IDENTIFICATION OF TRYPtic PEPTIDES UNIQUE
TO A 110,000-MOLECULAR WEIGHT
POLYPROTEIN ENCODED BY
THE T-8 ISOLATE OF MURINE LEUKEMIA VIRUS*

BY WIM J. M. VAN DE VEN, FRED H. REYNOLDS, JR.,
JONAS BLOMBERG, AND JOHN R. STEPHENSON

From the Carcinogenesis Intramural Research Program, and Laboratory of Cellular and Molecular Biology,
National Cancer Institute, Frederick Cancer Research Center, Frederick, Maryland 21701

Several type C RNA-transforming viruses have been shown to contain acquired cellular sequences within preexisting viral genes (1-3). In many instances expression of proteins encoded by such sequences occurs by inphase read through of translation initiated at the 5' terminus of the viral genome. Resulting polyprotein translational products contain type C viral gag gene amino terminal structural components covalently linked to acquired sequence-encoded components with probable transforming function (4-10). The nonstructural components of polyproteins encoded by several such virus isolates have been shown to resemble the well-characterized avian sarcoma virus-encoded transforming protein (11-13), pp60src, in that they are highly phosphorylated (14) and exhibit associated protein kinase activity (14, 15) with specificity for tyrosine acceptor sites (15-18). Transformation by viruses of this group is associated with elevated levels of total cellular phosphotyrosine (17, 18) and a reduction of epidermal growth factor (EGF) binding capacity (16, 19).

Staal et al. (20) have described the isolation of a weakly-transforming RNA type C virus from a thymoma suspension culture developed from a spontaneously lymphomatosis AKR/J mouse. This virus, designated T-8, is replication-defective (20), transforms mink cells in vitro (20), and encodes, as its major translational product, a highly phosphorylated 110,000 mol wt polyprotein containing AKR-murine leukemia virus (MuLV) amino terminal gag gene components, p15 and p12 (21). The functional significance and role, if any, of this protein in transformation is, as yet, unknown. In the present study, we have analyzed T-8 P110 for possible acquired sequence encoded component(s) and have examined levels of tyrosine-specific protein kinase activity and epidermal growth factor EGF binding by mink cell lines nonproductively infected by T-8.

* Supported under Public Health Service contract N01-CO-75380 from the National Cancer Institute, Bethesda, Md.

Abbreviations used in this paper: DME, Dulbecco's modification of Eagle's medium; EGF, epidermal growth factor; MCF, mink cell focus forming; MuLV, murine leukemia virus; PBSTDs, 10 mM sodium phosphate, pH 7.2, 0.9% NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TPCK, tolylsulfonfanyl phenylalanyl chloromethyl ketone.
Materials and Methods

Cells and Viruses. Cell lines were maintained in Dulbecco’s modification of Eagle’s medium (DME) supplemented with 10% calf serum (Colorado Serum Co., Denver, Co.). Cells used included a fetal mink lung cell line, CCL64, obtained from the American Culture Collection, Rockville, Md., and subclones of CCL64 nonproductively transformed by either T-8 virus (21) or AbLV (9) designated 64T8 and 64Ab-2, respectively. A Gardner FeSV transformed subclone of CCL64, designated 64F3, was generously provided by G. J. Todaro (National Cancer Institute, Bethesda, Md.). An Sc-1 subclone productively infected with AKR-MuLV was also used.

Immunoprecipitation, Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Two-Dimensional Tryptic Peptide Analysis. After labeling with [35S]methionine (1075 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) or [3H]lysine (78.1 Ci/mmol; New England Nuclear, Boston, Mass.), cells were washed twice with serum-free DME and disrupted in lysis buffer containing 10 mM sodium phosphate, pH 7.2, 0.9% NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) (PBS-TDS). After clarification of lysates, virus-specific proteins were immunoprecipitated and analyzed by SDS-PAGE on 5–20% polyacrylamide gradient slab gels as described previously (22). [35S]methionine- and [3H]lysine-labeled proteins purified by immunoprecipitation and SDS-PAGE were digested by incubation with tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK) trypsin (Worthington Biochemical Corp., Freehold, N. J.), and two-dimensional analysis of the tryptic peptides was performed by electrophoresis for 30 min at 500 V in electrophoresis buffer (acetic acid: formic acid: water, 15:5:80) and ascending chromatography in butanol, pyridine, acetic acid, and water (32:25:5:20).

Competition Immunoassays. Unlabeled antigens were assayed at serial twofold dilutions for the ability to compete with 125I-labeled AKR-MuLV p15 or p12 for binding limiting amounts of goat anti-serum directed against detergent-disrupted AKR-MuLV. Reaction mixtures contained 0.01 M Tris-HCl, pH 7.8, 1.0 mM EDTA, 0.4% Triton X-100, 1% bovine serum albumin, and 0.05 M NaCl in a total vol of 0.2 ml. In assays in which AKR-MuLV p15 was used as the 125I-labeled antigen, the NaCl concentration was increased to 0.2 M. Antiserum and unlabeled competing antigen were incubated at 37°C for 1 h, followed by the addition of 10,000 cpm 125I-labeled antigen. After further incubation, antigen-antibody complexes were immunoprecipitated, and radioactivity quantitated as previously described (22).

Assay for Protein Kinase Activity. Phosphorylation of proteins in an in vitro phosphotransferase reaction was performed as described by Blomberg et al. (16). Briefly, tissue culture cells (10⁶) were disrupted by repeated aspiration through a 25-gauge needle in 5 ml of 10 mM sodium phosphate, pH 7.2, buffer containing 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, and 1.0% Triton X-100, and clarified by centrifugation at 2000 g for 10 min. Supernates were preabsorbed by addition of normal goat serum (50 μl) and 0.5 ml of a 10% suspension of protein-A Sepharose CI-4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.), incubated overnight at 4°C, and reclarified by centrifugation at 100,000 g for 2 h. Viral proteins were immunoprecipitated by incubation with antiserum and protein-A Sepharose as previously described (16). Immunocomplexes were collected by centrifugation at 10 min at 2000 g and washed in PBS-TDS buffer. Protein kinase activity was assayed by resuspension of immunoprecipitates in 20 mM Tris-HCl, pH 7.2, 5 mM MgCl₂ buffer containing 1 μCi [γ³²P]ATP (2,000 Ci/mM; New England Nuclear) and incubation of reaction mixtures for 10 min at 30°C. Reactions were terminated by addition of 3 ml ice cold PBSTDS, nonprotein bound ³²P-label was removed by repeated centrifugation in excess PBSTDS, and ³²P-labeled proteins were analyzed by SDS-PAGE.

Phosphoamino Acid Separation. ³²P-labeled proteins contained in protein kinase reaction mixtures were purified by SDS-PAGE, and selected gel slices heated at 110°C for 1 h in 200 μl of 6 N hydrochloric acid. Hydrolyzed samples were filtered, lyophilized, resuspended in 1 ml deionized water, lyophilized a second time, and dissolved in buffer containing acetic acid: formic acid: water (15:5:80), pH 1.9. Phosphoamino acid standards including α-phospho-o-serine and α-phospho-o-threonine were purchased from Sigma Chemical Co., St. Louis, Mo. α-Phospho-l-tyrosine was synthesized as previously described (17). Hydrolyzed samples (3,000–10,000 cpm) containing 0.1 μmol of each phosphoamino acid standard were applied to 10 × 10
cm cellulose thin-layer chromatography plates. Two dimensional separation was performed by electrophoresis for 1 h in acetic acid: formic acid: water (15:5:80) buffer and chromatography in the second dimension in 0.5 M ammonium hydroxide: isobutyric acid (3:5). Unlabeled phosphoamino acid standards were detected by the ninhydrin reaction, and \(^{32}\)P-labeled amino acids visualized by autoradiography on X-Omat R film with the use of calcium tungstate intensifying screens (Eastman Kodak Co., Rochester, N. Y.).

**Epidermal Growth Factor Binding.** Cells were plated in 24 well cluster plates (Costar, Data Packaging, Cambridge, Mass.) at \(\sim 10^5\) cells/well. 48 h later, individual wells were washed once with DME containing 10% calf serum, incubated for 3 h at 37°C in serum-free DME, and for an additional 4 h at 4°C in 0.2 ml of Hepes-buffered (pH 7.4) DME containing 0.1% ovalbumin and 1.0–20 ng/ml mouse EGF (Collaborative Research, Inc., Waltham, Mass.) labeled with \(^{125}\)I at a specific activity of 20–30 \(\mu\)Ci/\(\mu\)g by the chloramine T method (23). Cells were subsequently washed three times in serum-free DME, dissolved in 1% SDS (1 ml), and radioactivity was measured in a gamma scintillation counter. Cell numbers were determined by staining with Sedi-stain (Becton-Dickinson & Co., Ltd., Drogheda, Ireland) and visual enumeration of three randomly chosen 0.03-mm\(^2\) fields in each of six wells/cluster plate.

### Results

**Characterization of T-8 P110 by Immunoprecipitation and SDS-PAGE Analysis.** Initial characterization of T-8 P110 was accomplished by immunoprecipitation and SDS-PAGE analysis. As shown in Fig. 1, T-8 P110 and a second protein of \(~55,000\) Mr were immunoprecipitated from the 64T8 mink cell line by anti-Rauscher MuLV, anti-Rauscher-MuLV p12, and to a lesser extent, by anti-Rauscher-MuLV p30. Neither P110 nor P55 were immunoprecipitable by a high titered anti-Rauscher-MuLV p30 serum. Because the anti-Rauscher-MuLV p30 serum used in this study has been shown to contain low level anti-p12 reactivity (24), this result does not necessarily indicate the presence of p30 determinants in P110. In contrast to sera with specificity for Rauscher-MuLV gag gene components, a previously-described (24) high-titered anti-Rauscher-MuLV gp70 serum precipitated AKR-MuLV Pr82\(^{env}\) and gp70 from AKR-MuLV-infected cells, but did not precipitate the 110,000 or 55,000 Mr, T-8 encoded polyproteins to a significant extent. In other studies, we have found both P110 and P55 to be precipitated by anti-Rauscher-MuLV p15 but not by anti-AKR-MuLV p15(E) (data not shown). The absence of detectable levels of either P110 or P55 expression in uninfected control mink cells argues for the viral specificity of both proteins.

**Identification of \[^{35}\]S\]Methionine-labeled Tryptic Peptides Unique to T-8 P110.** The above findings raise the possibility that P110 may consist of AKR-MuLV amino terminal gag gene structural (P15 and p12) components covalently linked to an acquired sequence-encoded nonstructural component. Alternatively, on the basis of molecular weight and antigenic criteria, the possibility that T-8 P110 might represent the translation product of an inphase deletion of the middle third of the AKR-MuLV gag/pol gene must be considered. In an effort to resolve these alternative models, \[^{35}\]S\]methionine-labeled T-8 P110 and AKR-MuLV translational products, including Pr180\(^{gag/pol}\), Pr65\(^{gag}\), p30, and Pr82\(^{env}\), were purified by immunoprecipitation and SDS-PAGE (Fig. 1 A and F), and subjected to tryptic peptide analysis. As shown in Fig. 2 A, AKR-MuLV Pr180\(^{gag/pol}\) contained twelve major well-resolved \[^{35}\]S\]methionine-labeled peptides. In contrast, in the case of Pr65\(^{gag}\) (Fig. 2 B) only two labeled peptides were seen, both of which were specific to its p30 component (Fig. 2 C). In a mixing experiment, these are shown to correspond to two of the Pr180\(^{gag/pol}\) peptides (Fig. 2 D), thus confirming its identity. Although by analysis of its total amino acid...
composition, a second AKR-MuLV gag gene component, p12, has been shown to contain a methionine residue (25), for reasons which are not apparent, we were unable to identify a \[^{35}S\]-methionine labeled tryptic peptide unique to p12.

Analysis of T-8 P110, under similar conditions, revealed a total of seven well-resolved methionine-containing peptides (Fig. 3A). In an effort to rule out the possibility that one or more of the labeled peptides observed might actually consist of multiple peptides migrating at identical positions, digests were analyzed using different separation conditions (Fig. 3B and C). In each case, a total of seven peptides were observed. Of these, two peptides, 4 and 5, which showed some overlap in the original separation shown in Fig. 2, panel A, were clearly resolved. To determine the representation of Pr65\textsuperscript{gp10} components in T-8 P110, a mixing experiment was performed. As shown in Fig. 4, one of the two p30-specific peptides was clearly separated from all seven T-8 P110 peptides, whereas the second p30 peptide, designated (a),
Fig. 2. \(^{[35}S\)methionine tryptic peptide compositions of AKR-MuLV Pr\(180^{+\text{pol}}\) (A), Pr\(65^{+\text{pol}}\) (B), p\(30\) (C), and identification of tryptic peptides shared by AKR-MuLV Pr\(180^{+\text{pol}}\) and Pr\(65^{+\text{pol}}\) (D). \(^{[35}S\)methionine-labeled Pr\(180^{+\text{pol}}\), Pr\(65^{+\text{pol}}\), and p\(30\) were isolated from Sc-1 cells productively infected with AKR-MuLV by immunoprecipitation and SDS-PAGE as described in Fig. 1. Proteins were digested with TPCK trypsin as described in Materials and Methods. Tryptic digests (4,000–12,000 cpm) were resuspended in electrophoresis buffer, spotted on cellulose thin layer glass plates (O), and separated in two dimensions by electrophoresis and ascending chromatography. The twelve major \(^{[35}S\)methionine-labeled peptides represented in AKR-MuLV Pr\(180^{+\text{pol}}\) are labeled a–l. Partially overlapped with P110 peptide 6. These findings establish that at least one, and probably both, of the AKR-MuLV Pr\(65^{+\text{pol}}\) methionine-containing tryptic peptides are unique to Pr\(65^{+\text{pol}}\) and not represented within T-8 P110.

To extend the above findings and test whether P110 might contain specific peptides not of Pr\(180^{+\text{pol}}\) origin, \(^{[35}S\)methionine-labeled tryptic peptides of the two proteins were analyzed in a mixing experiment. For this purpose, the separation conditions in Fig. 3C were used. As shown in Fig. 5A, the twelve AKR-MuLV Pr\(180^{+\text{pol}}\) methionine-containing tryptic peptides were well-resolved under these conditions, and the overall tryptic map was distinct from that exhibited by T-8 P110. In a mixing
FIG. 4. Comparison of T-8 P110 and AKR-MuLV Pr65\textsuperscript{gag} \textsuperscript{[35S]}methionine-labeled tryptic peptides. \textsuperscript{[35S]}methionine-labeled proteins including T-8 P110 and AKR-MuLV Pr65\textsuperscript{gag} were purified as described in Fig. 1, mixed at 1:1 ratio, and subjected to tryptic peptide analysis as described in the legend to Fig. 2. Individual peptides of AKR-MuLV Pr65\textsuperscript{gag} and T-8 P110 are labeled as described in Figs. 2 and 3, respectively.

Experiment, at least four of the seven methionine-containing P110 tryptic peptides are shown to be completely separable from peptides derived from Pr180\textsuperscript{gag/pol} (Fig. 5 B). The migration patterns of the three remaining P110 peptides and their relationship to Pr180\textsuperscript{gag/pol} peptides are inconclusive. These findings are summarized schematically in Fig. 5 C.

**Demonstration of Structural Components Common to AKR-MuLV Pr65\textsuperscript{gag} and T-8 P110.** The lack of conclusive evidence for methionine-containing tryptic peptides common to Pr180\textsuperscript{gag/pol} and T-8 P110 raised the possibility that T-8 P110 may be completely unrelated to AKR-MuLV. To examine this possibility, the p15 and p12 reactivities associated with P110 were analyzed with respect to immunologic type specificity. As shown in Fig. 6A, in a competition immunoassay in which anti-AKR-MuLV was used for precipitation of \textsuperscript{125I}-labeled AKR-MuLV p15, an extract prepared from mink cells nonproductively-infected with T-8 competed to the same final extent as detergent disrupted sucrose density gradient-purified AKR-MuLV. In contrast, an extract of uninfected control mink cells did not compete to significant extent in this assay. Of other virus isolates tested, mouse mammary tumor virus was completely nonreactive, whereas Moloney-MuLV competed to final extent of ~25%. Similar findings were obtained upon analysis of the p12 type specificity of T-8 P110. As with p15, the pattern of competition of T-8 P110 was distinct from that of Moloney-MuLV, but indistinguishable from AKR-MuLV. These findings thus provide strong immunologic evidence for the AKR-MuLV specificity of the T-8 P110 p15 and p12 structural components.

As an independent approach to establish the relatedness of T-8 P110 to AKR-MuLV Pr180\textsuperscript{gag/pol}, tryptic peptide analysis was performed following labeling with \textsuperscript{[3H]}lysine. The results indicate ~33 and 27 labeled peptides in Pr65\textsuperscript{gag} (Fig. 7 A) and
Fig. 5. Comparison of $[^{35}S]$methionine tryptic peptide compositions of AKR-MuLV Pr$^{180^{	ext{pol}}}$ and T-8 P110. $[^{35}S]$methionine-labeled AKR-MuLV Pr$^{180^{	ext{pol}}}$ and T-8 P110 were isolated and subjected to tryptic peptide digestion as described in the legend to Fig. 2. Electrophoretic separation of tryptic peptides was in buffer containing acetic acid, formic acid, and water (15:5:80), and ascending chromatography was in ethanol and water (70:30). Proteins tested included AKR-MuLV Pr$^{180^{	ext{pol}}}$ (A), and a 1:1 mixture of AKR-MuLV Pr$^{180^{	ext{pol}}}$ and T-8 P110 (B). Panel C represents a schematic drawing of the tryptic peptide migration patterns of a 1:1 mixture of AKR-MuLV Pr$^{180^{	ext{pol}}}$ (●) and T-8 P110 (○). Peptides presented in tryptic maps of the two proteins and not resolved by the separation conditions used are indicated as (●).
FIG. 6. Homologous competition immunoassays for AKR-MuLV p15 (A) and p12 (B). Virus and cell extracts prepared as described in the text were tested at serial twofold dilutions for ability to compete with $^{125}$I-labeled AKR-MuLV p15 or p12 for binding limited amounts of antibody to detergent-disrupted AKR-MuLV. Results are expressed as mean values of two separate determinations, and are normalized to 100% maximal binding at infinite antigen dilution. Viruses were tested at an initial concentration of 100 µg/ml and included AKR-MuLV (O), Moloney-MuLV (△), and mouse mammary tumor virus (■). Cell extracts were tested at initial dilutions of 1 mg/ml and included 6T8 (○) and CCL64 control mink cells (△).

P110 (Fig. 7 B), respectively. Moreover, in a mixing experiment (Fig. 7 D) ~6 of these peptides are seen to overlap, whereas the remaining 27 Pr65$^{env}$ and 21 T-8 P110 peptides are specific to their respective genomes. These findings are consistent with previous estimates of 33 lysine residues in AKR-MuLV Pr65$^{env}$, 8 of which are localized in its p15 and p12 components. Upon similar analysis, Pr82$^{env}$ is shown to contain ~26 well-resolved lysine-containing tryptic peptides, none of which correspond to those derived from either Pr65$^{env}$ or P110. The lack of representation of Pr82$^{env}$
Fig. 7. Two-dimensional tryptic peptide maps of in vivo-synthesized labeled AKR-MuLV and T-8 translational products. Tryptic peptide maps of [3H]lysine-labeled AKR-MuLV Pr65<sup>+</sup> (A), T-8 P110 (B), AKR-MuLV Pr65<sup>-</sup> (C), and a 1:1 mixture of Pr65<sup>+</sup> and P110 (D) were prepared as described in Fig. 2. Labeled peptides were visualized by autoradiography after spraying thin layer chromatography glass plates with ether containing 7% diphenyloxazole.
Identification of Tryptic Peptides

Fig. 8. Analysis of protein kinase activities associated with high molecular weight viral polyproteins in mink cells nonproductively infected with AbLV, Gardner FeSV, and T-8. Cells were disrupted, immunoprecipitated, assayed for $[\gamma^{32}P]ATP$ phosphotransferase activity, and analyzed by SDS-PAGE as described in Materials and Methods. Cell lines tested included AbLV (A, B), Gardner FeSV (E, F), and T-8 (C, D) nonproductively-infected and uninfected control (G, H) CCL64 mink cells. Sera used included goat anti-Moloney MuLV (A, C, G), goat anti-FeSV P115 (E), and normal goat serum (B, D, F, H).

Peptides in either Pr65$^{agg}$ or T-8 P110 was confirmed by appropriate mixing experiments (data not shown).

Analysis of T-8 P110 for Associated Protein Kinase Activity. High molecular polyprotein translational products of mammalian transforming viruses such as AbLV and the Gardner strain of FeSV are highly phosphorylated and exhibit associated protein kinase activities with specificity for tyrosine acceptor sites. In view of our previous results establishing T-8 P110 to be phosphorylated, it was thus of interest to analyze it for protein kinase activity. For this purpose, T-8 P110 was analyzed for autophosphorylation under in vitro conditions in the presence of $[\delta^{32}P]ATP$. As shown in Fig. 8, in contrast to AbLV P120, AbLV P80, Gardner FeSV P115, and a normal 150,000
FIG. 9. Identification of amino acid acceptor residues phosphorylated in an in vitro phosphotransferase reaction. Proteins were 32P-labeled, hydrolyzed, and subjected to two-dimensional analysis according to procedures described in Materials and Methods. Phosphoamino acid standards include phosphoserine (p-ser), phosphothreonine (p-thr), and phosphotyrosine (p-tyr). Equimolar concentrations of all three standards were mixed with a 32P-labeled hydrolysate of CCL64 mink cells (A), AbLV-transformed CCL64 mink cells (B), and T-8-transformed CCL64 mink cells (C). Positions of phosphoamino acids were visualized by both ninhydrin reaction and autoradiography.
IDENTIFICATION OF TRYPTIC PEPTIDES

Fig. 10. Comparison of EGF binding by AbLV (●), Gardner FeSV (▲), and T-8 (○) nonproductively-infected and uninfected control (□) CCL64 mink cells. Results are presented as mean values of triplicate binding determinations corrected for nonspecific adsorption by subtraction of the values from wells containing an additional 100 ng of unlabeled EGF as competitive inhibitor. This activity corresponded to 0.3-0.7 pg/well. Standard deviation values represent the sum of deviation of binding determinations and cell counts.

Mr mink cellular protein, which were efficiently labeled using these conditions, T-8 P110 was not detectably labeled.

In addition to the demonstration of tyrosine-specific protein kinase associated with AbLV and Gardner FeSV encoded polyproteins, cells transformed by these viruses have been shown to exhibit elevated levels of phosphotyrosine (15–18). It was thus of interest to analyze T-8 nonproductively-infected CCL64 mink cells with respect to total phosphoamino acid composition. As shown in Fig. 9, in addition to phosphoserine and phosphothreonine, AbLV nonproductive transformed mink cells exhibit readily detectable levels of phosphotyrosine. In contrast, by the procedures used in the present study, the levels of phosphotyrosine in uninfected or T-8 nonproductively infected mink cells were below detectability.

EGF Binding by T-8-infected Mink Cells. There is accumulating evidence for a decrease in EGF binding by type C viral transformed, as compared to uninfected control mink cells (19, 26). Moreover, in the case of AbLV transformation, there is evidence for a close association between cellular levels of tyrosine specific protein kinase activity and EGF binding (16). To explore the possible influence of T-8 P110 expression on EGF binding, T-8 nonproductively-infected and control uninfected mink cells were analyzed for binding of 125I-labeled EGF. As a positive control for transformation-associated loss of EGF binding, AbLV and Gardner FeSV transformed mink cells were similarly analyzed. As shown in Fig. 10, binding of 125I-labeled EGF by AbLV and Gardner FeSV transformed cells was much lower than the levels of binding exhibited by uninfected control mink cells. In contrast, binding by T-8 nonproductively-infected and control mink cells did not differ significantly.

Discussion

The spontaneous development of thymic tumors in high leukemia incidence strains of mice is etiologically associated with activation of endogenous ecotropic MuLV (27). The isolation, from preleukemic and leukemic thymus tissues, of MuLV variants with
altered host range (28, 29) and recombinant env genes (30, 31) has raised the possibility that alterations within env may be involved in tumor induction. This is supported by the ability of such isolates, designated as mink cell focus forming (MCF) viruses, to induce morphologic alteration of mink cells in tissue culture (29). The more recent isolation of transforming variants from thymus cell-derived MCF virus stocks, one of which has been shown to be replication-defective (20) and to encode, as its major translation product, a 110,000 $M_r$ polyprotein (21), suggested a model analogous to that described for other mammalian RNA transforming viruses such as avian acute leukemia virus (4, 7, 10), several independent isolates of FeSV (5, 6), and AbLV (8, 9).

In the present study, we have further characterized the 110,000 $M_r$ polyprotein encoded by the T-8 isolate of MuLV (21). This highly phosphorylated viral-encoded protein is shown to contain amino terminal gag gene structural components but to lack immunologic cross-reactivity with other known AKR-MuLV translational products. The possibility that T-8 P110 may contain a minor portion of p30 and thus the p12-p30 posttranslational cleavage site, cannot be excluded. However one, and probably both, of the AKR-MuLV p30 methionine-containing tryptic peptides are absent from T-8 P110. These findings, in combination with the demonstration of at least four well-resolved methionine-containing tryptic peptides unique to T-8 P110, are consistent with the possibility that all but the amino terminal portion of P110 is encoded by acquired sequences. These presumably are inserted at an inphase position within the gag/pol region of the AKR-MuLV genome. In this respect, the T-8 P110 polyprotein appears to resemble high molecular weight polyprotein translational products encoded by avian acute leukemia viruses, AbLV and various FeSV isolates.

The lack of detectable gp70 or pl5(E) expression in T-8 nonproductively-infected cells could be explained on the basis of such an insertion extending into the 5' terminus of the AKR-MuLV env gene.

An alternative, although less likely explanation of the present findings, is that T-8 P110 represents the translational product of a partially-deleted AKR-MuLV gag/pol gene. Such a deletion would presumably be inphase and extend from the 3' terminus of sequences encoding p12 to the middle of the pol gene. The identification of at least four methionine-containing tryptic peptides unique to T-8 P110 could be accounted for on the basis of the location of such peptides adjacent to deletion sites, or reflect major differences in posttranslational modification of T-8 P110 and AKR-MuLV Pr180$^{gag/pol}$. Implicit within such a model is the additional speculation that the deleted gag/pol gene product acquires transforming capacity as a consequence of an inphase deletion, or that an as yet unidentified T-8 gene product is responsible for transformation.

Although the T-8 nonproductively-infected mink cell line used in the present study was initially derived based on a capacity for growth in soft agar (20), the cells no longer exhibit this property nor appear morphologically-transformed. Moreover, T-8 P110 lacked detectable protein kinase activity, and T-8-infected mink cells exhibited neither the elevated levels of phosphotyrosine kinase (15, 17, 18, 32) activity nor reduction of EGF binding sites (16, 19) characteristic of cells transformed by avian sarcoma virus or by mammalian type C viruses such as AbLV and the Gardner and Snyder-Theilen strains of FeSV. Thus, as one possibility, transformation by T-8 may involve different mechanisms than transformation by other RNA type C viruses.
Alternatively, morphologic reversion of T-8 transformation subsequent to initial isolation of the 64T8 cell line may have been associated with loss of P110 protein kinase activity and restoration of EGF binding sites. In this respect it should be noted that transformation of mink cells by other RNA-transforming viruses such as AbLV (17, 33) and FeSV (34, 35) often reverts at high frequency and is associated with loss of polyprotein expression. In addition, transformation-defective mutants of AbLV have been described that express AbLV P120 at levels comparable to wild type AbLV transformants but lack tyrosine specific protein kinase activity (17). Elucidation of molecular events involved in loss of T-8 associated phenotypic transformation will require further study. By this means, it may be possible to gain insight into the functional significance of T-8 P110 and determine the role, if any, of its nonstructural component in T-8 transformation.

Summary

Mink cells nonproductively-infected with the weakly-transforming T-8 isolate of murine leukemia virus (MuLV) express a 110,000 mol wt polyprotein designated T-8 P110. By immunoprecipitation analysis, T-8 P110 is shown to contain AKR-MuLV amino terminal \( \text{gag} \) gene-specific components (p15, p12) but to lack p30, p10, gp70, and p15(E) antigenic determinants. These observations are further substantiated by tryptic peptide analysis indicating T-8 P110 to share approximately six lysine-containing tryptic peptides with AKR-MuLV Pr65\(^{\text{pol}}\) and none with AKR-MuLV Pr82\(^{\text{pol}}\). Furthermore, of seven methionine-containing T-8 P110 tryptic peptides, at least four can be conclusively shown not to be present in either AKR-MuLV Pr180\(^{\text{pol}}\) or Pr82\(^{\text{pol}}\). A clonal mink cell line nonproductively infected by T-8, and expressing high levels of P110, although not morphologically transformed, is shown to lack elevated levels of tyrosine-specific protein kinase activity and reduction of epidermal growth factor binding sites characteristic of cells transformed by many other RNA-transforming viruses. These findings argue either that the T-8 viral genome contains acquired cellular sequences encoding a portion of P110, or that T-8 P110 represents an inphase deletion of AKR-MuLV Pr180\(^{\text{pol}}\) with extensive posttranslational modification and that an as yet unidentified protein is responsible for T-8 associated transformation.

The authors thank R. P. Nalewaik and M. C. Nace for excellent technical assistance.

Received for publication 23 June 1980 and in revised form 4 September 1980.

References

1. Scolnick, E. M., E. Rands, D. Williams, and W. P. Parks. 1973. Studies on the nucleic acid sequences of Kirsten sarcoma virus: a model for formation of a mammalian RNA-containing sarcoma virus. J. Virol. 12:458.
2. Stehelin, D., H. E. Varmus, J. M. Bishop, and P. K. Vogt. 1976. DNA related to transforming gene(s) of avian sarcoma virus is present in normal avian DNA. Nature (Lond.). 260:170.
3. Fischinger, P. J. 1980. Type C RNA transforming viruses. In Molecular Biology of RNA Tumor Viruses, J. R. Stephenson, editor. Academic Press, Inc., New York. 163.
4. Bister, J., J. J. Hayman, and P. K. Vogt. 1977. Defectiveness of avian myelocytomatosis
virus MC29: isolation of long-term nonproducer cultures and analysis of virus-specific polypeptide synthesis. *Virology*. **82**:431.

5. Stephenson, J. R., A. S. Khan, A. H. Sliski, and M. Essex. 1977. Feline oncornavirus-associated cell membrane antigen (FOCMA): evidence for an immunologically cross-reactive feline sarcoma virus-coded protein. *Proc. Natl. Acad. Sci. U. S. A.* **74**:5608.

6. Sherr, C. J., A. Sen, G. J. Todaro, A. Sliski, and M. Essex. 1978. Pseudotypes of feline sarcoma virus containing an 85,000 dalton protein with feline oncornavirus-associated cell membrane antigen (FOCMA) activity. *Proc. Natl. Acad. Sci. U. S. A.* **75**:1505.

7. Duesberg, P. H., and P. K. Vogt. 1979. Avian acute leukemia viruses MC29 and MH2 share specific RNA sequences: Evidence for a second class of transforming genes. *Proc. Natl. Acad. Sci. U. S. A.* **76**:1633.

8. Witte, O. N., N. Rosenberg, M. Paskind, A. Shields, and D. Baltimore. 1978. Identification of an Abelson murine leukemia virus-encoded protein present in transformed fibroblasts and lymphoid cells. *Proc. Natl. Acad. Sci. U. S. A.* **75**:2488.

9. Reynolds, F. H., Jr., T. S. Sacks, D. N. Deobagkar, and J. R. Stephenson. 1978. Cells nonproductively transformed by Abelson murine leukemia virus express a high molecular weight polypeptide containing structural and nonstructural components. *Proc. Natl. Acad. Sci. U. S. A.* **75**:3974.

10. Roussel, M., S. Saule, C. Lagrou, C. Rommens, H. Beug, T. Graf, and D. Stahelin. 1979. Three new types of viral oncogene of cellular origin specific for haematopoietic cell transformation. *Nature (Lond.).* **281**:452.

11. Collett, M. S., and R. L. Erikson. 1978. Protein kinase activity associated with the avian sarcoma virus src gene product. *Proc. Natl. Acad. Sci. U. S. A.* **75**:2021.

12. Levinson, A. D., H. Oppermann, L. Levintow, H. E. Varmus, and J. M. Bishop. 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. *Cell.* **15**:561.

13. Hunter, T., and B. M. Sefton. 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc. Natl. Acad. Sci. U. S. A.* **77**:1311.

14. Van de Ven, W. J. M., F. H. Reynolds, Jr., and J. R. Stephenson. 1980. The nonstructural components of polyproteins encoded by replication-defective mammalian transforming retroviruses are phosphorylated and have associated protein kinase activity. *Virology*. **103**:185.

15. Witte, O. N., A. Dasgupta, and D. Baltimore. 1980. Abelson murine leukemia virus protein is phosphorylated in vitro to form phosphotyrosine. *Nature (Lond.).* **283**:826.

16. Blomberg, J., F. H. Reynolds, Jr., W. J. M. Van de Ven, and J. R. Stephenson. 1980. Abelson murine leukemia virus transformation: loss of epidermal growth factor binding sites. *Nature (Lond.).* **286**:504.

17. Reynolds, F. H. Jr., W. J. M. Van de Ven, and J. R. Stephenson. 1980. Abelson murine leukemia virus transformation defective mutants with impaired P120 associated protein kinase activity. *J. Virol.* **36**:374.

18. Reynolds, F. H. Jr., W. J. M. Van de Ven, and J. R. Stephenson. Feline sarcoma virus P115 associated protein kinase phosphorylates tyrosine: identification of a cellular substrate conserved during evolution. *J. Biol. Chem.* In press.

19. Todaro, G. J., J. E. DeLarco, and S. Cohen. 1976. Transformation by murine and feline sarcoma viruses specifically blocks binding of epidermal growth factor to cells. *Nature (Lond.).* **246**:26.

20. Staal, S. P., J. W. Hartley, and W. P. Rowe. 1977. Isolation of transforming murine leukemia viruses from mice with a high incidence of spontaneous lymphoma. *Proc. Natl. Acad. Sci. U. S. A.* **74**:3065.

21. Sacks, T. L., F. H. Reynolds, Jr., D. N. Deobagkar, and J. R. Stephenson. 1978. Murine
leukemia virus (T-8) transformed-cells: identification of a precursor polyprotein containing gag gene-coded proteins (p15 and p12) and a nonstructural component. J. Virol. 27:809.
22. Van de Ven, W. J. M., F. H. Reynolds, Jr., R. P. Nalewaik, and J. R. Stephenson. 1980. The nonstructural component of the Abelson murine leukemia virus polyprotein, P120, is encoded by newly acquired genetic sequences. J. Virol. 32:1041.
23. Stephenson, J. R., R. K. Reynolds, S. G. Devare, and F. H. Reynolds Jr. 1977. Biochemical and immunologic properties of gag gene coded proteins of endogenous type-C RNA tumor viruses of diverse mammalian species. J. Biol. Chem. 252:7818.
24. Reynolds, R. K., W. J. M. Van de Ven, and J. R. Stephenson. 1978. Translation of type C viral RNA's in Xenopus laevis oocytes: evidence that the 120,000 molecular weight polyprotein expressed in Abelson leukemia virus transformed cells is viral-coded. J. Virol. 28:665.
25. Oroszlan, S., L. E. Henderson, J. R. Stephenson, T. D. Copeland, C. W. Long, J. M. Ihie, and R. V. Gilden. 1978. Amino- and carboxy-terminal amino acid sequences of murine leukemia virus gag gene-coded proteins. Proc. Natl. Acad. Sci. U. S. A. 75:1404.
26. Todaro, G. J., and J. E. DeLarco. 1978. Growth factors produced by sarcoma virus-transformed cells Cancer Res. 38:4147.
27. Pincus, T. 1980. The endogenous murine type C viruses. In Molecular Biology of RNA Tumor Viruses. J. R. Stephenson, editor. Academic Press, Inc., New York. 78.
28. Kawashima, J., H. Ikeda, J. W. Hartley, E. Stockert, W. P. Rowe, and L. J. Old. 1976. Changes in expression of murine leukemia virus antigens and production of xenotropic virus in the late preleukemia period in AKR mice. Proc. Natl. Acad. Sci. U. S. A. 73:4680.
29. Hartley, J. W., N. K. Wolford, L. J. Old, and W. P. Rowe. 1977. A new class of murine leukemia virus associated with development of spontaneous lymphomas. Proc. Natl. Acad. Sci. U. S. A. 74:789.
30. Elder, J. H., H. W. Gautsch, F. C. Jensen, R. A. Lerner, J. W. Hartley, and W. P. Rowe. 1977. Biochemical evidence that MCF murine leukemia viruses are envelope (env) gene recombinants. Proc. Natl. Acad. Sci. U. S. A. 74:4676.
31. Rommelaere, J., D. V. Faller, and N. Hopkins. 1978. Characterization and mapping of RNase T1-resistant oligonucleotides derived from the genomes of Akv and MCF murine leukemia viruses. Proc. Natl. Acad. Sci. U. S. A. 75:495.
32. Sefton, B. M., T. Hunter, K. Beemon, and W. Eckhart. 1980. Evidence that phosphorylation of tyrosine is essential for transformation by Rous sarcoma virus. Cell 20:807.
33. Sacks, T. L., E. J. Hershey, and J. R. Stephenson. 1979. Abelson murine leukemia virus infected cell lines defective in transformation. Virology. 97:231.
34. Porzig, K. J., K. C. Robbins, and S. A. Aaronson. 1979. Cellular regulation of mammalian sarcoma virus expression: A gene regulation model for oncogenesis. Cell. 16:875.
35. Stephenson, J. R., A. S. Khan, W. J. M. Van de Ven, and F. H. Reynolds, Jr. 1979. Type C retroviruses as vectors for cloning cellular genes with probable transforming function. J. Natl. Cancer Inst. 63:1111.