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Proteins Encoded by the Bovine Papillomavirus E1 Open Reading Frame: Expression in Heterologous Systems and in Virally Transformed Cells

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The E1 open reading frame (ORF) of bovine papillomavirus type 1 is required for the persistence of viral genomes as multicopy plasmid molecules in transformed rodent fibroblasts. E1 has been reported to contain two separate complementation groups (M and R, corresponding to N- and C-terminal domains, respectively) which regulate viral replication. However, E1 behaves as a single gene with respect to cell transformation and viral transcription. We examined the proteins translated from the entire ORF by using three antisera raised against E1 peptide or bacterial fusion proteins. The capacity of the whole ORF to encode a 72-kDa protein was demonstrated by translation of synthetic RNA in a reticulocyte lysate system, by microinjection of RNA into *Xenopus* oocytes, and by expression in recombinant baculoviruses and vaccinia viruses. In eucaryotic cells, this protein was found to be phosphorylated and targeted to the cell nucleus. In vitro translation also produced shorter peptides, containing only the E1 C-terminal domain, because of internal translation starts on the third and fourth methionine codons within E1 ORF. On the other hand, mammalian cells infected by vaccinia E1 recombinant virus contained additional larger E1 phosphoproteins (transient 85-kDa and stable 88-kDa species), likely representing processed forms of the 72-kDa species. The E1 72-kDa nuclear phosphoprotein was detected in bovine papillomavirus type 1-transformed cells. We report the biochemical characteristics of full-sized and truncated E1 proteins: (i) the C-terminal half of E1 ORF contains a phosphorylation site(s); (ii) the full-sized E1, but not the C-terminal protein, binds DNA, without indication for recognition of defined sequences, and critical determinants for this activity are likely confined to an N-terminal domain of the protein; (iii) covalent affinity labeling experiments performed on vaccinia virus-encoded E1 proteins with an ATP analog confirmed our previous observation of sequence similarities between the E1 C-terminal domain and the ATPase domain of simian virus 40 large T antigen.

Bovine papillomavirus type 1 (BPV1) transforms rodent fibroblasts in culture towards a tumorigenic state by continuously maintaining viral genomes as free circular molecules (30). The persistence of 100 to 500 autonomously replicating viral DNA molecules in transformed cells mimics the situation of latent infection occurring in the basal layers of BPV1-induced fibropapillomas of cattle (see reference 10 for a discussion of this point) and differs from the vegetative replication which takes place with multiple successive rounds in vivo in differentiated keratinocytes. Latent replication in virally transformed cells is coupled to the S phase of the cell cycle (19), and evidence that all viral genomes sustain exact doubling at this stage of the cycle (43) has been provided by the use of a heterologous system. BPV1 plasmid replication thus offers a model system for the study of scheduled DNA replication in mammalian cells.

Genetic analyses have defined *cis*- and *trans*-acting elements that control the replication of BPV1 plasmids in transformed cells. Analysis of replicating DNA molecules located the starting point for bidirectional replication in the upstream regulatory region (52), close to what was defined as a sequence required in *cis* for plasmid maintenance (PMS1; 34). The study of hybrid replicons that combined simian virus 40 (SV40) origin of replication with BPV1 subgenomic fragments and were dependent on SV40 T antigen as a positive *trans*-acting factor to replicate in monkey cells allowed the isolation of BPV1 genomic elements controlling negatively the replication of BPV1 plasmids (42): two *cis*-acting sequences (NCOR) located in the vicinity of the previously defined PMS elements and a *trans*-acting protein whose critical determinants were contained in the N-terminal 40% of the E1 open reading frame (ORF) of the viral genome (Fig. 1). On the other hand, mutational analyses within the minimal subgenomic fragment capable of inducing cell transformation together with autonomous replication (32) have revealed that several ORFs encode proteins involved in BPV1 plasmid maintenance. Expression of E6/E7 ORFs has a limited role in affecting only the copy number of plasmid forms (35). Integration of viral DNA associated with E2 mutations (14, 15, 20, 40, 45) was generally believed to result from the loss of E2 transcriptional activation (48) required for appropriate expression of other viral genes, including E1. The E1 ORF was the only gene to be unambiguously defined as absolutely required, because any mutational interruption of the ORF led to integration of the viral DNA into the host genome (35, 40). This ORF is the longest and the most conserved in the genomes of all papillomaviruses sequenced to date. Moreover, we have observed that E1 polypeptide sequences contain an array of motifs also present in the sequence of the SV40 large T antigen (Fig. 1) and known to be important for the ATPase-helicase activity required in SV40 replication (7), and this suggested that an

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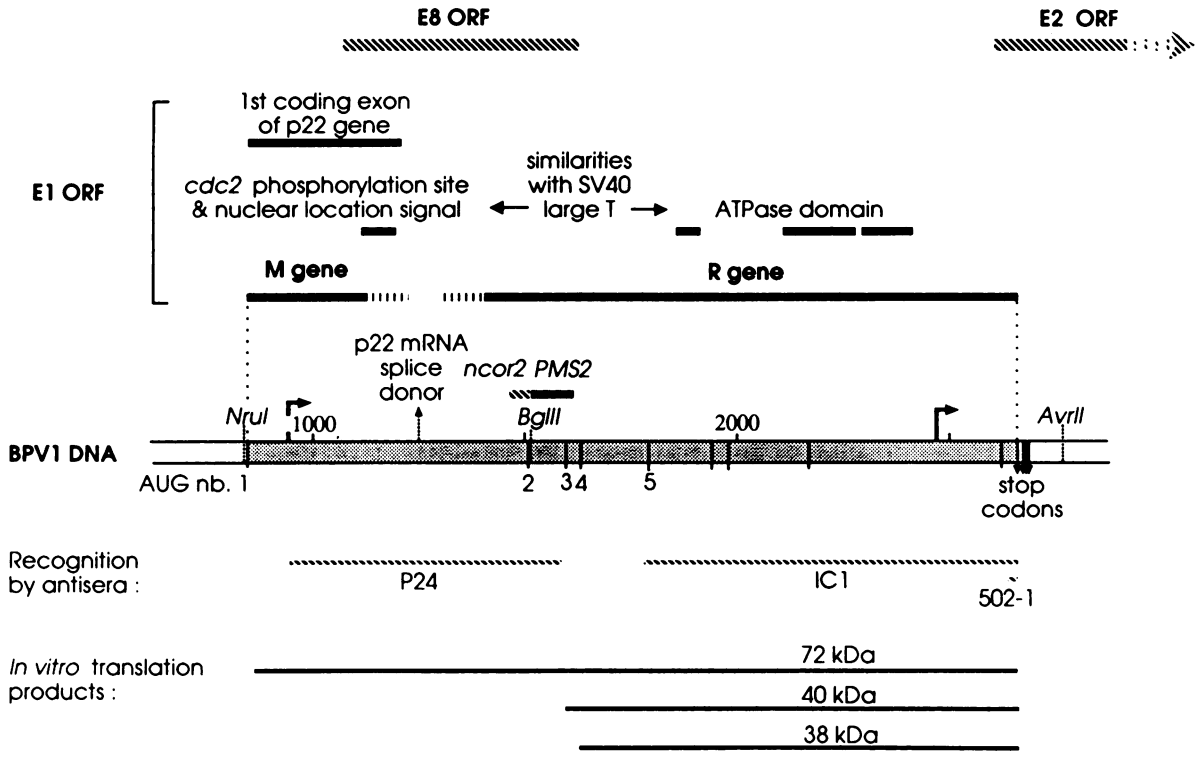

FIG. 1. Partial map of the BPV1 genome spanning the E1 ORF. The shaded area on the linear map of the DNA represents the E1 ORF, with methionine codons (numbered vertical dashes) and conventional nucleotide numbering on the BPV1 genome (starting at the unique *Hpa*I site [9]) indicated. Just above the DNA bar are marked the restriction sites used in this study to clone the E1 region or its R domain and the location of *cis*-acting elements: two promoters, an identified splice donor (51), and two regulatory elements acting in plasmid replication in either positive (PMS2 [34]) or negative (NCOR2 [42]) ways. Black bars indicate salient features of the E1 polypeptidic sequences: the locations of M and R mutants (36), that of the first exon of the mRNA coding for a 22-kDa protein (51), and similarities with the sequence of SV40 large T antigen (13). At the top are shown the two other ORFs present in this region. Below the DNA bar are aligned the E1 sequences contained in the bacterial proteins 1N and 1C used to produce the anti-E1 antibodies P24 and 1CI and the polypeptides produced by in vitro translation of E1 RNA (Fig. 2).

E1-encoded protein might function in the initiation of papillomavirus replication (13).

More detailed analysis allowed distinction between two classes of E1 mutants (Fig. 1), defined mainly in transient replication assays (37): M mutants, clustered in the N-terminal part of the E1 ORF, were still able to replicate transiently after transfection and did not present indications of *trans*-negative effect on the replication of BPV1 plasmids (5), while R mutants, spanning the distal 70% of the ORF, were totally replication defective. In addition, M and R mutants were reported to complement each other for the sum of E1 replicative functions, implying the existence of two separate genes within E1 ORF (36). On the other hand, somewhat contradictory evidence that E1 mutants present a unique phenotype, increasing both transformation frequency and viral transcription irrespective of the location of the mutational alteration in either the R or M domain (27, 46), has been presented. Furthermore, M and R mutants failed to complement each other for this property (46). This defined a third possible E1 gene, corresponding to the whole ORF.

The characterization of mRNAs spanning the E1 ORF helped to partially elucidate this rather complex situation. First attempts to identify E1 transcripts in BPV1-transformed cells failed, probably because of very low expression levels (1). The analysis of the cytoplasmic mRNAs present in virus-infected bovine cells led to the identification of a large transcript containing the whole ORF (8). More recently, Choe et al. (10) cloned a cDNA encoding a 22-kDa protein containing as 3′ exon the first 400 nucleotides of E1 ORF which represents a likely candidate for the M gene product (51). Scrutiny of their cDNA library led also to the identification of a second family of cDNAs covering the entire ORF with the exception of its first 220 nucleotides, presumed to have lost part of the 5′ end of the corresponding mRNA; this type of cDNA was interpreted as likely corresponding to the E1 R gene mRNA, since it was the only one with the capacity to encode the E1 R domain. This suggested that the E1 R gene might actually correspond to the entire ORF, and several reasons could be invoked to explain the discrepancy with the previous genetic observations restricting the R gene to the 5′ part of E1 ORF: intracistronic complementation, reinitiation of translation on internal methionine in the case of nonsense M mutants, and the existence of two R functions, one being sufficient for transient replication (R domain), while the other would be required to ensure stable plasmid replication (whole ORF).

We elected to study the protein(s) expressed by the entire E1 ORF with the help of recombinant viral vectors and by using antisera raised against artificial peptides or proteins. Internal initiation of translation was observed to occur in vitro, in agreement with one of the above hypotheses. The E1 protein expressed by heterologous systems in eucaryotic cells was found to be a nuclear 72-kDa protein subject to phosphorylation and with the capacity to bind ATP and, in a

nonspecific way, to DNA. Moreover, we were able to detect this protein in BPV1-transformed cells.

MATERIALS AND METHODS

Cells. *Spodoptera frugiperda* Sf9 cells were grown at 27°C in Grace medium with 10% fetal calf serum, 0.33% GIBCO Yeastolate, and 0.33% GIBCO casein hydrolysate, as described by Summers and Smith (50). Mammalian cell lines used for growing vaccinia viruses were human HeLa, simian CV1 and BSC40, mouse C127 and 3T6, and hamster BHK21 cells. BPV1-transformed mouse cell lines derived from C127 cells were ID14 transformed by wild-type BPV1 DNA (32), and two other lines were made by using E1 mutants 760 and 783 (frameshift by linker insertion at nucleotides 1019 and 1610, respectively [46]); scopT1 was a cell line transformed by the early region of the polyomavirus genome (41). These cell lines were grown at 37°C in Dulbecco modified Eagle medium with 10% fetal calf serum.

Solutions. Buffer A contained 0.5 M LiCl, 0.1 M Tris hydrochloride (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg of aprotinin per ml, and 1% Nonidet P-40. Buffer B contained 20 mM Tris hydrochloride (pH 8.5), 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 10 μg of aprotinin per ml. Buffer C was the same as buffer B but with added 0.5 M potassium acetate. Buffer D contained 50 mM NaCl, 20 mM Tris hydrochloride (pH 7.5), 1 mM EDTA, 1 mM DTT, and 5% glycerol. Buffer E contained 0.1 M KCl, 25 mM hydroxyethylpiperazine ethanesulfonate (K-HEPES) (pH 7.5), 3 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, and 5% glycerol. RIPA buffer contained 0.14 M NaCl, 20 mM Tris hydrochloride (pH 7.5), 1 mM EDTA, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). Loading buffer for protein electrophoresis contained 5% glycerol, 0.1 M DTT, 0.1 M Tris hydrochloride (pH 6.8), and 1% SDS.

Anti-E1 antibodies. Rabbits were immunized against bacterial E1 fusion proteins corresponding to the sequences presented on Fig. 1 (1N, amino acids 34 to 246; 1C, amino acids 314 to 605) to raise specific antisera in the way described elsewhere for the BPV1 E6 protein (4) (1C protein, 1CI antiserum; 1N protein, 1NB and P24 antisera). Antipeptide sera were raised against the E1 C-terminal decapeptide, either coupled to bovine serum albumin (502-1 serum) or limpet hemocyanin (150-1 serum). Full description of bacterial proteins and antibodies will be detailed elsewhere (E. Androphy, J. Schiller, and D. Lowy, data not published).

Antibodies were purified by immunoaffinity by using adsorption on the corresponding immobilized antigen-bacterial fusion proteins fixed on nitrocellulose (22) or the C-terminal decapeptide coupled to Sepharose. Antipeptide antibodies, although effective in enzyme-linked immunosorbent assays or Western blot (immunoblot) detection and well recognized by protein A, poorly immunoprecipitated the E1 proteins from solutions: less than 50 ng of bacterial E1 protein was retained by using 20 μg of specific 502-1 immunoglobulin G (IgG).

Recombinant vaccinia viruses. VV-E1 was constructed by R. Lathe and M. P. Kieny (39) by the procedure previously employed for the construction of vaccinia virus-polyomavirus recombinants (29). The *Nru*I-*Avr*II BPV1 DNA fragment was cloned within the polylinker of ptg186poly transfer plasmid, downstream from the vaccinia virus 7.5K promoter that had been inserted within the viral thymidine kinase gene. Recombination in vivo between this plasmid and wild-type vaccinia virus was followed by selection of thymidine kinase-deficient plaques; seven plaques were screened, all positive for expression of E1 proteins (see Fig. 4). With this virus, expression reached a plateau 10 to 15 h after infection. As controls, we used recombinants VV-E2 (38) and VVpyLT (21, 29), which encoded the BPV1 E2 transactivating protein and the polyomavirus large T antigen, respectively. All vaccinia virus stocks were prepared, as described previously, by low-multiplicity infection of the cell line to be used later in expression studies (21).

Recombinant baculovirus AcNPV-E1. Recombinant baculovirus was obtained by standard techniques (50). To express the E1 ORF from polyhedrin promoter, we inserted the *Nru*I-*Avr*II BPV1 E1 DNA fragment within transfer plasmid pVL941 (33) at its unique *Bam*HI site after having filled recessed termini with Klenow enzyme. Purification of polyhedrinless plaques, viral stocks, and infections were performed as described elsewhere (50). Screening for proper recombinants was directly performed by immunostaining of plaques obtained at the third round of plaque purification, with the use of anti-E1 1CI antiserum, phosphatase-coupled second antiserum, and fast red staining.

In vitro translation experiments. RNAs to be translated were synthesized in vitro by using the pBlueScript plasmid system (Stratagene): the *Nru*I-*Avr*II E1 DNA fragment was cloned between *Sma*I and *Xba*I sites in the polylinker of the plasmid pSK+, and the DNA of the resulting plasmid was cut with *Pvu*II to release the E1 ORF between the two opposite T3 and T7 phage promoters and transcribed in vitro separately by the two phage RNA polymerases (Stratagene mRNA capping kit), according to the recommendations of the supplier. Sense and antisense RNAs were then purified, and their sizes and concentrations were evaluated by agarose gel electrophoresis to check for complete transcription and absence of degradation.

In vitro translation was performed by using commercial rabbit reticulocyte lysate (Promega Biotec) with 0.2 μg of purified RNA and 5 μCi of [^{35}S]methionine (Amersham; 800 Ci/mmol) in a 50-μl final volume. The extent of translation was measured by counting the trichloroacetic acid-precipitable radioactivity of an aliquot, and the products were further analyzed by immunoprecipitation and denaturing polyacrylamide gel electrophoresis (PAGE) (26).

Immunoprecipitation techniques. (i) From in vitro translation experiments. Translation mixtures were made to 5 mM EDTA, diluted 10-fold with RIPA buffer, and mixed with antibodies and fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem) for 90 min at 0°C. After a short centrifugation, pellets were washed twice with RIPA and analyzed by reducing SDS-PAGE (26).

(ii) From vaccinia virus-infected or BPV1-transformed cells. Labeling of cells, extraction, and immunoprecipitation have been previously described (21). In most instances, a "two-step immunoprecipitation" (23) was performed by adding antiserum and Pansorbin to clarified cell extracts, incubating the cells under gentle mixing for 2 h at 4°C, and then washing them several times, first with buffer A and then with RIPA buffer. Immunoprecipitated complexes were resuspended in 50 μl of 8 M urea for 5 min at room temperature and centrifuged; supernatants were submitted to a second immunoprecipitation after a 20-fold dilution with buffer A, addition of fresh Pansorbin and antibody, and incubation overnight under the same conditions. Final pellets were washed four times with RIPA buffer and analyzed by reducing SDS-PAGE (26).

Immunostaining studies. For infection with vaccinia virus vectors, sparsely growing mouse C127 cells were fixed 8 h

after infection and stained with anti-E1 antiserum as first antibody, as previously described (21); second antibody was fluorescein-conjugated anti-rabbit IgG-specific IgG (BioSys Co., France). Baculovirus-infected Sf9 cells, grown attached to glass slides, were examined in the same manner, with minor modifications: second antibody was coupled to alkaline phosphatase, and staining was performed at pH 9.5 by the fast red technique (22).

Western blotting analyses. Electrotransfer of protein gels and immunostaining of the blots were performed as described elsewhere (21). Second antibody was anti-rabbit IgG-specific IgG coupled to either horseradish peroxidase or alkaline phosphatase (BioSys); staining was achieved by using Co-diaminobenzidine or bromochloroindolyl phosphate-Nitro Blue Tetrazolium, respectively, as substrate (22).

Cell fractionation. (i) Baculovirus-infected cells. At 40 h after infection, Sf9 cells were lysed in the cold by Dounce extraction in hypotonic buffer B (10 strokes with B pestle, 30 min at 4°C, 10^8 cells per ml) and centrifuged (10 min, 800 × *g*). The supernatant was stored in the cold as cytoplasmic fraction, actually containing not only cytoplasmic but also nuclear proteins already eluted from nuclear structures by high pH. The nuclear pellet was then extracted in the same way in buffer C, providing a second supernatant, the nuclear eluate. These two supernatants were adjusted by adding appropriate amounts of SDS, Tris, DTT, and glycerol for loading onto polyacrylamide gels for electrophoresis (26).

(ii) BPV1-transformed cells. Exponentially growing cells were lysed as described above by using hypotonic shock (same as buffer B but pH 7.5), producing cytoplasmic fraction and nuclear pellet. Further extraction of this pellet by buffer B gave pH 8.5 nuclear wash. Final elution from the nuclear pellet was performed by buffer B made 2 M potassium acetate (2 M salt nuclear elution). Total proteins of each fraction were precipitated with a methanol-chloroform mixture and analyzed by Western blots.

DNA-cellulose chromatography. Single-stranded and double-stranded DNA–cellulose was prepared by the method of Alberts and Herricks (2). Double-stranded DNA–cellulose was further treated with S1 nuclease to remove contaminating single-stranded DNA. Chromatography was performed as previously described (18), the columns being eluted by stepwise increase of NaCl concentration in buffer D. Eluted fractions corresponding to each step were pooled, concentrated by immunoprecipitation using 1CI serum, and analyzed by gel electrophoresis and autoradiography.

Affinity labeling using periodate-oxidized nucleotides. The 2′,3′-dialdehyde analog of ATP was prepared by oxidation with sodium periodate from [^{3}H]ATP (50 Ci/mmol) as previously described (11) and used without further purification. Tritiated analog was preferred to circumvent the possible β-elimination of phosphate groups, which might occur if a covalent bond formed between one of the reactive aldehyde and a lysyl group, as has been shown to be the case for mitochondrial ATPase (31). Labeling was performed under conditions previously defined as being optimal for labeling the polyomavirus large T antigen (buffer E [12]), i.e., absence of reagents possessing amino groups and presence of Mg ions. Labeling incubations utilizing crude extracts or immunoprecipitated material took place overnight at 4°C in the presence of $NaCNBH_3$ (12).

RESULTS

Translation started in vitro on first but also on third and fourth AUGs of full-sized E1 RNA. The BPV1 DNA *Nru*I-*Avr*II fragment encompassing the entire E1 ORF (Fig. 1) was placed under control of the phage T7 promoter and transcribed artificially using the corresponding purified RNA polymerase. Antisense RNA was made from an alternate promoter using T3 polymerase. Both synthetic RNAs were translated in vitro by using reticulocyte lysates. Only sense RNA allowed significant incorporation of radioactive methionine into acid-insoluble product. Translation products were examined for recognition by antisera raised against two bacterial proteins corresponding to distinct polypeptidic sequences within E1 ORF (Fig. 1) and to the C-terminal decapeptide (Fig. 2A). In the translation reaction made using sense RNA, all three anti-E1 sera recognized a major protein, the largest product, with an apparent molecular size of 70 to 72 kDa, consistent with the predicted translation product of E1 (69.3 kDa). Shorter peptides were also visible. Some, with apparent sizes between 42 and 50 kDa, were mainly recognized by the antibody raised against the N-terminal part of E1 (P24, lane 3) and were likely due to premature termination events. Two species, of 38 and 41 kDa (the latter being underrepresented), were recognized only by the two antisera directed against the C-terminal domain or the C-terminal peptide (lanes 2 and 4) and thus lacked approximately the 200 to 250 N-terminal amino acids of the E1 sequence. While these species could have been generated by specific proteolysis, this is unlikely because of the absence of any shorter fragments detected by the N-ter-

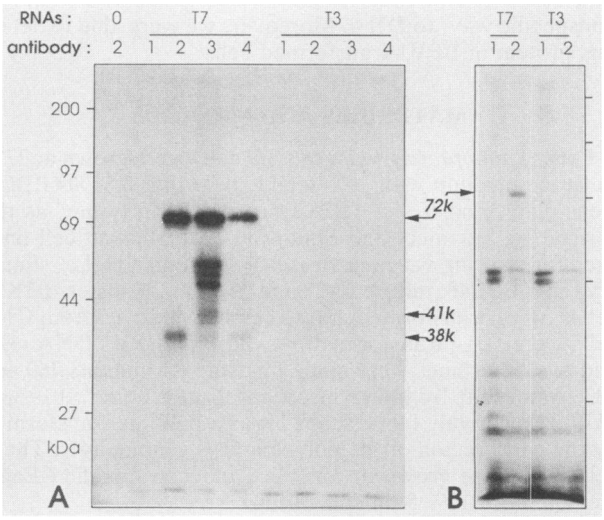

FIG. 2. Translation of synthetic E1 RNA. The BPV1 *Nru*I-*Avr*II DNA fragment containing the full-sized E1 ORF (Fig. 1) was cloned into pBlueScript plasmid between phage T3 and T7 promoters. Sense (T7) and antisense (T3) RNAs were transcribed from the resulting pBlueScript-E1 DNA by using the corresponding phage RNA polymerases and purified for further expression. (A) In vitro translation. E1 RNAs were incubated with rabbit reticulocyte lysate in the presence of [^{35}S]methionine under conditions of in vitro translation for 60 min at 30°C. Translation products were immunoprecipitated by using either rabbit nonimmune serum (lanes 1), anti-E1 antiserum IC1 (lanes 2), P24 (lanes 3), or 502-1 (lanes 4) and then analyzed by denaturing PAGE. (B) Microinjection into *Xenopus* oocytes. Oocytes were injected with E1 RNAs (10 μl per oocyte of an RNA solution containing 25 ng of RNA), maintained in Barth's solution, and incubated 20 h later in the presence of [^{35}S]methionine (50 μCi/100 μl of Barth's solution per oocyte) for 3 h. They were then lysed with buffer A (1 ml per oocyte), insoluble material was discarded by centrifugation, and the resulting supernatants were immunoprecipitated with the same sera as for panel A.

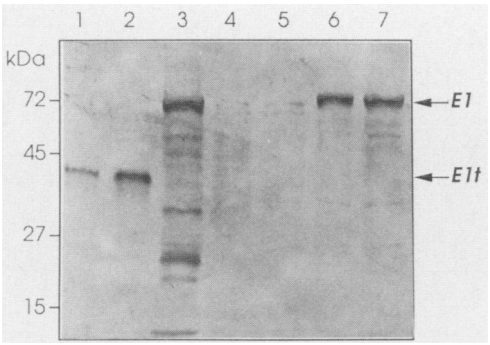

FIG. 3. E1 ORF is expressed from a recombinant baculovirus as a nuclear 72-kDa protein. Sf9 insect cells were infected at high multiplicity with the AcNPV-E1 virus for 40 h at 27°C and then extracted for detection of E1 protein by the Western blotting technique using 1C1 antibody. Lanes 1 and 2, 50 (lane 1) and 100 (lane 2) ng of 41-kDa bacterial truncated E1 protein D31 (E1t, C terminal domain; see text) purified by electroelution from gel bands as described in reference 22; lane 3, 2% SDS lysate of 5×10^5 infected Sf9 cells; lanes 4 and 5, supernatant of hypotonic extraction of the same cells with buffer B (cytoplasmic fraction) from 5×10^5 cells (lane 4) and 10×10^5 cells (lane 5); lanes 6 and 7, high-salt eluate from nuclei resulting from the hypotonic extraction with buffer C of 5×10^5 cells (lane 6) and 10×10^5 cells (lane 7). *E1* and *E1t* mark the proteins translated from the entire E1 ORF and from internal third and fourth methionines, respectively (Fig. 1).

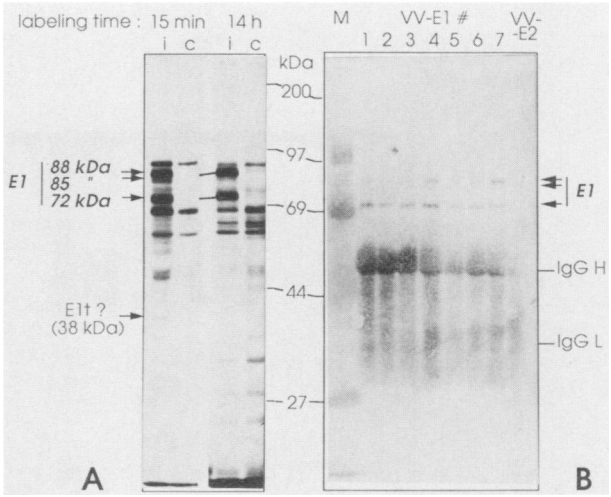

FIG. 4. E1 proteins expressed from vaccinia recombinant virus VV-E1. (A) Immunoprecipitation of metabolically labeled proteins. Mouse C127 cells were infected with VV-E1 virus (multiplicity of infection of 5 PFU per cell). After 6 h they were labeled with [^{35}S]methionine (1 mCi/ml), either for 15 min (in Dulbecco modified Eagle medium containing 1/100 of the usual methionine concentration [methionine/100 medium]) or for 14 h (methionine/10 medium). Cells were lysed with buffer A and submitted to two steps of immunoprecipitation with either a mixture of P24 and 502-1 antiserum (lanes i) or the corresponding preimmune serum (lanes c). (B) Western blotting analysis. Hamster BHK21 cells were separately infected with seven viral clones originating from seven distinct thymidine kinase-deficient plaques selected after homologous recombination between transfer plasmid ptg186poly-E1 and wild-type vaccinia virus (21; Materials and Methods). After 16 h of infection, cells were lysed with buffer A and the clarified lysates were immunoprecipitated with 1CI antibodies. Pellets (corresponding to 10^7 cells) were analyzed by denaturing PAGE and electrotransfer. Western blot was probed with 502-1 antibody. *E1* and *E1t*, As for Fig. 3; IgG H and IgG L, heavy and light chains of IgG, respectively.

minal antibody and because the kinetics of appearance and spontaneous degradation within the in vitro translation mixture were parallel for all three 72-, 41-, and 38-kDa proteins (data not shown).

While the major translation start was as expected from the first AUG (25), the 38- and 41-kDa species most likely arose by internal starts within the E1 ORF, presumably at the third or fourth methionine (Fig. 1). Since a *Bgl*II site was present immediately upstream from these positions, this could be verified by expressing in bacteria the *Bgl*I-*Avr*II BPV1 DNA fragment cloned in a bacterial expression plasmid (D31 construct; kindly provided by M. Lusky, Cornell University Medical School, New York, N.Y.). Two proteins comigrating with the 38- and 41-kDa bands were detected (data not shown; Fig. 3). Identical proteins were also produced by expressing the same DNA fragment from a vaccinia recombinant virus (VV-E1t) in mammalian cells (M. Lusky, personal communication; see also Fig. 5). Thus, despite lack of agreement with Kozak's consensus (25), the third or fourth methionine codons within E1 ORF can be used in vitro as internal translational start sites.

Expression of whole ORF in *Xenopus* oocytes or from a baculovirus recombinant produced only full-sized 72-kDa protein. The synthetic RNAs which were used in in vitro translation experiments were also microinjected into *Xenopus* oocytes (Fig. 2B). Anti-E1 antibodies precipitated a 72-kDa protein from oocytes injected with sense RNA but absent in controls (antisense RNA, nonimmune serum). No indication of any shorter E1 product was observed in this system.

Similarly, insertion of the E1 ORF behind the very strong polyhedrin promoter within the genome of *Autographa californica* nuclear polyhedrosis virus generated a recombinant baculovirus (AcNPV-E1) that efficiently expressed the E1 ORF. In Sf9 insect cells infected by this virus (Fig. 3), the E1 72-kDa protein was detected on Western blots of total cell SDS lysates by using any of the three anti-E1 antisera (not shown). Production was observed to reach peak levels within 35 and 45 h after infection, with a yield (1 mg/10^8 infected cells) similar to that obtained for SV40 large-T-antigen expression from similar recombinants (28, 39; our observations). Shorter products in the 38- to 40-kDa range were not detected under such conditions.

Expression in mammalian cells by using a vaccinia virus recombinant. For purposes of specific antitumoral vaccination experiments, our colleagues G. Meneguzzi, M. P. Kieny, and R. Lathe developed a series of recombinant vaccinia viruses encoding separately each of the BPV1 early ORFs (37a). We already had employed this type of construct to express the polyomavirus early proteins (29) and found that vaccinia virus-encoded large T and middle T antigens retained their biochemical characteristics (21). We thus decided to characterize the protein expressed in mammalian cells by infection with the vaccinia virus recombinant encoding the whole E1 ORF (VV-E1). Immunoprecipitation of metabolically labeled proteins from infected rodent cells (Fig. 4A) revealed several specific proteins recognized only by the anti-E1 sera. One had the apparent size of the primary translation product (72 kDa), while the other two migrated more slowly (apparent molecular sizes of 85 and 88 kDa). Moreover, the same three proteins were detected by immunoblot analysis of whole-cell content with anti-E1 sera, suggesting that the 85- and 88-kDa proteins were E1 products (Fig. 4B). We also excluded the possibility that these

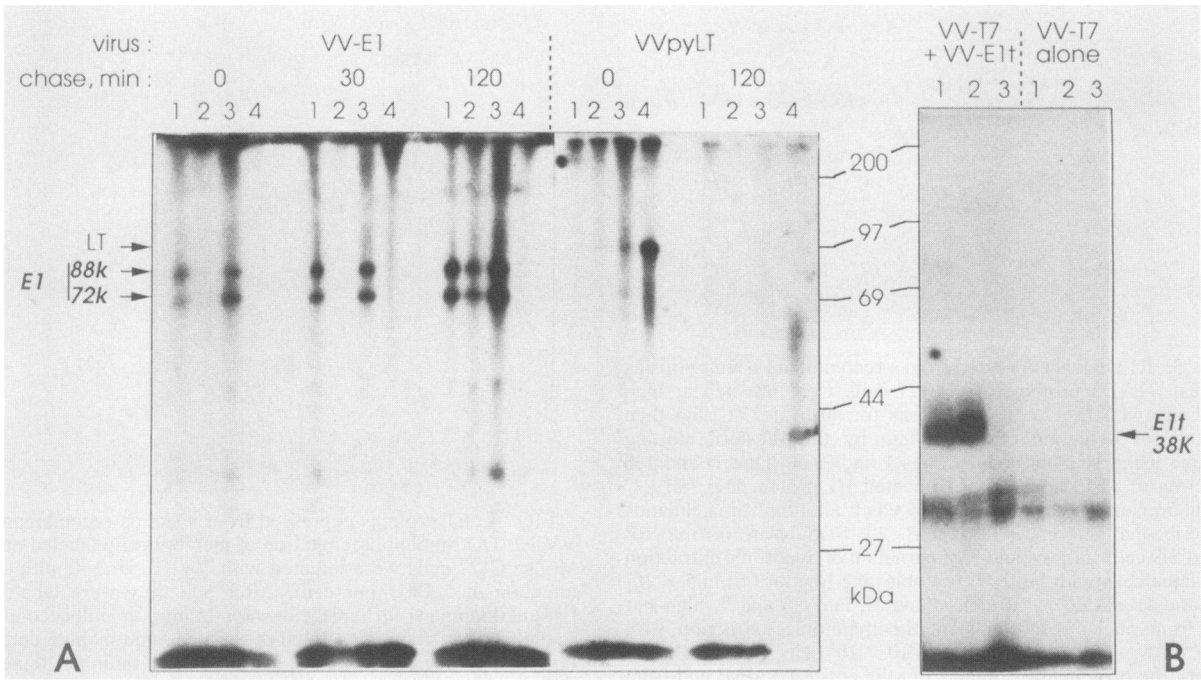

FIG. 5. Phosphorylation of E1 proteins expressed in mouse cells by infection with recombinant vaccinia viruses. (A) Mouse 3T6 cells were infected with vaccinia virus recombinants VV-E1 or VVpyLT at a high multiplicity of infection and 7 h later were labeled by a 15-min pulse with [^{32}P]phosphate (1 mCi/ml per petri dish) followed by a chase (dilution with 10 ml of normal medium) as indicated. Cells were then lysed with buffer A, and the clarified extracts were submitted to one-step immunoprecipitation by using anti-E1 P24 (lanes 1), 502-1 (lanes 2), 1CI (lanes 3), or control rat anti-polyomavirus T-antigen (lanes 4) antisera. (B) Mouse C127 cells were infected with vaccinia recombinant viruses VV-E1t (expressing the *BglII-KpnI* BPV1 DNA fragment [E1 R domain; see text and Fig. 1] under the control of T7 phage promoter) and VV-T7 (expressing the T7 RNA polymerase) (10 PFU per cell) for 24 h. They were then labeled with radioactive phosphate for 2 h, washed, lysed with buffer A, and immunoprecipitated as for panel A by using 1CI (lanes 1), 502-1 (lanes 2), or nonimmune rabbit (lanes 3) sera. *E1* and *E1t*, As for Fig. 3.

proteins resulted from abnormalities in the construction of the particular VV-E1 viral stock in use, because the identical three proteins were detected, with equivalent relative ratios, in seven different VV-E1 isolates originating from separate recombinational events.

The three proteins were recognized by all three anti-E1 sera (Fig. 4 and data not shown), demonstrating that all possess N-terminal and C-terminal epitopes. However, their half-lives were found to differ markedly (Fig. 4A and data not shown). Both the 72- and 88-kDa species, which were present as major species, appeared to be stable within vaccinia virus-infected cells (half-lives longer than 2 h), while the minor 85-kDa species disappeared very rapidly in pulse-chase experiments (not shown). Shorter products were also detected in immunoprecipitation experiments, likely the results of proteolysis (Fig. 4A). As noted above, one of these, migrating with an apparent molecular size of 38 kDa, might also have resulted from internal start of translation on the fourth methionine of the E1 ORF.

In contrast to the expression obtained with baculovirus recombinant in insect cells, VV-E1 vector expressed relatively low levels of E1 proteins. This was also evident when levels obtained by using the same type of construction for expressing other proteins were compared: for instance, ca. 50 ng of E1 protein per 10^7 VV-E1 infected cells instead of 1 μg of polyomavirus large T antigen per 10^7 cells infected with VVpyLT recombinant (21).

Phosphorylation of E1 proteins within the R domain. By labeling cells infected by VV-E1 vaccinia virus with [^{32}P]phosphate, we were able to immunoprecipitate with anti-E1 antibodies labeled 72- and 88-kDa E1 species (Fig. 5A). This indicated that the primary difference between these two forms of E1 protein cannot be attributed to phosphorylation of the larger species only. Although we cannot rule out differential phosphorylation (of distinct sites or to various extents), such a large difference favors the hypothesis of another type of posttranslational modification occurring to the 85- to 88-kDa species.

Phosphorylation of the E1 proteins in vaccinia virus-infected cells appeared to be a fairly stable process, in contrast to that of the polyomavirus large T antigen produced in the same context (Fig. 5A). Strikingly, however, the antipeptide antibody directed against the E1 C terminus (lanes 2) recognized the phosphorylated E1 proteins only after a 2-h chase, unlike what was observed with the two other anti-E1 antibodies (lanes 1 and 3). The antipeptide antibody did not exhibit this behavior with methionine-labeled E1 proteins, with which it gave case patterns comparable to those of the other antibodies, regardless of whether a chase was used (data not shown). This suggested a change in the availability of the C-terminal epitopes for interacting with antipeptide antibody which might be modulated by phosphorylation-dephosphorylation of the E1 protein.

The 22-kDa protein encoded by a cDNA spanning most of the M region of the E1 ORF has been shown to be a phosphoprotein (51). To determine whether other phosphorylation sites could be present on the E1 R domain, we examined shorter proteins. The same truncated E1 proteins (E1t) as those synthesized in vitro by internal initiation of

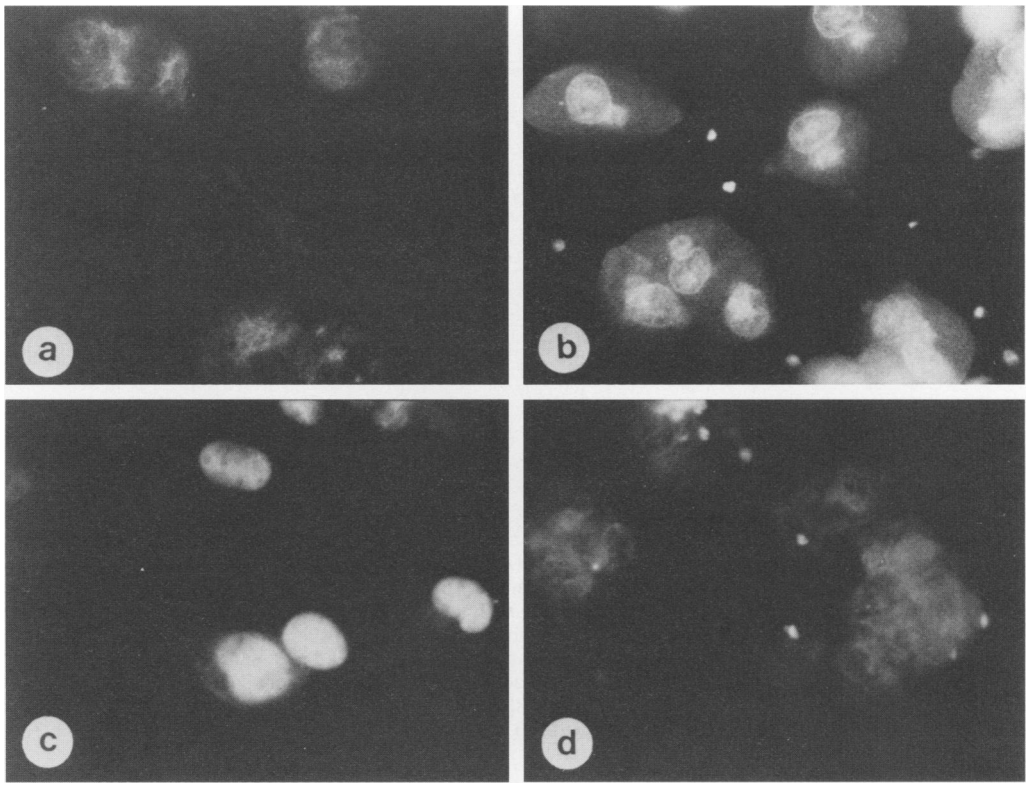

FIG. 6. Immunofluorescence studies on cells infected with the vaccinia recombinant virus expressing the whole E1 ORF. BHK21 cells were infected at high multiplicity with the vaccinia virus recombinants VVpyLT (a and c) or VV-E1 (b and d) expressing, respectively, the polyomavirus large T antigen or the whole E1 BPV1 ORF; 8 h after infection they were fixed and stained by using either rabbit anti-E1 P24 antiserum (a and b) or rat or anti-polyomavirus T-antigen antiserum (c and d) as a control. The second antibody was a mixture of immunoglobulins directed against rat and rabbit IgGs and coupled to fluorescein.

translation were produced in mouse cells after infection with a vaccinia virus recombinant (VV-E1t; kindly provided by M. Lusky) that expressed the *Bgl*I-*Avr*II BPV1 DNA fragment (see above and Fig. 1). Since expression from this recombinant was driven by T7 phage promoter (17), it was achieved by coinfection with a second vaccinia virus expressing the T7 RNA polymerase (VV-T7). Figure 5B shows the result of an experiment with phosphate labeling and immunoprecipitation analysis performed after coinfection of mouse cells. A 38-kDa protein analogous to E1t made in vitro by internal translation initiation (see above and Fig. 2) was clearly present as a labeled band in the lanes corresponding to anti-E1 sera and absent from the controls. This localized a phosphorylation site(s) downstream (3′) from the *Bgl*II site within the E1 ORF.

Nuclear location of E1 proteins. Mouse cells infected by vaccinia virus recombinant VV-E1 or, as a control, by the vaccinia recombinant encoding the polyomavirus large T antigen were examined by immunofluorescence staining (Fig. 6). In the control cells, anti-polyomavirus T-antigen antiserum produced a bright intranuclear fluorescence (Fig. 6c) (21). Negative controls presented only a few nonspecific patches of stain within the cytoplasm, possibly corresponding to "vaccinia factories" (Fig. 6a and d). Anti-E1 antiserum staining of VV-E1-infected cells produced a distinct pattern of fluorescence associated with the periphery of the nuclei (Fig. 6b), resembling association to nuclear matrix or membrane. This was confirmed by subcellular fractionation of VV-E1-infected cells, in which the 72-kDa protein could be detected by immunoprecipitation using anti-E1 antibodies in a nuclear fraction obtained after DNase treatment and high-salt washes (not shown).

Nuclear localization of E1 protein was also detected in insect cells infected with recombinant baculovirus AcNPV-E1 (Fig. 3). Hypotonic lysis of cells at pH 8.5, a technique known to elute many of the proteins associated with the nuclear compartment (for instance, SV40 large T antigen expressed from a similar vector [data not shown]) solubilized less than 10% of the E1 protein present in Sf9-infected cells (lanes 4 and 5). Quantitative elution from the resulting high-pH nuclear pellet was obtained by further treatment with high-ionic-strength buffer (lanes 6 and 7).

Existence of full-sized E1 protein in BPV1-transformed cells. We took advantage of these results to determine whether phosphate labeling would allow the detection of proteins encoded, at least in part, by the R domain of the E1 ORF in BPV1-transformed cells. Mouse ID14 cells, derived from the C127 cell line by transformation with BPV1 DNA (32), were chosen, as they were found to contain a high number of viral plasmids (ca. 200 wild-type viral DNA circular molecules per cell). They were labeled during exponential growth and lysed under conditions in which most of the E1 proteins from mouse cells infected with VV-E1 virus were extracted, and the cell lysate was analyzed by two-step immunoprecipitation. As a control, cells from the scopT1 polyomavirus-transformed C127 cell line (41) were treated in parallel. As shown in Fig. 7A, in addition to a nonspecific band of 80 kDa (marked B), two other bands that were not

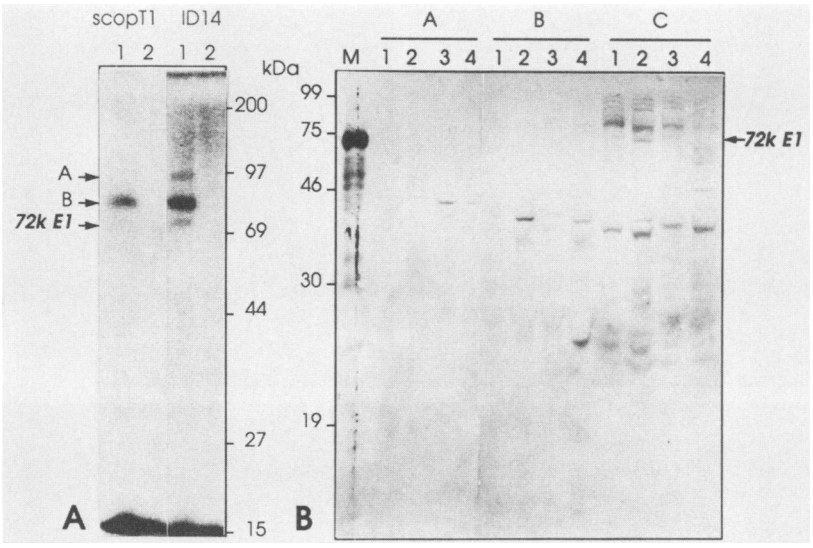

FIG. 7. E1 proteins detected in mouse BPV1-transformed cells. (A) Exponentially growing mouse cells transformed either with BPV1 DNA (ID14 cell line) or with the early region of polyomavirus genome (scopT1 cell line) were labeled by using [^{32}P]phosphate (1 mCi/ml, carrier free) for 1 h and then lysed with buffer A (10^7 cells per ml). Extracts were clarified by centrifugation and then subjected to two-step immunoprecipitation by using either anti-E1 P24 (lanes 1) or preimmune (lanes 2) antisera. (B) After cell lysis by hypotonic shock, fractionation between cytoplasmic fraction (lanes A), high-pH nuclear wash (lanes B), and high-salt nuclear eluate (lanes C) was performed as described in Materials and Methods. The same amounts of each fraction protein (100 μg) were submitted to SDS-PAGE and examined by Western blotting with anti-E1 1CI antiserum. Lanes 1, C127 untransformed mouse cells; lanes 2, ID14 wild-type BPV1-transformed C127 cells; lanes 3 and 4, C127 cells transformed by BPV1 E1 760 and 783 mutant DNAs, respectively.

present in control cells (lane 3) or with control serum (lanes 2) were recognized by the P24 antibody (lane 1). One of these had an apparent molecular size of 72 kDa, as expected for a protein expressed by translation of the entire ORF, the other being larger (band A, ca. 95 kDa).

The low level of expression of these proteins precluded their detection by [^{35}S]methionine labeling because of a much higher background, with numerous nonspecific proteins precipitating with the antibodies, especially around 70 and 90 kDa. However, fractionation of mouse C127 transformed cells followed by Western blot analysis performed by using another anti-E1 antibody (anti-C-terminal 1CI) allowed visualization of the 72-kDa species in ID14 cells (Fig. 7B). The corresponding band was, however, detected only in the fraction eluted from nuclei by high pH and high salt concentration (Fig. 7C, lane 2). Although present at the threshold level for detection among several other nonspecifically stained bands, it was the only one to be absent in the control untransformed C127 cells (lanes 1). Moreover, it was also absent in two other cell lines used as negative controls and obtained by BPV transformation using E1 mutants; the mutations, either in the M (760, lanes 3) or R (783, lanes 4) region, expected to stop E1 translation before the sequences recognized by 1CI antibody, had been shown to result in the same phenotype (high rate of transcription and transformation) suppressed by introduction of a construct encoding the entire E1 (46). Surprisingly, the 95-kDa protein observed by immunoprecipitation in ID14 cells was not detected in this type of analysis (lanes 2).

Thus, antibody recognition indicated that the 72-kDa band represented a bona fide E1 protein, containing both N-terminal and C-terminal epitopes, of the size corresponding to the primary translation product of the whole E1 ORF and present as a nuclear phosphoprotein in BPV1-transformed cells.

Amino-terminal domain plays a critical role in nonspecific DNA binding by E1 proteins. Chromatography on DNA-cellulose columns of the proteins produced by in vitro translation of E1 RNA (Fig. 8) showed that the 72-kDa full-sized E1 protein was partially retained on the columns, with a slight preference for double-stranded DNA. Bound E1 protein was eluted, mainly by applying 0.2 M salt to the columns, but some protein remained bound, even at 0.5 M salt in the case of double-stranded DNA. On the other hand, the 38- to 41-kDa E1t products could not be detected in the eluates but only in the flowthrough fractions. This indicated that binding to DNA by E1 protein required the presence of the N-terminal region.

We addressed the question of specific recognition of DNA sequences by the full-sized E1 protein. In performing immunoprecipitation of complexes between E1 protein and BPV1 DNA restriction fragments, similar to the technique used for the E2 protein (3), or gel-retardation assays using extracts of cells infected with VV-E1 recombinant vaccinia virus (as described in reference 38), we were unable to find any indication of specific binding to defined BPV1 DNA sequences (data not shown).

ATP-binding site within the R domain. Crude extracts of cells infected with VV-E1 vaccinia virus were incubated with periodate-oxidized tritiated ATP (oATP) under conditions that had previously been optimal for covalent affinity labeling of polyomavirus large T antigen (12). By immunoprecipitation with anti-E1 sera (Fig. 9, left panel), we were able to recover the three 72-, 85-, and 88-kDa proteins detected previously (Fig. 4) as labeled bands. Respective levels of labeling corresponded to their relative amounts as estimated by Western blots, indicating similar binding activities for all three species. Variation in the intensity of labeled E1 bands precipitated by the different serum likely reflected the fact that two of them do not quantitatively precipitate the E1 proteins. These three bands were not detected when a nonimmune rabbit serum was used (not shown). In addition

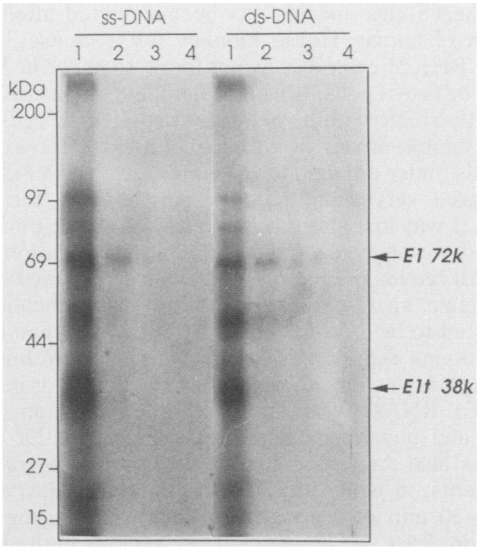

FIG. 8. DNA-cellulose chromatography of in vitro-translated E1 proteins. E1 synthetic RNA was produced and translated in vitro as described in the legend to Fig. 2. After 30 min of synthesis, translation was stopped by EDTA, and the reaction mixture was diluted fourfold with buffer D and centrifuged for 2 h at 40,000 rpm in a Beckman SW60 Ti rotor. The supernatant was directly applied on small DNA-cellulose columns (0.5 ml of packed matrix per 100 μl of in vitro translation mixture). Flowthrough (lanes 1) and successive eluates obtained with increasing NaCl (2:0.2 M, 3:0.5 M, and 4:2.0 M) were precipitated by using anti-E1 1CI antibodies, and the pellets were analyzed by reducing SDS-PAGE and fluorography. *E1* and *E1t*, As for Fig. 3.

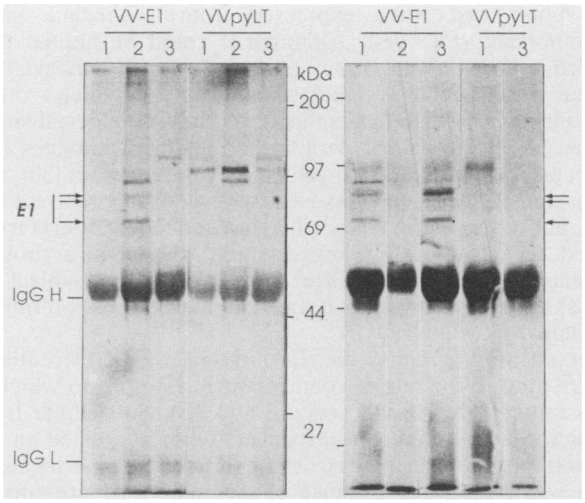

FIG. 9. Covalent affinity labeling of the vaccinia virus-encoded E1 proteins by using periodate-oxidized ATP. After 12 h of infection at high multiplicity with vaccinia virus recombinant VV-E1 or VVpyLT, mouse 3T6 cells were washed and lysed with buffer A (10^7 cells per ml) and the extracts were clarified by high-speed centrifugation and stored at −70°C. (A) By using gel filtration through Bio-Gel P30 spun columns, extracts were equilibrated into buffer E. Periodate-oxidized [^{3}H]ATP was added to 10 μM final concentration, and the reaction mixture was incubated for 15 h at 4°C in the presence of 10 mM $NaCNBH_3$. Reactions were stopped by adding EDTA to 10 mM and cold ATP to 1 mM and diluting the mixture with RIPA buffer. E1 proteins were immunoprecipitated by the addition of P24 (lanes 1), 1CI (lanes 2), or 502-1 (lanes 3) antisera in the presence of protein A-Sepharose for 2 h at 4°C. After several washes with RIPA buffer, pellets were analyzed by SDS-PAGE and fluorography. (B) Equal amounts of infected cell extracts as for panel A were first immunoprecipitated at 4°C by using the same antisera. Sepharose beads were washed in the cold five times, first with buffer A and then with buffer E. They were resuspended in 50 μl of buffer E and incubated overnight at 4°C in the presence of 10 μM ATP analog and 10 mM $NaCNBH_3$. The reaction was stopped as described in the legend to panel A, the beads were washed extensively again with RIPA buffer, and the immunoprecipitated material was analyzed. Nonspecific labeling of rabbit immunoglobulins used in large excess to precipitate the E1 proteins is indicated (IgG H and IgG L are, respectively, heavy and light chains) as the position of the labeled E1 proteins (arrows).

to nonspecific label of the heavy and light chains of the IgGs added in large excess for precipitating the E1 proteins, other nonspecifically labeled bands, in the range of 95 to 110 kDa, were present in the lanes corresponding to extracts from cells infected with a control vaccinia virus. It should be noted, however, that the labeling efficiency observed under these conditions (1 mM oATP) appeared to be weaker for E1 proteins (ca. 1 mol% of oATP per mol of protein) than that observed for a comparable amount of large T antigen (10%; not shown).

Since we had previously reported similarities of peptidic patterns within the E1 R domain with critical determinants for ATP binding of the SV40 large T molecule (13), we checked if we could affect in different ways the ATP binding by E1 proteins by using different antibodies, directed against either the E1 C- or N-terminal domain. We first immobilized the E1 proteins present in VV-E1-infected cells on three different immunoprecipitates made with the three anti-E1 antisera. The immunoprecipitates were incubated with the analog under the conditions described above. Analysis of the labeled material (Fig. 9, right panel) showed that the serum raised against protein 1C (amino acids 308 to 605) completely inhibited the labeling of E1 proteins, in contrast to the other sera (including the C-terminal decapeptide antibody). Critical determinants for ATP binding are thus localized between amino acids 308 and 595 on the E1 protein molecule.

DISCUSSION

The complexity of genetic data examining the functions of the E1 ORF and the mutants thereof, together with the uncertainty about the identity of E1 mRNAs, especially of those corresponding to the R gene, led us to address the identification of E1-encoded proteins with a direct approach. The full-length E1 protein (605 amino acids) was expressed in a variety of artificial expression systems after cloning at its translation initiation codon, enabling us to undertake its biochemical characterization. We unambiguously identified the primary product of translation of the entire ORF as a protein with an apparent molecular size of 72 kDa (Fig. 2) which was efficiently produced by using a baculovirus expression vector in insect cells (Fig. 3). This protein was subject to phosphorylation, both in insect cells (data not shown) and in mammalian cells infected by a vaccinia virus expression vector (Fig. 5). Furthermore, this full-sized protein was detected in BPV1-transformed cells in which the viral genome replicates as a plasmid (Fig. 7).

The phosphorylation of the protein expressed from the whole ORF is not surprising, since a cDNA containing the E1 M domain as a first exon was recently reported to encode a 22-kDa phosphoprotein (51). Phosphorylation is thus likely occurring on the same sites within the M region in both proteins. We found in addition that a truncated protein, limited to the carboxy-terminal two-thirds of E1, was also

phosphorylated when expressed from a vaccinia virus recombinant (Fig. 5B). Although it could be argued that vaccinia virus infection may affect protein phosphorylation in an artifactual way, this seems not very likely: other proteins known to be phosphorylated in physiological situations, like polyomavirus large T and middle T antigens (21) or capsid proteins (49) as well as BPV1 E2 protein (38), are phosphorylated when expressed from vaccinia virus vectors. The phosphorylation of vaccinia virus-encoded 38-kDa truncated E1 protein thus suggests that the 72-kDa protein detected in ID14 cells (see below) could be subject to phosphorylation on both its amino- and carboxyl-terminal domains.

In eucaryotic cells, the E1 protein was preferentially distributed to the nuclear compartment (Fig. 6), to which it was rather tightly bound (Fig. 3 and 7B). Subcellular fractionation and immunofluorescence studies suggested an association to nuclear matrix or membrane. Nuclear localization was anticipated because of the role of E1 proteins in viral DNA replication. Immunofluorescence studies performed on cells infected with the vaccinia virus vector encoding the truncated 38-kDa E1t C-terminal protein have shown accumulation of this protein into cytoplasmic aggregates (M. Lusky, personal communication; our observations). Although performed under conditions in which the infected cells underwent strong cytopathic effect, this would suggest that the E1 C-terminal half itself does not contain an appropriate nuclear location signal. We could speculate therefore that if E1 functions require nuclear localization, the E1 R protein, as defined by Lusky and Botchan (36), would not be replication competent but would require at minimum putative nuclear targeting sequences in the N-terminal region of E1. However, the limited size of the truncated C-terminal E1 might allow a limited access by diffusion to the nuclear compartment. To explain the absence of detection of E1t in the nuclei of vaccinia virus-infected cells, trivial explanations might also be put forward, like insolubility at high concentration or advanced cytopathic effect.

The full-sized E1 protein produced in mammalian cells by infection with VV-E1 vaccinia virus recombinant was present as the predicted 72-kDa species along with two other species, one transient and one stable, with respective molecular sizes of 85 and 88 kDa. The last two do not appear to possess polypeptide sequences different from that of the 72-kDa protein, since limited proteolysis by using V8 protease gave similar patterns for phosphate-labeled 88- and 72-kDa proteins (not shown). Although indeed we cannot exclude the possibility that the larger forms arise by distinct overphosphorylation patterns, this last observation is more in favor of a posttranslational modification by covalent addition of high-molecular-weight material, like glycosylation or ubiquitination, as a cause of such large migration changes. Occurring frequently for extracellular or intravesicular proteins, glycosylation has been described to occur also for nuclear material, like matrix-associated SV40 large T antigen (47) or Sp1 transcription factor (24). Another modification, which would increase the apparent molecular size by a multiple of 7 kDa, is ubiquitination, known to occur on chromatin proteins (53). The hypothetical possibility that the 88-kDa species is a ubiquitinated form of 72-kDa E1 protein is especially attractive. This suggests a specific degradative pathway for the E1 protein (16) and may explain how proteins triggering the initiation of DNA synthesis would be restricted to a precise phase of the cell cycle. This might be related to two intriguing observations. (i) This modification was found to take place in mammalian cells only. The 88-kDa species has been detected after VV-E1 infection of human HeLa, monkey CV1, mouse 3T6, and hamster BHK21 cells. It was not found to occur in *Xenopus* oocytes or insect cells. (ii) Although the 88-kDa species was apparently stable within vaccinia virus-infected cells, it was markedly labile under in vitro conditions. After extraction from cells under nondenaturing conditions, the 88-kDa form disappeared very rapidly in comparison with the 72-kDa protein. It was lost almost completely after one purification step, and we never detected it in cell fractionation experiments. Moreover, E1 expression levels determined by using the vaccinia virus expression vector in mammalian cells were found to be 2 orders of magnitude lower than those of other proteins expressed by the same vector. Although one could explain this by either poor translatability or instability of the E1 RNAs, we favor another explanation, that is, specific and physiologic instability of nascent E1 protein. This was also suggested to us by the in vitro translation experiments, in which the 72-kDa protein produced during the first 30 min of reaction was destroyed during further incubation. Last, we have noted two regions in the E1 ORF sequence (amino acids 85 to 105 and 578 to 592) that fit the criteria defined for PEST peptides (44), which may confer instability on the proteins. Taken together, the data suggest a model in which the nascent E1 protein molecules would be rapidly degraded unless stabilized by binding to an appropriate cellular target that might be nuclear or at an appropriate stage in the cell cycle prior to DNA replication.

Since the protein expressed from E1 ORF in heterologous systems was subject to phosphorylation, this observation allowed a means of detecting E1 proteins in BPV1-transformed cells. Our previous assays to immunoprecipitate [^{35}S]methionine-labeled E1 polypeptides from these cells had failed because of a high noise-to-signal ratio with multiple background protein bands. Two immunoreactive phosphoproteins with molecular sizes of 72 and 95 kDa were detected in ID14 cells (Fig. 7A). Although we used in this experiment an antiserum raised against a bacterial protein having a 92-amino-acid sequence shared with the 22-kDa phosphoprotein reported by Thorner et al. (51) (Fig. 1), we were unable to detect this protein, presumably because of the lack of strong epitopes on this common sequence. By Western blot analysis, the 72-kDa protein was also identified by using another anti-E1 serum (Fig. 7B), but no larger species could be detected. The significance of the 95-kDa phosphoprotein detected by immunoprecipitation using our amino-terminal P24 antibodies is unclear. Preliminary analysis by immunoprecipitation of the E1 proteins present in other transformed BPV1-transformed cell lines, of rat origin, allowed us to detect, in addition to the 72-kDa protein, other larger forms (>100 kDa) but no 95-kDa protein (data not shown). All these larger species might represent forms of the 72-kDa protein processed in the same manner as the 88-kDa protein produced in infection with vaccinia virus recombinant VV-E1. The absence of detection in the cell fractionation experiment is reminiscent of the behavior of the vaccinia virus-expressed 88-kDa species.

Much clearer was the case of the 72-kDa protein present in transformed cells, since recognition by two distinct antisera (P24 and 1CI) confirmed that it possessed both the C-terminal and N-terminal domains of the E1 ORF. This criterion, in addition to proper size, phosphorylation, and tight association to nuclear structures (Fig. 7B), was a firm indication that this protein was the product of expression of the complete E1 ORF, corresponding to translation of a continuous transcript spanning nucleotides 849 to 2664 on the viral genome.

A cDNA which might correspond to this type of messenger has been cloned from transformed mouse cells (10). Despite uncertainty about its 5′ end, the absence of any other E1 R transcript identified so far suggests that it may encode the 72-kDa protein detected in ID14 cells. This implies that its transcription has to start upstream from the first AUG of E1, excluding the promoter identified at position 890 (10) (Fig. 1). In this respect, it should be stressed that a mutation affecting the minor promoter located at nucleotide 7940 has been reported to result in the same phenotype as mutations within the E1 ORF; the sets of data for M and R mutants were indistinguishable (46). This suggested that expression of a gene corresponding to the entire E1 ORF was driven by this promoter. Further work is needed, however, to clarify this question.

A function ascribable with certainty to the 72-kDa E1 protein is the down-regulation of transcription from the viral promoter P89 (27) and, consequently, of the rate of transformation of C127 cells (46). This effect was confirmed by complementing E1 mutants, with respect to their high-transformation–high-transcription phenotype, with constructs in which the entire E1 ORF was placed under long terminal repeat control (46), thus utilizing constructs analogous to the viral vectors we used to overproduce the protein. There is no such direct proof for a replicative role for the 72-kDa E1 protein. The identification of a messenger containing E1 R sequence, which likely corresponds to the full-sized ORF (10), leads however to the prediction that the 72-kDa E1 protein might actually ensure the functions attributed to the R gene. At first site, this likely assumption seems to contradict the previous definition of two separate complementation groups within the E1 ORF (4). The observation of internal initiation of translation within the E1 ORF, occurring in vitro on the third or fourth methionine (Fig. 2A), suggested to us a hypothetical model reconciling both statements: in the case of the frameshift M mutants studied by Lusky and Botchan (36), reinitiation of the translation of E1 mRNA on internal methionine could produce truncated 38- to 40-kDa E1 R proteins sufficient to perform part of the replicative functions of the whole protein and to allow transient replication. Internal initiation of translation is actually a regular mechanism, required for the expression of important viral functions in transformed cells such as the production of E5-transforming protein from an E2-E5 messenger (54). Internal starts of translation within the E1 ORF, however, were not detected in vivo (Fig. 2B and 3), although they may be present at very low levels in the case of the vaccinia virus expression system (see, for instance, the 38-kDa band visible on Fig. 4 and 5 in association with the full-sized E1 protein). We propose that this mechanism should, however, work if translation, initiated regularly on the first E1 ATG, stops before reaching the second, third, or fourth methionine codon, as in the case of the frameshift mutants studied by Lusky and Botchan (36). The observation mentioned above that truncated E1 R expressed from vaccinia virus vector was mainly detected as cytoplasmic aggregates contradicts at first sight our hypothesis, but limited nuclear amounts of this protein might be sufficient to ensure proper replication. A possible way to verify the validity of our model would be to identify the truncated E1 R proteins supposed to be present in M^- mutans; however, preliminary examinations (Fig. 7B) did not allow us to detect such a truncated E1 protein in the M^- mutant 760 (lanes 3) when compared with the R^- mutant 783 (lanes 4).

The existence of a full-sized E1 translation product in BPV1-transformed cells raises another question: since the 72-kDa protein contains M polypeptide sequence, what is the function of the 22-kDa protein identified by Thorner et al. (51) as a product of the M gene? One may imagine that 73-kDa E1 and 22-kDa M proteins would play opposite roles in replication by competing for the same target, to which they bind through their common M molecular domain. If the entire E1 is required for viral DNA replication, then M could modulate by competing for factors that complex with the N terminus of E1 and thus down-modulate the E1 functions.

We have initiated the characterization of E1 protein biochemical activities most likely associated with the initiation of DNA replication. In agreement with the sequence similarities between E1 and SV40 large-T-antigen proteins (13), we detected a nucleotide-binding activity, which in the case of SV40 large T antigen reflects the function of its ATPase-helicase domain (7, 12). The binding of ATP to vaccinia virus-encoded E1 proteins was evidenced by a covalent affinity labeling assay (Fig. 9). Expectedly, the antibody raised against a bacterial fusion protein containing the putative nucleotide-binding site was the only one to inhibit labeling by the ATP analog. All three E1 proteins (72-, 85-, and 88-kDa species) bound the analog, thus confirming their identification as bona fide full-sized E1 polypeptides. Whether E1 proteins have ATPase-helicase activity, as is operative in the SV40 large T antigen (6), is currently under investigation.

In comparison with the SV40 large T antigen, the E1 protein appears to lack one major feature, that is, specific recognition of DNA sequences at which to initiate replication. We have not detected any indication of a specific recognition of defined BPV1 DNA sequences by E1 proteins expressed from VV-E1 vaccinia virus vector. The 72-kDa E1 protein was found to bind to DNA in a nonspecific manner (Fig. 8). The N-terminal domain is believed to be responsible for this property (Androphy et al., unpublished observations). We speculate that the E1-dependent initiation of BPV1 DNA replication involves the recognition of specific sequences at or near PMS1 (34, 52). If so, this recognition has to be mediated through interaction of E1 with another protein(s) of cellular or viral origin. This interaction might confer proper positioning for E1 to perform the second step in initiating DNA replication, as is the case for SV40 T-antigen ATPase-helicase, by unwinding the *ori* DNA for allowing DNA polymerase α-primase to start new DNA strands (6). Development of in vitro replication systems of BPV1 DNA using purified E1 protein might help to elucidate these unresolved questions.

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