordial gene from which CD2 and LFA-3 arose.

As shown, BLAST-1 has a *Bam*HI RFLP. Recent analysis of the *Bam*HI RFLP by nuclear family studies has confirmed the presence of a second allele and indicates that the two alleles are inherited in a codominant Mendelian fashion (37). The *Bam*HI RFLP may be a suitable marker for linkage mapping studies, since the gene has been previously mapped to the q21-q23 region of chromosome 1 (49).

In conclusion, the analysis of the BLAST-1 gene described here will now allow us to map regulatory regions responsible for tissue-specific and EBV-specific modes of regulation for a B-cell activation molecule.

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