Expression of the S-coded Genes of Lymphocytic Choriomeningitis Arenavirus Using a Baculovirus Vector

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SUMMARY

A DNA copy of the lymphocytic choriomeningitis virus (LCMV, WE strain) S RNA species has been inserted in both orientations into plasmids containing a 7.1 kb DNA sequence of the baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV). The inserts were placed behind the polyhedrin gene promoter of AcNPV and in the place of the nucleotide sequences including and flanking the polyhedrin gene translation initiation codon. The derived plasmids were used to obtain recombinant AcNPV viruses after transfection of Spodoptera frugiperda cells in the presence of infectious AcNPV DNA and the selection of polyhedrin-negative viruses. The expression of the two LCMV S-coded genes, the nucleoprotein and glycoprotein precursor, in S. frugiperda cells by the recombinant baculoviruses is described. Based on the results obtained with three different expression vectors, the site of insertion of the foreign genes in the 5' non-coding region of the polyhedrin gene appears to be an important determinant of the level of expression obtained.

INTRODUCTION

Sequence analyses of clones of DNA representing the S RNA species of the WE strain of lymphocytic choriomeningitis virus (LCMV, the prototype virus of the Arenaviridae) have shown that the viral nucleoprotein (N) is coded in a viral-complementary sequence and that the glycoprotein precursor (GPC) protein is coded in a viral-sense sequence (Romanowski & Bishop, 1985; Romanowski et al., 1985). Similar results have been obtained for the arenaviruses Pichinde virus (Auperin et al., 1984a, b) and Lassa fever virus (Clegg & Oram, 1985; D. D. Auperin, D. Saso & J. B. M. McCormick, unpublished results). The term ambisense RNA has been used to recognize the fact that the two proteins are coded on opposite strands of the arenavirus S RNA, a feature that is unique among RNA viruses, except for the S RNA of phleboviruses (Bunyaviridae family; Ihara et al., 1984). For Pichinde virus, it has been shown that there are subgenomic mRNA species corresponding to the two S-coded gene products (Auperin et al., 1984b), similar results have been obtained for LCMV (Romanowski et al., 1985, and unpublished data).

In order to synthesize enough of the two LCMV structural proteins for eventual functional analyses (investigation of the structure of the viral glycoproteins, in vitro assembly of N protein with the viral RNA, etc.) we have sought to express these gene products in eukaryotic cells by inserting the LCMV S DNA in either orientation in a baculovirus expression vector. The Autographa californica nuclear polyhedrosis virus (AcNPV) insect baculovirus vector was chosen in view of the reported high levels of foreign gene expression obtained using the AcNPV polyhedrin promoter (Smith et al., 1983). The results of expression analysis using AcNPV vectors and the LCMV N and GPC genes are described. Based on the results obtained, the level of expression obtained appears to be related to the site of insertion in the 5' non-coding region of the polyhedrin gene.
**METHODS**

**Viruses and cells.** AcNPV and recombinant virus stocks were grown and assayed in confluent monolayers of *Spodoptera frugiperda* cells in medium containing 10% foetal bovine serum according to the procedures described by Brown & Faulkner (1977). On occasion, virus stocks were obtained using spinner cultures of *S. frugiperda* cells. The WE strain of LCMV was grown in BHK-21 cells and titrated in Vero cells (Romanowski & Bishop, 1983).

**DNA manipulations, construction of DNA clones.** Plasmid DNA manipulations were carried out essentially as summarized by Maniatis et al. (1982). Restriction enzymes, T4 DNA ligase and the Klenow large fragment of DNA polymerase I were purchased from Biolabs (Beverly, Mass., U.S.A.). Calf intestinal alkaline phosphatase (Romanowski & Bishop, 1985; Romanowski et al., 1985). The construction of the complete LCMV DNA is illustrated in Fig. 1. From clone 39-50 a SalI- and PvuI-derived 1.8 kb restriction fragment was ligated to a PvuI- and SalI-derived 3.8 kb restriction fragment obtained from clone 81-268. After transformation of *Escherichia coli* MC1061 cells (Casadaban & Cohen, 1980) and the selection of drug-resistant colonies, followed by colony hybridization screening (Grunstein & Hogness, 1975) using nick-translated products of the same two precursor viral DNA fragments, clone B-13 was recovered and showed by restriction enzyme analyses to contain LCMV S residues 1 to 1248 (Fig. 1). A PstI 1.1 kb fragment from clone 86-139 (S residues 2286 to 3375) was recloned into the PstI site of pBR322 and clone 86-139-1 was obtained (Fig. 1) having the opposite orientation to the original 86-139 DNA as evidenced by the appropriate restriction enzyme analyses. From clone 83-174 a SalI and partial MstII digest was used to recover a 3.0 kb fragment which was then ligated to an MstII- and SalI-derived 3.9 kb fragment excised from clone 86-139-1. After transformation and colony screening, clone A-3 was obtained and shown by restriction enzyme analyses to have LCMV S residues 866 to 3375 (Fig. 1). To construct a clone (Y-1) containing the entire S sequence, a SalI- and MstII-derived 2.5 kb fragment from clone B-13 was ligated to a 5.2 kb SalI and partial MstII digest obtained from clone A-3 and the products used for transformation (Fig. 1). Finally, from the Y-1 clone a BamHI fragment representing LCMV S DNA residues 9 to 3362 was recloned into the BamHI site of pBR322 (Fig. 1, clone Y-1-A).

**Insertion of LCMV S DNA into transfer vectors containing a modified polyhedrin gene.** Transfer vector plasmids containing a 7.3 kb AcNPV *EcoRI* T fragment in pUC8 have been modified in a manner analogous to that described by Smith et al. (1983) by removal of the pUC8 BamHI site and replacement of the polyhedrin gene ATG codon and a number of nucleotides preceding (see text) and 175 nucleotides following the ATG codon with a BamHI linker (Possee, 1986). The transfer vectors therefore have a single BamHI restriction site for the insertion of new genes. In order to obtain transfer vector derivatives with LCMV S DNA inserts, plasmid DNA was digested to completion with BamHI, dephosphorylated and ligated to a 3.3 kb BamHI fragment recovered from clone Y-1-A. After transformation and screening with nick-translated LCMV DNA, plasmids were obtained (Fig. 2, pACRP5-LCM WE G and pACRP5-LCM WE N) that were characterized by restriction enzyme and sequence analyses (Maxam & Gilbert, 1980) and shown to have the viral insert in either orientation (see text).

**Transfection and selection of recombinant viruses.** *S. frugiperda* cells were transfected with mixtures of purified AcNPV DNA and either LCM WE G or LCM WE N transfer plasmid DNA by a modification of the procedures described by Smith et al. (1983). AcNPV DNA (1 µg), purified by the method of Smith & Summers (1978), was mixed with various concentrations of plasmid DNA (25 to 100 µg) and adjusted to 950 µl with HEPES-buffered saline (20 mM-HEPES, 1 mM-Na2HPO4, 5 mM-KCl, 140 mM-NaCl, 10 mM-glucose, pH 7.05). After precipitation with 50 µl 2.5 M-CaCl2, DNA was inoculated onto monolayers of 1 × 10⁶ *S. frugiperda* cells in 35 mm tissue culture dishes and incubated for 1 h at room temperature. The supernatant fluids were discarded and 1.5 ml of medium containing 10% foetal bovine serum was added. After 4 days of incubation at 28°C, the supernatant fluids were harvested and titrated in confluent monolayers of *S. frugiperda* cells. Plaques exhibiting no evidence of occlusion bodies (viral polyhedra, as determined by transmission light microscopy) were recovered and titrated on *S. frugiperda* cells to obtain recombinant, polyhedrin-negative, viruses. Following a third plaque picking, high-titered (10⁶ to 10⁸ p.f.u./ml) stocks of the recombinant viruses were obtained using spinner cultures of *S. frugiperda* cells.

**Extraction and characterization of viral and cellular nucleic acids.** To obtain virus DNA, *S. frugiperda* cells in 100 mm dishes were infected with virus at a multiplicity of 10 p.f.u./cell and incubated at 28°C for 48 h. The infected cells were harvested, briefly sonicated to obtain cell lysis and centrifuged for 10 min at 5000 g to remove cell debris. The supernatant fluids were recovered, centrifuged for 1 h at 75000 g, the pellets were resuspended in TE buffer (10 mM-Tris-HCl, 1 mM-EDTA, pH 7.5) and virus was isolated after further centrifugation for a similar period using linear gradients of 10 to 50% (w/v) sucrose in TE buffer. The visible bands of virus were harvested, diluted three- to fivefold in TE buffer, pelleted by centrifugation (75000 g, 1 h), the virus pellets were resuspended in TE buffer and DNA was extracted by addition of 10% (w/v) sodium N-lauryl sarcosinate, 10 mM-EDTA, pH 7.5, and...
incubation at 60 °C for 20 min, followed by phenol extraction and ethanol precipitation. Viral DNA was resuspended in water and stored at −70 °C. For Southern analyses, viral DNA preparations were digested to completion with BamHI and the products resolved by electrophoresis in 0.8% agarose (Bethesda Research Laboratories) then blotted to Genescreen (New England Nuclear, NEN). After drying, the membranes were baked at 80 °C and hybridized to nick-translated labelled LCMV WE DNA obtained from clone Y-1-A (Southern, 1975). The membranes were finally washed and autoradiographed.

To obtain infected cell RNA, S. frugiperda cells were inoculated with virus at a multiplicity of 10 p.f.u./cell and incubated for 24 h at 28 °C in medium. RNA was extracted from the infected cells by the procedure of Hefti & Bishop (1975). Total infected cell RNA was chromatographed on columns of oligo(dT)-cellulose to separate RNA species containing polyadenylic acid sequences from those which lacked polyadenylic acid. The RNA preparations were treated with 10 mM-methylmercuric hydroxide (Bailey & Davidson, 1976), resolved by electrophoresis in 1% gels of SeaKem agarose containing methylmercury and transferred by blotting to Genescreen. After blotting (Alwine et al., 1977), the membranes were dried, baked at 80 °C for 2 h, then hybridized to 32P-labelled nick translation products of the appropriate viral DNA as described by Denhardt (1966). Membranes were washed and autoradiographed.
Fig. 2. Construction of pACRP5-LCM WE G and pACRP5-LCM WE N transfer vectors. The LCMV S DNA coding region was inserted in both orientations into the pACRP5 transfer vector (Possee, 1986) as described in Methods. The following features are shown in the derived plasmids: the insertion site of the 7.1 kb EcoRI AcNPV fragment (open and speckled boxes) modified to remove the polyhedrin translation initiation site and flanking nucleotides (residues −32 to +175; Smith et al., 1983) and their replacement with a BamHI linker; the pUC8 plasmid sequence (single line) modified to remove a BamHI restriction site (pUC8-b, Possee, 1986; Smith et al., 1983); the position and orientation of the inserted sequences (cross-hatched boxes, see Fig. 3 and 4); the remaining polyhedrin coding region (speckled region); the putative AcNPV transcription initiation (residue approx. −58, Smith et al., 1983) and transcription termination sites; the WE translation initiation and termination sites; the putative AcNPV polyhedrin promoter.

Protein and immunofluorescence analyses. S. frugiperda cells were infected with virus at a multiplicity of 10 p.f.u./cell in 35 mm tissue culture dishes and labelled with 100 μCi [35S]cysteine (NEN, 1019 Ci/mmol) for 1 h at the indicated times using cysteine-free medium. Prior to labelling, the cells were incubated for 1 h in cysteine-free medium to reduce the intracellular pools of the precursor. In some experiments [3H]leucine (NEN, 58 Ci/mmol), or [35S]methionine (Amersham, 1131 Ci/mmol), or [3H]mannose (Amersham, 16 Ci/mmol) was employed using similar protocols. After the labelling periods, the media were removed, the monolayers rinsed three times with cold phosphate-buffered saline (PBS) and the cells lysed in 500 μl RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.15 M-NaCl, 0.05 M-Tris-HCl, 0.01 M-EDTA, 0.1% SDS, pH 7.4). Aliquots of 100 μl of the extracts were incubated with 5 to 10 μl of the appropriate antiserum for 1 h at 37°C before addition of 25 μl of a suspension of 100 mg Protein A-Sepharose CL-4B beads (Sigma) in RIPA buffer. Following a further 1 h incubation at 4°C, the beads were recovered by centrifugation, washed three times with cold RIPA buffer and the immune complexes that were bound to the beads were removed by boiling for 5 min in dissociation buffer (2.3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 62.5 mM-Tris–HCl, 0.01% bromophenol blue, pH 6.8) followed by centrifugation. Aliquots of the supernatant fluids were subjected to electrophoresis in discontinuous gels of 10% polyacrylamide as described by Laemmli (1970). After electrophoresis the gels were impregnated with 2,5-diphenyloxazole (Bonner & Laskey, 1974) and exposed at −70°C to X-ray film.

For immunofluorescence analyses, 10 μl aliquots of S. frugiperda cells infected 36 h previously at a multiplicity of 10 p.f.u./cell (or LCMV-infected Vero cells) were spotted onto glass slides and fixed with cold acetone for 10 min.
The cells were washed in PBS and incubated for 1h at 37 °C with 10 µl of a 10⁻³ dilution of LCMV N-specific monoclonal antibodies, or 10 µl of a 10⁻² dilution of GP2-specific monoclonal antibodies, both kindly provided by Dr M. Buchmeier (Scripps Clinic and Research Institute, La Jolla, Ca., U.S.A.). On occasion, cells were treated with antibodies without prior fixation. The slides were washed with PBS, the cells stained with fluorescein-conjugated swine anti-mouse IgG antibody for 1 h at 37 °C, washed again with PBS and examined for fluorescence.

RESULTS

Preparation of recombinant baculoviruses containing the LCMV N and GPC genes

Plasmids containing the complete LCMV S coding region in both orientations were constructed as described in Methods (see Fig. 1 and 2). Three transfer vectors (pACRP1, pACRP5, pACRP6) were employed representing different insertion sites in the 5' non-coding region of the AcNPV polyhedrin gene. In order to verify the orientation and sequence of the polyhedrin leader region relative to the LCMV genes, the nucleotide sequences spanning the 5' insertion sites were determined as exemplified for the pACRP5 vectors in Fig. 3. By comparison with the 5' sequence of the AcNPV polyhedrin gene (Smith et al., 1983) the insertion sites were identified as summarized in Fig. 4. As expected (Possee, 1986), the LCMV substitutions began at the AcNPV polyhedrin gene nucleotide −52 (pACRP1 vectors), −32 (pACRP5 vectors) and −8 (pACRP6 vectors), counting the polyhedrin ATG translation initiation codon as +1, +2 and +3 (Smith et al., 1983). The insertion sequences all started with CC residues derived from the BamHI linker used to construct the vectors (Possee, 1986) followed by the respective sequences of LCMV DNA (beginning in each case at LCMV viral, or viral-complementary, nucleotide residue number 9 (see Romanowski & Bishop, 1985; Romanowski et al., 1985)).

The plasmid DNA preparations were employed at various concentrations with 1 µg of infectious AcNPV DNA in transfection experiments using S. frugiperda cells. Putative recombinant viruses were isolated from the infected cells by selecting progeny viruses with a polyhedrin-negative plaque phenotype. After three successive cycles of plaque purification, stocks of virus were obtained and used to prepare viral DNA. The presence of LCMV DNA in the recombinant virus genomes was verified by Southern analyses as exemplified for the pACRP5-derived viruses in Fig. 5 and by comparison to AcNPV viral DNA as a negative control and a BamHI digest of clone Y-1-A as a positive control. Three recombinant viruses (YN6, YN12 and YN13) that were obtained from transfections initiated with the pACRP5-LCM WE N plasmid, as well as one recombinant virus (YG5) that came from the pACRP5-LCM WE G plasmid, were shown by these procedures to contain LCMV DNA sequences. Similar results were obtained using the pACRP1 and pACRP6 transfer vectors (data not shown). The frequency of recombinant virus isolation from the virus harvests was estimated to be of the order of 0.5% when 50 to 100 µg of plasmid DNA were employed in the transfection analyses (see Methods).

Expression of LCMV gene products by the recombinant baculoviruses

The presence of LCMV-related RNA species in cells infected with the recombinant baculoviruses was determined by Northern analyses. S. frugiperda cells were infected with the pACRP5-derived recombinant baculoviruses YN12 or YG5. As a control, S. frugiperda cells were infected with AcNPV. Infected cell nucleic acids were extracted at 24 h post-infection and chromatographed on columns of oligo(dT)—cellulose to select RNA having polyadenylic acid sequences. The poly(A)⁻ and poly(A)⁺ RNA preparations were treated with methylmercuric hydroxide and separated into size classes by agarose gel electrophoresis in the presence of methylmercuric hydroxide. RNA was blotted and fixed to Genescreen, then probed with the appropriate nick-translated DNA to identify LCMV-related RNA species (Fig. 6). On the basis of the presence of hybrids in the poly(A)⁺ lanes and absence of corresponding signals in the poly(A)⁻ lanes, it was concluded that various LCMV-related RNA species greater than 1 kb in length were synthesized in the recombinant virus-infected cells. In view of the size of the LCMV insert and the DNA constructions, the expected sizes of the mRNA species containing the LCMV sequences should have been of the order of 4.5 kb. In addition to such species, lower
Fig. 3. Sequences of the 5' insertion sites of (a) pACRP5-LCM WE N and (b) pACRP5-LCM WE G transfer vectors. Sequences were determined by the method of Maxam & Gilbert (1980) using for the pACRP5-LCM WE N vector a HindIII fill-in restriction fragment (LCM viral-complementary (vc) DNA residue no. + 84 (Romanowski & Bishop, 1985)) recut with EcoRV (AcNPV residue - 98) and for the pACRP5-LCM WE G vector a HinfI fill-in restriction fragment (LCM viral-sense (v) DNA residue no. + 59 (Romanowski et al., 1985)) also recut with EcoRV.

molecular weight bands were evident, presumably representing prematurely terminated transcription products. Several RNA bands were identified in the AcNPV-infected cells when a 7.3 kb probe that included the polyhedrin gene was employed (Fig. 6). When an internal polyhedrin-specific DNA probe was employed the principal RNA band was the expected 1.2 kb polyhedrin mRNA species (data not shown, see Howard et al., 1986). It was estimated that some 10-fold more LCMV-specific mRNA was obtained for the pACRP6-derived recombinant
**LCMV gene expression**

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Fig. 4. Comparison of the leader sequences of the LCMV genes inserted into the pACRP1, pACRP5 and pACRP6 transfer vectors to that of the AcNPV polyhedrin gene. The AcNPV leader sequence is the published data of Smith et al. (1983). The sequences are those read from the analyses including and similar to those described in Fig. 3, plus previously published data (Romanowski & Bishop, 1985; Romanowski et al., 1985).

Fig. 5. Southern blot analyses of recombinant baculovirus DNA. *BamHI* digests of viral DNA were recovered from AcNPV (NPV), clone Y-1-A (WE S cDNA), the recombinant viruses YN6, YN12 and YN13 derived from the pACRP5-LCM WE S transfer vector (see text), a recombinant obtained from the pACRP5-LCM WE G vector (YG5) and an LCMV N recombinant (A251) obtained from the pACRP1 transfer vector (see Fig. 4). The DNA products were resolved by agarose gel electrophoresis and probed with nick-translated WE S DNA. The probe was derived from the 3.4 kb *BamHI* restriction fragment recovered from clone Y-1-A.
Fig. 6. Northern blot analyses of RNA obtained from baculovirus-infected cells. Total cellular RNA was extracted from S. frugiperda cells infected with either recombinant YN 12, or recombinant YG 5, or wild-type AcNPV (NPV). After recovery of (a) the poly(A)- and (b) poly(A)+ RNA species (see text), the various RNA preparations were treated with methylmercuric hydroxide and resolved by electrophoresis in a 1% agarose gel containing 10 mM-methylmercuric hydroxide. After blotting and fixing to Genescreen, the RNA was hybridized to nick-translated probes. For the YN 12 lanes an MstII 1.1 kb DNA was used as a probe for LCMV WE N-specific sequences (Romanowski & Bishop, 1985). For the YG 5 lanes an MstII 0.9 kb DNA probe was used to detect LCMV G-specific sequences (Romanowski et al., 1985). For the AcNPV lanes an EcoRI 7.0 kb fragment was employed. In view of the size of the AcNPV probe, it is likely that it would react with sequences in addition to the polyhedrin mRNA species. Size markers (treated similarly) consisted of the LCMV WE S 3.4 kb BamHI fragment and 1.1 kb MstII fragment (left hand lane) and the AcNPV EcoRI 7.3 kb fragment I (right hand lane).

viruses by comparison with the pACRP5-derived recombinants; however, no LCMV-related mRNA bands were identified with the viruses derived from the pACRP1 transfer vectors (data not shown).

In order to determine whether LCMV N and G proteins were synthesized in the recombinant virus-infected cells, cells were pulse-labelled at various times post-infection and the labelled proteins immunoprecipitated with the appropriate antibody prior to resolution by polyacrylamide gel electrophoresis. The results of pulse-labelling pACRP5-derived virus-infected cells with [3H]leucine at 12 h post-infection followed by immunoprecipitation with either LCMV polyclonal serum or an LCMV monoclonal antibody specific for the N protein (Buchmeier et al., 1981) are shown in Fig. 7. Control lanes in the gel included immune precipitates of uninfected S. frugiperda (S.f.) or Vero cell extracts (Vero), as well as extracts of AcNPV-infected S. frugiperda cells (NPV) or LCMV-infected Vero cells (LCM). Both the polyclonal and N monoclonal antibodies precipitated the 62 × 103 mol. wt. N protein from LCMV (Buchmeier et al., 1981) and the YN 12 recombinant virus-infected cell extracts. No similar band was identified in the other lanes of the gel. These results indicated that LCMV N protein was expressed during the recombinant virus infection.

Protein bands corresponding to the approx. 72 × 103 mol. wt. GPC precursor to the two viral glycoproteins (Buchmeier & Oldstone, 1979; Buchmeier et al., 1981) were not evident in the YG 5 recombinant virus-infected cell extracts (Fig. 7). A reason may have been the time chosen for pulse-labelling. The time course of synthesis of GPC was therefore investigated using [35S]cysteine as the precursor amino acid in view of the relative abundance of cysteine in the viral glycoproteins (Romanowski et al., 1985). The results of immune precipitation of cell
LCMV gene expression

Fig. 7. Expression of LCMV proteins by recombinant baculoviruses derived from the pACRP5 transfer vector. *S. frugiperda* cells were infected with recombinant viruses derived from the pACRP5 transfer vector (see text, i.e. YN12 or YG5), or wild-type AcNPV (NPV) virus and labelled at 12 h post-infection for 1 h with [3H]leucine. The labelled proteins were recovered and precipitated with (a) anti-LCMV polyclonal antibodies or (b) anti-N monoclonal antibody preparations as described in Methods. For controls, uninfected Vero or *S. frugiperda* (S.f.) cell extracts and LCMV-infected Vero cell extracts (LCM) were processed similarly. The positions of the LCMV-induced N and GPC proteins are indicated by the arrows. In addition to N protein, the N-specific monoclonal antibody precipitated from the LCMV-infected cell extracts a protein band apparently slightly smaller than N in addition to a much smaller species (about 15 × 10^3 mol. wt.). Neither species was evident in the YN12 virus-infected cell extracts. Little GPC was detected in the YG5 virus-infected cell extracts using the polyclonal antibody.

Extracts obtained after 1 h pulse-labelling at 6, 12, 20 and 26 h post-infection are shown in Fig. 8, in comparison with a 12 h labelling period (12 to 24 h post-infection, Fig. 8 lane o/n), as well as the results obtained with extracts of LCMV-infected Vero cells and AcNPV-infected *S. frugiperda* cells. Only small quantities of GPC protein were observed at 12 h post-infection, whereas slightly more was detected at 20 h and in the extracts of cells labelled for 12 h.
Fig. 8. Expression of GPC by recombinant baculoviruses derived from the pACRP5 transfer vector. *S. frugiperda* cells were infected with the YG5 recombinant baculovirus (derived from pACRP5) and pulse-labelled with $[^{35}S]$cysteine for 1 h at 6, 12, 20 and 26 h post-infection, or overnight (o/n) for 12 h (12 to 24 h post-infection) and the derived cell extracts were immunoprecipitated with a GP2-specific monoclonal antibody (Buchmeier *et al.*, 1981) as described in Methods. For controls, $[^{35}S]$cysteine-labelled LCMV-infected Vero (LCM) and AcNPV-infected *S. frugiperda* cell extracts were treated similarly. The position of the 72 x 10^3 mol. wt. LCMV-induced GPC protein is indicated by an arrow. Although similar bands of labelled material were evident in the extracts of cells pulse-labelled at 12 and 20 h post-infection, more was present in the sample labelled overnight. The immunoprecipitated products that migrated faster than GPC may represent incompletely glycosylated species.

**Synthesis of LCMV proteins by different expression vectors**

The amounts of LCMV N or GPC proteins detected in cells infected with the recombinant viruses were found to depend not only on the time of labelling but also on the site of insertion of the foreign gene in the expression vector. These points are illustrated in Fig. 9(a) where the $[^{35}S]$methionine pulse-labelled proteins identified at 12, 24 and 48 h post-infection for recombinant viruses derived from pACRP5 (YN12) and pACRP6 (YN2) transfer vectors are compared to the labelled proteins obtained from uninfected (S.f.) or AcNPV-infected cells. No LCMV N protein was detected in the extracts of cells infected with recombinant viruses derived from the pACRP1 transfer vectors (data not shown). The amount of LCMV N protein identified in cells infected with the pACRP5-derived recombinant virus (YN12) was estimated to be 10- to 20-fold less than that obtained for the pACRP6-derived recombinant virus (YN2). For the latter, at 48 h post-infection the label incorporated into the viral N protein was estimated to be some 30 to 40% of the pulse-labelled products.

Similar results were obtained for the GPC recombinant viruses, as illustrated in Fig. 9(b). No GPC protein was detected in recombinant viruses derived from the pACRP1 transfer vector.
Fig. 9. Expression of LCMV proteins by recombinant baculoviruses derived from the pACRP5 and pACRP6 transfer vectors. *S. frugiperda* cells were infected with AcNPV, or recombinant viruses derived from the pACRP5 (YN12, YG5), or pACRP6 (YN2, YG6) transfer vectors (see Fig. 4) and pulse-labelled with (a) [3S]methionine, or (b) [3H]leucine at the indicated times (12, 24, 36, 48 or 60 h post-infection). The products were resolved by PAGE and the AcNPV polyhedrin protein (P), LCMV N protein (N) and GPC proteins were identified in the cell extracts (arrows). Uninfected cells (S.f.) served as controls.
Fig. 10. Immunofluorescence of recombinant baculovirus-infected *S. frugiperda* cells. *S. frugiperda* cells infected 36 h previously with recombinant baculoviruses derived from pACRP5 transfer vectors, namely YN12 (b) or YG5 (c), were fixed with acetone and examined as described in the text for immunofluorescence using N- or GP-specific monoclonal antibodies, respectively. Recombinant YG6 virus (derived from the pACRP6 transfer vector) was employed similarly (f) except that unfixed cells were used. As controls, AcNPV-infected *S. frugiperda* cells [fixed with acetone (d) or unfixed (e)] and LCMV-infected Vero cells (a, 24 h post-infection) were treated with a mixture of the two LCMV monoclonal antibodies and processed similarly.

Recombinant viruses obtained from the pACRP6 vector (YG6) synthesized GPC protein, as was evident from the [3H]leucine incorporation at 24, 36, 48 and 60 h post-infection (Fig. 9) and [3H]mannose at similar times (data not shown). As discussed above little GPC protein was detected by [3H]leucine (or [3H]mannose) incorporation for the recombinant virus (YG5) derived from the pACRP5 vector.

**Immunofluorescence analyses of recombinant virus-infected cells**

*S. frugiperda* cells were infected with the pACRP5-derived recombinant YN12 or YG5 viruses and prepared for immunofluorescence analyses 36 h post-infection using N- or GP2-specific monoclonal antibodies (Buchmeier et al., 1981). As controls 24 h infected cultures of LCMV-infected Vero cells and AcNPV-infected *S. frugiperda* cells were employed. The results, shown in Fig. 10, provided evidence for the expression of both LCMV N- and GP2-related antigens in the respective recombinant virus-infected cells. No LCMV-related antigen was detected in the AcNPV-infected cells (Fig. 10d). Viral proteins were evident in the LCMV-infected Vero cells (Fig. 10a). Similar results were obtained with the pACRP6-derived N and
GPC recombinants; however, no fluorescence was obtained with recombinant viruses derived from the pACRP1 transfer vectors (data not shown). In order to determine for the GPC recombinants whether glycoprotein was located on the surface of infected cells, fluorescence analyses were undertaken using unfixed cells. As illustrated in Fig. 10(f), a surface location of bound antibody was apparent for S. frugiperda cells infected with a GPC recombinant virus (YG6) derived from the pACRP6 transfer vector.

**DISCUSSION**

Recombinant baculoviruses with the LCMV S-coded genes in either orientation have been constructed and used to express the arenavirus antigens in S. frugiperda cells. The three insertion sites that have been employed are all located in the leader sequence of the AcNPV polyhedrin gene (residues -52 for pACRP1, -32 for pACRP5, and -8 for pACRP6). Therefore it is expected that the LCMV genes would be under the control of the baculovirus polyhedrin promoter. This has been the rationale for the expression of interferon and other foreign genes by baculovirus expression vectors (Smith et al., 1983; Miyamoto et al., 1985). Data to be presented elsewhere (Howard et al., 1986) have shown that the AcNPV polyhedrin mRNA transcripts are initiated in the region of polyhedrin gene residue -49 (see Fig. 4). The lack of LCMV protein expression or synthesis of LCMV-related mRNA species by the recombinant viruses derived from the pACRP1 transfer vector may be related to the fact that the site of insertion in that vector is before the polyhedrin mRNA transcription initiation site. The levels of expression of LCMV proteins by recombinant viruses derived from the other two transfer vectors differ by some 10- to 20-fold (Fig. 9), with more proteins synthesized by viruses derived from the pACRP6 vector than by viruses derived from the pACRP5 vector (see Fig. 4). Although the reason is not known, the most likely explanation is that the 5' leader sequence of the polyhedrin gene is important for the synthesis (or stability) of the mRNA species or influences their translation efficiencies. In this regard it is of interest to note that the two sites of insertion differ by a short sequence of only 24 nucleotides (see Fig. 4).

The levels of the LCMV N or GPC proteins synthesized by pACRP5- and pACRP6-derived recombinants do not appear to be as high as the level of polyhedrin protein synthesized by AcNPV (Fig. 9). Similar observations (including quantitative analyses) have been made by Possee (1986 and unpublished data) for influenza virus haemagglutinin expression by recombinant baculoviruses prepared using the same transfer vectors. In AcNPV-infected cells the polyhedrin protein is a major component of the cell by the end of the infection course. Why it is produced in such large quantities is not known; one possibility is that the promoter is activated late in the infection and to the exclusion of most of the other viral (and cellular) promoters. The polyhedrin protein is transported in AcNPV-infected cells to the nucleus, where it effectively crystallizes around virus particles. It is possible that it is thereby removed from cellular pools and that this facilitates its continued synthesis. This may not be the case for foreign proteins that accumulate in the cell, such as the LCMV proteins. These conclusions suggest that the constitution of the leader sequence and/or other regions of the polyhedrin gene are important for its expression (or the expression of a foreign gene), or that the constitution of the inserted gene has an effect upon expression. The transfer plasmids used to obtain the recombinants described here are the same as those reported by Smith et al. (1983). Their pAc380 plasmid contained a BamHI linker inserted at residue -58; in another plasmid (pAc360) it was positioned at +34. Both gave high yields of interferon (Smith et al., 1983) unlike the results reported here for recombinants derived from the pACRP1 and pACRP5 transfer vectors. Clearly, the importance of the leader sequences in the efficient expression of a foreign gene needs to be investigated further.

The size and sequences of the inserted LCMV DNA (3.3 kb) are also not comparable to those employed to express interferon (approx. 0.8 kb). The LCMV S species has an ambisense coding arrangement, with the N protein coded in a viral-complementary sequence corresponding to half of the viral RNA, and the GPC protein coded in a viral-sense sequence corresponding to the other half (Romanowski & Bishop, 1985; Romanowski et al., 1985). The intergenic region has an inverted complementary sequence (Romanowski & Bishop, 1985). The effect of these
arrangements on the transcription and stability of mRNA made from LCMV DNA is not known. LCMV has an RNA genome. It does not contain polyadenylation signals corresponding to the DNA polyadenylation signals of interferon or the polyhedrin gene of AcNPV. In fact, evidence has been presented that arenaviruses do not synthesize polyadenylated S mRNA species (Auperin et al., 1984b). It was expected therefore that in the baculovirus expression vector, transcription termination of mRNA containing LCMV information would rely on the polyhedrin gene polyadenylation signals (approx. 1 kb downstream from the end of the inserted genes). If this was the case then RNA transcripts of the order of 4 kb should have been seen in the Northern analyses. The observation that a variety of polyadenylated RNA species were identified in the recombinant baculovirus-infected cell extracts (greater than 1 kb, see Fig. 6) may indicate that there was premature termination of mRNA transcription (possibly due to inherent properties of the LCMV DNA) or that large transcripts were not stable. Clearly, it will be important to determine the effect of removal of the redundant LCMV (and polyhedrin gene) 3' sequences on both the synthesis of mRNA and the expression of these foreign genes.

REFERENCES


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