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# Characterization of Intracellular Precursor Polyproteins of Moloney Murine Leukemia Virus

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Intracellular Moloney murine leukemia viral precursor polyproteins were compared with mature viral proteins by immunoprecipitation and tryptic peptide mapping experiments. The results were consistent with precursor roles for $Pr65^{gag}$, $Pr200^{gag\text{-}pol}$, $Pr135^{pol}$, and $gPr83^{env}$. The glycosylated *gag* gene product $gPr85^{gag}$, although containing sequences characteristic of all four core proteins plus additional sequences not found in $Pr65^{gag}$, lacked a major tyrosine-containing p30 tryptic peptide, suggesting that $gPr85^{gag}$ is not processed to p30.

Reports from several laboratories have shown that the *gag*, *pol*, and *env* proteins (5) of murine leukemia viruses (MuLV's) are synthesized via higher-molecular-weight precursor polyproteins (2-4, 12, 21, 22, 25, 26, 30, 32, 33). In Rauscher MuLV (R-MuLV)-infected cells, *gag* proteins are primarily made via a 65,000-dalton polyprotein precursor, $Pr65^{gag}$ (3, 12), which is phosphorylated and cleaved to yield p30, p15, pp12, and p10, the mature virion core proteins (24). A unique precursor, termed $Pr200^{gag\text{-}pol}$ (12, 16, 17), is synthesized in R-MuLV-infected cells which contains the information from both the *gag* and *pol* genes. It appears that this large-molecular-weight read-through polyprotein is a precursor of virion reverse transcriptase (RT), but not a major source of the viral core proteins (12). The envelope proteins are initially synthesized as a glycosylated precursor of 90,000 daltons, $gPr90^{env}$, which is then cleaved and further glycosylated to yield gp69/71, p15E, and p12E (2, 4, 12, 23, 26, 30).

In this report, we present the antigenic and structural characterization of the proteins observed in Moloney MuLV (Mo-MuLV)-infected cells. Previous reports on the Moloney virus proteins have only compared the antigenic relationships of intracellular precursors to the mature proteins (25, 32). Our overall findings indicate that the mechanism of viral gene expression in Mo-MuLV-infected cells is similar in many respects to that observed in R-MuLV-infected cells. Furthermore, these results will be useful in the search for the transforming protein encoded by Moloney murine sarcoma virus because the sarcoma virus genome has extensive homology to the Mo-MuLV genome (9).

Mo-MuLV clone 1-infected NIH/3T3 cells were generated by infection at limiting dilution and were a kind gift of H. Fan (8). Cells were maintained in McCoy 5a medium supplemented with 15% fetal calf serum (27). Radioactive labeling of cells and virus was as previously reported (14). Monospecific antisera prepared against R-MuLV p30, p15, pp12, p10, gp69/71, and RT were obtained from the Logistics Program of the National Cancer Institute. All antisera were absorbed with excess uninfected mouse cytoplasmic extracts as described elsewhere (12), and anti-RT serum was further absorbed with excess disrupted R-MuLV as described (12). The Cowan I strain of *Staphylococcus aureus* was used as a second antibody for indirect immunoprecipitation (15). Immunoprecipitation and gel electrophoresis were as previously reported (12). Radioactive bands were visualized by fluorography with preflashed X-ray films (18). Peptide mapping involving cation-exchange chromatography or two-dimensional peptide fingerprinting was performed as previously reported (3, 17, 31).

Mo-MuLV-specific precursor polyproteins synthesized during a 15-min pulse-labeling period with [$^3$H]leucine are shown in Fig. 1 (lanes B to H). Anti-gp69/71 serum (lane C) precipitated a single band of radioactivity migrating at $\simeq$83,000 daltons ($gPr83^{env}$) as determined by using R-MuLV proteins as molecular weight standards (compare Fig. 1A and C). Note that the Mo-MuLV envelope precursor, $gPr83^{env}$, migrated faster in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) than its R-MuLV counterpart, $gPr90^{env}$. Antisera directed against each of the viral core proteins (Fig. 1E to H) precipitated bands of radioactivity migrating at 85,000 ($Pr85^{gag}$) and 65,000 daltons ($Pr65^{gag}$). Recent studies have shown that $Pr85^{gag}$ incorporated [$^3$H]mannose, bound to a

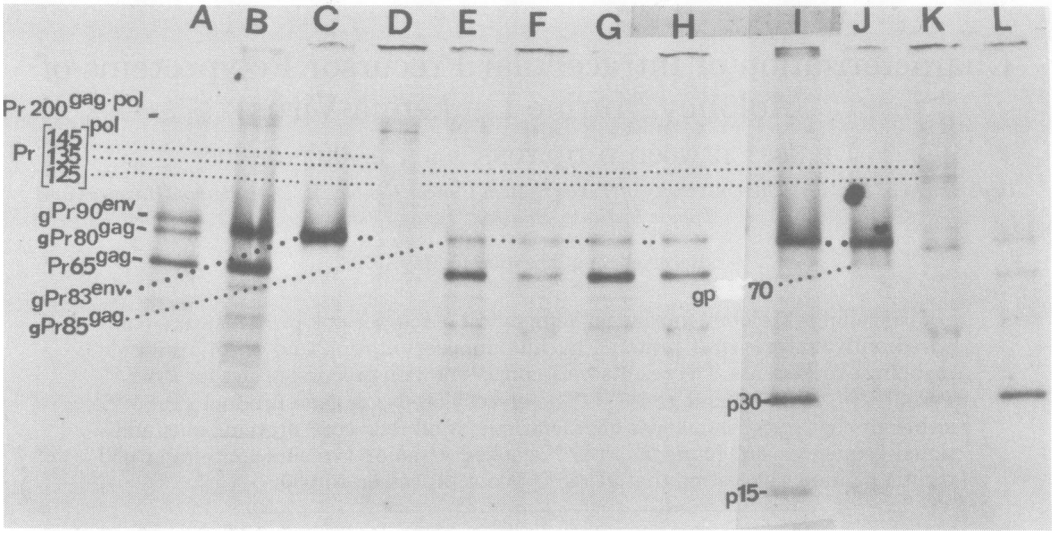


FIG. 1. *Pulse-chase analysis with monospecific antisera of virus-specific precursor polypeptides in Mo-MuLV-infected NIH/3T3 cells. Identical cultures of Mo-MuLV-infected NIH/3T3 cells were pulse-labeled for 15 min with [$^3$H]leucine in Earle salt solution. One culture was lysed immediately (lanes B to H), whereas the other was chased for 90 min in complete growth medium before lysis (lanes I to L). Cytoplasmic extracts were divided into equal portions and challenged with the following antisera: (B) anti-R-MuLV; (C) anti-gp69/71; (D) anti-RT; (E) anti-p30; (F) anti-p15; (G) anti-pp12; (H) anti-p10. Lanes I to L represent a chase experiment immunoprecipitated with: (I) anti-R-MuLV; (J) anti-gp69/71; (K) anti-RT; (L) anti-p30. Lane A represents a 15-min pulse-labeling of R-MuLV infected JLS-V16 cells immunoprecipitated with anti-R-MuLV. Immunoprecipitates were analyzed by 6 to 12% linear gradient SDS-PAGE.*

lectin column, and was eluted with α-methyl-D-mannoside (unpublished data). Thus, we shall refer to this polyprotein as gPr85$^{gag}$. Two minor bands in the 200,000-dalton region were also recognized by antisera to the viral core proteins (Fig. 1E to H) and are readily seen in the original fluorograph, but were not readily detected by photographic means. These two bands are more prominent in Fig. 1, lane D, in which anti-RT sera were used. These high-molecular-weight polyproteins represent the Mo-MuLV *gag-pol* polyprotein precursor and comigrate in SDS-PAGE with the *a* and *b* components of R-MuLV Pr200$^{gag-pol}$. Anti-RT serum also recognized bands at 145,000, 135,000, and 125,000 daltons (lane D), which correspond to the intermediate *pol* precursors.

A pulse-chase experiment was performed to determine the stability of the higher-molecular-weight proteins (Fig. 1I to L). p30 and p15 became labeled during the chase (lane I), and Pr65$^{gag}$ and gPr85$^{gag}$ largely disappeared (lane L). However, gPr83$^{env}$ did not show a significant reduction (lane J) although a small amount of radioactivity in gp70 could be detected. Pr200$^{gag-pol}$ and Pr145$^{pol}$ disappeared during the chase incubation, and several *pol*-related polypeptides, labeled Pr135$^{pol}$, Pr125$^{pol}$, and p80$^{pol}$, appeared (lane K). Similar sized RT-related polypeptides were found in R-MuLV-infected cells (12, 16, 17). A small amount of p30 was recognized by the anti-RT sera, even though this serum was absorbed with excess disrupted virus. Similar findings were made with R-MuLV-infected cells (12, 16) and suggest that a p30:RT complex exists within infected cells (12).

We further examined the relationships of these proteins by comparing the tyrosine-containing tryptic peptide maps of the precursor polyproteins with the tyrosine-containing tryptic peptide maps of the mature virion proteins. As has been demonstrated with R-MuLV (3), Mo-MuLV Pr65$^{gag}$ contained the structural determinants of p30, p15, pp12, and p10 as determined by cation-exchange chromatography of the tyrosine-containing tryptic peptides (unpublished data). We have been able to assign all but one tyrosine tryptic peptide within Pr65$^{gag}$ to a viral core protein. This unassigned tyrosine tryptic peptide is marked with an asterisk in Fig. 2.

Studies with R-MuLV have indicated an extremely close structural homology between gPr80$^{gag}$ and Pr65$^{gag}$ (3). R-MuLV-specified gPr80$^{gag}$ has also been shown to be analogous to Mo-MuLV gPr85$^{gag}$ (J. Kopchick, V. Ng, and R. Arlinghaus, unpublished data). As seen in Fig. 1, Mo-MuLV gPr85$^{gag}$ and Pr65$^{gag}$ contained the antigenic determinants of the four viral core

proteins. We compared the tyrosine-containing tryptic peptides of these *gag*-related, high-molecular-weight polypeptides by cation-exchange chromatography (Fig. 2). The results demonstrated extensive homology between $gPr85^{gag}$ and $Pr65^{gag}$. $gPr85^{gag}$ contained tryptic peptides characteristic of all four core proteins and had an additional broad peak at fractions 45 to 50. All of the tyrosine-containing tryptic peptides of $Pr65^{gag}$ coeluted with those of $gPr85^{gag}$.

To characterize further the relationship of these two Mo-MuLV *gag*-related polyproteins, we compared their two-dimensional peptide maps (Fig. 3). Analyses of the individual viral proteins alone and in various combinations have allowed us to identify the various spots in the maps of $gPr85^{gag}$ and $Pr65^{gag}$, as described in the legend of Fig. 3. As expected, the maps of $gPr85^{gag}$ and $Pr65^{gag}$ were very similar (compare Fig. 3). Tyrosine-containing tryptic peptides characteristic of each of the four core proteins were found in both $gPr85^{gag}$ and $Pr65^{gag}$. However, $gPr85^{gag}$ was significantly different from $Pr65^{gag}$. $gPr85^{gag}$ contained two additional peptides (spots 1 and 29) not found in $Pr65^{gag}$ or in any viral core protein, and $gPr85^{gag}$ lacked one tyrosine tryptic peptide characteristic of p30 which was found in $Pr65^{gag}$ (spot 28). A similar result was found in the R-MuLV system (17). These results indicate that the 85,000-dalton *gag* gene product has additional peptide sequence information over and above that of $Pr65^{gag}$, but surprisingly appears to lack some p30 information. The latter suggests that $gPr85^{gag}$ is not processed to $Pr65^{gag}$ and that p30 cannot be generated from $gPr85^{gag}$.

$Pr135^{pol}$ and cell-associated $p80^{pol}$ were isolated from infected cells by immunoprecipitation followed by SDS-PAGE. [$^3$H]tyrosine-labeled $Pr135^{pol}$ was digested with trypsin and mixed with [$^{14}$C]tyrosine-labeled $Pr65^{gag}$ tryptic peptides and co-chromatographed (Fig. 4A). $Pr135^{pol}$ lacked tryptic peptides of all four core proteins, but contained three $p80^{pol}$-specific tyrosine tryptic peptides (Fig. 4B). Thus, these experiments provide evidence that $Pr135^{pol}$ contains *pol* gene products but lacks *gag* gene products. It appears that $Pr135^{pol}$ has extra peptide information over and above that of $p80^{pol}$ similar to that seen in the R-MuLV system (17).

The antigenic relationship of the *env* gene products was also confirmed by peptide mapping. Tyrosine tryptic peptides of virion gp70 coeluted with tyrosine tryptic peptides of $gPr83^{env}$ upon ion-exchange chromatography, and two-dimensional fingerprinting of leucine-containing tryptic peptides demonstrated that p15E tryptic peptides comprise a subset of the $gPr83^{env}$ tryptic peptide fingerprint (data not shown). A common *env* gene product giving rise

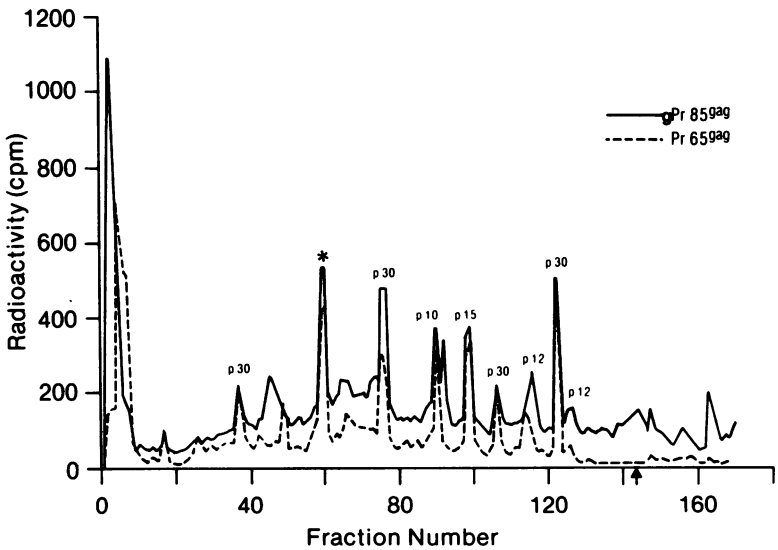


FIG. 2. *Comparison of tyrosine-labeled $gPr85^{gag}$ and $Pr65^{gag}$ by ion-exchange chromatography. Samples were prepared from Mo-MuLV-infected cells pulse-labeled for 15 min in the presence of [$^3$H]tyrosine, immunoprecipitated with anti-p30 serum, and purified by SDS-PAGE. The polypeptides were digested with trypsin, and the digest was fractionated on a Chromobead type P cation ion-exchange column (14). The peak fractions are marked to signify from which core protein they originate. The asterisk marks the tryptic peptide present in $gPr85^{gag}$ and $Pr65^{gag}$, but not present in any of the mature gag protein tryptic maps. The procedure is as described by Karshin et al. (14).*

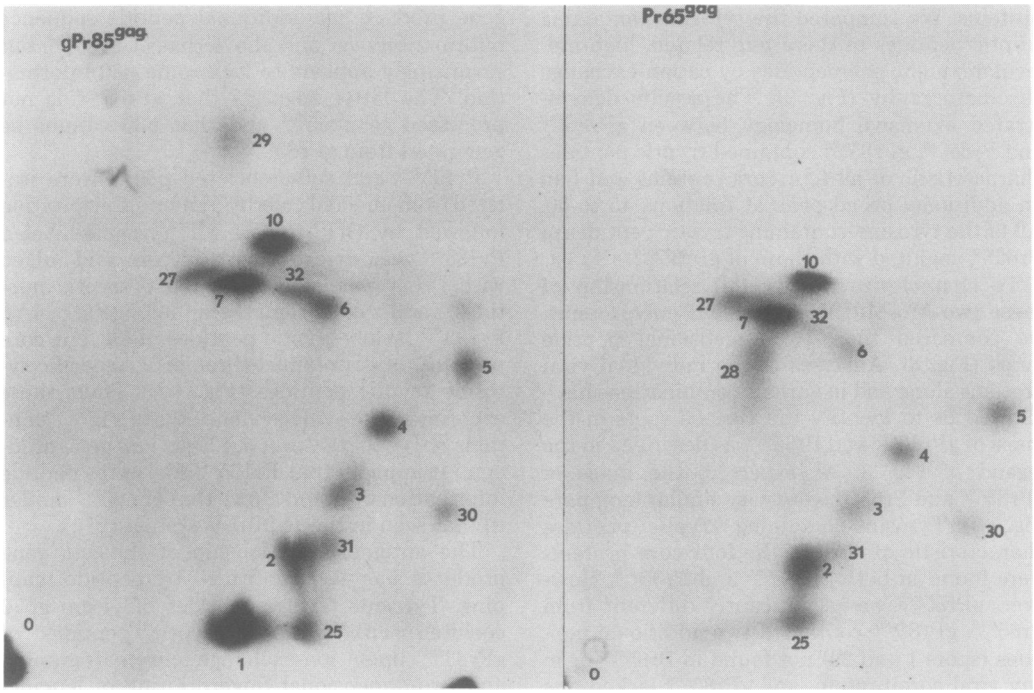


FIG. 3. *Two-dimensional peptide maps of [$^3$H]tyrosine-labeled gPr85$^{gag}$ and Pr65$^{gag}$ digested with trypsin. Samples were prepared as those shown in Fig. 2, but the tryptic peptides were fractionated in two dimensions on thin-layer cellulose plates, as in Kopchick et al. (17). The point of application of the sample is marked with zero. Spots 2, 4, 5, 7, 27, 28, and 30 are derived from p30; spot 6 is from p15; spots 25, 31, and 32 are from pp12; and spot 3 is from p10. The origin of spot 10 is as yet unknown.*

to the two mature virion envelope proteins, gp70 and p15E, is in agreement with observations in the R-MuLV system (14). Comparison of our peptide maps of gPr83$^{env}$ and gPr85$^{gag}$ by cation-exchange chromatography or two-dimensional fingerprints of tyrosine- or leucine-containing tryptic peptides demonstrated no structural homology.

The results presented in this report provide strong evidence that Mo-MuLV proteins derived from the *gag, pol,* and *env* genes are made from higher-molecular-weight polyproteins. The *gag* gene products gPr85$^{gag}$ and Pr65$^{gag}$ are unstable proteins that are rapidly formed. However, they are processed at different rates in that Pr65$^{gag}$ disappears more slowly than gPr85$^{gag}$. It seems quite likely from the results presented here and from studies by others (6, 21, 25, 26, 30, 32) that Pr65$^{gag}$ is the direct precursor to the viral core proteins. Our studies with the Rauscher system support this (3, 4, 11, 12), and it appears that some of the Pr65$^{gag}$ molecules are phosphorylated (24); a step which may facilitate its cleavage (1, 10, 11).

The role of gPr85$^{gag}$ in viral replication is, at this point, unclear; but several findings suggest that it may not be involved in formation of the four core proteins. First, Evans et al. (7) suggested that Friend-MuLV Pr75$^{gag}$ is processed by way of glycosylation to yield a 93,000-dalton glycosylated core polyprotein containing p30. Our past studies with R-MuLV gPr80$^{gag}$, which included pulse-chase experiments performed with amino acid analogs and protease inhibitors as well as peptide mapping (3, 12, 13), were consistent with the idea that it is the initial *gag* gene product leading to the formation of Pr65$^{gag}$. However, our recent findings now suggest a different interpretation. First, R-MuLV gPr80$^{gag}$ and Mo-MuLV gPr85$^{gag}$ are glycosylated as mentioned above; second, their peptide maps indicated that they lack one or two p30 tryptic peptides which are present in Pr65$^{gag}$ (17). In light of the findings of Tung et al. (28, 29) and Ledbetter and his colleagues (19, 20) that *gag* gene-derived glycosylated polyproteins are found on leukemic cell surfaces, it is tempting to postulate that R-MuLV gPr80$^{gag}$ and Mo-MuLV gPr85$^{gag}$ are further glycosylated and transported to the cell surface to perform some im-

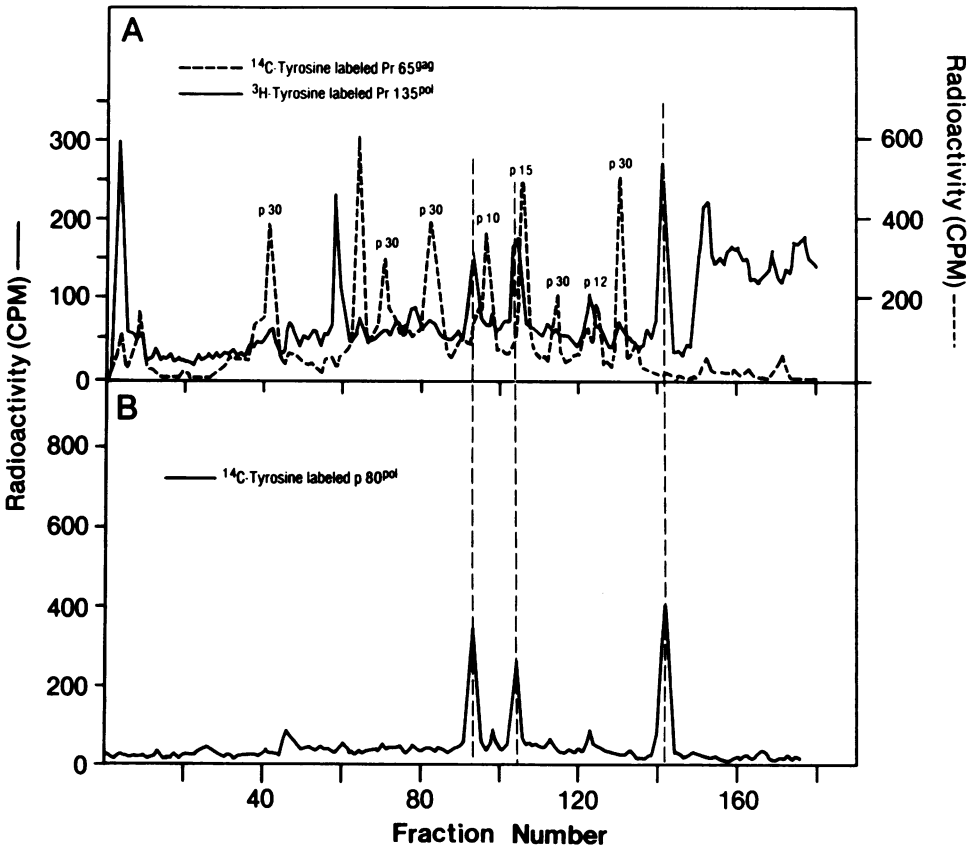


FIG. 4. *Ion-exchange column chromatography of tryptic digests of Pr135$^{pol}$ and cell-associated p80$^{pol}$. [$^{3}$H]tyrosine-labeled Pr135$^{pol}$ and [$^{14}$C]tyrosine-labeled p80$^{pol}$ were obtained from parallel cultures of Mo-MuLV-infected cells pulse-labeled for 15 min and chased for 90 min by immunoprecipitation with anti-RT serum followed by SDS-PAGE. [$^{14}$C]tyrosine-labeled Pr65$^{gag}$ was obtained from infected cells pulse-labeled for 15 min by anti-p30 precipitation and SDS-PAGE. The bands were digested with trypsin, and portions were mixed and fractionated on the Chromobead ion-exchange column as described in Karshin et al. (14). (A) [$^{3}$H]tyrosine-labeled Pr135$^{pol}$ versus [$^{14}$C]tyrosine-labeled Pr65$^{gag}$; (B) [$^{14}$C]tyrosine-labeled p80$^{pol}$.*

portant function. Studies are underway to elucidate the role of these *gag* gene-derived glycosylated polyproteins in virus replication.

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