P85<sup>gag-mos</sup> Encoded by ts110 Moloney Murine Sarcoma Virus: Rapid at the Restrictive Temperature

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SUMMARY

The stability of P85<sup>gag-mos</sup>, encoded by ts110 Moloney murine sarcoma virus, was studied. P85<sup>gag-mos</sup> was found to turn over more rapidly at 39 °C than 33 °C in two different cell lines. One of these is a non-producer cell clone (6m2) which appears only to express P85 at 33 °C. The other cell line (206-2IC) produces P85<sup>gag-mos</sup> at both 33 °C and 39 °C, although in lower amounts at the higher temperature. Both cell lines exhibit the normal morphological phenotype following a shift to the restrictive temperature. These data suggest that the presence of P85<sup>gag-mos</sup> alone is insufficient to cause transformation, and that some function(s) associated with the molecule may be heat-labile.

INTRODUCTION

Moloney murine sarcoma virus (Mo-MuSV) is a replication-defective virus that is capable of transforming cultured fibroblasts in vitro and causing fibrosarcomas in mice (Aaronson & Rowe, 1970). A region of 1-2 kilobases (kb) in the 3' half of the viral genome is responsible for the sarcomagenic properties of Mo-MuSV and is referred to as v-mos (Hu & Davidson, 1977; Van Beveren et al., 1981a, b). The v-mos region is similar to a normal cellular sequence (referred to as c-mos) which the virus acquired at the expense of viral envelope and polymerase genes. The entire nucleotide sequence of Mo-MuSV-124, including the v-mos region, has been determined (Van Beveren et al., 1981b). The nucleotide sequence would predict that a protein of approximately 41 000 daltons is encoded by v-mos. Translation in vitro of Mo-MuSV RNA generates a family of proteins ranging in molecular weights (Mr) from 42000 to 18000 (Papkoff et al., 1980, 1981; Murphy & Arlinghaus, 1982). Detection of a protein encoded by the v-mos gene in cells chronically infected by Mo-MuSV-124 has been reported using an antiserum directed against a synthetic peptide corresponding to the carboxy-terminal 12 amino acids of the predicted v-mos gene product (Papkoff et al., 1982). The protein, referred to as P37<sup>mos</sup>, was present in extremely small amounts.

We have concentrated our efforts in the study of Moloney sarcoma virus on a temperature-sensitive mutant of Mo-MuSV referred to as ts110-Mo-MuSV. Non-producer ts110-infected normal rat kidney (NRK) cells (6m2) are transformed at 33 °C but revert to a normal phenotype at 39 °C (Horn et al., 1980). At 33 °C, 6m2 cells synthesize an 85 000 mol. wt. gag-mos fusion polyprotein (P85<sup>gag-mos</sup>) and a 58 000 mol. wt. gag protein (P58<sup>gag</sup>) (Horn et al., 1981). At 39 °C, P85<sup>gag-mos</sup> is not detectable, whereas P58<sup>gag</sup> is still present (Horn et al., 1980; Brown et al., 1981). Immunoprecipitation experiments and tryptic peptide mapping studies (Horn et al., 1980) indicate that both P58<sup>gag</sup> and P85<sup>gag-mos</sup> contain antigenic sequences related to p15, p12 and p30 but no p10-related sequences. The non-gag portion of P85<sup>gag-mos</sup> has been analysed by peptide mapping studies and contains sequences derived from the v-mos region of the viral genome (Horn et al., 1981; Murphy & Arlinghaus, 1982). In addition, we have demonstrated that an anti-mos serum generated by Papkoff et al. (1982) specifically precipitates P85<sup>gag-mos</sup> but not P58<sup>gag</sup> in a non-producer cell line (Stanker et al., 1983a, b). Similar results were observed by Papkoff &
Hunter (1983) using a producer cell clone infected with ts110-Mo-MuSV. The amount of mos present in P85\textsuperscript{gag-mos} has not yet been clearly established. However, our tryptic peptide mapping studies (Murphy & Arlinghaus, 1982) and heteroduplex analysis (Junghans \textit{et al.}, 1982) indicate that P85\textsuperscript{gag-mos} contains p15, p12 and part of p30 sequences fused to a v-mos sequence which may lack the first 20 to 30 codons of v-mos. Thus, P85\textsuperscript{gag-mos} appears to be a truncated gag protein fused to a truncated v-mos protein.

NRK 6m2 cells rescued with MuLV produce ts110-Mo-MuSV pseudotype viruses which package a 3.5 kb RNA and a 4.0 kb RNA (Horn \textit{et al.}, 1981; Junghans \textit{et al.}, 1982). Non-producer 6m2 cells also produce a 3.5 kb and a 4.0 kb RNA, and both RNAs contain mos sequences (unpublished results). Our studies (Junghans \textit{et al.}, 1982) indicate that the 3.5 kb RNA codes for P85\textsuperscript{gag-mos} while the 4.0 kb RNA codes for P58\textsuperscript{gag}. The nature of the temperature-sensitive lesion(s) in 6m2 cells has yet to be clearly resolved. We have proposed that one defect in ts110-Mo-MuSV-infected 6m2 cells is an inability to produce the 3.5 kb RNA via a splicing reaction (Junghans \textit{et al.}, 1982). In this communication we report on the temperature lability of P85\textsuperscript{gag-mos}.

\textbf{METHODS}

Cells, virus and antisera. A non-producer clone of NRK cells (6m2) infected with ts110-Mo-MuSV and a ts110 NRK producer cell line (designated 206-2IC), generated by superinfection of 6m2 cells with the IC strain of Mo-MuLV, were maintained in McCoy’s 5a medium supplemented with 15% foetal calf serum as described earlier (Horn \textit{et al.}, 1981). Monospecific goat antiserum prepared against Rauscher leukaemia virus p15, p12, p30 and p10 were obtained through the Office of Program Resources and Logistics, Viral Oncology, NIH, and were absorbed with excess uninfected ILS-V16 cell cytoplasmic extracts as previously described (Jamjoom \textit{et al.}, 1977).

Labelling of cells. Seventy to eighty percent confluent, 6m2 and 206-2IC cells in T-25 flasks (Corning) were labelled with L-\textsuperscript{3H}leucine (Horn \textit{et al.}, 1981). The cells were rinsed in Earle’s balanced salt solution and then incubated for 20 min in Earle’s salt solution containing 500 \textmu Ci/ml of L-\textsuperscript{3H}leucine (sp. act. approx. 100 Ci/mmol). In chase experiments, the isotope solution was decanted, the cells were rinsed with Earle’s salt solution and incubated in McCoy’s 5a medium.

Immunoprecipitation and gel electrophoresis. Cell lysis was performed in a detergent-containing buffer (Jamjoom \textit{et al.}, 1977). Indirect precipitation was carried out using formalin-killed \textit{Staphylococcus aureus} (Cowan strain) as described by Kessler (1975). The precipitates were washed and processed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970). Radioactive proteins were visualized by fluorography using preflashed films (Laskey & Mills, 1975).

\textbf{RESULTS}

Previous studies from this laboratory have demonstrated that non-producer, ts110-Mo-MuSV-infected 6m2 cells maintained at 33 °C have a transformed phenotype and synthesize two virus-coded proteins: an 85000 mol. wt. gag–mos fusion polyprotein (P85\textsuperscript{gag-mos}) and a 58000 mol. wt. gag protein (P58\textsuperscript{gag}). In contrast, cells grown at 39 °C have a normal phenotype and synthesize P58\textsuperscript{gag}; however, P85\textsuperscript{gag-mos} has never been detected (Horn \textit{et al.}, 1980; Brown \textit{et al.}, 1981) in 6m2 cells grown at 39 °C. Furthermore, P85\textsuperscript{gag-mos} was the only mos-encoded protein we have detected in 6m2 cells using an antiserum to different portions of the v-mos protein (Stanker \textit{et al.}, 1983a; G. Gallick & R. Arlinghaus, unpublished results). Thus, P85\textsuperscript{gag-mos} appears to be the active transforming protein in 6m2 cells. In the following experiments we have investigated the stability of P85\textsuperscript{gag-mos} in both 6m2 cells and the ts110-Mo-MuSV producer cell line, 206-2IC, in an effort to define more clearly the nature of the temperature-sensitive lesion(s) in ts110-Mo-MuSV.

\textit{Temperature-dependent morphological changes to 6m2 and 206-2IC cells}

6m2 cells have a transformed phenotype when grown at 33 °C but assume a normal morphology when grown at 39 °C. The rate at which this morphological change occurs was investigated. Cells maintained at 33 °C were shifted to 39 °C for 1, 2, 4, 6 and 24 h (Fig. 1a to f). By 1 h after the temperature shift, the cells began to flatten out and assume a more 'normal' morphology (b). By 4 h after a 33 °C to 39 °C temperature shift (d), the cells had undergone a
Fig. 1. 6m2 cells: 33°C to 39°C temperature shift. 6m2 cells were grown at 33°C for 48 h (a), then shifted to 39°C for 1 h (b), 2 h (c), 4 h (d), 6 h (e), and 24 h (f).

A dramatic change in morphology and were assuming the non-transformed morphology observed at 24 h following the temperature shift (compare Fig. 1d with f). The results of an identical temperature shift experiment using the ts110 virus producer 206-2IY cell line are shown in Fig. 2.
Fig. 2. 206-2IC cells: 33 °C to 39 °C temperature shift. 206-IC cells were grown at 33 °C for 48 h (a), then shifted to 39 °C for 1 h (b), 2 h (c), 4 h (d), 6 h (e), and 24 h (f).

(a to f). The results clearly demonstrate that the 206-2IC producer cell line maintained a temperature-sensitive phenotype for transformation and that a rapid morphological change occurs following a shift-up to 39 °C. The temperature sensitivity of the 206-2IC clone was not expected since we have previously shown that at 39 °C, 206-2IC cells, unlike 6m2 cells,
synthesize P85\textsuperscript{gag-mos} (Horn et al., 1981). Furthermore, the presence of P85\textsuperscript{gag-mos} in 206-2IC cells grown at 33 °C or 39 °C has been confirmed by Papkoff & Hunter (1983) using an anti-\textit{mos} serum.

**Temperature-lability of P85\textsuperscript{gag-mos}**

Having obtained the above results, we initiated several experiments designed to evaluate the stability of P85\textsuperscript{gag-mos} at the elevated temperature. P85\textsuperscript{gag-mos} pulse-labelled at 33 °C in 6m2 cells was stable in chase experiments at 33 °C for up to 4 h (Fig. 3a, lanes 3 to 5) when immunoprecipitated with anti-gag antiserum. This result confirms earlier studies which indicated that P85\textsuperscript{gag-mos} was stable for up to 2 h in chase experiments at 33 °C (Horn et al., 1980). Some reduction in the amount of P85\textsuperscript{gag-mos} was, however, noted in cells chased for 4 h (Fig. 3a, lane 5). A similar reduction was also observed for P58\textsuperscript{gag}. Anti-p10 antiserum, as expected, did not precipitate either P85\textsuperscript{gag-mos} or P58\textsuperscript{gag} (Fig. 3a, lane 2). In contrast, P85\textsuperscript{gag-mos} pulse-labelled at 33 °C disappeared rapidly when the cells were chased at 39 °C (Fig. 3b). After a 2 h chase at 39 °C, the amount of P85\textsuperscript{gag-mos} is greatly reduced (Fig. 3b, lane 4) and is not detectable after a 4 h chase at 39 °C (Fig. 3b, lane 5). In contrast, the stability of P58\textsuperscript{gag} was not similarly affected by temperature shift.

Similar experiments using the 206-2IC producer cell line are presented in Fig. 4 (a, b). Cytoplasmic extracts from cells pulse-labelled at 33 °C were immunoprecipitated with anti-p10 and anti-p15 sera (Fig. 4a, lanes 2 and 3, respectively). As expected, P85\textsuperscript{gag-mos} and P58\textsuperscript{gag} were detected by anti-p15 serum but not by anti-p10 serum, whereas gPr85\textsuperscript{gag} was detectable with either serum. In addition, other helper MuLV gag proteins were also detected with both anti-p10 and anti-p15 antisera. It should be emphasized that P85\textsuperscript{gag-mos} and gPr85\textsuperscript{gag} can, in some gel experiments, be resolved as in Fig. 4, but in other experiments they co-migrate (as in Fig. 5). The ts110-Mo-MuSV-encoded P85\textsuperscript{gag-mos} and P58\textsuperscript{gag} proteins pulse-labelled at 33 °C were detected for up to 4 h (Fig. 4a, lanes 4 to 6) when chased at 33 °C. However, Pr65\textsuperscript{gag} from the helper MuLV was processed in a normal manner. In contrast, P85\textsuperscript{gag-mos} pulse-labelled at 33 °C rapidly
Fig. 4. Heat lability of P85 gag-mos in 206-21C cells at 39 °C. (a) 206-21C cells were pulse-labelled with L-[3H]leucine at 33 °C, lysed immediately and the cytoplasmic extract immunoprecipitated with anti-p10 serum (lane 2) and anti-p15 serum (lane 3). The pulse medium was decanted from companion flasks, replaced with complete growth medium and the cells incubated at 33 °C for 1 h (lane 4), 2 h (lane 5) and 4 h (lane 6), at which time they were lysed and the cytoplasmic extracts immunoprecipitated with an anti-p15 serum. (b) The experiment shown in (a) was repeated except that, following the pulse label at 33 °C, the cells were chased at 39 °C. Pulse-labelled extracts were immunoprecipitated with anti-p10 and anti-p15 serum (lanes 2 and 3, respectively). The anti-p15 immunoprecipitation of the 1 h, 2 h and 4 h chases are shown in lanes 4, 5, and 6, respectively. Lane 1 serves as a marker.

decreased in cells incubated at 39 °C and was virtually undetectable after a 1 h and a 2 h chase at 39 °C (Fig. 4b, lanes 4 and 5). As was shown above in 6m2 cells, the level of P58 gag was not similarly affected by incubation of cells at 39 °C. The helper virus-coded Pr65 gag was similarly processed at either temperature.

The above experiment clearly indicates that P85 gag-mos is unstable at 39 °C relative to 33 °C. This event is quite specific, since other viral proteins are not similarly affected. A comparison of the time course of morphological changes observed in ts110-infected 6m2 cells and its virus producer counterpart with the rate of disappearance of P85 gag-mos in pulse–chase experiments suggests a correlation between these two events (Fig. 1 to 4). Evidence has recently been provided which indicates that the kinase function associated with P85 gag-mos is also heat-labile in vitro (Kloetzer et al., 1983) as well as in vivo (W. Kloetzer & R. Arlinghaus, unpublished results). Whether the observed morphological changes are a result of the temperature-sensitivity or heat lability of this kinase function remains to be shown.

Lability of P85 gag-mos in 206-21C cells maintained at 39 °C

We next investigated the lability of P85 gag-mos in 206-21C cells maintained at 39 °C. 206-21C cells were maintained at 39 °C for 48 h and had a non-transformed morphology (see above). The cells were pulse-labelled for 20 min and the cytoplasmic extracts immunoprecipitated with anti-p10 and anti-p15 serum (Fig. 5, lanes 2 and 3, respectively). Anti-p15 serum precipitated P85 gag-mos and P58 gag from the pulse-labelled cells (Fig. 5, lane 3) and neither was recognized by anti-p10 serum (lane 2). The 85000 mol. wt. protein seen in lane 2 (anti-p10 serum) is not P85 gag-mos but gPr85 gag of the helper virus which sometimes co-migrates with P85 gag-mos on our gels. Close inspection of the fluorograph reveals a closely spaced 85K doublet in lane 3 and a single band in lane 2. After a 1 h chase, the upper band still remained (P85 gag-mos) as detected by anti-p15 (lane 4) but not anti-p10 (lane 9). Less P85 gag-mos was observed in the 2 h chase samples (lane 5) and was virtually undetectable after a 4 h chase (lane 6). Thus, the heat lability of P85 gag-mos at 39 °C in 6m2 and 206-21C cells is quite similar.
DISCUSSION

The results presented here show that P85ag-mos encoded by ts110 Moloney MuSV is a heat-labile protein. Non-producer 6m2 cells produce P85ag-mos at 33 °C but not at 39 °C (Horn et al., 1980). Temperature shift experiments in which 6m2 cells were shifted from 33 °C to 39 °C show a relatively rapid disappearance of P85ag-mos at 39 °C. Thus, P85ag-mos was found to be more stable at 33 °C, whereas P58ag stability was unaffected by temperature. The disappearance of P85ag-mos at 39 °C in 6m2 cells coincides in time with the morphological changes observed as these cells acquire a normal phenotype. Such results indicate that P85ag-mos was required to maintain the transformed phenotype. However, results from MuLV-infected 6m2 cells (206-21C cells), which show that P85ag-mos is synthesized at restrictive temperatures, suggest that the presence of P85ag-mos alone is insufficient for transformation.

206-21C cells produce P85ag-mos at both 33 °C and 39 °C. Yet, these cells undergo phenotypic changes upon temperature shift which are similar to those occurring in 6m2 cells, which make no detectable P85ag-mos at 39 °C. Furthermore, P85ag-mos produced in 206-21C cells is also heat-labile at 39 °C. It should be noted that the level of P85ag-mos synthesis in 206-21C cells is always somewhat greater than in 6m2 cells under equivalent conditions. These observations suggest that not only the presence of P85ag-mos but also some property of P85ag-mos is important for transformation.

The heat lability of P85ag-mos may reflect a change in conformation of the protein with temperature, thus allowing cellular proteases to degrade it rapidly. Furthermore, temperature-induced changes of P85ag-mos might also be expected to affect a function(s) associated with this protein. Studies are in progress to determine whether the increased turnover of P85ag-mos at
39 °C is an important primary event causing cells to take on the normal phenotype or whether it is a terminal event that follows an earlier conformational change leading to loss of a function(s) associated with P85ag-mos. In this regard, we have recently shown that P85ag-mos has an associated immune complex kinase activity (Kloetzer et al., 1983), and that this kinase activity is sharply reduced in 6m2 cells following a 30 min shift to 39 °C (W. Kloetzer, S. Maxwell & R. Arlinghaus, unpublished results). In addition, the phosphorylation of P85ag-mos in 206-2IC cells is also drastically reduced in cells maintained at 33 °C compared to 39 °C (unpublished results).

One explanation for the heat lability of P85ag-mos is that the mos portion of this protein may lack the amino terminus of P37 mos (Murphy & Arlinghaus, 1982; Junghans et al., 1982). Therefore, we suggest that it is the inactivation of a functional P85 ga~mos at 39 °C that accounts for the temperature-sensitive nature of 206-2IC cells as compared to 6m2 cells which lack P85ag-mos at 39 °C.

A possible explanation for the synthesis of P85 at the restrictive temperature in 206-2IC cells is that the 3.5 kb messenger RNA that codes for this molecule is overproduced in these cells relative to 6m2 cells at 33 °C, and that a lower level of this 3.5 kb mRNA synthesis continues at 39 °C. In support of this idea, Southern blot analysis indicates that cloned 6m2 cells contain one (mos-specific) provirus, and suggests that 206-2IC cells contain two (mos-specific) proviruses (E. Murphy, unpublished results). We hypothesize that the provirus in 6m2 cells codes for a 4-0 kb mRNA, and that splicing of that primary transcript yields a 3-5 kb RNA (Junghans et al., 1982). Preliminary evidence (G. Gallick & R. Arlinghaus, unpublished results) indicates that the splicing event is, in fact, temperature-sensitive. We further propose that the infection of 6m2 cells with MuLV (yielding 206-2IC cells) has allowed for reverse transcription of the 3-5 kb RNA, and integration of this RNA results in the second provirus found in 206-2IC cells. At 33 °C both proviruses are transcribed and some of the 4.0 kb RNAs are spliced to yield the 3.5 kb RNA. At 39 °C, both genomes are transcribed but the splicing of the 4.0 kb genome does not occur, thus reducing the steady-state level of the 3.5 kb RNA (E. Murphy & R. Arlinghaus, unpublished results). Studies to test this working model are under way.

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