A Subset of Pr65^{gag} Is Nucleus Associated in Murine Leukemia Virus-Infected Cells

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Nuclei of cells infected with Moloney murine leukemia virus (MoMuLV) were examined for the presence of *gag* proteins. This analysis was performed in conjunction with other studies suggesting a possible role for *gag* proteins in regulating nuclear events relating to processing and/or transport of viral genomic RNA. We detected Pr65^{gag} and a p30-related protein in a nuclear fraction of infected cells. We also found evidence that a highly conserved amino acid sequence, which is shared by p30 and U1 small nuclear ribonucleoprotein 70-kDa protein, is a component of the nuclear targeting sequence for Pr65^{gag}. Immunoelectron microscopy studies with a monoclonal anti-p12 antibody established that approximately 18% of *gag*-containing proteins of MoMuLV are located in the nucleus. Such *gag*-containing proteins from a mutant MoMuLV that lacks N-terminal myristic acid had greater affinity for the nucleus, suggesting that fatty acid acylation of Pr65^{gag} plays a role in overcoming the proposed nuclear transport signal. The possible roles that nuclear *gag* proteins may play in retroviral replication are discussed.

The primary translation product of the *gag* gene of all retroviruses is a precursor polypeptide that is processed by the virus-encoded protease into individual proteins (6). The *gag* precursor of type C murine leukemia viruses (MuLV), termed Pr65^{gag}, is composed of p15 (MA), p12, p30 (CA), and p10 (NC) (1). Pr65^{gag} is cleaved in a stepwise manner to yield intermediate cleavage products of Pr27 (p15 and p12) and Pr40 (p30 and p10) (19, 24), which are further cleaved in assembled viral particles to yield the four structural proteins. Though each protein has its separate role, the *gag* proteins are involved collectively in the assembly of viral components at the cell membrane prior to and during packaging of virions and also comprise the core of the new virus particle (6). Our unpublished findings have suggested to us that the *gag* gene products of MuLVs have other functions that may involve transport and/or splicing of viral RNAs. Therefore, we have searched for the presence of *gag* protein in the nucleus of cells infected with Moloney MuLV (MoMuLV). We report the presence of MoMuLV *gag* protein in the nucleus of infected cells and also present data that indicate that the p30^{gag} protein contains a candidate nuclear targeting signal (NTS).

MATERIALS AND METHODS

Cell lines and viruses. Uninfected NIH 3T3 cells and TR239A cells infected with an MoMuLV mutant (a Gly-2→Ala substitution) that cannot make myristylated *gag* (29) were maintained in Dulbecco modified Eagle medium supplemented with 10% calf serum. KBM NIH 3T3 cells (a gift from Karen Beemon, Johns Hopkins University) infected with wild-type clone 1 MoMuLV and normal rat kidney (6m2) cells infected with Moloney murine sarcoma virus (MuSV) strain ts110, which is temperature sensitive for transformation (2), were maintained on McCoy's medium supplemented with 12.5% fetal calf serum. The TR239A cells

were provided by Alan Rein (National Cancer Institute, Frederick Cancer Research Facility, Frederick, Md.).

Antisera. Monoclonal mouse anti-p12 serum was obtained from Bruce Chesebro (4). Polyclonal goat anti-p30 and anti-p10 sera were obtained from the National Cancer Institute. Mouse monoclonal antibody Y12 (18), directed against Sm antigen (the major antigenic component of small nuclear ribonucleoproteins [snRNPs] involved in splicing), was provided by Joan Steitz (Yale University, New Haven, Conn.). The mouse monoclonal antibody and the rabbit polyclonal anti-p10 antibody were partially purified by ammonium sulfate precipitation (12). Anti-phosphoglycerate mutase (anti-PGM) serum was provided by Robert J. Schwartz (Baylor College of Medicine, Houston, Tex.).

Western immunoblot assay. The procedure described previously by Tobin et al. (34) was used in these studies.

Radioimmunoprecipitation of viral proteins and preparation of cell extracts. The procedure was performed on cells radiolabeled by incubation in medium containing [³⁵S]methionine/cysteine translabel (ICN, Costa Mesa, Calif.) basically as described previously (23). Briefly, cells were rinsed with phosphate-buffered saline (PBS) and scraped into lysis buffer containing 30 mM Tris (pH 7.5), 125 mM KCl, 5.7 mM MgCl₂, 3.6 mM CaCl₂, 0.5 mM EDTA, 0.5% Nonidet P-40 (NP-40), and 0.1% sodium deoxycholate (DOC). Protease inhibitors trasylol (100 U/ml) and sometimes phenylmethylsulfonyl fluoride (1 mM) were added to the lysis buffer before use. The cells were lysed by 10 strokes with a tight-fitting pestle in a Dounce homogenizer, and the nuclei were pelleted by centrifugation at 12,000 × g. The nuclear fraction was either boiled in sample buffer (17) for 5 min (for Western assays) or sonicated in lysis buffer for three 20-s bursts at a setting of 4 in a Virtis Virsonic 300 sonicator (for immunoprecipitation experiments). The cytoplasmic fraction was either used as described (for immunoprecipitation assays) or precipitated with acetone for 10 min at 4°C, washed with 80% acetone, and boiled in sample buffer (for Western assays). Samples were separated on polyacrylamide gels

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FIG. 1. (A) Western blot analysis of nuclear and cytoplasmic extracts of MoMuLV-infected cells with a monoclonal antibody against p12. NIH 3T3 cells (lane 3) and MoMuLV-infected KBM cells were fractionated into cytoplasmic (lanes 1, 4, and 6) and nuclear (lanes 2, 5, and 7) fractions. The cytoplasm was precipitated with 80% acetone; the pelleted cytoplasmic fraction and nuclei were solubilized in Laemmli sample buffer and separated on a 10% polyacrylamide gel. The proteins were electroblotted onto nitrocellulose and reacted with monoclonal anti-p12 (lanes 1 to 3), monoclonal anti-Sm (lanes 4 and 5), and polyclonal antivimentin (lanes 6 and 7) sera. (B) Nuclear and cytoplasmic fractions of MoMuLV-infected cells were analyzed with various antisera. Cytoplasmic (lanes 1, 3, 5, and 7) and nuclear (lanes 2, 4, 6, and 8) fractions were analyzed with polyclonal anti-Gag sera made against p10 (lanes 1 and 2) and p30 (lanes 3 and 4), and with a monoclonal anti-p12 antibody (lanes 5 and 6). Polyclonal antiserum made against the cytoplasmic protein PGM was also used (lanes 7 and 8). Lane M, molecular size standards (in kilodaltons).

(17), treated with 0.5 M sodium salicylate (3), and exposed to preflashed Kodak X-Omat X-ray film (Kodak, Rochester, N.Y.).

A second method of cell fractionation was sometimes used. It is basically the method described by Lerner and Steitz (18) but with modifications. Cells were scraped, washed by centrifugation twice in PBS, and suspended in buffer A (140 mM NaCl, 10 mM Tris-HCl [pH 7.5], 1.5 mM MgCl₂) at 5×10^6 cells per 0.3 ml. The cells were lysed by 10 strokes of a Dounce homogenizer (tight pestle), incubated for 3 min on ice, and given 10 more strokes in the homogenizer. The mixture was underlayered with 1 ml of buffer B (10 mM Tris-Cl [pH 7.5], 5 mM MgCl₂, 0.8 M sucrose, 0.1% Tween 40, 0.05% DOC) and spun for 0.5 min in a microcentrifuge. We modified this procedure to use the detergent mixture favored by Feeney and Zieve for purification of nuclei (8). The cytoplasmic upper phase was retained and prepared for analysis as described above. The pellet was washed by centrifugation successively with 1 ml of buffer C (0.3 M sucrose, 8 mM KCl, 1.5 mM MgCl₂, 15 mM Tris-Cl [pH 7.5]) and buffer D (100 mM NaCl, 1 mM MgCl₂, 10 mM Tris-Cl [pH 7.5]). The pellet was then suspended in Laemmli sample buffer and prepared as described above.

Immunoelectron microscopy. The procedure was performed as described previously (5). Cell pellets were fixed with 1% glutaraldehyde–1% paraformaldehyde, dehydrated in dimethylformamide, and embedded in Lowicryl K4M for thin sectioning. The sections were reacted with monoclonal anti-p12 antibody and then again with gold-conjugated anti-mouse serum. The sections were examined with a JEOL 1200EX transmission electron microscope.

RESULTS

Pr65^{gag} cofractionates with both nuclear and cytoplasmic fractions. Before we could attempt to detect *gag* gene-encoded proteins in the nucleus of virus-infected cells, we first had to determine the most advantageous method of

preparing nuclei. Previously, it has been shown that use of detergent in nuclear preparations results in the leakage of nuclear proteins into the cytoplasmic fraction (8), which we also observed in our own work (see below). Thus, our initial experiments were performed with nuclei prepared in the absence of (or in the presence of very low concentrations of) detergents. However, these preparations proved unsatisfactory, as they obviously were contaminated with cytoplasmic proteins. Despite the nuclear leakage problem, we then used a rather harsh method to prepare nuclei that involves treatment with NP-40 and DOC (see Materials and Methods). The cytoplasmic and nuclear fractions were analyzed by Western immunoblotting, using various antisera to detect *gag*-containing proteins. We were somewhat surprised to detect a substantial amount of the *gag* precursor polypeptide (Pr65^{gag}) in the nuclear fraction from infected cells (Fig. 1A, lane 2) despite the use of a harsh method for preparing nuclei. No similar protein was detected in extracts made from unfractionated, uninfected NIH 3T3 cells (lane 3). The presence of Pr65^{gag} in these nuclear preparations was not the result of cytoplasmic contamination, as identically prepared nuclei were free of detectable contamination of vimentin (lane 7), a highly insoluble cytoskeleton protein that was easily detected in the cytoplasm (lane 6). No other *gag* protein was detected in the nuclei with this antibody (monoclonal anti-p12) even though there was a substantial amount of the precursor protein Pr27^{gag} (p15–p12) present in the cytoplasmic fraction (lane 1). This finding is relevant because the N-terminal part of Pr27^{gag} (p15) is highly hydrophobic (containing myristic acid at its amino terminus) and hence tightly bound to cellular membranes. Thus, the absence of Pr27^{gag} in the nuclear fraction (lane 2) is further proof that our nuclear preparations are free from cytoplasmic protein or membrane protein contamination. It was also interesting to note that no Sm protein was detectable (lane 5) in the nuclear fraction prepared in this manner (23) but was readily detected in the cytoplasm (lane 4). Sm proteins are

FIG. 2. Radioimmunoprecipitation of MoMuLV-infected cells. MoMuLV-infected cells were labeled for 5 min with 500 μ Ci of 35 S translabel per ml and then incubated with unlabeled growth medium for 0 min (lanes 1, 3, 5, and 7) or 15 min (lanes 2, 4, 6, and 8). Cytoplasmic (lanes 1 to 4) and nuclear (lanes 5 to 8) extracts were made and immunoprecipitated with anti-p30 serum (lanes 1, 2, 5, and 6) or anti-p10 serum (lanes 3, 4, 7, and 8). Lane M, molecular size standards (in kilodaltons).

the core components of snRNPs and are very abundant in the nucleus (18). These data confirm an earlier report that nuclear proteins are easily removed from the nucleus by detergent treatment (8) and also suggest that Pr65^{gag} is tightly associated with structures in the nucleus, preventing its exit to the cytoplasm.

The question of cytoplasmic contamination was examined further in a second experiment (Fig. 1B). Nuclear (lanes 2, 4, 6, and 8) and cytoplasmic (lanes 1, 3, 5, and 7) fractions of KBM cells were assayed by immunoblotting, using three different anti-*gag* sera and antiserum that detects the cytoplasmic protein PGM. Again the Pr65^{gag} precursor polyprotein was seen in the nuclear fraction (lanes 2, 4, and 6). However, we also detected appreciable amounts of p30 (lane 4) in the nuclear fraction. Again, lack of cytoplasmic contamination was demonstrated, because a strong Pr27 band was detected with anti-p12 in the cytoplasmic fraction (lane 5) but was not seen in the corresponding nuclear fraction (lane 6). Of interest, trace amounts of the p30-p10 precursor Pr40^{gag} were also detected in the nuclear fraction with p30 and p10 antisera (longer exposure of lanes 2 and 4). Also, PGM is clearly seen in the cytoplasm (lane 7) but is totally absent in the nuclear fraction (lane 8). The presence of some but not all *gag*-containing viral proteins in the nuclear fraction will be discussed later.

Kinetics of nuclear *gag* formation. We then performed an experiment designed to assess the kinetics of Pr65^{gag} migration into the nucleus. Infected cells were labeled with [35 S]methionine for 5 min and incubated with isotope-free growth medium for 0 or 15 min (Fig. 2). Nuclear and cytoplasmic preparations were made in the presence of both NP-40 and DOC (23) and were immunoprecipitated with one of two anti-*gag* sera, anti-30 serum (lanes 1, 2, 5, and 6), or anti-10 serum (lanes 3, 4, 7, and 8). Note the presence of p30 in the 15-min chase sample immunoprecipitated with anti-p30 (lane 2) but not with anti-p10 (lane 4). Longer chase periods (data not shown) reveal that p30 enters the nucleus

FIG. 3. Western blot analysis of nuclear and cytoplasmic extracts of a myristylation mutant of MoMuLV. Immunoblots were made by using extracts of TR239A cells expressing the myristylation mutant virus prepared as described in the legend to Fig. 1. Cytoplasmic fractions (lanes 2, 4, and 6) and nuclear fractions (lanes 1, 3, and 5) were reacted with polyclonal anti-p30 serum (lanes 1 and 2), monoclonal anti-12 serum (lanes 3 and 4), and polyclonal anti-PGM serum (lanes 5 and 6). Lane M, molecular size standards (in kilodaltons).

within an hour and reaches the steady-state level that we detect by immunoblotting within this time. These data indicated a rapid movement of Pr65^{gag} into the nucleus, as it was detected in the nuclear fraction within 15 min of being synthesized (lanes 6 and 8). Also, the glycosylated *gag* precursor protein (gPr85^{gag}) and P55^{gag} (present in longer exposures) were predominantly cytoplasmic. Moreover, the virtual absence of Pr65^{gag} in the nuclei at 0 min (lanes 5 and 7) indicates that the nuclear Pr65^{gag} observed was not the result of cytoplasmic contamination, since as much, if not more, Pr65^{gag} was seen in the cytoplasm at 0 min (lanes 1 and 3) as at 15 min (lanes 2 and 4).

Pr65^{gag} is known to be myristylated at its N terminus (12a, 29, 31). This myristylation event has been shown to result in the directed transport of the protein to the cell membrane where viral assembly occurs (29). We were curious about how the migration of Pr65^{gag} into the nucleus would be affected if myristylation of Pr65^{gag} was prevented. Thus, we performed Western immunoblotting assays on extracts from TR239A cells expressing the mutant MoMuLV which, because of a Gly-2→Ala mutation (29), is unable to make myristylated *gag* protein (Fig. 3). Pr65^{gag} was seen in the nuclear preparation from cells infected with mutant virus (lanes 1 and 3). Again, when we used anti-PGM to test our nuclear extracts, we observed no contamination by cytoplasm (lane 5).

Pr65^{gag} enters nuclei, as judged by immunoelectron microscopy. Our results are consistent with a recent publication regarding baculovirus-produced *gag* protein of human immunodeficiency virus type 1 (HIV-1) that could be detected by immunoelectron microscopy in the nuclei of infected insect cells (30). Also, when the signal for myristylation was removed, even more HIV *gag* protein was found in the nucleus. This finding is consistent with the fact that targeting signals are N-terminal dominant (10). Thus, removal of the N-terminal myristylation signal, which targets *gag* protein to the cell membrane (29), should then result in increased migration of Pr65^{gag} to the nucleus, presumably as a result of the now heightened influence of a downstream NTS(s).

Because our Western immunoblot data for a similar mutant (Fig. 3) were not quantitative, we performed essentially the same experiment with MoMuLV as was performed with HIV-1. Thin sections were made of cells infected with wild-type virus and of cells (TR239A) infected with virus carrying the mutation that has been shown to prevent myristylation of Pr65^{gag}. The sections were first reacted with monoclonal anti-12 antibody and then with gold-conjugated second antibody and examined under the electron microscope. After scoring the results from observations made from 12 representative cell sections, we found our electron microscope findings to be in complete agreement with those of the previously mentioned HIV study (30). We detected nuclear *gag* protein in cells infected with wild-type virus (Fig. 4C), which also supported our previous data. Approximately 18% of the intracellular immunogold was in the nucleus (Table 1). However, cells infected with the mutant virus exhibited significantly greater numbers of nuclear *gag* protein (Fig. 4B), as about 32% (or nearly twice the wild-type level) of the intracellular immunogold particles were found in the nucleus. Very little gold staining of uninfected NIH 3T3 cells (Fig. 4A) was seen, nor was there an obvious nuclear preference seen in these cells (Table 1). Our experiments reveal that nonmyristylated MoMuLV *gag* also migrates to the nucleus but in greater amounts than that of the wild type, as was reported for HIV-1 (30).

Does Pr65^{gag} contain an NTS? We have detected significant concentrations of two MoMuLV *gag* proteins, i.e., Pr65^{gag} and p30-related protein, in the nuclei of infected cells. No non-p30-containing *gag* proteins were found in the nucleus even though such proteins as Pr27^{gag} were very abundant in the cytoplasm. This finding seemed relevant because of the report that demonstrated that both p30 and the 70-kDa protein associated with U1 snRNP contain a common antigenic epitope (28). This epitope is highly conserved in primary sequence in the U1 snRNP and in the p30 of a number of mammalian retroviruses, yet it has been assigned no known function for either class of protein. Also, it has recently been shown that all of the U1 snRNP-specific proteins migrate to the nucleus prior to snRNP assembly (8), indicating that they must contain an NTS of their own. Therefore, we began to consider the possibility that this epitope (28), common to both proteins, may serve either p30 or the 70-kDa protein in the capacity of an NTS. This hypothesis is supported by the fact that this amino acid sequence is very basic, a prime requirement of virtually all previously identified NTSs. We have in our possession a strain of MuSV that we could use to examine that question. The ts110 mutant of MuSV exhibits a temperature-sensitive phenotype; i.e., it is temperature sensitive for cellular transformation (2). It is also a temperature-sensitive splicing mutant of MuSV (16, 11, 21). It has been shown to contain a central deletion of parental MuSV sequence such that the *gag* portion of the genome is fused out of frame with that of sequences of the *mos* proto-oncogene (which is the transforming agent of MuSV and its derivatives) and thus makes as the sole translation product from its primary transcript a truncated *gag* polypeptide, designated P58^{gag} (14, 22). Splicing of the 4-kb viral RNA genome (also a temperature-sensitive event) results in an in-frame fusion such that translation of the spliced RNA produces the transforming protein product, P85^{gag-mos} (13, 33). Also, both P58^{gag} and P85^{gag-mos} are myristylated (9) and presumably are targeted to the cytoplasmic membrane as is Pr65^{gag} of parental MoMuLV (12a, 29, 31). Primer extension sequencing experiments indicate that the P58^{gag} truncated *gag* polypeptide

would contain the purported NTS, while P85^{gag-mos} would not (22) (Fig. 5). Therefore, we were interested in what Western blot analysis of nuclear and cytoplasmic extracts from MuSV ts110 infected cells would reveal.

We observed that P58^{gag}, which contains one of the proposed NTSs (Fig. 5), was found in the nucleus at temperatures both permissive and nonpermissive for splicing (Fig. 6, lanes 2 and 4). However, P85^{gag-mos}, which is expressed in a temperature-sensitive manner and thus is not synthesized at the nonpermissive temperature (lanes 1 and 2), lacks all of the proposed NTSs and was not found in the nuclear fraction of cells maintained either at the nonpermissive temperature (lane 2) or at the permissive temperature (lane 4). P85^{gag-mos} was present, however, in the cytoplasmic sample isolated from cells grown at the permissive temperature (lane 3). Since both *gag* and viral *mos* sequences are known to be cytoplasmic, the presence of P58^{gag} but not P85^{gag-mos} in the nucleus suggests that the NTS responsible for *gag* migration into the nucleus resides on the C-terminal portion of p30 (sequence 3 of Fig. 5) and overlaps with the homologous sequence found in U1 snRNP 70-kDa proteins.

DISCUSSION

The retroviral *gag* gene plays a dominant role in the assembly of new virus particles at the cell membrane, and its proteins comprise the core components of the new virion (6). We are now the second laboratory to report the presence of a retroviral *gag* gene product in the nuclei of infected cells. The first laboratory studied HIV-1 and found the HIV *gag* polypeptide in the nuclei of cells when overexpressed in insect cells (30). Our results, on the other hand, were obtained with mouse cells productively infected with MoMuLV. The presence of *gag* protein in the nucleus of infected cells would suggest another role for that protein in the life cycle of retroviruses. Because of previously published work (15) suggesting a possible role for *gag* gene expression in regulating splicing of murine retrovirus genomic RNA, we undertook a variety of studies that led to the results described in this report. This proposed new role for the *gag* gene product has been buttressed recently by the report that a single nucleotide modification in the three-nucleotide stop codon between the *gag* and *pol* genes, resulting in the exclusive production of Pr200^{gag-pol} and no synthesis of Pr65^{gag}, also greatly reduces *env* protein production (26). We have obtained similar results (19a). Recent work has demonstrated that p10, the C-terminal component of Pr65^{gag}, is responsible for the dimerization of genomic RNA, a step essential for production of infectious virus because it is a prerequisite for packaging (27). These authors also reported finding dimer viral RNA in the infected cells but showed no data. In 1973, Fan and Baltimore published similar findings (7a). Also, an earlier report proposed that dimerization of viral RNA may occur in the nucleus (20). It is not clearly understood when and where dimerization of retroviral RNA occurs. Even if the final formation of dimer RNA occurs during maturation of the virus particle as proposed by Canaani et al. (2a), the process could initiate anywhere in the cell, even within the nucleus. However, no mechanism has been proposed to explain how such *gag*-initiated nuclear dimerization of viral RNA could occur. Therefore, there are at least two possible functions for the *gag* precursor polypeptide to perform in the nucleus of infected cells, i.e., to provide a *trans* regulatory role in splicing of viral RNA to create *env*-specific mRNA and to

FIG. 4. Immunoelectron microscopic analysis of *gag* antigen in the nucleus and cytoplasm of virus-infected cells. Uninfected NIH 3T3 cells (A), TR239A cells infected with the myristylation mutant virus (B), and KBM cells infected with wild-type MoMuLV (C) were fixed with glutaraldehyde-paraformaldehyde, embedded in Lowicryl KM4, sectioned, and reacted with monoclonal anti-p12 serum. Bound antibody was detected by using gold-conjugated second antibody. The regions of the cytoplasm (cyto), nucleus (nuc), and nuclear membrane (nm) are indicated. Arrows indicate representative gold particles in each field. The inset in panel C is a higher magnification indicating staining of virus particles with our antibody.

TABLE 1. Quantitation of *gag* protein content in the nucleus and cytoplasm by immunoelectron microscopy

Cells ^a	Avg no. of gold particles/cell section				% Nuclear
	Total (SD)		–BKG (3T3) ^b		
	Nucleus	Cytoplasm	Nucleus	Cytoplasm	
NIH 3T3	4.2 (2.6)	5.5 (2.1)			
TR239A	8.8 (4.5)	15.3 (6.2)	4.6	9.9	31.7
KBM	13.4 (6.6)	46.2 (19.6)	9.2	40.8	18.4

^a KBM cells are NIH 3T3 cells productively infected with MoMuLV. TR239A cells are cells expressing an MoMuLV mutant that fails to add myristate fatty acid to the amino terminus of Pr65^{gag}.

^b Data obtained by subtracting the corresponding gold particles per section of NIH 3T3 cells from virus-infected cell sections.

provide a nuclear source of p10 for initiating dimerization of viral RNA.

Proteins are directed to the nucleus as the result of NTSs. Many NTSs have been characterized (for recent reviews, see references 10 and 25). Originally, the NTS for simian virus 40 T antigen (Fig. 5) was taken as the canonical consensus sequence for the NTS. However, it is now thought to be the exception rather than the rule. Another review (7) discusses a bipartite consensus sequence that appears to be more representative of the NTS for the majority of nuclear targeted proteins. This sequence consists of 2 basic amino acids followed by a spacer of 10 amino acids and then a 3-of-5 basic amino acid motif. Also, less than 5% of nonnuclear targeted proteins examined in the SwissProt data base contained this bipartite NTS (7).

Evidence was obtained for the presence of an NTS in the C-terminal domain of HIV p24 (30). Our data also suggest that the C-terminal portion of the viral *gag* p30 very likely

FIG. 5. Comparison of proposed nuclear targeting sequences in p30 with published consensus targeting sequences. B, basic amino acid; X, any amino acid. Boxes indicate basic regions of the bipartite consensus (2 basic amino acids followed by a spacer of 10 amino acids and a second group in which 3 of 5 amino acids must be basic); underlining indicates basic amino acids in the MoMuLV or MuSV ts110 sequence. The number 454 corresponds to the amino acid residue of Pr65^{gag}; residue 433 begins the first of three tandem repeats that share homology with a similar tandem repeat in the 70-kDa protein present in U1 RNA snRNPs. In P58^{gag}, the *gag* gene sequence is interrupted at residue 454. In P85^{gag-mos}, *gag* residue 325 is fused in frame to viral *mos* residue 21 (22, 35). SV40, simian virus 40.

FIG. 6. Western blot analysis of nuclear and cytoplasmic extracts of MuSV ts110-infected cells for detection of a possible NTS within p30 sequences. Immunoblots of cells infected with MuSV ts110 were prepared as described in the legend to Fig. 1. Cytoplasmic (lanes 1 and 3) and nuclear (lanes 2 and 4) fractions of cells incubated at the nonpermissive (lanes 1 and 2) and permissive (lanes 3 and 4) temperatures for splicing were reacted with monoclonal anti-p12 serum. Lane M, molecular size standards (in kilodaltons).

contains an NTS. Moreover, the sequence shared by p30 and the 70-kDa protein of U1 snRNPs (28) was a likely NTS candidate, since apparently both proteins are targeted to the nucleus and both contain this highly conserved amino acid sequence (whose function has as yet not been determined). This region of the viral protein, starting with *gag* amino acid 454 (32), contains two perfect fits with the reported bipartite consensus sequence for an NTS, as shown in Fig. 5 (MoMuLV sequences 1 and 2). Finally, because a spacer of eight amino acids is also functional (7), a third fit with the bipartite consensus sequence is found in the same region (Fig. 5, sequence 3). This finding is of interest because the deletion in *gag*, which created the MuSV ts110 genome, occurred within the other two NTS consensus sequences (22). However, this third putative sequence would satisfactorily explain the presence of P58^{gag} in nuclear extracts as well as the absence of P85^{gag-mos} (Fig. 6), since it is present in P58^{gag} but would be removed by the RNA splicing event that forms the MuSV ts110 mRNA for P85^{gag-mos} (22). It could be argued that the failure to detect P85^{gag-mos} in the nucleus was due to enhanced cytoplasmic targeting supplied by the addition of *mos* sequences, but its C-terminal addition to *gag* sequences should not play a dominant targeting role to the hybrid protein if an NTS is included in the remaining *gag* sequence, since targeting sequences have been shown to be N-terminally dominant (10). The presence of P58^{gag} in the nucleus again argues for the presence of a p30-specific NTS, since no *gag* protein lacking p30 sequences was detected in any nuclear extracts (i.e., Pr27, the p15 and p12 precursor). Finally, had P85^{gag-mos} been detected in the nucleus, this finding would have totally refuted the hypothesis that the 70-kDa-like region of p30 was functioning as an NTS. Since P85^{gag-mos} was not found in the nucleus, that hypothesis remains viable. Though the 70-kDa protein homologous sequence present in p30 (Fig. 5, residues 435 to 456) does not itself contain a bipartite NTS, it therefore cannot provide this function for the 70-kDa protein.

We do not see de novo-synthesized p30 in nuclear preparations (Fig. 2), which supports the notion that nuclear-associated p30-related protein seen in Western immunoblots

is the product of proteolytic processing. Since the normal processing of *gag* polypeptide occurs in budding virus particles, we assume that nuclear p30 must be the product of cytoplasmic proteolytic processing by unencapsidated viral protease. Further studies are under way to pursue the role of Pr65^{gag} and the p30-related protein in the nucleus.

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