Bovine papillomavirus type 1 E1 ATPase activity does not depend on binding to DNA nor to viral E2 protein

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Replication of bovine papillomavirus type 1 (BPV-1) DNA has been shown to require two viral proteins known to interact in a molecular complex: E2, a transcription activator, and E1, another nuclear phosphoprotein, which binds to the replication origin and for which helicase/ATPase activities have previously been reported. Here we characterize the BPV-1 E1 ATPase activity. In contrast to Seo et al. (Proceedings of the National Academy of Sciences, USA, 90, 702-706, 1993), we were able to detect this activity in the absence of nucleic acid in partially purified preparations of either E1 protein or of E1–E2 protein complex. Measurements of specific activity and kinetic parameters gave similar values for preparations of various kinds. ATPase activity was quantitatively retained by immunoprecipitates obtained by using anti-E1 or, in the case of E1-E2 complex, anti-E2 antibodies. Significantly, preparations of bacterially expressed glutathione S-transferase–E1 fusion protein exhibited levels of DNA-independent ATPase activity comparable to those of baculovirus-expressed E1. The presence of nucleic acids of various types, including stoichiometric amounts of a BPV-1 ori DNA fragment containing E1 and E2 binding sites, did not grossly affect E1 ATPase activity, the most notable effect being a 2-fold stimulation by unspecific ssDNA. Altogether, our results indicate that BPV-1 E1 possesses an intrinsic ATPase activity which does not depend on the presence of nucleic acid; moreover, they render unlikely any modulation of E1 ATPase activity due to binding either E2 protein or target DNA sequences, or as a result of protein phosphorylation.

Introduction

The molecular mechanisms controlling the initiation of DNA replication in mammalian cells are still largely unknown, with most of our knowledge being limited to viral models, and especially those offered by polyomavirus and simian virus 40 (SV40) (Challberg & Kelly, 1989; Stillman, 1989). DNA replication initiates at a precisely defined origin on the viral genome (ori), at which regulatory proteins bind and thus engage the replication machinery. A central element within the replication origin (core ori) has to be recognized specifically by a critical protein, such as the SV40 large T antigen, corresponding to the ‘initiator’ whose absolute requirement for replication was postulated more than 30 years ago (Jacob et al., 1963). Other factors must also cooperate with the initiator, by binding to enhancer elements adjacent to the core origin of replication (DePamphilis, 1993), but they are not required under all circumstances. In vitro cell-free systems permit viral DNA replication in an enhancer-independent manner, and have greatly aided study of the molecular interactions taking place at the SV40 core ori during the initial steps of DNA synthesis (Borowiec et al., 1990). The initiation of DNA replication requires two essential processes to take place at the replication origin: DNA melting and opening, and the positioning of the replication fork machinery. Origin recognition, which depends on ATP binding, results both in the specific formation of T antigen hexamers and in local DNA melting. Then T antigen helicase activity unwindss DNA on both sides of the ori. In addition, T antigen specifically interacts with both DNA polymerase α and RPA/ssDNA binding protein, helping thus to position the first elements of the replication fork machinery (reviewed in Waga & Stillman, 1994). Although the start of DNA synthesis clearly requires all these molecular functions, only one of them, local DNA melting (Baker et al., 1988), is performed in bacteria by the DnaA protein in order to initiate the synthesis of the E. coli chromosome; even ATP hydrolysis by DnaA protein is not required per se in this process, but only ATP binding (Sekimizu et al., 1987; Bramhill & Kornberg, 1988). Polyomaviruses have thus condensed several functions into a unique position, probably as a strategy to save space within their genome and/or to bypass host mechanisms controlling DNA replication.
Papillomaviruses offer another model worth investigating, because their genomes are maintained as plasmids in transformed cells and during the early phase of their multiplication cycle, being replicated to controlled levels during the S phase of the cell cycle. Their replication requires two viral proteins, the transcription activator E2 and the full-length product of the E1 gene (Ustav & Stenlund, 1991; Chiang et al., 1992). The precise identification of the E1 product as a nuclear phosphoprotein (Sun et al., 1990; Santucci et al., 1990) allowed its overexpression and purification (Mohr et al., 1990; Santucci et al., 1992; Yang et al., 1993) and led to the recent development of an in vitro system for bovine papillomavirus I (BPV-1) DNA replication (Yang et al., 1991; Müller et al., 1994). As far as they have been characterized, the biochemical properties of the E1 protein parallel those identified for the SV40 large T antigen: (i) recognition of a palindromic sequence which constitutes most of the core ori identified on the papillomavirus genomes (Wilson & Ludes-Meyers, 1991; Ustav et al., 1991; Yang et al., 1991; Holt et al., 1994), and (ii) ATP-dependent helicase/DNA unwinding activities (Seo et al., 1993a, b; Yang et al., 1993). Although necessary in vivo for the replication of BPV plasmids, the viral transcription factor E2 is not required in vitro to obtain efficient DNA synthesis (Müller et al., 1994; Bonne-Andréa et al., 1995a). Its role during the process of initiating DNA replication is thus unclear. By establishing a molecular association with E1 (Mohr et al., 1990; Blitz & Laimins, 1991; Lusky & Fontane, 1991), E2 has been shown to improve E1 binding to the ori (Seo et al., 1993b; Lusky et al., 1993). In addition, as suggested by recent in vitro replication experiments (Li & Botchan, 1993, 1994), acidic transcription factors could increase the efficiency of DNA replication, if appropriate binding sites are replacing the E2 responsive elements that normally are located adjacent to the ori. This is a similar situation to that observed both in vivo for polyomavirus DNA replication (He et al., 1993) and in vitro for SV40 DNA synthesis (Cheng et al., 1992): in both cases, cell transcription factors probably help to maintain the ori region free of nucleosomes and to engage replication factors into molecular association with ori-bound large T antigen molecules. In the case of BPV, however, E2 has also to be present also to achieve such a scenario. Many details remain to be elucidated: (i) how does the E1 protein act to unwind the ori DNA sequences or to interact with cell proteins in order to build the first elementary replication fork? (ii) What are the molecular controls that down-regulate these processes, since in vivo the extent of papillomavirus DNA replication is very limited when compared to that of polyomaviruses? In particular, does the low level of BPV plasmid replication reflect molecular differences in the structure and functions of the viral initiator proteins? (iii) What is the purpose of E1–E2 interactions?

We have previously shown the association of a DNA-independent ATPase activity with the BPV-1 E1 protein in partially purified preparations (Santucci et al., 1992). More recently, Seo et al. (1993a) were able to detect ATP hydrolysis catalysed by BPV-1 E1 preparations purified nearly to homogeneity, but only in the presence of ssDNA; they thus concluded that E1 ATPase activity was strictly DNA-dependent. Their result was, however, at variance with two recent observations on human papillomavirus E1 proteins. Immunoprecipitates of baculovirus-expressed HPV-11 E1 were reported to be able to hydrolyse ATP in the absence of DNA (Bream et al., 1993). More significantly, a bacterially expressed fusion protein, made between E. coli maltose-binding protein and HPV-6b E1 and affinity-purified, was also shown to hydrolyse ATP in the absence of any added nucleic acid (Hughes & Romanos, 1993); moreover, this property was clearly affected by a specific point mutation.

These conflicting results prompted us to re-examine the status of BPV-1 E1 ATPase, for the following reasons. Sequence similarities exist between the T antigen ATPase domain of polyomaviruses (Bradley et al., 1987) and a region thought to have the same function within the E1 molecules of papillomaviruses (Clertant & Seif, 1984). Although DNA-dependent ATPase activity is taken as a hallmark of DNA helicases (Matson & Kaiser-Rogers, 1990), SV40 large T antigen is peculiar amongst these enzymes in being able to hydrolyse ATP in the complete absence of nucleic acids: only one sort of nucleic acid [poly(dT)] enhances large T ATPase activity to limited extent (less than 5-fold), while other nucleic acids have only a marginal effect (Giachero & Hager, 1979; Clark et al., 1981; our observations). In addition, the phosphorylation state of the large T molecule does not affect its ATPase and helicase activities, in contrast to its other molecular functions (Mohr et al., 1987, 1989; McVey et al., 1989). It would be interesting to know whether these characteristics are shared by the papillomavirus E1 protein. Here we document that this is the case, showing that, very like SV40 large T antigen, BPV-1 E1 possesses an intrinsic DNA-independent ATPase activity which does not seem to be modulated either by binding to its ori DNA target, or by phosphorylation or by association with E2 protein.

Methods

Plasmid and viral expression vectors. In order to construct a plasmid that would express a fusion protein between BPV-1 E1 and Schistosoma japonicum glutathione S-transferase (GST), the BPV-1 DNA NruI–StuI restriction fragment (nt 838–3351) was ligated to BamH1 linkers, and then inserted into the unique BamH1 site of plasmid pGEX-3X.
(Pharmacia), thus producing an in-frame fusion of the whole E1 ORF 3' to the GST sequence, with an insertion of six extra amino acids (-Gly-Ile-Arg-Ala-Ser-Val-) between the factor Xa proteolytic signal and the E1 initiator methionine (plasmid pGEX-E1).

Recombinant baculoviruses expressing either E1 or E2 proteins were previously described (Santucci et al., 1990; Monini et al., 1991). They were used at high multiplicity to infect SF9 insect cells for 40-48 h at 26 °C (Summers & Smith, 1987).

Antibodies. Rabbit polyclonal anti-E1 antibodies were raised against either the N-terminal (P24) or C-terminal part of the E1 protein (1C1, 26 °C (Summers & Smith, 1987).

Extraction and purification procedures. The selective extraction of E1 protein from baculovirus-infected cells and details of its purification will be given elsewhere (Bonne-Andréa et al., 1995b). Briefly, quantitative extraction from nuclei isolated by hypotonic shock was performed by an incubation in a high salt/high pH medium (300 mM-potassium acetate, 50 mM-MgSO4, pH 8.3). After centrifugation, the supernatant (containing at least 75% E1 protein, amounting to 5-10% of the total protein in the extract) was adjusted to pH 8.0 and 150 mM-potassium acetate and submitted to chromatographic fractionation, as follows. Firstly a DEAE-cellulose column allowed us to quantitatively recover E1 in the flow-through, as detected by Western blotting. E1 was then bound by an heparin-agarose column, from which it was eluted by 500 mM-potassium-acetate buffer. Peak fractions, adjusted to 200 mM-potassium acetate, were loaded onto a 1 ml FPLC MonoQ column (Pharmacia), from which E1 was eluted by 1-0 mM-potassium-acetate buffer. Peak fractions, containing E1 as the major protein (up to 40% total protein), were dialyzed against 20 mM-Tris-acetate pH 8.0, 200 mM-potassium-acetate, 10 mM-MgSO4, 5 mM-DTT, 10% glycerol, and stored in aliquots at -70 °C. In some experiments, partially purified E1 was obtained by a single-step procedure: the nuclear eluate, obtained as described above, was passed through an FPLC column filter (Pharmacia) and then directly applied to the 1 ml FPLC MonoQ column, from which E1-containing fractions were recovered as described above.

For E2 extraction, baculovirus-infected cells were pelleted 48 h post-infection and frozen in liquid nitrogen. Cells were resuspended in buffer A (20 mM-MES pH 6.2, 10 mM-NaCl, 1 mM-DTT, 1 mM-PMSF, 10 µg/ml leupeptin and aprotinin, 106 cells/ml) and incubated for 30 min on ice. The nuclear pellet was recovered by 5 min centrifugation at 2000 rpm, then resuspended in buffer A containing 0.5 mM-NaCl 1 mM-EDTA, 1% NP40 and kept on ice for 45 min. The resulting nuclear eluate was adjusted to 0.25 mM-NaCl and 10% glycerol by slow stepwise dilution in the cold with buffer A containing 20% glycerol, and was centrifuged at high speed (35000 rpm, 30 min, 4 °C, Beckman 60Ti rotor). The supernatant was then subjected to further chromatographic fractionation.

When starting from cells doubly infected by both recombinants, E1 was quantitatively recovered together with E2 by using the E2 extraction procedure described above: the nuclear eluate obtained at pH 6.2 with 0.5 mM-NaCl was found to contain E1 or E2 levels comparable to those of extracts made from single infections. Double-sandwich ELISA assays performed by using anti-E1 and anti-E2 antibodies established that E1 and E2 were mostly present in the eluate as a molecular complex (Santucci et al., 1992). Significantly, the behaviour of both proteins through further purification steps did not differ from what was observed in parallel for the E2 protein, similar to what has previously been reported (Li et al., 1989; Monini et al., 1991). The nuclear eluate from 106 cells was passed through an FPLC column filter (Pharmacia) and directly applied onto either 1 ml FPLC MonoS (Pharmacia) column or an S-Sepharose column (both previously equilibrated with buffer B: 20 mM-MES pH 7.0, 0.25 mM-NaCl, 10% glycerol, 1 mM-DTT). After washing, gradient elution was performed with buffer B containing increasing salt concentrations. E1 and E2 were eluted together as a single peak by 0.6 M-NaCl. For further purification, peak fractions, adjusted to 0.25 mM-NaCl, pH 8.0, were applied onto a 1 ml FPLC MonoQ column (equilibrated with buffer C: 20 mM-Tris-HCl pH 8.0, 0.25 mM-NaCl, 10% glycerol, 1 mM-DTT) and eluted with an NaCl gradient). E1 and E2 were recovered as a single peak at 0.55 M-NaCl. Examination of this material by SDS-PAGE revealed E1 and E2 bands, with Coomassie staining intensities in a 2:1 ratio, amounting to ca. 40% of the total protein content (Santucci et al., 1992). Fractions containing E1 and E2 were pooled, dialysed extensively against 0.1 M-NaCl buffer C, and stored as aliquots at -70 °C for as long as 6 months without observing significant ATPase decay. Only freshly thawed aliquots were used, after which they were discarded.

Purification of glutathione S-transferase–E1 fusion protein and of bacterial E1. To express GST and GST–E1 fusion proteins, plasmids pGEX-3X and pGEX-E1 were introduced in parallel into E. coli (HB101 strain). Induction was performed as previously described (Smith & Johnson, 1988), by adding 1 mM-IPTG for 90 min at 37 °C. Induced cells were harvested by centrifugation at 4 °C, frozen in liquid nitrogen and stored at -70 °C. The cell pellet was resuspended in ice-cold buffer D (20 mM-Tris–HCl pH 8.0, 0.4 M-NaCl, 50 mM-MgSO4, 5 mM-DTT, 1% Triton X-100, 1 mM-PMSF, 10 µg/ml leupeptin and aprotinin; 1 vol. buffer D per 10 vol bacterial culture) and lysed on ice by sonication (10 s pulses with 10 s rest for 2 min). The lysate was cleared by centrifugation (30 min, 35000 r.p.m., 4 °C, Beckman 60Ti rotor), and the supernatant was incubated with glutathione–Sepharose beads (Pharmacia), previously equilibrated in buffer D, with gentle agitation for 1 h at 4 °C. Complete retention of the GST–E1 protein was obtained by mixing 160 µg GST–E1 (i.e. 17 nmol) with 10 ml 50% glutathione–Sepharose beads suspension; parallel controls were performed with the unfused GST protein, 45 µg (17 nmol) of which was bound by the same amount of beads. After incubation, beads were pelleted by a brief centrifugation and washed successively twice with buffer D, and twice with the same buffer containing 1 M-NaCl. Beads to be assayed directly for ATPase activity were then equilibrated in ATPase buffer (see below). Before specifically eluting or cleaving the GST proteins, glutathione–Sepharose beads were first equilibrated in buffer E (50 mM-Tris–HCl pH 8.0, 0.1 M-NaCl, 10 mM-MgSO4, 5 mM-DTT) by two more washes. Elution was then performed by incubating the bead suspension in buffer E containing 10 mM-reduced glutathione (Sigma) for 10 min on ice, in a final volume two- to threefold that of the packed beads. Cleavage with Factor Xa was also directly carried out on the beads, by incubating overnight at 4 °C in buffer E containing 1 M-NaCl, and Factor Xa (in an enzyme/substrate ratio of 1:50). Eluted GST–E1 or GST proteins, or E1 protein resulting from Factor Xa cleavage were recovered from the bead mixture by briefly centrifuging and were immediately assayed for ATPase activity.

Immunodetection of E1 and E2 proteins. E1 and E2 proteins were detected in infected cells, in extracts and through purification steps, by using Western- or dot-blot techniques, as already described (Santucci et al., 1990, 1992). As primary antibody we employed polyclonal anti-E1 sera (1:800 dilution), or monoclonal anti-E2 IgG (2 µg/ml). Secondary antibodies coupled to alkaline phosphatase (Bio-Rad) were used at a 1:2000 dilution, and staining was performed using NBT and BCIP substrates (Harlow & Lane, 1988).

Determination of protein content. Protein concentrations were measured by using the Bio-Rad assay. Specific estimation of amounts of E1 and/or E2 were performed by densitometric determination of Coomassie Blue staining of the E1 (70 KDa) or E2 (55 KDa) bands in polyacrylamide gels, referred to a standard curve obtained on the same gel with serial amounts of bovine serum albumin, using an LKB Ultroscan apparatus. The value measured for a specific band could also be compared to the sum of the staining intensities of all peptide bands
in the lane of interest, allowing estimation of the E1 or E2 content as the percentage of total proteins.

Detection of contaminant nucleic acids. The absence of any significant contamination of E1 preparations by nucleic acids was checked by the specific 5’-end-labelling technique. Protein preparations (10–100 µg E1) were first treated with proteinase K (Boehringer; 50 µg/ml) in the presence of 10 mM-EDTA and 1% SDS for 45 min at 37 °C, then deproteinized by phenol–chloroform extraction. Nucleic acids were pelleted by ethanol precipitation with the help of glycogen as a carrier and dried; samples were then dephosphorylated with calf intestine alkaline phosphatase (Boehringer; 2 U/100 µl 50 mM-Tris pH 9.0, 1 mM-MgCl₂, 0.1 mM-ZnCl₂, 1 mM-spermidine for 1 h at 37 °C). Phosphatase was destroyed by a 70 °C treatment and phenol-extracted. After a second ethanol precipitation, samples were incubated in the presence of T₄ polynucleotide kinase and [γ-³²P]ATP (3000 Ci/mmol, 0.1 µCi) for 30 min at 30 °C in the appropriate buffer (New England Biolabs). Labelling was measured by scintillation counting of TCA precipitates and analysed by gel electrophoresis examination, after occasional digestion with RNase or DNase. The efficiency of 5’-end labelling was measured by adding known amounts of defined DNA fragments as internal standards to some of the samples that were to be examined.

Determination of ATPase activity. This was usually performed by the charcoal technique (Clark et al., 1981). Unless stated otherwise, assays were performed at 30 °C, in 20 µl 20 mM-Tris pH 8.0, 5 mM-MgSO₄, 100 mM-NaCl, 1 mM-DTT, 1–10% glycerol, 5 µM-ATP and with the addition of 0.5 µCi [γ-³²P]ATP (Amersham; 3000 Ci/mm). After various incubation times, 5 µl aliquots were removed and immediately added to 250 µl of charcoal suspension; after vortexing and centrifuging, the radioactivity present (as soluble inorganic phosphate) in 125 µl of the supernatant was counted. Rates of hydrolysis vs time were determined as the slopes of three-plot kinetics.

When assays were performed by using γ-³²P-labelled substrates, time-point aliquots from each incubation were spotted onto polyethyleneimine (PEI)-cellulose thin-layer chromatography plates. Plates were developed with 1.2 M-LiCl (Penningroth et al., 1980), dried and submitted to autoradiography; the radioactive spots were then excised and counted.

Assaying ATPase activities retained in immunoprecipitates was performed as described previously (Clerant et al., 1984). Protein A-Sepharose was first loaded with antibody by incubation under gentle agitation for 1 h (1 µg IgG/µl packed beads), then the beads were washed six times with the same buffer as that of the protein preparations under study. They were then incubated under gentle agitation for 2 h at 4 °C in the presence of partially purified E1 preparations, under conditions of antibody excess, in a final volume twice that of the antigen solution under assay. The supernatant was recovered by centrifugation and assayed as described above. Beads were washed twice with 0.1 M-Tris–HCl pH 8.0, 0.5 M-NaCl, 1 mM-DTT buffer, then three times with the buffer used for ATPase assays (see above), and resuspended in this buffer. Assays for ATPase activity were performed by adding labelled ATP and incubating the bead suspension under gentle agitation, in a final volume equal to that of the protein sample that was submitted to immunoprecipitation. The ATPase activity of GST–E1 fusion protein bound to glutathione-Sepharose beads was assayed in the same manner.

**Results**

**Partially purified E1 or E1–E2 proteins exhibit ATPase activity**

In order to study the biochemical properties of viral proteins required for BPV-1 DNA replication, we overexpressed both the E1 and the E2 proteins by the use of appropriate recombinant baculoviruses (Santucci et al., 1990; Blitz & Laimins, 1991). BPV proteins were then selectively extracted from the nuclei of infected insect cells, and partially purified. In order to investigate whether molecular association with E2 modified the properties of E1, we employed coinfection with both viruses to purify an E1–E2 complex. As previously reported (Santucci et al., 1992) and described in Methods, the E1–E2 complex was efficiently purified by following the E2 purification scheme. We assayed for enzyme activity various preparations of partially purified E1 protein or E1–E2 complex: all were found to exhibit ATPase activity, while a preparation containing E2 alone was unable to hydrolyse ATP to a significant level (Table 1). Measurements of the rate of ATP hydrolysis gave values close to each other relative to the quantity of E1 protein under assay, whatever the preparation employed. By performing assays at 30 °C in the presence of 5 µM-ATP, we obtained consistent values for specific activity in the range of 6–9 pmol ATP hydrolysed/h/pmol E1 protein (Table 1, and data not shown).

| Table 1. ATPase activity present in various E1 or E1/E2 preparations |
|-----------------------|-----------------|-----------------|
| Preparation* | Protein† (mg/ml) | E1 content† (% total protein) | ATPase activity‡ (ATP hydrolysed, /pmol E1/h) |
| E1 | A | 0.20 | 18 | 4.3 ± 0.4 | 8.4 ± 0.8 |
| B | 0.20 | 20 | 5.1 ± 0.3 | 8.9 ± 1.8 |
| E1–E2 | C | 0.95 | 13 | 7.5 ± 0.2 | 58.9 ± 0.8 |
| D | 0.30 | 10 | 3.0 ± 0.3 | 91.2 ± 7.0 |
| E | 0.95 | 12 | 6.0 ± 0.8 | 54.9 ± 0.8 |
| F | 0.12 | 30 | 4.6 ± 0.5 | 89.9 ± 1.0 |
| E2 | G | 0.10 | – | 0.3 ± 0.1 | – |

* Protein preparations were obtained as described in Methods. Preparations A and B were two parallel chromatographic fractionations of the same nuclear eluate prepared from cells infected with the E1 recombinant baculovirus, obtained as peak fractions eluted from either heparin–agarose (A) or MonoQ columns (B). Preparations C–F were from cells co-infected by both E1 and E2 viruses: C was the peak fraction eluted from a MonoS column, while D and E were two distinct fractions obtained by Sepharose chromatography of low pH–high salt nuclear eluate, and were, respectively, from the front (D) and the tail (E) of the E1–E2 peak; central fractions of this peak were pooled and applied to a MonoQ column, from which E1 and E2 proteins were still eluted as a single peak, presented here as preparation E. Preparation F was obtained by submitting the nuclear eluate to a single step of chromatography on a MonoS column; as a control directly comparable to F, preparation G was obtained in parallel from cells infected by the E2 virus only, by the same nuclear extraction and MonoS chromatography, and contained 40% pure E2 protein (Santucci et al., 1992).

† Protein content and ATP hydrolysis were measured as described in Methods. Assays were performed in 20 µl volumes in the presence of 5 µM-ATP, by using the charcoal technique. ATPase values were calculated as percentage ATP hydrolysis relative to the amount of E1 (1 pmol E1 = 70 ng).
various concentrations of either unlabelled ATP (lanes 1–4) or dATP mixtures without protein addition. After 2 h, 1 μl aliquots from each protein were incubated, as described in Methods, in the presence of unlabelled nucleotides added as carriers was visualized under UV light. incubation were spotted onto a PEI–cellulose plate. The migration of nucleotide, as indicated. Lanes 1 and 5 correspond to incubation complex (preparation F, Table 1) containing 140 ng (i.e. 2 pmol) E1 protein. Moreover, the presence of E2 protein only partially purified, these results suggested that E1 protein. Additionally, the presence of E2 protein associated with E1 did not seem to affect this activity.

Table 2. E1-associated ATPase activity is immunoprecipitated by specific antibodies

<table>
<thead>
<tr>
<th>Antibodies used for immunoprecipitation</th>
<th>ATPase recovery (% input)</th>
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<tr>
<td></td>
<td>Supernatant</td>
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<tr>
<td>Polyclonal rabbit IgGs</td>
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<tr>
<td>Anti-E1 (IC1)</td>
<td>10–15</td>
</tr>
<tr>
<td>Non-immune</td>
<td>116–107</td>
</tr>
<tr>
<td>Monoclonal mouse IgGs</td>
<td>9–5</td>
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* Aliquots (20 μl) of partially purified E1–E2 complex (Table 1, preparation F) were submitted to immunoprecipitation, as described in Methods, by using 20 μl of a suspension of Protein A–Sepharose beads preloaded with an excess of antibodies, as indicated. Supernatants were kept for assay; bead pellets were washed and brought to 40 μl with buffer. Identical volumes of either bead suspension (pellet) or supernatant were assayed for ATPase activity, as described in Table 1. For each immunoprecipitation, two aliquots of bead pellets and supernatants were assayed, corresponding respectively to 4 and 8 μl input E1–E2 preparation. Indicated here are the two relative values measured for ATP hydrolysis rate, as compared to input ATPase activity, corresponding respectively to the smallest and largest input.

Although preparations of E1 and E1–E2 proteins were only partially purified, these results suggested that ATPase activity is solely determined by the presence of E1 protein. Moreover, the presence of E2 protein associated with E1 did not seem to affect this activity.

We verified that ATP hydrolysis was due to a bona fide ATPase, and not to a nonspecific phosphatase, by performing assays with α-32P-labelled ATP and dATP substrates and analysing the reaction products by PEI–cellulose thin-layer chromatography. Fig. 1 presents assays of a preparation of E1–E2 complex, but equivalent results were obtained with every preparation examined that contained either E1 or E1–E2 protein (data not shown). Since the label was always observed to be released as nucleoside diphosphate, and not as AMP/dAMP or phosphate spots (as visible in Fig. 3), the activity found in association with E1 protein was clearly an ATPase and not a phosphatase. As also seen in Fig. 1, this activity cleaves dATP as well as ATP. Other experiments showed that it also hydrolyses, to a much lower extent, GTP, but not other nucleoside triphosphates (not shown).

E1-associated ATPase activity was examined under a variety of conditions in order to optimize the assays. The enzyme exhibited a broad optimum of ionic strength (0.10 M to 0.25 M salt), no significant variation within physiological pHs (from 6.8 to 8.2) and a marked dependence upon divalent cations (optimum: 1–2 mM for Ca2+ and Mn2+, 2–10 mM-Mg2+, with Mn2+ >> Mg2+ >> Ca2+).

E1 ATPase activity is immunoprecipitated by specific antibodies

To further analyse the association between ATPase activity and E1 protein, we examined the effect of various antibodies raised against defined portions of the E1 molecule (Santucci et al., 1990) on ATPase activity. None was found to neutralize ATPase activity (data not shown). For two of them, directed against the N-terminal part of the molecule (P24) or the C-terminal decapeptide (P20-1), this was not unexpected, because similarities with the SV40 large T ATPase domain were located elsewhere in the E1 sequence. However the third antibody, raised against the C-terminal half of the E1 molecule (IC1), had previously been shown to inhibit covalent affinity labelling of E1 by an ATP analogue (Santucci et al., 1990); this is similar to what has been observed in the case of polyomavirus large T antigen with anti-T antibodies (Clerc et al., 1984).

As expected, immunoprecipitating E1 by any of these antibodies efficiently depleted E1 preparations of ATPase activity. Table 2 presents the results of experiments performed with partially purified E1–E2 complex: after incubation with either the non-neutralizing IC1 anti-E1 antibody or a monoclonal anti-E2 antibody bound to Protein A–Sepharose beads (see Methods), less than 15% of the initial ATPase remained in the supernatant. Significantly, a large portion of the input ATPase was recovered in the precipitates obtained with either of the specific antibodies, in contrast to what was found with control antibodies. Immunoprecipitation experiments performed using the two other anti-E1 antibodies gave similar results (not shown). Thus, the E1-associated ATPase present in E1–E2 complex preparation could be precipitated as active enzyme by either anti-E1 or anti-E2
antibody. Consistently anti-E1, but not anti-E2 antibodies, also immunoprecipitated active ATPase from E1 preparations (not shown). These experiments indicated the existence of a physical link between the observed ATPase activity and the E1 molecule; in addition, they verified the existence of a molecular association between co-purified E1 and E2 proteins.

It is worthy of note (see below), that we did not detect in these immunoprecipitates significant amounts of nucleic acids: less than 5 ng/μg E1, mainly due to large DNA fragments (data not shown); similar levels of DNA were also found in non-specific immunoprecipitates, suggesting that contaminating nucleic acids were not specifically bound to the E1 protein. Moreover, from what we know of the size of DNA E1-binding sites (at least 18 bp; Holt et al., 1994), a simple calculation suggests that, in addition to being nonspecific, this level of contamination is probably much too low to permit quantitative binding of E1 to DNA in the immunoprecipitates.

A GST–E1 bacterial fusion protein has ATPase activity

The previous experiments did not exclude the possibility that ATPase activity could result from a specific and stable association of E1 with an irrelevant insect protein. Unfortunately, using baculovirus-expressed proteins and classic chromatographic procedures, we failed to obtain completely purified active E1 protein; rather, after two chromatographic steps, E1 replicative activity, as assessed in an *in vitro* system, was completely lost while ATPase was still present (Bonne-Andréa et al., 1995b). We thus decided to express E1 in bacterial cells as a fusion protein with glutathione S-transferase (GST), in order to purify it by single-step affinity chromatography. *E. coli* cells harbouring the plasmid pGEX-E1 produced
upon induction a polypeptide of the expected size (98 kDa) which reacted with E1 antisera (Fig. 2, lanes 1). In addition to GST–E1 protein, several polypeptides, present in variable proportions in the soluble extract prepared from induced bacterial cells, also reacted specifically with anti-E1 antibodies (Fig. 2b, lane 1), indicating that various GST–E1 proteolytic products were also produced. Extracts prepared from induced cultures were submitted to affinity chromatography, and the material bound to the beads or recovered from them by either eluting with reduced glutathione or cleaving with Factor Xa was analysed by SDS-PAGE (Coomassie blue staining, Fig. 2a; Western blotting using E1 antisera, Fig. 2b). In addition to full-length GST–E1 protein fixed on beads or present in glutathione eluates, we observed several minor proteins (Fig. 2a, lanes 1 and 2), probably degradation products since they were also detected in soluble extracts by Western blotting (as discussed above). Factor Xa cleavage generated an immunoreactive E1 polypeptide of normal size (72 kDa: Fig. 2a, lane 6; Fig. 2b, lanes 2 and 3). Induced bacterial cells harbouring control plasmid pGEX-3X produced, as expected, a 26 kDa GST protein, also purified by using glutathione–Sepharose beads (Fig. 2a, lanes 3 and 4).

Eluted GST–E1 protein, E1 protein prepared by Factor Xa cleavage, and GST protein as a control were all assayed for ATPase activity. Fig. 3 shows a representative assay performed by using dATP as a substrate. We observed formation of dADP in reactions containing either GST–E1 or mature E1 protein, while in the presence of control GST protein, the level of hydrolysis was insignificant. Similar results were obtained by using ATP as a substrate (data not shown).

By performing several ATPase assays with various preparations of GST–E1 protein, we could measure a constant specific activity, slightly lower (ca. 4 pmol ATP hydrolysed/h/pmol E1) than that measured under the same conditions for the E1 protein expressed in insect cells (see Table 1). It has to be stressed, however, that the enzyme activity of bacterial E1 protein seemed to be unstable: for instance, storage of glutathione-Sepharose bound GST–E1 at 4 °C for 48 h reduced the ATPase level to 20% of that found initially. Measured activities might have depended upon the total duration of the experiment, including extraction, purification and (possibly) proteolysis steps. Our results would thus represent underestimates of the actual specific activity, which clearly does not differ much from that of baculovirus-expressed E1. More importantly, this finding, in conjunction with those obtained with baculovirus-expressed E1 protein, excluded the possibility that ATPase activity detected in all E1 preparations could be due to a minor
contaminant constantly present in association with E1, under all the conditions tested. Rather, the BPV-1 E1 protein possesses an intrinsic ATPase activity, able to function in the absence of any nucleic acids. To further assess the latter point, we performed sensitive assays to reveal the presence of nucleic acids in the material eluted from glutathione–Sepharose beads (see Methods); they failed to detect anything other than small RNA pieces (less than 25 nucleotides long) present at low levels (0.4% contamination by weight) in both GST and GST–E1 material (data not shown). Here also, in addition to being nonspecific, this level of contamination (more than 10 GST–E1 molecules for every RNA oligonucleotide) was much too low to allow quantitative binding of E1 to contaminating RNA.

Nucleic acids affect E1 ATPase activity only slightly

In order to determine whether E1 ATPase could be modulated by interacting with DNA, enzyme assays were performed in the presence of various nucleic acids. The presence of M13 ssDNA stimulated E1 or E1–E2 ATPase activity 1.5–3-fold (Figs 4b and 5). Under the same conditions, we did not observe any stimulation by DNA in the case of the GST–E1 fusion protein (Fig. 4a). On the other hand, E1, E1–E2 and GST–E1 ATPase activities were not affected by the presence of salmon sperm dsDNA, poly(dT), poly(U) or tRNA (Fig. 5, and data not shown).

Both E1 and E2 proteins have been reported to bind specific sequences on the viral genome: E1 recognizes a palindromic motif localized in the BPV-1 minimal ori (Holt et al., 1994; Ustav et al., 1991; Wilson & Ludes-Meyers, 1991; Yang et al., 1991), and the transcriptional activator E2 binds to a set of responsive elements present in the vicinity of viral promoters, some of them being close to the E1 binding site. It has been reported that the E1–E2 complex is able to bind to both types of sequences (Mohr et al., 1991; Yang et al., 1991). In order to check a possible effect of E1 or E2 specific DNA binding on E1 ATPase activity, we prepared large amounts of a 200 nucleotide BPV-1 subgenomic dsDNA fragment (nt 7841–100) spanning the BPV-1 minimal ori (Ustav et al., 1993) and containing the E1 binding site, a flanking low-affinity E2 binding site, and a second E2 responsive element recognized with high affinity (BS11 and BS12; Li et al., 1990). As shown in Fig. 5, the addition of stoichiometric quantities of this DNA fragment relative to E1 protein under assay resulted in a slight stimulation of ATPase activity (1.5-fold higher compared to activity measured in the absence of any DNA). Surprisingly, this stimulation was no longer observed with excess DNA. Addition to the assays of a control DNA fragment of similar size did not produce any effect. The same small enhancement of E1 ATPase activity by the ori DNA fragment was observed at low and high ATP concentrations; this suggests that this small increase does not correspond to important changes in the affinity of the enzyme towards ATP, but merely to the stabilization of E1 molecules in an active form due to specific binding to DNA.

Kinetics of E1 ATPase activity

In order to fully characterize the effect of DNA on E1 ATPase activity, we performed enzyme assays over a wide range of ATP concentrations, either in the presence or absence of M13 ssDNA. The assays were done in parallel on both E1 and E1–E2 preparations, so as not to miss any modulation of the activity by E2 binding. Varying ATP concentration affected hydrolysis rates in a non-Michaelian way. When plotted on Hill’s coordinates (Fig. 6), the values obtained both for E1 or E1–E2 ATPase were an approximate fit to parallel straight lines with a slope of less than 0.8. This should indicate an anti-cooperative behaviour of E1-associated enzyme. However, imprecision of the assays at high ATP concentrations, where hydrolysis rates became close to background values, rendered the estimation of $V_{max}$ values difficult, especially because the data were also consistent with biphasic response curves, with a transition zone between 10 and 100 $\mu$M-ATP. Bisphasic behaviour, also seen when plotting the data by the
Eadie–Hofstee representation (not shown), might be explained by contamination of E1 ATPase preparations by another irrelevant enzyme activity of lower affinity towards ATP, and thus detected mainly at high ATP concentrations. However, the close similarity between ATPase plots for E1 and E1–E2 materials, prepared by very different extraction/purification procedures, rendered this hypothesis unlikely. In the same way, when assaying bacterially made E1 (as shown in Fig. 3) we found almost constant ATP hydrolysis rates up to substrate concentrations as high as 125 μM-dATP; this result implies still higher $K_m$ values, thus excluding the hypothesis of a contaminant accounting for the upper part of the response curves. By thus considering each of the four sets of plots present in Fig. 6 as resulting from a unique enzymatic activity, we extrapolated $K_{cat}$ and $K_m$ values. In the absence of DNA, the values were very close to each other for E1 and E1–E2 enzymes (3 and 5 min$^{-1}$, and 0.75 mm-ATP for both). As seen in Fig. 6, the presence of M13 ssDNA slightly improved the affinity for ATP, but only by 2-fold without changing the $V_{max}$. Despite some imprecision in the data, the value we calculated for the E1 ATPase catalytic constant is low, but well in the range of that reported for SV40 large T antigen (1.8 min$^{-1}$; Clark et al., 1981; Clertant et al., 1984).

**Discussion**

We have confirmed in this work that the BPV-1 E1 protein possesses an intrinsic ATPase activity, by demonstrating similar levels of specific activity in various preparations of E1 or E1–E2 proteins, including bacterially made molecules. Importantly, this activity was detected without addition of any nucleic acid to the assays. Although it could still be argued that our baculovirus-expressed E1 preparations contain trace amounts of stimulating DNA (but at levels lower than one DNA binding site for every 10 E1 molecules in immunoprecipitated material), this is not the case for affinity purified GST–E1 fusion protein (Fig. 4). By using the very sensitive technique of polynucleotide kinase 5’ end-labelling, we were able to detect only small RNA contaminants, present at low levels in the material bound by glutathione–Sepharose beads (less than 0.05 pmol 5’ ends/pmol GST–E1); moreover, being present in both GST and GST–E1 preparations, these contaminants were not specifically associated with the E1 protein. Our results are thus consistent with recent observations made on HPV E1 proteins (Bream et al., 1993; Hughes & Romanos, 1993), but are at variance with a previous report on BPV E1 (Seo et al., 1993a). This discrepancy might be explained in two ways: (i) by using different conditions of cell lysis and extraction and a complex fractionation procedure, Seo et al. might have purified a subpopulation of E1 molecules with peculiar properties; (ii) by using a longer purification scheme, they might have prepared a partially denatured E1 protein, able to recover activity by binding to ssDNA or during incubations for replication assays. Indeed, E1 behaves in our purification schemes as an unstable protein. By using rapid cell fractionation followed by only one MonoQ chromatographic step, we prepared partially purified baculovirus-expressed E1, which was found to be very active in an *in vitro* BPV DNA replication system, exhibiting a replicative efficiency comparable to that of SV40 T antigen (Bonne-Andréa et al., 1995a); however, we almost completely lost any replicative activity when further purifying E1 through a second chromatographic step. ATPase activity behaved more stably, remaining unchanged after the second column (data not shown). Conversely, as already mentioned, we observed that the ATPase activity of bacterially produced E1 purified almost to homogeneity by factor Xa cleavage was also very unstable. Once lost, however, the addition of M13 ssDNA did not help to recover E1 ATPase (not shown), an observation which is not consistent with one of the above hypotheses.

E1 ATPase activity was stimulated by DNA, but only in its single-stranded form and to a small extent, its affinity towards ATP being increased by a factor of only 2-5. No other variation could be observed for E1 ATPase activity, when exploring the effect of its binding either to its viral molecular partner, E2 protein, or to its DNA target at the replication origin on the viral genome. For bacterially expressed E1 protein ATPase activity was also found, with a specific activity close to that of its eukaryotic counterpart, but in this case it was not stimulated by the presence of M13 DNA, as discussed below. The weakness or even the absence of stimulation of E1 ATPase activity by ssDNA is a characteristic shared with large T antigen (see Table 3): for instance, while for SV40 large T antigen ATPase activity was stimulated, but only by poly(dT), to a maximum of 4-fold, in the case of polyomavirus no stimulation could be observed in the presence of various nucleic acids, including M13 ssDNA and poly(dT) (Gaudray et al., 1980; P. Gaudray & P. Clertant, unpublished data). Another important similarity with large T antigen is the lack of any effect on ATPase activity of the presence of ori DNA sequences to which the initiator protein binds specifically (Clark et al., 1980; Gaudray et al., 1980).

ATPase activity was observed in the case of bacterially expressed E1, with characteristics similar to those found for the protein produced in insect cells. This implies that E1 ATPase activity is not affected by post-translational modifications specific to eukaryotic cells. The E1 molecule is subject to phosphorylation on several sites,
Table 3. Characteristics of E1 and large T antigen ATPases*

<table>
<thead>
<tr>
<th>Virus</th>
<th>SV40 large T</th>
<th>Polymavirus large T</th>
<th>BPV-1 E1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affinity, $K_m$ (μM)</td>
<td>5</td>
<td>150</td>
<td>750</td>
</tr>
<tr>
<td>Turn-over number, $K_{cat}$ (s⁻¹)</td>
<td>0.03</td>
<td>4</td>
<td>0.05 (0.09)</td>
</tr>
<tr>
<td>Catalytic efficiency, $V_{max}/K_{cat}$ (M⁻¹ s⁻¹)</td>
<td>6000</td>
<td>26000</td>
<td>70 (120)</td>
</tr>
<tr>
<td>Hill number</td>
<td>~1</td>
<td>~1</td>
<td>≤ 0.8</td>
</tr>
<tr>
<td>Stimulation by nucleic acids:</td>
<td>Yes</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Large T kinetic parameters, determined in the absence of nucleic acids, and T ATPase stimulation by DNA were as described previously (Clark et al., 1981; Clertant et al., 1984; Giachero & Hager, 1979; P. Gaudray & P. Clertant, unpublished observations). E1 data are derived from the experiment in Fig. 6; values in parentheses are for the E1-E2 complex, when they differed from those for uncomplexed E1.

located on both its C- and N-terminal halves (Santucci et al., 1990; Sun et al., 1990; Lusky & Fontane, 1991); these phosphorylations thus do not appear to be involved in modulating E1 ATPase activity. This is one more similarity with SV40 large T antigen: in both cases, the same value for ATPase specific activity was found for proteins produced in either bacteria or in eukaryotic cells (Mohr et al., 1987, 1989; McVey et al., 1989). A similar observation was made for large T helicase (Mohr et al., 1989), and more recently for E1 helicase activities (Müller et al., 1994). The ATPase activity of bacterially expressed E1 is, however, peculiar, in not being stimulated by ssDNA. The difference with its counterpart produced in eukaryotic cells might be explained by a change in the ability to bind ssDNA: ssDNA binding, known to occur with E1 protein translated in reticulocyte lysates (Santucci et al., 1990), might be affected by post-translational modifications. In the case of SV40 T antigen, the question of a possible lack of an effect of DNA on underphosphorylated large T ATPase activity has not been documented; as already mentioned, variations in the phosphorylation state of the protein affect its DNA-binding capacity, but not its ATPase activity (Mohr et al., 1987, 1989; McVey et al., 1989). It is also known that the same molecular domain of large T is involved both in nonspecific binding to DNA and in specific recognition of ori sequences (Simmons et al., 1990); altogether this suggests that the stimulation of large T ATPase by ssDNA might also depend on the phosphorylation state of the protein.

When comparing the replicative efficiencies of bacterially expressed large T antigen and E1 protein, a difference can be noted. A bacterially produced E1 protein, expressed by the same GST–E1 fusion gene, was recently reported to be active in in vitro replication systems, even when using reconstituted mixtures of purified replication proteins (Müller et al., 1994). Post-translational modifications of the E1 molecule specific to eukaryotic cells thus do not affect its basic ATPase activity nor its replicative activity in in vitro systems. On the contrary, bacterially produced large T is known to be 10-fold less active in an in vitro replication system than its eukaryotic counterpart (Mohr et al., 1989). This might well be due to differences in the way phosphorylations might affect other functions of E1 and large T in initiating DNA replication, such as the recognition of ori DNA (McVey et al., 1989). Unvarying ATPase activity would, on the contrary, reflect the fact that in both cases ATP hydrolysis is catalysed by a separate molecular domain, kept free of any influence from other parts of the molecule.

As initiators of viral DNA replication, SV40/ polyomavirus large T antigen and papillomavirus E1 proteins are believed to start DNA synthesis by similar mechanisms. Both bind the origin of replication on the viral genome, but mechanisms underlying this property, such as the dependence upon ATP or the role of E2 protein, are still unclear and may differ in certain respects. In addition to amino acid sequence homology within similar ATP-binding domains (Clertant & Seif, 1984; Bradley et al., 1987; Sun et al., 1990; Bream et al., 1993), these proteins share ATPase activity with a number of characteristics in common (also summarized in Table 3). (i) The ATPase is linked to DNA helicase activity, whose function requires ATP hydrolysis for melting the DNA duplex by moving along in the 3' → 5' direction, thus being able to act during DNA replication as a leading-strand helicase (Wiekowski et al., 1988; Seo et al., 1993a; Yang et al., 1993). (ii) ATPase activity is, however, poorly stimulated by the presence of helicase substrate DNAs, in contrast to most known eukaryotic DNA helicases, whose ATPase activity is either undetectable in the absence of nucleic acids (see for instance helicase $\alpha$: Seo et al., 1991), or stimulated more than 10-fold by addition of ssDNA (as is helicase $\alpha$: Seo & Hurwitz, 1993). (iii) ATPase activity is catalysed by an autonomous molecular domain, acting in a manner totally independent of the rest of the molecule, being, for instance, unaffected by ori recognition (conversely, however, ATP binding modulates ori recognition: Borowiec et al., 1990). (iv) ATPase activity is very slow, hydrolysing less than 5 ATP molecules /min/ molecule of monomeric protein. Another possible similarity between E1 and large T ATPase might be its active quaternary structure. Both monomeric and hexameric forms of large T antigen have been reported to exhibit ATPase activity, to the same specific activity (Dean et al.,
One of the characteristics shared by T antigen and E1 ATPases, i.e. their low turn-over number, raises an intriguing question about the requirement of ATP hydrolysis for fork progression during DNA replication. When comparing SV40 large T antigen ATPase to helicase activity (Wiekowski et al., 1988), it is striking that it melts DNA in a processive manner at a much greater rate than it hydrolyses ATP (respectively more than 75 bp DNA melted/min/fork, compared to less than 2 ATP molecules hydrolysed/min/T monomer: see Table 3). One has to take in account, however, the fact that T helicase activity is probably catalysed by T antigen hexamers, since it has been shown that partially duplex oligonucleotides, devised to provide an efficient helicase substrate and thus behaving as synthetic replication forks, bind T hexamers with a probable ratio of one hexamer per fork (SenGupta & Borowiec, 1992). This increases the rate of ATP hydrolysis, up to a maximum of 12 ATP molecules/min/fork, thus implying that T helicase melts at least 6 bp DNA for every ATP molecule hydrolysed. Nothing is yet known about how large T antigen uses ATP hydrolysis to move along and melt DNA; as an explanation for the coupling of slow ATPase with efficient DNA melting, one may imagine that ATP hydrolysis is required just in order to induce a conformational change in the large T molecule, resulting in specific sliding of T hexamer bound to the replication fork along the leading strand, so as to melt the DNA duplex ahead the fork. Obviously, similar studies have to be performed with the E1 protein, before ascertaining the generality of such a model for viral DNA helicase initiators.

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