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Expression of largest RNA segment and synthesis of VP1 protein of bluetongue virus in insect cells by recombinant baculovirus: association of VP1 protein with RNA polymerase activity

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Abstract

The bluetongue virus core particles have been shown to contain an RNA-directed RNA polymerase (1). To identify the protein responsible for the virion RNA polymerase activity, the complete 3.9 Kb DNA clone representing the largest RNA segment 1 (L1) of bluetongue virus (BTV-10) was placed under control of the polyhedrin promoter of *Autographa californica* nuclear polyhedrosis virus (AcNPV). The derived recombinant virus was used to infect *Spodoptera frugiperda* cells. As demonstrated by stained polyacrylamide gel electrophoresis and by the use of bluetongue virus antibody, infected insect cells synthesized the largest protein of BTV-10 (VP1, 150 k Da). Antibody raised in rabbit to recombinant VP1 protein recognized bluetongue virus VP1 protein. The recombinant virus infected cell lysate had significantly inducible levels of RNA polymerase enzymatic activity as determined by a poly (U)-oligo (A) polymerase assay. The availability of enzymatically active bluetongue virus RNA polymerase provides a system in which we can precisely delineate the role this protein plays in the regulation of bluetongue replication.

INTRODUCTION

The nucleocapsid of bluetongue virus (BTV) is an icosahedral core particle composed of two major proteins (VP3 and VP7), three minor proteins (VP1, VP4 and VP6) and 10 double-stranded RNA genome segments (2,3). The purified nucleocapsid was shown to contain an RNA-directed RNA polymerase (transcriptase) (4) although the protein or proteins that constitute the viral transcriptase have not been identified. However, the location, size and molar ratio of the VP1 protein in the virus particle, as well as our recent prediction of the protein sequence, indicate that the VP1 protein is the most likely candidate polymerase of the virion (5). Since determination of the nature of RNA polymerase is essential for understanding the viral infection process and in order to establish any direct relationship of VP1 protein with virion polymerase activity we have expressed BTV-10 RNA segment L1 gene (encoding VP1 protein) in a baculovirus expression vector. In our previous reports we have demonstrated that the biologically active bluetongue viral proteins of the correct molecular weights could be obtained using these expression vectors (6,7,8). In this report

we present data indicating that the biologically active VP1 protein can be synthesized at a high level using the same expression system and that the largest BTV protein is indeed able to catalyze the polymerization reaction *in vitro*.

METHODS

In order to obtain a complete BTV-10 L1 gene, two overlapping L1 DNA clones representing nucleotides 1-2105 (pD105) and 1821-3944 (pSB5) were manipulated as illustrated in Fig. 1 (9,5). Both clones were digested with *Mlu*I and *Nhe*I and the fragments of plasmid DNAs containing L1 sequences were recovered followed by ligation as shown in Fig. 1. After transformation of *Escherichia coli* MC1061 cells, with pBR322 plasmid containing the complete L1 sequence, drug-resistant colonies were screened using nick-translated products of L1 DNA derived from clones pD105 and pSB5 (10). After identification, clone pBTV10-1 was confirmed by the appropriate restriction enzyme and sequence analyses (11) to contain BTV-10 L1 residues 1-3944.

To isolate the complete L1 DNA fragment from pBR322, the plasmid pBTV 10-1 was digested with *Pst*I, electrophoresed and the fragment was recovered by electroelution. Since extra dCdG and dAdT sequences had been introduced in the termini during the cloning process (5), *Bal* 31 exonuclease was used to eliminate these terminal sequences. Finally the entire coding region of L1 DNA including 3 bases upstream from the ATG initiation codon and 24 bases downstream from the TAG stop codon was ligated to the symmetrical polylinker of the plasmid pUC 4K (see Fig. 1; 12).

The coding sequence of BTV-10 L1 DNA was then inserted into the *Bam* HI site of the transfer vector pAcYM1 as described previously (Fig. 1; 6,7,11,12,13). The derived recombinant plasmid (pAcDT10-1) was analysed by restriction endonuclease digestion and the junction sequences determined (Fig. 1; 11). *S. frugiperda* cells were transfected with mixtures of wild-type infectious AcNPV DNA and the plasmid DNA pAcDT10-1. Recombinant viruses (e.g., AcBTV 10-1) were identified by their polyhedrin-negative plaque phenotypes and plaque purified three times on monolayers of *S. frugiperda* cells.

In order to demonstrate that BTV-10 VP1 protein was synthesized in recombinant baculovirus infected cells, *S. frugiperda* cells in 35 mm tissue culture dishes were infected with recombinant virus at a multiplicity of 5 PFU/cell and the cells incubated at 28°C for 3 days. At the end of the incubation period, cells were rinsed three times with phosphate buffered saline (PBS) and resuspended in 100 µl of 10 mM Tris-HCl buffer (pH 7.4). Extracts were also made from wild-type AcNPV and mock-infected cells as well as BTV-10 infected BHK-21 cells. After

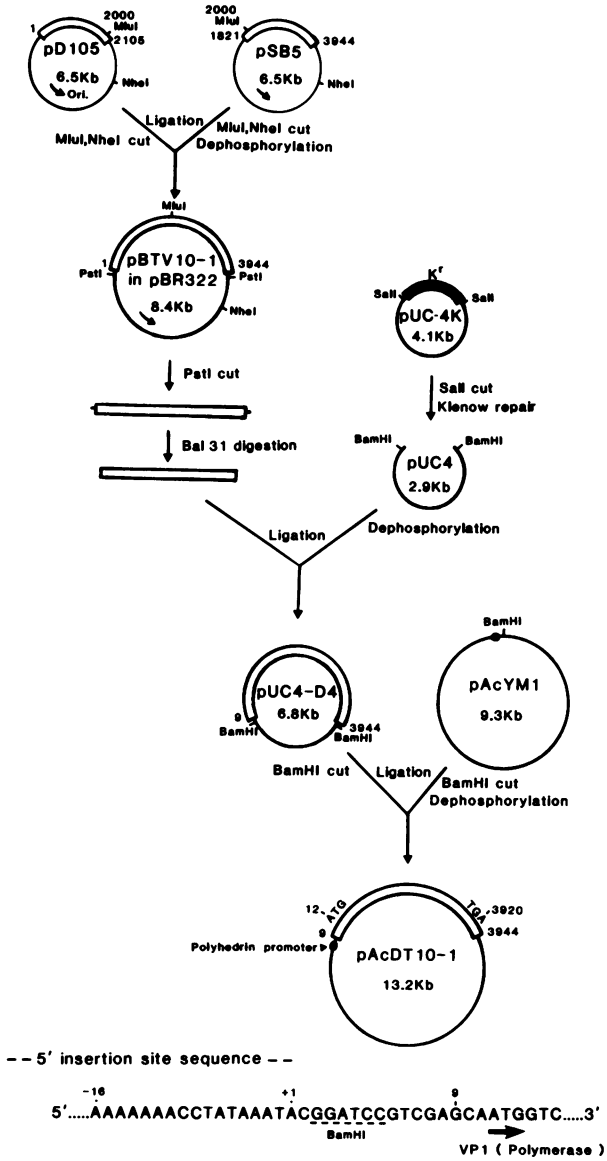

Fig. 1 Construction of transfer vector: A plasmid (pBTV10-1) containing the full length segment 1 of BTV-10 DNA (1-3944 bp) was constructed using two partial clones, pD105 (1 to 2105bp) and pSB5 (1821-3944 bp) as described in the text. After removing the homopolymeric tails (e.g., A and C) with exonuclease *Bal* 31, the *Pst*1 excised fragment (3.9 kb) was subcloned into pUC4K subcloning vector. A candidate plasmid, pUC4-D4 containing BTV segment 1, (9-3944 bp), was then ligated to the *BamH*1 site of pAcYM1 vector as described in the text. Subsequently, a recombinant transfer vector (pAcDT10-1) containing segment 1 DNA of BTV-10 was selected.

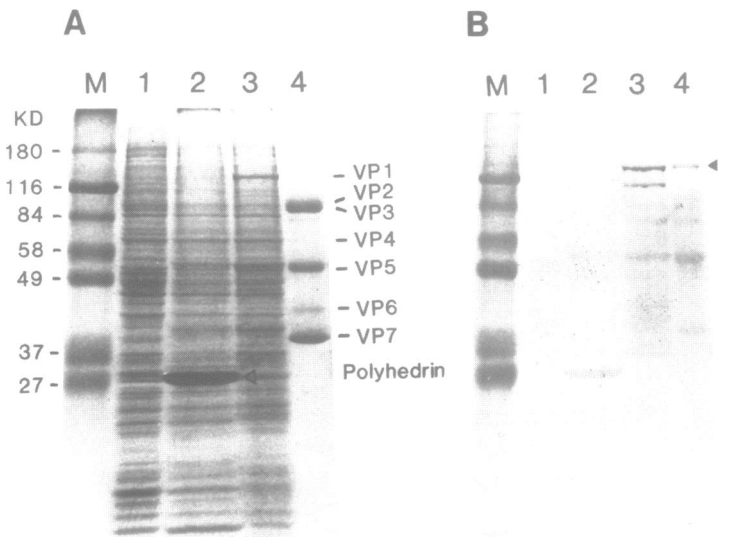

Fig. 2 Expression of VP1 in *S. frugiperda* cells by recombinant baculovirus derived from pAc DT10-1 transfer vector: Protein samples were resolved by 10-20% linear gradient SDS-PAGE and stained with Coomassie brilliant blue (A). Blotted immobilon transfer membrane was reacted with anti-VP1 monospecific rabbit antibodies using an alkaline phosphatase conjugate detection procedure (Western blot, B). M; standard protein markers, 1; mock infected *S. frugiperda* cell lysate, 2: wild type AcNPV infected *S. frugiperda* cell lysate, 3; AcBTV 10-1 infected *S. frugiperda* cell lysate, 4; purified United States prototype BTV 10 virions (17)..

adding the protein dissociation buffer, followed by heating at 100°C for 10 minutes, an aliquot of each preparation was analysed by electrophoresis in a 10-20% linear gradient polyacrylamide gel (14). Prestained protein markers (Sigma Co.) were included as molecular weight standards.

RESULTS

As shown in Fig. 2A (lane 3), the recombinant virus synthesized a protein with a molecular size of *ca* 150 kD, in agreement with the estimated size (149,588 D) of the BTV-10 VP1 protein (5,2) and migrated to the same position as VP1 protein of purified BTV-10 virions (Lane 4).

In order to confirm that the 150 kD protein was VP1, a sample of each protein extract from the above preparations were electrophoresed, transferred onto a Immobilon membrane (Millipore Co.) and subjected to Western analyses using anti-VP1 rabbit monospecific serum. To raise the monospecific antiserum cell lysate of AcBTV 10-1 recombinant virus infected cells was resolved by 10%

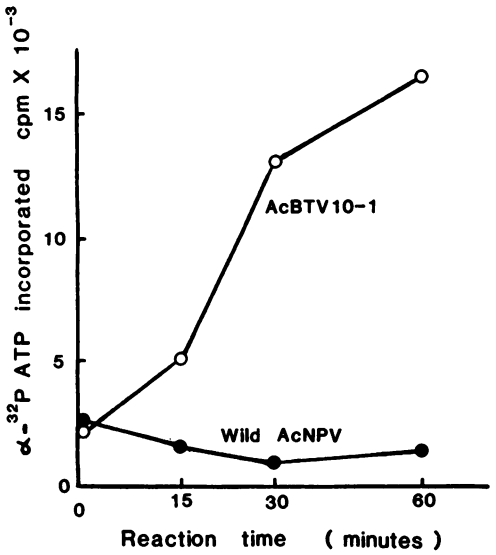

Fig. 3 Polymerase activity of a recombinant baculovirus containing VP1 protein of BTV-10: In a standard reaction, a 25 µl aliquot of supernatant recovered from infected cell lysates was assayed in 200 µl of a solution containing 50 mM Hepes buffer (pH 8.0), 8mM Mg $(CH_3COO)_2$, poly (U) (20 µg/ml), oligo (A)12-18 (10 µg/ml), 32Pα-ATP 400 Ci/mM, 4 mM phosphoenolpyruvic acid and pyruvate kinase (3u/ml). The assay was at 37°C for various times. The enzyme activity was measured as the amount of labelled product collected on 0.45 µm membrane filter after precipitation by 7% TCA with 10 µg of carrier tRNA and 0.2 M sodium pyrophosphate and dried. The radioactivity was determined as Čerenkov radioactivity using a scintillation counter. Poly (A) polymerase activity was tested in the recombinant virus infected cell lysate (o) or in the wild-type AcNPV-infeced cell lysate (●).

SDS-PAGE and the VP1 band identified by 0.25 M KCl precipitation of the SDS-protein complexes as described by Hager and Burgess (1980; 15). The VP1 protein band was excised and used to immunize a rabbit. IgG was purified from the crude serum on a protein A-sepharose CL-4B column after absorbing the rabbit serum with acetone powder of *S. frugiperda* cells. As shown in Fig. 2B by Western blot analysis, anti-VP1 antibodies recognized mainly the VP1 protein of bluetongue virus (lane 4) and the expressed VP1 protein of the recombinant virus (lane 3). However, a second minor protein (ca. 100 Kd) was also identified along with the expressed VP1 protein (lane 3). This protein which was not analysed further and was probably a degraded form of VP1. The other faint bands visualized in lanes 2, 3 and 4 were non-specific, since they were also reacted with the control rabbit serum (data not shown).

To test whether the VP1 protein is indeed the virion polymerase enzyme,

recombinant cell lysate containing BTV VP1 protein was tested for polymerase activity in an *in vitro* assay as described by Flanagan and Baltimore (16). Supernatant (30% of expressed VP1 was solubilized) of the infected cell lysate (25 µl) was mixed with 200 µl reaction mixture containing poly (U) (20 µg/ml) as a template, oligo $(A)_{12-18}$ (10 µg/ml) as a primer and ^{32}P-α-ATP (400 Ci/mM) as the labelled nucleotide and incubated at 37°C for various times. Aliquots of extract-reaction mixture were assayed for trichloroacetic acid precipitable material. The *in vitro* synthesized product was precipitated by 7% trichloroacetate acid with 10 µg of carrier tRNA in 0.2 M sodium pyrophosphate and collected on 0.45 µm Millipore HA filter; radioactivity was determined in a scintillation counter. The results of this experiment are illustrated in Fig. 3. It was evident that ^{32}P-α-ATP was incorporated into the acid precipitatable products by the reaction mixtures containing the recombinant virus infected cell lysates. In contrast, reaction mixtures containing the mock-infected (data not shown) or AcNPV- infected cell lysates showed little or no ^{32}P-labelled products. Thus, we believe that the activity detected from extracts of the recombinant lysate specifically results from the expression of an enzymatically active BTV RNA polymerase. No activity was detected in the absence of the primer or the template (data not shown) indicating that both a template and a primer are necessary for the polymerase activity.

DISCUSSION

The purpose of this study was to develop a system in which we could identify the bluetongue virus protein that is involved in virion polymerase activity. The experiments described here demonstrated that not only can the largest viral encoded polypeptide be expressed at a high level by recombinant baculovirus but also that the expressed protein is enzymatically active and capable of inducing the RNA polymerase activity *in vitro*. Currently we are in the process of purifying the VP1 protein and developing systems to establish whether VP1 protein is associated with other enzymatic activities (e.g., capping and/or methylation of the mRNA etc.). The expression of enzymatically active BTV RNA polymerase in AcNPV provides a foundation for further experimentation on the mechanisms of BTV replication. Since the replication of the genome of members of the Reoviridae appear to involve two distinct processes, transcription of the dsRNA genome segments to mRNA copies and the replication of mRNA segments to negative sense ssRNA segments, VP1 protein may contain both activities as alternative functions. Further studies involving BTV dsRNA and ssRNA templates as well as other minor nucleocapsid proteins (e.g., VP4 and VP6) are in progress.

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