P85: a *gag-mos* Polyprotein Encoded by *ts110* Moloney Murine Sarcoma Virus

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Antibody to a synthetic peptide (anti-C3 serum) with the predicted sequence of the C terminus of the Moloney murine sarcoma virus (strain 124) *v-mos* gene was used in immunoprecipitation experiments with cytoplasmic extracts of a clone of NRK cells infected with *ts110* Moloney murine sarcoma virus, termed 6m2 cells. *ts110* Moloney murine sarcoma virus codes for two viral proteins of 85,000 and 58,000 *M_r*, termed P85 and P58, respectively, in nonproducer 6m2 cells maintained at 33°C. Anti-C3 serum specifically recognized [³H]leucine-labeled P85, but not P58, from infected cells maintained at 33°C, whereas antiserum prepared against murine leukemia virus p12 recognized both proteins. Normal serum and anti-C3 serum pretreated with excess C3 peptide did not precipitate P85. Immunoprecipitation experiments after metabolic labeling of 6m2 cells with ³²P_i showed that P85 is phosphorylated. Both anti-C3 and anti-p12 sera specifically detected ³²P-labeled P85. Cell-free translation of *ts110* murine sarcoma virus/murine leukemia virus RNA produces P85, P58, and helper virus protein Pr63^{gag}. Anti-C3 serum specifically precipitated P85 but neither P58 nor Pr63^{gag}. We conclude from these studies that P85 is a product of both the *gag* and *mos* genes of *ts110* murine sarcoma virus, and, therefore, it is referred to as P85^{*gag-mos*}. We have not detected any other *v-mos* gene product in *ts110*-infected cells.

Moloney murine sarcoma virus (Mo-MuSV) was initially isolated from sarcomas induced in BALB/c mice after infection with Moloney murine leukemia virus (Mo-MuLV) and is a prototype mammalian transforming virus (18). Previous studies have demonstrated that clonal isolates of Mo-MuSV contain a 1.2-kilobase genetic region (9), derived from the host genome (27), which has been termed *v-mos*^{Mo} (mouse sarcoma virus, Moloney strain). Based on the nucleotide sequence of Mo-MuSV (26-28), a protein of approximately 40,000 daltons (40K) could be encoded by *v-mos*^{Mo}. Translation of the 3' half of the MuSV genome in *in vitro* translation systems generates a family of proteins ranging in size from about 18K to 40K (8, 17, 20, 23, 24). Antisera directed against synthetic peptides corresponding to the carboxy terminus of the predicted *v-mos*^{Mo} gene product have been generated (24). Antisera to peptides C1 and C3, corresponding to the 6 amino acids and 12 amino acids, respectively, starting from the C terminus, of the *v-mos*^{Mo} gene product with the addition of an N-terminal tyrosine, were shown to precipitate four *in vitro*-synthesized *v-mos*^{Mo} proteins (P37, P33, P24, and P18).

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Furthermore, hybrid arrest translation experiments indicate that these four proteins are encoded within the *v-mos*^{Mo} gene, since translation was inhibited by DNA sequences from within the *v-mos*^{Mo} gene. Recently, this same antiserum to the C3 peptide detected a P37 *mos*-encoded protein in low amounts in cells transformed by Mo-MuSV 124 (25).

A temperature-sensitive mutant (*ts110*) of Mo-MuSV, having a lesion in the function necessary for the maintenance of transformation, was generated by mutagenesis of Mo-MuSV 349 (4). A nonproducer cell clone (6m2) infected by *ts110* Mo-MuSV has transformed properties (i.e., growth in soft agar) when maintained at 33°C, but reverts to a normal phenotype at 39°C. Studies from this laboratory have demonstrated that 6m2 cells grown at 33°C express two virus-specific proteins of 85K (P85) and 58K (P58). In contrast, only P58 was detected in cells maintained at 39°C (12, 13, 29). Immunoprecipitation experiments and tryptic peptide mapping studies have demonstrated that P58 contains *gag* sequences related to p15, p12, and p30. P85 contains the above *gag* sequences plus additional non-*gag* sequences (12, 29). Some of the additional tryptic peptides observed in P85 were seen in a P23 believed to be derived from the 3'

half of the *mos* gene and generated by in vitro translation of either Mo-MuSV 124 (2) or Mo-MuSV 349 (13). In addition, in vitro translation of *ts110* virus RNA generated a P85 in addition to a P33 and a P23; the latter share tryptic peptides with Mo-MuSV 349 *mos*-encoded P33 and P23 as well as the non-*gag* portion of P85 (20). The above results, in addition to temperature-shift experiments (5), provide evidence suggesting that P85 is a hybrid polypeptide synthesized from *gag* and *v-mos*^{Mo} genes. This conclusion is further supported by heteroduplex studies using *ts110* Mo-MuSV RNA and cloned Mo-MuSV 124 DNA, which indicate that a portion of p30, p10, and *pol* sequences usually observed in Mo-MuSV 124 have been deleted from *ts110* genomic RNA and that *v-mos*^{Mo} is adjacent to the remaining *gag* sequences (15).

In this study, we investigated the proteins synthesized by *ts110* Mo-MuSV-infected cells and the in vitro translation products generated from *ts110* viral RNA by immunoprecipitation with the above-mentioned *v-mos*^{Mo}-specific anti-C3 serum.

ts110 Mo-MuSV, produced by UV irradiation of Mo-MuSV 349, was used in these studies (4). Mo-MuSV 349 was derived by infecting mouse TB cells with Mo-MuSV 124 (see Table 7 of reference 3). A nonproducer clone of NRK cells (6m2) infected with *ts110* Mo-MuSV and a *ts110* producer cell line (designated NRK-206-2IC) generated by superinfection of 6m2 cells with the IC strain (10) of Mo-MuLV were maintained in McCoy 5a medium supplemented with 15% fetal calf serum as described previously (13). Mo-MuSV 349 and Mo-MuSV 124 were obtained from J. Ball (University of Western Ontario, London, Ontario, Canada) and maintained as previously described (3).

Previous tryptic peptic mapping experiments (13, 20) indicated that P85 contains *mos* as well as *gag* peptides. In contrast, P58 contained only *gag*-related peptides. The availability of an anti-*mos* serum (anti-C3 serum [24]) allowed for a method to independently determine whether P85 contains *v-mos*^{Mo} peptide sequences. In these experiments, 6m2 cells were pulse-labeled with [³H]leucine for 20 min, and the cytoplasmic extract was divided into several equal portions before immunoprecipitation with various antisera. To prove that anti-C3 serum is recognizing a *mos*-related protein, anti-C3 serum was pretreated with an excess of synthetic peptide before use to block precipitation of C3-related products. Studies by J. Papkoff and T. Hunter, who supplied both the anti-C3 serum and the C3 peptide used, recommended using 3 µg to 5 µg of peptide per µl of serum in blocking experiments (24); our own studies verified these results. Anti-C3 serum (Fig. 1A, lane A) recog-

nized a band in the 85K size class that comigrated with P85^{*gag-mos*} and was detected by anti-p12 serum (lane C-2) but not by anti-p10 serum (lane C-1). This protein was not detected upon pretreatment of anti-C3 serum with 3 µg of C3 peptide (Fig. 1A, lane B). Lane C represents a preimmune serum control. In another experiment, 6m2 cells were labeled with ³²P_i for 2 h, and the effect of blocking anti-C3 serum with C3 peptide was again tested (Fig. 1B). As was the case with the [³H]leucine-labeled 85,000-dalton protein, the ³²P-labeled protein was recognized by anti-C3 serum (Fig. 1B, lane A) but was not immunoprecipitated when the serum was pretreated with excess C3 peptide (Fig. 1B, lane B). Thus, antibodies to the 12 amino acids from the C terminus of the *v-mos*^{Mo} gene product specifically recognize P85^{*gag-mos*}. Similar results were obtained by Papkoff and Hunter in their study of *ts110*-infected producer cells derived from the 6m2 cell clone used in our studies (22).

The blocking experiments described above provide good evidence that anti-C3 serum specifically recognizes P85^{*gag-mos*}. To provide further evidence that anti-C3 and anti-p12 sera were detecting the same 85K polypeptide, we compared the V8 protease peptides from each by the method of Cleveland et al. (6). In this experiment, 6m2 cells maintained at 33°C were pulse-labeled for 2 h with ³²P_i. Samples of the cytoplasmic extract were immunoprecipitated with either anti-C3 or anti-p12 serum. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the 85K bands were analyzed by the procedure of Cleveland et al. (6). The banding pattern from the 85K protein recognized by anti-p12 serum (Fig. 1C, lane A) was very similar to the pattern of the 85K protein recognized by anti-C3 serum (Fig. 1C, lane B). The peptide fragment at 68K visible in lane B is also present in lane A but in a lower amount and therefore is not observed in lane A of Fig. 1C.

To confirm the identity of the ³²P-labeled 85K protein recognized by anti-C3 serum, a second experiment (Fig. 2A) was performed in which samples of the extract were immunoprecipitated with anti-p12 serum (lane A), anti-p10 serum (lane B), preimmune serum (lane C), and anti-C3 serum (lane D). Bands corresponding to P85^{*gag-mos*} and P58^{*gag*} were detected by anti-p12 serum but not by anti-p10 serum, whereas anti-C3 serum recognized only P85^{*gag-mos*}.

The phosphoamino acids present in P85^{*gag-mos*} were analyzed by a previously published procedure (21) in which the hydrolysis procedure was changed to detect phosphotyrosine (Fig. 2B). The results showed that P85^{*gag-mos*} contains predominantly phosphoserine and smaller amounts of phosphothreonine but lacks phosphotyrosine.

We have previously shown that the viral RNA

FIG. 1. Analysis of *ts110*-infected 6m2 cells for *mos* gene products. 6m2 cells were rinsed in Earle salt solution and then incubated at 33°C for 20 min in Earle balanced salt solution (1) containing 1 mCi of L-[³H]leucine (specific activity, 100 Ci/mmol) per ml. Cells metabolically labeled with ³²P were preincubated for 30 min in phosphate-free Dulbecco minimal essential medium supplemented with 10% dialyzed fetal calf serum. The cells were then incubated for 2 h in fresh phosphate-free medium containing 2 mCi of ³²P_i (carrier-free) per ml. Monospecific goat antiserum prepared against Rauscher MuLV p12 and p10 were obtained through the Office of Program Resources and Logistics, Viral Oncology, National Institutes of Health, and were absorbed with excess uninfected JLS-V16 cell cytoplasmic extracts as previously described (14). The C3 peptide and rabbit antiserum (termed anti-C3 serum) prepared against the 12-residue synthetic peptide corresponding to the C-terminal end of the predicted *v-mos*^{Mo} gene product were generated as described previously (24) and generously supplied by Tony Hunter and Jackie Papkoff of the Salk Institute, San Diego, Calif. The sequence of the C3 peptide is NH₂-Tyr-Gln-Arg-Asp-Leu-Lys-Ala-Phe-Arg-Gly-Thr-Leu-Gly-COOH. The N-terminal tyrosine was added for coupling purposes. Cell lysis was performed in a detergent-containing buffer (21). Indirect precipitation was carried out using Formalin-killed *Staphylococcus aureus* (Cowan strain) as described by Kessler (16). The precipitates were washed and processed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1). ³H-labeled proteins were visualized by fluorography with preflashed films (14); ³²P-labeled proteins were detected by autoradiography with enhancing screens. Samples of cytoplasmic extracts were immunoprecipitated with either anti-C3 serum (lane A in panels A and B) or anti-C3 serum pretreated with excess C3 peptide (lane B in panels A and B). Serum at 1 μl/3 μg of C3 peptide was used for blocking treatment. The M lanes are marker proteins. (A) Lanes C-1, C-2, and C are anti-p10, anti-p12, and preimmune immunoprecipitations, respectively. (B) Numbers refer to *M_r*s of standard marker proteins. The samples were fractionated on a 6 to 12% polyacrylamide gel containing sodium dodecyl sulfate. (C) The 85K protein labeled with ³²P_i was isolated by immunoprecipitation with either anti-C3 or anti-p12 serum. The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and digested with 15 μl of V8 protease (10 μg/ml) according to the method of Cleveland et al. (6). Lane A, anti-p12-precipitated P85; lane B, anti-C3-precipitated P85. The numbers are molecular weight markers.

coding for P85^{gag-mos} is packaged into virus (13). The *ts110* MuSV/MuLV viral RNA contains three RNA size classes: 4.0- and 3.5-kilobase *ts110* RNAs as well as an 8.3-kilobase MuLV RNA (13). This mixture yields P85^{gag-mos}, Pr72^{gag} (the apoprotein of the glycosylated *gag* polyprotein gPr85^{gag}), Pr63^{gag} (the *gag* gene polyprotein that is the precursor to the four core proteins), and P58^{gag}, in addition to the presumed *mos*-related polypeptides P33 and P23 (20). The results of a cell-free translation experiment followed by immunoprecipitation with anti-C3 serum and other antisera are shown in

Fig. 3. The supernatant fluid obtained by high-speed centrifugation (lane C) was immunoprecipitated with normal serum (lane D) and with anti-C3 (lane E), anti-p12 (lane F), and anti-p10 (lane G) sera. Anti-C3 serum specifically recognized P85^{gag-mos} as well as P33 and P23 bands (lane E). Additional bands of about 55K and 13K were also detected by anti-C3 serum (lane E). Interestingly, the 55K band detected by anti-C3 serum (lane E) was not recognized by anti-p12 (lane F) or anti-p10 (lane G) serum, suggesting that the 55K protein is the result of ribosomal attachment and protein synthesis ini-

FIG. 2. Phosphorylation of virus proteins in 6m2 cells maintained at 33°C. Transformed 6m2 cells were metabolically labeled with $^{32}\text{P}_i$ and the cytoplasmic extracts were immunoprecipitated and analyzed on 6 to 12% acrylamide gels. (A) Gel treated with 1 N KOH at 55° for 2 h according to the method of Cooper and Hunter (7). Equal aliquots of extract were immunoprecipitated with anti-p12 serum (lane A), anti-p10 serum (lane B), preimmune serum (lane C), and anti-C3 serum (lane D). (B) Phosphoamino acids of $\text{P85}^{\text{gag-mos}}$ were separated by one-dimensional electrophoresis on thin-layer plates as described previously (21).

tiation within the p30 gene. In fact, all of the bands in lane E of less than 85K are most likely the result of internal initiation downstream from the methionine codon at the beginning of the *gag* gene that yields $\text{P85}^{\text{gag-mos}}$, as predicted from the results of Papkoff et al. (23, 24) with Mo-MuSV 124 translation experiments. In contrast to the results obtained with anti-C3 serum, anti-p12 serum (lane F) recognized $\text{P85}^{\text{gag-mos}}$ and P58^{gag} as well as the helper virus *gag* proteins (Pr63^{gag} and Pr72^{gag}), whereas anti-p10 serum (lane G) only recognized the helper virus proteins and not $\text{P85}^{\text{gag-mos}}$ and P58^{gag} .

As a positive control, we have shown that anti-C3 serum recognized the *mos*-encoded products (P38, P23, and P18) of the wild-type virus Mo-MuSV 349. Figure 3, lane H, shows the translation products of the wild-type virus Mo-MuSV 349, which include P63^{gag} and P38, P33, and P23 *mos* products. The *mos* gene products were recognized by anti-C3 serum (lane J), as expected from the results of Papkoff et al. (24).

The data presented here provide convincing evidence that the 85K product encoded by *ts110* Mo-MuSV is the product of both the *gag* and the *mos* genes. The *gag* portion of $\text{P85}^{\text{gag-mos}}$ has

been shown to contain p15, p12, and p30 antigenic determinants, together with tyrosine-containing tryptic peptides found in these mature core proteins (12, 29). The ability of anti-C3 serum to specifically precipitate $\text{P85}^{\text{gag-mos}}$ and not P58^{gag} or helper virus-encoded *gag* gene products indicates that $\text{P85}^{\text{gag-mos}}$ contains determinants from the predicted C terminus of Mo-MuSV 124 *v-mos*. The specificity of the recognition of the 85K protein by anti-C3 serum is established, since the immunoprecipitation of the 85K band could be blocked by competition with excess C3 peptide, and preimmune sera failed to recognize the 85K protein. In addition, the 85K protein detected by anti-C3 serum in 6m2 cells maintained at 33°C was not seen in 6m2 cells maintained at 39°C (unpublished data). It has been established previously that $\text{P85}^{\text{gag-mos}}$ is not detected in 6m2 cells maintained at 39°C (12, 29).

Our previous peptide mapping studies have shown that some of the non-*gag* tyrosine-containing tryptic peptide sequences in $\text{P85}^{\text{gag-mos}}$ are also found in Mo-MuSV 349-encoded P23 (13), a product believed to be derived from the 3' half of the *v-mos* gene of Mo-MuSV 124 in vitro (8, 24). Furthermore, most but possibly not all of the *v-mos* gene product of wild-type MuSV is present in $\text{P85}^{\text{gag-mos}}$ (15, 20). Thus, it is clear from a variety of different types of evidence that $\text{P85}^{\text{gag-mos}}$ represents a truncated *gag* gene product fused to a large portion of the *mos* gene product of Mo-MuSV 124.

We have failed to detect any other *mos* gene product in *ts110*-infected cells besides $\text{P85}^{\text{gag-mos}}$. Sequence studies of *v-mos* of Mo-MuSV 124 (26–28) as well as cell-free translation studies (8, 24) and more recent intracellular studies (25) indicate that *v-mos* of Mo-MuSV 124 generates a 37K to 44K polypeptide. Our translation experiments using Mo-MuSV 349 RNA yielded a P38 product precipitated by anti-C3 serum (Fig. 3). However, we have had difficulty detecting the full-length *mos* gene product in Mo-MuSV 349-transformed cells. A faint band of the size of P37^{mos} can be detected, using anti-C3 serum, which appears to be blocked by excess C3 peptide (unpublished data). No such protein was detected in *ts110*-infected 6m2 cells. However, the level of P37^{mos} that we have been able to demonstrate in Mo-MuSV 349 cells makes it difficult to completely eliminate the existence of any separate *mos* product in *ts110*-infected cells.

Papkoff and Hunter (22) have examined *ts110*-MuSV/MuLV producer cells derived from 6m2 cells by MuLV infection. These cells produce $\text{P85}^{\text{gag-mos}}$ at 33°C, but smaller amounts are seen at 39°C (13). Papkoff and Hunter have confirmed that $\text{P85}^{\text{gag-mos}}$ is specifically recognized by anti-

FIG. 3. Immunoprecipitation of translation products of *ts110* and wild-type virus with anti-C3 serum. Viral RNA from 30S to 70S *ts110* Mo-MuSV (13) and wild-type Mo-MuSV 349 were translated in an mRNA-dependent protein synthesis system (13, 19). The products of the translation reaction were treated with 50 μ g of RNase A per ml and 2 mM EDTA in a buffer containing 0.5% Nonidet P-40, 0.5% deoxycholate, 50mM NaCl, and 20mM Tris-hydrochloride (pH 7.5). The protein products were immunoprecipitated after high-speed centrifugation at 30,000 rpm for 1 h in an angle 50 Ti rotor (Beckman Instruments, Inc.) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes C–G are products derived from *ts110* RNA. Lane B, minus RNA; lane C, supernatant fluid (78,000 $\times$ g) of the total cell-free translation product. Lanes D–G are immunoprecipitations of the supernatant fluid with normal serum (lane D), anti-C3 serum (lane E), anti-p12 serum (lane F), and anti-p10 serum (lane G). Lanes H–J were derived from translation products of Mo-MuSV 349 50S RNA. Lane H, total cell-free product remaining in the supernatant fluid after high-speed centrifugation; lanes I and J, equal aliquots immunoprecipitated with normal serum and anti-C3 serum, respectively. Lane A, marker proteins from Rauscher MuLV-infected cells.

C3 serum. No other *mos* product was detected in these cells. In their studies $P85^{gag-mos}$ was resolved into two bands, the upper one being phosphorylated. We have recently used the same gel conditions described by Papkoff and Hunter and also have detected a $P85^{gag-mos}$ doublet in 6m2 nonproducer cells. Both are recognized by anti-C3 serum and both are removed by competition with C3 peptide (L. Stanker and R. Arlinghaus, unpublished data). Papkoff and Hunter (22) found, however, that in acutely infected NIH 3T3 cells, $P85^{gag-mos}$ and a trace amount of a P33 *mos*-related protein were detected by anti-C3 immunoprecipitation. The origin of the trace level of the P33 protein is unknown, but it could result from cleavage of $P85^{gag-mos}$ or possibly from the integration of a variant MuSV produced during the acute infection as a result of recombination between the helper MuLV and *ts110* Mo-MuSV. In considering all of these data, it appears most likely that $P85^{gag-mos}$ is the sole initial product of

the transforming gene in *ts110* Mo-MuSV-infected 6m2 cells.

$P58^{gag-mos}$, like many of the currently characterized viral transforming proteins, is phosphorylated. Phosphoamino acid analyses showed that the phosphorylated amino acids are serine and threonine. No phosphotyrosine was detected. Similar results were obtained by Papkoff and Hunter (22).

An important difference in transformation by *ts110* versus wild-type Mo-MuSV is the increased production of the *gag-mos* protein in 6m2 cells compared with that of $P37^{mos}$ in cells transformed by wild-type virus. Recently, Papkoff and Hunter (25) have identified low levels of a protein doublet of approximately 37K from [35 S]methionine-labeled Mo-MuSV 124-transformed producer cells (25). The upper band is phosphorylated in phosphoserine. The ability to more easily detect $P85^{gag-mos}$ in 6m2 cells provides a system whereby both the expression and the mechanism of action of a murine transform-

ing protein can be investigated. In this regard, we have recently obtained evidence that P85^{gag-mos}, although efficiently recognized in pulse-chase experiments by anti-p15 or anti-p12 serum, is poorly recognized in 2-h chase experiments immunoprecipitated with anti-C3 serum (L. Stanker and R. Arlinghaus, unpublished data). Experiments are underway to determine where P85^{gag-mos} is processed or modified in some way after its synthesis, as is the case with a retrovirus structural protein (11). In this regard, similar observations were made on examination of another Mo-MuSV variant expressing a P100^{gag-mos} protein (L. Stanker and R. Arlinghaus, unpublished data).

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