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Detection of Mouse Mammary Tumor Virus RNA in BALB/c Tumor Cell Lines of Nonviral Etiologies

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A complementary DNA (cDNA) probe to mouse mammary tumor virus (MMTV) RNA was synthesized using calf thymus DNA oligonucleotides as a random primer. This probe was then used to study the expression of MMTV RNA in cell lines from BALB/c tumors induced in vivo either spontaneously or in response to viral, chemical, or hormonal stimuli. The cDNA had a length of approximately 400 to 500 nucleotides and specifically hybridized to MMTV RNA and BALB/c lactating mammary gland RNA, but not to Moloney leukemia virus RNA. Calf thymus DNA-primed cDNA could protect 50% of iodinated MMTV RNA from S1 nuclease digestion at cDNA-RNA ratios of 1:1 and 90% of labeled viral RNA at ratios of 10:1. Thermal denaturation of MMTV RNA-cDNA hybrids yielded a T_m of 88.5°C, indicative of a well-base-paired duplex. Screening of mouse mammary tumor cells for MMTV sequences revealed that three out of five lines of BALB/c origin had undetectable levels of viral RNA (<three molecules per cell) by RNA excess hybridization. Two of the three "virus-negative" cell lines were derived from tumors induced by the chemical carcinogen 7,12-dimethylbenz(α)anthracene, whereas the third tumor occurred spontaneously. Two lines from tumors induced by either viral (mammary tumor virus) or hormonal (17-β-estradiol) stimulus contained between three and nine molecules of MMTV RNA per cell by both RNA excess and cDNA excess hybridization. Clonal derivatives of these tumor lines had levels of viral RNA comparable to those of their parental lines. Therefore, it appears that the presence of detectable MMTV RNA sequences is not a necessary requirement for the maintenance of all murine mammary gland neoplasias.

It is well established that some strains of mouse mammary tumor virus (MMTV) are exogenously infective oncogenic agents, as demonstrated by foster-nursing experiments (13). However, the involvement of endogenous MMTV sequences in mouse mammary tumorigenesis under conditions in which virus production may not be overt is unknown. One question which can be directly approached is the following: is the continuous presence of MMTV RNA necessary for the maintenance of transformation in all mouse mammary cancers regardless of their derivation?

Previous reports agree that exposure to chemical carcinogens and/or irradiation can activate MMTV expression (11, 14, 26, 29). However, whether the activation phenomenon is causally related to the neoplastic process or is secondary to other changes in cell regulation during tumorigenesis has not been determined.

The present investigation was designed to detect MMTV RNA sequences in a series of tumor cell lines derived from the low mammary cancer mouse strain BALB/c. These lines had been established from tumors arising in vivo either spontaneously or in response to viral, chemical, or hormonal stimuli (4). MMTV complementary DNA (cDNA) was synthesized using random calf thymus oligodeoxynucleotide primers and was shown to be of sufficient length and specificity to be used in long-term hybridization experiments. In addition, this cDNA was able to protect 100% of iodinated viral RNA sequences from S1 nuclease digestion. Annealing of tumor cell RNA to the representative MMTV probe demonstrated sequences in tumor lines with viral or hormonal etiologies, but little or no detectable viral sequences were observed in spontaneous and 7,12-dimethylbenz(α)anthracene (DMBA)-induced tumor derivatives.

MATERIALS AND METHODS

Reagents, cell lines, and virus. Virus released by Mm5mt/cl cells at 12 to 24-h intervals was provided

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by the Viral Resources Laboratory of the Viral Oncology Program. Moloney leukemia virus 70S RNA and the MMTV producer cell line designated H-1 were kindly provided by Wade Parks; the cells were maintained as previously described (4). BALB/c tumor cell lines were derived and maintained as described by Butel et al. (4). The MTV-L/BALB cell line originated from the first transplant generation of a tumor arising in an MTV-L(NIV) BALB/c breeder, and the ESD/BALB line was derived from the second transplant generation of a tumor arising from neonatal injection of a BALB/c female mouse with 17-β-estradiol. Sp/BALB cells were derived from a spontaneous tumor arising from transplantation of the hyperplastic alveolar nodule line (D1) (17). The DMBA/BALB cell line was established from the first transplant generation of a tumor arising from a DMBA-induced hyperplastic alveolar nodule line (15). The DMBA-2/BALB line and its clonal derivatives were established from the fourth transplant generation of a tumor arising from transplantation of a DMBA-induced alveolar hyperplasia (15, 16; J. P. Dudley et al., manuscript in preparation).

Oligo(dT)$_{12-18}$ was purchased from Miles Laboratories (Elkhart, Ind.), and calf thymus DNA was from P-L Biochemicals (Milwaukee, Wis.). Deoxyribonucleotides were from P-L Biochemicals or Sigma Chemical Co. (St. Louis, Mo.). Na-^{125}I (551 mCi/ml) was purchased from New England Nuclear (Boston, Mass.). [5-^{3}H]dCTP or [8-^{3}H]dGTP (17 to 32 Ci/mmol) were products of Schwarz/Mann Radiochemicals (Orangeburg, N.Y.). Avian myeloblastosis virus RNA-dependent DNA polymerase was provided by J. N. Beard (Life Sciences Inc., St. Petersburg, Fla.) in conjunction with the Virus Cancer Program. S1 nuclease was produced by Miles Laboratories, and proteinase K was obtained from E. M. Biochemicals (Elmsford, N.Y.). DNase I was purchased from Worthington Biochemicals (Freehold, N.J.).

Extraction of nucleic acids. Viral RNA and cellular RNA extracts were prepared as described by Rosen et al. (23, 24). Purified (sucrose gradient-banded) MMTV from tissue culture supernatants (approximately 10 ml or 5 mg of protein) was pelleted at 100,000 $\times$ *g* for 1 h in a Beckman 50 Ti rotor. Pellets were resuspended in 0.5 ml of 0.01 M Tris-hydrochloride, pH 7.8, and 1 mM dithiothreitol and adjusted to 0.5% sodium dodecyl sulfate (SDS) and 0.01 M Na_2-EDTA. Proteinase K was added to the virus suspension to a final concentration of 100 μg/ml and incubated at 37°C for 30 min. The reaction was terminated by the addition of 2 volumes of a solution containing buffer-saturated, redistilled phenol (0.01 M Tris-hydrochloride, pH 8.0, 25 mM Na_2-EDTA, 75 mM NaCl, and 0.5% SDS), chloroform, and isoamyl alcohol (25:24:1, vol/vol/vol). The aqueous layer obtained after agitation for 5 min and centrifugation at 10,000 $\times$ *g* for 10 min was pooled with the supernatant subsequently obtained by re-extraction of the protein interface. A final phenol-chloroform-SDS extraction was performed, and the aqueous phase was adjusted to 0.2 M sodium acetate (NaAc), pH 5.0. RNA was recovered by overnight precipitation at −20°C after the addition of 2 volumes of 100% ethanol. The viral RNA was subsequently pelleted by centrifugation at 10,000 $\times$ *g* for 30 min, resuspended once in 95% ethanol, and dissolved in water. Yields of RNA were 1.0 to 1.5% (wt/wt) of the original protein. Viral RNA was subsequently heat denatured at 80°C for 3 min before rapid cooling on ice and extraction of low-molecular-weight RNA and DNA by the NaAc extraction procedure described by Palmiter (19). The size of viral RNA obtained by this method was estimated to be 10-30S by sedimentation on neutral gradients (0.3 to 1.0 M) containing 0.1 M NaCl, 0.01 M NaAc, and 1 mM Na_2-EDTA for 4 h at 250,000 $\times$ *g* in a Beckman SW56 rotor.

Cell lines were grown to confluency in Dulbecco medium containing 10% fetal bovine serum and 5 μg of insulin per ml before harvesting of cell monolayers by trypsinization.

DNA was removed from nucleic acid extracts by extraction with 3.0 M NaAc, pH 6.0, as described by Palmiter (19). Three successive acetate extractions removed greater than 95% of the DNA (as determined by diphenylamine analysis [3]) present in nucleic acid preparations.

Preparation of calf thymus oligonucleotide primer. Calf thymus DNA fragments were isolated by a modification of the methods of Taylor et al. (28) and W. P. Parks (personal communication). Calf thymus DNA (5 mg/ml) was sheared by passage through a 25-gauge needle and alkali digested (0.5 N NaOH) for 4 h at 37°C. The concentration of DNA was then adjusted to 2 to 3 mg/ml, and the solution was neutralized by the addition of 0.1 M Tris-hydrochloride, pH 7.4; 0.01 M magnesium acetate, 0.01 M $CaCl_2$, and 60 μg of DNase I per ml were then added. After incubation for 4 h at 37°C, SDS and proteinase K were added to final concentrations of 0.2% and 10 μg/ml, respectively, and the incubation was continued at 37°C for 30 min. The reaction mixture was then extracted by the phenol-chloroform-SDS procedure previously described. The aqueous phase was dialyzed against water and lyophilized preceding chromatography on Sephadex G-75 in 0.1 M NaAc, pH 5.0. Fractions of the included peak were pooled and precipitated by the addition of 2 volumes of ethanol at −20°C. The size of the oligonucleotide primers was estimated by electrophoresis on 12% polyacrylamide gels to be approximately six to eight nucleotides in length.

Synthesis of cDNA. Conditions for cDNA synthesis were modifications of those described by Monahan et al. (18) and Young et al. (35). The reaction contained heat-denatured, NaAc-treated viral RNA (35 μg/ml or greater), avian myeloblastosis virus polymerase (125 U/ml), 150 mM KCl, 1 mM unlabeled triphosphates, 200 μM [^{3}H]dCTP, 4 mM sodium pyrophosphate, 14 mM magnesium acetate, 20 mM dithiothreitol, and calf thymus DNA fragments at a ratio of 40:1 (primer to template) in a total reaction volume of 100 μl. Incubations were performed at 46°C for 20 min.

The cDNA was purified using the method of Harris et al. (12). Average specific activities for tritium-labeled probes were 1×10^7 to 2×10^7 cpm/μg; calculations were based on an average cytosine content of 25%. Yields of cDNA were based on calculated specific activities. Alkaline sucrose gradient analysis of viral cDNA was performed according to Monahan et al. (18).

RNA excess hybridization. Hybridizations were performed as described by Rosen and Barker (22).

Each hybridization sample contained approximately 1,500 cpm of tritium-labeled cDNA, varying amounts of unlabeled viral or cellular RNA (0.2 ng/ml to 9.7 mg/ml), 20 μg of hen oviduct rRNA carrier, 0.6 M NaCl, 2 mM Na_2-EDTA, and 0.01 M HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid), pH 7.0, in a final volume of 50 μl. After denaturation at 100°C for 1 min, samples were incubated for up to 120 h at 68°C. The reaction was terminated by freezing in dry ice-alcohol. Hybrid formation was assayed using S1 nuclease digestion, as described by Monahan et al. (18). Control samples lacking RNA were tested in duplicate for the recovery of radioactivity in both the presence and the absence of S1. The average S1 resistance of ^{3}H-labeled cDNA routinely observed was between 5 and 10% of the radioactivity added after a 96-h incubation and was subtracted from each sample value. The data are expressed as the percentage of duplex formation versus log equivalent R_0t (the concentration of RNA in nucleotides per liter multiplied by time [seconds]), i.e., corrected for standard conditions in 0.12 M phosphate buffer (0.18 M Na^+) at 62°C (2).

cDNA excess hybridizations. Hybridization reactions contained 0.01 M HEPES (pH 7.0), 0.6 M NaCl, 2 mM Na_2-EDTA, 1.3 ng (~19,000 cpm) of calf thymus (CT)-primed cDNA, and varying amounts of viral or cellular RNA (RNA/cDNA = 0.1 to 76,000) in a total volume of 10 μl. Samples were incubated at 68°C for 96 h ($C_0t = 7.48 \times 10^{-3}$) before addition of 8 μg of oviduct rRNA and S1 digestion as described above. A background S1 resistance of 3.4% was subtracted from each experimental value before calculation of percent hybrid formation. The data were plotted as the percentage of duplex formation versus the RNA-cDNA ratio, as described by Young et al. (34), and the slope of the line obtained by linear regression analysis of the initial rate of hybridization was calculated. Correlation coefficients of 0.98 or greater were routinely observed. The percentage of viral RNA in mammary tumor cell lines was determined by the ratio of the slopes obtained for purified MMTV RNA-cDNA and cellular RNA-cDNA hybrids.

Thermal denaturation of cDNA-RNA hybrid. The melting profile of cDNA-RNA hybrids was determined by a modification of the method of Rosen and Barker (22). The denaturation of hybrids was monitored by the use of S1 nuclease subsequent to RNA excess hybridization to an R_0t of 1.25×10^{-1}.

Iodination of viral RNA. Viral RNA was iodinated by the technique of Commerford (5) with the modifications of Woo et al. (33) and Prensky et al. (21). Five microliters of Na-^{125}I in 0.1 N NaOH was added to 2 μl of 0.3 mM Na_2SO_3 in 0.3 N H_2SO_4 and allowed to stand at room temperature for 30 min. This solution was subsequently adjusted to 0.1 M NaAc (pH 5.0), 0.05 mM potassium iodide, 68 μg of 50-70S viral RNA per ml, and 0.5 mM $TlCl_3$ and incubated at 60°C for 15 min. The reaction was chilled and terminated by the addition of 5 μl of 0.01 M tyrosine and 15 μl of 2.0 M sodium phosphate buffer, pH 7.0. Heating was continued at 60°C for a second 15-min period followed by rapid cooling in ice and the addition of 100 μl of a column buffer containing 0.01 M Tris-hydrochloride (pH 7.6), 0.1 M NaCl, and 2 mM Na_2-EDTA. The iodinated product was separated from unbound radioactivity by chromatography on Sephadex G-50. The specific activity of the labeled RNA was estimated to be 1.5×10^7 cpm/μg. The size of viral RNA was estimated by centrifugation on neutral sucrose gradients as described above. Fractions used for the protection experiment were calculated to contain RNA with sedimentation values of 10-20S and were essentially free from contaminating low-molecular-weight cellular RNAs.

Nuclease protection assays. Samples containing 0.25 ng of [^{125}I]RNA were incubated with increasing ratios of tritium-labeled cDNA for 96 h at 68°C in a total volume of 10 μl ($R_0t = 1.5 \times 10^{-1}$). Hybrids were digested with S1 nuclease and precipitated by addition of 20% trichloroacetic acid before membrane filtration (Millipore Corp.; as described above). Quantitation of radioactivity was performed in a Nuclear-Chicago 1185 Series automatic gamma counter (Des Plaines, Ill.).

RESULTS

Characterization of MMTV cDNA probe. The cDNA preparations used in this study were carefully characterized for several reasons. Previous investigations of MMTV expression in mouse mammary tumor cells used cDNA probes which represented undefined portions of the MMTV genome (25, 26). Therefore, it has not been possible to determine whether there are qualitative as well as quantitative differences in the expression of type B virus in the mammary tissues of various mouse strains. Lack of detectable hybridization to MMTV cDNA can be interpreted to mean that no type B viral sequences are present in the nucleic acid preparation being tested or, alternatively, that MMTV sequences present in the preparation are not represented within the cDNA probe. cDNA preparations were, therefore, analyzed for representation of viral sequences using an S1 nuclease protection assay. The specificity and fidelity of hybridization were also evaluated.

(i) Representation of viral genome by cDNA. The ability of cDNA to protect labeled 50-70S viral RNA from nuclease digestion permits an estimation of the representativeness of copying by reverse transcriptase. An experiment using iodinated MMTV RNA and increasing ratios of CT-primed cDNA is depicted in Fig. 1. Equal ratios of cDNA to viral RNA resulted in 50% double-stranded material, whereas 90% protection could be achieved at ratios of 10:1 (cDNA-viral RNA). Complete hybridization, indicative of 100% copying of the template MMTV RNA, was obtained at ratios of 20:1 or greater. Therefore, although the MMTV genome was completely represented by the cDNA preparation, there appeared to be an unequal representation of MMTV RNA sequences. Cox (6), Getz et al. (10), and Davis and Nayak (7) have re-

ported nuclease protection results using CT-primed cDNA's similar to those described here.

(ii) Specificity of cDNA in hybridization reactions. The specificity of CT-primed cDNA hybridization with homologous RNA sequences was determined by RNA excess hybridization. The hybridization kinetics observed when cDNA was hybridized to its high-molecular-weight RNA template are shown in Fig. 2. A $R_0t_{1/2}$ value of 5.0×10^{-2} was obtained for homologous MMTV RNA. Thermal denaturation of hybrids formed between MMTV CT-primed cDNA and high-molecular-weight viral RNA revealed a T_m of 86.4°C, a value consistent with other well-base-paired hybrids (18, 22). No hybridization was observed with heterologous Moloney leukemia virus RNA approaching an equivalent R_0t equal to 1. Hybridization analysis was also performed with cellular RNA containing relatively large amounts of MMTV RNA. After growth in dexamethasone-containing medium for 96 h, H-1 cellular RNA had a $R_0t_{1/2}$ value of 3, similar to the value previously reported by Scolnick et al. (27). In addition, RNA extracted from 9- to 11-day lactating mammary glands of BALB/c mice was found to contain low levels of MMTV sequences, as previously reported by Varmus et al. (30). Control preparations which showed no appreciable hybridization to CT-primed cDNA included polyadenylic acid, yeast RNA, and RNA extracted from simian virus 40-transformed mouse fibroblasts (VLM cells), BALB/c liver tissue, and a BALB/c liver cell line (data not shown). In addition, the sizes of cDNA preparations were evaluated by alkaline sucrose sedimentation. Average sedimentation values obtained ranged from 6.0–7.0S (approximately 400 to 550 nucleotides) (data not shown).

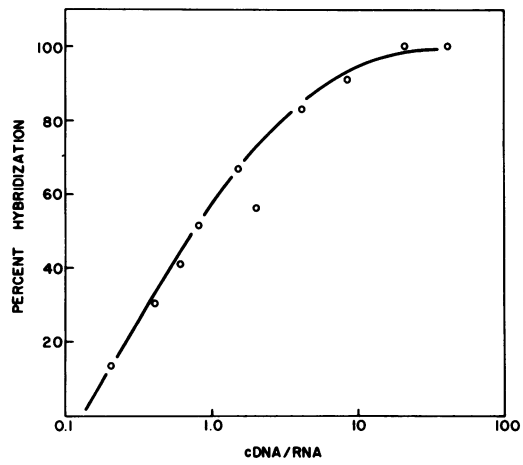

FIG. 1. *Protection of ^{125}I-labeled MMTV RNA by ^{3}H-labeled, CT-primed MMTV cDNA. Samples containing 0.25 ng of viral RNA were incubated with increasing ratios of cDNA for 96 h at 68°C in a total volume of 10 μl (R_0t = 1.5 × 10^{-1}). Hybrids were digested with S1 nuclease and precipitated by addition of 20% trichloroacetic acid before membrane (Millipore Corp.) filtration and quantitation in a gamma counter.*

The preceding analyses indicated that the CT-primed cDNA was of sufficient length and specificity for use in hybridization studies, as well as being representative of the entire MMTV genome. The CT-primed cDNA, therefore, was used to quantitate MMTV sequences in BALB/c mouse mammary tumor cell lines of diverse etiologies.

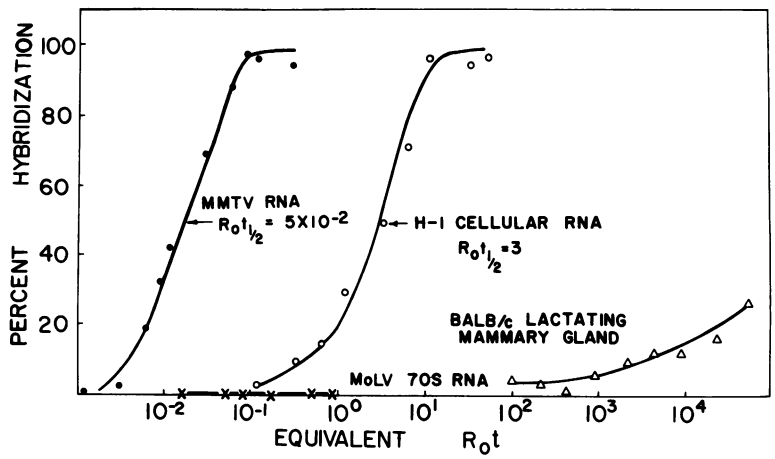

FIG. 2. *Specificity of hybridization to ^{3}H-labeled MMTV cDNA. Hybridizations were performed as described in the text for 96 h, with the exception of reactions containing Moloney leukemia virus RNA (1 to 4 h). cDNA used for hybridization analysis was prepared with calf thymus DNA fragments.*

Detection of MMTV-specific RNA in tumor cell lines. (i) RNA excess hybridizations. The hybridization kinetics observed when mammary tumor cell RNA derived from five different parental cell lines were annealed to CT-primed cDNA are depicted in Fig. 3. Hybridization to ESD/BALB and MTV-L/BALB cell lines was readily detected, with Sp/BALB yielding a very low level of duplex formation at the highest R_0t value tested. No specific hybridization could be demonstrated with either DMBA/BALB or DMBA-2/BALB cellular RNA.

The amount of virus-specific RNA detected in ESD/BALB and MTV-L/BALB can be estimated by comparing the R_0t values obtained at 20% hybridization, similar to that described by Varmus et al. (31). Such an analysis revealed that 2.8×10^{-4}% of cellular RNA is virus specific. Using an RNA-DNA ratio of 2.0, 4.9 pg of DNA per cell, and a molecular weight of 3.0×10^6 for MMTV RNA, there are approximately 6.0 molecules of viral RNA per cell. The level of viral sequences in Sp/BALB cells is probably at least 40-fold lower (i.e., approximately 0.1 molecule per cell). Therefore, the cDNA probe displayed a high degree of both sensitivity and specificity.

One clone derived from each parental cell line was also analyzed by RNA excess hybridization to CT-primed cDNA (Fig. 4). There appears to be only minor differences between these particular clones and the hybridization data given for the parental lines in Fig. 3. No detectable annealing was observed with Sp/BALB Cl 7, DMBA/BALB Cl 2, and DMBA-2/BALB Cl 2, whereas ESD/BALB Cl 3 and MTV-L/BALB Cl 2 had 1.1×10^{-4} and 2.3×10^{-4}% viral-specific sequences, respectively. It was estimated that there are 2.0 and 4.5 molecules per cell in ESD/BALB Cl 3 and MTV-L/BALB Cl 2 cells, respectively, as opposed to 6.0 molecules per cell for their parental lines. It appears that there is a general trend for clonal lines to contain slightly decreased levels of MMTV RNA; this may be a

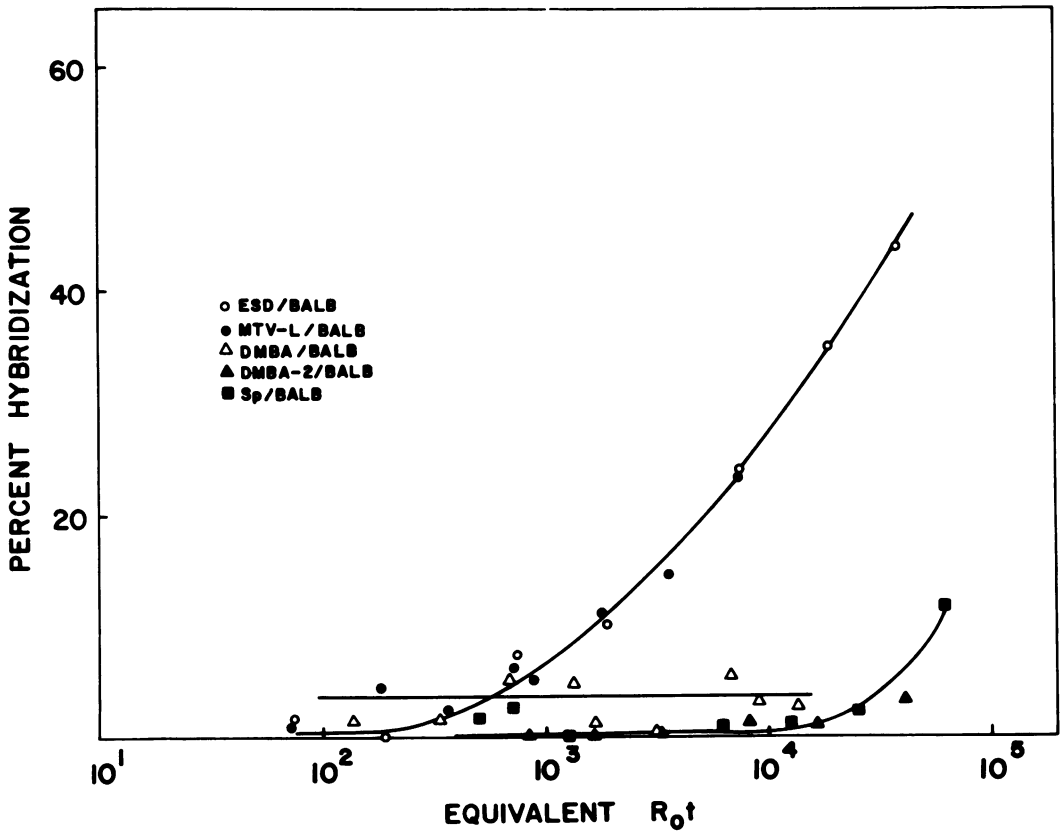

FIG. 3. *Detection of MMTV sequences in mammary tumor cells by RNA excess hybridization. Parental cell lines were grown in medium containing 10 μg of insulin per ml and 10% fetal bovine serum and extracted for total cellular RNA. Samples were hybridized with CT-primed cDNA for up to 120 h and subsequently assayed for hybrid formation, using S1 nuclease.*

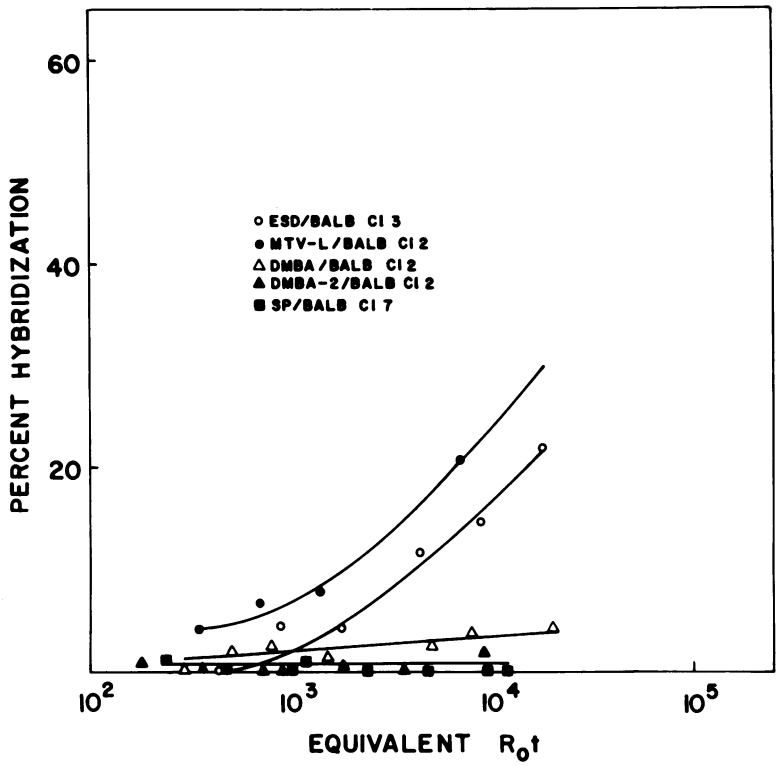

FIG. 4. *MMTV expression in clonal mammary tumor cell lines. Clones of each of the respective BALB/c parental tumor cell lines were grown in medium containing 10% fetal bovine serum and 10 μg of insulin per ml for 96 h before extraction of total cellular RNA. Samples were hybridized to a representative CT-primed cDNA for 96 h at 68°C.*

reflection of their higher passage level in culture (20).

(ii) cDNA excess hybridization. Due to changes in the kinetics of hybridization that occur at low complementary sequence ratios and the very high R_0t values using RNA excess hybridization, a technique initially described by Young et al. (34) was used to allow a more quantitative estimate of the number of viral sequences present in the mammary tumor cell lines. This method depends upon comparisons of the initial rates of cDNA hybridization observed with the cell line RNA and with homologous template RNA under conditions where both the concentration of cDNA and the time of hybridization are sufficient to allow all reaction to go to completion.

Figure 5 demonstrates the specificity of CT-primed cDNA excess hybridizations using template MMTV RNA and a C3H virus producer cell line grown in the presence and absence of dexamethasone. Comparison of the slopes of the lines obtained for the H-1 cell line with MMTV RNA revealed that 0.019% of cellular RNA was virus specific in cells grown in insulin alone, whereas 0.22% of the RNA was virus specific when cells were exposed to glucocorticoids. This represents 374 and 4,373 molecules per cell, respectively, approximately a 12-fold induction. A 10- to 20-fold induction level has been noted previously for these cells (27).

The quantitation of endogenous sequences in the BALB/c mouse mammary tumor lines is shown in Fig. 6. The ESD/BALB cell line contained 4.4×10^{-4}% MMTV (~9 molecules per cell), whereas MTV-L/BALB Cl 2 cells had 3.5×10^{-4}% viral RNA (~7 molecules per cell).

The results of MMTV sequence quantitation in the BALB/c tumor cell lines by RNA excess and cDNA excess hybridization are summarized in Table 1. Data obtained by the two methods are comparable within a twofold range. It should be noted that RNA extracted from DMBA/BALB and DMBA-2/BALB cells or their clonal derivatives did not exhibit detectable hybridization to CT-primed cDNA. The RNA obtained from the Sp/BALB line showed only low level hybridization (~10%) with MMTV cDNA,

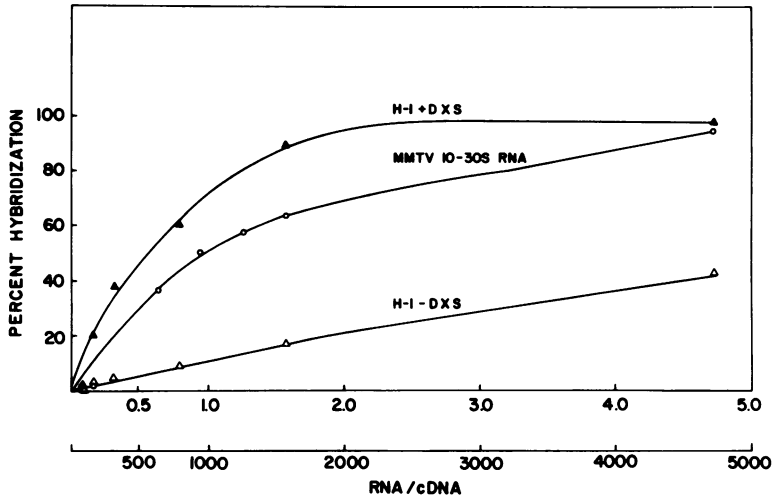

FIG. 5. *Specificity of cDNA excess hybridizations. NaAc-treated 10-30S MMTV RNA was hybridized at ratios of 0.16 to 4.70 with cDNA (1.3 ng) in a total reaction volume of 10 μl (○). Total RNA extracted from cells grown in the presence (▲) or absence (△) of dexamethasone (1 μg/ml in Dulbecco medium containing 10% fetal bovine serum) was annealed to cDNA at ratios varying from 79 to 4,700. Hybridizations were incubated for 96 h at 68°C.*

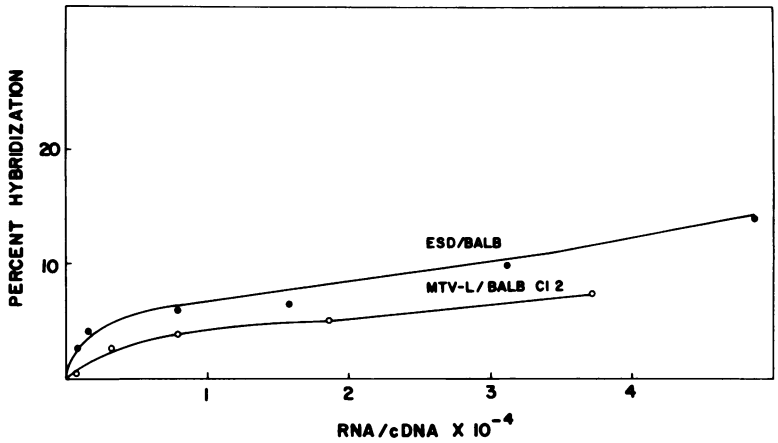

FIG. 6. *Quantitation of endogenous MMTV sequences by cDNA excess hybridization. The ESD/BALB and MTV-L/BALB Cl 2 cell lines were grown in Dulbecco medium containing 10% fetal bovine serum and 10 μg of insulin per ml before extraction of total cellular RNA. Increasing amounts of RNA were added to 1.3 ng of cDNA in a total reaction volume of 10 μl and hybridized for 96 h at 68°C. Hybrid formation was assayed using S1 nuclease.*

whereas RNA from Sp/BALB Cl 7 demonstrated no hybridization over background levels with this probe.

DISCUSSION

This study revealed that three out of four transplantable BALB/c cell lines derived from tumors of nonviral etiology (i.e., derived from mice which had not been exposed to virus-containing milk or purified virus preparations) contained less than three copies of MMTV RNA per cell as detected by a representative cDNA. With the Sp/BALB line, a very low level of hybridization (<20%) was demonstrated in tumor cells at R_0t values of >10,000. This level of hybridization was probably significant since comparable levels of annealing were not detected in simian virus 40-transformed mouse cells, normal liver tissue, or a normal BALB/c mouse liver cell line. Of the three tumor lines,

TABLE 1. *Summary of detection of MMTV sequences in mouse mammary tumor cell lines by RNA and cDNA excess hybridization*

Cell line	RNA excess[a]		cDNA excess	
	% Cellular RNA ($\times 10^{-5}$)	No. of molecules/cell[b]	% Cellular RNA ($\times 10^{-5}$)	No. of molecules/cell
MTV-L/BALB	28.0	6.0		
MTV-L/BALB Cl 2	23.0	4.5	35.0	7.0
ESD/BALB	28.0	6.0	44.0	9.0
ESD/BALB Cl 3	11.0	2.0		
Sp/BALB	<2.4[c]	<0.5		
Sp/BALB Cl 7	<13.0[c]	<2.5		
DMBA/BALB	<8.8[c]	<2.0		
DMBA/BALB Cl 2	<7.5[c]	<1.5		
DMBA-2/BALB	<3.7[c]	<1.0		
DMBA-2/BALB Cl 2	<16.0[c]	<3.0		

[a] Based on values of R_0t (concentration of RNA in nucleotides per liter multiplied by time [seconds]) obtained at 20% hybridization.

[b] Calculations were based on a RNA-DNA ratio of 2.0 (derived from analysis of ESD/BALB cells), a DNA content of 4.9 pg/cell, and a molecular weight of 3.0×10^6 for MMTV RNA.

[c] No hybridization above background levels noted in these samples. These values are the highest values of R_0t tested.

two were induced by the administration of the chemical carcinogen DMBA (DMBA/BALB and DMBA-2/BALB), whereas the third line (Sp/BALB) was derived from a spontaneous tumor. Neither glucocorticoid stimulation (J. P. Dudley and J. S. Butel, submitted for publication) nor the use of a 3'-specific probe (J. P. Dudley, J. M. Rosen, and J. S. Butel, Proc. Natl. Acad. Sci. U.S.A., in press) was successful in detecting viral sequences in these cell lines. Results obtained in this study agree with those of other investigators who have found no evidence for selective transcription of murine sarcoma virus (8) or murine leukemia virus (9) in chemically transformed cells. It is apparent that there is no "overall" derepression of gene expression in transformed mouse cells, as has been previously suggested in other systems (10, 32).

In contrast, two tumor cell lines, ESD/BALB and MTV-L/BALB, contained readily detectable amounts of MMTV RNA which were approximately 200-fold less than in dexamethasone-treated C3H tumor cell (H-1) cultures. The ESD/BALB line originated from a tumor arising in an animal subjected to neonatal estrogen treatment, whereas MTV-L/BALB cells were derived from a tumor appearing in a C+ breeder. The C+ subline of BALB/cCrgl mice was established from an animal that developed a spontaneous, B-particle-positive tumor; this animal was subsequently bred, and her progeny were kept separate from the "virus-free" mice. The origins of these latter two tumors suggest that accumulation of endogenous MMTV RNA sequences of BALB/c mice can be stimulated under certain circumstances.

If it were assumed that MMTV information is responsible for the maintenance of all murine mammary cancers, several explanations can be offered for the lack of detectable RNA in these transplantable mammary cell lines.

(i) The cell lines lacking MMTV sequences may have been derived from normal fibroblasts surrounding the original tumor tissue which became spontaneously transformed in vitro. Several lines of evidence suggest that this possibility is unlikely. Morphologically, all of the cell lines in culture appear to be epithelioid, and tumors induced in syngeneic animals by the cultured cell lines are carcinomas (4; Dudley et al., manuscript in preparation). Although some of the inbred tumors are anaplastic, the cell line which displays the most classical in vitro and in vivo epithelial morphology (DMBA-2/BALB) contains almost undetectable amounts of MMTV RNA.

(ii) The MMTV probe used in these studies may be incapable of detecting partial transcription by the endogenous mammary tumor virus. Considerable effort was directed toward proving the representative nature of the probe, and there is no evidence to suggest otherwise. Results indicate that even when there is partial transcription (as detected by 3'-cDNA [Dudley et al., in press]) of the viral genome (e.g., lactating tissue), CT-primed cDNA can be used to demonstrate low levels of hybridization. However, it cannot be ruled out that the endogenous BALB/c virus shares little or no homology with the exogenous C3H virus over a portion of its genome, and, if so, transcription of the nonhomologous region would go undetected.

(iii) It can be hypothesized that MMTV is involved in the initiation of mammary neoplasias and that a chemical carcinogen, hormone, or other "promoter" maintains the transformed state. In this respect, it is interesting to note that relatively high levels of MMTV RNA can be detected in preneoplastic lesions which give rise to tumors at a high frequency (R. Pauley, personal communication).

(iv) Transcription of MMTV RNA may be repressed in vitro during cell passage (20). However, this effect would have to be both very rapid and complete, since DMBA-2/BALB Cl 2 cells were tested for MMTV RNA at passage 9 and were highly tumorigenic in syngeneic, immunocompetent mice at passage 8.

(v) Finally, there is no direct evidence (except by analogy with other virus systems) that a viral protein product, and therefore an mRNA, is

necessary for the transformation process. Transformation of mammary gland cells could theoretically be accomplished through the process of provirus integration per se. However, there is no known virus system where such a phenomenon has been documented.

In summary, the data presented in this report suggest that there is no requirement for the continuous expression of MMTV RNA in the maintenance of transformation of all murine breast cancers. Results indicate that viral sequences normally present at low levels in lactating BALB/c mammary glands are actually decreased in amount or undetectable in DMBA-induced tumor cells. This appears to be in contrast to several reports of "MMTV activation" by chemical agents such as urethane (11, 26, 29). Alternatively, it may simply reflect a difference between the mechanisms of action of these two agents. Further experiments are necessary to resolve this question.

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