Journal of Virology, June 1990, p. 2759–2767
0022-538X/90/062759-09$02.00/0
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Vol. 64, No. 6

Identification of Epstein-Barr Virus Terminal Protein 1 (TP1) in Extracts of Four Lymphoid Cell Lines, Expression in Insect Cells, and Detection of Antibodies in Human Sera

BARBARA FRECH,[1] URSULA ZIMBER-STROBL,[2,3] KARL-OTTO SUENTZENICH,[2,3] OLEG PAVLISH,[2]† GILBERT M. LENOIR,[4] GEORG W. BORNKAMM,[2,3] AND NIKOLAUS MUELLER-LANTZSCH[1]*

Abteilung Virologie, Institut für Medizinische Mikrobiologie, Universitätsklinikum, Haus 47, D-6650 Homburg/Saar,[1] Abteilung Virologie, Institut für Medizinische Mikrobiologie der Universität Freiburg, D-7800 Freiburg,[2] and Institut für klinische Molekularbiologie und Tumorgenetik, Hämatologikum der GSF, 8000 München 70,[3] Federal Republic of Germany, and International Agency for Research on Cancer, 69372 Lyon, France[4]

Received 31 October 1989/Accepted 1 March 1990

The terminal proteins TP1 and TP2 are putative products of Epstein-Barr virus (EBV) genes expressed during the latent cycle of the virus. They are predicted to code for 53- and 40-kilodalton integral membrane proteins. We used the baculovirus *Autographa californica* nuclear polyhedrosis virus as an expression vector to produce TP1 in large amounts in insect cells. The DNA sequences used to express TP1 originated from a TP1 cDNA derived from an M-ABA/CBL1 cDNA library. Rabbit antisera raised against procaryotic TP1 fusion proteins recognized a monomer and a dimer of the recombinant TP1 protein in the infected insect cells. Immunofluorescence studies of living insect cells showed that the recombinant protein is located in the plasma membrane. The insect cells infected with the recombinant baculovirus producing TP1 provided a test system to screen human antisera for TP1 antibodies. A total of 168 human EBV-positive and EBV-negative antisera were studied. TP1 antibodies were detected only in sera from nasopharyngeal carcinoma patients (16 out of 42). Rabbit antiserum raised against the recombinant TP1 protein expressed in the baculovirus system specifically recognized a protein of about 54 kilodaltons in the lymphoblastoid cell lines M-ABA and M-ABA/CBL1 and in the Burkitt's lymphoma cell lines BL18 and BL72. This protein could be located in the total membrane fraction of M-ABA cells and is upregulated by treating the cells with 12-*O*-tetradecanoylphorbol-13-acetate.

Epstein-Barr virus (EBV), an ubiquitous human herpesvirus, is the etiological agent of infectious mononucleosis. It is closely associated with two human tumors, nasopharyngeal carcinoma (NPC) and Burkitt's lymphoma (BL) (7). EBV infects and immortalizes B lymphocytes in vitro (11).

The linear viral genome is organized as two regions of unique sequences, U_S (short) and U_L (long), separated by an array of internal 3.1-kilobase (kb) repeats and flanked by direct terminal repeats (2). After infection, the EBV genome normally circularizes at its terminal repeats and the viral DNA is maintained as multiple copies of episomal molecules in the immortalized cells (19). Evidence for integration of viral copies into the cellular genome has also been provided for some cell lines (10, 22). EBV-immortalized cells are latently infected by the virus since the lytic cycle of EBV is suppressed and only a subset of viral genes is expressed in the cells. These are the genes coding for the nuclear antigens (6, 8, 24, 26, 30, 32), the latent membrane protein (12), and the terminal proteins (14, 17, 27). The products of the genes expressed in latently infected cells are of particular interest because they are believed to be essential for the growth transformation of B lymphocytes by EBV and the maintenance of the latent state of the virus.

It is a unique feature of the terminal genes that their transcripts cross the terminal repeats and that the coding sequences of the genes are created only by circularization or multimerization of the linear viral genome. Two poly(A)$^+$ RNAs, 1.7 and 2.0 kb in length, have been described as being constitutively expressed from the short unique region of B95-8 virus (14). They are present in a wide variety of EBV-infected B-cell lines, including nonproducer cell lines. Analysis of two cDNAs showed that both transcripts consist of nine exons (17, 27). They share the eight C-terminal exons located in the short unique region of EBV and differ only in the N-terminal first exon located in the long unique region close to the right-hand end of the linear genome (17, 27).

The putative translation products of the 2.0- and 1.7-kb RNAs are designated terminal protein 1 (TP1) and terminal protein 2 (TP2), respectively (17; G. Laux and P. J. Farrell, personal communication). According to its amino acid sequence, TP1 is a mainly hydrophobic protein with a hydrophilic amino terminus and a molecular mass of about 53 kilodaltons (kDa). TP2 lacks the hydrophilic amino terminus and has a molecular mass of about 40 kDa. Both proteins are predicted to be integral membrane proteins with 12 transmembrane domains.

In vitro translation experiments showed that the 2.0- and 1.7-kb transcripts are translated into a 54- and 40-kDa protein, respectively (27). Both in vitro-synthesized proteins could be shown to be membrane associated. However, neither of the proteins could so far be found in latently infected B cells, nor could antibodies be detected in human sera which specifically recognized the terminal proteins synthesized in vitro.

We have expressed TP1 in large amounts in the eucaryotic baculovirus expression vector system. Using the recombinant protein as an antigen, we were able to detect antibodies to TP1 in human sera. By expressing TP1 in a procaryotic system and generating TP-specific rabbit antisera we could identify TP1 as a fusion protein of about 56 kDa in insect

* Corresponding author.
† Present address: Moscow Oncological Research Center, Moscow 115478, USSR.

cells infected with the recombinant baculovirus as well as a protein of about 54 kDa in the EBV-positive B-cell lines M-ABA, M-ABA/CBL1, BL18, and BL72.

MATERIALS AND METHODS

Cell lines and virus. BJAB and BL41 are EBV-negative BL cell lines (15, 18). BL18, BL72, BL74, Ly91, and Jijoye are EBV-positive BL cell lines (3, 13, 18). M-ABA is a marmoset cell line established by cocultivation of marmoset lymphocytes with EBV derived from an NPC, and B95-8 is a marmoset cell line established by cocultivation with EBV derived from a patient with infectious mononucleosis (5, 23). M-ABA/CBL1 and B95-8/CBL are cell lines established by immortalization of human cord blood lymphocytes with either of the EBV strains. All lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 40 U of penicillin per ml, 50 μg of streptomycin per ml, 10 U of moronal per ml, and 10 μg of neomycin sulfate per ml. For induction of the cells 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was added to the culture medium at a concentration of 20 ng/ml.

The continuous insect cell line SF158 was derived from *Spodoptera frugiperda* (fall armyworm) and was maintained in TNM-FH medium supplemented in the same way as the RPMI 1640 medium (34).

Wild-type baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) was amplified by infection of SF158 cells. Extracellular virions and viral genomic DNA were prepared as previously described (34).

Construction and induction of recombinant procaryotic expression vectors. pM G27-14 is a *Hin*dIII-*Sal*I subclone of M-ABA virus DNA cloned into pBR327 (map coordinates of B95-8 virus DNA, 166,481 to 166,644; 2, 25). It contains the first exon of TP1 (map coordinates 166,548 to 166,916 of B95-8 DNA). Part of the first exon was cloned into the procaryotic expression vector pATH1 by digestion of pM G27-14 DNA with *Bam*HI, followed by filling in the 5′ overhanging ends. After digestion with *Bgl*II, the fragment was isolated and cloned into pATH1 digested with *Bam*HI and *Sma*I (T. J. Koerner, personal communication).

A *Sma*I-*Bam*HI fragment of a TP1 cDNA isolated from an M-ABA/CBL1 cDNA library (K.-O. Suentzenich, unpublished data) was isolated and cloned into *Cla*I-digested pATH10, which was treated with Klenow polymerase and subsequently cut with *Bam*HI.

The resulting pATH constructs encode fusion proteins containing 37 kDa of the amino terminus of the anthranilate synthetase (TrpE) of *Escherichia coli* and either 11 (first exon) or 51 kDa of TP1. *E. coli* C600 was transformed with the expression vectors, and induction of the fusion proteins was achieved as previously described (31). Bacterial extracts were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by staining with Coomassie brilliant blue.

Immunizations. Procaryotic and eucaryotic fusion proteins were purified by electrophoresis in 10% SDS-polyacrylamide gels, and the gels were stained as previously described (37). Fusion protein bands used for immunizations were excised, minced, and eluted with a buffer containing 50 mM NH_4HCO_3 and 0.5% mercaptoethanol as previously described (28). Samples (300 μg) of the freeze-dried protein were emulsified in 1 ml of phosphate-buffered saline (PBS) and 1 ml of complete Freund adjuvant. Antisera were obtained from rabbits immunized by intradermal injection at 20 different sites. The animals were boosted 4 and 8 weeks after the first immunization by subcutaneous injection, and antisera were drawn 2 weeks thereafter (36).

Construction of baculovirus transfer vectors and generation of recombinant baculoviruses. The TP1 cDNA derived from the M-ABA/CBL1 cDNA library was cut with *Pst*I, and blunt ends were generated by T4 DNA polymerase. After digestion with *Sma*I, the fragment was isolated and cloned into a *Sma*I-digested pAC 409 vector (20). In parallel, the same cDNA was digested with *Bam*HI and cloned into a pAC 360 vector cut with *Bam*HI (20).

Baculovirus plasmid transfer vectors were designed in a way that they contained the polyhedrin-encoding sequences of AcNPV flanked by several kilobases of AcNPV sequences which allow recombination with the wild-type virus in cotransfection experiments. pAC 409 and pAC 360 carry the restriction sites for cloning downstream of the polyhedrin initiation site. Thus, recombination results in the production of fusion proteins with 9 and 12 N-terminal polyhedrin amino acids, respectively.

SF158 cells were cotransfected with 1 μg of baculovirus genomic DNA and 2 μg of recombinant transfer vector DNA by the calcium phosphate transfection method (34). At 4 days after transfection, extracellular virions were collected and allowed to form plaques as previously described (34). The cells were transferred to nitrocellulose filters and lysed by placing the filters in a 5% SDS solution for 10 min. The filters were baked for 1 h at 50°C. Subsequently, SDS was electrophoretically removed from the filters in a transfer buffer (25 mM Tris hydrochloride, 19 mM glycin, 20% [vol/vol] methanol; pH 8.8) containing no SDS. Plaques were screened by immunocolonyblot analysis with rabbit antisera raised against the procaryotic TP1 fusion proteins. Recombinant virus was further purified by performing two more rounds of plaque purification.

Immunofluorescence. SF158 cells were washed once in PBS and fixed at −20°C in acetone. The cells were incubated for 30 min with a rabbit or a human antiserum (1:40 diluted in PBS) and stained by indirect immunofluorescence with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (IgG) or goat anti-human IgG, respectively.

The staining of living cells was performed in suspension. Cells (10^6) were washed three times in PBS. The cells were incubated on ice in 50 μl of 1:40 diluted rabbit antiserum for 30 min and then incubated with 1:40 diluted fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibodies under the same conditions. After each incubation step, the cells were washed three times in ice-cold PBS.

Immunoblots. SF158 cells and B cells were washed in PBS and then disrupted by sonication either under reducing conditions in a sample buffer containing 6% SDS and 10% mercaptoethanol or (additionally) 8 M urea or in the absence of reducing agents under alkylating conditions in a sample buffer containing 6% SDS and 0.05 M iodoacetate. The proteins were separated on polyacrylamide gels and electrophoretically transferred to Immobilon membranes (Millipore Corp.) as previously described (35). The blots were incubated with rabbit antisera at a dilution of 1:400 and then stained indirectly either by using peroxidase-conjugated goat anti-rabbit antibodies or by using the peroxidase-antiperoxidase technique.

In competition assays, the antisera used for staining were preincubated on ice with 2 μg of isolated fusion protein or anthranilate synthetase protein per ml for 1 h and 30 min.

Total membrane preparation. The total membrane preparation was based on a method previously described (21).

Cells (10^7) were pelleted, washed in PBS, and suspended in cold lysing buffer (10 mM Tris [pH 7.5], 1 mM KCl, 1 mM $MgCl_2$). The cell suspension was sonicated, and the nuclei were pelleted by centrifugation at 800 × *g* for 15 min. The nuclear pellets were washed twice. Supernatants from individual washing steps were pooled and centrifuged at 100,000 × *g* for 1 h to pellet the total membrane fraction. Soluble proteins in the resulting supernatant were precipitated with absolute ethanol at −20°C. The pellets of the three subcellular fractions were suspended by sonication in sample buffer as mentioned above and analyzed by SDS-PAGE and immunoblotting.

RESULTS

Expression of procaryotic TP1 fusion proteins. A procaryotic expression vector encoding part of the first exon of TP1 was obtained by cloning a 2.4-kilobase-pair *Bam*HI-*Bgl*II fragment of the genomic M-ABA DNA clone 961-20 in frame with the anthranilate synthetase-coding sequences contained in the pATH1 vector. Of the genomic fragment, 303 base pairs were derived from the first exon of TP1. The 18 N-terminal amino acids of the first exon of TP1 were missing in this fragment. The intron sequence contained a stop codon directly adjacent to the coding sequences so that only TP1 (101 amino acids) sequences were expressed. The observed electrophoretic mobility of the fusion protein was about 50 kDa, which is in the expected range of 48 kDa (data not shown).

Additionally, a *Bam*HI-*Sma*I fragment of the M-ABA/CBL1 cDNA (Fig. 1) was cloned in pATH10. The cDNA fragment contained nearly the complete reading frame of TP1, again lacking the 18 N-terminal amino acids. The resulting fusion protein showed a relative molecular mass of about 90 kDa and a high degree of spontaneous degradation (data not shown).

Expression of TP1 fusion proteins in the baculovirus system. A *Pst*I-*Sma*I fragment of the M-ABA/CBL1 cDNA which contained the complete coding sequences of TP1 was cloned into the pAC 409 vector, and a *Bam*HI fragment of the same cDNA which lacked the 18 N-terminal amino acids was cloned into the pAC 360 vector as described in Materials and Methods (Fig. 1B).

SF158 cells were cotransfected with AcNPV DNA and a recombinant transfer vector. Recombination between AcNPV sequences present in the transfer vectors and viral DNA resulted in polyhedrin-negative plaques expressing TP1 which were identified by immunocolonyblot analysis as described in Materials and Methods.

SF158 cells infected with the recombinant baculoviruses were examined at 8, 12, 16, 20, 25, 36, and 48 h postinfection for the expression of TP1 fusion proteins. The cells were fixed in acetone and stained by indirect immunofluorescence with rabbit antisera raised against the two procaryotic fusion proteins. Both antisera exhibited strong reactions with the infected SF158 cells from 20 h postinfection and later (Fig. 2A).

The rabbit preimmune sera did not react with the recombinant protein expressed in SF158 cells (data not shown), nor did the immune sera react with cells infected by wild-type AcNPV only (Fig. 2B).

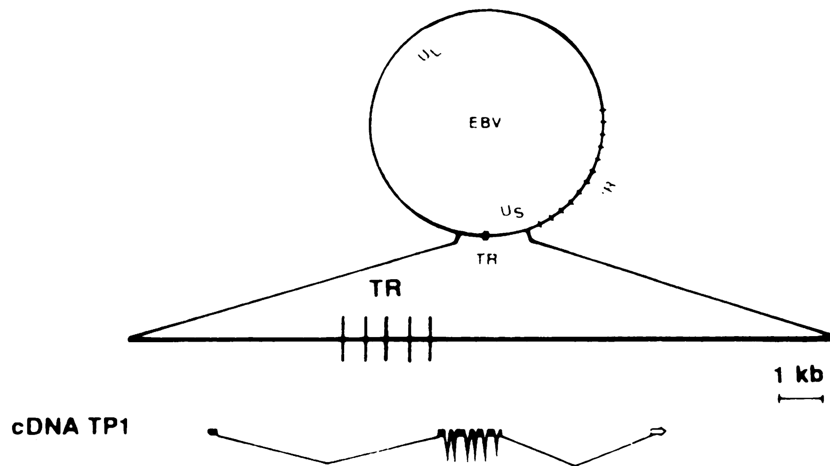
Cellular lysates were prepared by sonication of infected SF158 cells under reducing or alkylating conditions and analyzed by SDS-PAGE and immunoblotting. At 20 h after infection, TP1-specific protein bands could be detected. Under reducing conditions, the protein appeared to aggregate and stuck to the top of the gel. Under alkylating conditions in the absence of reducing agents, bands of about 56 and 110 kDa and, additionally, high-molecular-weight aggregates could be detected (Fig. 3A). The larger band might have been a dimer resulting from a very stable aggregation of two fusion proteins. To show that the larger band and the high-molecular-weight aggregates consisted of the TP1 fusion protein only, the 56-kDa band was excised from the gel, suspended in alkylating buffer, and again subjected to SDS-PAGE. A picture very similar to that from total cellular extracts was obtained, indicating that a certain percentage of the electrophoretically purified protein again formed aggregates of 110 kDa and more (Fig. 3C). The aggregation of the protein under reducing conditions and the partial disaggregation under alkylating conditions in the absence of reducing agents thus seem to reflect a physical property of the recombinant protein observed with proteins from both recombinant baculoviruses. Nevertheless, these data demonstrate that the EBV-encoded protein TP1 is produced in large amounts in the baculovirus expression system.

Plasma membrane localization of the TP1 fusion protein in SF158 cells. Living SF158 cells infected with the recombinant baculovirus carrying the complete TP1 reading frame were stained by an indirect immunofluorescence procedure as described in Materials and Methods. The cell surfaces were brilliantly stained with the rabbit antiserum generated against the hydrophilic amino terminus of TP1 (Fig. 2D). This result indicates that the recombinant protein is located in the plasma membrane of the insect cell and that the hydrophilic amino terminus, which is predicted not to be located in the membrane, is accessible for antibodies.

Serological response to TP1 in EBV-seropositive individuals. A total of 168 antisera from healthy individuals and from patients with BL, NPC, infectious mononucleosis, or other EBV-unrelated diseases (miscellaneous diseases) were studied with respect to antibody responses to TP1. Patients with EBV-unrelated diseases were especially selected for high titers against EBV antigens. The antibodies were detected by indirect immunofluorescence of acetone-fixed SF158 cells infected with the recombinant baculovirus carrying the complete reading frame of TP1 (Fig. 2C). These cells synthesized TP1 in large amounts (Fig. 3A) and could therefore be used for detection of anti-TP antibodies in human sera. The immunofluorescence pattern of TP-positive human sera fit with the pattern obtained with the rabbit antisera (Fig. 2A) and could be specifically inhibited by preincubation of the sera with the procaryotic 90-kDa TP1 fusion protein (data not shown). Insect cells infected with the wild-type baculovirus served as a negative control (data not shown). Antibodies to TP1 could be detected only in sera from NPC patients and were of the IgG type. An indirect immunofluorescence with fluorescein isothiocyanate-conjugated anti-IgA antibodies was negative for all NPC sera. The data are summarized in Table 1. The NPC sera were derived from two different serum collections, one from Germany and one from Tunisia. In both collections, about 40% of the NPC patients had antibodies to TP1. No correlation was found between a TP1 antibody response and high titers to other EBV proteins.

Detection of a TP1-specific protein in lymphoid cell lines. The recombinant TP1 fusion protein expressed in the baculovirus system was excised from polyacrylamide gels, and a rabbit antiserum was raised against the fusion protein as

A



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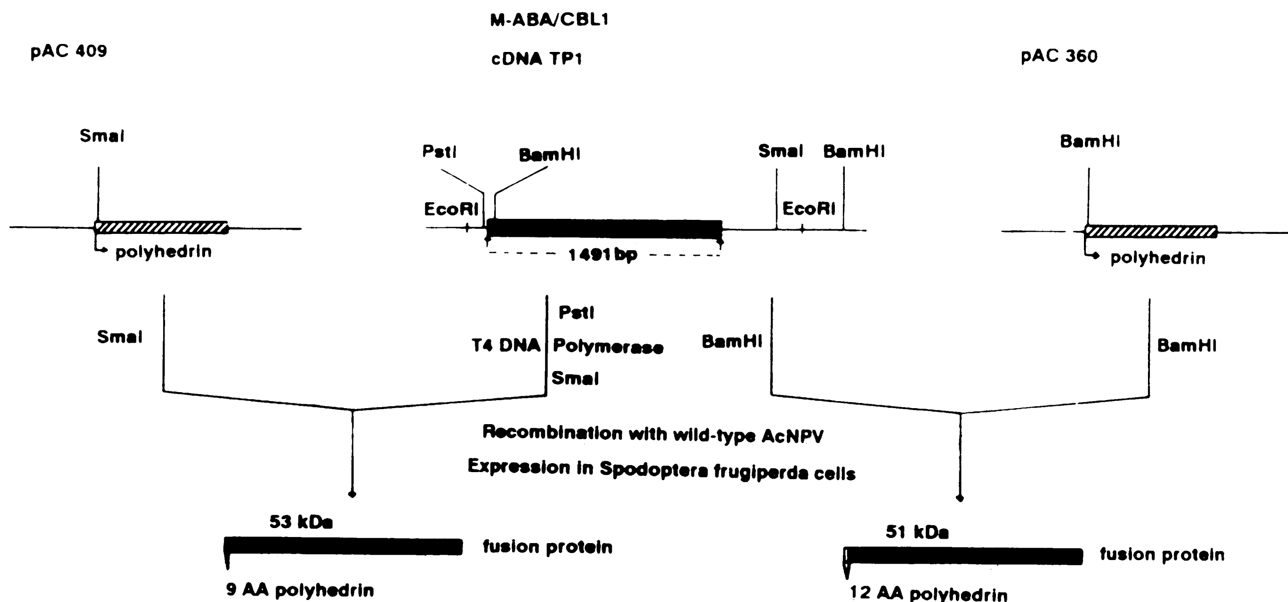

FIG. 1. (A) Schematic representation of the EBV genome circularized at the terminal repeats (TR). The unique sequences (U_S and U_L) and the position of the internal repeats (IR) are indicated. The structure of the TP1 cDNA isolated from the M-ABA/CBL1 cDNA library is shown below. The TP1 cDNA corresponds to the 2.0-kb transcript. Solid and open boxes represent coding and noncoding sequences, respectively. (B) Schematic representation of the TP1 cDNA isolated from an M-ABA/CBL1 cDNA library and the strategy of construction of recombinant baculoviruses expressing TP1 fusion proteins. ■, Sequence coding for TP1; AA, amino acids. The restriction sites used for cloning are indicated.

described in Materials and Methods. With this antiserum, different EBV-positive and EBV-negative B-cell lines were analyzed by immunoblotting for expression of the terminal proteins. A band with a relative molecular mass of about 54 kDa which specifically reacted with the rabbit antiserum could be detected in total cellular extracts of the cell lines M-ABA and M-ABA/CBL1 under reducing conditions (Fig. 4A). It turned out that a sample buffer containing 8 M urea increased the solubility of the specific protein (data not shown). For a demonstration of the subcellular localization of the specific protein band, BL41 and M-ABA cells were fractionated into nuclear, total membrane, and soluble fractions by using differential centrifugation of lysed cells. The different fractions were suspended in alkylating sample buffer and analyzed by SDS-PAGE and immunoblotting. A strong 54-kDa band and, as in the baculovirus system, a weak band of about 110 kDa could be detected only in the total membrane fraction of the cell line M-ABA (Fig. 5A). The staining of both bands could be specifically inhibited by preincubation of the serum with the procaryotic 90-kDa TP1 fusion protein (Fig. 5B). By fractionating other B-cell lines, TP1 could also be detected in the total membrane fractions of the two cell lines, BL18 and BL72 (Fig. 4B). Furthermore, it could be demonstrated that the terminal protein is upregulated to certain amounts by treating M-ABA cells with TPA after subcultivation (Fig. 6). The results in Fig. 6 indicate

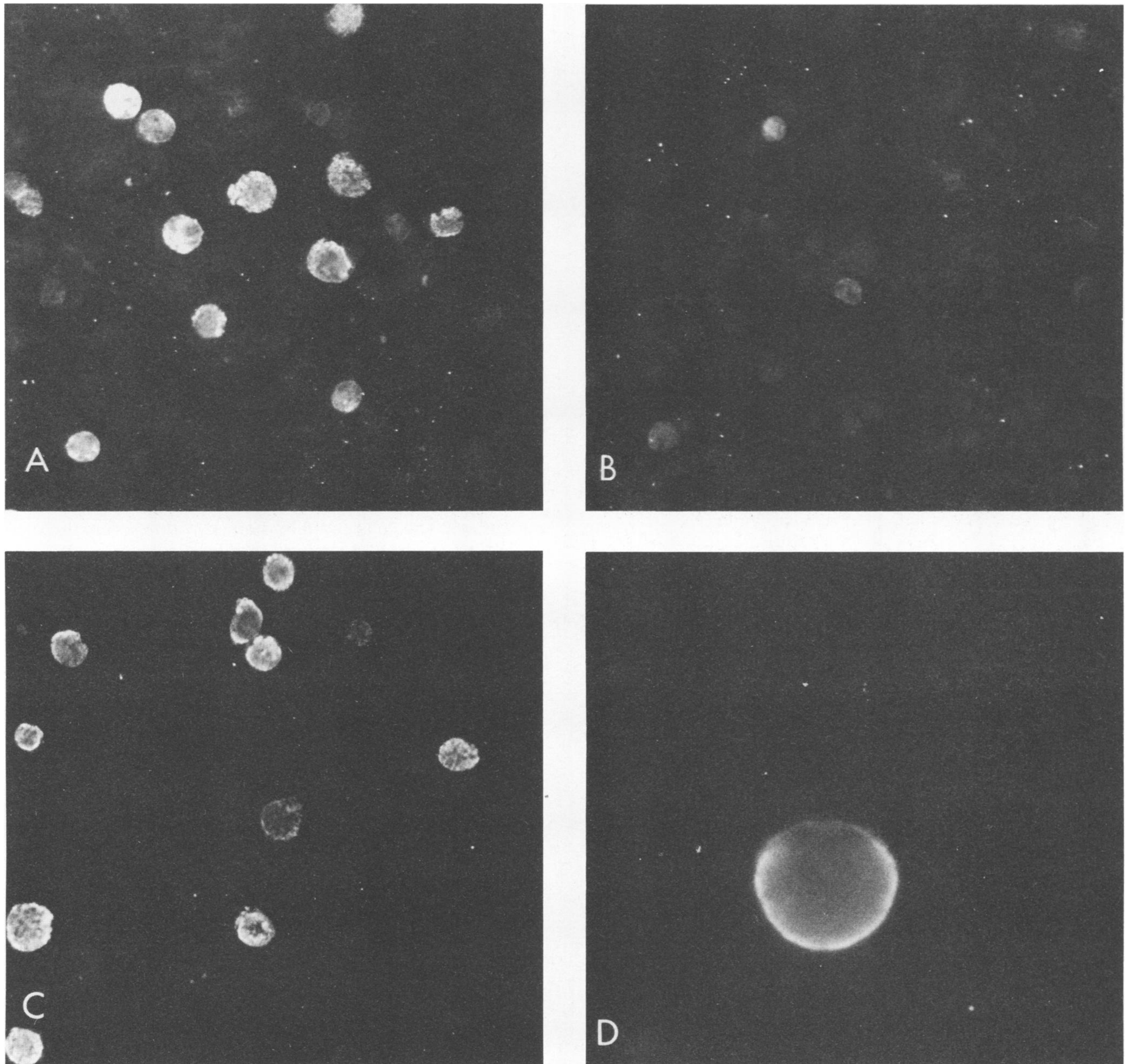

FIG. 2. Indirect immunofluorescence of acetone-fixed (A, B, and C) or living (D) SF158 cells infected with recombinant baculovirus carrying the complete reading frame of TP1 (A, C, and D) or the wild-type AcNPV (B). For immunofluorescence of the fixed cells, infected and uninfected cells were mixed at a ratio of 1:10. The cells were stained either with the exon 1-specific rabbit antiserum (A, B, and D) or with a TP1-positive human antiserum (C). Antisera were used at dilutions of 1:40.

that a maximum of induction of both the dimer and the monomer could be achieved between 12 and 24 h after the addition of TPA.

DISCUSSION

The terminal proteins of EBV cannot easily be detected in EBV-positive cell lines. This may, at least in part, be due to the low abundance of the transcripts of the terminal genes. The overexpression of proteins in eucaryotic expression systems by genetic engineering provides a tool to study the properties and perhaps the function of proteins which are expressed in low amounts in vivo. We chose the baculovirus expression vector system because several mammalian proteins expressed in that system show posttranslational modifications as observed for the same proteins in vivo (16, 29). Two TP1 fusion proteins, one containing the complete reading frame of TP1 and one lacking 18 N-terminal amino acids, were expressed in the insect cells in high quantities. Analysis of the recombinant protein under reducing conditions on SDS-PAGE resulted in the formation of aggregates which stuck to the tops of the polyacrylamide gels. This problem could be partly overcome by preparation of the cellular extracts in alkylating sample buffer in the absence of

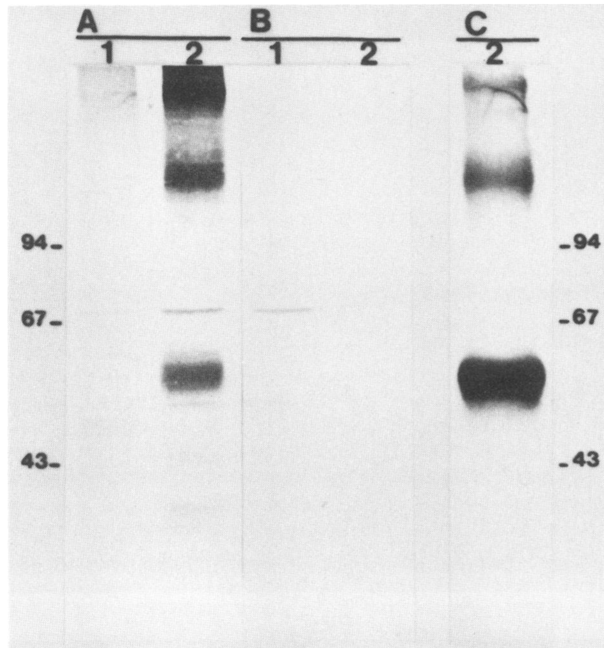

FIG. 3. Immunoblot analysis of SF158 cells infected with recombinant baculovirus carrying the complete reading frame of TP1 (A) or with wild-type AcNPV (B). Cells were lysed under reducing (lanes 1) or alkylating (lanes 2) conditions and analyzed by SDS-PAGE (10% acrylamide) and immunoblotting. (C) The 56-kDa band which was excised from the gel in panel A, resuspended in alkylating buffer, and again loaded on a 10% SDS-polyacrylamide gel. The blots were probed with exon 1-specific rabbit antiserum at dilutions of 1:400. The apparent molecular masses were calculated from comigrating molecular weight standards and are given on the sides in kilodaltons.

reducing agents. The low solubilization of the protein in SDS sample buffer under reducing conditions probably is a matter of the extreme hydrophobicity of the proteins and has already been observed with other membrane proteins (9). Under alkylating conditions, a TP1 fusion protein of about 56 kDa and a dimer of that protein of about 110 kDa could be detected by immunoblot analysis. We could demonstrate that the high-molecular-weight aggregates and the dimer consist of the 56-kDa fusion protein and are not due to an aggregation with other protein species, since excision of the 56-kDa band and its subsequent resuspension in alkylating sample buffer, analysis in SDS-PAGE, and immunoblotting resulted to a small percentage in the formation of high-molecular-weight aggregates and dimerization of the protein. The observed dimerization of the recombinant TP1 molecule could well reflect the in vivo situation, since similar results were obtained when cells immortalized by M-ABA EBV were analyzed.

The protein bands detected in the immunoblot were rather broad, suggesting a posttranslational modification. To test this possibility, we labeled infected SF158 cells in vivo with [^{3}H]myristic acid or [^{3}H]palmitic acid. However, we could not detect any incorporation of labeled fatty acids (data not shown). A treatment of the infected SF158 cells with tunicamycin did not result in a shift of the TP1 bands in the immunoblot, nor could the bands be stained with peroxidase-conjugated lectins (data not shown). Both results indicate that the recombinant terminal protein is not glycosylated.

The primary structure of TP1 suggests a membrane local-

TABLE 1. Antibody response to the terminal protein of EBV in different groups of individuals[a]

Serum source[b]	No. of sera tested	No. of TP-positive sera	% TP-positive sera
EBV SN	15	0	0
Healthy SP	11	0	0
NPC	42	16	38
BL EBV positive	8	0	0
BL EBV negative	6	0	0
IM	11	0	0
Miscellaneous diseases	75	0	0

[a] The test system was indirect immunofluorescence on *S. frugiperda* cells infected with a recombinant baculovirus carrying the reading frame of TP1.

[b] EBV SN, Individuals seronegative for EBV viral capsid antigens; Healthy SP, healthy individuals seropositive for EBV antigens; IM, infectious mononucleosis.

ization. This was supported by in vitro translation data from TP1 and TP2 (27). Since the surfaces of living insect cells could be stained by indirect immunofluorescence, we suggest that the recombinant protein is located in the plasma membrane. The cells could be stained with a serum directed against the hydrophilic amino terminus, which is predicted not to be located in the membrane. As intact cells are usually impermeable for antibodies, this result at least suggests the possibility that the amino terminus is exposed on the outer surface of the plasma membrane. Yet it cannot be excluded that the inner surfaces of plasma membranes of baculovirus-infected cells are accessible for antibodies. As for the latent membrane protein of EBV, the structure of TP1 derived from the amino acid sequence and the plasma membrane localization of the protein is suggestive of an ion channel or a cellular receptor (1, 33).

TP1 was expressed in high quantities in the insect cells, providing a test system to screen human antisera for antibodies to TP1 by indirect immunofluorescence of infected insect cells. TP1-positive human sera exhibited a specific fluorescence pattern very similar to the pattern obtained with the rabbit antisera. The detection of TP1-positive human antisera is the first proof that TP1 is expressed during the life cycle of EBV in vivo. All TP1-positive antisera were sera from NPC patients. Since NPC sera exhibited very high antibody titers against EBV proteins, control sera were especially selected for high titers against EBV antigens to make sure that the positive immunofluorescence was not merely the effect of high antibody titers against viral proteins. The results indicate that approximately 40% of the NPC patients possessed antibodies against TP1. In further studies, it will be interesting to analyze whether antibody titers develop at a precancerous state or during tumor progression. A more detailed follow-up of NPC patients might reveal whether TP1 is expressed at certain stages of tumor development and thus may serve as a marker for NPC or the prognosis of the tumor. Furthermore, it might be interesting to look for TP1 expression in NPC tumor biopsies in order to clarify the role of TP1 in tumor development.

Finally, we could detect a 54-kDa protein band in total cellular extracts of the lymphoblastoid cell lines M-ABA and M-ABA/CBL1 and in total membrane fractions of the BL cell lines BL18 and BL72, which specifically reacted with a TP1-positive rabbit antiserum. As in the baculovirus system, a larger band of about 110 kDa which is probably a dimer of TP1 could be found in the cell line M-ABA under alkylating conditions. In agreement with the results of in vitro transla-

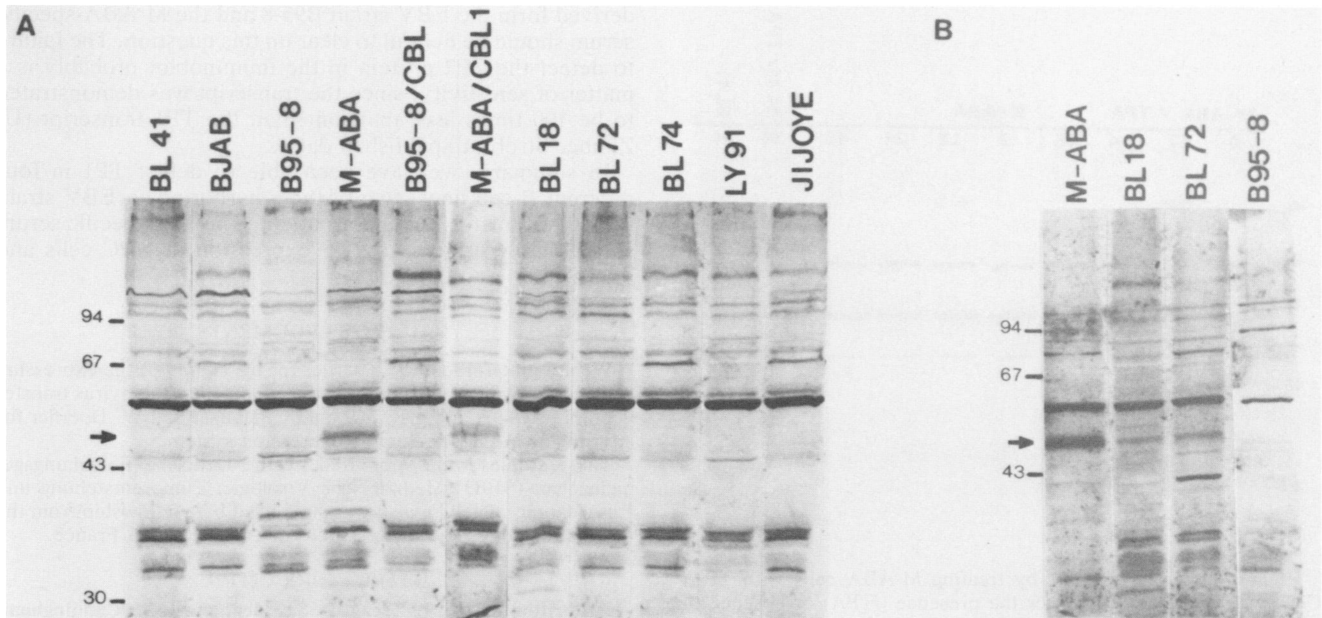

FIG. 4. Immunoblot analysis of extracts of EBV-positive and EBV-negative cell lines. (A) Immunoblot analysis of total cellular extracts. Cells were harvested at 24 h after subcultivation as described in Materials and Methods. (B) Immunoblot analysis of total membrane fractions. Cells were harvested at 24 h after subcultivation and fractionated as described in Materials and Methods. Cellular extracts and membrane fractions were suspended in reducing sample buffer containing 8 M urea, loaded on 10% SDS-polyacrylamide gels, and probed with rabbit antiserum raised against the eucaryotic TP1 fusion protein. The 54-kDa band is indicated by an arrow. The apparent molecular masses were calculated from comigrating molecular weight standards and are given on the left in kilodaltons.

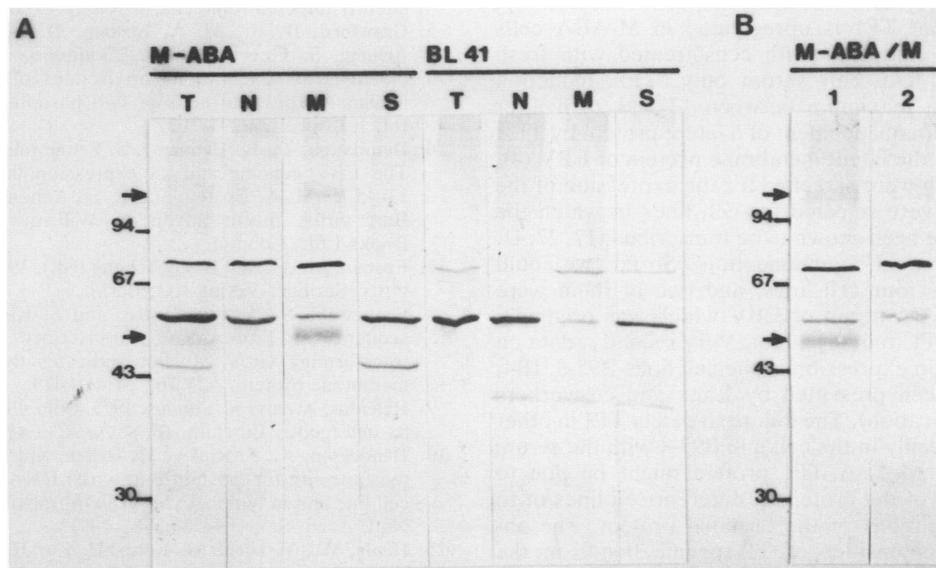

FIG. 5. (A) Immunoblot analysis of subcellular fractions of BL41 and M-ABA. Cells were treated with TPA for 24 h and then fractionated as described in Materials and Methods. Total cellular extracts (T), nuclear fractions (N), total membrane fractions (M), and soluble protein fractions (S) were suspended in alkylating sample buffer, loaded on a 10% SDS-polyacrylamide gel, and probed with rabbit antiserum raised against the eucaryotic TP1 fusion protein. (B) Competition for anti-TP1 antibodies by preincubation of rabbit serum raised against the eucaryotic TP1 fusion protein. Total membrane fractions of M-ABA cells were probed with rabbit antiserum preincubated with the 90-kDa procaryotic TP1 fusion protein (lane 1) or the anthranilate synthetase protein alone (lane 2). The 54- and 110-kDa protein bands are indicated by arrows. Apparent molecular masses were calculated from comigrating molecular weight standards and are given on the left in kilodaltons.

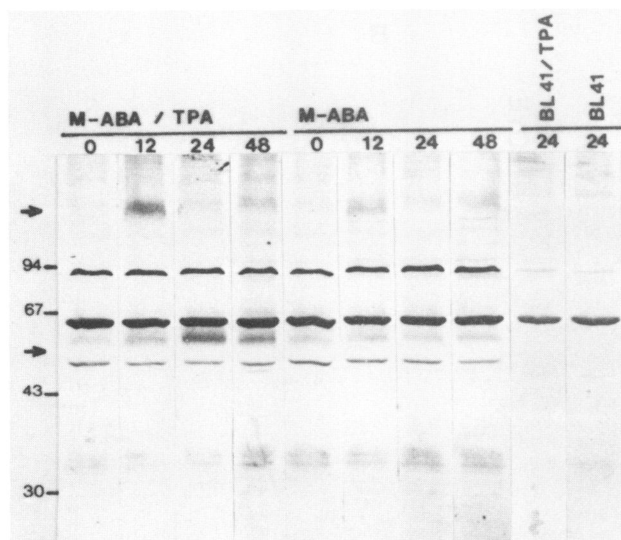

FIG. 6. Induction of TP1 by treating M-ABA cells with TPA. Cells were cultivated in either the presence (/TPA) or absence of TPA. Cells were harvested at different times (given in hours above the lanes) after subcultivation, suspended in alkylating buffer, loaded on 10% SDS-polyacrylamide gels, and probed with rabbit serum raised against the eucaryotic TP1 fusion protein. Extracts of BL41 cells cultivated in the presence or absence of TPA are given as controls. The 54- and 110-kDa protein bands are indicated by arrows. Apparent molecular masses were calculated from comigrating molecular weight standards and are given on the left in kilodaltons.

tion experiments (27) and our data from the baculovirus system, TP1 was found only in the membrane fraction of M-ABA cells, indicating that it is a membrane protein in EBV-immortalized lymphoblastoid cells, too. Additionally, we could show that TP1 is upregulated in M-ABA cells treated with TPA compared with cells treated with fresh medium and 10% fetal calf serum only. TPA induction seemed to reach a maximum between 12 and 24 h after induction. A similar enhancement of a latent protein by TPA was observed with the latent membrane protein of EBV (4). The cell lines which were screened for the expression of the terminal proteins were selected for cell lines in which the terminal genes have been shown to be transcribed (17, 27; U. Zimber-Strobl, personal communication). So far, we could detect TP1 in only four cell lines, and two of them were harboring the M-ABA strain of EBV which was originally isolated from an NPC tumor patient. Very recently, data on TP1 and TP2 protein expression in the cell lines B95-8, IB4, and X50-7 have been presented by Laux and co-workers (personal communication). The failure to detect TP1 in other cell lines and especially in the cell line B95-8 with the serum raised against the M-ABA TP1 protein might be due to different quantities of the protein in different cell lines or to immunological variations of the terminal protein. The absence or varying intensities of TP-specific bands in the immunoblot might reflect either case or a combination of both. Sequence data from the M-ABA/CBL1 TP1 cDNA show a number of point mutations compared with the sequence data from B95-8, resulting in five amino acid changes which, according to their hydrophobicity profiles, could well change the immunological specificity of TP1 (Zimber-Strobl, personal communication). A comparison of the specificity of a serum raised against a TP1 protein derived form the EBV strain B95-8 and the M-ABA-specific serum should be helpful to clear up this question. The failure to detect the TP2 protein in the immunoblot probably is a matter of sensitivity, since the transcript was demonstrated to be 100 times less abundant than the TP1 transcript (U. Zimber-Strobl, unpublished data).

In summary, we have been able to detect TP1 in four different B-cell lines, two of them harboring an EBV strain derived from an NPC tumor patient. The TP1-specific serum should allow us to study TP expression in NPC cells and biopsies.

ACKNOWLEDGMENTS

We are grateful to T. J. Koerner for his supply of the expression vector pATH, M. P. Summers for providing the baculovirus transfer vectors pAC 409 and pAC 360, and C. Hauser and W. Doerfler for advising us in the baculovirus expression system.

These studies were supported by the Deutsche Forschungsgemeinschaft (SFB31-Medizinische Virologie: Tumorentstehung und Entwicklung). Oleg Pavlish was supported by a fellowship from the International Agency for Research on Cancer, Lyon, France.

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