Identification of the Epstein-Barr Virus Terminal Protein Gene Products in Latently Infected Lymphocytes

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The terminal protein (TP) gene produces two overlapping mRNAs in latently infected lymphocytes that are predicted to encode the similar polypeptides TP1 (497 amino acids) and TP2 (378 amino acids), with TP1 exon 1 providing 119 extra unique residues at the N terminus. Rabbit antisera were raised to procaryotic fusion proteins and used to detect expression of a predicted 53-kilodalton (kDa) TP product in transfected 293 cells and latently infected lymphocytes. Fractionation of transfected 293 cells showed this protein to be localized to an integral membrane preparation. The same fraction of latently infected lymphocytes contained proteins of 53 and 27 to 39 kDa as determined by Western immunoblotting with the TP-specific rabbit antisera. Immunoprecipitation of TP products from ³⁵S-labeled human lymphoblastoid cells (CR/B95-8) was used in pulse-chase experiments and showed that TP1 was a labile protein with a half-life of approximately 2 to 4 h. The anti-fusion protein serum detected a 53-kDa TP1 and degradation products in the range of 25 to 35 kDa. A panel of Burkitt's lymphoma cell lines and cell lines established with virus recovered from the BL cells were analyzed by Western immunoblotting and found to contain the 53-kDa TP1 product, its degradation products, or both. Only two EBV-positive BL cell lines (BL72 and Wewak II) were negative in this assay. The results suggest that a labile TP1 protein may be expressed by most, if not all, EBV-infected cell lines.

The Epstein-Barr virus (EBV) is a ubiquitous lymphotropic human herpesvirus. It is the causative agent of infectious mononucleosis and is closely associated with African Burkitt's lymphoma, undifferentiated nasopharyngeal carcinoma, and B-cell lymphomas in immunodeficient patients (for a review, see reference 9). The virus also efficiently immortalizes human B lymphocytes in tissue culture (13). The EBV genome is a linear double-stranded DNA molecule of approximately 172 kilobases (kb) characterized by terminal tandem repeats (TR) and a variable number of 3.1-kb internal repeats (IR) joining a short and a long unique region. After infection, the viral genome circularizes via its terminal repeats, becomes amplified in copy number, and is maintained latently as an episome in the immortalized cells (17, 39). Integration of the viral genome into host DNA has been observed in a few cell lines (1, 12, 20a, 24, 41).

In immortalized cells, latent viral genomes may express up to six nuclear proteins (EBNA1, -2, -3A, -3B, -3C, and -4) and one membrane protein (LMP) (2, 3, 7, 8, 14-16, 26, 27, 30, 32, 33, 38, 42). Some, and possibly all, of these genes are expected to have roles in the immortalization process. EBNA1 is required for maintenance of episomes and binds to oriP, the episomal origin of replication (23, 29, 45). In addition, spontaneous deletion events in long-term-cultured Burkitt's lymphoma cell lines have produced nonimmortalizing mutants of EBV that lack the EBNA2 open reading frame and the 3' unique part of the EBNA4 (also called LP

or EBNA5) coding region (4, 25, 28). Although the effects of EBNA4 expression on lymphocytes remain unclear, a number of significant biological effects have been attributed to EBNA2 expression. EBNA2 enhances serum independence in rodent cells (6) and induces expression of the CD23 activation antigen on the surfaces of infected lymphocytes (43). No function has been described for the EBNA3 family of proteins (14, 15, 18, 26, 27, 30). The latent membrane-associated protein (LMP), when expressed in RAT-1 cells, can lead to morphological transformation and a tumorigenic phenotype (42).

Recently, transcripts from a gene that crosses the fused termini of the viral episome have been identified (21, 36), and from cDNA analysis they appear to encode two potential membrane proteins. Two messages of 2.0 and 1.7 kb have been detected which have unique 5' ends and encode proteins of 497 (TP1) and 379 (TP2) residues. The RNA from this gene has been detected in a wide range of EBV-positive lymphoid cell lines, including productive and nonproductive in vitro-immortalized lymphocytes and Burkitt's lymphoma lines of the restricted and nonrestricted expression phenotypes (21, 34, 35). This suggests that an important role may be served by the TP gene products, although the proteins have not been demonstrated to exist in lymphocytes infected with EBV. In this report we demonstrate, by using monospecific sera raised to TP fusion proteins, that the TP1 mRNA in fact encodes a labile protein expressed in latently infected lymphocytes and that it can be recovered from integral membrane fractions.

MATERIALS AND METHODS

Cell culture. Lymphoblastoid cell lines and Burkitt's lymphoma cell lines were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU of penicillin per ml, and 100 µg of streptomycin per ml. 293

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cells were grown in Dulbecco modified Eagle medium with the same supplements.

DNA clones. For eucaryotic expression, the TP1 cDNA was cloned as a *Bam*HI fragment into the *Bam*HI site of the M13 clone B8.H (21), with the EBV genomic sequence providing the initiator methionine of TP1. The 2.0-kb *Hind*III-*Sma*I fragment of the resulting M13 clone was ligated into *Hind*III-*Hpa*I-digested pMLP-CAT, giving rise to pMLTP1 (see Fig. 1C). This construct contains the TP1 cDNA with its initiator methionine downstream of the adenovirus type 2 major late promoter and upstream of the simian virus 40 polyadenylation signal. The deletion mutant pMLTP1del was created by digestion of the plasmid pMLTP1 with *S*tyI and subsequent religation in a reaction favoring circularization of the plasmid (Fig. 1C). The TP2 cDNA (20a) was cloned as a blunt-ended *Eco*RI fragment into *Sma*I-digested pMLP10 (M. Laverno, O. Jean-Jean, P. Balsano, H. Will, and M. Perricaudet, Virology, in press).

For procaryotic expression, the TP1 cDNA was cloned as a *Bam*HI fragment into the *Bgl*III site of pLEX-O (31). The fragment was recovered with flanking *Bam*HI sites from pLEX-O and cloned into the *Bam*HI site of pRIT2T (Pharmacia) to create the procaryotic inducible expression plasmid pREXTP1. A 300-base-pair *Bam*HI fragment from the B95-8 M13 clone PMO37.SET, representing TP1 exon 1, was cloned into the *Bgl*III site of pLEX-O, and the recovered *Bam*HI fragment was cloned into the *Bam*HI site of pRIT2T (Pharmacia) and designated pREX24.

Purification of bacterial fusion proteins. Plasmids pREXTP1 and pREX24 were used to transform expression strains of *E. coli* N4830, which were grown as overnight cultures, diluted 1:100, and grown to an A_{600} of 0.7 in 2× TYE medium (Difco Laboratories). An equal volume of medium preheated to 65°C was added, and cultures were incubated at 42°C for a further 2 h. The cells were pelleted, lysed by lysozyme treatment, and dissolved in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 7.5, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 0.2% sodium dodecyl sulfate [SDS], and 1 mM phenylmethylsulfonyl fluoride [PMSF]) to a final volume 20% of the original induced culture. The lysate was treated with DNase I and sonicated to reduce viscosity. Fusion proteins were recovered by incubating extracts with 0.2 ml of immunoglobulin G-Sepharose 6 beads (Pharmacia) for 12 h at 4°C with agitation and detached from the beads by the addition of 2.0 ml of 10 mM Tris, pH 8.0–8 M urea. The purified fusion proteins were dialyzed against 10 mM Tris, pH 8.0–100 mM NaCl, and purity was assessed by Coomassie blue staining of samples electrophoresed on SDS-polyacrylamide gels.

Transfection of 293 cells. 293 cells were transfected by the calcium phosphate precipitation technique (11). Briefly, the cells were passaged 1 day before transfection to give half-confluent plates on the day of transfection. The DNA precipitate was made by adding, dropwise, 500 μ l of 2× HBS (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.12, 1.5 mM Na_2HPO_4 , 280 mM NaCl) to 25 μ g of plasmid DNA in 1 mM Tris hydrochloride, pH 7.4–0.1 mM EDTA–250 mM CaCl_2 . After 20 min at room temperature, the precipitate was added to the plates without medium. After 30 min, medium was added and the precipitate was incubated at 37°C. After 16 h, the plates were washed and refilled with medium. The cells were harvested 48 h afterwards.

Cell fractionation. To prepare whole-cell extracts, 10^7 cells were lysed in 0.3 ml of sample buffer (125 mM Tris, pH 6.8, 2% SDS, 0.2% 2-mercaptoethanol, 10% glycerol, 1 mM

PMSF, 0.02 mM bromphenol blue). Membrane fractions were prepared by a modification of the procedure described by Warren (44). A total of 10^7 cells were suspended in 1 ml of 10 mM Tris, pH 7.5–25 mM NaCl–5 mM ZnCl_2 –1 mM PMSF and incubated for 20 min on ice. Cells were disrupted by Dounce homogenization (20 strokes) and centrifuged at $3,000 \times g$ for 10 min. The pellet, designated crude nuclear pellet, was suspended in 10 mM Tris, pH 7.5–1 mM PMSF, incubated for 20 min on ice, and disrupted with 30 strokes in a Dounce homogenizer. The extract was centrifuged at $3,000 \times g$ for 10 min, and the resulting pellet was designated the nuclear pellet. The nuclear membrane supernatant was centrifuged at $100,000 \times g$ at 4°C for 1 h and dissolved in sample buffer. The crude membrane supernatant was adjusted to 1 M NaCl by addition of an appropriate volume of 5 M NaCl. After incubation for 4 h at 0°C, the membrane preparation was centrifuged at $100,000 \times g$ for 1 h at 4°C. The membrane pellet was suspended in sample buffer and sonicated in 15-s intervals for 3 min at 0°C.

Immunoprecipitation. Immunoprecipitations were carried out by a modification of the procedure described by Schaffhausen et al. (37). Briefly, extracts from 10^7 cells were sonicated (Heat Systems W-375, microprobe, setting 5 for 30 s) in RIPA buffer (50 mM Tris, pH 7.5, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 0.2% SDS) and incubated with 30 μ l of equilibrated, settled protein A-Sepharose 4B beads (Pharmacia). After at least 6 h at 4°C with constant mixing, the beads were recovered by low-speed centrifugation, washed twice with RIPA buffer and twice with 10 mM Tris, pH 7.5–150 mM NaCl, and suspended in 50 μ l of electrophoresis sample buffer.

SDS-polyacrylamide gel electrophoresis and immunoblotting. Proteins were separated by the discontinuous gel electrophoresis technique (20). Gels, 1.5 mm thick and 180 mm long, consisted of a 10% resolving gel (acrylamide-bis-acrylamide, 30:0.8) and a 5% stacking gel. Generally, 20 μ l of sample representing 2×10^6 to 3×10^6 cells was electrophoresed at 60 V for 12 h and blotted onto nitrocellulose by procedures which have been described previously (5, 10). After blotting, the nonspecific antibody-binding sites on the filter were blocked with dilute skim milk (19). The immunoreagents, when bound to the nitrocellulose filters, were detected with ^{125}I -labeled staphylococcal protein A (Amersham Corp.). Molecular weight determinations were made by using prestained high- and low-molecular-weight standards (Bio-Rad Laboratories) run on the same gels.

Sera. Monospecific sera were prepared against fusion proteins AXTP1 and AX24 (the products of pREXTP1 and pREX24) in Sandy Half-flop rabbits. Animals were immunized with 500 μ g of fusion protein in complete Freund adjuvant (Difco) administered intradermally at four sites. Subsequently, boosts were at 2-week intervals and were composed of 500 μ g of fusion proteins in incomplete Freund adjuvant. Test bleeds, 7 days after boosting, were performed after the third and subsequent boosts.

RESULTS

Expression of TP in 293 cells. Preparation of immunological reagents for the detection of the putative TP proteins is essential to any characterization of the function of this novel latent gene. Two distinct mRNAs have been cloned and sequenced, revealing the primary structures of two potential membrane proteins, TP1 (497 amino acids) and TP2 (378 amino acids) (Fig. 1A and B). These polypeptides differ only in the N-terminal hydrophilic domain (119 amino acids),

FIG. 1. Schematic representation of the TP gene and constructs made for this study. (A) Bar represents the duplex viral DNA, with the internal boxes as the major open reading frames showing direction and with shading to indicate expression class (■, latent; □, late; ▨, early). Above and below the bar, small vertical and bent arrows represent polyadenylation signals and TATA boxes, respectively. The rightward extent of the Raji deletion and the scale are shown below the genome. Above are the exons of the two DNAs shaded to indicate the open reading frame (■) and noncoding regions (□) of the mRNA. (B) Putative structure of the TP1 and TP2 polypeptides, shown as integral membrane proteins with 12 spanning segments. (C) Constructs used for transient expression of the cDNAs. pMLP expression vector consisted of the adenovirus 2 E1A enhancer (■) linked to the major late promoter (■). The TP1 and TP2 cDNAs (□) were cloned into sites between the promoter and the simian virus 40 polyadenylation signal (□). A region in pMLPTP1del (▨) has been thrown out of phase by the *S*ylI deletion of pMLPTP1. A region at the 5' end of pMLPTP2 (▨) represents the noncoding TP2 exon 1'. (D) Structure of fusion proteins (□, TP1 sequences; ■, FX recognition sequence; □, CII sequence; ▨, *S. aureus* protein A sequences).

which is encoded by exon 1 of TP1 and is missing from TP2 (Fig. 1B). The TP2 polypeptide is expected to use as an initiation codon the first in-phase methionine, which occurs in exon 2 at residue 120. Initially, on the basis of the derived amino acid sequence, several peptides were synthesized, coupled to keyhole limpet hemocyanin or to tuberculin purified protein derivative and used to raise anti-peptide sera in rabbits. When these sera were tested on EBV-positive lymphocytes, equivocal or negative results were obtained. The TP1 cDNA was therefore cloned into procaryotic expression vectors, and fusion proteins were synthesized (Fig. 1D). The TP1-containing fusion proteins (CXTP1 and AXTP1) were recovered in a mostly degraded form. The intact TP1 fusion AXTP1 and its degradation products were used to immunize rabbits. The fusion proteins CX24 and AX24, which contained only the hydrophilic N-terminal exon 1 sequences of the TP1 protein, were stable when synthesized in bacteria, and AX24 was purified and used to immunize rabbits.

Early test bleeds (after three boosts) of these animals showed the presence of antibodies to fusion proteins at 1:100 dilutions on Western immunoblots. To test the reactivities of these antibodies to eucaryotically expressed TP gene products, we expressed the TP1 and TP2 cDNAs transiently by transfection of 293 cells with constructs bearing these cDNAs behind a strong promoter, the adenovirus major late promoter (Fig. 1C). In addition, the TP1-containing construct pMLPTP1 was digested with *SlyI* and religated to produce a frameshift mutation after residue 269 (between the fifth and sixth putative membrane-spanning segments) that replaces the COOH terminus with 13 novel amino acids. Initially, 293 cells transfected with various DNA concentrations of pMLPTP1 or pMLPTP2 were Western blotted onto nitrocellulose filters and probed with antisera raised against the AX24 and AXTP1 fusions.

A major band of 53 kDa was detected in the cells transfected with 20 μ g of pMLPTP1 with both antisera (Fig. 2). An additional band of 60 kDa was sometimes detected with the anti-AXTP1 sera, as were other low-molecular-weight species. Cells transfected with pMLPTP2 or the pMLP plasmid bearing no insert did not show any specific banding. Since the transient expression of pMLPTP1 showed a dependence on DNA concentration, transfections over a wide range of pMLPTP2 DNA concentrations (5 to 100 μ g) using different clones were attempted, but no TP2 product was ever detected with our antisera. Restriction analysis with several enzymes showed the TP2 expression plasmids to be of the correct size, insert orientation, and structure. The antisera therefore only appeared to recognize epitopes contained within, or conformationally influenced by, the unique sequences of TP1 exon 1. The low-molecular-weight species detected in pMLPTP1-transfected cells might be degradation products of TP1 rather than TP2-related species. A similar though not identical pattern of bands was detected in the CR/B95-8 lymphoblastoid cell line included in the panel for comparison.

Fractionation of 293 cells expressing pMLPTP1. In order to determine whether the transiently expressed TP products localized to membranes as predicted, a membrane fractionation was performed on pMLPLP1-transfected 293 cells (Fig. 3). TP1 was recovered in approximately equal amounts from the initial crude nuclear pellet and the processed integral membrane fraction, although the major putative degradation product was present preferentially in the nuclear pellet. The localization of the *SlyI* deletion mutant product of pMLPTP1del was similar to that of the intact TP1,

FIG. 2. Transfections of 293 cells with the pMLPTP expression vectors were harvested after 72 h and analyzed by Western immunoblotting with rabbit anti-fusion protein sera at a 1:100 dilution. Numbers above each lane represent DNA concentrations used in the transfection (micrograms per 60-mm dish of 75% confluent cells). pMLP lanes were transfected with vector containing no insert. CR/B95-8 EBV-immortalized lymphoblastoid cell line is included for comparison. Positions of molecular size markers are indicated on the right (in kilodaltons). The Western blots were probed with anti-AXTP1 (A) and anti-AX24 (B).

indicating that membrane localization was not dependent upon the seven COOH-terminal putative membrane-spanning segments. The deleted product, predicted to have a molecular mass of 30 kDa, migrated anomalously slowly on the polyacrylamide gels for reasons that are unclear. The deletion enhanced the relative proline content of the polypeptide and may introduce conformational anomalies that result in slower migration. Nevertheless, the disappearance of the 53-kDa band and the presence of a faster-migrating species provided additional evidence for the TP-specific nature of the antisera and the identity of the bands observed on Western blots. No bands were detected in the size range of the strongest immunoreactivities with our anti-TP sera in 293 cells mock transfected with pMLP bearing no insert. The high-molecular-weight bands in Fig. 3 are nonspecific and have been detected in EBV-negative lymphocytes (BJAB) with both the preimmunization control serum and the early test bleeds of the AXTP1-immunized rabbits.

Expression of TP1 in lymphocytes. Although CR/B95-8

FIG. 3. Membrane localization of TP1 protein in 293 cells after transfection with pMLPTP1del (lanes A) and pMLPTP1 (lanes B). The whole cells (WC) were fractionated into a crude nuclear pellet (NUC) and an integral membrane fraction (MEMB). The fractions were analyzed by polyacrylamide gel electrophoresis and immunoblotting with rabbit anti-TP1 sera developed with ^{125}I -labeled protein A. Protein size markers are indicated (in kilodaltons) to the right of the autoradiograph. Specific reactivities with the polyvalent rabbit sera are shown to the left of the autoradiograph.

produced a readily detected 53-kDa band, many other EBV-positive cell lines, which we knew expressed TP RNA from previous studies (21), were negative for the 53- and 39-kDa bands by Western blotting with the anti-AX24 or anti-AXTP1 serum used on the blot in Fig. 2. Indeed, during the course of immunizing boosts, the avidity of anti-AXTP1 response shifted away from the 53-kDa band and towards the recognition of a heterogeneously migrating population of species around 27 kDa. These bands appeared to be specific to EBV-positive cells and were only detected with sera from immunized animals after a long course of immunization. Two distinct patterns of reactivity among EBV-positive cell lines emerged and depended upon the immunogen used to raise the response (Fig. 4). Antisera against the AX24 (exon 1) fusion recognized the 53-kDa TP1 in whole-cell extracts of CR/B95-8 (and many other cell lines [see below]) but failed to detect the protein in Jijoye or its subclones (P3HR1, C5, and C16), in three strains of Raji, and in Namalwa. The serum produced intense bands of 38 and 21 kDa in P3HR1 and C5, respectively, and a weak band of 28 kDa in Namalwa. A second pattern was produced by antisera from animals immunized repeatedly (more than eight boosts) with AXTP1-degraded fusion products. Bands were detected in most cell lines in the range of 20 to 35 kDa and varied in size, intensity, and number from cell line to cell line. With few exceptions (for example, P3HR1) these were not the same species as were detected with the anti-AX24 sera. One notable exception was the 38-kDa band in Namalwa, which was the only product detected in this cell line and was detected by both kinds of sera. In the Namalwa cell line, the EBV genome is present as a head-to-head linearly cointegrated double insert into the cellular genome and therefore does not possess left-to-right fused termini (i.e., no intact terminal gene). The Namalwa integration is at a single site with the right ends of the EBV genome (and thus the TP1 promoter and exon 1-containing sequence) oriented towards

FIG. 4. Western immunoblot detection of terminal proteins in lymphocytes using antisera against the exon 1 fusion protein AX24 (A) and an antiserum against AXTP1 which detects putative degradation products (B). The 53-kDa TP1 band was detected only in CR/B95-8 cells, whereas both sera detected low-molecular-weight species in the cell lines. Only the Raji strains were negative for both TP1 and the low-molecular-weight bands. The 28-kDa band detected in Namalwa cells is predicted to arise from a transcript which contains both viral and cellular sequences. Molecular masses are indicated (in kilodaltons) on the left.

cellular sequences (22). The product detected with the anti-TP sera may therefore result from a chimeric RNA containing TP exon 1 and downstream cellular sequences. Raji viral genomes, on the other hand, are circular but have a deletion of sequences near the right end that includes the promoter and upstream region of the TP1 gene, and the absence of a TP1 banding pattern is thus expected and consistent with Northern (RNA) blot analysis.

We reasoned that the low-molecular-weight species might be degradation products of TP rather than unique species or unrelated cross-reacting molecules since (i) they were detected only with immune sera and not with preimmune sera, (ii) the immunogen was a fragment of TP (AX24) or a bacterially generated degradation product of AXTP, and (iii) there was no evidence from cDNA or Northern analyses that indicated the presence of mRNA expressed from the TP gene that had structures other than the full-length 2.0- and 1.7-kb messages (20a, 21, 36).

Subcellular fractionation of TP1 in lymphocytes. The intracellular localization of TP in lymphocytes was investigated

FIG. 5. Fractionation of CR/B95-8 lymphoblastoid cell line into cytoplasmic membranes (CYT. MEMB), cytosol, nuclear membranes (NUC MEMB), and nuclear pellet (NUC PEL) fractions of extracts representing equivalent cell numbers were analyzed by Western immunoblotting with anti-AX24 (A), anti-AXTP1 (B), anti-CS1-4, a monoclonal antibody recognizing LMP (C), and anti-EBNA1 (D), a human anti-EBNA serum. Specific bands are indicated on the left side of each panel, and molecular masses (in kilodaltons) are indicated on the right.

in CR/B95-8 cells. This cell line has consistently given the strongest signals for the 53-kDa TP1 band and the low-molecular-weight putative degradation products. Two main low-molecular-mass species (36 and 25 kDa) predominated in most preparations of CR/B95-8 cells, with considerable variation in the relative intensities of the bands.

Localization was first investigated by immunofluorescence of cytospin cell preparations fixed with acetone or acetone-methanol (1:1). No specific fluorescence was detected when the cells were incubated with the immune rabbit sera and fluorescein-conjugated goat anti-rabbit serum. Biochemical fractionation was performed, and samples representing equivalent cell numbers were loaded onto polyacrylamide gels. Western blots were probed with the AX24 and AXTP1 sera (Fig. 5A and B). The 53-kDa protein was detected in the cytoplasmic membrane, cytosol, and nuclear pellet fractions with an approximately equal distribution, unlike the low-molecular-weight species, which were mostly present in the cytoplasmic membrane fraction. The localiza-

tion of LMP and EBNA1 in these extracts is shown for comparison (Fig. 5C and D). In contrast to the cytosolic localization of the TP1 product, no LMP was released from the high-salt-treated crude membrane preparation into the cytosol fraction. This raises interesting questions about the nature of the free and membrane-bound forms of TP1 that challenge the predicted integral membrane structure depicted in Fig. 1B.

Stability of TP in CR/B95-8 cells. If the low-molecular-weight species detected in the EBV-positive cell lines were degradation products, then it would be reasonable to suspect that the TP proteins had short half-lives in lymphocytes. An experiment involving short pulse-labeling with [³⁵S]methionine in methionine-deficient medium followed by incubation for various lengths of time in medium containing excess cold methionine was performed on growing CR/B95-8 and EBV-negative RAMOS cells. Extracts made from pulse-labeled and chased cells were immunoprecipitated with control and anti-AXTP serum (Fig. 6). The 53-kDa TP1 protein was detected in cells continuously labeled for 8 h and in cells pulsed for periods as short as 1 h. Upon chase with cold methionine-containing medium, the incorporated label disappeared rapidly and was undetectable after 4 h. In the initial experiments, long continuous pulses of 8 h, which allowed for better accumulation of [³⁵S]methionine in intracellular pools, produced better labeling of TP1. The low-molecular-weight species that were detected in these extracts were thought to be degradation products (Fig. 6) but were not found in the chases of cells pulse-labeled for 1 h or in immunoprecipitates with the anti-AXTP-sera (not shown). When these experiments were repeated and the detergents were omitted from the bead-washing procedures of the immunoprecipitations, low-molecular-mass bands of 27 and 30 kDa were detected with anti-AXTP sera (Fig. 7). The 30-kDa species increased in intensity after 2 h of chase, corresponding to the decrease in the intensity of the TP1 band detected by the anti-AX24 serum in the same extracts. Although these data are not conclusive, the lability of the TP1 protein in CR/B95-8 (Fig. 6 and 7), the cofractionation of the putative fragment bands with TP1 (Fig. 5), and the EBV-specific serological evidence all suggest that the low-molecular-weight species are degradation products of TP.

Expression of TP in Burkitt's lymphoma cell lines. Burkitt's lymphoma cell lines have been classified into four groups (I, I/II, II, and III) on the basis of their *in vitro* growth characteristics and cell surface phenotypes (34, 35). The group I lines are most like their parental biopsy cells, whereas the group III lines have progressed in culture to take on a phenotype which is characteristic of lymphoblastoid cell lines established with the Burkitt's lymphoma virus *in vitro*. The expression of EBV latent genes is more restricted in group I lines (particularly EBNA2 and LMP) compared with the full complement of latent gene expression in the group III lines. We wished to determine whether this restricted gene expression phenotype extended to the TP protein.

Protein extracts of Burkitt's lymphoma and lymphoblastoid cell line pairs were Western blotted and probed with anti-AX24, anti-AXTP1, and anti-LMP sera (Fig. 8). The 53-kDa TP1 band was detected in two of the Burkitt's lymphoma lines, Wewak 1 (WW1) and BL74, and three of the lymphoblastoid cell lines, BL18, BL29, and Cheptages (Chep), when the blot was probed with the anti-AX24 serum. The levels of expression appeared to be similar, except for WW1 lymphoblastoid cell line, in which the level of expression was much lower. The anti-AXTP1 serum which recog-

FIG. 6. Immunoprecipitation of TP1 metabolically labeled with [35 S]methionine. CR/B95-8 and Ramos cells were pulse-labeled for either 8 or 1 h. Cells pulse-labeled for 1 h were incubated in chase medium for 1, 2, 4, or 8 h. (A) Immunoprecipitates of CR/B95-8 with control preimmune serum. (B) Immunoprecipitates of CR/B95-8 with anti-AX24 serum. (C) Immunoprecipitates of Ramos with anti-AX24 serum.

nizes putative degradation products detected bands of different sizes and intensities in the range of 25 to 35 kDa in most of the cell lines. A pattern of various intensities of protein banding from line to line has also been observed in blots probed with anti-LMP sera (34, 35) (Fig. 8), although there was no correlation between the levels of LMP (Fig. 8) and the levels of TP or the degradation fragments for the cell

lines used in this study. The results of Western blot experiments performed with Burkitt's lymphoma and lymphoblastoid cell line pairs derived from all phenotypic categories of Burkitt's lymphoma expression are summarized in Table 1. In general, the 53-kDa TP1 protein (AX24 column) has been easier to detect in lymphoblastoid cell lines than in Burkitt's lymphoma cell lines, but both types contained the degradation fragments (AXTP1 column). Two lines (BL72 and Wewak II) have yielded negative results for both the anti-AX24 and anti-AXTP1 sera.

DISCUSSION

We have raised monospecific rabbit antisera that recognize the TP1 gene product expressed transiently in 293 cells and constitutively in EBV-infected lymphocytes. The protein expressed in the 293 cells was of 53 kDa, as estimated by relation to standard size markers. The predicted molecular weight of 53,012 is remarkably close to the observed size and suggests that this product might not be significantly modified by glycosylation or other posttranslational adducts that alter gel mobility. In both the transient expression assays and lymphocytes, the TP products were recovered from high-salt-extracted cytoplasmic membranes. The nuclear localization we observed may presumably also be membrane mediated, as the fraction contains nuclear membranes and residual endoplasmic reticulum. The membrane localization was not affected by the deletion of the seven carboxyl-terminal putative membrane-spanning segments (Fig. 4). Although we cannot determine the orientation of the polypeptide in the membrane from the present studies, TP1 does not contain a typical amino-terminal signal sequence, and we would therefore expect the exon 1 sequences to be on the cytoplasmic face of cellular membranes. TP2, however, begins with the first hydrophobic domain which could possibly serve as a signal sequence, thus orienting TP1 and TP2

FIG. 7. Immunoprecipitations of TP1 and putative degradation fragments in CR/B95-8 pulse-labeled with [35 S]methionine for 1 h and chased for 1, 2, 4, and 8 h in chase medium. The upper panel shows the kinetics of the 53-kDa TP1 detected with anti-AX24 serum, and the lower panel shows the kinetics of low-molecular-weight species detected by anti-AXTP1 in the same extracts. For this experiment, detergents were omitted from the bead-washing procedure.

FIG. 8. Western immunoblots of six Burkitt's lymphoma and lymphoblastoid pairs of cell lines. The virus recovered from the Burkitt's lymphoma cell line was used to establish the lymphoblastoid cell line. The TP1 protein was recognized by the anti-AX24 serum (A) as a 53-kDa band in some of the lysates. This serum also detected low-molecular-weight bands presumed to be degradation products. The AXTP1 serum (B) detected bands in most of the cell lines in the 25- to 35-kDa range, which varied in size and intensity from cell line to cell line. The reactivities of the same cell extracts with the CS1-4 anti-LMP monoclonal antibody (C) against a different latent viral membrane protein is shown for comparison.

oppositely in membranes. Further experiments are required to address this question. The presence of TP1 in the cytosol (a fraction which contained no LMP) was unexpected because of the very hydrophobic nature of the protein and its predicted multiple membrane-intercalating segments. The 1-h, $100,000 \times g$ centrifugation is calculated to pellet all particles with an S value greater than 42S. It is possible, therefore, that the protein may persist in suspension in very small membrane micelles or protein aggregates.

The use of rabbit antisera to detect TP expression in a large number of cell lines, some of which had been previously analyzed by Northern analysis and were shown to contain TP RNAs, gave results which were not consistent. This was mostly resolved by probing Western blots with anti-AXTP1 sera from rabbits that had received 8 to 10 boosting inoculations. These sera reacted with a range of low-molecular-mass species between 25 and 38 kDa which we have interpreted as being degradation products. Pulse-chase experiments confirmed that the TP1 protein was rapidly turned over in the CR/B95-8 cell line and that it was possible to detect label entering low-molecular-weight spe-

TABLE 1. Expression of TP1 in Burkitt's lymphoma cell lines

Phenotypic group	Cell line	Virus type	Expression of:			
			TP1 (αAX24)	TP1 (αAXTP1)	EBNA2	LMP
Burkitt's lym- phoma cell lines						
I	Cheptages	B		+		
I	Makau	A		+		
I	Wewak II	B				
I/II	BL29	B		+		
I/II	BL72	A			+	+
II	Wewak I	A	+	+	+	+
II	BL74	A	+	+		
III	BL16	B		+	+	+
III	BL18	A		+	+	+
III	Mwika	A	+	+	+	+
BL virus-trans- formed cell lines						
III	Cheptages	B	+	+	+	+
III	Makau	A		+	+	+
III	Wewak II	B		+	+	+
III	BL29	B	+	+	+	+
III	BL72	A		+	+	+
III	Wewak I	A	+	+	+	+
III	BL74	A		+	+	+
III	BL16	B		+	+	+
III	BL18	A	+	+	+	+
III	Mwika	A	+	+	+	+

cies with the appropriate kinetics. The sera, however, detected a large range of product sizes and intensities in different cell lines. Such differences may be due to variations in turnover rates and stabilities of products (e.g., Jijoye and its subclones [Fig. 4]) or to amino acid sequence variations within the TP products of different viruses that affect gel migration, fragment stability, and/or antigenicity.

Both anti-AXTP1 and anti-AX24 sera recognized the same low-molecular-mass bands in Namalwa cells (28 kDa) and BL18 cells (27 to 24 kDa), pointing to another potential source for these products as virus host fusion proteins. For Namalwa cells, the two EBV genomes are linear head-to-head integrated molecules (12, 22, 24) which could give rise to fused messages expressed from TP1 exon 1 and the downstream cellular sequences. Fusion transcripts of this type have been detected in IB4 cells (36) and recently in Namalwa (U. Strobl, personal communication). Since the 28-kDa band in Namalwa cells is the only product detected by both sera, it seems likely that it represents a fusion protein containing N-terminal TP1 exon 1 sequences and C-terminal cellular sequences.

These considerations make it extremely difficult to use serological tests to identify TP products in cell lines or biopsy specimens. Our analyses of the Burkitt's lymphoma and lymphoblastoid paired cell lines revealed that fewer than half of the lines listed in Table 1 contained intact TP1 detectable by using the antiserum specific for the hydrophilic N-terminal exon 1. Yet when the antiserum which detected degradation products was used, nearly all lines scored positive. No obvious pattern has emerged which would point to some posttranslational mechanism for controlling TP levels in different cell lines. This finding is similar in some respects to the large-scale variations in banding intensity of LMP that have been documented (34, 35), although these

variations do not correlate with the results obtained for the TP products in this study. It is difficult, at present, to draw conclusions regarding the Wewak II and BL72 cell lines that have scored negative. BL72 has previously been reported to express TP RNAs at normal levels (20a); thus, the absence of product does not reflect a transcriptional block on TP gene expression. It may be that the amount of protein is too low to be detected by the present assay because of high rates of turnover and/or low rates of translation in these cells. It seems more likely that the product will be found in these cells when more sophisticated monoclonal reagents have been made and the sensitivity of the assay is improved. These results lead us to suspect that the TP gene products may have a crucial role in the processes of EBV immortalization and latency.

LITERATURE CITED

- Andersson-Anvret, M., and T. Lindahl. 1978. Integrated viral DNA sequences in Epstein-Barr virus-converted human lymphoma lines. *J. Virol.* **25**:710-718.
- Bodescot, M., O. Brison, and M. Perricaudet. 1986. An Epstein-Barr virus transcription unit is at least 84 kilobases long. *Nucleic Acids Res.* **14**:2611-2620.
- Bodescot, M., B. Chambraud, P. Farrell, and M. Perricaudet. 1984. Spliced RNA from the IRI-U2 region of Epstein-Barr virus: presence of an open reading frame for a repetitive polypeptide. *EMBO J.* **3**:1913-1917.
- Bornkamm, G. W., J. Hudwenz, U. K. Freese, and U. Zimmer. 1982. Deletion of the nontransforming Epstein-Barr virus strain P3HR1 causes fusion of the large internal repeat to the subregion. *J. Virol.* **43**:952-968.
- Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulphate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radio-iodinated protein A. *Anal. Biochem.* **112**:195-203.
- Dambaugh, T., F. Wang, K. Hennessy, A. Rickinson, and E. Kieff. 1986. Expression of the Epstein-Barr virus nuclear protein 2 in rodent cells. *J. Virol.* **59**:453-462.
- Dillner, J., B. Kallin, H. Alexander, I. Ernberg, M. Uno, Y. Ono, G. Klein, and R. A. Lerner. 1986. A novel Epstein-Barr virus determined nuclear antigen (EBNA-5) partly encoded by the transformation associated Bam WYH region of EBV DNA. *Proc. Natl. Acad. Sci. USA* **83**:6641-6645.
- Dillner, J., B. Kallin, G. Klein, H. Jorvall, H. Alexander, and R. Lerner. 1985. Antibodies against synthetic peptides react with the second EBV nuclear antigen. *EMBO J.* **4**:1813-1818.
- Epstein, M. A., and B. G. Achong. 1979. *The Epstein-Barr virus*. Springer-Verlag KG, Berlin.
- Fischer, D., M. Robert, D. Shedd, W. Summers, J. Robinson, J. Wolak, J. Stefano, and G. Miller. 1984. Identification of Epstein-Barr nuclear antigen polypeptide in mouse and monkey cells after gene transfer with cloned 2.9-kilobase-pair subfragment of the genome. *Proc. Natl. Acad. Sci. USA* **81**:43-47.
- Graham, F., and A. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**:456-467.
- Henderson, A., S. Ripley, M. Heller, and E. Kieff. 1983. Chromosome site for Epstein-Barr virus DNA in a Burkitt tumor cell line and in lymphocytes growth-transformed in vitro. *Proc. Natl. Acad. Sci. USA* **80**:1987-1991.
- Henle, W., V. Diehl, G. Kohn, H. zur Hausen, and G. Henle. 1967. Herpes-type virus and chromosome marker in normal leukocytes after growth with irradiated Burkitt cells. *Science* **157**:1064-1065.
- Hennessy, K., S. Fennewald, M. Hummel, T. Cole, and E. Kieff. 1984. A membrane protein encoded by Epstein-Barr virus in latent growth transforming infection. *Proc. Natl. Acad. Sci. USA* **81**:7202-7211.
- Hennessy, K., S. Fennewald, and E. Kieff. 1985. A third viral nuclear protein in lymphoblasts immortalized by Epstein-Barr virus. *Proc. Natl. Acad. Sci. USA* **82**:5944-5948.
- Hennessy, K., F. Wang, E. Woodland-Bushman, and E. Kieff. 1986. Definitive identification of a member of the Epstein-Barr virus nuclear protein 3 family. *Proc. Natl. Acad. Sci. USA* **83**:5693-5697.
- Hurley, E. A., and D. A. Thorley-Lawson. 1988. B cell activation and the establishment of Epstein-Barr virus latency. *J. Exp. Med.* **168**:2059-2077.
- Joab, I., D. T. Rowe, M. Bodescot, J.-C. Nicolas, P. J. Farrell, and M. Perricaudet. 1987. Mapping of the gene coding for Epstein-Barr virus-determined nuclear antigen EBNA3 and its transient overexpression in a human cell line by using an adenovirus expression vector. *J. Virol.* **61**:3340-3344.
- Johnson, D. A., J. W. Gautsch, J. R. Sportsman, and J. H. Elder. 1984. Improved technique using non-fat milk for analysis of proteins transferred to nitrocellulose. *Gene Anal. Tech.* **1**:3-8.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
- Laux, G., A. Economou, and P. J. Farrell. 1989. The terminal protein gene 2 of Epstein-Barr virus is transcribed from a bidirectional latent promoter region. *J. Gen. Virol.* **70**:3079-3084.
- Laux, G., M. Perricaudet, and P. J. Farrell. 1988. A spliced Epstein-Barr virus gene expressed in immortalized lymphocytes is created by circularization of the linear viral genome. *EMBO J.* **7**:769-775.
- Lawrence, J. B., C. A. Villnave, and R. H. Singer. 1988. Sensitive, high resolution chromatin and chromosome mapping *in situ*. Presence and orientation of two closely integrated copies of EBV in a lymphoma line. *Cell* **52**:51-61.
- Lupton, S., and A. J. Levine. 1985. Mapping genetic elements of Epstein-Barr virus that facilitate extrachromosomal persistence of Epstein-Barr virus-derived plasmids in human cells. *Mol. Cell. Biol.* **5**:2533-2542.
- Matsuo, T., M. Heller, L. Petti, E. O'Shiro, and E. Kieff. 1984. Persistence of the entire Epstein-Barr virus genome integrated into human lymphocyte DNA. *Science* **226**:1322-1325.
- Miller, G., J. Robinson, L. Heston, and M. Lipman. 1974. Differences between laboratory strains of Epstein-Barr virus based on immortalization, abortive infection and interference. *Proc. Natl. Acad. Sci. USA* **71**:4006-4010.
- Petti, L., and E. Kieff. 1988. A sixth Epstein-Barr virus nuclear protein (EBNA3B) is expressed in latently infected growth-transformed lymphocytes. *J. Virol.* **62**:2173-2178.
- Petti, L., J. Sample, F. Wang, and E. Kieff. 1988. A fifth Epstein-Barr virus nuclear protein (EBNA3C) is expressed in latently infected growth-transformed lymphocytes. *J. Virol.* **62**:1330-1338.
- Rabson, M., L. Gradoville, L. Heston, and G. Miller. 1982. Nonimmortalizing P3J-HR-1 Epstein-Barr virus: a deletion mutant of its transforming parent, Jijoye. *J. Virol.* **44**:834-844.
- Rawlins, D. R., G. Milman, S. D. Hayward, and G. S. Hayward. 1985. Sequence specific DNA binding of the Epstein-Barr virus nuclear antigen (EBNA1) to clustered sites in the plasmid maintenance region. *Cell* **42**:859-868.
- Ricksten, A., B. Kallin, H. Alexander, J. Dillner, R. Fahraeus, G. Klein, R. Lerner, and L. Rymo. 1988. BamHI E region of the Epstein-Barr virus genome encodes three transformation-associated nuclear proteins. *Proc. Natl. Acad. Sci. USA* **85**:995-1000.
- Rowe, D. T., and J. Clarke. 1989. The type-specific epitopes of the Epstein-Barr virus nuclear antigen 2 are near the carboxy terminus of the protein. *J. Gen. Virol.* **70**:1217-1229.
- Rowe, D. T., P. J. Farrell, and G. Miller. 1987. Novel Epstein-Barr virus nuclear antigens recognized by human sera in latently infected lymphocytes. *Virology* **156**:153-162.
- Rowe, D. T., J. Metlay, L. Heston, and G. Miller. 1985. Identification and expression of a nuclear antigen from the genomic region of the Jijoye strain of Epstein-Barr virus that is missing in its non-immortalizing deletion mutant P3HR1. *Proc. Natl. Acad. Sci. USA* **82**:7429-7433.

34. Rowe, D. T., M. Rowe, G. I. Evans, L. E. Wallace, P. J. Farrell, and A. B. Rickinson. 1986. Restricted expression of EBV latent genes and T-lymphocyte detected membrane antigen in Burkitt's lymphoma cells. *EMBO J.* **5**:2599-2607.
35. Rowe, M., D. T. Rowe, C. D. Gregory, L. S. Young, P. J. Farrell, H. Rupani, and A. B. Rickinson. 1987. Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells. *EMBO J.* **6**:2743-2753.
36. Sample, J., D. Liebowitz, and E. Kieff. 1989. Two related Epstein-Barr virus membrane proteins are encoded by separate genes. *J. Virol.* **63**:993-997.
37. Schaffhausen, B. S., J. E. Silver, and T. L. Benjamin. 1978. Tumor antigens in cells productively infected with wild type polyoma virus and mutant N 6-18. *Proc. Natl. Acad. Sci. USA* **75**:79-83.
38. Shimizu, N., M. Yamaki, S. Sakuma, Y. Ono, and K. Takada. 1988. Three Epstein-Barr virus (EBV)-determined nuclear antigens induced by the BamHI E region of EBV DNA. *Int. J. Cancer* **41**:744-752.
39. Sugden, B., M. Phelb, and J. Domoradzki. 1979. Epstein-Barr virus DNA is amplified in transformed lymphocytes. *J. Virol.* **31**:590-595.
40. Summers, N., E. Grogan, D. Shedd, M. Robert, C.-R. Liu, and G. Miller. 1982. Stable expression in mouse cells of nuclear neoantigen after transfer of a 3.4-megadalton cloned fragment of Epstein-Barr virus DNA. *Proc. Natl. Acad. Sci. USA* **79**:5688-5692.
41. Teo, C. G., and B. E. Griffin. 1987. Epstein-Barr virus genomes in lymphoblastoid cells: activation in mitosis and chromosomal location. *Proc. Natl. Acad. Sci. USA* **84**:8473-8477.
42. Wang, D., D. Liebowitz, and E. Kieff. 1985. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. *Cell* **43**:831-840.
43. Wang, F., C. D. Gregory, M. Rowe, A. B. Rickinson, D. Wang, M. Birkenbach, M. H. Kikutani, and E. Kieff. 1987. Epstein-Barr virus nuclear antigen 2 specifically induces expression of the B-cell activation antigen CD23. *Proc. Natl. Acad. Sci. USA* **43**:3452-3457.
44. Warren, L. 1974. Isolation of plasma membranes from tissue cultures of L cells. *Methods Enzymol.* **31**:156-162.
45. Yates, J., N. Warren, D. Reisman, and B. Sugden. 1984. A cis-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. *Proc. Natl. Acad. Sci. USA* **81**:3806-3810.