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# Quantitation of Murine Mammary Tumor Virus-Related RNA in Mammary Tissues of Low- and High-Mammary-Tumor-Incidence Mouse Strains

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Lactating mammary glands and hormonally induced mammary tumors of BALB/c mice from three geographically separated breeding colonies were examined by molecular hybridization, using murine mammary tumor virus (MuMTV) cDNA representing the entire viral genome to determine the amount of MuMTV-related RNA expressed in these tissues. The RNA extracted from these tissues by the classical sodium dodecyl sulfate-pronase, phenol-chloroform procedure (method 1) contained barely detectable levels of MuMTV-related sequences. In contrast, both normal lactating mammary glands and hormonally induced mammary tumors of these mice were found to contain approximately one to two copies of the MuMTV genome per cell by using a new procedure in which the RNA was extracted with guanidine derivatives (method 2). No significant differences in the MuMTV-related RNA content of the BALB/c mammary tissues were observed regardless of their colony of origin. Our results suggest that expression of MuMTV RNA does not change in BALB/c mammary glands during transformation to a malignant state and that MuMTV expression does not play a role in tumorigenesis in these mice. In view of the increased recovery of MuMTV-related RNA from BALB/c mice with method 2, we compared the level of MuMTV RNA expression in lactating mammary glands and mammary tumors of other mouse strains, including C57BL/6 and RIII, using both extraction methods. Yields of MuMTV-related RNA from mammary tissues increased by as much as 35- to 40-fold, using method 2 as compared with method 1. Therefore method 2, involving guanidine derivatives, appears to be the method of choice for MuMTV-related RNA extraction from the mammary tissues of certain strains of mice, particularly those expressing relatively low levels of MuMTV RNA.

The murine mammary tumor virus (MuMTV) is unique among murine oncornaviruses in its ability to cause mammary adenocarcinomas (11). MuMTV-induced mammary tumor incidence in various mouse strains may be affected by a number of factors, including hormones, genetic background, diet, and parity number (10). Nucleotide sequences related to the MuMTV genome have been detected by nucleic acid hybridization in the somatic cell DNA of all inbred mouse strains tested, as well as in feral mice (9). Significant expression of MuMTV RNA, however, has been shown to occur only in mammary glands and virus-induced mammary tumors. Recent studies with representative MuMTV cDNA ($cDNA_{rep}$) have shown that in high-mammary-tumor-incidence strains of mice such as C3H and GR, mammary tumors may contain quantities of MuMTV-related RNA approximately equal to those of normal lactating mammary glands (2, 4-7).

The BALB/c mouse strain in various colonies has been reported to demonstrate a mammary tumor incidence widely varying from 1% to as high as 35% in certain sublines (8). All mammary tumors arising spontaneously from this strain, however, appear late in the life of the mouse at 16 to 24 months of age. The frequency of mammary tumor incidence, as well as the time of appearance of the tumors, can be increased through the use of chemical carcinogens (2, 6, 11) or by hormonal induction with pituitary isografts (4, 7, 8, 12, 13). The involvement of MuMTV in the production of BALB/c spontaneous mammary tumors has not been established, and even in sublines with a tumor incidence of 35%, no evidence of MuMTV antigens or type B oncornaviral particles could be detected in milk or tumor extracts, nor could biological evidence of tumorigenic activity be established when the extracts were inoculated into various mouse strains (8). In other laboratories

in which MuMTV-related RNAs in BALB/c mammary glands and tumors were quantitated, the quantity of such RNA appeared to vary, depending on the colony from which the mice were selected (1, 2, 4–7, 13). We examined this possibility by using an MuMTV $cDNA_{rep}$ probe and BALB/c mice from three U.S. laboratories in three different locations. BALB/c lactating mammary glands and hormonally induced tumors, when extracted by a procedure utilizing proteinase treatment and phenol extraction (20; method 1) were found to have similar, barely detectable levels of MuMTV RNA. The use of a new RNA extraction procedure specifically developed for tissues rich in RNase (18; method 2) greatly increased the apparent yield of MuMTV-related RNA in BALB/c mammary glands and hormonally induced mammary tumors. However, we found no significant differences in the quality or quantity of MuMTV-related RNA, regardless of the colony of origin of the mice or whether the tissue was normal or malignant.

Extraction of RNA by method 2 rather than method 1 increased the yields of MuMTV-related RNA from known MuMTV RNA-containing mammary tissue by 3- to 40-fold, depending on the mouse strain examined. The general pattern of MuMTV RNA expression (as cDNA-RNA hybridization) in mammary glands from high- and low-mammary-tumor-incidence mouse strains GR, C57BL, C3H, and RIII/fC57BL was found to be identical whether RNA was extracted by method 1 or method 2. RNA-cDNA hybridization patterns from RIII mouse mammary glands, however, varied, depending on the method of RNA extraction. RIII mammary glands extracted by method 1 appeared to contain two distinct populations of MuMTV-related RNA, with the most abundant class subgenomic in nature and the less abundant class containing the entire genome. RIII mammary tumors extracted by method 1 contained a single-component class of MuMTV-related RNA containing all of the genome sequence. Both RIII mammary glands and tumors extracted by method 2 appeared to contain single-component MuMTV-related RNA with complete genome representation.

## MATERIALS AND METHODS

**Cell culture.** The GR-3A (14), MuMTV-73 (15), and C57BL (19) cell lines were grown in monolayer culture in Eagle minimal essential medium supplemented with 10% fetal calf serum. For the measurement of MuMTV-related RNA and protein in virus-producing cells, the cultures were treated with dexamethasone (1 μg/ml for 18 h before extraction.

**cDNA preparation and hybridization.** Single-stranded cDNA representative of the entire MuMTV genome was prepared, using avian myeloblastosis virus reverse transcriptase and MuMTV subunit genomic RNA, with calf thymus oligonucleotide DNA primers. The representative nature of this cDNA preparation ($cDNA_{rep}$) has been described previously (16). Hybridization of extracted cellular or viral RNAs with [$^3$H]cDNA was carried out under mineral oil in 0.6 M NaCl. Percent hybridization was calculated by determining the quantity of cDNA remaining after treatment with S1 nuclease (15). RNA $C_0t$ values ($C_rt$) were determined as the product of RNA concentration in moles of nucleotide and incubation time in seconds, and corrections were made for salt concentration (16).

**Mice.** BALB/c mice were obtained from three sources: (i) the Institute for Medical Research in Camden, N.Y. (IMR mice) from J. Holben; (ii) the Cancer Research Laboratory in California through the kindness of S. Nandi (CRL mice); and (iii) the Michigan Cancer Foundation in Detroit, Mich., through the courtesy of C. McGrath (MCF mice). The spontaneous incidence of mammary tumors in all three colonies was <10%. BALB/c mice carrying hormonally induced mammary tumors and pituitary isografts for maintenance of those tumors were kindly provided by S. Nandi and C. McGrath. RIII and C57BL/6 and RIII/fC57BL mice were purchased from J. Holben at the Institute of Cancer Research. C3H mice were the gift of G. Fernandes of this institute.

**RNA extraction.** RNA extraction from purified C3H MuMTV obtained through the courtesy of the National Cancer Institute Research Resources Agency and RIII MuMTV purified from mouse milk was carried out as previously described (10). Total cellular RNA extraction from mid-lactating (unless otherwise indicated) mammary glands and mammary tumors was carried out by one or both of the following procedures. All buffers were either autoclaved or treated with diethyl pyrocarbonate before use.

**Method 1 (SDS-pronase, phenol-chloroform).** This procedure is a modification of that described previously (10). Tissues were homogenized in 10 mM Tris-hydrochloride (pH 8) containing 0.1 M NaCl and 1 mM EDTA (TNE buffer), using a Sorvall Omnimixer at full speed for 2 min while the homogenizing cup was kept in ice. Sodium dodecyl sulfate (SDS) and previously autodigested pronase were then immediately added to a final concentration of 1% (wt/vol) and 1 mg/ml, respectively. After digestion was carried out for 30 min at 37°C, the sample was extracted with 1 volume of phenol which had been previously saturated with TNE together with 0.8 volume of chloroform. The aqueous layer was then removed, and total nucleic acid was precipitated by the addition of 0.1 volume of 5 M LiCl and 2.5 volumes of absolute ethanol followed by incubation at −40°C overnight. A nucleic acid pellet was obtained by centrifugation in the Sorvall HB-4 rotor for 20 min at 10,000 × *g*. The pellet was washed twice with 80% ethanol, dried, and redissolved in 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM $MgCl_2$ (DNase buffer), and DNase I (Worthington Diagnostics, Freehold, N.J.) was added to the solution to a final concentration of 50 μg/ml. After incubation at 37°C for 15 to 30 min, SDS and pronase were added to 1% (wt/vol) and 0.5 mg/ml,

respectively, and incubation was continued for an additional 30 min. Phenol-chloroform extraction and ethanol precipitation of RNA were then carried out as described above, and the resulting RNA preparations were dissolved in water.

**Method 2 (guanidine thiocyanate).** This procedure is essentially a modification of that described by Robertson and Varmus (14) and Ullrich et al. (18). Immediately after being removed from the animal, the tissue was placed in 4 M guanidine thiocyanate containing 25 mM sodium citrate (pH 7.2), 0.1 M 2-mercaptoethanol, and 1% (wt/vol) sodium *N*-lauroyl sarcosinate and homogenized, using a Sorvall Omnimixer at full speed for 1 min at room temperature. After centrifugation of the homogenate at 8,000 × *g* for 10 min at 10°C, the supernatant was collected, and nucleic acid was precipitated by the addition of 0.025 volume of 1 M acetic acid and 0.75 volume of absolute ethanol and subsequent incubation at −20°C overnight. The nucleic acid pellet obtained by centrifugation at 6,000 × *g* for 10 min at −10°C was redissolved in half its original volume, using 7.5 M guanidine hydrochloride containing 25 mM sodium citrate (pH 7.2) and 5 mM dithiothreitol. After the solution was shaken vigorously for 5 min at room temperature, the RNA was precipitated by the addition of 0.025 volume of 1 M acetic acid and 0.5 volume of absolute ethanol and placing the solution at −20°C overnight. A second precipitation of RNA from guanidine hydrochloride yielded an RNA pellet after centrifugation which was redissolved in a minimal volume of water. After removal of undissolved material by centrifugation in a Beckman Microfuge for 5 min, the RNA was precipitated one final time by addition of LiCl and absolute ethanol as described for method 1. The final RNA pellet was dissolved in a minimal volume of sterile water.

**Radioimmunocompetition assays.** MuMTV p28 and gp52 were purified to homogeneity by hydrophobic, ion-exchange, and gel filtration chromatography and iodinated with $^{125}I$ as previously described (3). Competing antigen preparations from mammary gland or tumor tissue, as well as cell cultures, were prepared by homogenizing material, after fine mincing, in buffer consisting of 50 mM Tris-hydrochloride (pH 8.0), 0.4 M KCl, 5 mM sodium acetate, and 0.5% (vol/vol) Triton X-100. Nuclei and cell debris were sedimented at 5,000 × *g* in the Sorvall HB-4 rotor for 10 min, and sodium deoxycholate was added to the supernatant fraction to a final concentration of 0.5% (wt/vol). The solution was then extracted twice with 2 volumes of diethyl ether, the ether was evaporated, and the water phase was centrifuged at 10,000 × *g* for 15 min at 4°C. The resulting supernatant was then dialyzed for 18 h against 5 mM Tris-hydrochloride (pH 7.8) containing 0.005% Triton X-100 and lyophilized, and the resulting powder was taken up in a minimal volume of water. Radioimmunocompetition assays to determine the quantity of gp52 and p28 present in tissue and cell extracts were carried out as previously described (3).

## RESULTS

**MuMTV-related RNA in BALB/c mouse mammary glands and tumors.** RNA from lactating mammary glands of IMR, CRL, and MCF BALB/c mice, as well as hormonally induced mammary tumors carried in mice with pituitary isografts, was extracted by a procedure utilizing proteinase digestion of SDS-solubilized tissue, followed by phenol-chloroform extraction (method 1, see Materials and Methods). C3H mouse mammary glands and tumors were also extracted as positive controls, since they had previously been shown to contain large quantities of MuMTV-related RNA (20). The high-incidence-mammary-tumor RIII mouse strain offspring, when suckled on a low-mammary-tumor-incidence strain (C57BL/6), no longer produce significant quantities of MuMTV in their milk and also demonstrate a much-decreased mammary tumor incidence (11). Lactating mammary glands from RIII/fC57BL mice therefore were extracted to serve as possible negative controls. The results of the RNA MuMTV $cDNA_{rep}$ hybridization studies are shown in Fig. 1A. No significant differences in the hybridization patterns of RNA from BALB/c mammary glands were observed, regardless of their colony of origin. At $C_rt$ values of up to $10^5$, <20% hybridization was obtained with RNA from both BALB/c mammary glands and hormonally induced tumors. This value was not significantly higher than that obtained with our negative control (RIII/fC57BL lactating mammary gland RNA), which hybridized to <10% of the $cDNA_{rep}$ at a $C_rt$ of $10^5$. RNA extracted from C3H mouse mammary tumors, and lactating mammary glands, however, produced hybridization kinetics indicating $C_rt_{1/2}$ values of 110 and 150, respectively. We therefore attempted to determine whether the chemical method of RNA extraction which we used for this study might be the reason for the apparent absence of MuMTV-related RNA in BALB/c mouse mammary tissues. A procedure utilizing guanidine derivatives for the extraction of RNA from tissues rich in RNase activity was recently described (18; method 2, see Materials and Methods), but had not yet been compared with other procedures for the extraction of MuMTV-related RNA from mouse mammary glands with respect to possible increased yields of such RNA. Representative hybridization results for MuMTV-related RNA content of BALB/c mammary glands and hormonally induced tumors and RIII/fC57BL lactating mammary glands extracted by method 2 are shown in Fig. 1B. Yields of MuMTV-related RNA from BALB/c mammary glands and tumors were greatly increased. The $C_rt_{1/2}$ value for MuMTV-related RNA from BALB/c mouse tissues could be determined as approximately $10^5$ mol·s/liter. Maximal $cDNA_{rep}$ hybridization of

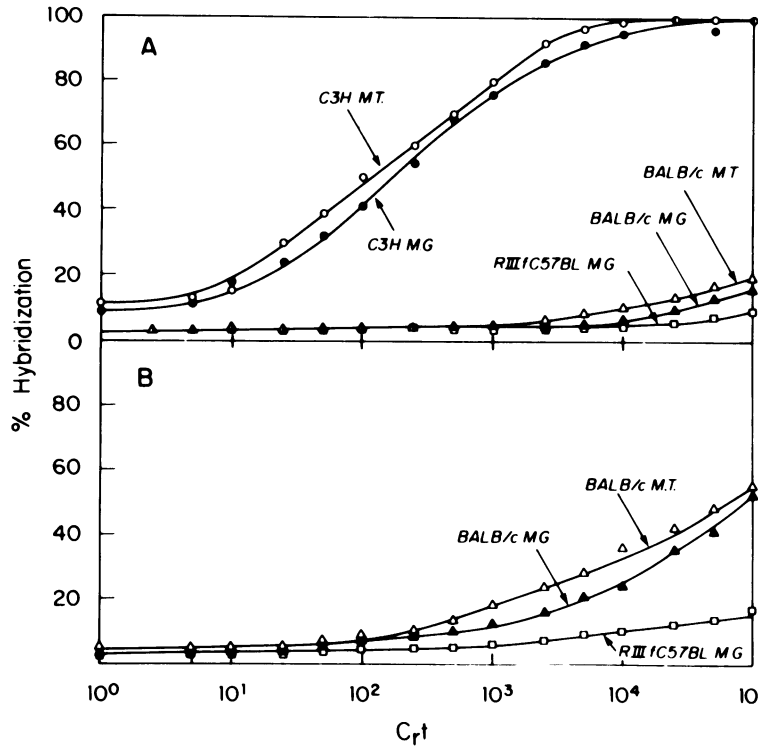


FIG. 1. *Hybridization of MuMTV cDNA$_{rep}$ to RNA extracted by method 1 (A) or method 2 (B) from mid-lactating mammary glands of BALB/c (▲), RIII/fC57BL/6 (□), and C3H (●) mice. A single representative line is shown for the BALB/c mice, as mice from all three colonies when examined gave virtually identical results. Hybridization was also carried out on RNA extracted from spontaneous mammary tumors of C3H (○) mice and on hormonally induced tumors from MCF and CRL BALB/c mice (△) maintained in mice with pituitary isografts. Hybridization was carried out as described in the text, and no normalization of values was performed.*

RIII/fC57BL mammary gland RNA extracted by method 2, however, only increased to 16% at a $C_rt$ value of $10^5$, suggesting that the increased yields of RNA seen in the other tissue sources were, in fact, due to better preservation of pre-existing cellular RNA. Thus, we found that BALB/c mammary glands and hormonally induced tumors contained similar, very low quantities of MuMTV-related RNA and that the quantity of such RNA appeared not to vary with the colony of origin. Although the use of different methods of RNA extraction altered the apparent yield of MuMTV-related RNA quantities of both the glands and tumors, the concentration of such RNA in the different tissues appeared equivalent. Additional studies showed no significant differences in MuMTV-related RNA content in BALB/c mammary glands from mid-lactating mice of first, second, fourth, or fifth lactation (data not shown).

In view of the increased recovery of MuMTV-related RNA from BALB/c mammary glands and tumors obtained with method 2 as compared with method 1, we determined whether increased yields would also be obtained with other high- and low-mammary-tumor-incidence strains of mice known to express varying quantities of MuMTV RNA. The C57BL/6 mouse strain demonstrates low mammary tumor incidence and has been reported to contain significant quantities of MuMTV-related RNA in mammary glands extracted with guanidine thiocyanate (14). We found that RNA extracted by method 2 contained approximately 40-fold greater quantities of MuMTV-related RNA ($C_rt_{1/2}$ = 1,600) than did RNA extracted by method 1 ($C_rt_{1/2}$ = 70,000) from lactating mammary glands of this mouse strain (Fig. 2B and A, respectively). That the use of method 1 alone did not necessarily produce low quantities of MuMTV-related RNA is shown in Fig. 1A and 2A, in which RNA extracted by method 1 from mid-lactating mammary glands of the high-mammary-tumor-incidence MuMTV-producing C3H and GR mouse strains may be seen to have a $C_rt_{1/2}$ of 150 and 170 mol·s/liter, respectively.

The RIII mouse strain also possesses a high MuMTV-dependent spontaneous mammary tumor incidence, but no information on the quantity of MuMTV-related RNA present in the mammary glands of this strain has yet appeared in the literature. The kinetics of MuMTV $cDNA_{rep}$ hybridization to RNA extracted from RIII mouse mid-lactating mammary glands and early-arising tumors extracted by method 1 are shown in Fig. 2A. The RIII mammary gland MuMTV-related RNA appeared to consist of two distinct populations, the most abundant of which ($C_rt_{1/2}$ = 200, component I) contained approximately 30% of the information present in the $cDNA_{rep}$ probe. A minor population of MuMTV-related RNA ($C_rt_{1/2}$ = 40,000, component II) contained the complete $cDNA_{rep}$ sequence. It is interesting to note that RNA extracted from RIII mammary tumors by method 1 (Fig. 2A) appeared to consist of a single population of RNA containing the entire probe sequence, as did the MuMTV-related RNA from all other mouse strains examined. Rather than the biphasic pattern of hybridization kinetics exhibited by RIII mammary gland method 1-extracted RNA with MuMTV cDNA, RNA from the same source extracted by the guanidine procedure (method 2) showed a single RNA component. Even if the more abundant "component I" RNA (Fig. 2A) is used for comparison, a significantly greater quantity of MuMTV-related RNA was present in RIII lactating mammary glands when extraction was by method 2 rather than by method 1, whereas quantities of MuMTV-related RNA in RIII mammary tumors appeared identical, regardless of the method of extraction. Our complete hybridization studies, including 70S MuMTV RNA standard hybridizations, as well as the number of hybridizations performed for each mouse strain and with each RNA extraction method, are summarized in Table 1. The results indicate that method 2 should be the method of choice for providing high yields of MuMTV-related RNA from the mammary tissues of certain strains of mice, particularly those expressing low levels of such RNA (i.e., BALB/c mice).

**Analysis of MuMTV protein content from various cell sources.** The differing concentrations of MuMTV-related RNA seen in tissues

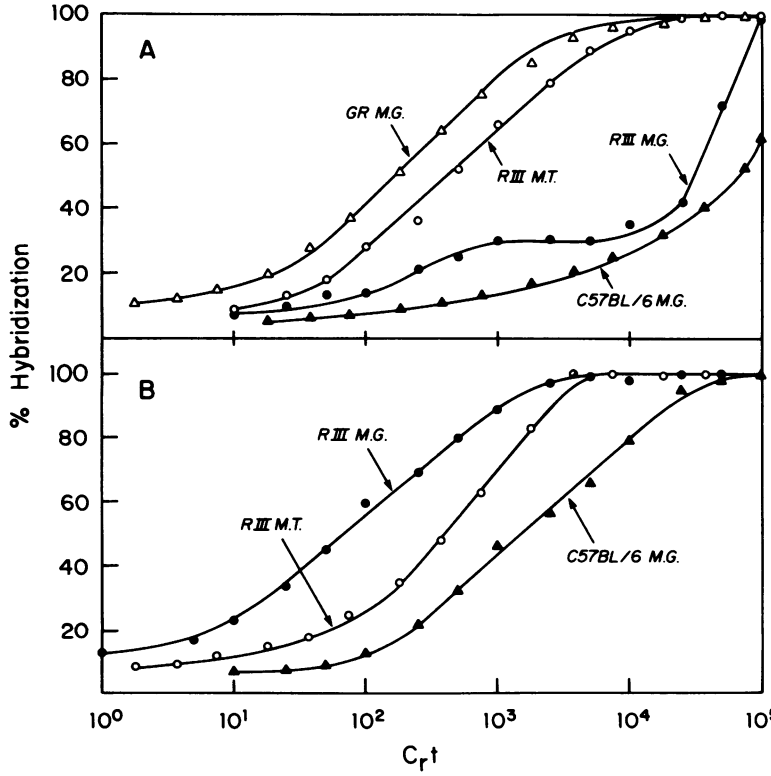


FIG. 2. *Hybridization of MuMTV $cDNA_{rep}$ to RNA extracted by method 1 (A) or method 2 (B) from C57BL/6 (▲), RIII (●), and GR (△) mouse mammary glands dissected at mid-lactation and also to RNA extracted from RIII mammary tumors* (○).

TABLE 1. *MuMTV-related RNA in murine cellular RNA*

| RNA source[a] | Method of extraction | $C_rt_{1/2}$ value | % MuMTV-specific RNA | Approx no. of molecules/cell[b] |
|---|---|---|---|---|
| C3H MuMTV 70S RNA | Method 1 (3)[c] | $2.5 \times 10^{-2}$ | 100 | — |
| RIII MuMTV total RNA | Method 1 (3) | $2.8 \times 10^{-2}$ | 89 | — |
| GR-3A cells | Method 1 (3) | $1.1 \times 10^{1}$ | 0.23 | 8,680 |
| GR mammary gland | Method 1 (3) | $1.7 \times 10^{2}$ | 0.015 | 564 |
| C57BL/6 mammary gland | Method 1 (4) | $7.0 \times 10^{4}$ | 0.00004 | 2 |
| C57BL/6 mammary gland | Method 2 (3) | $1.6 \times 10^{3}$ | 0.0016 | 80 |
| BALB/c mammary gland | Method 1 (6) | $>10^{5}$ | $\ll$0.000002 | $\ll$0.2 |
| BALB/c mammary gland | Method 2 (6) | $9.0 \times 10^{4}$ | 0.000027 | 1 |
| BALB/c mammary tumor | Method 1 (6) | $>10^{5}$ | $\ll$0.000002 | $\ll$0.2 |
| BALB/c mammary tumor | Method 2 (6) | $6.0 \times 10^{4}$ | 0.000042 | 2 |
| C3H mammary gland | Method 1 (3) | $1.5 \times 10^{2}$ | 0.017 | 497 |
| C3H mammary tumor | Method 1 (3) | $1.1 \times 10^{2}$ | 0.023 | 868 |
| RIII mammary gland | Method 1 (10) | | | |
| | Component I | $2.0 \times 10^{2}$ | 0.0125 | 469[d] |
| | Component II | $4.0 \times 10^{4}$ | 0.000063 | 2 |
| RIII mammary gland | Method 2 (4) | $7.0 \times 10^{1}$ | 0.036 | 1,353 |
| RIII mammary tumor | Method 1 (4) | $5.0 \times 10^{2}$ | 0.005 | 250 |
| RIII mammary tumor | Method 2 (4) | $4.0 \times 10^{2}$ | 0.006 | 300 |
| RIIIf/C57BL/6 mammary gland | Method 1 (3) | $>10^{5}$ | $\ll$0.000002 | $\ll$0.2 |
| RIIIf/C57BL/6 mammary gland | Method 2 (2) | $>10^{5}$ | $\ll$0.000002 | $\ll$0.2 |

[a] All mammary glands used were in mid-lactation and obtained from mice of the second or third parity unless otherwise indicated.

[b] Calculated on the basis of one equivalent of 35S RNA weighing $0.53 \times 10^{-11}$ μg, and assuming $2 \times 10^{-5}$ μg of total RNA per cell (14). —, None.

[c] Numbers in parentheses refer to the number of individual preparations carried out. Each extraction was performed on the mammary tissue from either a single mouse or a pool of tissue from two or more mice (as was sometimes required with mammary tumors).

[d] Adjusted to a molecule of 35% of the sequence complexity of 35S RNA, as indicated by the hybridization kinetics of component I.

from various mouse strains (Table 1) might also indicate strain-specific differences in the relative expression of MuMTV *env* and *gag* gene products. Indeed, Teramoto et al. (17) have reported that such noncoordinate gene expression can occur in mammary tissues. To examine this possibility, we measured individually the concentrations of the major MuMTV core protein, p28, and those of the external MuMTV glycoprotein, gp52, by competition radioimmunoassays (3). Extracts were prepared from GR and RIII mouse mammary glands; from RIII mammary tumors; and from the MuMTV-producing tissue culture cell lines MuMT-73 (C3H tumor-originated; 15), GR-3A (from a GR mouse mammary tumor; 14), and C57BL MG (infected with and producing RIII MuMTV; 19). The uninfected C57BL MG line served as a negative control, and solubilized C3H MuMTV served as a positive control and standard of reference. Although the levels of MuMTV protein expressed by the different sources varied greatly, it is interesting to note (Table 2) that the mid-lactating GR mouse mammary gland contained the greatest concentration of MuMTV proteins, whereas the gp52-to-p28 ratios of the GR mammary gland and the GR tumor-originated cell line were both higher than that of the RIII mouse tissues and the RIII MuMTV-producing cell line. When comparing individual mice of the same strain, the glycoprotein-to core protein ratio was found to vary by less than twofold. The differences observed in these ratios when comparing the GR and RIII MuMTV-producing cells may be indicative of the coordinate *gag* and *env* gene product expression specific to each MuMTV strain.

## DISCUSSION

We investigated, using molecular hybridization techniques with MuMTV $cDNA_{rep}$, the quantity and MuMTV genome sequence representation in mammary glands and tumors of a variety of mouse strains. In this study we also used two different methods of RNA extraction—one (method 1) a classical SDS-protease-phenol extraction procedure (20) and the other (method 2) a recently developed method making use of the chaotropic properties of guanidinium derivatives to minimize RNase activity during extraction (18). Using low-tumor-incidence BALB/c mice from three different sources, we attempted to reconcile differences reported in the literature

TABLE 2. *Concentration of MuMTV gp52 and p28 in murine cells*[a]

| Protein source | gp52 (ng/mg of protein) | p28 (ng/mg of protein) | gp52/p28 |
|---|---|---|---|
| MuMTV | 300,000 | 170,000 | 1.8 |
| GR mammary gland | 4,900 | 1,060 | 4.6 |
| GR-3A cell line | 780 | 160 | 4.9 |
| MuMT-73 cell line (C3H mammary tumor originated) | 3,600 | 820 | 4.4 |
| RIII mammary gland | 108 | 73 | 1.5 |
| RIII mammary tumor | 1,420 | 490 | 2.9 |
| C57BL MG cells (uninfected) | <1.5 | <0.2 | —[b] |
| C57BL MG cells (infected with and producing RIII MuMTV) | 112 | 56 | 2.0 |

[a] Proteins were purified, iodinated, and used to assay protein concentrations in competition radioimmunoassays as previously described (3). Tissue extracts were prepared as described in the text. The GR mammary gland was obtained from a mid-lactating mouse from a second parity, whereas the mammary gland of the RIII mouse was from a late-lactating animal. All tissue culture cell lines were exposed to 1 μg of dexamethasone per ml for 24 h before RNA extraction.

[b] —, None.

on the quantity of MuMTV-related RNA in mammary glands and hormonally induced mammary tumors spontaneously occurring in mice with hypophyseal isografts (1–7, 12, 13, 20). Our results indicate that no significant differences in the MuMTV-related RNA concentration from mammary glands obtained from three widely geographically separated colonies exist when Method 1 is used for RNA extraction. Furthermore, the quantity of MuMTV-related RNA, which was barely detectable when method 1 was used for extraction, was significantly increased if method 2 was used for obtaining RNA from mammary glands or tumors. This result suggests that the presence of RNase in both BALB/c mammary glands and tumors may have resulted in the failure to report measurable MuMTV-related RNA levels by investigators using procedures similar to method 1 (1, 2, 6, 7, 12, 13, 20). Our results are in general agreement with those of Michalides et al. (6, 7), Dudley et al. (1), and Morris et al. (10) for the quantity of MuMTV-like RNA in BALB/c mammary glands of low-tumor-incidence lines of BALB/c mice. However, McGrath et al. (4, 5) found 100- to 1,000-fold more MuMTV-related RNA in mid-lactating BALB/c mammary glands of first parity MCF mice than that which we report. Since we used the mice from the MCF colony, as well as those from the parent colony (CRL mice), the discrepancy in our findings may be explained by differences in the representative nature of cDNA used for each study, indicated by the fact that a 10:1 cDNA/RNA mass ratio protected only 76% of labeled RNA from RNase digestion in the previous study (5) whereas the $cDNA_{rep}$ used in this study protected over 90% of template RNA from RNase digestion at a cDNA/RNA ratio of 4:1 (16). Dudley et al. (1) have shown that BALB/c mammary glands produce a 20-fold excess of polyadenylic acid-adjacent MuMTV RNA sequences (detected by oligodeoxythymidylic acid-primed cDNA synthesis) over sequences representative of the entire MuMTV genome. The probe used by McGrath et al. (5) may have contained an excess of 3′-adjacent sequences if the endogenously primed reaction produced a majority of short products which extended only part way into the 3′ region, with the rest being strong-stop sequences from the 5′ terminus.

Our finding that BALB/c mammary gland tumors induced by hormonal stimulation contain an amount of MuMTV-related RNA equivalent to that found in normal lactating mammary glands of these mice (Fig. 1), regardless of whether the RNA was extracted by method 1 or method 2, suggests that exposure of BALB/c mice to hormones does not increase the expression of MuMTV genes in preneoplastic or neoplastic mammary tissues. We therefore believe that the induction of hormonally induced BALB/c mouse mammary tumors occurs by a mechanism independent of MuMTV involvement. It should be noted, however, that our results discussed above contrast with the previous findings of Pauley et al. (12, 13) and Michalides et al. (6, 7), which indicated that BALB/c hormonally induced mammary tumors contain significantly greater quantities of MuMTV RNA than do normal lactating mammary glands. However, great variation in the quantity of MuMTV-related RNA has been found in cells obtained from BALB/c mammary tumors (12, 13). Our data show that there is significant variation in the apparent amount of MuMTV-like RNA in both mammary glands and tumors, depending upon the method of extraction. It is therefore possible that uniform extraction procedures, utilizing the method of extraction which precludes the possibility of RNase action on RNA contents of tissues (method 2), will clarify the observed differences in RNA content.

Extension of studies comparing the MuMTV-related RNA content of mammary glands extracted by method 1 or method 2 to other strains of mice (Table 1) revealed that for the low-mammary-tumor-incidence C57BL/6 strain, as

well as for the high-mammary-tumor-incidence RIII mouse strains, method 2 is superior for recovery of MuMTV-specific RNA. During this study, the RIII mouse strain revealed itself to be unique in a quite unexpected manner. When extracted by method 1, RIII mouse mid-lactating mammary glands (of any parity) appeared to contain two populations of MuMTV-related RNA, as indicated by the biphasic nature of hybridization kinetics (Fig. 2A). Differences observed in the MuMTV-related RNA content of mammary glands extracted by method 1 or 2 may be due to differences in the action of endogenous RNase, which is minimized by method 2 (18). The biphasic hybridization pattern observed with RIII strain mammary gland RNA extracted by method 1 may be due, in part, to the presence of an excess of subgenomic RNA, perhaps a naturally occurring RNA species such as 24S *env* MuMTV mRNA (16). In that case, the action of endogenous RNase would greatly decrease the concentration of full-sequence MuMTV RNA, while the low-molecular-weight fragments generated by the digestion of 35S RNA might add to the "subgenomic RNA" population, producing a biphasic curve when hybridized to $cDNA_{rep}$. If an excess of functional 24S subgenomic *env* mRNA existed, the ratio of envelope glycoprotein gp47 to the core polypeptide p27 would be much higher than that of strains in which hybridization of extracted RNA to MuMTV $cDNA_{rep}$ showed a single RNA component. Teramoto et al. (17) have recently reported that noncoordinate expression of MuMTV *gag* and *env* proteins can occur in mammary tissues. The results of our studies (Table 2) indicate that, in fact, GR MuMTV-producing mouse cells (which show a single-component RNA-cDNA hybridization curve, Fig. 2) appear to have a slightly higher gp52/p28 ratio than do RIII MuMTV-producing cells. Therefore, an excess of functional *env* mRNA (16) does not appear to be the explanation for the aberrant results obtained with method 1-extracted RNA from RIII mouse mammary glands. The reason for the biphasic RIII mammary gland RNA-cDNA hybridization pattern, reproducibly obtained only with method 1-extracted RNA, and its biological significance, if any, remain to be elucidated.

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## ADDENDUM IN PROOF

Since the submission of this paper we received a preprint of a manuscript (J. Natl. Cancer Inst., in press) in which the authors (C. M. McGrath, W. A. Press, T. M. Maloney, and R. F. Jones) report that, with the guanidine method of RNA extraction, the MuMTV-related RNA content of hormonally induced BALB/c mammary tumors varies greatly from tumor to tumor and that the expression of MuMTV-related RNA in BALB/c mammary gland cells may not be sufficient to cause tumor development.

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