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Epstein-Barr Virus Nuclear Antigen 2 Activates Transcription of the Terminal Protein Gene

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Transcription of the terminal protein (TP) gene of Epstein-Barr virus (EBV) in Burkitt's lymphoma cells, in EBV-negative Burkitt's lymphoma cells converted with transformation-defective (P3HR1) and transformation-competent (B95-8, AG876) EBV strains, and in EBV-immortalized cell lines was studied. A TP1 cDNA probe spanning the boundary between exons 1 and 2 and discriminating between TP1 and TP2 transcripts was used for S1 analysis. TP RNA expression varied widely in Burkitt's lymphoma cells. TP-specific transcripts were not detectable or only hardly detectable in Burkitt's lymphoma cells with the group I phenotype ($CD10^+$ $CD77^+$ $CD21^-$ $CD23^-$ $CD30^-$ $CDw70^-$) as well as in P3HR1 virus-converted Burkitt's lymphoma lines. TP expression was high in Burkitt's lymphoma lines with the group II and group III phenotypes ($CD21^+$ $CD23^+$ $CD30^+$ $CDw70^+$), in B95-8 and AG876 virus-converted lines, and in EBV-immortalized cells. Detection of TP1 RNA correlated with EBNA2 expression. TP1 transcription was shown to be dependent on EBNA2 expression by stable transfection of an EBNA2 expression vector into P3HR1 virus-converted BL41 cells. EBNA2 is activating the TP1 as well as the TP2 promoter, as shown by the analysis of TP promoter-chloramphenicol acetyltransferase constructs transiently transfected into EBNA2-positive and EBNA2-negative Burkitt's lymphoma cells.

Epstein-Barr virus (EBV), a human B-cell lymphotropic herpes virus, is widespread in all human communities. The virus causes infectious mononucleosis and is associated with two human cancers, Burkitt's lymphoma (BL) and nasopharyngeal carcinoma. EBV is able to transform normal resting B cells into permanently growing lymphoblastoid cell lines (LCL) in vitro. In EBV-positive tumors and in LCLs the virus is maintained in an episomal state (23), usually at high copy number. Viral integration into the cellular genome has also been observed (4) but has been studied in detail only in the BL line Namalwa, which has no episomal but two integrated copies (21, 26).

EBV-immortalized cells usually do not produce virus particles. The virus is in a latent state, with only a small number of genes expressed (for a review, see reference 18). These genes code for at least six nuclear (EBNAs) and three membrane proteins, latent membrane protein (LMP) and terminal proteins 1 and 2 (TP1 and TP2), which are alternatively designated LMP1, LMP2A, and LMP2B, respectively, by some authors. All these genes may be important for the establishment and maintenance of the immortalized state of B lymphocytes. Little is known about the function of these genes. EBNA1 is necessary for the extrachromosomal replication of the viral episomal DNA (29, 40). EBNA2 appears to be absolutely required for the establishment of B-cell immortalization. The EBV strain P3HR1, with a 6.6-kb deletion (9, 17) which has removed the EBNA2 and part of the EBNA-LP gene, has lost the ability to immortalize B cells. Reintroduction of EBNA2 into the P3HR1 viral DNA restores the transforming potential of the virus (15). Several lines of evidence indicate that EBNA2 is also a modulator of expression of viral as well as cellular genes. In EBV-negative BL cells infected in vitro by the EBNA2-defective P3HR1 virus, LMP expression is down regulated (27). EBNA2 precedes the expression of the other viral latent antigens upon infection of B lymphocytes (2, 30) and is supposedly involved in activation of LMP expression (1, 14). B-cell activation markers such as CD21 and CD23 are up regulated in EBV-negative BL cells which are infected by the transformation-competent B95-8 virus but not by the EBNA2-defective P3HR1 virus (10). Transfection of the EBNA2 gene into P3HR1 virus-converted BL cells and into EBV-negative BL cells has established the role of EBNA2 in the induction of CD21 and CD23 (12, 39).

The LMP is also associated with phenotypic changes in B cells (38). Additionally, it might be an effector protein in cell transformation, since rodent fibroblasts transfected with the LMP gene lose their anchorage dependence and acquire oncogenicity (7, 37).

Nothing is known about the biological and biochemical function of the other viral latent genes coding for additional nuclear (EBNA3a, EBNA3b, EBNA3c, and EBNA-LP) and membrane (TP1 and TP2) proteins. The TPs are unique in that their gene is composed of exons located at both ends of the linear viral DNA and requires circularization of the viral genome to become a functional gene (19, 20, 36). The TP gene gives rise to two RNAs (2.0 and 1.7 kb) with coding potential for membrane proteins with molecular masses of 54 and 40 kDa (13, 24, 32). TP1 and TP2 RNAs have different promoters. Both have a unique exon 1 located in the U_L region but share exons 2 to 9 in the U_S region (Fig. 1a). TP2 RNA has a potential translation initiation codon at the beginning of exon 2 and codes for an extremely hydrophobic protein which lacks the hydrophilic N terminus encoded by exon 1 of TP1 RNA. Recombinant TPs are only weakly

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immunogenic, presumably because of the high degree of hydrophobicity (13). So far, nothing is known about the biological function of the TP gene. Recently it has been shown that TP and LMP colocalize in the plasma membrane of EBV-infected LCL (24), indicating that TP and LMP could function together in the process of immortalization.

Even though the viral gene products of the latent state play an essential role in virus-induced growth transformation in vitro, their contribution to the malignant phenotype is unclear. BL cells established as cell lines do not necessarily represent the tumor cells in vivo with regard to cell surface phenotype and pattern of viral gene expression (31, 33, 35). When freshly seeded in culture, BL cells have the phenotype of tumor cells in biopsies. They grow in single-cell suspension and do not express EBNA2 and LMP. Upon prolonged cultivation in vitro, they become EBNA2 and LMP positive and start to grow in clumps. The change in growth pattern is associated with the appearance of activation markers on the cell surface (groups II and III, according to Rowe et al. [34, 35]). A minority of BL cells, however, remain EBNA2 and LMP negative and maintain their original tumor cell phenotype and single-cell growth pattern upon establishment as cell lines in vitro (group I). Such cell lines may therefore be regarded as a model with which to study the mode of EBV latency in BL cell lines in vivo. It is particularly important to learn whether the newly described TP gene is expressed in such cells.

Because antisera with high affinity are not available, nothing is known about the pattern of TP expression in EBV-associated tumors. Therefore, we have attempted to study TP gene expression at the level of RNA. In this study we found that TP RNA expression is highly variable in BL cell lines depending on the phenotype, whereas TP is regularly expressed in EBV-immortalized cells. We also found that the TP1 and TP2 promoters are regulated either directly or indirectly by EBNA2 in lymphoid cells.

MATERIALS AND METHODS

Culture conditions. All cells 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 at 37°C in an atmosphere of 5% CO_2. Cultures were fed by dilution with one-half or two-thirds the total volume of fresh medium once or twice a week.

Cell lines. Most cell lines used in these experiments were established at the International Agency for Research on Cancer, Lyon, France (22), and in the laboratory of A. B. Rickinson, Department of Cancer Studies, Birmingham, England (31). The converted cell lines are single-cell clones obtained after infection of EBV-negative BL cell lines with the virus strains P3HR1, B95-8, and AG876 (10). P3HR1-converted cells transfected with EBNA2 were described by Cordier et al. (12). LCL were spontaneously established (IARC-LCL261, IARC-LCL139) or obtained by immortalization of peripheral B lymphocytes with either B95-8 virus (IARC305, IARC277, IARC100, IARC176A) or virus of the cell line indicated (LCL-ELI). The CBL cell lines are cord blood lymphocytes immortalized with EBV M-ABA (M-ABA/CBL) or B95-8 (B95-8/CBL).

RNA preparation. Total cellular RNA was extracted by the lithium chloride-urea method described by Auffray and Rougeon (5). Some of the RNAs were prepared after treatment of cells with cycloheximide. In this case the cells were grown for 16 h in culture medium supplemented with 50 μg of cycloheximide per ml. Cells were collected by centrifugation and washed once in ice-cold phosphate-buffered saline (PBS). The pellet was resuspended in 3 M LiCl–6 M urea and pressed through a syringe needle until the viscosity disappeared. The solution was kept overnight at 0°C. The RNA was pelleted by centrifugation for 1 h at 6,000 × *g*. The pellet was dissolved in 10 mM Tris hydrochloride (pH 7.5)–0.5% sodium dodecyl sulfate (SDS) and extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and then once with an equal volume of chloroform-isoamyl alcohol (24:1). The extracted RNA was precipitated with 70% ethanol.

Plasmid construction. We have isolated TP1-specific clones from a lambda gt10 cDNA library of the M-ABA/CBL cell line (36a). The longest insert (positions 34 to 2010 of Laux et al. [20]) was sequenced and subcloned into the *Eco*RI site of pSPT19 to generate pU516-1. pU516-1 was digested with *Eco*RI and *Nde*I, and the 5′ protruding *Nde*I ends were filled in with the Klenow fragment of DNA polymerase I. The resulting DNA fragment, containing positions 34 to 575, was isolated and cloned into the *Eco*RI and *Sma*I site of pUC19 to generate pU587-14. pU587-14 was digested with *Bam*HI and *Ava*II, the protruding *Ava*II ends were made blunt ended, and the fragment which contained the TP1 cDNA from positions 248 to 575 was isolated. This cDNA fragment was cloned into the *Bam*HI and *Sma*I site of M13mp19 to create pU662-19.

The TP1 promoter-chloramphenicol acetyltransferase (CAT) construct contains the promoter and upstream region of TP1 (positions 165696 to 166559 of the EBV sequence according to Baer et al. [6]) in front of the CAT gene. The *Sph*I-*Pst*I EBV fragment was inserted in the *Hin*dIII and *Pst*I site of the vector pBLCAT5. The TP2-CAT and LMP-CAT constructs are described elsewhere (19).

The EBNA2 expression vector used in the cotransfection contains an EBV fragment from positions 35448 to 54360. The clone was generated by deleting the *Not*I repeats of pM780-28 described by Polack et al. (28).

The CMV-LTR-CAT construct was obtained from I. S. Y. Chen (11).

S1 analysis. A single-stranded probe was prepared by primer extension of the M13 clone pU662-19 to detect TP1 and TP2 in cellular RNA by S1 analysis. After the labeling reaction, the clone was digested with *Hin*dIII to give a 374-bp uniformly labeled fragment, which was isolated from a denaturating 5% polyacrylamide gel.

Hybridization of labeled DNA fragments to total RNA was carried out by a modification of the method of Berk and Sharp (8). Hybridization mixtures of 20 μl containing 70,000 cpm of the probe, 50 μg of RNA, 90% formamide, 400 mM NaCl, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.5), and 1 mM EDTA were denaturated at 90°C for 5 min and immediately transferred to 52°C. After 15 h the hybridization was terminated by addition of 180 μl of ice-cold buffer containing 250 mM NaCl, 30 mM sodium acetate (pH 4.5), 2 mM zinc acetate, 5% glycerol, and 400 U of nuclease S1 (Boehringer, Mannheim, Germany). The samples were then incubated at room temperature for 1 h, extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1), and precipitated with ethanol. Protected DNA fragments were separated on 5% denaturating polyacrylamide gels. The gels were dried and autoradiographed.

As a internal control, a 5′-labeled synthetic oligonucleotide of the human glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used (3). The 5′ nucleotide of the oligonucleotide is the first complementary T of the polyadenylation signal of the GAPDH gene (position 1187 according

to the published sequence) and is followed by 87 bases which are complementary to the GAPDH gene. At the 3' end of the synthetic oligonucleotide, 13 noncomplementary bases were added. The 5' labeling of the probe was performed with polynucleotide kinase (25).

Transfection of the cells. The cells were transfected by electroporation by the method of Cann et al. (11). Briefly, 10^7 cells with a viability of more than 90% were washed once and resuspended in 0.25 ml of fresh RPMI 1640 medium with 10% calf serum. The cells were placed on ice in a gene pulser cuvette, and 20 μg of the corresponding DNA was added. Immediately after addition of DNA, cells were electroporated with the Bio-Rad gene pulser at 0.25 V and 960 μF. At 10 min after electroporation, cells were resuspended in 10 ml of RPMI with 20% fetal calf serum.

CAT assays. Cells were harvested 24 h after transfection, washed once with PBS and resuspended in 50 μl of 0.25 M Tris hydrochloride (pH 7.8). They were lysed by three cycles of freezing and thawing. Debris were removed by centrifugation at 14,000 × *g* for 10 min and the supernatants were heated to 65°C and held there for 10 min. For standardization of the CAT assays, protein concentrations were determined by the Bio-Rad protein assay. Equal protein concentrations were used in each assay. To 25 μl of protein extract was added 25 μl of a mixture containing 5 mM acetyl coenzyme A and 0.05 μM [^{14}C]chloramphenicol, and the resulting

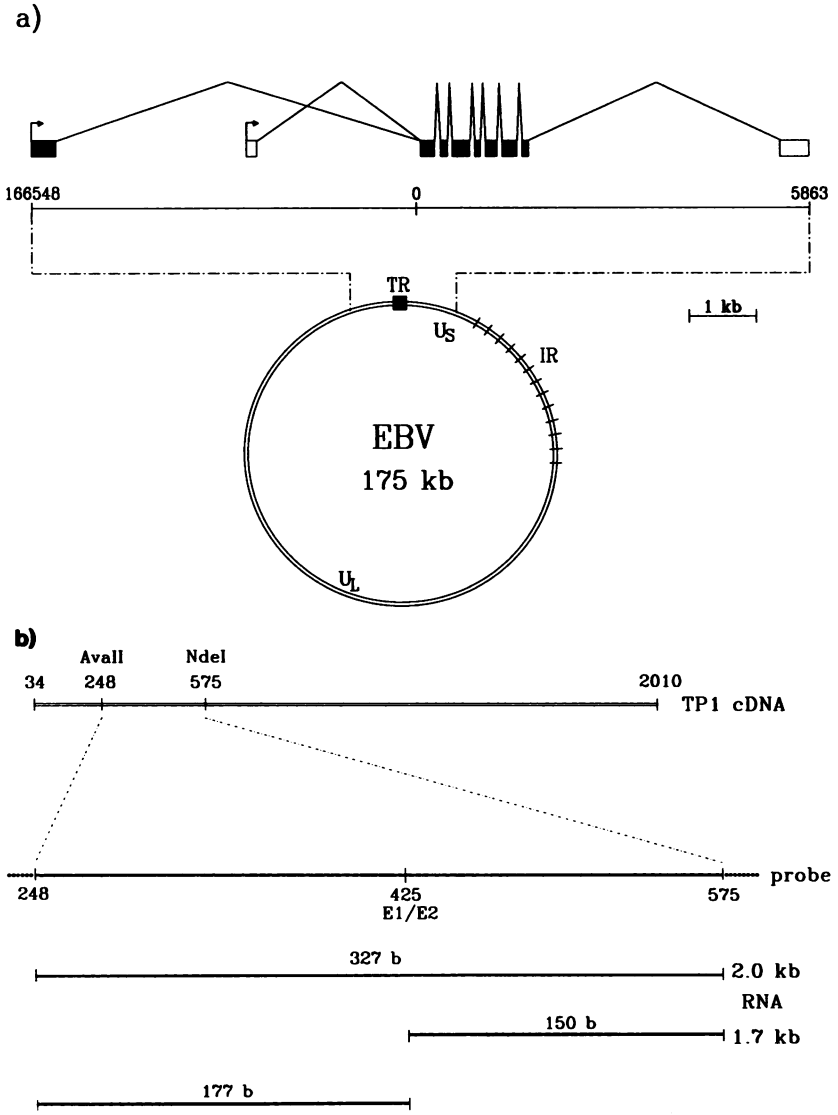

FIG. 1. (a) Schematic representation of the EBV genome and the TP gene. The EBV genome is circularized through the terminal repeats (TR). The internal repeats (IR) divide the genome into a short unique region (U_S) and a long unique region (U_L). The TP gene gives rise to a 2.0-kb TP1 mRNA and a 1.7-kb TP2 mRNA. Both RNAs are transcribed across the terminal repeats, share eight exons in the U_S region, and each have one unique exon in the U_L region. The promoters of the TP1 and TP2 RNA are located at positions 166526 and 169795, respectively, of the B95-8 virus DNA sequence (6). Symbols: ■, translated exons; □, nontranslated exons. (b) Schematic representation of the TP1 cDNA clone isolated from a M-ABA/CBL cDNA library. For detection of TP1 and TP2 transcripts by S1 analysis, positions 248 to 575 (20) of this cDNA were cloned in M13mp19 and uniformly labeled. The dotted lines flanking the cDNA probe represent M13 sequences. Protected fragments are shown below the probe. The 327-base fragment corresponds to the 2.0-kb RNA, and the 150-base fragment corresponds to the 1.7-kb TP2 RNA.

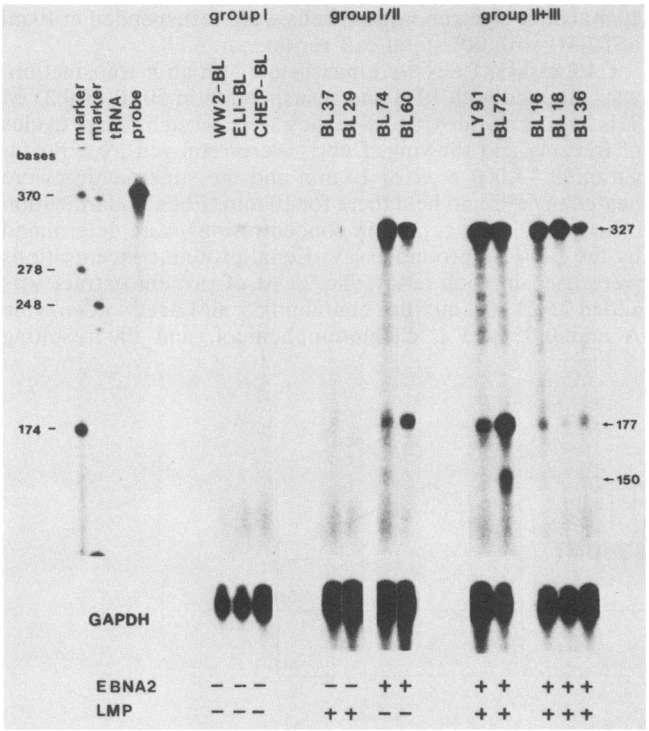

FIG. 2. S1 analysis of TP expression in BL cell lines. A 50-μg portion of RNA from each cell line was hybridized to the single-stranded probe shown in Fig. 1b. Protected fragments were separated on a 5% denaturating polyacrylamide gel. The sizes are given in bases. As internal control, a GAPDH probe (described in Materials and Methods) was added to the hybridization mixture. The phenotype of the cells (groups I to III) is given by using the convention of Rowe et al. (34, 35).

mixture was incubated at 37°C for 1 h. Acetylated products were assayed by thin-layer chromatography with chloroform-ethanol (19:1) as the running medium. CAT assays were quantitated by counting the amount of acetylated chloramphenicol in a Berthold radioactivity thin-layer scanner (TCL-510).

RESULTS

TP RNA expression in BL cells. S1 analyses were performed to study a large panel of cell lines for TP expression. A uniformly labeled, single-stranded probe was prepared that carried parts of exons 1 and 2 of a TP1 cDNA. After hybridization and S1 digestion, fragments of 327 bases (corresponding to TP1) and 150 bases (corresponding to TP2) were protected (Fig. 1b). Additionally, a synthetic oligonucleotide was prepared for detection of GAPDH mRNA which gives rise to a protected fragment of about 80 bases. The GAPDH gene is a housekeeping gene expressed in all cells at a constant level and can therefore be used as an internal standard.

TP transcription was highly variable in different EBV genomes carrying BL cell lines, varying from nonexpression or marginal expression in WW2-BL, ELI-BL, CHEP-BL, BL37, BL29, BL78, and P3HR1 cells to very high expression in Maku, JI, LY47, LY67, LY91, BL72, and BL74 cells, with intermediate expression in the remaining lines (Fig. 2; Table 1). The dominant fragment had, as expected, a size of 327 bases, corresponding to TP1 RNA. However, a 150-base fragment corresponding to TP2 RNA was prominent in only two cell lines (BL72 and JI) and was only marginally present in a number of others. This is compatible with Northern (RNA) blot analyses in which TP2 RNA was weakly detectable in poly(A)$^+$ RNA of most of the cell lines studied (20). Unexpectedly, an additional fragment of 177 bases was protected in most cases. This fragment could be derived neither from intact TP1 nor from intact TP2 transcripts. It corresponds exactly to the size of the part of exon 1 of TP1 RNA present in the probe. Remarkably, this fragment was readily detectable also in Namalwa cells which had been reported to be TP RNA negative by Northern blot analysis (36). S1 analysis with additional probes revealed that this fragment represents TP1 exon 1 spliced to unknown sequences and not an unspliced precursor RNA (data not shown).

Realizing the enormous variability in TP RNA expression in EBV-positive BL lines, we attempted to correlate the pattern of TP expression with the growth pattern and phenotype of the cells. EBV-positive BL cell lines exhibit a high degree of variability in their growth pattern and phenotype. A minority of cell lines grow in single-cell suspension,

TABLE 1. Phenotype and expression of viral latency genes in BL lines

Cell line	Phenotype[a]	Expression of:				
		TP1	TP2	177-base TP gene	EBNA2	LMP
WW2-BL	I	−	−	−	−	−
ELI-BL	I	±	−	−	−	−
CHEP-BL	I	±	−	−	−	−
BL29	I/II	−	−	−	−	+
BL37	I/II	±	−	−	−	−
BL60	I/II	+	−	+	+	−
BL74	I/II	+	−	+	+	−
WW1-BL	II	+	−	?	+	+
LY91	II	+	−	+	+	+
BL72	II	+	+	+	+	+
BL16	III	+	−	+	+	+
BL18	III	+	−	+	+	+
BL36	III	+	−	+	+	+
KIE-BL	III	+	−	+	+	+
LY47	III	+	−	+	+	+
LY67	III	+	−	+	+	+
BL78	ND[b]	±	−	−	ND	ND
BL137	ND	+	−	+	ND	ND
KK124	ND	+	−	+	ND	ND
JI	ND	+	+	+	+	+
Maku	ND	+	−	+	+	+
Namalwa	ND	−	−	+	+	+
P3HR1	ND	−	−	−	−	+
Jijoye	ND	+	−	+	+	+
BL30–P3HR1	ND	−	−	−	−	−
BL30–B95-8	ND	+	−	+	+	+
BL40–P3HR1	ND	±	−	−	−	−
BL40–B95-8	ND	+	+	−	+	+
BL41–P3HR1	ND	−	−	−	−	−
BL41–B95-8	ND	−	−	+	+	+
BL41–AG876	ND	+	−	−	+	+

[a] Phenotypes are those of Rowe et al. (34, 35).
[b] ND, Not done.

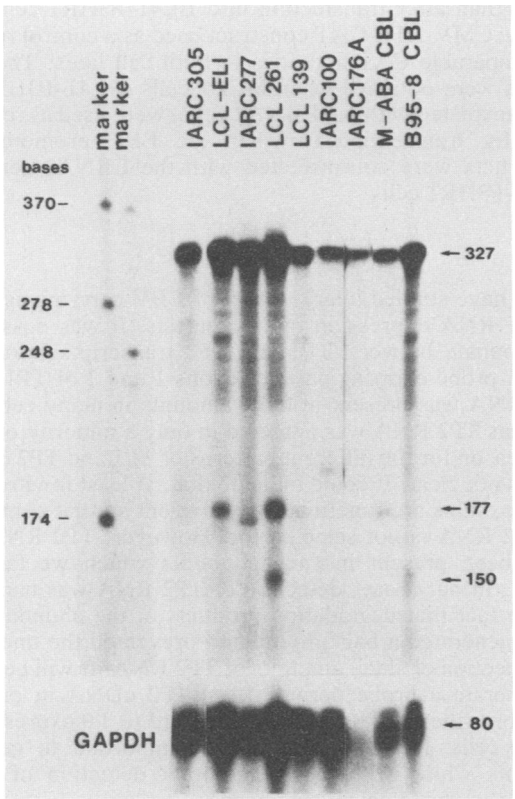

FIG. 3. TP expression in LCL. S1 analysis was performed as described in the legend to Fig. 2.

similar to most EBV-negative BL lines, and show a low expression of both adhesion molecules (LFA1 and LFA3) and B-cell activation markers (CD21, CD23, CD30, and CDw70) (group I phenotype, according to Rowe et al. [34, 35]). Most BL lines, however, grow in clumps, similar to EBV-immortalized primary lymphocytes, and exhibit a high density of adhesion molecules as well as activation markers on their surface. There is a good correlation between phenotype and TP RNA expression (Fig. 2; Table 1). Cell lines which have been classified as group I expressed low (ELI-BL, CHEP-BL) or undetectable (WW2-BL) levels of TP RNA. Cell lines classified as group I/II (BL29, BL37, BL60, BL74) did not present a consistent pattern. BL29 and BL37 cells, which still grew in single-cell suspension in our hands, exhibited undetectable and low TP RNA levels, respectively. BL60 (also growing in single-cell suspension) and BL74 (growing in clumps) showed relatively high TP RNA levels, as did all cell lines with group II and group III phenotypes.

TP expression in LCL. The pattern of TP expression in EBV-immortalized LCL is shown in Fig. 3. TP1 RNA was highly expressed in all cell lines tested. TP2 RNA, however, was easily detectable only in IARC-LCL261 cells and hardly or not detectable in the other cell lines. A fragment of 177 bases was found in about half of the LCL studied.

The TP gene is transcribed only in cells containing the EBNA2 gene. To study whether TP RNA expression is influenced by the type of virus used to convert EBV-negative BL lines, we analyzed TP RNA expression in BL30, BL40, and BL41 cells after infection with the EBNA2-defective P3HR1 virus, the EBNA2A-carrying B95-8 virus, and the EBNA2B-containing AG876 virus (Fig. 4). None of the three P3HR1 virus-converted lines expressed TP RNA. Within the group of B95-8 and AG876 virus-converted lines, the pattern of TP expression was not homogeneous. BL30–B95-8 cells expressed significant levels of TP1 RNA as well as of the 177-base fragment, whereas BL40–B95-8 cells and BL41–AG876 expressed only TP1 RNA in easily detectable amounts. Surprisingly, in BL41–B95-8 cells neither TP1 nor TP2 RNA was found; however, the 177 bases fragment was detected as the only TP transcript, a pattern similar to that observed in Namalwa cells (Fig. 5). These analyses revealed a correlation between TP and EBNA2 expression. All cell lines infected with virus strains expressing EBNA2 revealed at least one TP-specific transcript, whereas all cell lines infected with the EBNA2-defective P3HR1 virus were negative for TP RNA expression. A correlation between EBNA2 and TP transcription is also evident in group I/II BL cells (Fig. 2) since BL60 and BL74 cells are EBNA2 and TP RNA positive and BL29 and BL37 cells are EBNA2 and TP RNA negative.

TP transcription is activated after stable transfection of EBNA2 in BL41-P3HR1 cells. To further evaluate the role of EBNA2 in TP transcription, we analyzed TP RNA in P3HR1 virus-converted BL41 cells which had been stably transfected with an episomal vector carrying the EBNA2 gene (12). The cell clones MCB1-9 and MCB2, which expressed the exogeneous EBNA2 gene, also expressed TP RNA, whereas the cells transfected with the vector alone (MCN1-1) did not show the TP-specific band (Fig. 5). A cell

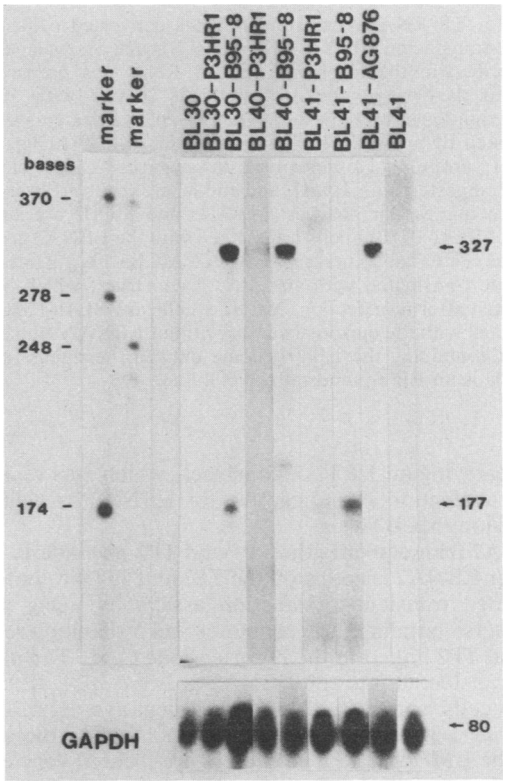

FIG. 4. TP expression in EBV-converted cell lines. TP expression in BL30-, BL40-, and BL41-converted cell lines is shown. The indicated EBV-negative cell lines were infected in vitro by P3HR1, B95-8, or AG876 virus. S1 analysis was performed as described in the legend to Fig. 2.

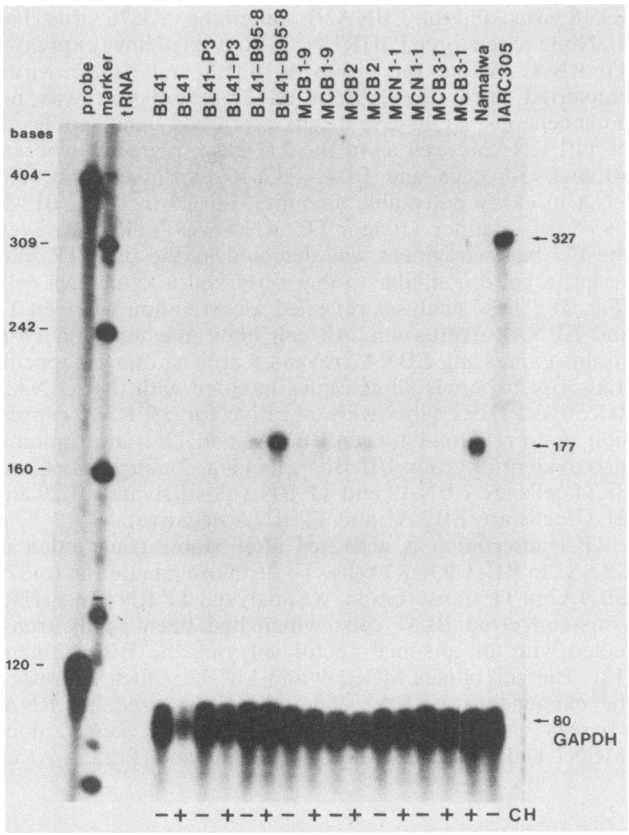

FIG. 5. TP RNA expression in BL41-converted cell lines and EBNA2-transfected BL41–P3HR1 cells. S1 analysis was performed as described in the legend to Fig. 2. RNA was prepared from untreated (lanes −) cells or from cells treated with 50 μg of cycloheximide per ml (lanes +). The protected fragments (in bases) are marked by arrows. The first lane shows the TP1 (Fig. 1b) and GAPDH probe. Lymphotropic papovavirus DNA cloned in pBR322, digested with *Hpa*II, and end labeled with ^{32}P was used as the molecular weight marker. MCB1-9 and MCB2 are single-cell clones of BL41-P3HR1 cells transfected with the EBNA2 gene on an episomal vector and expressing EBNA2. MCB3-1 is a BL41–P3HR1 cell clone transfected with an EBNA2 construct, which has been rearranged after transfection. MCN1-1 cells are BL41–P3HR1 cells transfected with the episomal vector without EBNA2. Namalwa is a BL line containing the EBV genome only in the integrated form. IARC305 is an EBV-immortalized cell line.

clone carrying an EBNA2 construct, which was rearranged after transfection, was negative for EBNA2 as well as TP expression (MCB3-1).

EBNA2 transactivates the TP1 and TP2 promoters. To find whether EBNA2 is acting on the TP1 and TP2 promoters, we performed transient-transfection assays by using plasmid constructs containing the promoter and 5′-flanking region of TP1 and TP2 linked to the reporter gene CAT. The plasmids were transfected into the EBNA2-positive BL41–B95-8 and MCB2 cells and into the EBNA2-negative BL41–P3HR1 cells. The LMP promoter was also included in the analysis since the LMP and TP2 promoters are located very close to each other and might contain common regulatory elements. A cytomegalovirus long terminal repeat-CAT construct (11) was used as a positive control. With all three EBV promoter-CAT constructs, CAT activities were significantly higher after transfection into the EBNA2-positive cell line BL41–B95-8 than after transfection into BL41–P3HR1 cells (Fig. 6). The CMV-LTR-CAT construct used as a control resulted in comparable CAT activities in both cell lines. The same results were obtained when MCB2 cells (BL41–P3HR1 stably transfected with the EBNA2 gene) were used as recipient cells for transfection or when the EBV promoter-CAT constructs were cotransfected with the EBNA2 gene into BL41–P3HR1 cells.

DISCUSSION

We have studied a large panel of EBV-carrying cell lines for TP RNA expression by S1 analysis. It was possible to discriminate between TP1 and TP2 transcripts by using a cDNA probe carrying parts of exons 1 and 2 of TP1 RNA. TP1 RNA was present in large amounts in many cell lines, whereas TP2 RNA was detected in only a minority of lines. The reason for the different patterns of TP1 and TP2 expression is not clear. It could indicate that, at least in vitro, both viral proteins are functionally equivalent so that expression of TP2 RNA is not selected for. However, TP2 RNA may have been present in small amounts which we failed to detect. In our assay, detection of TP2 RNA was hampered by the fact that degradation products of the abundant TP1 RNA generated a background and prevented the unequivocal detection of small amounts of TP2 RNA. It will be useful to generate a probe derived from TP2 cDNA in order to overcome these difficulties. With regard to TP expression in tumor cells, it will be particularly important to generate reagents which will allow the direct detection of TP in biopsies or histological specimens.

Unexpectedly, the S1 analysis revealed a 177-base fragment which was present in most of the cell lines studied. This type of TP transcript has also been detected in Namalwa cells. Namalwa cells contain only integrated and no episomal copies of the viral genome (16, 21, 26). These cells have been reported to be negative for expression of TP1 and TP2 RNA (36), which is consistent with our observation that the 2.0- and 1.7-kb transcripts could not be detected by Northern blot (data not shown) and S1 analyses. Since the viral genome is known to be integrated in Namalwa cells via the terminal repeats (26), the transcript probably represents a fusion transcript between TP1 exon 1 and cellular sequences at the site of viral integration. It is interesting in this context that in BL41 cells converted by B95-8 virus, only the 177-base fragment could be detected whereas the 327- and 150-base fragments were missing. This indicates that B95-8 virus-converted BL41 cells contain only integrated and no episomal copies of the viral genome. This is in agreement with data of Hurley and Thorley-Lawson (16a), who found that viral DNA is only integrated and not maintained episomally in this particular cell line. There is no consistent pattern with regard to the presence or absence of the 327- and 177-base fragments in different EBV-negative BL cell lines converted by EBV. Apparently, the presence of the 327-base fragment may be taken as evidence for episomal copies in BL41–AG876, BL40–B95-8, and BL30–B95-8 cells. In BL41–P3HR1 cells stably transfected with an EBNA2-expressing plasmid (MCB2), only the 177-base fragment could be detected, indicating that these cells harbor only integrated copies, similar to their B95-8 virus-converted counterpart.

The analyses of TP RNA in BL lines revealed a remarkable heterogeneity, from no or very low expression in some cell lines to intermediate or high expression in others. The variability of TP transcription in BL cells contrasted with

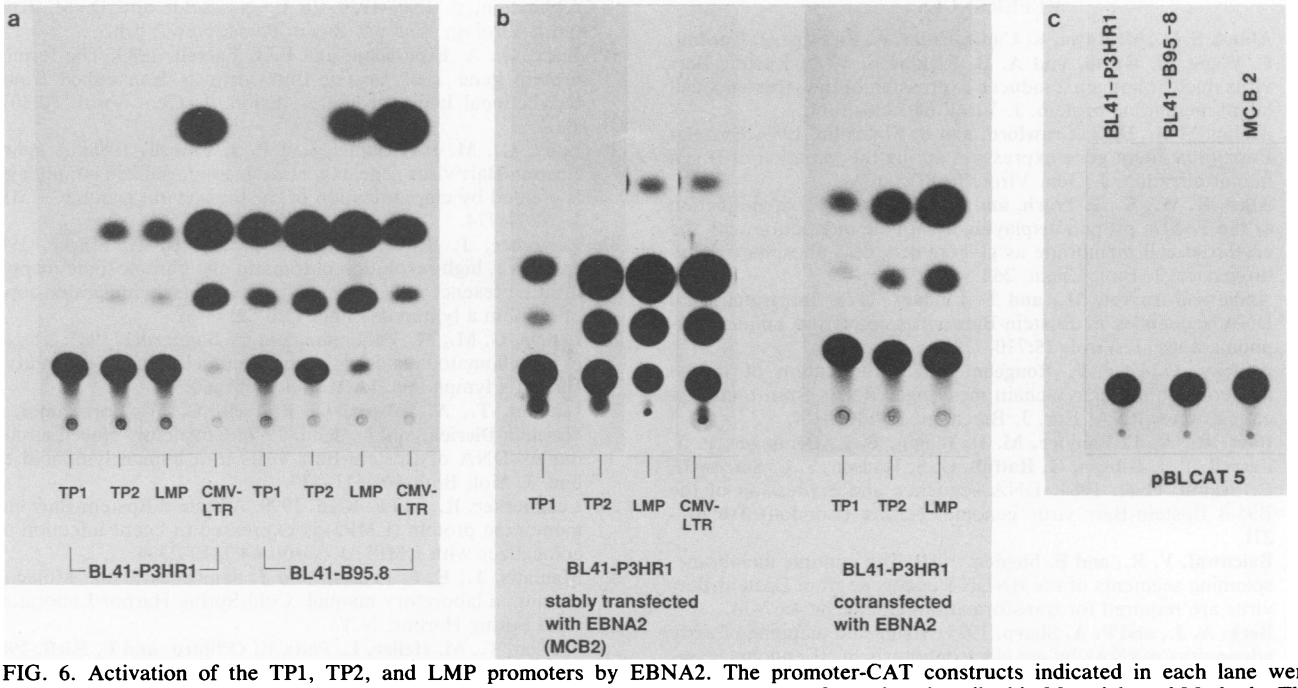

FIG. 6. Activation of the TP1, TP2, and LMP promoters by EBNA2. The promoter-CAT constructs indicated in each lane were transfected into BL41–P3HR1, MCB2, and BL41–B95-8 cells, and CAT assays were performed as described in Materials and Methods. The cytomegalovirus long terminal repeat (CMV LTR) construct was included as a positive control. (a) CAT activity after transfection of the reporter plasmids into the EBNA2-negative BL41–P3HR1 cell line and the EBNA2A-positive BL41–B95-8 cell line. CAT activity increased 75-, 30-, and 39-fold in BL41–B95-8 cells compared with BL41–P3HR1 cells after transfection of TP1, TP2, and LMP promoter-CAT constructs, respectively. (b) CAT activity after transfection of the promoter-CAT constructs in BL41–P3HR1 cells stably transfected with EBNA2 (MCB2) and after cotransfection of the promoter-CAT constructs into BL41-P3HR1 cells together with an EBNA2A expression vector. CAT activity increased 5-, 6-, and 17.3-fold in MCB2 versus BL41–P3HR1 cells after transfection of TP1, TP2, and LMP promoter-CAT constructs, respectively. EBNA2 cotransfection resulted in a 6-, 3.3-, and 7.6-fold increase of CAT activity after transfection of TP1, TP2, and LMP promoter-CAT constructs, respectively. (c) CAT activity after transfection of the promoter-negative CAT plasmid pBLCAT5 into the cell lines indicated.

invariably high TP RNA levels in EBV-immortalized LCL. Attempting to correlate the level of TP transcription with the phenotype of BL cell lines, it became apparent that TP RNA expression is low or absent in group I and some group I/II BL cell lines and high in group II and III BL cell lines which have acquired the phenotype of lymphoblastoid cells. A change in phenotype of the cells in vitro has been demonstrated to be associated with expression of EBNA2 and LMP in culture, suggesting that TP RNA expression may be dependent on one or both of these viral antigens. Two lines of evidence correlate TP expression with EBNA2 rather than LMP. First, TP RNA is absent from BL29 cells expressing low levels of LMP but not EBNA2 and is detectable in BL60 cells expressing EBNA2 but not LMP. Second, the TP gene is not transcribed in P3HR1 cells but is transcribed in the parental Jijoye line. Likewise, TP RNA is absent from P3HR1 virus-converted cells and is present in large amounts in the B95-8 and AG876 virus-converted counterparts. Introduction of an EBNA2 gene into P3HR1 virus-converted BL41 cells led to induction of TP-specific transcripts. We have shown previously that the transfected clones (MCB1-9 and MCB-2) express EBNA2 but not EBNA-LP (12), thus establishing the link between TP and EBNA2 expression and ruling out EBNA-LP as the cause of TP gene activation. Additionally, transfections of TP1 and TP2 promoter-CAT constructs showed that the increase in TP RNA levels caused by EBNA2 is mediated by promoter activation. The reason for the lower activity of the TP1 promoter compared with the TP2 promoter in CAT assays is unclear. We assume that negative regulatory sequences which contribute to the low TP1 promoter activity in transient-transfection assays are missing on the TP2 promoter-CAT construct. Negative regulatory elements have recently also been found in the 5′-flanking region of the LMP promoter (14). It will now be important to see whether EBNA2 is also capable of transactivating the TP promoters in an EBV-negative background, when the EBNA2 gene is cotransfected. However, it is unclear whether EBNA2 is directly involved in the increase of TP RNA levels or whether EBNA2 operates by inducing or modifying a viral or cellular intermediate. This question is now being addressed by studying DNA-protein interactions of the relevant regulatory elements. Finally, it will be interesting to see whether the expression of the LMP and TP genes underlies a similar regulation, assuming that the proteins act in combination in the plasma membrane.

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