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Detection and Characterization of a Glycoprotein Encoded by the Mouse Mammary Tumor Virus Long Terminal Repeat Gene

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Mouse mammary tumor virus (MMTV) is a retrovirus that causes mammary tumors in susceptible mice. MMTV contains a unique open reading frame (ORF) in the unique 3′ region of the proviral long terminal repeat (LTR) with the potential to encode a 36-kDa protein. However, the ORF gene product has not been detected in murine mammary tissues or cell lines. We utilized the baculovirus expression vector system to generate large amounts of the ORF protein. Putative ORF gene products of 36 and 45 kDa were detected as unique proteins in extracts of insect cells infected with recombinant baculovirus (LTR-ORF BV), and the identities of these proteins as viral gene products were confirmed immunologically. Antipeptide antisera were generated in rabbits against peptides chosen from computer-predicted hydrophilic regions of the ORF coding sequence. These antisera reacted specifically by immunoprecipitation and by immunoblot with the proteins expressed in LTR-ORF BV-infected insect cells, as well as with MMTV LTR ORF in vitro translation products. Polyclonal antisera were raised against two putative ORF protein species partially purified from insect cells. These sera specifically immunoprecipitated viral protein products translated in vitro. In vitro translation of MMTV LTR ORF transcripts in the presence of canine pancreatic microsomal membranes generated a higher-molecular-weight ORF gene product, indicating that the ORF protein is modified by N-linked glycosylation. This glycosylated ORF product comigrated with the larger ORF protein species produced in infected insect cells. The gp45 product was metabolically labeled with [^{3}H]mannose, [^{3}H]galactose, and [^{3}H]*N*-acetyl-D-glucosamine in insect cells, whereas this incorporation was inhibited in the presence of tunicamycin. Digestion of gp45 with endoglycosidase H yielded the lower-molecular-weight ORF protein p36. These observations suggest that the ORF glycoprotein contains hybrid N-linked oligosaccharides. Demonstration of the modified nature of the ORF gene product will facilitate characterization of ORF protein expression in murine tissues.

Mouse mammary tumor virus (MMTV) contains an unusual open reading frame (ORF) in the unique 3′ (U3) region of the proviral long terminal repeat (LTR) in addition to the standard retroviral replicative genes *gag*, *pol*, and *env* (4, 6, 7, 10, 16). This unique ORF extends for approximately 960 nucleotides, corresponding to a maximum coding capacity of 36,000 to 37,000 M_r. An ORF gene product has not been detected in murine mammary cells or tissues, although four glycoproteins that were postulated to be truncated ORF products were detected in EL-4 T-cell leukemia cells treated with 12-*O*-tetradecanoylphorbol-13-acetate (29).

Several lines of evidence suggest that the ORF encodes a functional protein that may be involved in the viral life cycle, in mammary tumorigenesis, or in both. The ORF sequence is highly conserved among all MMTV strains sequenced (at both the nucleotide and amino acid levels) (1–3, 6, 18, 28). In vitro translation of the 3′ end of viral RNA (4, 28, 30) or in vitro-transcribed ORF RNA (5) produces proteins of 36,000, 24,000, 21,000, and 18,000 M_r. The smaller products presumably arise from internal methionine residues acting as internal translation start sites. Transcripts that correspond to a 1.6- to 1.7-kb spliced ORF mRNA have been detected in vivo in mouse mammary tissues, including lactating mammary gland, preneoplastic lesions, and various murine mammary tumors (19–21, 37, 38).

The ORF product has been identified as a potential transcriptional transactivator (36) or a negative-acting regulator (31). More recently, genetic studies have mapped mouse superantigens or minor lymphocyte stimulatory (MLS) antigens, which can eliminate T cells expressing specific T-cell receptor variable β chain (Vβ) elements, to various endogenous MMTV proviruses (9, 11, 39, 40). Marrack et al. (27) showed that maternally transmitted (exogenous) MMTV encoded a superantigen that could clonally delete Vβ14-bearing T cells. Transfection experiments (2) and transgenic mouse analyses (1) specifically demonstrated that the 3′ LTR ORF does code for an MLS antigen or MLS-like structure.

To understand the function of the ORF gene product, it is necessary that the protein be identified and characterized. We have employed the baculovirus expression vector system (BEVS) to produce large quantities of the ORF gene product. BEVS represents an extremely powerful system for foreign protein production and can express large amounts of recombinant gene products that appear to be antigenically, immunogenically, and functionally similar to the authentic proteins (23). BEVS has an additional advantage in that it carries out glycosylation and other eukaryotic posttranslational modifications that may be important for the immunogenicity or functional activity of a protein.

This report describes the synthesis of ORF proteins in the BEVS, coupled with immunological confirmation that the proteins are MMTV LTR ORF gene products. In addition, the data demonstrate that one form of the ORF gene product is a glycoprotein, probably of the hybrid asparagine-(N-) linked type.

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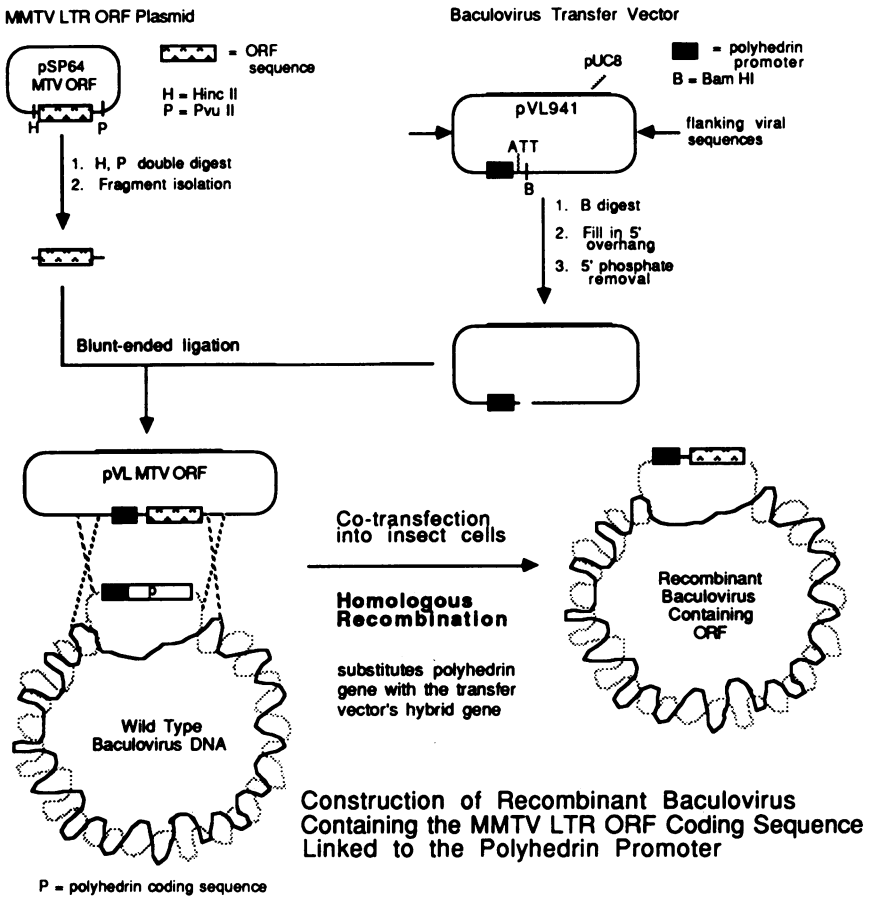

FIG. 1. Construction of recombinant baculovirus containing the MMTV LTR ORF coding sequence juxtaposed to the polyhedrin promoter. The ORF sequence-containing fragment was excised from pSP64 MTV ORF and purified by SeaPlaque (FMC Bioproducts) agarose gel electrophoresis. It was subcloned into the linearized, blunt-ended, dephosphorylated baculovirus transfer vector pVL941. WT baculovirus DNA and purified pVL MTV ORF plasmid DNA were cotransfected into insect cells by the calcium phosphate precipitation method. Note that generation of recombinant baculovirus requires homologous recombination within the insect cell.

MATERIALS AND METHODS

Viruses, cells, and plasmids. *Autographa californica* nuclear polyhedrosis virus (AcNPV) represents the prototypic baculovirus. Sf9 cells are a continuous cell line established from *Spodoptera frugiperda*, the fall armyworm (34). They are grown at 27°C in TNM-FH medium (12, 34), which consists of Grace's medium (JRH Biosciences, Lenexa, Kans.), lactalbumin hydrolysate, TC Yeastolate (Difco Laboratories, Detroit, Mich.), 10% fetal calf serum (FCS; JRH Biosciences), and 0.1% Pluronic F-68 (BASF Corp., Parsippany, N.J.). Plasmid pSP64 MTV LTR ORF, containing the exogenous C3H virus 3′ LTR ORF plus 5′ and 3′ flanking LTR sequences, was generously donated by Larry Donehower. The C3H-derived ORF-containing *Bgl*II-*Sac*I restriction fragment was subcloned from the right *Eco*RI fragment (which contains the 3′ LTR) from the line 15 provirus (26) into the *Bam*HI and *Sac*I restriction sites located in the polylinker region of the plasmid pSP64. The baculovirus transfer vector pVL941 (kindly provided by Max Summers; 25) contains several kilobases of AcNPV DNA, both 5′ and 3′ to the polyhedrin gene, which has a mutated initiation codon (ATT) to preclude production of fusion proteins.

Generation of recombinant transfer vector. The MMTV LTR ORF coding region was subcloned from pSP64 MTV LTR ORF as summarized in Fig. 1. All enzymes were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.), unless otherwise noted, and were used according to the manufacturer's suggestions. A blunt-ended DNA fragment containing the ORF sequence was obtained by a double restriction endonuclease digest with *Hinc*II (GIBCO BRL, Gaithersburg, Md.), and *Pvu*II and was subsequently purified by SeaPlaque (FMC Bioproducts, Rockland, Maine) agarose gel electrophoresis. The ORF insert was ligated to the baculovirus transfer vector pVL941, which had been linearized, made blunt ended, and dephosphorylated with *Bam*HI, the Klenow fragment of DNA polymerase I (New England BioLabs, Inc., Beverly, Mass.), and calf intestinal alkaline phosphatase, respectively. Restriction digests confirmed the proper insert orientation in the bacterial clone pVL941-MMTV ORF (Fig. 1).

Generation and isolation of recombinant baculovirus. Wild-type (WT) AcNPV DNA and the recombinant transfer vector pVL941-MMTV ORF were cotransfected into Sf9 cells by calcium phosphate precipitation. Virus was harvested from the insect cell cultures 5 days after transfection and was used to infect Sf9 cells in 96-well tissue culture plates. Dot-blot hybridizations of DNA isolated from infected cells provisionally identified recombinant viruses.

ORF insert DNA was labeled by random oligonucleotide primers (Boehringer Mannheim) with [^{32}P]dCTP (~3,000 Ci/mmol; ICN Biomedicals, Inc., Irvine, Calif.) for use as the probe. Plaque assays (34) were used to further select recombinant viruses, because recombinant plaques are polyhedrin occlusion negative. Several clones of recombinant virus (LTR-ORF BV) were plaque purified at least three times to eliminate contaminating WT AcNPV.

Cell infection and labeling conditions. Suspension or monolayer cultures of Sf9 cells were infected with a multiplicity of infection (MOI) of 5 to 10 PFU of baculovirus per cell. Sf9 cells were starved for 30 min with methionine-free TNM-FH medium plus 0.5% FCS and then labeled for 2 h with 30 to 75 μCi of Tran^{35}S-label (>1,000 Ci/mmol; ICN Biomedicals) per ml. Alternatively, cells were labeled for 3 h with 100 μCi of [^{3}H]mannose ([^{3}H]Man; 28 Ci/mmol), 200 μCi of [^{3}H]galactose ([^{3}H]Gal; 25 Ci/mmol), or 200 μCi of [^{3}H]*N*-acetyl-D-glucosamine ([^{3}H]GlcNAc; 30 Ci/mmol) (all obtained from ICN Biomedicals) per ml following a 60-min starvation period with glucose-free Grace's medium plus 0.5% FCS. Tunicamycin (Sigma Chemical Co., St. Louis, Mo.), an inhibitor of N-linked glycosylation, was used at 5 μg/ml.

Protein extraction conditions. Infected Sf9 cells were extracted at 34 to 38 h postinfection (p.i.). Then 1% Trasylol (Mobay/FBA Pharmaceuticals, West Haven, Conn.) and 0.2 mM leupeptin (Boehringer Mannheim) were added to all extraction buffers as protease inhibitors. Infected insect cells were treated with Empigen BB (Albright and Wilson, Ltd., Whitehaven, Cumbria, England) extraction buffer, pH 9.0 (1% Empigen BB, 50 mM Tris hydrochloride, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA); the nonsolubilized proteins and cell components were further extracted with RIPA buffer, pH 8.0 (1% Nonidet P-40, 1.0% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, 50 mM Tris hydrochloride, 5 mM EDTA, 150 mM NaCl). Nucleic acids were removed by high-speed centrifugation (~150,000 × *g*) for 45 to 60 min, using a TL100 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.).

Cytoplasmic RNA purification. Cytoplasmic RNA was harvested at 21 h p.i. from infected insect cells by using a modified version of the Luckow and Summers method (24). Dilutions of the RNA preparations were dotted onto nitrocellulose (Schleicher & Schuell, Keene, N.H.) and probed with heat-denatured purified ORF insert that had been labeled with ^{32}P, using random oligonucleotide primers. Mock- and WT-infected Sf9 cells served as negative controls.

Hybridizations. Hybridizations were performed by using the following protocol. The baked nitrocellulose filter was rehydrated with 2× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate) and then prehybridized for 15 min at room temperature with solution N (10% dextran sulfate, 40% formamide, 4× SSC, 7 mM Tris hydrochloride [pH 7.4], 0.02% Ficoll 400 [Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.], 0.02% bovine serum albumin fraction V [Sigma], and 0.02% polyvinylpyrrolidone 360 [Sigma]) plus freshly denatured sheared calf thymus DNA (final concentration, 0.5 mg/ml). The prehybridization buffer was replaced by fresh solution N plus denatured calf thymus DNA and denatured, ^{32}P-labeled probe DNA (10^7 cpm per blot), and the filter was incubated overnight at 42°C. The filter was then washed with 2× SSC–0.1% SDS for 5 min, twice with 2× SSC–0.1% SDS at room temperature for 20 min, and twice with 0.1× SSC–0.1% SDS at 65°C. Hybridization signals were visualized by autoradiography at −70°C, using XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) and intensifying screens (Dupont, Wilmington, Del.).

Production of antisera. Two peptides, corresponding to two computer-predicted major hydrophilic regions of the ORF protein, were synthesized by the Baylor College of Medicine Protein Chemistry Facility. The sequence of peptide M (18-mer) was as follows: CYGMGIENRKRRSTS VEE; amino acids 4 to 18 correspond to residues 163 to 177 from the middle of the ORF gene product; three amino acids (CYG) were added to the amino-terminal end of peptide M (C to facilitate conjugation to the carrier, Y to provide a site for iodination, and G to aid peptide flexibility following conjugation). The sequence of peptide C (18-mer) was CVL TQEEKDDIKQQVHDY from residues 263 to 280 near the carboxy terminus of the ORF product. The initial cysteine residue provided the sulfhydryl group necessary for conjugation to the carrier molecule, which was activated by using the heterobifunctional cross-linking reagent, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS; Pierce, Rockford, Ill.). The peptides were then individually conjugated to the MBS-activated carrier molecule keyhole limpet hemocyanin (KLH; Sigma). A small amount of peptide was iodinated by using Enzymobeads (Bio-Rad Laboratories, Richmond, Calif.). The ^{125}I-labeled peptide was conjugated to KLH by adding it to an aliquot taken from the conjugation reaction mixture containing unlabeled peptide and MBS-activated KLH. The degree of coupling was monitored by separating ^{125}I-labeled peptide-KLH from free (uncoupled) ^{125}I-labeled peptide on Bio-Gel P-2 columns (Bio-Rad Laboratories). The degree of conjugation was estimated at 45 to 50% for each peptide. The amount of coupled peptide C or peptide M equaled 20 to 25 molecules of peptide per 100,000 Da of KLH.

New Zealand White rabbits were immunized intramuscularly with 0.5 ml of conjugation mixture (0.5 mg of peptide total) mixed with complete Freund's adjuvant (initial injection) or incomplete Freund's adjuvant (subsequent injections). Rabbits were inoculated at 1-month intervals. Test blood samples were drawn approximately 10 and 24 days after the third and subsequent injections. Antisera also were raised against baculovirus-expressed proteins. The ORF proteins were semipurified from whole-cell extracts by one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (8), using a Protean II (Bio-Rad Laboratories) gel unit. Autoradiograms and Coomassie blue staining indicated that at least one WT baculovirus protein comigrated with p45 and another with p36. Protein localization in a gel was accomplished by identifying the appropriate metabolically labeled protein bands. Minced gel fragments were combined with equal volumes of Freund's adjuvant for injections, timed as above. At least 30 to 40 μg of gp45 and 80 to 100 μg of p36 were prepared for each injection. Rabbit antisera were designated as follows: antipeptide C, rabbit 336; antipeptide M, rabbit 337; anti-p36, rabbits 388 and 389; anti-gp45, rabbits 390 and 391.

In vitro transcription and translation. MMTV LTR ORF RNA was transcribed from pSP64 MTV LTR ORF with SP6 polymerase and subsequently treated with RNase-free DNase (RQ1 DNase; Promega Corp., Madison, Wis.). ^{35}S-labeled proteins were translated upon addition of the RNA and Tran^{35}S-label (ICN Biomedicals) to the rabbit reticulocyte lysate reaction mixture by following the instructions given by the manufacturer (Promega). Canine pancreatic microsomal membranes (Promega) were added to the translation reactions to investigate N-linked glycosylation. The in

vitro-translated proteins were analyzed by SDS-PAGE after mixing 5 to 7 μl of the reaction mixture with 2× disruption buffer (4% SDS, 4% β-mercaptoethanol, 10% glycerol, 50 mM Tris hydrochloride [pH 6.8] plus bromophenol blue) or after diluting the reaction mixture 1:10 with 1 M Tris hydrochloride (pH 8.0) and subsequently immunoprecipitating the proteins (see below).

Immunoprecipitation and immunoblot. Immunoprecipitations were performed as described previously (14) with the following modifications. Infected Sf9 cell extracts were diluted with 1 M Tris hydrochloride (pH 8.0) to a final SDS concentration of 0.05 to 0.1% prior to antibody addition. All samples were precleared with normal rabbit serum followed by formalin-inactivated, heat-killed *Staphylococcus aureus* Cowan I (SACI) (17) to remove nonspecific protein binding. The SACI used to collect primary antibody-antigen complexes was pretreated with unlabeled protein extracts from WT baculovirus-infected insect cells to further decrease nonspecific background. The SACI-antibody-antigen pellets were washed three times with RIPA buffer containing 0.5% SDS (pH 8.0). Samples were analyzed by SDS-PAGE, and the separated radiolabeled proteins were detected by autoradiography. Blocking experiments were performed by incubating antipeptide antisera with either heterologous or homologous peptide for 1 to 2 h at room temperature prior to addition to cell extracts.

For immunoblot (Western) assays, unlabeled protein extracts from infected Sf9 cells were immunoprecipitated with either antipeptide or antiprotein antiserum and then separated by SDS-PAGE. The proteins were electrophoretically transferred to nitrocellulose (32, 35) overnight, using a stepwise increase from 0.20 to 0.70 A; individual protein lanes were visualized by staining the nitrocellulose with Ponceau S solution (Sigma). Filter strips were treated with blocking buffer (0.25% gelatin, 0.1% Nonidet P-40, and 0.05% Tween 20 in Tris-buffered saline) before incubation with antibody dilutions that had been preabsorbed with nitrocellulose treated with blocking buffer. After being washed three times with blocking buffer, the individual strips were incubated with ^{125}I-labeled protein A (radiolabeled by Bolton-Hunter reagent; ICN Biomedicals) (32). The strips were rinsed three times with blocking buffer and twice with Tris-buffered saline before being air dried and autoradiographed.

Antipeptide antisera were tested for reactivity by immunodot blots as described above for Western blots. Fixed amounts of homologous or heterologous peptides (25 to 100 ng) were dotted onto nitrocellulose by using a dot-blot apparatus. Peptides were diluted with distilled water, and antisera were diluted with blocking buffer. Preimmune sera and a fibronectin fragment (Sigma) served as negative controls.

Endo H digestion. Infected Sf9 cells were metabolically labeled for 3 h with 75 μCi of Tran^{35}S-label per ml after a 30-min starvation period. Cell extracts were immunoprecipitated with anti-gp45 antibodies as described above. The SACI-antigen-antibody pellets were washed three times with RIPA buffer containing 0.1% SDS (pH 8), were resuspended in 0.1 M sodium phosphate buffer (pH 5.8), were pooled, and were then subdivided into equivalent fractions. Each fraction was incubated with recombinant endoglycosidase H (Endo H) (Boehringer Mannheim) for 1 h at 37°C, and then 5× disruption buffer was added to stop the reaction. Digested immunoprecipitates were analyzed on an SDS–12.5% polyacrylamide gel.

Pulse-chase experiments. At 36 h p.i., LTR-ORF BV-infected insect cells were starved for 30 min with methionine-free TNM-FH medium plus 0.5% FCS, were pulse-labeled with 100 μCi of Tran^{35}S-label per ml for 7 or 30 min, and were then chased with TNM-FH medium containing unlabeled methionine plus 10% FCS for various lengths of time before the cells were harvested. Cell extracts were immunoprecipitated with anti-gp45 sera and analyzed by SDS-PAGE.

RESULTS

Recombinant baculovirus production, detection, and analysis. Recombinant baculoviruses that carry the MMTV LTR ORF coding sequence were successfully generated by homologous recombination within cotransfected insect cells. Because different recombinant virus clones can vary in their expression levels of a recombinant gene, several LTR-ORF BV clones were analyzed at the RNA level. Sf9 cells were infected at equivalent MOIs, RNA extracts were prepared 21 h later, and the expression of ORF RNA was compared by dot-blot hybridization. The majority of recombinant baculovirus clones expressed similar ORF transcript levels (data not shown). Clone 20 was selected for use in subsequent experiments.

Detection of the putative ORF proteins. To test recombinant gene expression at the protein level, Sf9 cells were infected at an MOI of 5 to 10 PFU of clone 20 LTR-ORF BV per cell. Infected cells were metabolically labeled with a [^{35}S]methionine-[^{35}S]cysteine mixture and were extracted as described in Materials and Methods. Two putative ORF proteins (at approximately 36,000 and 45,000 M_r) were identified by SDS-PAGE by comparing profiles of proteins extracted from insect cells infected with either WT or recombinant baculovirus. p36 and p45 appeared by 26 h p.i. and expression peaked at about 34 h p.i. (data not shown), kinetics that resembled the synthesis of the polyhedrin protein (13). Thus, p36 and p45 appeared only in LTR-ORF BV-infected cells and their synthesis followed the kinetics of expression of the polyhedrin promoter, making these proteins likely candidates for ORF gene products. The smaller 36-kDa protein had the expected molecular size for a 319-amino-acid ORF gene product. The larger molecular size of the 45-kDa protein suggested the presence of posttranslational modification(s).

Computer-assisted selection of peptide sequences for antibody production. The hydrophilic portions of a protein represent the regions most likely to generate antipeptide antibodies able to react against the whole protein. To determine hydrophilic ORF sequences, we utilized the Kyte and Doolittle (22) hydropathy analysis program in the Baylor College of Medicine Molecular Biology Information Resource Center. Two peptides were synthesized that represented especially hydrophilic regions of the putative ORF gene product (see Materials and Methods). Peptide M corresponded to ORF amino acids 163 to 177, and peptide C corresponded to ORF amino acids 263 to 280.

Generation and analysis of antipeptide antisera. Antisera were generated in rabbits against the selected peptides to prepare ORF protein immunological reagents. Four rabbits produced antisera that specifically recognized the homologous peptide on immunodot blots (data not shown). However, antisera from rabbits 337 (antipeptide M) and 336 (antipeptide C) reacted best by immunoprecipitation and by immunoblot with proteins extracted from LTR-ORF BV-infected insect cells. Both putative ORF proteins, p36 and p45, were recognized by both antisera. The antipeptide M

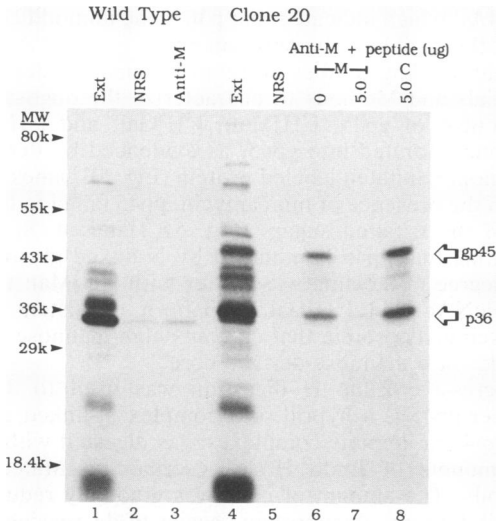

FIG. 2. Specificity of reactivity of antipeptide antisera with LTR ORF proteins. Peptide blocking experiments confirmed the specificity of reactivity of the antipeptide antisera with ORF proteins. LTR-ORF BV clone 20-infected cells were extracted with Empigen BB buffer followed by RIPA buffer containing 1% SDS as detailed in Materials and Methods. Infected cell extracts (equivalent to 3×10^6 cells) were processed for immunoprecipitation, and the immunoprecipitates were analyzed on a 12.5% SDS gel. Blocking experiments utilized antipeptide antiserum that had been preincubated with 5 μg of either heterologous (peptide C, lane 8) or homologous (peptide M, lane 7) peptide for immunoprecipitation of clone 20-infected insect cell extracts (equivalent to 3×10^6 cells). The anti-M serum failed to immunoprecipitate any WT-infected cell proteins (lane 3). The positions of p36 and gp45 are indicated on the right by open arrows, and molecular weight (MW) markers are shown on the left. Aliquots equivalent to 1.5×10^4 extracted cells of nonimmunoprecipitated infected cell extracts were electrophoresed for reference (lanes 1 and 4). Note that the homologous peptide (lane 7) abrogated the ability of the anti-M peptide antibody to immunoprecipitate both ORF proteins, whereas the heterologous peptide had no effect (lane 8).

antisera immunoprecipitated greater amounts of protein and demonstrated stronger recognition of both ORF proteins by immunoblot than did the antipeptide C antisera (see below). This may indicate that anti-M antibodies intrinsically possess higher affinities for ORF gene products or that they recognize more accessible epitope(s).

To prove that the antipeptide antisera were reacting specifically, WT-infected cell protein extracts were examined by immunoprecipitation and SDS-PAGE (Fig. 2). Neither antiserum immunoprecipitated protein species that migrated in the M_r ranges of the ORF proteins. Small amounts of polyhedrin protein were present in both the normal and immune serum precipitates, presumably due either to polyhedrin protein sticking to the bacterial immunoadsorbent or to pelleting of the large polyhedrin occlusions during centrifugation of the antigen-antibody complexes.

The specificities of the antipeptide antisera were further tested by blocking experiments. Antipeptide M antiserum was preincubated with 5 μg of either homologous peptide or heterologous peptide prior to immunoprecipitation of clone 20-infected cell protein extracts. Preincubation with the homologous M peptide abolished the ability of the antiserum to precipitate both ORF gene products, p36 and p45 (Fig. 2, lane 7), whereas heterologous C peptide failed to diminish reactivity (Fig. 2, lane 8). Similar results were obtained in an analogous blocking experiment using the antipeptide C antiserum (data not shown). Thus, the specific reactivity of the two different antipeptide antisera confirmed immunologically that the baculovirus-expressed proteins p36 and p45 were ORF gene products.

Reactivity of polyclonal antiprotein antisera. Putative ORF proteins p36 and p45 were extracted from LTR-ORF BV-infected insect cells, were separated by one-dimensional SDS-PAGE, and were used to immunize rabbits as detailed in Materials and Methods. Antisera were generated that recognized the respective immunizing protein from LTR-ORF BV-infected insect cell extracts. Antiserum against p45 not only recognized itself, but it also reacted with p36 by immunoprecipitation (Fig. 3B, lanes 3 and 7) and by immunoblot analysis (Fig. 3A, lanes 7 and 8, and Fig. 3B, lanes 5 to 8). These observations substantiated results obtained with the antipeptide antisera (Fig. 2) that suggested that p36 and p45 were different forms of the same ORF protein. The anti-p36 serum immunoprecipitated a small amount of p45 that was detectable by subsequent immunoblot analysis using antipeptide sera (data not shown) and anti-p45 sera (Fig. 3B, lane 6). Although antiserum against p36 recognized the homologous protein on immunoblots (Fig. 3A, lanes 5 and 6, and Fig. 3B, lanes 1 to 4), it did not immunoprecipitate p36 very well from infected insect cell protein extracts (data not shown) and it failed to react with p45 on Western blots (Fig. 3A, lanes 5 and 6, and Fig. 3B, lanes 1 to 4).

Evidence for glycosylation of the ORF gene product from in vitro translation experiments. One possibility considered was that p45 might represent a glycosylated form of p36. This hypothesis was tested by adding canine pancreatic microsomal membranes to in vitro rabbit reticulocyte lysate translation reactions containing in vitro-transcribed ORF RNA (see Materials and Methods). Aliquots of the translation mixtures were analyzed by SDS-PAGE. A prominent product of 36,000 molecular size was detected in the absence of microsomes (Fig. 4A, lane 1). Smaller protein species also were present. Translation in the presence of microsomes yielded a new doublet at 45 and 47 kDa, with a concomitant decrease in the level of the 36-kDa protein product (Fig. 4A, lane 2).

The in vitro translation reaction products were immunoprecipitated with anti-gp45 antisera (Fig. 4A, lanes 4 and 5). The antisera reacted with all the ORF proteins, including the higher-molecular-weight glycoproteins and the lower-molecular-weight species. The smaller proteins represented truncated products resulting from internal methionine residues acting as translation start sites (4, 5, 30) (Fig. 4B). This immunoreactivity by anti-gp45 further confirms the identity of the baculovirus-expressed ORF proteins, because the antibodies raised against the insect cell-produced products recognized in vitro-translated ORF gene products.

Investigation of ORF protein glycosylation in vivo. To confirm that gp45 was glycosylated in vivo, infected insect cells were treated with an inhibitor of N-linked glycosylation, tunicamycin (Fig. 5A). Tunicamycin (5 μg/ml) was present during the 30-min starvation period in methionine-free medium, as well as throughout a 2-h metabolic labeling time. The Sf9 cell extracts were immunoprecipitated with anti-gp45 antibody. The amount of gp45 synthesized in the presence of tunicamycin was dramatically reduced, with a concomitant increase in the amount of p36 relative to levels in the untreated samples (Fig. 5A, lanes 3 and 6). This observation is consistent with the in vitro translation results

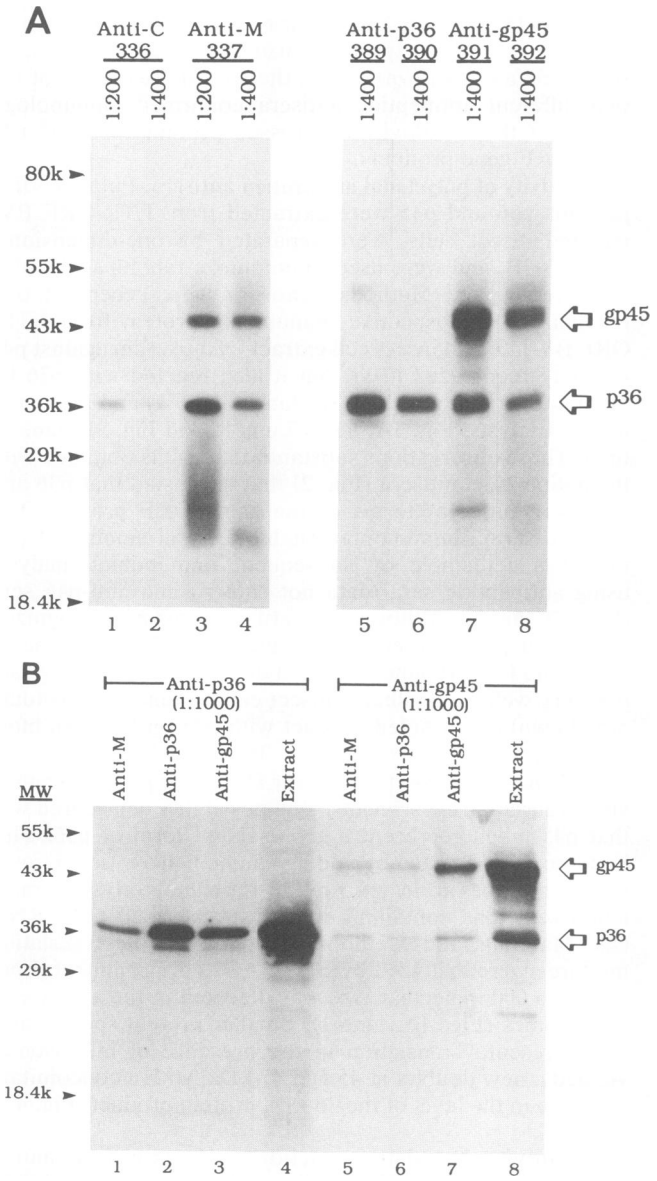

FIG. 3. Recognition of both LTR ORF protein species by coupled immunoprecipitation-immunoblot analysis. Unlabeled extracts of approximately 1.5×10^6 LTR-ORF BV clone 20-infected Sf9 cells were immunoprecipitated, proteins were resolved on preparative SDS–12.5% polyacrylamide gels and transferred to nitrocellulose, and immunoblot analyses were performed as described in Materials and Methods. Antiserum dilutions used for immunoblot are indicated at the top of each figure. Bound antibodies were visualized by using ^{125}I-labeled protein A. The antipeptide serum-treated blots were exposed to film for 16 h, whereas antiprotein serum immunoblots were exposed for 4 h. (A) Extracts were immunoprecipitated with a mixture of anti-p36 and anti-gp45 sera. The four types of anti-ORF antisera were then used for immunoblot analysis. Note that the anti-M peptide serum (panel A, lanes 3 and 4) reacted more strongly than did the anti-C peptide serum (panel A, lanes 1 and 2). All four types of antisera recognized both p36 and gp45, with the exception that anti-p36 failed to react with gp45 by immunoblot. (Reactivity with gp45 by the anti-C serum was visible with longer exposures.) The positions of p36 and p45 are indicated on the right, and molecular weight (MW) markers are indicated on the left. (B) Infected insect cell lysates were immunoprecipitated with anti-M, anti-p36, or anti-gp45 sera; precipitated ORF proteins were detected by immunoblot with anti-p36 (panel B, lanes 1 to 3) or anti-gp45 (panel B, lanes 5 to 7) serum. ORF proteins from nonimmunoprecipitated extracts (equivalent to 7.5×10^5 cells) were detected by immunoblot with anti-p36 or anti-gp45 serum (panel B, lanes 4 and 7, respectively). Different rabbit sera directed against p36 and gp45 were used for the homologous immunoprecipitation and immunoblot treatments. Note that the antiprotein sera immunoprecipitated and recognized by immunoblot both ORF gene products better than did the antipeptide sera. All the antisera were able to immunoprecipitate both ORF protein species, but the anti-p36 serum failed to recognize gp45 by immunoblot. The positions of the ORF proteins p36 and gp45 are indicated on the right and the molecular weight (MW) markers are indicated on the left.

(Fig. 4A), which indicated that p36 is the unmodified precursor of the N-linked glycoprotein gp45.

Sugar-labeling studies were performed as described in Materials and Methods to characterize the oligosaccharide component of gp45. [^{3}H]Man, [^{3}H]Gal, and [^{3}H]GlcNAc were incorporated into gp45, as evidenced by detection of immunoprecipitated labeled protein (Fig. 5B, lanes 2, 6, and 10). In the presence of tunicamycin, gp45 failed to label with any of the tritiated sugars (Fig. 5B, lanes 4, 8, and 12), indicating that gp45 is modified by N-linked glycosylation. The degree of labeling was higher with [^{3}H]Man than with [^{3}H]GlcNAc and [^{3}H]Gal, a pattern consistent with an N-linked glycoprotein that contains high-mannose oligosaccharides or a mannose-derived core.

Progressive Endo H digestion was used to determine whether gp45 is a hybrid or a complex N-linked glycoprotein. gp45 in immune complexes was digested with increasing amounts of Endo H as described in Materials and Methods. The amount of gp45 was gradually reduced, and several different glycoprotein species of decreasing molecular sizes (between 45 and 36 kDa) were produced (Fig. 6, lanes 2 to 7). Endo H cleaves between the two GlcNAc residues that connect the mannose-containing core to the asparagine residue in the protein backbone, leaving a GlcNAc residue attached to the protein. This final cleavage product was apparently generated, as manifested by the protein band which migrated a little more slowly than p36 (Fig. 6, lanes 3 to 7). Because Endo H cleaves high-mannose or hybrid oligosaccharides, but not complex oligosaccharides, gp45 appears to be an N-linked hybrid glycoprotein (15).

The kinetics of modification of the ORF protein were determined by using pulse-chase and immunoprecipitation experiments. gp45 was detectable immediately after a 7-min pulse (data not shown), suggesting that glycosylation of p36 occurs either cotranslationally or soon after protein translation. It appeared that complete trimming of the oligosaccharide side chains (based on molecular weight) was not achieved until at least 30 min after synthesis. Both p36 and gp45 appeared to have a half-life between 6 and 12 h in insect cells.

DISCUSSION

The MMTV LTR ORF gene product has not been detected in murine mammary tissues despite intensive efforts. However, in vitro-translated ORF gene products have been recognized by using antipeptide antisera. Racevskis and Prakash (30) determined that a single ORF protein of 36,000 M_r was translated in vitro from hybrid-selected mRNAs isolated from lactating mammary glands or from 7,12-dimethylbenz(α)anthracene-induced mammary tumors of BALB/c mice. This was in contrast to the multiple ORF protein species produced by in vitro translation of subge-

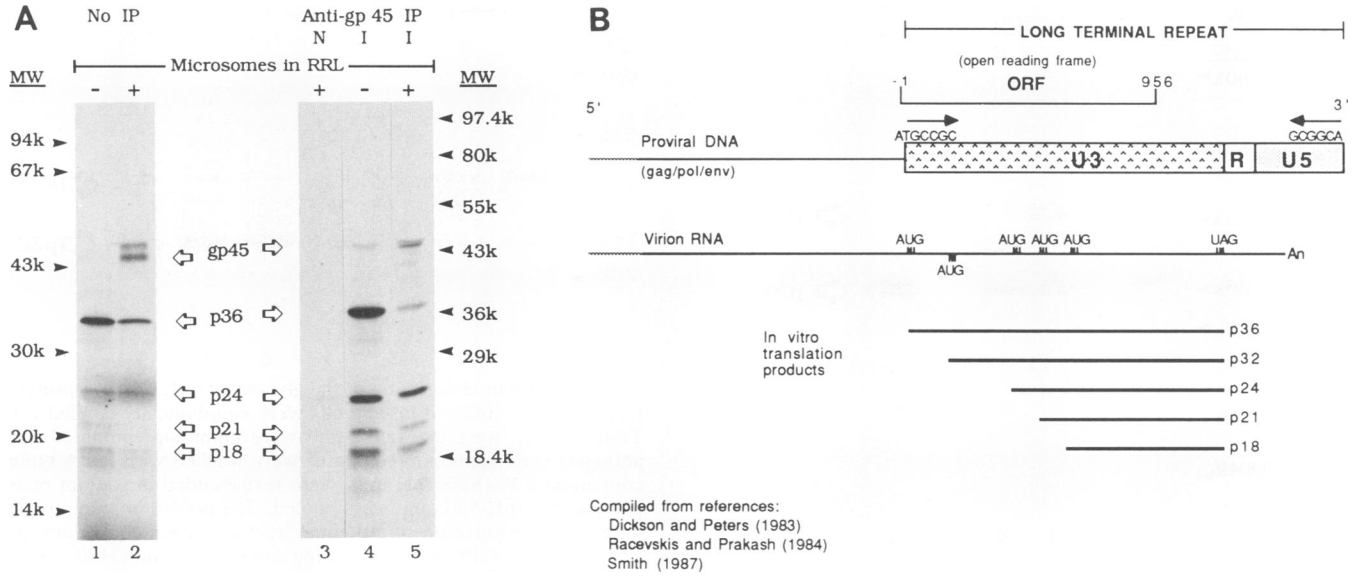

FIG. 4. Appearance of higher-molecular-weight LTR ORF-encoded proteins by the addition of microsomal membranes to in vitro translation reactions. (A) In vitro translation was performed as described in Materials and Methods by using in vitro-transcribed ORF RNA. Canine pancreatic microsomal membranes were added to the rabbit reticulocyte lysate mixture to investigate the glycosylation of synthesized proteins. Translation products were immunoprecipitated (IP) with anti-gp45 sera (N, normal; I, immune; lanes 3 to 5). The crude translation reaction products, as well as specific immunoprecipitates, were analyzed on a 12.5% SDS gel. ORF protein species are indicated by the open arrows; the positions of the molecular weight (MW) markers are shown. The 45,000-M_r species appeared only in the presence of microsomes, suggesting it was a glycosylated product (lanes 2 and 5). The gp45-gp47 doublet is compressed by the immunoglobulin G heavy chain band in the immunoprecipitate shown in lane 5. (B) Translation products of the LTR ORF coding region. The position of the ORF relative to the entire MMTV LTR is shown. The first start codon begins outside the U3 region in the *env* gene. The inverted direct repeats are indicated by inverted arrows. The small ORF protein species detected in in vitro translation systems are depicted. The multiple LTR ORF products result from internal methionine residue start site usage.

nomic or cloned LTR sequences (4, 5, 30). Hybrid-selected RNAs from lactating mammary glands of GR mice and an MMTV antigen-expressing, transplantable T-cell lymphoma of DBA/2 mice supported in vitro translation of MMTV structural proteins, in addition to the p36 LTR ORF gene product (30). A major ORF glycoprotein of 37,000 M_r, and three minor ORF glycoproteins of 31,000, 34,000, and 39,000 M_r, were identified in EL-4 cells treated with 12-*O*-tetradecanoylphorbol-13-acetate (29). All four glycoproteins were derived from a nonglycosylated apoprotein of 21,000 M_r that was detected after endoglycosidase F digestion (endoglycosidase F cleaves high-mannose and biantennary hybrid or complex oligosaccharides) (15) or tunicamycin treatment. Phorbol ester treatment of the C57BL/6 T-cell lymphoma cell line induced expression of an MMTV-specific transcript containing *env* and incomplete 3′ LTR sequences. The LTR region contained a 491-nucleotide deletion in the U3 region of the LTR. The ORF proteins were immunoprecipitated by using antipeptide antiserum, and the identities were confirmed by tryptic peptide mapping. Although these ORF gene products were detected in nonmammary cells and were not full length because of the deletion in the LTR, they demonstrated the capability of ORF gene products to be glycosylated in vivo.

Smith et al. (33) concluded that LTR ORF-encoded proteins were closely associated with MMTV intracytoplasmic A particles, yet were not present in mature virions. The most prevalent intracytoplasmic A particle proteins that were recognized by anti-LTR peptide antisera migrated at 68,000 and 72,000 M_r, much more slowly than the predicted ORF gene products, and possibly represented a *gag*-ORF fusion protein. However, three different antipeptide antisera reacted weakly on immunoblots with smaller proteins associated with intracytoplasmic A particles, including species at 43,000, 36,000, and 32,000 M_r. In addition, one antipeptide antiserum recognized higher-molecular-weight proteins (68,000 to 80,000) extracted from mammary tumors of C3H/Sm mice.

The failure to detect reproducibly MMTV LTR ORF gene products of the proper sizes in murine mammary cell lines and tissues emphasizes the need for more effective ORF protein reagents and better insight into the nature of the ORF gene products. The BEVS enabled production of large amounts of the LTR ORF proteins and subsequent generation of specific immunological reagents. Antipeptide antisera also were prepared, and they provided immunological confirmation that ORF gene products were being expressed in the LTR-ORF BV-infected insect cells.

Antiserum prepared against gel-purified gp45 is especially useful, because it recognizes both the glycosylated and nonglycosylated forms of the ORF gene product. In contrast, antiserum raised against gel-purified, nonglycosylated p36 displays relatively weak reactivity against the higher-molecular-weight form of the ORF protein; conformational changes or epitope masking by protein modification may be responsible for its inability to recognize gp45. The more limited specificity of the anti-p36 reagent may be helpful in differentiating subcellular localizations of the two forms of ORF protein.

The expression system described here allowed characterization of an ORF gene product. Tunicamycin inhibition and sugar-labeling studies demonstrated that a high-molecular-

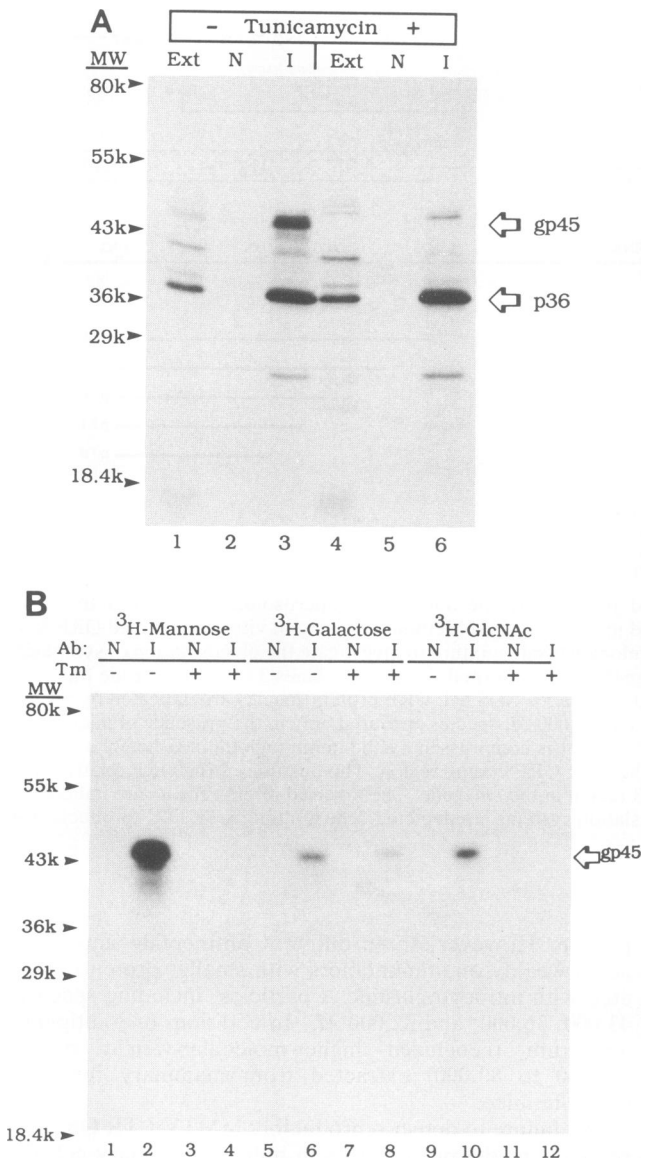

FIG. 5. (A) Evidence for glycosylation of the ORF protein in vivo. Monolayer cultures of Sf9 cells were infected with an MOI of 10 PFU per cell and were treated with tunicamycin (5 μg/ml) for 30 min prior to and during metabolic labeling as described in Materials and Methods. Tunicamycin-treated (+) or untreated (−) cell extracts (3×10^6 cell equivalents) were immunoprecipitated with anti-gp45 sera (N, normal rabbit sera; I, immune sera). Immunoprecipitates were analyzed by SDS-PAGE on a 12.5% gel. Nonimmunoprecipitated cell extract aliquots (ext; equivalent to 1.5×10^5 cells) were run for reference (lanes 1 and 4). Note the disappearance of gp45 and the concomitant increase in the amount of p36 in the immunoprecipitates of tunicamycin-treated cell extracts (compare lanes 3 and 6). Positions of molecular weight (MW) markers are shown on the left. (B) Characterization of LTR ORF protein glycosylation in vivo by tritiated sugar labeling. Clone 20-infected Sf9 cell monolayers (6×10^6 cells) were metabolically labeled with tritiated sugars in the presence or absence of tunicamycin as described in Materials and Methods. Labeled extracts were immunoprecipitated with anti-gp45 sera, and the immunoprecipitates were analyzed on a 12.5% SDS gel that was enhanced with Amplify (Amersham Corp., Arlington Heights, Ill.) during autoradiography. gp45 incorporated [^{3}H]Man, [^{3}H]Gal, and [^{3}H]GlcNAc (lanes 2, 6, and 10), whereas in the presence of tunicamycin it failed to label with the tritiated sugars (lanes 4, 8, and 12). A faint band in the immunoprecipitate from tunicamycin-treated, [^{3}H]Gal-labeled cells (lane 8) represents a comigrating, contaminating glycoprotein that is present in WT-infected cells.

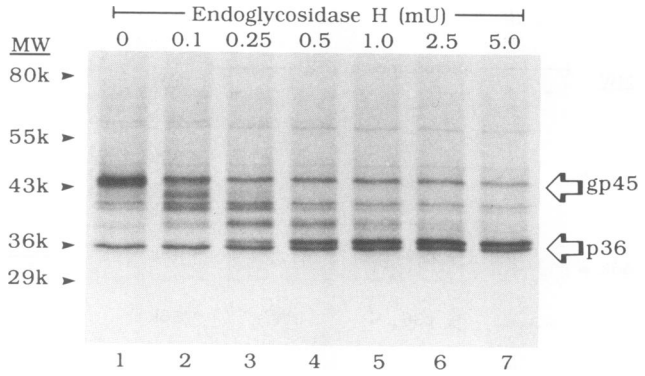

FIG. 6. Endo H digestion of the glycosylated LTR ORF protein. LTR-ORF BV-infected insect cells were metabolically labeled with Tran^{35}S-label, were extracted, and were immunoprecipitated with anti-gp45 sera. Immunoprecipitates were washed with RIPA buffer containing 0.1% SDS (pH 8.0), were resuspended in sodium phosphate buffer (pH 5.8), and were pooled. The pooled immunoprecipitate was then partitioned into equal fractions, each equivalent to a sample from 3×10^6 cells. Increasing amounts of Endo H (0.1 to 5.0 mU) were added to fractions, the mixtures were incubated for 1 h at 37°C, and the digested immunoprecipitates were analyzed on a 12.5% SDS gel. Note the gradual reduction in the amount of gp45 and the appearance of several partially digested glycoprotein species of between 36 and 45 kDa (lanes 2 to 7). The upper protein of the doublet at 36 kDa (lanes 3 to 7) probably represented the final digestion product, the backbone p36 plus attached GlcNAc residues. The positions of the ORF proteins, p36 and gp45, and molecular weight (MW) markers are indicated with open arrows and arrowheads, respectively.

weight form of the ORF gene product is modified with N-linked oligosaccharides. The predicted amino acid sequence from the exogenous C3H MMTV LTR ORF contains five potential N-linked glycosylation sites (NXT/S), but we do not know how many of those sites actually get modified. The high degree of labeling of gp45 by [^{3}H]Man is compatible with the presence of N-linked oligosaccharides, which all have a mannose-containing core sequence in common [Manα1→3(Manα1→6)Manβ1→4GlcNAcβ1→4GlcNAc-Asn] (15). Furthermore, incorporation of [^{3}H]GlcNAc was expected because GlcNAc represents the saccharide moieties which join core mannose residues to asparagine residues in the protein backbone and which also initiate antenna formation (15).

The presence of galactose, as evidenced by labeling with [^{3}H]Gal, suggests that gp45 is either a hybrid or a complex glycoprotein. These two types of structures differ by the type of sugar residue attached to the α1,6-linked core mannose. Both types of oligosaccharides have at least one GlcNAc residue attached to the α1,3-linked core mannose. Hybrid structures have only mannose residues attached to the α1,6-linked core mannose, rather than additional GlcNAc residues present in complex oligosaccharides. Glycosidase Endo H can discriminate between hybrid and complex sugar modification because it cleaves between the GlcNAc residues that connect high-mannose and hybrid oligosaccharides to the protein backbone but not between

those that attach complex oligosaccharides. The gp45 ORF product appears to be a hybrid N-linked glycoprotein because it was cleaved by Endo H and was labeled with [^{3}H]Man, [^{3}H]GlcNAc, and [^{3}H]Gal in the absence of tunicamycin. These results are consistent with the ORF glycoproteins detected in the EL-4 lymphoma cells (29). In addition, the results strongly suggest that the ORF gene product lacks a cleavable signal sequence, because the final digestion product had a slightly larger molecular weight than the unmodified precursor p36.

The existence of more than one form of ORF gene product may indicate separate or cooperating functions for the proteins or both. Previous reports have suggested that the ORF gene product may be either a transcriptional transactivator (36) or a negative-acting factor that decreases initiation of transcription (31). Although these effects appear to be contradictory, it is possible that both functions coexist in MMTV-infected cells and are mediated by the different forms of the ORF protein. The ORF proteins active in transcriptional regulation would probably reside in the nucleus; the nonglycosylated p36 represents a good candidate for nuclear localization. In contrast, the glycosylated form, gp45, may reside in a membrane compartment. A 22-amino-acid hydrophobic stretch located near the amino terminus of the ORF protein may represent a transmembrane-spanning domain, as predicted by computer analysis (data not shown). This glycoprotein may function independently from other ORF gene products and may elicit its effects from the membrane or through the generation of second messengers.

Recent investigations have determined that the MMTV LTR ORF protein functions as an MLS antigen or superantigenlike structure (1, 2, 27). Superantigen molecules combine with major histocompatibility complex class II molecules to form ligands that complex with a specific subset of T-cell receptors via a targeted Vβ element. T lymphocytes that express these specific Vβ elements are clonally deleted in the thymus during T-cell maturation and establishment of self-tolerance. The protein interactions required for such complex formation strongly suggest that the MLS antigen ORF gene product is exposed on the cell surface or is secreted to allow presentation of the MLS antigen by B cells in the context of major histocompatibility complex class II molecules. We propose that the expressed MLS antigen encoded by exogenous and endogenous MMTV is the murine equivalent of our baculovirus-expressed gp45. It is possible that the glycosylated ORF gene product gp45 may mediate the MLS antigen phenomenon.

The identification and partial characterization of the glycosylated ORF product, coupled with the immunological tools generated here, make it now possible to further address the function(s) of the ORF gene product(s). Important questions concern the expression and modification of ORF proteins in murine mammary and lymphoid tissues, whether the ORF product(s) has a functional role(s) in the virus life cycle or in mammary tumorigenesis, and whether the ORF protein(s) is directly involved in the murine superantigen response.

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