Increased Concentration of an Indigenous Proviral Mouse Mammary Tumor Virus Long Terminal Repeat-Containing Transcript Is Associated with Neoplastic Transformation of Mammary Epithelium in C3H/Sm Mice

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Increased amounts of mouse mammary tumor virus (MMTV) proviral transcripts were found in RNA dot blots from MMTV-negative, C3H/Sm mouse mammary tumors which arose spontaneously or were induced by hormonal or chemical carcinogens or both. Other dot blots probed with a long terminal repeat (LTR) probe showed that LTR (MMTV)-containing transcripts were disproportionately represented in these tumor RNAs. Different segments of the MMTV genome were used in sequential hybridizations to Northern blots to determine relative sequence content and size of MMTV transcripts in transformed mammary tissues, as compared with those in lactating mammary glands. Increased amounts of 4.4-kilobase *env* and 8.1-kilobase genomic MMTV transcripts were detected with an *env* probe in many of the tumor RNAs examined. Hybridization of the same Northern blots containing tumor RNAs with an LTR probe revealed a 2.2-kilobase transcript which was prominent in RNAs from chemically-induced, hormonally-induced, and spontaneous mammary tumors relative to those from lactating mammary glands. The LTR-containing transcript did not possess significant homology to either *env* or *gag-pol* probes. This distinctive, transformation-enhanced, 2.2-kilobase transcript may contain mouse cellular sequences in addition to LTR sequences or it may represent the message for a nonstructural viral protein encoded within the LTR open reading frame of one or more of the four C3H/Sm MMTV proviral genes.

The link between mammary tumorigenesis and mouse mammary tumor virus (MMTV) proviral expression has not been established. Recent evidence concerning MMTVs and similar retroviruses has centered interest on the long terminal repeat (LTR) as a critical agent in the induction of tumorigenesis. LTRs from exogenous MMTVs are strong, corticosteroid-responsive promoters of gene expression in tissue culture (24, 27, 28) or in recombinant plasmids (1, 5, 15, 16). It has been suggested that MMTV LTRs may induce malignant transformation either by insertion as promoters into host DNA sites proximal to mouse cellular oncogenes (23, 26) or as remote enhancers of the expression of nonadjacent oncogenic sequences. A long open reading frame has been found in the MMTV LTR; polypeptides which may be its products have been observed after *in vitro* translations of viral RNAs (3, 4, 6, 7, 17).

In C3H mice free of exogenous MMTV, MMTV proviral genes are demonstrably active in producing frequently arising mammary tumors. The C3H/Sm (formerly C3H/StWi) subline spontaneously lost its exogenous MMTV infection in 1958 and became a low mammary cancer strain (1.6% of breeders had mammary tumors at 23 months) (29). These mice do not express their endogenous proviral MMTVs as virions. In C3H/Sm mice, MMTV proviral transcription occurs in normal and neoplastic mammary tissues (31, 32), but viral structural antigens are undetectable and spontaneous mammary tumorigenesis is rare. As a result of the absence of virion production, C3H/Sm MMTV sequence-containing RNAs represent only the result of transcription from proviral sequences, unobscured by the presence of

RNAs synthesized and encapsidated during vegetative growth.

Mammary tumors can be induced in this subline by a variety of agents. These mice are still highly susceptible to reinfection by exogenous MMTV (C3H) and are thus converted to a high mammary tumor line (100% of breeders had mammary tumors at 8 to 10 months). Our previous studies have shown that C3H/Sm virgin females develop mammary tumors as a result of treatment with 7,12-dimethyl(α)-benzanthracene (DMBA; 46%) or by chronic hormonal stimulation (18%) (33). MMTV-specific proteins were not found in any of these C3H/Sm tumors (33).

In contrast to the C3Hf/He and C3H/He sublines, which have three MMTV proviral genes (units I, II, and V) (2), C3H/Sm mice have four proviral genes (units I, II, III, and IV) (2, 8). C3H/Sm mice lack proviral unit V, the inheritance of which has been linked to endogenous MMTV expression (*Mtv-1*) and mammary tumorigenesis in C3Hf/He and DBA/ mice (21). In addition, the new proviral MMTV insertions characteristic of mammary tumorigenesis in the presence of MMTV infection (8, 23, 26) were undetectable in cellular DNAs from uninfected C3H/Sm tumors induced by a variety of agents (8).

This work describes analyses of RNAs from normal and neoplastic C3H/Sm mammary tissues. These experiments were undertaken in an effort to determine the pattern of C3H/Sm proviral RNA expression. In the course of these studies it became clear that typical MMTV transcripts are present in C3H/Sm mammary tissues. Examination of uninfected C3H/Sm mammary tumors revealed increased levels of MMTV RNAs and a novel 2.2-kilobase (kb) LTR (MMTV)-containing transcript which was present in all

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mammary tissues and whose concentration was increased in every C3H/Sm tumor RNA examined.

MATERIALS AND METHODS

Mice. The origin of the C3H/Sm strain has been reported previously (30, 32, 33). Mice were bred and maintained in a closed colony, given water and food ad libitum, and housed in a temperature- and light cycle-controlled room. DMBA (Calbiochem-Behring) was administered to C3H/Sm virgin mice in the presence or absence of chronic hormonal stimulation of the mammary gland by implantation of pituitary isografts (33). A 1-mg amount of DMBA dissolved in 0.2 ml of cottonseed oil was fed intragastrically once weekly until a dosage of 2 mg of DMBA was achieved (one animal received a single dose of 0.5 mg of DMBA). Tumor incidence and the distribution of MMTV *env* (gp52) and *gag* (p28) antigens in these tumors have been reported previously (33).

RNA isolation. Normal lactating mammary glands were obtained from multiparous mice 3 to 6 days postparturition. Further tissue information is given in Table 1. After tissues were dissected, they were immediately frozen in liquid nitrogen and stored at very low temperatures (less than -55°C) until RNA isolation.

RNAs were isolated from relatively small amounts of tissue by a much modified guanidinium hydrochloride procedure (D. Roop, personal communication). Tissues were homogenized (after a short time [60 to 90 s] for thawing) in 6 volumes (original volume) of 8 M guanidinium hydrochloride (Bethesda Research Laboratories)–0.2 M KCl, using a Polytron homogenizer. Immediately after homogenization, an equal volume of IAC (isoamyl alcohol-chloroform [1:24]) was added, and the mixture was briefly rehomogenized. The organic and aqueous phases were separated by centrifugation, and each phase was reextracted; the aqueous phase was extracted with IAC, and the organic phase was extracted with 8 M guanidinium hydrochloride–0.2 M KCl. After centrifugation, the aqueous phases were pooled, and one-half volume of 95% ethanol was added. Precipitation was overnight at -20°C . The precipitate was collected by centrifugation and resuspended in one-half original volume of 7 M guanidinium hydrochloride–0.02 M KCl. After resuspension, KCl was added to a final concentration of 0.2 M, and EDTA was added to 0.025 M. One-half volume of ethanol was added, and precipitation was for 1 h at -25°C . This step was repeated twice more, reducing volumes by one-half each time. The final precipitate was washed twice with 70% ethanol and finally resuspended in sterile water.

RNA gel electrophoresis and blotting. For RNA dot blots, RNAs were spotted onto nitrocellulose (Schleicher & Schuell, Inc.) by the method of Thomas (34). For Northern blots, RNAs were dissociated in a mixture of 16.7% formaldehyde, 41% formamide, and 1× MOPS (3-[N-morpholino]propanesulfonic acid) for 10 min at 60°C ; chilled; and loaded onto 1.5% agarose gels made up in 16.5% formaldehyde–1× MOPS (pH 7.0). Electrophoresis was in 1× MOPS (pH 7.0) for 6 or more h at 45 to 70 V. After electrophoresis, the gels were stained with 15 µg of ethidium bromide per ml in 1× MOPS for 15 min and then destained in 1× MOPS for 15 min for better visualization. Gels were photographed with a ruler placed directly on top of the agarose. Gels were blotted without further treatment. RNAs were transferred overnight to Gene Screen (New England Nuclear Corp.), using 1× SSPE (180 mM NaCl, 10 mM sodium phosphate buffer [pH 6.8], 1 mM EDTA) as the transfer buffer. The Gene Screen was briefly rinsed with 1× SSPE, air dried, and baked at $\geq 80^{\circ}\text{C}$ for 2 h. To remove residual ethidium

bromide, the Gene Screen was washed in formamide washing solution (96% formamide [FLUKA], 10 mM EDTA, 10 mM Tris [pH 7.4]) for 30 min at 60°C . The Gene Screen was subsequently washed with 2× SSPE and either used immediately or frozen.

Hybridization. Prehybridization was for at least 3 h at 41°C in a heat-sealed bag in a solution containing 50% F-D solution, 10% Denhardt solution, 5× SSPE, 1% sodium lauryl sulfate, and 250 µg of dissociated, sheared salmon sperm DNA per ml. (F-D is a solution of formamide [FLUKA] and dextran sulfate prepared as follows. Ten grams of finely powdered dextran sulfate was brought to a volume of 50 ml by the addition of formamide. This solution was heated to 60°C for 2 to 3 h to dissolve the dextran sulfate. After thorough mixing, it was filtered twice through a 0.45-µm Millex-HA filter [Millipore Corp.] and stored in the refrigerator. The use of the F-D solution appears to reduce background significantly [M. Harper, personal communication].) To start the hybridization, dissociated, labeled probe (1×10^5 to 50×10^5 cpm/ml) was thoroughly mixed with the prehybridization solution before allowing it to touch the Gene Screen. After hybridization for 16 to 17 h, the Gene Screen was washed twice with 2× SSPE–0.4% sodium lauryl sulfate at room temperature (total time, 90 min) and then twice with 0.1× SSPE at 60°C (total time, 30 min). The Gene Screen was briefly dried with paper towels, wrapped in Saran Wrap, and autoradiographed for a period of time ranging from 4 h to 5 days. To reuse the Gene Screen, it was washed with formamide washing solution (see above) at 60°C for 30 min to remove the hybridized probe, rinsed with 2× SSPE, and then prehybridized and hybridized as described above. One major advantage of blotting onto Gene Screen was that blots could be rehybridized a number of times, after gentle but thorough removal of the previous probe. This

TABLE 1. Description of tissues

Mouse tissue	MMTV virions	History
Normal		
C3H/Sm liver	–	Uninfected
C3H/Sm lactating mammary gland	–	Uninfected
C3H/Sm lactating mammary gland	+	Infected with MMTV (C3H)
C3Hf/He lactating mammary gland	+	Endogenous infection
Adenocarcinoma		
C3H/Sm	–	DMBA + hormone induced; first transplant generation; arose from C3S-6 HAN line (142F)
C3H/Sm	–	Hormone induced; arose from C3S-10 HAN line (142J)
C3H/Sm	–	Hormone induced; line 6666
C3H/Sm	–	Hormone induced; line 7771
C3H/Sm	–	Arose spontaneously; 6th and 8th tumor transplant generations; line C37-1
C3H/Sm	–	Induced by DMBA; primary tumor
C3H/Sm	+	Tumor induced by exogenous MMTV infection; arose from C3Sv-1 HAN line (145A)

allows direct relative quantitation of the amounts of RNA actually bound to the Gene Screen, using rDNA probes in the case of total RNAs. The use of a rehybridizable blot allows analysis with a series of probes, whose hybridization to transcripts of known sizes gives marker bands. Rehybridization of blots can be used to compare the degree of hybridization to different probes of related interest directly and to determine relative amounts or sizes or both of different messages. Because the same blot can be used, differences in electrophoresis conditions and amounts of RNA actually transferred are eliminated. All three of these applications were utilized in this work. There was no detectable loss of RNA on subsequent hybridizations; a test blot with Gene Screen was probed with five different probes without loss of RNA and without significant carry-over from preceding probes. (Experiments were planned such that hybridizations with probes homologous to less abundant transcripts preceded hybridizations with probes representative of more abundant transcripts.) Densitometric analysis of the amount of hybridization to the rDNA probe was used in quantitating directly the amount of RNA bound to the Gene Screen. The relative amounts of RNA determined in this manner were consistent with the amount of ethidium bromide staining of the gel.

Probes. Probes were derived from pBR322 plasmids containing inserts of MMTV (C3H) (clone p7-1A; gift of J. Majors and H. Varmus [9]), LTR (MMTV [C3H]; gift of J. Majors and H. Varmus), mouse rDNA (clone A22A2; a 6.6-kb insert from gtWESMr100 [10, 35] subcloned into pBR322, gift of B. Soellner-Webb), and mouse α -casein (clone pCM α 11; gift of J. Rosen [11]). The MMTV insert (removable from pBR322 by using *EcoRI*) was further digested with *PstI* (see summary shown schematically in Fig. 1). The LTR (MMTV) and casein inserts were removed by using *PstI*; the mouse rDNA insert was removed by using *EcoRI*. Inserts were isolated by electrophoresis, extraction from the agarose with NaClO₄, and then binding to glass-fiber filters. The *env* probe used to analyze MMTV fragments and to confirm hybridization was the kind gift of D. Wheeler.

Nick translation. Insert (50 to 100 ng) was labeled by using a standard nick-translation procedure. The labeled DNA was isolated from the reaction mix by spermine precipitation (14). Immediately after nick translation, the 60- μ l reaction mix was diluted to 500 μ l with water, and 40 μ l of LS buffer (10 mM KCl, 10 mM Tris [pH 7.4]) and 20 μ l of 100 mM spermine were added. The DNA was incubated for 15 min on ice and then was collected by a 10-min centrifugation in an Eppendorf centrifuge in a cold room. To remove the spermine, the labeled precipitates were mixed with a solution of 75% ethanol, 0.3 M sodium acetate, and 0.05 M magnesium acetate and precipitated for 1 h in ice. After a 5-min centrifugation, the precipitated, labeled insert was resuspended in 50 μ l of 20 mM EDTA. After counting a 5% sample, the required amount of probe (original volume) was dissociated by mixture with an equal volume of 100 mM NaOH. After neutralization of the probe with a 0.33 (original) volume of 500 mM NaH₂PO₄, the probe was used directly for hybridization as described above.

RESULTS

Previous evidence from liquid hybridization with a cDNA MMTV probe had indicated that the majority of MMTV sequences were represented in total RNAs from C3H/Sm mammary tissues (31, 32), although MMTV structural antigens were undetectable (32, 33). It was possible that the absence of MMTV proteins in C3H/Sm tissues was the result

FIG. 1. Anatomy of p7-1A MMTV clone. The top schematic indicates the generalized structure of a C3H exogenous virus. Its single *EcoRI* site is indicated by $\blacktriangle$; *PstI* restriction sites are indicated by ∇ . The bottom schematic shows the structure of the p7-1A clone. Fragments resulting from a double digestion with *EcoRI* and *PstI* are indicated by capital letters. The *env* fragment had a slight hybridization to LTR-containing fragment C. Fragments A and B were both used in the hybridizations as the *env* probe. Fragment D was used as the *gag-pol* probe.

of irregular or imperfect MMTV proviral gene transcripts. Ultimately, we wanted to determine the sizes of the C3H/Sm proviral MMTV transcripts by using Northern blots. The concentration of MMTV-specific sequences in total C3H/Sm RNAs has been shown to be low, about 0.00035% in retired breeder females and 0.00043% in DMBA-induced mammary tumors (32, 33). To determine whether sufficient MMTV sequences were present in total RNA to allow their detection when bound to nitrocellulose, RNAs from normal and neoplastic C3H/Sm tissues, uninfected or infected with exogenous MMTV (C3H), were spotted onto nitrocellulose and hybridized to a cloned 6.6-kb insert comprising most of the MMTV genome (Fig. 1; Table 2) (9).

MMTV transcripts were readily detectable in the RNAs from tumors and lactating mammary glands taken from C3H/Sm mice infected with exogenous MMTV (C3H) (Fig. 2A). MMTV transcripts were also detectable in an uninfected mammary tumor which had arisen from a C3S-6 hyperplastic alveolar nodule (HAN) line (142F) originally induced by treatment with DMBA in the presence of a pituitary isograft (Fig. 2A, DMBA+Pit). No MMTV-specific transcripts were found in liver RNA (Fig. 2A), even on autoradiographic overexposure. However, the same overexposure did reveal small amounts of MMTV RNA in the uninfected C3H/Sm lactating mammary gland and in a tumor which arose within a C3S-10 HAN line (142J) originally induced by a pituitary isograft (Fig. 2A, Pit). The extent of hybridization to the rDNA probe (Fig. 2D) indicated that smaller amounts of RNA from the last two preparations had been spotted onto the nitrocellulose, partially accounting for the low MMTV signal in these RNAs. Hybridization with the α -casein probe

TABLE 2. Description of the p7-1A clone

Fragment ^a	Assignment	Size (kb)	Hybridized to:		Adjacent to pBR322
			LTR	<i>env</i>	
A	<i>pol</i>	0.7	—	—	Yes
B	<i>env</i>	1.8	—	+	No
C	LTR	0.9	+	—	No
D	<i>gag-pol</i>	3.4	—	—	Yes

^a Fragments resulted from a double digestion with *EcoRI* and *PstI*.

FIG. 2. Assay for MMTV-specific sequences in RNAs from C3H/Sm lactating mammary glands and mammary tumors. RNAs were spotted in a volume of 5 μ l onto nitrocellulose. Each RNA was serially diluted by a factor of two. The initial spot of liver RNA contains ca. 6 μ g of RNA. RNAs were from liver, mammary tumors (MT), or lactating mammary glands (LMG); in some cases, RNAs were from animals infected with exogenous MMTV (MMTV+) or from uninfected tumors induced by DMBA plus pituitary isografts (DMBA+Pit) or pituitary isografts alone (Pit). Probes used in each hybridization are indicated above each set of dot blots. Autoradiography was for a period of time ranging from 4 h to 3 days.

provided a positive hybridization pattern for a different tissue-specific RNA expression (Fig. 2C). Low levels of MMTV transcripts were also detected in RNAs from C3H/Sm HAN lines grown in virgin mice, although no MMTV transcripts were detected in virgin mammary glands (data not shown).

By autoradiographically exposing the dot blots such that the relative amount of signal for the MMTV-infected C3H/Sm tumor RNA (MT, MMTV+) was similar for the hybridization to either the MMTV or LTR probe, an increase was observed in the ratio of LTR to MMTV hybridization in three of the RNA preparations (Fig. 2A and B). This increase in LTR hybridization was disproportionately larger with the RNA from the uninfected tumor induced by DMBA plus pituitary isograft (Fig. 2B, MT, DMBA+Pit) as compared with the RNA from MMTV-infected lactating mammary gland (Fig. 2B, LMG, MMTV+). The increase in the ratio of LTR to MMTV hybridization was especially striking since the 6.6 kb MMTV probe contains a partial LTR sequence ca. 1 kb in length. In further analyses of MMTV transcripts, we employed a fraction of the 6.6 kb MMTV probe which was primarily *env*, since this gene sequence would hybridize with both *env* and genomic messages. In this manner, enhanced expression of typical proviral RNAs could be distinguished from unique expression of transcripts containing LTR sequences.

Nature of transcripts in nontransformed tissues of C3H/Sm mice. Northern blots were used to ascertain the size distribution of MMTV transcripts in normal mammary gland RNAs from strain C3H/Sm. C3Hf/He lactating mammary gland RNA was used as a control indicative of complete proviral MMTV expression (20, 21). The C3Hf/He mouse has a moderately high rate of mammary tumorigenesis (50% at 18 months). Mammary tumorigenesis has been linked to the dominant expression of proviral unit V of C3Hf/He, which is genetically linked to the *Mtv-1* gene (2, 21). Lactating mammary gland from C3H/Sm mice infected with exoge-

nous MMTV was used as a control indicative of unimpaired exogenous MMTV RNA expression in C3H/Sm mammary tissues. MMTV transcripts in the RNAs of lactating mammary glands of uninfected C3H/Sm mice were found to be similar in size and relative proportions to those found in C3Hf/He mice or C3H/Sm mice infected with exogenous MMTV (C3H) (Fig. 3). The probe used to characterize MMTV transcripts was homologous to *env* but also contained a small amount of *pol* sequences (Fig. 1; Table 2). The

FIG. 3. Comparison of MMTV transcripts in RNAs from normal lactating mammary glands. RNAs were isolated, electrophoresed, blotted onto Gene Screen, and hybridized as described in the text. The hybridization probe was the MMTV *env* fragment (5×10^6 cpm/ml). RNAs were from lactating mammary glands from (A) C3H/Sm mice infected with exogenous MMTV, (B) C3Hf/He mice, and (C) uninfected C3H/Sm mice.

FIG. 4. Hybridization of an *env* (MMTV) probe to RNAs from lactating mammary glands and C3H/Sm mammary tumors. RNAs from various tissues were electrophoresed and hybridized as described in the text. Amounts of RNA bound in each lane were (A) 24 μ g in C3H/He lactating mammary gland; (B) 20 μ g in C3H/Sm lactating mammary gland; (C) 20 μ g in a tumor induced by DMBA plus pituitary isograft (142F); (D) 15 μ g in a tumor induced by pituitary isograft (142J); (E) 20 μ g in a tumor induced by pituitary isograft (6666); (F) 10 μ g in a tumor induced by pituitary isograft (7771); and (G) 11 μ g in a spontaneous tumor line (C37-1). (a) An autoradiograph of hybridization, using 5×10^6 cpm of *env* probe per ml, is shown. Markers are indicated by open arrows. (b) A histogram of these data, normalized for the amount of each RNA bound to the Gene Screen and compared to the relative amount of signal for the 4.4-kb transcript for the C3H/Sm lactating mammary gland (indicated by *), is shown.

strongest hybridization with this probe was to the 4.4-kb transcript, with a smaller amount of hybridization to the 8.1-kb transcript. An additional band could be distinguished at 2.2 kb but only after autoradiographic overexposure. There was no obvious defect in the size of MMTV transcripts or in their relative abundance in C3H/Sm mammary tissue which might explain the absence of MMTV antigens and virions in these tissues.

Increased amounts of MMTV transcripts observed in mammary tumors. The *env* probe was hybridized to RNAs from mammary tumors induced by several different carcinogens. In each case, we observed the same three transcripts, two major species at 8.1 and 4.4 kb and a minor band at 2.2 kb, that were present in normal mammary glands (Fig. 4A). As expected, the 4.4-kb transcript preferentially hybridized to the *env* probe. A 1.5- to 3.5-fold increase in the amounts of 4.4-kb MMTV transcript was observed in most of the mammary tumor RNAs over the levels found in the C3H/Sm lactating mammary gland RNA.

After the hybridizations of interest, the blot was hybridized to an rDNA probe to determine the relative amounts of each RNA preparation bound to the Gene Screen. The relative amounts of hybridization were determined, using a recording microdensitometer. The intention was to load 20 μ g of RNA per slot. As measured by rDNA hybridization, the actual amounts of RNA transferred to the Gene Screen varied, sometimes significantly. If we assume the amount of C3H/Sm lactating mammary gland RNA bound to be 20 μ g, then we may determine the relative amounts of each RNA bound from rDNA hybridization. In addition to this correction, we arbitrarily set a value of 1 for the autoradiographic signal for the hybridization of the *env* probe to the 4.4-kb transcript in the C3H/Sm lactating mammary gland RNA. The amounts of signal compared to RNA content were then

calculated for each RNA relative to C3H/Sm lactating mammary gland. The histogram prepared in this manner shows clearly the increase in the amount of MMTV transcripts in a series of C3H/Sm mammary tumors (Fig. 4B). There was little or no increase in the amount of 4.4-kb MMTV transcript over lactating mammary gland in the tumor derived from a C3S-10 HAN line induced by pituitary isograft (Fig. 4B, bar D), the spontaneously arisen, serially transplanted tumor line (Fig. 4B, bar G), a primary spontaneous tumor, or primary tumors induced by DMBA alone (data not shown). This last observation is consistent with previous results, using liquid hybridization, in which similar levels of MMTV RNAs were detected in RNAs from C3H/Sm mammary glands or primary mammary tumors induced by DMBA (32).

Enhanced concentrations of an LTR-containing transcript in mammary tumors. All three transcripts were observed when the same blot was rehybridized to the LTR probe (Fig. 5A). However, hybridization of the LTR probe to the 8.1-kb transcript was considerably diminished. The LTR probe hybridized strongly to the 2.2-kb transcript in both lactating mammary gland and mammary tumor RNAs compared with the hybridization of the *env* probe to this 2.2-kb transcript. A marked increase in the amount of the 2.2-kb LTR-containing transcript was observed in the RNAs from several mammary tumors (Fig. 5A). A histogram of these data was prepared as described above (Fig. 4B), except that the value of 1 was assigned to the amount of autoradiographic signal for the hybridization of the LTR probe to the 2.2-kb transcript in uninfected C3H/Sm lactating mammary gland RNA (Fig. 5B). The relative amounts of hybridization of the LTR or *env* probes to the same Northern blot are compared directly in Fig. 5C. In addition, RNAs from three independent primary mammary tumors induced by DMBA showed substantially higher amounts of LTR-containing transcripts (data not

FIG. 5. Hybridization of the LTR (MMTV) probe to RNAs from lactating mammary glands and C3H/Sm mammary tumors. RNAs are the same as those described in the legend for Fig. 4. The same Northern blot on Gene Screen was used for this hybridization. (a) An autoradiograph of hybridization, using 2×10^5 cpm of LTR probe per ml, is shown. Markers are indicated by open arrows. (b) A histogram of these data, normalized for the amount of each RNA bound to the Gene Screen and compared to the relative amount of signal for the 2.2-kb transcript for the C3H/Sm lactating mammary gland (indicated by *), is shown. (c) A direct comparison of autoradiographs from the sequential hybridization of *env* (Fig. 4A) and LTR (Fig. 5A) probes to these RNAs is shown.

shown). The most pronounced increase in the concentration of the 2.2-kb LTR-containing transcript was observed in RNA from a C3H/Sm mammary tumor line (C37-1) derived from a spontaneous tumor found in a very old (23-month) retired breeder (Fig. 5, bar G). This high concentration of the 2.2-kb LTR-containing transcript was reproducibly observed in subsequent Northern blots, using this and another RNA preparation from a different transplant generation of the C37-1 mammary tumor line (Fig. 6).

The slight hybridization to the 2.2-kb sequence by the *env* probe may be the result of the ability of the *env* probe to hybridize to a limited extent to LTR sequences (Fig. 1; Table 2). Alternatively, a short, poorly hybridizable stretch of *env* or *pol* sequences present in the probe may also be present in the LTR transcript. The 2.2-kb transcript was also not homologous to the *gag-pol* probe (Fig. 6). Therefore, it seems likely that the 2.2-kb sequence represents some part of the LTR and sequences either from parts of the MMTV genome not tested (this would include the leader sequence [37] and a large portion of the *gag* sequence) or from the mouse genome.

An unusual transcript was observed in this hybridization: a 5.2-kb transcript homologous to the *gag-pol* probe (Fig. 6). The expectation was that the *gag-pol* probe would hybridize to the 8.1-kb genomic transcript. Careful measurement indicated that this transcript was indeed slightly larger than the 4.4-kb transcript, although it was much smaller than the genomic transcript. LTR and *env* probes did not hybridize to this 5.2-kb transcript.

DISCUSSION

It is clear from the data that typical, tissue-specific gene regulation of at least one MMTV provirus occurs in C3H/Sm mice, although no MMTV proteins or virions are detectable. The deficiency of MMTV protein products could result from a flaw in C3H/Sm proviral MMTV sequences or from the action of two genes in C3H/Sm mice which appear to

suppress endogenous MMTV-induced mammary tumorigenesis and antigen production in crosses between C3H/Sm and C3Hf/He mice (*Smt-1* and *Smt-2*) (31).

Through the use of Northern blots, a more sensitive technique than hybridization in solution which also permits measurement of the transcripts, we found increased steady-state levels of MMTV transcripts in C3H/Sm mammary tumors induced by pituitary isografts. The increased amounts of MMTV transcripts observed in C3H/Sm mammary tumors is also consistent with the observation that some hypomethylation of MMTV proviral sequences occurs in non-virally induced C3H/Sm mammary tumors (8). This finding is also in agreement with a previous report which determined that increased levels of MMTV RNAs were found in lactating mammary glands of C3Hf/He mice bearing pituitary isografts, although this increase was not maintained in C3Hf/He mammary tumors induced by pituitary isografts (20). The increased levels of MMTV RNA in the C3Hf/He mammary glands, in which virions were also increased significantly, most probably represented vegetative reproduction of endogenous MMTV, as opposed to the nonencapsidated proviral transcripts observed in C3H/Sm mammary tissues.

The 5.2-kb *gag-pol* transcript observed in three different C3H/Sm tumor RNAs (Fig. 6) has not been previously described. This observation may be due to the type of probe used (Fig. 1; Table 2) and may be typical of viral expression or the transcript may represent a typical proviral message whose expression is repressed or masked by vegetative reproduction of the virus in other mouse strains.

The 2.2-kb transcript is unlikely to be the result of transcription from a previously undetected provirus, since the transcript was detected in lactating mammary gland RNAs from two different strains (C3H/Sm and C3Hf/He). Furthermore, the genomes of both strains have been carefully analyzed for proviral sequences (2, 8). The 2.2-kb transcript is unlikely to be the product of transcription from a newly acquired proviral sequence, since it is observed in

FIG. 6. Sequential hybridization of different MMTV specific sequences to RNAs from C3H/Sm mammary tumors. RNAs were electrophoresed and hybridized as described in the text. RNAs were isolated from (A) a tumor induced by DMBA, 12 µg of RNA; (B) a spontaneous tumor line C37-1, 13 µg of RNA; and (C) a tumor arising in C3S-6 HAN line (142F), 20 µg of RNA. Probes used are indicated under each slot; they represent the result of hybridization to 2.1×10^6 cpm of *env* per ml, 4.5×10^5 cpm of LTR per ml, and 3.1×10^6 cpm of *gag-pol* per ml. The *gag-pol* probe is fragment D shown in Fig. 1 and Table 2. The hybridized probe was removed with formamide washing solution before each new hybridization as described in the text.

normal mammary tissues. In addition, C3H/Sm DNAs from these tumor lines and from chemically induced mammary tumor types have been examined by Southern blot analysis and have been shown to lack any additional proviral sequences (8).

In vitro translation products of MMTV RNAs that may result from transcription from the open reading frame (*orf*) of the LTR (MMTV) have been described (3, 4), although such polypeptides have not been detected in MMTV-infected cells. Recently an LTR-containing RNA reported in certain BALB/c mouse mammary tumors by Wheeler and co-workers (37) has been proposed as a candidate *orf* transcript. It differs in several respects from the 2.2-kb sequence described here. First, the BALB/c LTR-containing transcript described by Wheeler and co-workers is 1.6 kb and thus smaller than that observed in C3H/Sm mammary tissues. This difference might be ascribed to variance in the methods used in determining molecular weights, but the C3H/Sm LTR-containing transcript described here is significantly larger than the RNA for α -casein (1.6 kb) (13) and is also slightly larger than 18S rRNA (2.1 kb). Second, *env* probes hybridize significantly better to the 1.6-kb (BALB/c) transcript than to the 2.2-kb (C3H/Sm) transcript. The difference in these two sets of observations is not due to the use of different *env* probes, because we have hybridized our RNAs to the same *env* probe used by Wheeler and co-workers, which they graciously provided. Using either *env* probe, we obtained relatively little hybridization to the 2.2-kb (C3H/Sm) transcript. Third, Wheeler and co-workers observed the 1.6-kb (BALB/c) transcript only in certain HANs and tumors (37). This transcript was consistently absent in RNAs from BALB/c tissues infected with exogenous MMTV. In contrast, the 2.2-kb (C3H/Sm) transcript was observed in every tissue examined, whether or not exogenous or endogenous MMTV was present.

The 2.2-kb LTR (MMTV)-containing transcript appears to have some characteristics in common with a 20S (ca. 2.2 kb) LTR (murine leukemia virus [MuLV])-containing transcript observed in C3H 10T_{1/2} mouse cells transformed by chemicals or radiation (18). The 20S LTR (MuLV), transformation-specific transcript had no homology to MuLV *env*, *gag*, or *pol* probes. Its hybridization to the LTR (MuLV) probe was strong but not so great that hybridization to the other MuLV-specific sequences was overshadowed. Interestingly, the 20S LTR (MuLV)-containing transcript did not hybridize to a probe representing the U3 region of the LTR (MuLV). Since the 20S transcript did hybridize to the whole LTR (MuLV) probe, the assumption would be that the short U5 region was responsible for the hybridization. It has been suggested that promotion could occur from the 3' LTR (12, 25) as well as from the 5' LTR. Since transcription through the U5 region (but not through *env* or *gag-pol* regions) would be initiated at the end of the U3 region of the 3' LTR and would continue into the mouse genome, it is conceivable that the 3' LTR could have a role in oncogenesis in these cells by promotion of a proximal cellular oncogene, similar to the role proposed for the LTR of the avian leukosis virus (22). Promotion at the 3' LTR may be the cause of the appearance of the 2.2-kb LTR (MMTV)-containing sequence. Alternatively, the 2.2-kb RNA may result from transcription of an open reading frame (3, 4, 6, 7, 17) in the LTR (MMTV), as has been suggested for the 1.6-kb (BALB/c) transcript (37). Finally, it may represent an abnormal splice into the MMTV genome which bypasses the sequences that we used as probes (*env*, a small part of *gag*, and the majority of *pol*).

A 20S LTR (MMTV) sequence has also been observed in

the C57BL subline (19, 36). Just as in the case of the C3H/Sm subline, this strain produces MMTV RNAs but no MMTV proteins and has a low rate of mammary tumorigenesis.

The finding of this sequence raises many questions. Is it transported to the cytoplasm, and if so, is it translatable into protein, either in vivo or in vitro? Are non-LTR sequences on this transcript derived from the MMTV or mouse genome? If from the mouse genome, is the transcript present as the result of readthrough or of a downstream promotion? Is the provirus from which this LTR is derived located in either the *int-1* or *int-2* regions? These portions of the mouse genome are enriched for MMTV insertion sites in tumors which arose after exogenous MMTV infection (23, 26).

Like the 4.4-kb transcript, the 2.2-kb RNA is absent in liver and virgin mammary gland and present in lactating mammary gland RNAs. Unlike the 4.4-kb RNA, the concentration of the LTR-containing transcript was increased in all of the tumors examined. In addition, RNAs from some tumors (DMBA-induced and spontaneous) had disproportionately larger increases in the amount of LTR transcript compared with the relative amount of 4.4-kb transcript. If increased concentration of the LTR transcript is related to transformation, this observation may indicate that its expression is more critical than other proviral messages for progression and maintenance of tumors of nonviral etiology.

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