Expression of Polyomavirus Large T Antigen by Using a Baculovirus Vector

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A gene encoding the large T antigen of polyomavirus was inserted into the baculovirus *Autographa californica* nuclear polyhedrosis virus so that gene expression was under the control of the strong, very late polyhedrin gene promoter. Significantly more large T antigen was produced in recombinant virus-infected insect cells than was observed in polyomavirus-transformed mouse cells. The insect-derived T antigen exhibited polyomavirus origin-specific DNA binding. The baculovirus expression system provides a convenient source of T antigen for in vitro studies.

Polyomavirus large T antigen (PyTag) is a specific DNA-binding protein (3-5, 24) which is involved in the regulation of viral gene expression (6, 8), the initiation of viral DNA replication (7, 20), and the oncogenic transformation of primary rat embryo cells (23). The protein is present in low quantities in both lytically infected and transformed cells. To obtain higher quantities of the protein for in vitro studies, helper-independent adenovirus vector systems have been developed which produce approximately fivefold more PyTag than do wild-type polyomavirus-infected cells (13, 14).

A helper-independent baculovirus vector system has been developed for the high-level expression of foreign genes in a eucaryotic cell environment (11, 16-19, 22, 27). It was of interest to determine whether this insect virus vector system can efficiently express biologically active PyTag. We now report that a baculovirus expression system is capable of providing sufficient quantities of biologically active T antigen for in vitro studies.

A recombinant baculovirus expressing the PyTag gene was constructed by allelic replacement of wild-type baculovirus polyhedrin gene sequences with sequences of a recombinant plasmid, pEV51LT, which was constructed as outlined in Fig. 1. An intronless construct of the PyTag gene was obtained from plasmid pspLT5 (28) by *Bam*HI and *Xho*I digestion, purified by gel electrophoresis, and inserted into *Kpn*I- and *Xho*I-digested pEV-51 (16), a plasmid which facilitates the insertion of foreign genes into the polyhedrin region of the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV). Cotransfection of the resulting plasmid pEV51LT with wild-type AcNPV DNA into a permissive host cell line, *Spodoptera frugiperda* IPLB-SF21, resulted in allelic replacement of the wild-type polyhedrin gene of AcNPV with the PyTag gene under the control of the abundantly expressed polyhedrin promoter.

Recombinant virus plaques were visually selected by their occlusion-negative phenotype (16, 25), resulting from polyhedrin replacement with the PyTag gene region of pspLT5. A stock of recombinant virus was developed, and the viral DNA was isolated as previously described (16). The

nature of the recombinant virus DNA was confirmed by digestion with *Eco*RI, *Pst*I, and *Eco*RI-*Pvu*II and by Southern blotting with labeled pEV51LT as a hybridization probe. The restriction map of the *Eco*RI-I region of the recombinant virus and the predicted nucleotide sequence of the leader region of the resulting fusion are shown at the bottom of Fig. 1. The *Bgl*II site of pEV-51 is positioned within the polyhedrin leader sequence 22 base pairs upstream of the polyhedrin translational start site (approximately 33 base pairs downstream of the 5' end of the transcriptional start site), and the *Xho*I site of the PyTag gene is 24 base pairs upstream of the initiating ATG of PyTag. The recombinant AcNPV is heretofore referred to as vEV51LT.

To monitor the synthesis of PyTag in vEV51LT-infected cells, *S. frugiperda* cells were infected with either wild-type or recombinant virus and pulse-labeled with [³⁵S]methionine at various times postinfection (p.i.). Proteins from infected cell lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The accumulation of polyhedrin protein is visible in wild-type-infected cells beginning 18 h p.i. by Coomassie brilliant blue staining of the gels (data not shown). Increased synthesis of this protein, beginning at 18 h and extending through 36 h p.i., is observed by use of autoradiography (Fig. 2). This is a normal pattern for polyhedrin synthesis (22). Polyhedrin synthesis is not observed in vEV51LT-infected cells (Fig. 2); another protein expressed earlier in infection comigrates in the 32-kilodalton (kDa) region of the gel (22). A 100-kDa protein is observed in both wild-type lanes and vEV51LT lanes at 12 h p.i. by autoradiography (Fig. 2), but a stronger signal is observed in the vEV51LT lanes from 18 through 36 h p.i. This observation is consistent with the interpretation that PyTag synthesis begins at 18 h in vEV51LT-infected cells and comigrates with another protein of similar size that decreases after 18 h. Unlike polyhedrin synthesis in wild-type (L-1)-infected cells, the 100-kDa protein does not accumulate through 36 h p.i., as indicated by the absence of significant Coomassie blue staining in this region (data not shown). In contrast to the expression usually noted for foreign genes expressed under polyhedrin promoter control, the amount of 100-kDa protein synthesized during the pulse is maximal at 18 h p.i. and declines through 36 h in vEV51LT-infected cells (Fig. 2).

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FIG. 1. Construction of the transplacement plasmid pEV51LT, which contains the PyTag gene under AcNPV polyhedrin promoter control, and a map of the *EcoRI*-I fragment of the recombinant baculovirus vEV51LT containing the PyTag gene. The PyTag gene (■) was removed from plasmid pspLT5 (28) and inserted (12) into the multicloning site (MCS) of pEV-51 (16). The resulting plasmid, pEV51LT, containing the PyTag gene in the appropriate orientation and position with respect to polyhedrin promoter control, was selected with ampicillin in transformed *Escherichia coli* JM83. To facilitate allelic replacement of pEV51LT sequences with the corresponding regions of homology in the AcNPV genome (□□□□), pEV51LT was digested with *Sfi*I and *Not*I before cotransfection with wild-type AcNPV DNA into *S. frugiperda* cells (16). Plaques with occlusion-negative phenotypes were selected and replaques, and virus stocks were prepared. The restriction pattern of the recombinant AcNPV *EcoRI*-I fragment (bottom of figure) and the predicted nucleotide sequence at the *Xho*I fusion site are presented. Kb, Kilobase pairs.

Correspondence of the 100-kDa protein with PyTag is shown by immuno precipitation of the protein from vEV51LT-infected but not from wild-type-infected 24-h cell lysates by using a polyclonal anti-PyTag antibody (Fig. 3). Cell lysates were prepared from monolayers of *S. frugiperda* cells (1.5×10^7 cells per 1,000-mm-diameter dish) which were infected at a multiplicity of infection of 20 with vEV51LT or wild-type (L-1) virus and pulse-labeled with [35 S]methionine (22) at 24 h p.i. The medium was removed

FIG. 2. Autoradiogram of proteins synthesized in recombinant and wild-type virus-infected *S. frugiperda* cells. Cells (10^8 per 35-mm-diameter dish) were infected (20 PFU per cell) with either recombinant vEV51LT or wild-type (L-1) AcNPV and pulse-labeled with [35 S]methionine at selected times p.i. as previously described (22). The cells were harvested, pelleted, and suspended in water, and an equal volume of solubilization solution (2% SDS-2% 2-mercaptoethanol-1 M urea) was added. The proteins were separated by SDS-PAGE (10) and visualized by autoradiography. Molecular-weight (10^3) markers are indicated on the left; PyTag (100 kDa) and the polyhedrin protein (32 kDa) are indicated on the right.

from the dishes, the dishes were placed on ice, and the cells were washed twice with 5 ml of cold phosphate-buffered saline per plate. A 0.75-ml sample of lysis buffer (50 mM Tris [pH 8]-150 mM NaCl-1% Nonidet P-40-0.1% aprotinin [Sigma Chemical Co.]) was distributed over the cell surfaces. The cells were scraped into cold microcentrifuge tubes and kept at 0°C for 30 min. The tubes were centrifuged at $15,000 \times g$ for 2 min, and the supernatants (cell extracts) were stored in liquid N_2 until use. Extracts were incubated with anti-PyTag antiserum, and antibody-antigen complexes were precipitated, washed, and suspended as previously described (22).

To quantitate the amount of PyTag made in vEV51LT-infected cells, PyTags from lysates of a polyomavirus-transformed mouse cell line, PYT-54, and vEV51LT-infected *S. frugiperda* cells collected 24 h p.i. were immunoprecipitated with a monoclonal antibody. The immunoprecipitated proteins were separated by SDS-PAGE and visualized by silver staining (Fig. 4A). On the basis of Lowry protein determinations and quantitation of silver-stained PyTag bands on SDS-polyacrylamide gels, approximately 10-fold more PyTag was produced per milligram of infected insect cell extract than PYT-54 cell extract. The amount of PyTag produced in the recombinant virus-infected cells corresponds to approximately $15 \mu\text{g}/10^7$ infected *S. frugiperda* cells. The size of PyTag produced in

baculovirus-infected cells is similar if not identical to that of mammalian cell-derived PyTag, on the basis of their migration in SDS-polyacrylamide gels (Fig. 4).

Since PYT-54 cells express large T antigen at 70 to 100% of the level found in polyomavirus lytic infections (24), the data indicate that the levels achieved with the baculovirus-based expression system are equal to or higher than the levels achieved with the adenovirus-based expression systems (13, 14). To address this point directly, we purified PyTag simultaneously from the adenovirus recombinant AdSVR587-infected CV1 (monkey kidney) cell extracts and from vEV51LT-infected *S. frugiperda* cell extracts. Approximately two to five times more PyTag was obtained from infected insect cells than from infected monkey cells, as determined by silver staining of SDS-polyacrylamide gels (Fig. 4B); the levels were then normalized for equivalent quantities of protein, as determined by Lowry protein analyses of initial cell extracts.

To determine whether the insect cell-derived PyTag has DNA-binding properties similar to those of PyTag from infected or transformed mouse cells, a modified McKay assay (15) was used. PyTag from vEV51LT-infected *S. frugiperda* cells was purified by using an anti-PyTag immunoaffinity column (20) of monoclonal antibody F-4 (21, 24) covalently cross-linked to anti-mouse immunoglobulin G

FIG. 3. Immunoprecipitation of PyTag from vEV51LT-infected cells. Immunoprecipitated proteins (I) from [35 S]methionine-labeled vEV51LT- or wild-type (L-1)-infected cell extracts (see text) were compared with total proteins (C) in the cell extracts by SDS-PAGE. An autoradiogram of the resulting gel is presented; the positions of the polyhedrin and PyTag (100 kDa) proteins are noted.

FIG. 4. Polyacrylamide gel analysis of immunoprecipitated PyTag from vEV51LT-infected cells, polyomavirus-transformed PYT-54 cells, and AdSVR587-infected CV1 cells. (A) PYT-54 cell extract (2 mg) was precipitated with monoclonal control pab419T (lane a) or with anti-PyTag monoclonal antibody F-4 (lane b). Extracts (0.8 mg) of *S. frugiperda* cells infected with wild-type (lane c) or recombinant (lane d) AcNPV were prepared as described in the legend to Fig. 3 and were precipitated with antibody F-4. The crude extract of vEV51LT-infected cells (lane e) is also shown. (B) Extracts of wild-type-infected *S. frugiperda* cells (lane a), vEV51LT-infected *S. frugiperda* cells (lane b), and AdSVR587-infected CV1 cells (lane c) were immunoprecipitated with F-4 antibody and analyzed by SDS-PAGE. In both panels, protein bands were visualized by silver staining; the arrowheads indicate the position of PyTag. Protein size markers (lanes m) of 92.5, 65, and 46 kDa are indicated.

agarose beads. Four immunopurified PyTag fractions were incubated with 32 P-labeled, *Hinf*I-digested p37.3A₂ DNA, a plasmid which contains the entire polyomavirus genome inserted into the plasmid pAT153 (3); *Hinf*I digestion produces 23 fragments ranging in size from 1,801 to 18 base pairs. The nature of the DNA in PyTag-DNA complexes precipitated with F-4 monoclonal antibody was determined by gel electrophoresis. Specific precipitation of the 604-base-pair *Hinf*I fragment containing the origin of replication of the polyomavirus genome was observed by autoradiography of gels containing the immunoprecipitated DNA (Fig. 5). Thus, the insect-derived PyTag possesses specific DNA-binding properties.

Insect baculovirus expression vector systems have been shown to produce moderate to high levels of a variety of different foreign genes (1, 9, 11, 16, 22, 26, 27). The AcNPV vector system can produce low quantities of PyTag. Nevertheless, the levels of PyTag produced in vEV51LT-infected insect cells are significantly higher than those obtained in polyomavirus-infected cells or -transformed cells. Baculovirus-mediated PyTag expression compares favorably with adenovirus-mediated expression (13). The helper-independent vEV51LT is easily propagated, reaches high titers in the media of infected cells ($>10^8$ PFU per ml), and is stable. A safety feature of the baculovirus expression system is the barrier to entry and expression of baculovirus genes in mammalian cells (2).

The AcNPV expression system, using the polyhedrin promoter, usually produces much higher levels of foreign gene products (1, 9, 16, 22, 26, 27) than those we have observed for PyTag. The low level of expression of PyTag may be related to the observation that synthesis of the protein appears to decrease from 18 to 36 h p.i., whereas

FIG. 5. Specific binding of immunoaffinity-purified baculovirus-derived PyTAG to the polyomavirus DNA origin of replication. PyTAG from infected cells was purified from cellular extracts by immunoaffinity column chromatography by using goat anti-mouse agarose coupled to F-4 monoclonal antibody as previously described (20). Samples were tested for specific polyomavirus DNA binding in a nitrocellulose filter-binding assay (see below). Peak fractions of HS and EG eluates containing greater (EG1, HS1) and lesser (EG2, HS2) DNA-binding activity were pooled, dialyzed (20), and stored at -20°C . The DNA-binding immunoassay was a modification of procedures described by Cowie and Kamen (3) in which immunoaffinity-purified PyTAG (5 μl) was incubated with 20 ng of ^{32}P -labeled *Hinf*I-digested p37-3A₂ DNA at room temperature for 1 h, followed by incubation with F-4 monoclonal anti-PyTAG antibody (30 min). Immune complexes were precipitated with immunoglobulin G sorbent. The DNA fragments were released and analyzed on agarose gels as previously described (24). Lanes 1 to 4 are DNA fragments bound by immunopurified fractions of PyTAG HS1, HS2, EG1, and EG2, respectively. Outer lanes (M) are total ^{32}P -labeled *Hinf*I fragments of p37.3A₂. The fragment containing the polyomavirus DNA replication origin (ori) is indicated.

expression of proteins under polyhedrin control generally increases and accumulates during this time (22). It is possible that the T antigen is exerting some negative effect on virus expression. A contributing factor to low expression may be the use of the pEV-51 vector instead of pEV-55 (16); pEV-51 lacks 22 base pairs of the polyhedrin mRNA leader, whereas pEV-55 includes the entire leader (16).

The PyTAG produced in baculovirus-infected insect cells is biologically active with respect to its origin-specific DNA-binding properties. Ongoing experiments show that the PyTAG produced in this system is active in initiating DNA replication *in vitro* and possesses ATPase activity (Y. Murikami, C. Prives, and J. Hurwitz, work in progress). Since T antigens undergo extensive posttranslational modifications in mammalian cells, it will be interesting to determine what types of posttranslational modifications are present in insect-derived protein and to correlate modifications with the numerous functions of this complex protein.

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