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Development of baculovirus triple and quadruple expression vectors: co-expression of three or four bluetongue virus proteins and the synthesis of bluetongue virus-like particles in insect cells

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ABSTRACT

Baculovirus multiple gene transfer vectors pAcAB3 and pAcAB4 have been developed to facilitate the insertion of three or four foreign genes respectively into the *Autographa californica* nuclear polyhedrosis virus (AcNPV) genome by a single co-transfection experiment. The pAcAB3 vector contains a polyhedrin promoter and two p10 promoters on either side of the polyhedrin promoter but in opposite orientations. The pAcAB4 vector has an additional polyhedrin promoter in opposite orientation to the first copy that is in juxtaposition to the first p10 promoter. Each of these derived vectors (pAcAB3, pAcAB4) have been used for the simultaneous expression of three or four bluetongue virus (BTV) genes respectively. When *Spodoptera frugiperda* cells were infected with the recombinant virus (AcBT-3/2/7/5) expressing the four major structural genes of BTV, double-capsid, virus-like particles consisting of VP2, VP3, VP5 and VP7 of BTV were assembled.

INTRODUCTION

Recent advances in gene manipulation have made it possible to express foreign genes in heterologous systems. In general, recombinant gene expression has two particular goals: (i) the efficient production of large amounts of authentic protein products (e.g., for diagnostic or therapeutic use, and (ii) the study of the biological functions of the expressed products. The productivity and flexibility of baculovirus expression vectors (1, 2) and the ability of the baculovirus genome to incorporate (and express) large amounts of foreign DNA have permitted this system to be used for both single and dual gene expression (3, 4, 5). To accomplish this, several expression vectors have been developed based on the resident promoters of *Autographa californica* nuclear polyhedrosis virus. One which has found particular favour is the strong promoter of a so-called 'very late' polyhedrin gene (*ph*). The promoter of the *ph* gene has been exploited to express one or two foreign gene products either by replacing the *ph* gene or by duplicating the promoter together with appropriate transcription termination signals (see review, Bishop, 1992) (2). A second very late gene, p10, which is responsible for the synthesis of a non-structural protein, has been successfully utilized to express foreign proteins either by replacing the resident p10 gene (5, 6) or by duplicating the p10 promoter and inserting it elsewhere in the AcNPV genome, eg, in proximity to the *ph* gene site (4).

The synthesis of complex structures such as viruses often requires the synthesis and interaction of several gene products. For example, for bluetongue virus (Orbivirus, Reoviridae) seven viral proteins are incorporated into virions. Of these, three are minor components (VP1, VP4, VP6) that together with the ten double stranded RNA segments of the virus genome are enclosed within the core which is composed of two major proteins (VP3, VP7), that in turn is enclosed by the two outer virion proteins (VP2, VP5) to form the complete virus particle (7, 8).

Recently, we have reported the construction of a dual recombinant baculovirus containing the gene sequences coding for VP3 and VP7 (9). Insect cells infected with this recombinant were shown to assemble core-like particles (CLPs) composed of VP3 and VP7 alone, which were identical in size and shape to authentic BTV cores. In further studies, a second dual recombinant baculovirus was constructed to express VP2 and VP5 (10). When insect cells were co-infected with these two dual recombinants, all four proteins were synthesized, and morphological structures resembling BTV particles, double-shelled virus-like particles (VLPs) were recovered (10).

In this report, we describe the construction of triple (pAcAB3) and quadruple (pAcAB4) expression vectors utilizing p10 and polyhedrin promoters. Each pair of identical promoters were placed in opposite orientation to the other copy and in segregated

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fashion to minimize the possible gene elimination by recombination between repeated sequences. The vectors were utilized to express bluetongue virus proteins. Using the quadruple vector, double shelled VLPs consisting of four proteins were synthesized.

METHODS

Viruses and cells

Spodoptera frugiperda cells (IPLB-SF21), AcNPV and recombinant viruses were propagated as described previously (11, 12).

Construction of plasmid transfer vectors

Standard procedures were used for plasmid DNA manipulations (13). For the construction of multiple transfer vectors, the *Ppu*MI–*Bgl*II fragment of AcNPV, containing 114 bp of the p10 promoter and the *Eco*RV–*Bam*HI fragment containing 95 bp of the *ph* promoter of AcNPV were purified from pAcUW3 and pAcYM1 vectors respectively (4, 14). Subsequently, a fragment containing both p10 and *ph* promoters in juxtaposition was generated by ligating the two fragments using a *Ppu*MI-blunt end adapter. This fragment was then religated into the *Xba*I site of pAcUW3 dual vector using *Xba*I–*Spe*I–*Sma*I–*Bam*HI and *Xba*I–*Stu*I–*Xba*I–*Bam*HI adapters (Figure 1). These manipulations produced a vector designated pAcAB4 containing duplicated copies of p10 and *ph* promoters in both orientations. For construction of a triple transfer vector, pAcAB3, one of the two *ph* promoters of pACAB4 was excised using SmaI and BamHI enzymes, and followed by religation using a *Sma*I–*Bam*HI-linker.

Construction and purification of recombinant viruses

For construction of recombinant transfer vectors, various BTV genes were selected. Each BTV DNA fragment was excised by digesting with *Bam*HI enzyme from the single transfer vectors described previously (15–19). Each fragment was directly cloned into the *Bam*HI or *Bgl*II sites of the triple or quadruple vectors. For insertion into other sites (eg, *Xba*I/*Stu*I or *Spe*I/*Sma*I) each fragment was first subcloned into the *Bam*HI site of the multiple cloning vector pUC19 and subsequently isolated as a *Xba*I–*Sma*I fragment. The transfer vectors containing BTV genes cloned in pAcAB3 and pAcAB4 vectors were co-transfected with *Bsu36*I cut BacPAK6 DNA, white plaques were selected and purified by two sequential plaque assays (20).

SDS–polyacrylamide gel electrophoresis

S.frugiperda cells were infected at a multiplicity of 10 PFU per cell either with a recombinant baculovirus or with wild-type AcNPV. Cells were harvested at 48 h post-infection, washed with phosphate-buffered saline, and lysed at 4°C in 50 mM Tris hydrochloride (pH 8.0)–150 mM NaCl–0.5% Nonidet P-40. Protein dissociation buffer (10% β-mercaptoethanol, 10% sodium dodecyl sulfate [SDS], 25% glycerol, 10 mM Tris hydrochloride [pH 6.8], 0.02% bromophenol blue) was added to each sample, and the mixture was incubated at 100°C for 5 min. Proteins were resolved by SDS–10% polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie brilliant blue.

Purification of assembled BTV particles from insect cells

The expressed virus-like particles (VLPs) were purified by lysing infected cells as described above followed by centrifugation on a discontinuous sucrose gradient 30%:50% [wt/vol] in 0.2 M Tris hydrochloride [pH 8.0] at 85,000 *g* for 3 h. The particles were analyzed by SDS–PAGE (as above) or were examined by electron microscopy.

Western immunoblot analysis

Proteins were separated by SDS–PAGE and electroblotted onto Immobilon-P (Millipore Corp., Bedford, MA) using a Sartorius semi-dry electroblotter. The BTV derived protein bands were identified by probing with BTV-10 antisera as described previously (10).

Immunoprecipitation

Purified VLPs and core-like particles (CLPs) were diluted to the concentration of 0.1 mg/ml with 50 mM Tris hydrochloride (pH 8.0) — 150 mM NaCl — 5 mM $MgCl_2$ — 0.5% Nonidet P-40, 6 μl of anti-VP2 monoclonal antibody was added to each 200 μl of VLPs or CLPs and incubated for 5 h at 5°C. To these samples, 20 μl of pre-swollen protein A–Sepharose beads were added and the mixture incubated for 1 h at room temperature. After centrifugation and three washes of the beads, proteins were solubilized by heating for 5 min in protein dissociation buffer, separated by 10% SDS–PAGE and detected by Western immunoblot analysis with rabbit anti-BTV-10 serum.

Electron microscopy

Expressed CLPs and VLPs were absorbed onto copper 400-mesh Formvar carbon-coated grids by floating on droplets of the samples for 2 min. After washing twice in 0.2 M Tris hydrochloride (pH 8.0), the particles were stained for 20 s on droplets of 2% uranyl acetate. All grids were examined in a JEOL electron microscope.

RESULTS

Construction of the triple and quadruple expression vectors and preparation of recombinant viruses

To insert three or four foreign genes simultaneously into the AcNPV genome the recombinant vectors pAcAB3 and pAcAB4 were constructed as described in Methods. Figure 1A shows the schematic organization of pAcAB3. Figure 1B shows the nucleotide sequence of its promoter cassette. This vector was designed to insert three foreign gene sequences into unique *Bam*HI–*Sma*I (p10 promoter), *Xba*I–*Stu*I (*ph* promoter), *Bgl*II (p10 promoter) sites. Figure 1 C and D show the general organization of pAcAB4 and the nucleotide sequence of its promoter cassette. In this vector foreign gene sequences can be inserted into unique *Bam*HI (*ph*), *Spe*I–*Sma*I (p10), *Xba*I–*Stu*I (*ph*), and *Bgl*II (p10) sites. These restriction enzyme sites were chosen to facilitate recloning DNA fragments from the pUC-based plasmids.

When BTV VP6, or VP7 or the non-structural NS1 protein was expressed by single gene recombinant baculoviruses, all three proteins were synthesized in abundance, reaching up to 50 to 70% of the total protein mass of the infected *S.frugiperda* cells (16, 17, 19). Therefore, the genes encoding these proteins were chosen for insertion into the triple gene transfer vector. Each DNA fragment was recovered from the corresponding single gene recombinant transfer vector and was either directly cloned into the pAcAB3 vector, or via subcloning into the multiple cloning vector pUC19 (as described in Methods). By this procedure the transfer vector pAcAB3. VP6/NS1/VP7 was constructed.

To express four proteins in the quadruple vector we selected the genes that were either expressed at a high level in insect cells (eg, VP2, VP6, VP7, NS1) by single expression vector systems or can be assembled into a morphological structure in a native configuration (10, 16, 17, 19). For high level expression VP2, VP6, VP7 and NS1 were isolated from the corresponding single gene recombinant expression vector and cloned into pAcAB4 and the transfer vector pAcAB4.VP2/VP6/NS1/VP7 was constructed. By a similar procedure a vector encoding the four major structural proteins of BTV, namely VP2, VP3, VP5 and VP7, pAcAB4.VP3/VP2/VP7/VP5 was constructed since these proteins can self-assemble into VLPs in insect cells (10). The

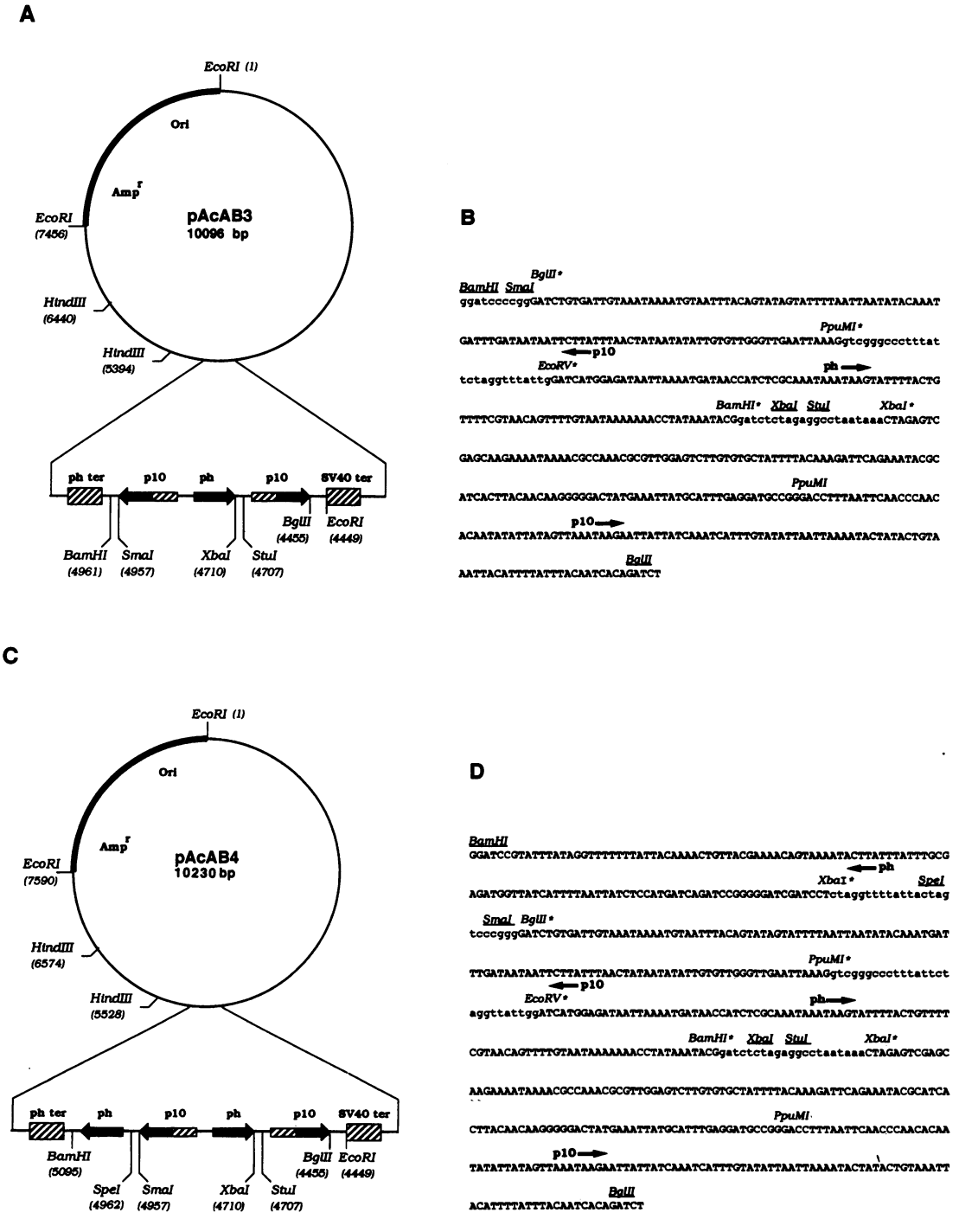

Figure 1. General organization and the DNA sequence of vectors pAcAB3 (A, B) and pAcAB4 (C, D). Black arrows represent promoters and indicate the direction of transcription. Hatched areas show potential transcription termination signals. The DNA sequences of the pAcAB3 (B) and pAcAB4 (D) promoter cassettes are shown below. The synthetic linker and adapter sequences are shown in small letters, sequences derived from parental pAcUW3 and pAcYM1 vectors are represented in capital letters. Arrows indicate the direction of transcription. Incomplete restriction sites produced on the junctions with linkers and adapters are indicated by enzymes with asterisks.

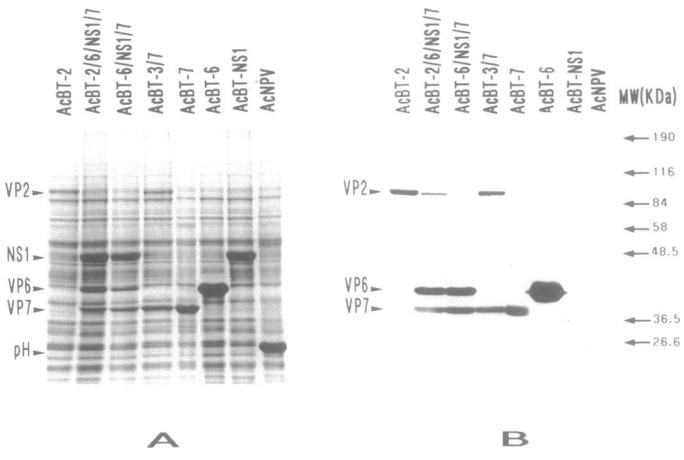

Figure 2. Expression of BTV proteins in insect cells by recombinant baculoviruses. *S.frugiperda* cells were infected at a multiplicity 10 PFU per cell with recombinant baculoviruses or wild-type AcNPV. Cells were harvested at 48 h post infection. Proteins were separated by 10% SDS−PAGE and stained with Coomassie brilliant blue (A) or electroblotted onto Immobilon-P membrane and reacted with rabbit antiserum raised to purified BTV-10 particles. Bound antibodies were detected with an alkaline phosphatase conjugate by standard methods. The serum contained antibodies to the virus proteins VP2, VP6 and VP7, but not to the non-structural protein NS1.

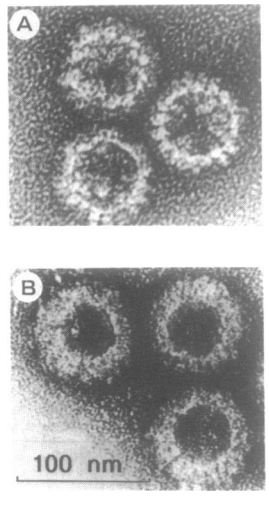

Figure 3. Electron micrographs of baculovirus-expressed particles. (A) Single-shelled CLPs composed of VP3 and VP7 were produced in insect cells infected with dual recombinant baculovirus expressing VP3 and VP7 (9). (B) Double-shelled VLPs consisting of VP2, VP3, VP5 and VP7 were produced in insect cells infected with quadruple recombinant baculovirus AcBT-3/2/5/7.

order of genes in these transfer vectors was the same as the order of cloning sites from left to right in pAcAB3 and pAcAB4 (Figure 1).

Spodoptera frugiperda cells were transfected with mixtures of each transfer vector and linearized BacPAK6, as described elsewhere (20, 21). Recombinant plaques with a white phenotype were isolated. Three recombinant viruses, designated AcBT-6/NS1/7, AcBT-2/6/NS1/7 and AcBT-3/2/7/5 were obtained from their respective vectors.

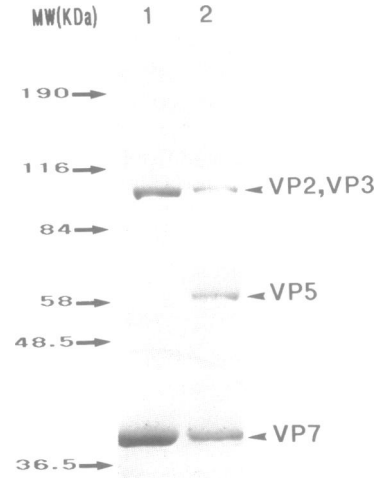

Figure 4. SDS−PAGE analysis of CLPs and VLPs. CLPs were obtained using dual recombinant baculovirus expressing BTV VP3 and VP7 proteins as described previously (9). VLPs were obtained using the quadruple recombinant baculovirus AcBT-3/2/5/7 as described in Methods. Lane 1, CLPs; Lane 2, VLPs. The positions of BTV VP2, VP3, VP5 and VP7 proteins are indicated.

Expression of BTV genes by triple and quadruple recombinant viruses

To assess whether these multiple recombinant baculoviruses synthesized BTV proteins in *S.frugiperda* cells, RNAs and mRNAs made in infected cells were identified by Northern Blot hybridization analysis (data not shown), and the proteins made in infected cells were analysed by SDS−PAGE as described in Methods. As shown in Figure 2A, cells infected with AcBT-6/NS1/7, synthesized three additional proteins with M_r of 64 kDa, 38.5 kDa and 36 kDa respectively *in lieu* of the 29 kDa polyhedrin protein synthesized in wild-type AcNPV-infected cells. All three proteins were expressed in significantly higher levels than the level of expression of any other cellular or AcNPV derived protein. AcBT-2/6/NS1/7 expressed BTV proteins although the level of VP2 expression was lower than the other three proteins as shown in Figure 2. Nevertheless, the presence of VP2 protein in the infected cells was identifed by Western analyses (see Figure 2B).

We have demonstrated previously that *S. frugiperda* cells, co-infected with two dual recombinant baculoviruses expressing VP2/VP5 and VP3/VP7 produce BTV-like particles consisting of the core which is formed by VP3 and VP7, and the outer shell which consists of VP2 and VP5 (10). To assess the ability of the quadruple gene expression vector to make virus-like particles, subconfluent monolayers of *S.frugiperda* insect cells were infected with the recombinant AcBT-2/3/7/5 virus, harvested 48 h post-infection, lysed with the nonionic detergent Nonident P-40, and released particles were purified on discontinuous sucrose gradients as described in Methods. When examined under the electron microscope, typical empty double-shelled particles consisting of a core surrounded by a thick outer capsid were observed (Figure 3). Contrary to experiments in which BTV-like particles are produced by co-infection of insect cells with two dual expressing viruses, all the produced particles appeared to be double-shelled VLPs and no CLPs were observed. When analysed by SDS−PAGE, the VLPs consisted of four major BTV

structural proteins (Figure 4). Since VP3 and VP2 co-migrated in this gel system, the presence of VP2 was confirmed by immuno-precipitating the VLPs with monoclonal VP2 antibody (data not shown).

The stability of triple and quadruple recombinant viruses

To determine whether recombinant viruses expressing multiple BTV proteins are genetically stable, each was passaged at high m.o.i. nine times successively. Fifty clones of each recombinant virus were isolated and individually propagated in *S.frugiperda* cells. Each isolate was analysed by SDS−PAGE and Western blot using BTV serum. In each case, the level of expression of all three or four proteins were equivalent to that of the original isolates (data not shown). To confirm the stability at the genetic level, both restriction endonuclease digestion and Southern blot analyses using ^{32}P-labelled probes to the individual BTV genes were performed for all three recombinant viruses after four passages. As in the case of protein profiles, the DNAs of these recombinant viruses revealed no signs of instability, indicating that the triple and quadruple recombinant viruses are stable at least for several passages.

DISCUSSION

This report describes the construction of triple, pAcAB3 and quadruple pAcAB4 transfer vectors, to accommodate the insertion of three or four foreign genes into the baculovirus genome in a single cotransfection experiment. To address the possibility that recombinant baculoviruses containing duplicated promoters might be unstable, we avoided the juxtaposition of identical promoters by arranging and segregating them as illustrated in Figure 1. The triple and quadruple recombinant baculoviruses proved to be stable for up to nine passages.

We have utilized both SV40 and polyhedrin terminators to terminate the transcription on both sides of the expression cassette. It has been demonstrated previously that p10 promoter is able to terminate upstream transcription (22, 23), and therefore, we have used p10 promoter to terminate transcription from the polyhedrin promoter in pAcAB3 vector. Thus, each promoter has a separate terminator. Using this transfer vector, three BTV proteins, VP6, VP7 and NS1, were expressed at high level. In pAcAB4 vector, three different terminators were employed for four promoters. In this vector, the left-hand polyhedrin (*Bam*HI site) and p10 (*Spe*I/*Sma*I sites) promoters shared the same polyhedrin terminator. We have compared the expression levels of VP6 and NS1 proteins in alternative sites by cloning one into the *Bam*HI site and the other in the *Spe*I/*Sma*I sites or vice versa. In each case the expression of the gene placed at the *Spe*I/*Sma*I site was significantly higher than that of the gene placed in the *Bam*HI site. In this context it is noteworthy that other investigators have previously indicated that over-running transcripts from an upstream promoter might down-regulate transcription from the downstream promoter (24, 25). Our data suggest that in the quadruple vector the p10 promoter probably makes transcripts that over-run and down-regulate the downstream *ph* promoter products. However, it will be necessary to confirm such a possiblily by inserting a transcription terminator between these two promoters, which we are currently investigating. Right-hand oriented p10 promoter (*Bgl*II site) in both triple and quadruple vectors provides strong expression despite the presence of the upstream *ph* promoter, probably because the former has sequences for termination of upstream transcription (22, 23, 26).

In the quadruple expression vector that made the 4 structural proteins of BTV, the weaker site was used for expression of VP3, since the molar proportion of VP3 in VLPs is much less than the other three major proteins of BTV (27, 28). BTV particles are calculated to be composed of 60 copies of VP3, 780 copies of VP7, 120 copies of VP5 and 180 copies of VP2. The recombinant baculovirus AcBT-3/2/7/5 made virtually homogenous VLPs. This is in contrast to the results from coinfection with two dual viruses AcBT-2/5 and AcBT-3/7 which produces mixtures of VLPs and CLPs (10). More efficient production of BTV VLPs will not only facilitate our ongoing research on defining the three-dimensional structure of the particles but also the use of VLPs as candidate vaccines against bluetongue disease of sheep (29).

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