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Murine Mammary Tumor Virus Expression During Mammary Tumorigenesis in BALB/c Mice

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Steady-state levels of murine mammary tumor virus (MuMTV) RNA were quantitated during mammary tumorigenesis in BALB/c mice by molecular hybridization with a representative MuMTV complementary DNA (cDNA) probe. Hyperplastic alveolar nodule (HAN) lines are preneoplastic mammary lesions that were induced in BALB/c mice by hormones alone or in combination with 7,12-dimethylbenz(a)anthracene and give rise to mammary tumors. The hormone-induced HAN lines D1 and D2 contained detectable amounts of hybridizable MuMTV sequences. MuMTV RNA sequences were also observed in five of the six transplanted BALB/c mammary tumors that were examined. Similar levels of hybridizable MuMTV RNA were observed between the D1 or D2 HAN line and mammary tumors derived from each HAN line. The D2 HAN line as well as D2, C4, and CD8 mammary tumors accumulated RNA that was apparently homologous to most of the MuMTV genome. Thermal denaturation of hybrids indicated extensive sequence homology between the MuMTV cDNA and hybridizable RNA in the BALB/c HAN lines and mammary tumors. A low level of type C viral RNA was observed in the BALB/c HAN lines and most mammary tumors by molecular hybridization with a cDNA to Moloney murine leukemia virus. These data demonstrate that MuMTV sequences are frequently expressed in hormone-induced BALB/c HAN lines and mammary tumors derived from HAN lines or ductal hyperplasias induced in BALB/c mice by hormones and/or a chemical carcinogen. The transition from the preneoplastic to the neoplastic state in BALB/c mice does not appear to be due to a change in the steady-state levels of MuMTV RNA since the hormone-induced HAN lines and mammary tumors had similar levels of hybridizable MuMTV RNA.

Murine mammary tumorigenesis is influenced by several factors including the murine mammary tumor virus (MuMTV), hormones, the genetic complement, and environmental agents (2, 24, 25). BALB/c mice have been used to study mammary tumorigenesis because this strain has a low incidence of mammary tumors in virgin mice and retired breeders (14, 25). Mammary tumors and hyperplastic alveolar nodules (HAN), the morphological precursor to mammary tumors (8, 9), have been induced in BALB/c mice by prolonged hormonal stimulation alone or in combination with 7,12-dimethylbenz(a)anthracene (DMBA) (17-21). HAN are alveolar hyperplasias that morphologically resemble the midpregnant mammary gland (8, 9). HAN lines with stable morphological, biological, and tumorigenic properties have been established by serial transplantation of primary HAN in the gland-free mammary fat pad of syngeneic mice (16-21). These HAN lines give rise to mammary adenocarcinomas at a frequency of from 4 to 86% (16-21). In addition, DMBA induces ductal hyperplasias and ductal carcinomas in virgin BALB/c mice (21; D. Medina, J. Natl. Cancer Inst., in press).

Several lines of evidence have demonstrated that BALB/c mice contain an endogenous MuMTV. First, cellular DNA from normal BALB/c organs contains five to nine copies of DNA homologous to the MuMTV genome per diploid cell (23). Second, BALB/c mammary glands and spontaneous tumors contain very low levels of MuMTV RNA (15, 28, 32, 38). Third, infectious MuMTV virions were detected in a low percentage of spontaneous BALB/c mammary tumors (3).

The role of the endogenous MuMTV in the etiology of hormone- and chemical carcinogen-induced HAN and mammary tumors in BALB/c mice is unknown. In these studies, hormone-induced BALB/c HAN lines, which were established from primary HAN induced in BALB/c mice by hormonal stimulation alone or in combination with DMBA, and mammary tumors that arose in the HAN lines were examined for

MuMTV expression. MuMTV expression was quantitated by molecular hybridization with a complementary DNA (cDNA) probe representative of the MuMTV genome. Appreciable levels of hybridizable MuMTV RNA were observed in BALB/c HAN lines and most mammary tumors. Importantly, similar levels of hybridizable MuMTV RNA were observed between a given hormone-induced HAN line and mammary tumors that arose from the HAN line.

MATERIALS AND METHODS

Virus and reagents. MuMTV, Moloney strain murine leukemia virus (MoMuLV), and avian myeloblastosis virus reverse transcriptase were obtained through the Office of Resources and Logistics of the Virus Cancer Program, National Cancer Institute. MuMTV was isolated and purified by zonal centrifugation of the culture supernatant from Mm5mt/c1 cells, a cell line derived from a C3H mouse mammary tumor (26). The purity of the MuMTV preparations was evaluated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of viral polypeptides and by determining the cation preference of the endogenous reverse transcriptase activity. The preferential cation requirement for magnesium (11) and the electrophoretic mobilities of viral polypeptides (36) demonstrated that the virus preparation was type B MuMTV. MoMuLV was isolated from the culture supernatant of MoMuLV-infected NIH 3T3 cells and purified by zonal centrifugation.

All chemicals were of reagent grade including deoxyribonucleotides, [5-^{3}H]deoxycytidine-5'-triphosphate (21 Ci/mmol) and ribonuclease-free sucrose (Schwarz/Mann, Orangeburg, N.Y.), actinomycin D (Calbiochem, San Diego, Calif.), calf thymus DNA and polyriboadenylate (P-L Biochemicals, Milwaukee, Wis.), S_1 nuclease and $\phi X174$ DNA (Miles Laboratories, Elkhart, Ind.) and proteinase K (EM Biochemicals, Elmsford, N.Y.). Glassware was alkali washed and baked; solutions were made ribonuclease (RNase)-free by filtration through membrane filters (0.45 μm, type HAWP, Millipore Corp.).

Nodules and tumors. Mice of strain BALB/cCrglMe and BALB/cfC3HCrglMe were bred and maintained in a controlled environment at the mouse colony of the Department of Cell Biology. A BALB/cCrgl breeding pair was obtained from the Cancer Research Laboratory, Berkeley, Calif., in 1970 and were brother × sister mated. The mammary tumor incidence in BALB/cCrglMe retired breeders between 1975 and 1977 was 1% (2 out of 206); the mean age of retired breeders was 15 months and the mean number of litters was 9.1. HAN lines were maintained by serial transplantation in the gland-free, mammary fat pads of 3-week-old BALB/cCrglMe mice. The origin and tumor-producing characteristics of the BALB/c outgrowth lines are shown in Table 1. The D1 and D2 HAN lines used in this study were in transplant generations 45 to 48. Mammary tumors were maintained by serial transplantation in virgin 8- to 12-week-old BALB/cCrglMe mice.

Preparation of cellular RNA. Following sacrifice of the animals by cervical dislocation, tissues were excised, areas of necrosis and hemorrhagia were removed, and the tissues were frozen and stored in liquid nitrogen. Total cellular RNA was isolated by direct homogenization of pulverized, frozen tissue in equal volumes of a buffer containing 0.5% (wt/vol) SDS, 25 mM Na_2EDTA and 75 mM NaCl (pH 8.0), and buffer-saturated redistilled phenol (pH 8.0) containing 1 mg of 8-hydroxyquinoline per ml (29). RNA extracts were treated with proteinase K at 40 μg/ml for 30 min at 37°C (30). In addition, extraction with 3 M sodium acetate was performed to remove contaminating DNA (27). RNA samples of nodules and mammary tumors prepared by this method contained less than 1% DNA by chemical analysis (5). The amount of RNA was determined from the absorbance at 260 nm. The ratio of the absorbance of the RNA samples at 260 nm to 280 nm or 230 nm was 1.8 to 2.2.

Isolation of viral RNA. Purified MuMTV or MoMuLV virions were concentrated by centrifugation for 1 h at 84,000 × g in a Beckman SW50.1 rotor at 4°C. Virus was suspended in a solution of 0.1 M NaCl, 1 mM Na_2EDTA, and 10 mM Tris-hydrochloride (pH 7.0) to a concentration of approximately 10 mg of protein per ml. SDS and proteinase K were added to a final concentration of 0.5% (wt/vol) and 100 μg/ml, respectively. Following incubation at 37°C for 30 min, viral RNA was extracted three times with an equal volume of buffer-saturated phenol (pH 8.0) and chloroform-isoamyl alcohol (24:1 [vol/vol]). The aqueous phase was adjusted to 0.25 M NaCl and the RNA was precipitated by addition of two volumes of ethanol. After storage at −20°C overnight, RNA was pelleted

TABLE 1. *Origin and tumor-producing capabilities of BALB/c mammary hyperplastic outgrowth lines*

Outgrowth line	Type of outgrowth[a]	Origin of outgrowth		Tumor-producing capability (%)[c]
		Hormone[b]	DMBA[b]	
D1	HAN	+		12
D2	HAN	+		51
C3	HAN	+	+	81
C4	HAN	+	+	84
C5	HAN	+	+	85
CD8	DH		+	72

[a] Outgrowths were either HAN or ductal hyperplasias (DH).

[b] D1 and D2 HAN originated in BALB/c mice that had pituitary isografts alone (D2 [21]) or in combination with estradiol-17β (D1 [19]). C3, C4, and C5 HAN originated in BALB/c mice bearing pituitary isografts that had received DMBA (17). The CD8 DH occurred in virgin BALB/c mice treated with DMBA (Medina, in press).

[c] Determined by transplanting outgrowths into the cleared mammary fat pads of syngeneic female mice. The number of outgrowths that produced tumors within 12 months, expressed as a percentage of the total number of transplanted outgrowths, is defined as the tumor-producing capability (17, 18a; Medina, in press).

by centrifugation at 15,000 × *g* for 30 min and resuspended in a small volume of 0.01 M NaOAc (pH 5.0). The recovery of viral RNA following extraction was 1 to 2% by weight of the initial viral protein.

Viral RNA was applied to a linear 10 to 35% (wt/vol) sucrose density gradient in a buffer containing 0.1 M NaCl, 0.01 M NaOAc (pH 5.0), and 1 mM Na_2EDTA. Centrifugation was performed at 4°C for 2 h at 297,000 × *g* in a Beckman SW56 rotor. Sedimentation values were estimated on the basis of the sedimentation of 18*S* and 28*S* rRNA centrifuged in parallel gradients. Fractions in the 70*S* region of the gradient were pooled and the RNA was recovered by ethanol precipitation. Approximately 25% of the total RNA applied to the gradient was recovered from the 70*S* region of the gradients.

Synthesis of cDNA. Representative cDNA probes to 70*S* MuMTV RNA and 70*S* MoMuLV RNA were synthesized in vitro by using calf thymus DNA fragments (35) as primer for avian myeloblastosis reverse transcriptase (13). Viral 70*S* RNAs were heated at 80°C for 2 min and quick cooled immediately before addition to the reaction mixture. The reaction mixture contained 50 mM Tris-hydrochloride (pH 8.3); 20 mM dithiothreitol; 150 mM KCl; 14 mM $Mg(OAc)_2$; 60 μg of actinomycin D per ml; 0.4 mM each dATP, dGTP, and dTTP; 0.1 mM [5-^{3}H]dCTP (21 Ci per mmol); 50 μg of viral RNA per ml; 2,000 μg of calf thymus DNA fragments per ml (6 to 10 nucleotides in length); 4 mM $Na_4P_2O_7$ (Chelex-100 treated); and 125 U of avian myeloblastosis virus reverse transcriptase per ml. Synthesis was at 46°C for 20 min. The reaction was stopped by the addition of SDS and Na_2EDTA to final concentrations of 1% (wt/vol) and 20 mM, respectively. Polyriboadenylate carrier was then added to 150 μg per ml.

The cDNA product was purified by chromatography on Sephadex G-50, treated with Chelex-100 to remove heavy metals, precipitated with ethanol and placed in 200 μl of 10 mM Na_2EDTA. RNA was digested by treatment with 0.1 N NaOH at 60°C for 1 h. The alkali-digested cDNA was applied directly to linear, alkaline sucrose density gradients, 0.3 to 1.0 M sucrose, in 0.9 M NaCl, 0.1 N NaOH, and 5 mM Na_2EDTA. Gradients were centrifuged at 150,000 × *g* for 24 h in a Beckman SW40 rotor. Parallel gradients contained 6.8*S* sheared *Escherichia coli* DNA in addition to 16.1*S* linear and 18.4*S* circular φχ174 DNA (34) as standards. Both the MuMTV cDNA and MoMuLV cDNA sedimented as broad peaks approximately 6.5*S*. Gradient fractions containing cDNA estimated to be 5*S* or greater were pooled and neutralized with 0.2 volumes of 1 M NaOAc (pH 5.0). The cDNA was recovered by precipitation with ethanol in the presence of 40 μg of polyriboadenylate per ml. The specific activity of the cDNA synthesized under these conditions was 15,000 cpm per ng, assuming a 50% (C + T) content in the cDNA's. Following sucrose density gradient fractionation, the yield of cDNA was approximately 6%, relative to the input RNA in the cDNA synthesis reaction.

The DNA fragment-primed MuMTV cDNA has been shown to be representative of the entire MuMTV genome on the basis of its ability to protect [^{125}I]-MuMTV RNA from RNase digestion (13). Approximately 63% of the [^{3}H]MuMTV cDNA was protected from S_1 nuclease digestion at a ratio of 70*S* MuMTV RNA to MuMTV cDNA of one (Fig. 1). At a ratio of 70*S* MuMTV RNA to MuMTV cDNA of 3.5 or greater, 95% of the [^{3}H]MuMTV cDNA probe was protected from S_1 nuclease digestion. The specificity of the MuMTV cDNA probe was demonstrated by the observations that no hybridization between the MuMTV cDNA and BALB/c or BALB/cfC3H liver RNA was detected (data not shown). Approximately 7% of the [^{3}H]MuMTV cDNA probe hybridized to 70*S* MoMuLV RNA at a concentration ratio of RNA to cDNA of 300, indicating that 0.02% of the 70*S* MoMuLV RNA hybridized to the MuMTV cDNA probe (data not shown).

The type C virus cDNA probe used in these studies was synthesized from 70*S* MoMuLV RNA with DNA fragments as random primers. Approximately 60 and 95% of the [^{3}H]MoMuLV cDNA was hybridized with 70*S* MoMuLV RNA at concentration ratios of RNA to cDNA of 1 and 3, respectively (see Fig. 7). Approximately 8% of the [^{3}H]MoMuLV cDNA probe was hybridized with 70*S* MuMTV RNA at a concentration ratio of RNA to cDNA of 300, indicating that 0.03% of the 70*S* MuMTV RNA hybridized to [^{3}H]MoMuLV cDNA (see Fig. 7). Both the MuMTV and MoMuLV cDNA probes contained less than 5% double-stranded material on the basis of S_1 nuclease resistance under the hybridization conditions used in these studies.

Nucleic acid hybridization. Nucleic acid hybridizations were performed by the titration method (40). Hybridization reactions were carried out in a final volume of 20 μl containing 0.6 M NaCl, 0.01 M HEPES (*N* - 2 - hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.0), 2 mM Na_2EDTA, 8 μg of chick liver

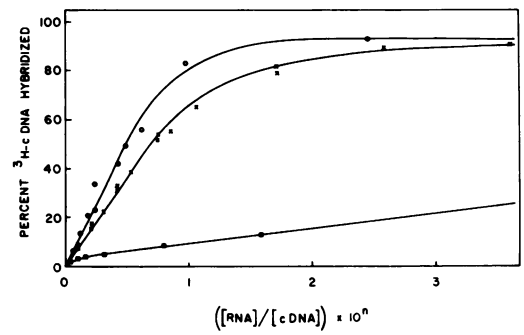

FIG. 1. *MuMTV expression in spontaneous BALB/c and BALB/cfC3H mammary tumors. The representative MuMTV cDNA probe was hybridized with various amounts of total cellular RNA from spontaneous BALB/c and BALB/cfC3H mammary tumors and assayed as described in the text. The ratio of RNA to cDNA concentration used in these hybridizations ranged from 0 to 3 with 70S MuMTV RNA* (×), *from 0 to 3 × 10^3 with BALB/cfC3H mammary tumor RNA* (○), *and from 0 to 3 × 10^5 with BALB/c mammary tumor RNA* (■). *The mean percent MuMTV RNA in total cellular RNA from two BALB/c and two BALB/cfC3H mammary tumors ± one standard error was 0.0007 ± 0.0002% and 0.1014 ± 0.0428%, respectively.*

RNA, and 0.6 ng of [^{3}H]cDNA. Increasing amounts of RNA were added to each hybridization reaction in tapered reaction vials. After heat denaturation at 100°C for 1 min, hybridization was carried out at 68°C for 48 h. The extent of hybridization was determined by digestion with S_1 nuclease, 900 U, in 200 μl of 0.4 M NaOAc, pH 4.5, 0.8 M NaCl, and 5 mM $ZnCl_2$ for 1 h at 37°C. Bovine serum albumin, 50 μg, was added and the S_1 nuclease-resistant cDNA-RNA hybrids were precipitated with cold 20% Cl_3CCOOH, collected on membrane filters (Millipore Corp.), rinsed, and dried. Filters were dissolved in 3 ml of ethylene glycol monomethyl ether and counted in 10 ml of liquifluor (Beckman)-toluene. All data have been corrected for S_1 nuclease-resistant [^{3}H]cDNA.

The concentration of virus-specific RNA in cellular RNA extracts was determined by comparing the kinetics of hybridization between [^{3}H]cDNA and cellular RNA to that observed between [^{3}H]cDNA and 70*S* viral RNA as described for the titration method (40). The method involves hybridizing RNA to a fixed amount of cDNA for sufficient time to assure completion of the annealing reaction. In these studies the equivalent C_0t, the product of the cDNA concentration in moles per liter × the time in seconds, normalized to 0.12 M phosphate buffer (4), was 0.09. To verify that the product of the MuMTV cDNA concentration and time were sufficient to assure completion of the hybridization reaction, hybridizations were performed for 48 h with a 10-fold greater concentration of MuMTV cDNA. No increase in the amount of hybridization was observed as a function of the concentration ratio of input RNA to cDNA (data not shown). Therefore, under these annealing conditions the amount of hybrid formed during the initial portion of the hybridization reaction is proportional to the concentration of hybridizable RNA present in the annealing reaction as predicted for the titration method (40).

Thermal denaturation of cDNA-RNA hybrids. The thermal stability of cDNA-RNA hybrids was analyzed to assess the fidelity of base pairing in the hybrids. Briefly, following hybridization the NaCl concentration was adjusted to 0.2 M by diluting the reaction mixture with 2 volumes of 0.01 M HEPES (pH 7.0) and 2 mM Na_2EDTA. Portions containing approximately 1,500 cpm were removed, heated at the designated temperature for 7 min, and quick cooled. The amount of hybrid remaining was determined by S_1 nuclease digestion. Data are presented as the fraction of the hybridized [^{3}H]cDNA that was digested by S_1 nuclease at a specified temperature.

Estimation of MuMTV RNA equivalents per 2c amount of DNA. The number of MuMTV RNA equivalents per 2c amount of DNA was calculated from the following formula: MuMTV RNA equivalents per 2c amount of DNA = (fraction of MuMTV RNA in total cellular RNA) × (picograms of total cellular RNA per 2c amount of DNA) × (equivalents of MuMTV RNA per picogram of MuMTV RNA). The fraction of MuMTV RNA in total cellular RNA was calculated from the hybridization data. The amount of DNA and RNA per gram of tissue was determined by direct chemical analysis of the tissue for total DNA (5) and RNA (7). The picograms of total cellular RNA per 2c amount of DNA was calculated from the amount of DNA and RNA per gram of tissue based upon an estimated 2c amount of DNA in mouse tissues of 6 pg (39). The equivalents of MuMTV RNA per picogram of MuMTV RNA were 93,500 based upon the molecular weight for MuMTV RNA of 6.45×10^6 (10).

RESULTS

MuMTV RNA in spontaneous BALB/c mammary tumors. BALB/cCrglMe mice have a low incidence of mammary tumors, less than 1%, in virgin mice and retired breeders. Spontaneous mammary tumors that occurred in BALB/cCrglMe retired breeders had very low levels of MuMTV RNA, 0.0007% of total cellular RNA (Fig. 1). Varmus et al. (38) observed similar low levels of MuMTV RNA (0.0005%) in BALB/c mammary tumors. BALB/cfC3H mice have a high incidence of mammary tumors in retired breeders due to the presence of MuMTV-S, the exogenous milk-transmitted virus. Spontaneous mammary tumors from BALB/cfC3H retired breeders had higher levels of MuMTV RNA (0.1014% of the total cellular RNA or about 570 equivalents of MuMTV RNA per 2c amount of DNA) than did the spontaneous BALB/c mammary tumors (Fig. 1). In contrast to the limited extent of hybridization observed with spontaneous BALB/c mammary tumor RNA, even at concentration ratios of RNA to cDNA of 150,000, spontaneous BALB/cfC3H tumor RNA hybridized to the MuMTV cDNA to the same extent as MuMTV RNA. Expression of the exogenous MuMTV-S was apparently not restricted by the BALB/c genetic background since C3H and BALB/cfC3H spontaneous mammary tumors contained similar levels of MuMTV RNA (38; data not shown).

MuMTV RNA in hormone-induced HAN lines and tumors. Since spontaneous mammary tumors in BALB/c retired breeders had very low levels of MuMTV RNA, it was of interest to determine if HAN lines or mammary tumors induced by hormones had similar levels of MuMTV RNA. A rapid initial increase in the amount of hybridization was observed with D1 HAN line and D1 mammary tumor RNA at concentration ratios of RNA to cDNA from 0 to about 20,000 (Fig. 2). However, even at concentration ratios of RNA to cDNA of 200,000, only a small increase in the extent of hybridization was observed (Fig. 2; Table 2). Analysis of the initial portion of the hybridization curve indicated that approximately 0.011% of the total cellular RNA in D1 HAN and mammary tumors was MuMTV-specific RNA. The limited extent of the hybridization with the D1 HAN line and D1 tumor RNA may be due to the accumulation of MuMTV RNA in these tissues that is homol-

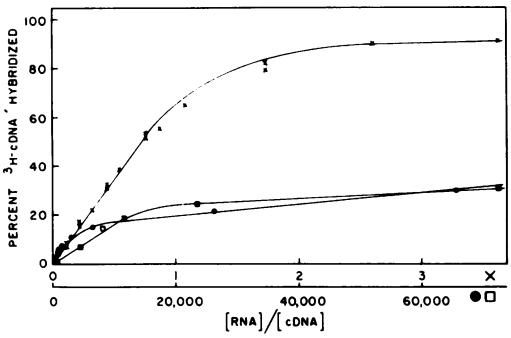

FIG. 2. *MuMTV expression in the D1 HAN line and D1 mammary tumors. The representative MuMTV cDNA probe was used to determine the level of MuMTV RNA in total cellular RNA by molecular hybridization (see text). The percentage of [^{3}H]-MuMTV cDNA hybridized is shown as a function of the ratio of the RNA to cDNA concentration for D1 HAN line RNA (●), D1 mammary tumor RNA (□), and 70S MuMTV RNA (×).*

ogous to only a portion of the MuMTV genome. Importantly, the level of MuMTV RNA in the D1 HAN line and D1 tumors was not significantly different on the basis of a pooled *t* test.

The D2 HAN line and D2 mammary tumors contained appreciable amounts of MuMTV RNA, approximately 0.12 and 0.14% of the total cellular RNA, respectively (Fig. 3; Table 2). As was observed in the D1 series of nodules and tumors, no significant difference, determined by a pooled *t* test, in the amount of hybridizable MuMTV RNA was observed in the progression of nodules to tumors. RNA from these tissues consistently hybridized to the MuMTV cDNA to approximately the same extent as the template RNA (Fig. 3; Table 2), indicating that most, if not all, of the MuMTV genome was expressed in these tissues. The number of equivalents of MuMTV RNA per 2c amount of DNA ± one standard error of the mean in D2 HAN and D2 tumors was estimated to be 702 ± 75 and 924 ± 351, respectively. Similar levels of MuMTV-specific RNA were observed in D2 tumors of the first and subsequent transplant generations (Table 2), indicating that the level of MuMTV RNA in BALB/c mammary tumors was maintained through many transplant generations.

The thermal stability of the MuMTV cDNA-RNA hybrids was determined to assess the fidelity of base pairing between [^{3}H]MuMTV cDNA and the HAN line or mammary tumor RNA (Fig. 4; Table 2). Changes in the T_m, the mean thermal dissociation temperature, of 1 to 2°C are not considered significant. The T_m of the hybrid between MuMTV cDNA and 70S MuMTV RNA (Fig. 4) or BALB/cfC3H mammary tumor RNA (data not shown) was 88°C. Hybrids between MuMTV cDNA and D1 HAN line RNA, D1 tumor RNA, or D2 tumor RNA had a T_m within 2°C of the T_m of the back hybrid (Fig. 4). D2 HAN line RNA hybrids with MuMTV cDNA had a T_m of 84.5°C, indicating 2 to 3% base-pair mismatching (37). Less than 3% base-pair mismatching was observed between the MuMTV cDNA and cellular RNA

TABLE 2. *MuMTV-specific sequences in hormone-induced BALB/c HAN lines and mammary tumors*

Tissue[a]	MuMTV RNA (% cellular RNA)[b]	Maximum % hybridization of MuMTV cDNA[c]	T_m (°C)[d]
D1 nodule			
TG-46	0.0040	25 (1×10^4)	—
TG-46	0.0096	35 (4×10^4)	—
TG-46	0.0044	37 (6×10^4)	—
TG-48	0.0262	38 (1×10^5)	86
Mean	0.0111 ± 0.0052		
D1 tumor			
TG-1	0.0080	32 (1×10^5)	—
TG-1	0.0022	20 (1×10^5)	—
TG-1	0.0034	21 (2×10^4)	—
TG-1	0.0053	28 (2×10^4)	—
TG-1	0.0197	32 (3×10^4)	—
TG-1	0.0076	60 (2×10^5)	87
Mean	0.0110 ± 0.0032		
D2 nodule			
TG-45	0.1320	≥90 (4×10^3)	—
TG-47	0.1140	≥90 (1×10^3)	—
TG-48	0.1585	≥90 (2×10^3)	84,85
TG-48	0.0954	≥90 (3×10^3)	—
Mean	0.1250 ± 0.0134		
D2 tumor			
TG-1	0.3665	≥90 (7×10^2)	—
TG-1	0.0455	≥90 (4×10^3)	86
TG-23	0.0840	≥90 (2×10^3)	—
TG-24	0.0678	≥90 (2×10^3)	—
TG-31	0.2360	≥90 (1×10^3)	—
TG-36	0.0492	≥90 (5×10^3)	—
Mean	0.1415 ± 0.0536		

[a] The transplant generation of each nodule line and tumor is specified. First transplant generation (TG-1) D1 tumors arose from the D1 nodule line TG-43 or TG-45. TG-1 D2 tumors originated from the D2 nodule line TG-48. TG-23, -24, -31 and -36 D2 tumors originated from the D2 HAN line TG-31.

[b] The level of MuMTV RNA was determined by hybridization of total cellular RNA with [^{3}H]MuMTV cDNA as described in the text. For each group of nodules and tumors the mean ± one standard error was calculated.

[c] The maximum hybridization observed at the concentration ratio of RNA to cDNA that is specified.

[d] —, Not determined. See text for details.

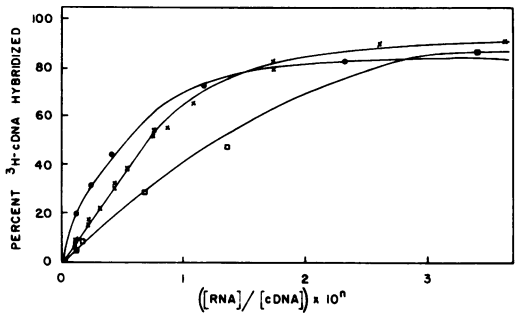

FIG. 3. *MuMTV expression in the D2 HAN line and D2 mammary tumors. The level of MuMTV RNA in total cellular RNA from the D2 HAN line and D2 mammary tumors was determined by hybridization with the representative [³H]MuMTV cDNA probe (see text). The ratio of the RNA to cDNA concentration used for hybridization ranged from 0 to 3 with 70S MuMTV RNA* (×) *and from 0 to 3 × 10³ with both D2 HAN line* (●) *and D2 mammary tumor* (□) *RNAs.*

from HAN lines or mammary tumors induced by hormones, indicating extensive homology between the MuMTV-specific sequences expressed in these tissues and the MuMTV cDNA probe. All hybrids had sharp thermal transitions and the slopes of the thermal denaturation profiles were similar, suggesting that the small decrease in the T_m was due to limited base-pair mismatching throughout the hybridizable MuMTV sequences.

MuMTV expression in carcinogen-induced mammary tumors. Mammary tumors that originated from HAN lines induced by DMBA in BALB/c mice bearing pituitary isografts (17) were assayed for MuMTV RNA to determine if the MuMTV genome is expressed in chemical carcinogen-induced mammary adenocarcinomas. Analysis of the initial portion of the hybridization curve between the MuMTV cDNA and C3 or C4 mammary tumor RNAs (Fig. 5) indicated that 0.0034% and 0.0129%, respectively, of the total cellular RNA was MuMTV-specific RNA (Table 3). A rapid initial increase in hybridization was observed in concentration ratios of C3 mammary tumor RNA to MuMTV cDNA of between 0 and 40,000. At concentration ratios of C3 mammary tumor RNA to MuMTV cDNA of even 200,000, only a small increase in the extent of hybridization was observed. The C4 mammary tumors contained approximately fourfold higher levels of MuMTV RNA than the C3 tumors, and about 90% of the MuMTV cDNA was hybridized to C4 tumor RNA. The number of equivalents of MuMTV RNA per 2c amount of DNA ± one standard error of the mean was 64 ± 22 in the C4 tumors. The level of hybridizable MuMTV RNA in C5 mammary tumors was below the reliable lower limit of detection, that is, less than 0.0005% of the total cellular RNA (Fig. 5; Table 3). Even at concentration ratios of RNA to cDNA of 200,000, only about 10% hybridization was observed with RNA from C5 mammary tumors. These observations demonstrate MuMTV expression in some but not all mammary tumors derived from BALB/c HAN lines that were induced by DMBA.

The fidelity of base pairing in hybrids between [³H]MuMTV cDNA and hybridizable RNA from C3 or C4 mammary tumors was determined by thermal denaturation (Fig. 6). The T_m's of the hybrids were 86 and 84°C with C3 and C4 mammary tumor RNAs, respectively (Table 3). The T_m of the hybrid with C3 mammary tumor RNA, relative to the T_m of the back hybrid with 70S MuMTV RNA, was within experimental

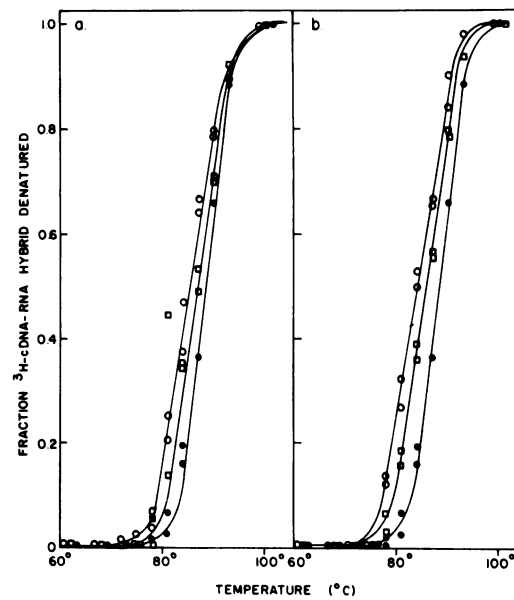

FIG. 4. *Analysis of the thermal stability of hybrids between [³H]MuMTV cDNA and hormone-induced BALB/c HAN line or mammary tumor RNAs. (a) Thermal denaturation of MuMTV cDNA hybrids with 70S MuMTV RNA* (●), *D1 HAN line RNA* (○), *and D1 mammary tumor RNA* (□). *(b) Thermal denaturation of MuMTV cDNA hybrids with 70S MuMTV RNA* (●), *D2 HAN line RNA* (○), *and D2 mammary tumor RNA* (□). *Hybridizations and thermal denaturations were carried out as described in the text. The percentage hybridization observed and the ratio of the RNA to cDNA concentration used for hybridizations were 99.9% and 21.6 with 70S MuMTV RNA, 55.6% and 171,400 with D1 HAN line RNA, 44.5% and 217,400 with D1 mammary tumor RNA, 92.8% and 21,430 with D2 HAN line RNA, and 92.9% and 23,100 with D2 tumor RNA.*

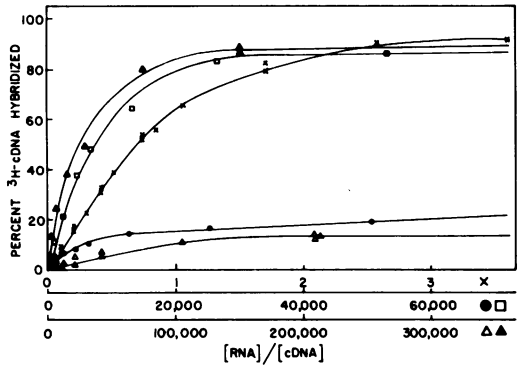

FIG. 5. *MuMTV expression in carcinogen-induced BALB/c mammary tumors. The level of MuMTV RNA in total cellular RNA was determined by molecular hybridization with the representative [³H]-MuMTV cDNA (see text). Hybridizations were performed with RNA from C3 (●), C4 (□), C5 (△), and CD8 (▲) mammary tumors and 70S MuMTV RNA (×).*

TABLE 3. *MuMTV specific sequences in carcinogen-induced BALB/c mammary tumors*

Tumor line[a]	MuMTV RNA (% cellular RNA)[b]	Maximum % hybridization of MuMTV cDNA[c]	T_m (°C)[d]
C3			
(TG-1)	0.0034	18 (5×10^4)	—
(TG-1)	0.0007	17 (5×10^4)	—
(TG-1)	0.0088	25 (2×10^5)	86
(TG-1)	0.0008	19 (1×10^5)	—
Mean	0.0034 ± 0.0019		
C4			
(TG-1)	0.0054	90 (9×10^4)	—
(TG-1)	0.0194	90 (2×10^4)	—
(TG-1)	0.0117	85 (6×10^4)	—
(TG-1)	0.0021	64 (1×10^5)	—
(TG-1)	0.0260	87 (2×10^4)	84
Mean	0.0129 ± 0.0044		
C5			
(TG-1)	<0.0005	8 (2×10^4)	—
(TG-1)	<0.0005	12 (2×10^5)	—
CD8			
(TG-9)	0.0054	86 (2×10^5)	—
(TG-9)	0.0048	78 (2×10^5)	—
(TG-9)	0.0031	77 (1×10^5)	—
Mean	0.0044 ± 0.0007		

[a] The transplant generation (TG) of each mammary tumor that was assayed is specified. First transplant generation (TG-1) tumors arose from the C3 nodule line TG-23 or TG-24, the C4 nodule line TG-18, and the C5 nodule line TG-18 or TG-20.

[b] The level of MuMTV is expressed as a percentage of MuMTV RNA in total cellular RNA from each tumor. The mean percent MuMTV RNA ± one standard error for each group of tumors was calculated.

[c] The percentage of hybridization that was observed at the specified concentration ratio of RNA to cDNA.

[d] See text for details. —, Not determined.

error. However, a 4°C lowering in the T_m was observed with C4 mammary tumor RNA, indicating almost 3% base-pair mismatching in the hybridized molecules. The thermal denaturation profiles showed sharp thermal transitions and similar slopes indicating that limited base-pair mismatching was distributed throughout the hybridizable sequences.

The predominant type of mammary lesions in virgin BALB/c mice treated with DMBA alone are ductal hyperplasias that give rise to ductal adenocarcinomas (22; Medina, in press). A low but detectable amount of MuMTV RNA, approximately 0.004% of the total cellular RNA, was observed in mammary tumors derived from ductal line CD8 (Fig. 5; Table 3). These observations suggest that endogenous MuMTV sequences are expressed in chemical carcinogen-

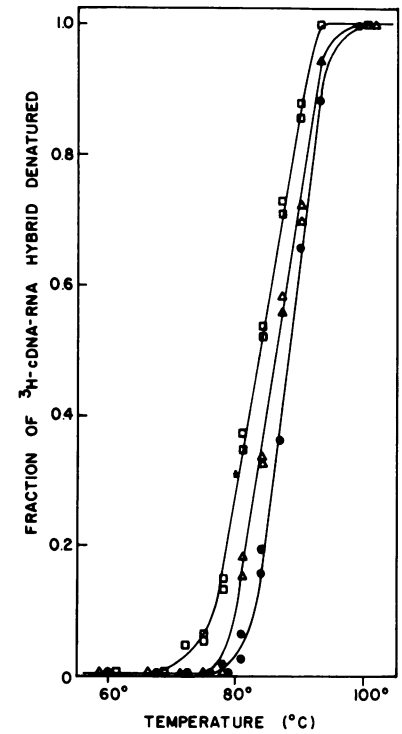

FIG. 6. *Analysis of the thermal stability of hybrids between [³H]MuMTV cDNA and carcinogen-induced BALB/c mammary tumor RNAs. Thermal denaturation of the MuMTV cDNA hybrids with 70S MuMTV RNA (●), C3 mammary tumor RNA (△), and C4 mammary tumor RNA (□). Hybridizations and thermal denaturations were performed as described in the text. The percentage of hybridization observed and the ratio of the RNA to cDNA concentration used for the hybridization were 99.9% and 21.6 with 70S MuMTV RNA, 24.9% and 203,000 with C3 mammary tumor RNA, and 89.9% and 98,600 with C4 mammary tumor RNA.*

induced ductal tumors. CD8 mammary tumor RNAs hybridized to approximately 80% of the [^{3}H]MuMTV cDNA at concentration ratios of RNA to cDNA of approximately 100,000. Additionally, the CD8 tumors were estimated to contain 19 equivalents of MuMTV RNA per 2c amount of DNA.

Type C virus expression in BALB/c HAN lines and mammary tumors. Type C virus expression was examined to determine whether the changes observed in MuMTV expression in the BALB/c HAN lines and mammary tumors relative to normal BALB/c mammary glands are specific for type B viral sequences. Also, it was of interest to learn if DMBA-induced mammary tumors in BALB/c mice contained type C virus RNA since DMBA-induced mammary tumors in C3H/StWi mice have type C virus particles and type C viral RNA (32a).

Low levels of RNA hybridizable to the MoMuLV cDNA probe, less than 0.002% of the total cellular RNA, were observed in D1 and D2 HAN lines as well as the D1 tumor, whereas the D2 tumor contained an appreciable amount of hybridizable RNA, 0.0797% of the total cellular RNA (Table 4). These observations indicate that appreciable type C expression does not occur in the D series of preneoplastic mammary lesions. The level of cellular RNA hybridizable to the MoMuLV cDNA probe in the DMBA-induced mammary tumors, C3, C4, and C5 (Fig. 7; Table 4), was similar to the level of MuMTV RNA in the same tumors (Table 3). Even in mammary tumors with an appreciable amount of RNA hybridizable to the MoMuLV cDNA probe, less than 60% of the cDNA was protected from S_1 nuclease digestion (Fig. 7; Table 4).

TABLE 4. *Expression of type C viral sequences in BALB/c HAN lines and mammary tumor*

Line	Tissue	Type C RNA (% cellular RNA)[a]
D1	HAN	0.0007 ± 0.0001% (2)
	Tumor	0.0017 ± 0.0009% (5)
D2	HAN	0.0007 ± 0.0002% (2)
	Tumor	0.0790 ± 0.0350% (4)
C3	Tumor	0.0024 ± 0.0016% (2)
C4	Tumor	0.0116 ± 0.0098% (2)
C5	Tumor	<0.0005 (1)

[a] The level of type C viral RNA is expressed as a percentage of the total cellular RNA. The concentration of type C RNA was determined by hybridization of total cellular RNA with [^{3}H]MoMuLV cDNA. The mean percentage of type C viral RNA ± one standard error for the number of determinations (in parentheses) was calculated.

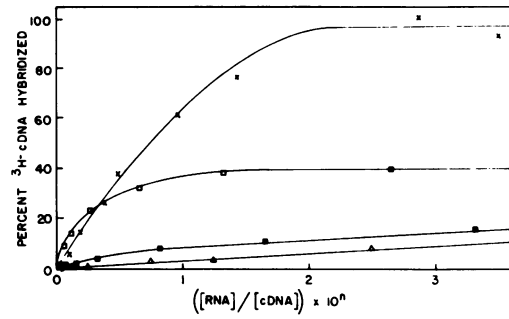

FIG. 7. *Type C viral RNA in BALB/c mammary tumors. The representative [^{3}H]MoMuLV cDNA was hybridized with various amounts of 70S MoMuLV RNA (×), 70S MuMTV RNA (△), and total cellular RNA from two C4 mammary tumors (□, ■). The ratio of the RNA to cDNA concentration for hybridization ranged from 0 to 3 with 70S MoMuLV RNA, from 0 to 3 × 10^2 with 70S MuMTV RNA, and from 0 to 3 × 10^4 with each C4 mammary tumor RNA. Hybridizations were performed and assayed as described in the text.*

DISCUSSION

Genetic and biochemical studies have demonstrated that mammary tumorigenesis in mice is influenced by MuMTV (2, 24, 25). In low mammary tumor incidence strains, such as BALB/c, MuMTV sequences are present in cellular DNA (23) and transmitted as a germinal provirus (3). To quantitate steady-state levels of MuMTV RNA during mammary tumorigenesis in BALB/c mice, a cDNA probe to MuMTV RNA was synthesized in vitro with DNA fragments as a random primer (35). The DNA fragment primed MuMTV cDNA probe is a specific probe and a representative copy of the MuMTV genome (13).

Our report as well as previous studies (32, 38) have demonstrated a very low level of MuMTV expression in spontaneous BALB/c mammary tumors arising in retired breeders presumably as a result of the changes in the hormonal environment during pregnancy and lactation. On the other hand, mammary tumors occurring in BALB/cfC3H retired breeders infected with the exogenous virus MuMTV-S, the milk-transmitted virus, had high levels of MuMTV expression. The question that has been addressed in this study concerns the influence on MuMTV expression of agents, other than the exogenous mammary tumor virus, that cause neoplastic development of the mammary gland in BALB/c mice.

The hormone-induced BALB/c D1 HAN line and mammary tumors that arose from the D1 HAN line contained approximately 0.01%

MuMTV-specific RNA sequences. The level of MuMTV RNA, approximately 0.13% of the total cellular RNA, in the hormone-induced D2 HAN line and D2 mammary tumors was similar to the level of MuMTV RNA in spontaneous mammary tumors from BALB/cfC3H mice. Three of the four mammary tumors that were derived from carcinogen-induced alveolar or ductal hyperplasias contained detectable levels of MuMTV RNA, ranging from 0.003 to 0.013% of the total cellular RNA. These observations indicate that there is no correlation between the tumor-producing capability of the mammary outgrowth lines (Table 1) and the level of MuMTV RNA (Tables 2 and 3) in mammary tumors derived from these HAN lines. Additionally, the observation that the C5 mammary tumors did not contain detectable MuMTV RNA suggests that MuMTV expression may not be required for maintenance of the mammary tumor phenotype in BALB/c mice. This is consistent with our conclusion that MuMTV production is not essential for DMBA-induced mammary tumorigenesis in C3H/StWi mice (32a).

Both D2 nodules and D2 mammary tumors appeared to express the complete MuMTV genome since RNA from these tissues hybridized to the MuMTV cDNA to approximately the same extent as the template RNA. RNA from C4 and CD8 mammary tumors hybridized to 80 to 90% of the MuMTV cDNA, indicating that the MuMTV sequences expressed in these tumors were homologous to most of the MuMTV genome. Analysis of the initial portion of the hybridization curve indicated that the D1 HAN line, D1 tumor, and C3 tumor contained similar or greater amounts of MuMTV RNA than did the CD8 tumor. However, the CD8 tumor RNA hybridized to most of the MuMTV cDNA, whereas only about 30% of the MuMTV cDNA was hybridized with D1 HAN line, D1 tumor, or C3 tumor RNAs.

A possible explanation for the limited extent of hybridization between the MuMTV cDNA and D1 HAN line, D1 tumor, and C3 tumor RNAs is that RNA homologous to different portions of the MuMTV genome may not accumulate to the same steady-state level in these tissues. Importantly, BALB/c mammary glands and BALB/c mammary tumor cell lines contain higher levels of MuMTV sequences complementary to the 3′ portion of the MuMTV genome than to other regions of the genome (J. P. Dudley et al., Proc. Natl. Acad. Sci. U.S.A., in press). The presence of MuMTV sequences in our cDNA probe that are not found in BALB/c cellular DNA (12, 31) may account for our inability to reach the same extent of hybridization with C4 and CD8 tumor RNAs as was achieved with the template 70*S* MuMTV RNA. However, the limited difference between the germinally transmitted MuMTV sequences in BALB/c mice and the MuMTV sequences present in the MuMTV cDNA probe (12, 31) would not seen to account for the low level of hybridization observed with D1 nodule, D1 tumor, or C3 tumor RNAs. Therefore, it will be necessary to attempt to identify which portions of the MuMTV genome are expressed in BALB/c HAN and tumors, and to determine if different segments of the MuMTV genome are expressed in the D1 HAN line and D1 mammary tumors.

In nearly all of the BALB/c HAN lines and mammary tumors that were examined, type C viral RNA was detected with the [^{3}H]MoMuLV cDNA probe; in most cases, the level of type C viral RNA was low, less than 0.002% of the total cellular RNA. BALB/c cells contain three host range classes of endogenous murine type C viruses (1, 33). The type C viral sequences in BALB/c mice are related to each other and to leukemogenic murine type C viruses (6). The observation that RNA from BALB/c mammary tumors only hybridized to a portion of the MoMuLV cDNA is probably due to partial homology between MoMuLV cDNA and type C viral sequences expressed in BALB/c mice (6). A high proportion of DMBA-induced mammary tumors in C3H/StWi mice have type C viral RNA and contain type C virus particles (32a). The significance of type C virus expression in mammary tumors, particularly DMBA-induced mammary tumors in C3H/StWi mice, is undefined at present.

Mammary tumorigenesis has been viewed as a progression from the normal state through a preneoplastic stage to mammary neoplasia (16, 18). Our observations demonstrate that the MuMTV genome may be expressed in the most common type of hormonally induced preneoplastic lesion in the BALB/c mammary gland, the hyperplastic alveolar nodule (8, 9, 14, 25). McGrath and co-workers (15) observed MuMTV RNA sequences in hormone-induced nodules and reported that mammary tumors had higher levels of MuMTV RNA than nodules. In our studies, no statistically significant change in the steady-state level of MuMTV RNA was observed during the progression from the preneoplastic stage to mammary neoplasia in hormone-induced D1 and D2 HAN lines. These observations suggest that an increase in MuMTV expression does not appear to be required for the progression of nodules to tumors in BALB/c mice.

The population of cells in a given HAN line are stable but heterogeneous (16, 18). Presumably, the characteristics of HAN-derived mammary tumors are due to the properties of the subpopulation of cells in the nodule that give rise to the tumor. In view of the heterogeneity of cell populations in the nodules, it was surprising that the hormone-induced HAN lines and mammary tumors had similar levels of MuMTV RNA. Also, approximately the same level of type C virus expression was observed in the D1 HAN line and D1 mammary tumors. These observations suggest a similarity in the cell subpopulations within nodules and mammary tumors. On the other hand, a difference in the cell subpopulations between the D2 nodule line and tumors was suggested by the observation that D2 tumors had appreciably higher levels of type C virus expression than the D2 HAN line. The heterogeneity of BALB/c mammary tumor cell populations is suggested by the observation that cells selected during cell culture of hormone and carcinogen-induced BALB/c mammary tumors did not contain detectable MuMTV RNA (13). On the basis of these observations it is important to determine if MuMTV expression is restricted to a particular subpopulation of cells within the BALB/c HAN lines and mammary tumors.

The strongest evidence that the low tumor incidence strain BALB/c contains MuMTV is that MuMTV sequences are found in cellular DNA (23). However, previous evidence has indicated that only a low level of MuMTV RNA in present in BALB/c mammary glands and spontaneous mammary tumors (15, 28, 32, 38). Our studies demonstrate that HAN lines and mammary tumors, derived from HAN induced in BALB/c mice by hormone and/or DMBA, may express the endogenous MuMTV genome, but expression is apparently not required for mammary tumor progression. Future studies concerning MuMTV expression in BALB/c mice might focus on a number of problems such as the expression of MuMTV RNA in the mammary gland immediately after chemical carcinogen treatment and in mammary tumors induced directly by chemical carcinogens; the cellular as well as subcellular distribution of MuMTV RNA in BALB/c HAN lines and tumors; and the determination of whether the MuMTV RNA is translated.

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