Expression of bovine leukemia virus *ENV* glycoprotein in insect cells by recombinant baculovirus

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Abstract The gp51-p30 glycoprotein constituting BLV envelope was expressed in Sf-21 insect cells by means of recombinant baculoviruses. Post-infection cell lysates were analyzed, in order to define the immunologic reactivity of recombinant products. Oligosaccharide chains, containing *N*-acetylglucosamine, mannose, galactose and sialic acid were found on recombinant gp51-p30. In order to investigate the timing of transcription and translation of the glycoprotein, kinetic assays were carried out on cell lysates and directly in situ on Sf-21 cells during the course of baculovirus infection. The use of different solubilizing reagents was also evaluated in order to rescue recombinant glycoprotein from its subcellular location.

Key words: Baculovirus; Biotechnology; Bovine leukemia virus; Glycosylation; Recombinant glycoprotein

1. Introduction

Baculovirus expression vector system (BEVS) is generally regarded as one of the most promising and versatile expression systems currently available [1]. Insect cell lines usually allow production of recombinant proteins with very large yields, usually not reached with other expression systems. A peculiar feature of this expression system is its ability to carry out post-translational modifications on recombinant proteins [2,3]. Particularly relevant to the study presented here is the research on the expression of fowl plaque virus hemagglutinin [2].

Bovine leukemia virus (BLV) is a retrovirus, belonging to BLV-HTLV genus, responsible for enzootic bovine leukosis (EBL), one of the most diffused retroviral infections of domestic cattle. The disease is characterized by evolution of persistent lymphocytosis, and in some cases B-cell lymphomas may develop [4,5]. BLV envelope proteins can elicit a strong and long-lasting immune response in infected cattle [4]. Indeed, earliest phases of BLV infection can be diagnosed by serological techniques, based on gp51 antibody detection [6], as well as by molecular techniques, using DNA probe hybridization [7] and in vitro amplification of viral genome sequences [8,9]. Moreover, gp51 was also used to produce vaccines against BLV infection. Howewer, a fully protective vaccine against BLV has not been obtained yet [10,11].

One of the main topics currently under investigation in biotechnology is the search for eukaryotic expression systems able to synthesize glycoproteins carrying oligosaccharide chains very similar to those found on native glycoproteins. Glycosylation is known to play a main role in conferring biological properties to *env* proteins of BLV [12,13], so this protein can be considered a suitable model to be used in this study. The aim of this paper is the analysis of recombinant *env* protein of BLV, expressed by engineered BEVS. We chose this model to evaluate expression of glycosylated proteins in insect cells and to compare recombinant gp51-p30 to the native BLV*env* glycoprotein synthesized by mammalian cells.

2. Materials and methods

2.1. Bacterial strains and plasmids

pGEM-1224 plasmid, containing a *Bgl*II-*Bgl*II fragment (nucleotides 5084–6308) of BLV*env* gene, was kindly provided by S. Gaudi (Fondation Merieux, Lyon, France). TA-cloning of the PCR product was carried out in pCRII plasmid (Invitrogen). Both pCRII and pGEM-1224 plasmids were amplified in JM 109 bacterial strain, while the baculovirus shuttle plasmid used in this work, pBlueBacHis (Invitrogen), was amplified in Top 10 bacterial strain (Invitrogen), according to current protocols [14,15].

2.2. Insect cell lines and viral vector

Sf-9 or Sf-21AE cells [16] were grown in Grace's medium (Invitrogen) or in SF900 medium (Gibco), supplemented with 5% fetal calf serum (FCS) and gentamycin (50 mg/ml). In some experiments, a *Trichoplusia ni* cell line, High 5, [17] was also used. Wild-type AcMNPV (*Autographa californica* multiple nuclear polyhedrosis virus) stocks (Invitrogen), as well as recombinant baculoviruses produced in this experiment, were propagated in Sf-9 or Sf-21 cells as described by Summers and Smith [18].

2.3. Cloning in pBlueBacHis vector

BLV*env* insert (nucleotides 5131–6158) was amplified by polymerase chain reaction (PCR) from pGEM-1224 plasmid [7] by means of two synthetic oligonucleotides: SP6 (Promega) and a specific BLV*env* (6158-CCGATTATCCTTATCCGAATCTTGTTC-6131) primer. Amplified DNA, cut by *Nco*I-*Hin*dIII restriction endonucleases (1100 bp), was cloned in pBlueBacHis-B shuttle vector, downstream the *polh* promoter cassette. DNA ligation, transformation and selec-

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Abbreviations: AcMNPV, *Autographa californica* multiple nuclear polyhedrosis virus; BCA, bichinchoninic acid protein quantitative assay; BEVS, baculovirus expression vector system; BLV, bovine leukemia virus; bp, base pair; BSA, bovine serum albumin; DEPC, diethyl-pyrocarbonate; FCS, fetal calf serum; FLK, fetal lamb kidney cell line; FLK-gp51, gp51 purified from persistently infected FLK cell; gp51-p30, glycoproteins constituting BLV envelope; kbp, 10^3 base pairs; kD, 10^3 daltons; MAb, monoclonal antibody; MOI, multiplicity of infection; *occ*$^-$, AcMNPV plaques showing *polh*$^-$ phenotype; PBS, phosphate buffered saline; PCR, polymerase chain reaction; p.i., post infection; *polh*, polyhedrin coding gene of AcMNPV; PRINS, primed in situ labelling of nucleic acids; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis

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tion of recombinant Top 10 bacteria were carried out according to current protocols [15]. Before cotransfection, recombinant plasmids were extracted, analyzed and sequenced, in order to verify proper insert positioning in shuttle plasmid [19].

2.4. Production, screening and purification of recombinant baculovirus strains

Linearized AcMNPV DNA and pBlueBacHis-*env* plasmid were cotransfected into Sf-21 cells by cationic liposomes [20]. Recovered supernatants underwent plaque screening by a colorimetric method based on *lac*Z gene expression, looking for *polh*$^-$ (*occ*$^-$) and blue plaques [21]. Supernatants were also assayed for recombinant DNA by PCR, using a pair of commercial 'F' and 'R' primers (Invitrogen) or a specific 'SecBac' (CTAGCATGACTGGTGGACAG) and 'R' primers pair. Plaque assays and PCR amplification were carried out twice on putative positive supernatants.

2.5. Small scale infections

Sf-21 or Sf-9 cells were plated and infected at low multiplicity of infection (MOI) with aliquots of recombinant viral supernatants (A1, A2, 23), and rescued at 72 h post infection (p.i.) or at 90% cell lysis. In kinetic experiments, infections were stopped 6–96 h p.i., and samples were processed as described below.

2.6. SDS-PAGE, Western and lectin blot analysis

Cell lysates were resolved on 14.9% acrylamide gels according to Neville's protocol [22]. Cell pellets and lyophilized infection supernatants were directly solubilized in sample buffer (final concentrations: 10 mM Tris-HCl, pH 8, sodium dodecylsulfate 2%, β-mercaptoethanol 2%). Uninfected Sf-21 cells as well as Sf-21 cells infected with wild-type AcMNPV were similarly processed and run as negative controls. Purified gp51, obtained from persistently infected fetal lamb kidney cells (FLKgp51), was kindly provided by M. Merza (Dept. of Veterinary Virology, Uppsala, Sweden), and used as positive control in some experiments. Equal amounts of each sample were run on SDS-PAGE gels and analyzed by Coomassie blue or silver staining. Similar SDS-PAGE gels were blotted on nitrocellulose sheets as described [23], and probed overnight with MAb 51/05 (1:1000–1:4000) [24] or with pools of BLV-positive or negative bovine sera, previously collected from dairy cattle in some Northern Italy farms. Reactions were revealed by secondary peroxidase-conjugated antibodies (1:500–1:1000), and colorimetric reactions were developed with H_2O_2 and α-chloro-naphthol (Bio-Rad). A complete series of protein lysates, similarly blotted on nitrocellulose filters, was assayed with a panel of 17 biotinylated lectins (Vector) [25], in order to assess the type of glycosylation on recombinant molecules. Lectins and their specificities are presented in Table 1.

2.7. In situ analysis: primed in situ labelling (PRINS) on specific mRNAs and indirect immunofluorescence analysis

$8–10\times10^5$ Sf-9 cells were allowed to adhere on sterile glass slides pretreated overnight with 20% FCS in SF900 medium and infected at a low MOI with recombinant baculovirus strain 23. Slides were fixed at different times p.i. (0, 6, 12–96 h p.i.) with 4% paraformaldehyde in PBS, in order to permeabilize the cell membranes. Slides were washed three times with PBS plus 0.5% Triton X-100, air dried and frozen at −20°C in sealed polyethylene bags. All reagents were treated with 0.1% diethylpyrocarbonate (DEPC) and current cautions to prevent mRNA degradation were also taken [15]. Uninfected cell samples were similarly processed. Samples, predigested with RNase-free DNase, underwent PRINS reaction by applying primer-mediated reverse transcriptase mRNA labelling [26]. Biotinylated cDNAs were revealed in situ by a colorimetric reaction developed according to manufacturer's instructions (Gene-Tect, Clontech). Images were acquired and analyzed by means of both light and confocal microscopy. A similar complete series of permeabilized cell samples, collected from recombinant and wild-type infections, was assayed with MAb 51/05 to evaluate recombinant protein localization. Samples were treated with blocking solution (BSA 3% and 10% normal rabbit serum in PBS) before incubation with MAb 51/05 (1:100–1:500 in PBS) in a moistened chamber. Reactions were developed with a rabbit FITC-conjugated secondary antibody and analyzed by both fluorescent and confocal microscopy.

2.8. Antigen solubilization assays

On the basis of kinetic assay results, Sf-21 cells infected with recombinant viral strain 23 were recovered at 72 h p.i. and lysed by different reagents: 3 M urea; 3 M urea plus 4% Tween-20; 8 M urea; 8 M urea plus 4% Tween-20; 4% Tween-20 in PBS; 10% Tween-20 in PBS; 2% octyl-β-D-glucopyranoside in PBS. After treatment, cell lysates, clarified by low speed centrifugation, were extensively dialyzed against distilled H_2O, lyophilized and resuspended in distilled sterile H_2O. Protein concentration was determined by BCA assay (Pierce), and equal amounts of proteins were run on 14.9% SDS-PAGE gels, as described. After silver staining, recombinant protein was quantified by densitometry (Cream 4.0; Chem-en-tec).

3. Results and discussion

3.1. Recombinant gp51-p30 expression

The BLV*env* gene codes an *env* precursor protein, gp72. The N-terminus is processed to form gp51, external glycoprotein, while the C-terminus corresponds to the transmembrane protein p30 [5]. The cloning strategy is shown in Fig. 1. The insert, corresponding to nucleotide position 5431–6158, was cloned in pBacBluHis (polylinker version 'B'), maintaining the primitive *env* reading frame. Nucleotide sequence of recombinant plasmids was determined before cotransfection.

A 1:3 mix of linearized AcMNPV [20] and pBlueBacHis-

Table 1
Lectin blot assays

Lectin	Abbreviation	Binding specificity	A1	A2	23	FLK-gp51
Concanavalin A	ConA	α-Man	+	+	+	+
Pisum sativum agglutinin	PSA	α-Man	+	+	+	+
Lens culinaris agglutinin	LCA	α-Man	+	+	+	+
Peanut agglutinin	PNA	Galβ1-3GalNAc (*O*-linked)	−	−	−	−
Ricinus communis agglutinin	RCA	β-Gal, β-GalNAc	−	−	−	+
Sophora japonica agglutinin	SJA	Terminal β-Gal, β-GalNAc	−	−	−	−
Soybean agglutinin	SBA	Terminal β-Gal, β-GalNAc	−	−	−	−
Dolichos biflorum agglutinin	DBA	α-GalNAc	−	−	−	−
Bandeiraea simplicifolia agglutinin	BSA	α-Gal, α-GalNAc	−	−	−	+
Wheat germ agglutinin	WGA	Terminal NeuNAc (GlcNAcβ1-4)	+	+	+	+
Triticum vulgaris agglutinin	SuccWGA	*t*-[(β-1,4)GlcNAc]$_2$	−	−	−	+
Ulex europeas agglutinin	UEA	α-Fuc	−	−	−	−
Phaseolus vulgaris agglutinin	PHA-E	Galβ1-4GalNAc	+	+	+	+
Phaseolus vulgaris agglutinin	PHA-L	Tri-tetra-antennary complex *N*-linked oligosaccharides	−	−	−	+
Datura stramonium agglutinin	DSA	Galβ1-4GalNAc	+	+	+	+
Maachia amurensis agglutinin	MAA	α-2,3 Sialic acid	+	+	+	+
Galanthus nivalis agglutinin	GNA	Terminal α-Man	−	−	−	−

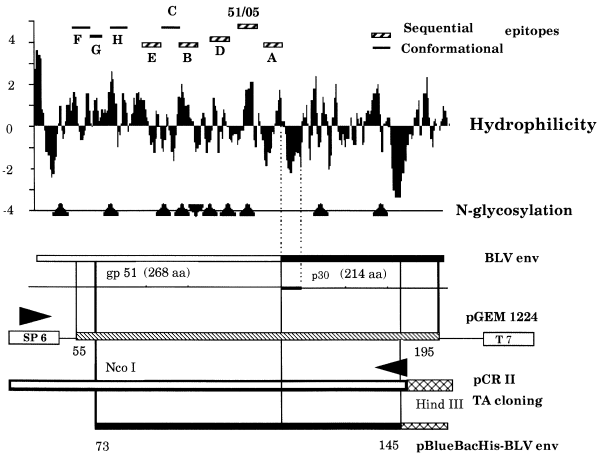

Fig. 1. Molecular organization and cloning strategy of BLV env glycoproteins. From the top: Hydrophilicity-hydrophobicity plot, also displaying major epitopes and *N*-glycosylation sites mapped on env glycoproteins; BLV*env* gene map, showing leader peptide and gp51-p30 boundary; env region cloned in AcMNPV vector.

BLV*env* DNA was mixed with cationic liposomes [21]. Liposome-mediated Sf-21 cell infection was allowed to proceed for 48 h. Putative recombinant baculoviruses were screened by plaque assay on agarose-X-gal medium [21].

PCR analysis with two semi-nested primer pairs (F+R, 1100 bp; SecBac+R, 838 bp) confirmed that the BLV*env* insert had successfully replaced *polh* sequence in three independent viral plaques, indicated as A1, A2 and 23 (clone 23 is shown in Fig. 2A).

SDS-PAGE analysis of three recombinant cell lysates showed a 51–55-kDa band that was absent in wild-type baculovirus-infected cells as well as in the same uninfected insect cell line (Fig. 2B). Control samples from wild-type infected cells showed only a 30-kDa band, corresponding to *polh* gene product. While gp72, glycosylated precursor of BLV envelope protein, is proteolytically processed into gp51 and p30 in mammalian cells, recombinant protein is not cleaved in insect cells. Bands at low molecular weight, detected by Western blot, might be produced by unspecific intracellular proteolysis.

3.2. Recombinant gp51-p30 analysis

Western blot analysis of A1, A2, 23 cell lysates showed that the 51–55-kDa band specifically reacted with MAb 51/05, a monoclonal antibody able to recognize a sequential epitope mapped at the C-terminus of gp51 BLV antigen. Eight major epitopes have been mapped on gp51 (Fig. 1): conformational epitopes C, F, G, H, are located at the gp51 N-terminus, while A, B, D, E sequential epitopes are found at the C-terminus [27]. Humoral immune responses raised by BLV infection are directed against gp51 conformational epitopes [28]: in fact, A1, A2, 23 and FLK-gp51 failed to react with a pool of bovine BLV-positive sera in Western blot assay (Fig. 3).

3.3. Recombinant gp51-p30 glycosylation pattern analysis

Glycosylation of gp51 antigen has been shown to play a

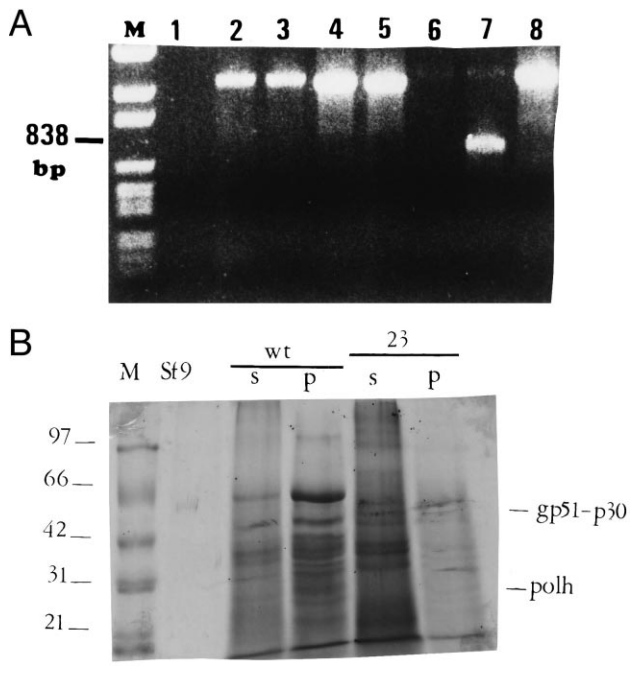

Fig. 2. Confirmation of a positive recombinant plaque (recombinant viral strain 23). A: PCR amplification of recombinant plaques, using the primer pair SecBac-R. M: Molecular weight standards (2600–60 bp); lane 1: mock reaction without viral DNA; lanes 2–6, 8: negative reactions; lane 7: positive amplification (838 bp). B: SDS-PAGE analysis of Sf-21 cell lysates. M: Molecular weight standards (97–14 kDa); Sf-9 uninfected cell lysate; Sf-9 cells infected by wild-type AcMNPV (s: supernatant; p: pellet); Sf-9 cells infected by recombinant baculovirus (strain 23); *polh*: polyhedrin (31 kDa); gp51-p30: recombinant product (51–55 kDa).

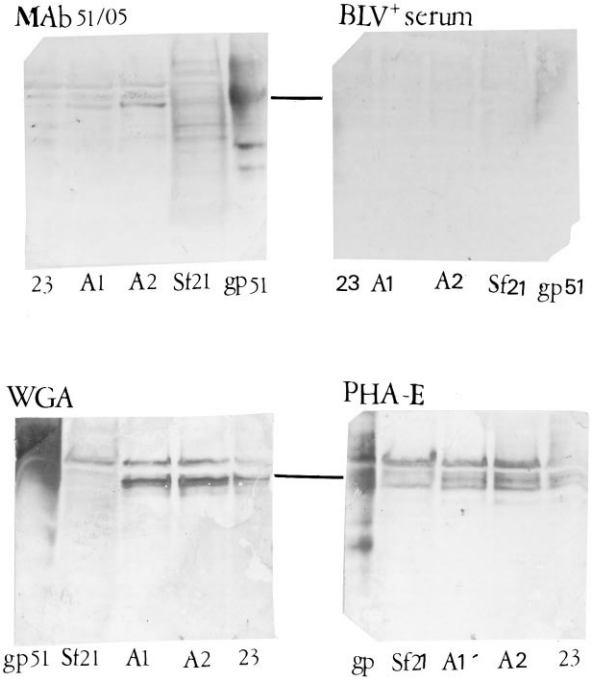

Fig. 3. Western blot and lectin blot analysis of recombinant glycoprotein gp51-p30. Top: Western blot analysis of cell lysates with the Mab 51/05 and a BLV-positive bovine serum. From the left, in both panels: Sf-21 cells infected by recombinant strains 23, A1, A2; uninfected Sf-21 cells and native gp51 from FLK cells. Bottom: Lectin blot analysis of recombinant gp51-p30 glycoprotein with biotinylated lectins WGA (wheat germ agglutinin) and PHA-E (erythrocyte-phyto-hemo-agglutinin). From the left, in both panels: native gp51 from FLK cells, uninfected Sf-21 and Sf-21 cells infected by recombinant strains A1, A2 and 23.

critical role in maintaining native conformation of this BLV antigen, as shown by immunological studies [29] and, more strikingly, by failure in protective immunity due to deglycosylated antigens assayed as subunit vaccines [12,13]. All native gp51 antigens produced in different BLV-infected mammalian cells were shown to be *N*-glycosylated, while *O*-glycosylation was never described on this molecule [25]. Recombinant gp51-p30 produced in *Spodoptera frugiperda* cells underwent glycosylation, as shown by lectin blot experiments (Fig. 3). As shown in Table 1, *N*-acetylglucosamine, mannose, galactose and sialic acid residues were found on recombinant protein. These sugars have also been found on native gp51 purified from FLK cells, as well as on gp51 expressed in eukaryotic cell lines [25]. Fucose and *O*-linked oligosaccharides were not found on gp51 from FLK cells, as well as on recombinant gp51-p30. This latter result may indicate that sugar residues

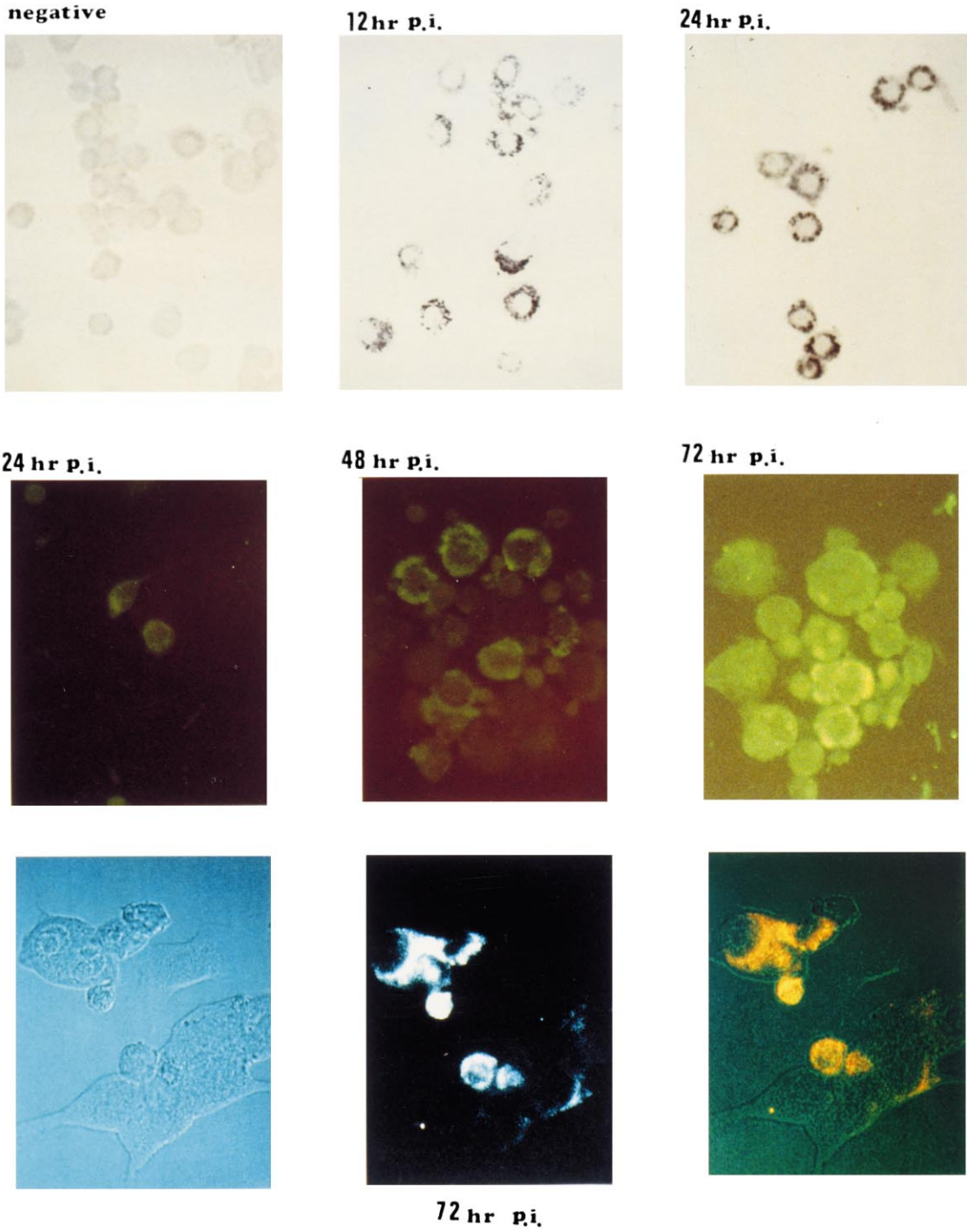

Fig. 4. In situ analysis of *env* mRNA by PRINS and intracellular staining of recombinant gp51-p30 glycoprotein by indirect immunofluorescence. Top, from the left: PRINS labelling of negative cells; PRINS labelling of mRNAs in infected cells (strain 23, 12 and 24 h p.i.). Second row: Intracellular detection of gp51-p30 recombinant glycoprotein by fluorescence microscopy, at 24–72 h p.i. Third row: Confocal image analysis. From the left: Cell morphology; immunostaining of the same field; superimposed images.

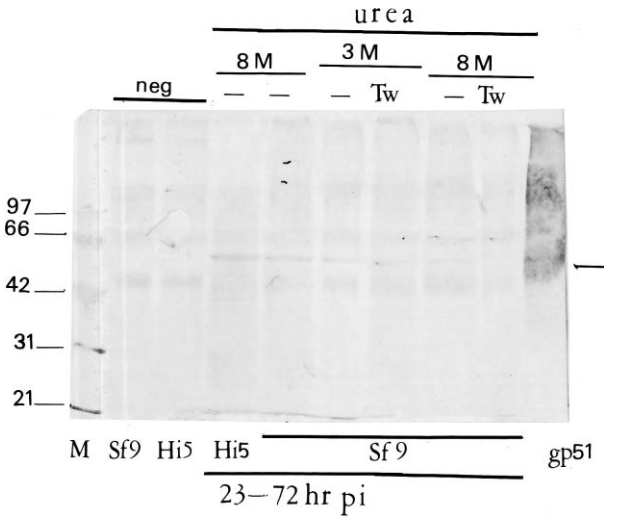

Fig. 5. Western blot analysis of cell lysates infected by recombinant strain 23, after solubilization assays. From the left: M: molecular weight standards; Sf-9 and High5 uninfected cell lysates; High5 and Sf-9 infected cell lysates, solubilized in 8 M urea; Sf-9 infected cell lysates solubilized in 3 M urea and in 3 M urea plus 4% Tween-20; Sf-9 cell lysates solubilized in 8 M urea and 8 M urea plus 4% Tween-20; untreated FLK-gp51.

on recombinant glycoproteins are more likely arranged in *N*-linked mono- or bi-antennary chains, and, as shown by PHA-L lectin lacks reactivity.

3.4. Kinetic analysis of expression

Kinetic analysis of cell lysates showed that recombinant proteins were poorly detectable at all infection stages, even though they accumulated mostly in 'late' infection phases (48–72 h p.i.) (data not shown).

Scarce reactivity of recombinant gp51-p30 is not imputable to ineffective transcription and translation, as shown by both PRINS and immunofluorescence results (Fig. 4). gp51-p30 mRNA could be already detected at 12 h p.i. and, more clearly, at 24 h p.i. The recombinant protein became fully detectable by immunofluorescence at 48 h p.i. These results are consistent with current data on *polh* gene regulation and expression, suggesting that the formation of intracellular 'inclusion bodies' may entrap the recombinant antigen in an insoluble form.

3.5. Solubilization of inclusion bodies

Inclusion bodies are more likely formed when expression levels of heterologous proteins are very high [30], or in response to toxic effects exerted by the expressed product on host cell metabolism. These considerations are particularly valid for baculovirus-infected cells, because the *polh* can drive the expression of heterologous genes to considerably high levels (up to one third of total cell proteins). Moreover, the presence of hydrophobic stretches of amino acids in heterologous proteins, or their improper folding might cooperate in promoting the intracellular precipitation of recombinant antigens: particularly in late phases of baculovirus infection, when host cell functions decline [31,32].

In order to improve the detection of recombinant gp51-p30, crude cell lysates underwent different solubilization protocols: urea at different concentrations (3 M and 8 M), detergents (4% and 10% Tween-20; 2% octyl-β-D-glucopyranoside) and combination of urea and detergents (3 M urea plus 4% Tween-20; 8 M urea plus 4% Tween-20) (Fig. 5).

Best results in glycoprotein solubilization, as determined by densitometric determination after SDS-PAGE and silver staining, were achieved by 3 M urea lysis buffer (10.1%), followed by 4% Tween-20 (5.59%), 10% Tween-20 (5.17%) and 2% octyl-β-D-glucopyranoside (5.41%).

4. Conclusions and perspectives

Recombinant gp51-p30 appears to be glycosylated: the sugar residues on this product are very similar to those identified on native glycoproteins [25]. *O*-linked glycans were neither found on native gp51 produced in mammalian cells nor on recombinant gp51-p30. Carbohydrates are more likely arranged in *N*-linked oligosaccharides, albeit displaying more simple structures than glycans found on native glycoprotein. However, due to the essential role played by glycosylation in maintaining the conformation of major epitopes present on native gp51, even small differences in glycosylation patterns might influence reactivity and biologic activity of recombinant protein [33]. This will require a more detailed analysis on recombinant gp51-p30.

In the present experiment, both the high efficiency of *polh* promoter and the presence of C-terminal hydrophobic amino acids stretches might have cooperated to determine the gp51-p30 precipitation in cytoplasmic aggregates. Urea and detergents proved to be useful to solubilize recombinant glycoprotein, the former displaying the highest effectiveness. In spite of several drawbacks, insect cells should be considered a feasible eukaryote host to express glycoproteins. Our results show gp51-p30 sugar residues are very similar to those found on native gp51 expressed in mammalian cells, while nearly all recombinant and even native glycoproteins 'made in insect cells' were shown to carry *N*-acetylglucosamine and mannose residues only [2,3]. To date, human plasminogen appears to be the unique other recombinant glycoprotein expressed by BEVS carrying complex-type *N*-glycans [34]. Present result might be due to gp51-p30 precipitation in insoluble aggregates, which helps recombinant protein to escape lytic enzymes abundantly produced by insect cells [3]. Such a result will require further investigation, not only to analyze fine glycosylation patterns on recombinant gp51-p30 but also to throw new light on insect cells potential in glycan synthesis and oligosaccharide trimming.

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