The genetic information for RNA C-type viruses is naturally integrated within the DNA of mouse cells (1-4). In tissue culture, unexpressed virus can be activated spontaneously (5) or by treatment with different chemical inducers (6-8). It has recently been shown that one class of induced virus is oncogenic when inoculated into animals (9). Thus, the study of cellular controls over the expression of integrated C-type viral genes may help in determining the mechanisms of development of naturally occurring cancer.

The identification of translational products of endogenous viral genetic information in virus-negative mouse cells would be useful in an analysis of the cellular restrictions to virus expression. It is known that in the absence of virus production, normal mouse cells contain detectable levels of an antigen that cross-reacts immunologically with the major structural polypeptide (p30) of mouse C-type viruses (10-12). However, it has yet to be shown biochemically that this activity represents the p30 polypeptide. Recently, a sensitive immunologic test for another virion polypeptide, p12 (13), has been reported. Further, type-specific immunologic assays for p12 and p30 polypeptides allow classification of mouse C-type viruses into different subgroups (14). In the present report, methods have been developed to purify these antigens from virus-negative mouse cells. It has been possible to identify them immunologically and biochemically as virion structural polypeptides, and to compare their antigenic specificities with those of known murine leukemia virus (MuLV) isolates.

Materials and Methods

Cells.—Liver or spleen cell suspensions were prepared from NIH Swiss mice obtained from colonies of the National Institutes of Health, Bethesda, Md. The continuous mouse cell lines, BALB/3T3 (15) and NIH/3T3 (16), were grown in Dulbecco's modification of Eagle's medium supplemented with 10% calf serum (Colorado Serum Co., Denver, Colo.).

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Abbreviations used in this paper: GuHCL, guanidine hydrochloride; K1, Kirsten strain of MuLV; MuLV, murine leukemia virus; R, Rausher strain of MuLV.
C-TYPE VIRION POLYPEPTIDES IN MOUSE CELLS

$^{125}$I-Labeled C-type Virus Polypeptides.—$^{125}$I-labeled 30,000 mol wt (p30) and 12,000 mol wt (p12) antigens of the Rauscher strain (R) and Kirsten strain (Ki) of MuLV were prepared as reported previously (12-14).

Assays.—Species- and type-specific competitive immunoprecipitation assays for the p30 and p12 antigens of Ki-MuLV and R-MuLV (14), and the reverse transcriptase assay for RNA C-type virus in tissue culture fluids (17) have been described. Protein was measured by the method of Lowry et al. (18) using bovine serum albumin as a standard.

Preparation of Cell Extracts.—Mouse spleen, liver, or tissue culture cell extracts were prepared by homogenization in 2 vol 0.01 M Tris-HCl buffer, pH 7.8 with a Ten Broeck homogenizer (Corning Glass Works, Corning, N. Y.), followed by sonication for approximately 60 s at a setting of 70 with a Biosonic II sonic oscillator (Will Scientific, Inc., Rochester, N. Y.). The resulting extracts were treated with 2 μg/ml DNase (bovine pancreas, DNase I, Worthington Biochemical Corp., Freehold, N.J.) at 4°C for 4 h, dialyzed overnight against 0.01 M Tris-HCl (pH 7.8), and clarified by centrifugation at 3,000 rpm in an IEC PR 6000 centrifuge (International Equipment Co., Needham Heights, Mass.) for 30 min.

Viral Antigen Purification from Cell Extracts.—Cell extracts were subjected to purification procedures as described below. Further details are provided in the results.

Acid precipitation: Around 500–1,500 mg cell extract in 100 ml 0.01 M Tris-HCl buffer was adjusted to pH 6.0 with 1.0 N HCl. The extract was incubated at 4°C for 1 h and the resulting precipitate pelleted by centrifugation at 30,000 rpm for 60 min in a Beckman type 30 rotor (Beckman Instruments Inc., Palo Alto, Calif.).

DE 52 ion exchange chromatography: The acid-soluble fraction was exhaustively dialyzed against 0.01 M Tris-HCl, 1.0 mM EDTA buffer, pH 7.8 (buffer A) and applied to a 1.5 × 25 cm Whatman DE 52 (H. Reeve Angel & Co., Inc., Clifton, N. J.) column equilibrated with the same buffer. The column was washed with 100 ml buffer A and the bound proteins eluted with a 200-ml linear gradient of 0.0–0.4 M KCl in buffer A. Columns were run at 4°C at a flow rate of 10 ml/h and 4-ml fractions collected.

Phosphocelulose ion exchange chromatography: Fractions containing the appropriate antigenic reactivity from DE 52 columns were pooled and dialyzed against 0.01 M Imidazole, 1.0 mM EDTA buffer, pH 6.0 (buffer B). This material was applied to a 1.5 × 25 cm phosphocelulose (Whatman p11) column equilibrated with buffer B. The column was washed and the protein eluted as described for the DE 52 column with the exception that buffer B was used.

Gel filtration chromatography: Fractions from phosphocelulose columns were dialyzed against 0.01 M Tris-HCl buffer, pH 8.0, lyophilized, resuspended in 0.4 ml of 6 M guanidine hydrochloride (GuHCl), 0.01 M DTT, 0.01 M EDTA buffer, pH 8.5 (buffer C), and heated at 45°C for 30 min. This material was chromatographed on an agarose (Bio Gel A-5m, 200–400 mesh, Bio-Rad Laboratories, Richmond, Calif.) column (1.5 × 90 cm) at room temperature using buffer C as eluant at a flow rate of 1.5 ml/h, and 1-ml fractions collected.

RESULTS

Purification of Viral-Specific Antigens from Normal Mouse Cells.—In attempts to purify viral-related antigens from cells, it was considered important to use methods that would not select for properties such as a molecular size or antigenicity. These latter parameters could then be used in the identification of the purified material. Thus, in the present studies, the approach utilized was to add tracer amounts of $^{125}$I-labeled virion polypeptides to cell extracts, and to develop methods to obtain optimal recovery and separation of the labeled polypeptide from the bulk of the cellular protein. These procedures included acid precipitation and ion exchange chromatography. At each step, the recovery
of the labeled marker could then be compared with that of the corresponding cellular immunologic reactivity. The amounts of $^{125}$I-labeled polypeptide markers were below levels detectable by competition immunoassay.

**Partial purification of p30**: 1,200 mg NIH Swiss liver cell extract was mixed with 12,000 cpm $^{125}$I-labeled p30 (<5 ng protein) and subjected to acid precipitation as described in Materials and Methods. As shown in Table I, 70% of the $^{125}$I-p30 but only 25% of the total cellular protein remained in the supernate, providing a nearly threefold enrichment of $^{125}$I-p30. Portions of the total and acid-soluble fractions were also assayed for mouse viral p30 antigen. The results (Table I) indicate that the cellular p30 cross-reacting material showed a two- to threefold increase in specific activity.

The acid-soluble fraction was subjected to DE 52 ion exchange chromatography as described in Materials and Methods. As shown in Fig. 1 A, $^{125}$I-p30 eluted at 0.1 M KCl, whereas the protein eluted over a much broader range with peaks at 0.05 M and 0.13 M KCl. Fractions 32–37, which contained the $^{125}$I-p30 peak, were pooled and assayed for $^{125}$I-p30 radioactivity, p30 cross-reacting material, and total protein. From the results (Table I), it is seen that both $^{125}$I-p30 and p30 cross-reacting material were enriched a further four- to fivefold. When the material in these pooled fractions was subjected to phosphocellulose chromatography, the $^{125}$I-label eluted at 0.16 M KCl (Fig. 1 B). $^{125}$I peak fractions (45–50) were pooled and assayed for $^{125}$I-p30 radioactivity, p30

### Table I

**Partial Purification of p30 Antigen from Mouse Cells**

| Purification step       | Total protein | Cellular p30 antigen | $^{125}$I-labeled p30 |
|-------------------------|---------------|----------------------|----------------------|
|                         | mg            | %                    | ng (X10^-3)/mg       | %                    | cpm (X 10^4)/mg     |
| Original extract        | 1200          | 100                  | 2                    | 100                  | 1.0                 |
| Acid precipitation (pH 6.0) | 312          | 70                   | 5.3                  | 66                   | 2.5                 |
| DEAE 52                 | 48            | 35                   | 18                   | 40                   | 10                  |
| Phosphocellulose        | 13            | 22                   | 40                   | 20                   | 18                  |
| Guanidine HCl           | 0.4           | 7.4                  | 492                  | 9.3                  | 310                 |

* 12,000 cpm $^{125}$I-labeled R-MuLV p30 (<5 ng) was added to 1,200 mg NIH Swiss mouse liver cell extract prepared as described in Materials and Methods, adjusted to pH 6.0, and centrifuged at 30,000 rpm for 90 min. p30 antigen in the acid-soluble supernatant extract fraction was purified further by DEAE 52, phosphocellulose, and agarose-GuHCl chromatography as described in the legends to Figs. 1 and 2. Total cellular protein concentrations were measured by the method of Lowry et al. (18). A heterologous intraspecies immunoassay with anti-AKR-MuLV sera and $^{125}$I-labeled R-MuLV p30 antigen was used for p30 antigen determinations.
Fig. 1. Partial purification of $^{125}$I-labeled RNA C-type viral polypeptides from NIH Swiss mouse liver cell extracts. (A) DE 52 chromatographic analysis of $[^{125}$I]R-MuLV p30 antigen (12,000 cpm) mixed with 312 mg “acid-soluble” liver cell extract as described in Materials and Methods. (B) Phosphocellulose chromatographic analysis of fractions 32-37 from (A) as described in Materials and Methods. (C) DE 52 chromatography of $[^{125}$I]R-MuLV p12 antigen (18,000 cpm) and 133 mg acid-soluble liver cell extract. (D) Phosphocellulose chromatography of fractions 30-37 from (C).

cross-reacting material, and total protein. As shown in Table I, the pooled fractions were enriched a further two- to threefold for both $[^{125}$I]p30 and cellular p30 cross-reacting material. These sequential purification steps thus resulted in a 18–20-fold increase in specific activity of both $^{125}$I-labeled p30 and un-
labeled p30 cross-reacting antigen. The fact that the enrichments of \(^{125}\)I-labeled p30 and cellular p30 cross-reacting antigen were comparable at each step of purification indicated that the cellular antigenic reactivity was biochemically similar to the virion p30 polypeptide.

**Partial purification of p12:** Extracts of NIH Swiss mouse cells were found to contain barely detectable reactivity in the immunoassay for p12 antigen. Attempts were made to purify this antigenic activity using similar procedures except for the use of \(^{125}\)I-labeled p12 as marker. As shown in Table II, 65% of the \(^{125}\)I-p12, but only 25% of the total protein in the extract remained in solution at pH 6.0 (Table II). When analyzed by ion exchange chromatography, \(^{125}\)I-p12 eluted at 0.15 M KCl from DE 52 (Fig. 1 C), and in the wash-through of the phosphocellulose column (Fig. 1 D). The relative enrichment of \(^{125}\)I-p12 and of cell-associated p12-like antigen was similar at each stage of the purification (Table II). These sequential procedures provided a 9–10-fold increase in specific activity of p12 cross-reacting antigen.

**Molecular Weight Analysis of Partially Purified Viral Polypeptides.—**The antigenic reactivities isolated from NIH Swiss mouse liver cells were subjected to agarose: GuHCl chromatography to determine their molecular sizes. Fractions were tested for total cellular protein, \(^{125}\)I radioactivity, and p30 and p12 immunologic cross reactivities. As shown in Fig. 2, the p30-like cellular activity chromatographed with \(^{125}\)I-labeled p30; similarly the p12 antigen-like reactivity and \(^{125}\)I-labeled p12 antigen comigrated. From these findings, it is clear that the molecular weights of the cellular immunological reactivities were indistinguishable from those of the corresponding viral structural polypeptides. In addition, agarose: GuHCl chromatography provided a further 10–20-fold

### Table II

**Partial Purification of p12 Antigen from Mouse Cells**

| Purification step | Total protein | Cellular p12 antigen | \(^{125}\)I-labeled p12 antigen |
|------------------|---------------|---------------------|-----------------------------|
|                  | mg % | Sp act (X10^3)/mg | Yield | Sp act (X10^3)/mg | Yield |
| Original extract | 500 | 100 | 0.6 | 100 | 3.6 |
| Acid precipitation (pH 6.0) | 133 | 72 | 1.1 | 78 | 7.5 |
| DE 52 | 35 | 51 | 4.3 | 56 | 29 |
| Phosphocellulose | 2.5 | 35 | 4.9 | 39 | 33 |
| Guanidine HCl | 1.9 | 18 | 39 | 22 | 208 |

* Purification procedures were performed as described for the p30 in Table I with the exception that a heterologous intraspecies immunoassay with anti-AKR-MuLV sera and \(^{125}\)I-labeled R-MuLV p12 antigen (14) was used for p12 antigen determinations.
purification of each, resulting in final purifications of cellular p12 and p30 by 50–60-fold and 200–300-fold, respectively (Tables I and II).

Analysis of Other Virus-Negative Cells for p30 and p12 Viral Polypeptides.—The above studies demonstrate the presence of two C-type viral polypeptides in NIH Swiss mouse liver cells. In other studies, NIH Swiss spleen, NIH/3T3, and BALB/3T3 cultures, subjected to the same purification procedures, were
also found to contain both viral polypeptides. In contrast, purified extracts of African green monkey and human liver cells were nonreactive in immunoassays for the p30 and p12 mouse C-type viral polypeptides. The results of the above biochemical and immunological studies provide strong evidence that the low levels of immunological cross-reactivity with two virion polypeptides detected in normal virus-negative mouse cells represent those structural polypeptides.

Comparison of the Levels of p30 and p12 in Normal and Virus-Infected Mouse Cells.—The levels of the two viral polypeptides present in normal mouse cells were compared with the levels of each found in whole virions and in virus-producing cells. As shown in Table III, the ratio of p30-p12 in a purified preparation of Ki-MuLV was 3.2. A similar ratio was detected in NIH/3T3 cells producing either Ki-MuLV or R-MuLV. While the levels of the two polypeptides in uninfected NIH/3T3 cells were considerably lower, the ratio of their concentrations (3.1) was comparable to that observed in whole virions.

It was possible to study the relative concentrations of viral-specific polypeptides in normal cells and in the same cells exogenously infected with, and chronically producing, MuLV. At the same time, the level of virus production in each case was compared by reverse transcriptase assay. This latter assay provides a measure of physical particles released into the supernate (17). As shown in Table IV, with MuLV-infected NIH/3T3 and BALB/3T3 cells, the ratio of virus released to the level of cell-associated p30 antigen ranged from 53 to 75 pmol viral polymerase/ng cellular p30. In contrast, this ratio was less than 0.1 in uninfected NIH/3T3 or BALB/3T3 cells. These findings suggest that the level of this cell-associated viral polypeptide in normal cells was disproportionately high for the amount of virus released.

Immunological Type Specificities of Partially Purified Cell-Associated Viral Polypeptides.—It has been previously demonstrated that the p30 and p12 polypeptides of mouse C-type viruses possess strain- or type-specific antigenic determinants (14). It was possible to test partially purified p30 and p12 polypeptides...
TABLE IV
Relative Amounts of Cell-Associated p30 and Virus Released by Infected and Uninfected Mouse Cells

| Cells     | p30/10^6 Cells* | Virus released into supernatant 24 h/10^6 cells† | PMol supernatant viral polymerase/µg cellular p30 |
|-----------|-----------------|-----------------------------------------------|-----------------------------------------------|
| NIH/3T3   |                 |                                               |                                               |
| Uninfected| 1.8 x 10^6      | <2.0 x 10^-1                                  | <0.1                                          |
| +KI-MuLV  | 1.9 x 10^6      | 1.0 x 10^4                                   | 53                                            |
| +R-MuLV   | 2.1 x 10^9      | 1.2 x 10^4                                   | 59                                            |
| BALB/3T3  |                 |                                               |                                               |
| Uninfected| 1.5 x 10^9      | <2.0 x 10^-1                                  | <0.1                                          |
| +R-MuLV   | 1.2 x 10^9      | 9.0 x 10^3                                   | 75                                            |

* Quantitation of cell-associated p30 was performed as described in the legend to Table I. Results are the mean values from three separate determinations.
† Amount of virus in supernatant fluids was determined by methods described previously (17). Results are expressed as picomoles of [3H]thymidine monophosphate incorporated by 24-h harvests of tissue culture fluids per 10^6 cells and are mean values from three separate determinations.

peptides from NIH Swiss cells for their abilities to compete in type-specific immunoassays for R-MuLV or AKR-MuLV p30 or p12 polypeptides, and thus, to compare the cell-associated viral polypeptides with those of known MuLV(s). As shown in Fig. 3, p30 and p12 polypeptides of R-MuLV reacted preferentially in R-MuLV homologous immunoassays, while unlabeled p30 and p12 of AKR-MuLV competed preferentially in homologous assays for those components of AKR-MuLV. The patterns of reactivity of isolated p30 and p12 from NIH Swiss cells in corresponding R-MuLV and AKR-MuLV type-specific assays were intermediate in each case (Fig. 3), making them readily distinguishable from both R-MuLV and AKR-MuLV polypeptides. In these same assays, the two polypeptides of previously studied inducible C-type viruses were indistinguishable from those of AKR-MuLV (14). Thus, the present findings suggest that the p30 and p12 polypeptides of NIH Swiss cells may represent components of a new class of endogenous C-type virus.

DISCUSSION

The present studies show that it is possible to achieve a marked enrichment of antigenic activities from normal mouse cells that cross-react with two mouse C-type viral polypeptides. The partially purified antigens when studied by biochemical and immunological techniques, independent of the methods utilized in their isolation, had properties very similar to those of virion p30 and p12 polypeptides. The fact that the recovery of viral polypeptides from normal cells at each step in the purification closely resembled the recovery of labeled poly-
peptide marker suggests that the majority of the antigenic activity is in a form similar to that observed in the virus. If larger precursor molecules exist, such as those synthesized by certain RNA viruses (19), these must either be immunologically distinct or represent only a minor fraction of the total viral polypeptides of the cell.

Viral antigens were obtained from cells in sufficient quantities to allow subgroup classification in type-specific competition immunoassays for p30 and p12 polypeptides (14). The patterns of reactivities of both antigens in these assays were similar to each other but were clearly distinct from those of either R-MuLV or AKR-MuLV. Previous studies have shown that two inducible viruses of
BALB/c mouse cells and an inducible virus of C58 cells, while biologically distinguishable (1, 3, 17), contain p30 and p12 polypeptides that are very similar to those of AKR-MuLV (14). While corresponding cell-associated viral polypeptides of NIH Swiss mouse cells may be the composite of gene products of more than one endogenous virus, the present studies indicate that the dominant antigenic activities in these preparations are different from those of any other MuLV strain so far examined. Thus, the viral antigens of NIH Swiss cells may reflect those of a new class of endogenous virus.

Recent studies indicate that NIH Swiss mouse cells contain C-type viral genetic material within their high molecular weight DNA (4). The present report provides clear evidence that at least two structural polypeptides are fully translated in these cells in the absence of detectable virus release. Further, the ratio of intracellular concentrations of these polypeptides closely resembled that observed in whole virions. Thus, the cellular regulatory factors that are involved in restriction of complete virus expression do not lead to disproportionate levels of either molecule. Further, the amounts of cell-associated p30 and p12 in normal mouse cells were disproportionately high in relation to the amount of virus released by the cells. These findings suggest that the block to synthesis of endogenous NIH Swiss virus may involve a replication step(s) other than the translation of either of these two virion polypeptides. In other studies in which uninfected mouse cells have been examined for the presence of two other virion polypeptides, the reverse transcriptase (20) and a 69,000-70,000 mol wt glycoprotein (21), neither has been detected. This may be due to differences in sensitivity of the assays or, alternatively, may reflect the existence of regulatory controls at the level of translation.

The methods developed in the present study have provided a 50–60-fold purification of the p12 viral polypeptide and nearly 300-fold purification of the C-type viral p30 antigen from virus-negative mouse cells. This was possible because of the availability of highly purified 131I-labeled viral polypeptide markers and of very sensitive immunologic techniques for detection of the corresponding cell-associated polypeptides. While specific viral polypeptide markers are as yet unavailable from many species, broadly reactive and very sensitive immunoassays which detect all known mammalian C-type p30 antigens (12) have been developed. Further, using the same isolation procedures, primate C-type viral p30 antigens appear to behave similarly to mouse viral p30 (unpublished observations). Thus, it may be possible to use these methods to enrich for C-type viral-related antigens from species in which the levels in crude cell extracts are presently below detection (12).

**SUMMARY**

Antigens which immunologically cross-react with two mouse C-type viral polypeptides, p30 and p12, are present at very low levels in normal virus-negative mouse cells. These two antigens have been purified by 50–300-fold
from cell extracts and shown to cochromatograph with the corresponding labeled viral polypeptides in several systems. Their type-specific antigenicities are shown to be distinct from those of previously tested MuLV isolates suggesting that they may be components of a new class of endogenous C-type virus. The methods utilized in the present studies for concentration of virus-specific antigens of normal mouse cells provide an approach for detection of C-type viral antigens in cells of other species.

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