Regulation of Endogenous Murine Mammary Tumor Virus Expression in C57BL Mouse Lactating Mammary Glands: Transcription of Functional mRNA with a Block at the Translational Level

AKHIL B. VAIDYA,* NANCY E. TARASCHI, STEPHEN L. TANCIN, AND CAROLE A. LONG

Department of Microbiology and Immunology, Hahnemann University, Philadelphia, Pennsylvania 19102

Received 20 January 1983/Accepted 9 March 1983

Expression of endogenous murine mammary tumor viruses (MuMTVs) in various mouse strains is regulated in different ways, and in the absence of exogenous MuMTV, this regulation influences the incidence of spontaneous mammary tumors. Two mouse strains with low mammary tumor incidence, BALB/c and C57BL, control endogenous MuMTV expression at different stages. Neither of the strains had any detectable MuMTV polypeptides in its lactating mammary glands (LMG). However, in C57BL LMG, substantial amounts of MuMTV RNA were present, whereas very little viral RNA was detected in BALB/c LMG. By determining MuMTV RNA levels in LMG of hybrids and backcrosses of BALB/c and C57BL mice, we found that there are three unlinked, independently segregating genetic loci in C57BL mice that are responsible for the presence of moderately high amounts of MuMTV RNA in LMG. The viral RNA in C57BL LMG was processed and transported to the cytoplasm where it was found to cosediment with EDTA-sensitive polysomes. No viral proteins were detected in run-off reactions that permit completion of nascent polypeptide synthesis with polysomes from C57BL LMG, and sensitive radioimmunoassays failed to detect any MuMTV proteins in these tissues. In contrast, MuMTV mRNA purified from C57BL LMG did direct the synthesis of both *gag* and *env* MuMTV polypeptides when added to a heterologous rabbit reticulocyte lysate cell-free translation system. We propose that MuMTV mRNA in C57BL LMG, for unknown reasons, is blocked at the translational level.

Murine mammary tumor viruses (MuMTVs), the type B retroviruses, form a unique group, their main pathological manifestation being a tissue-specific, hormonally influenced carcinoma (2, 20). MuMTVs can be transmitted either horizontally through milk or vertically through endogenous proviruses. Whereas milk-transmitted MuMTVs are responsible for the high mammary tumor incidence of most mouse strains, endogenous MuMTVs can also be highly tumorigenic when inoculated into young mice (19). All inbred strains of mice carry endogenous MuMTV genes, and acquisition of these proviruses seems to have occurred through relatively recent MuMTV infections of germ-line cells followed by segregation and stabilization (6).

Several aspects of MuMTVs make them interesting model systems for investigation. First, MuMTVs cause mammary carcinoma after a substantially long incubation period, and they do not carry an apparent oncogene as a part of their genome. The current theory of enhanced expression of a cellular oncogene mediated by a viral

promoter—put forward as an explanation for the oncogenesis of avian leukosis viruses (11, 23)—is being tested for its applicability to MuMTV-induced tumorigenesis (22). Second, the rate of MuMTV gene expression is increased 10- to 100-fold by glucocorticoids (for a review, see reference 25), providing an excellent model system to study steroid regulation of gene expression. The large terminal repeats of MuMTV DNA appear to carry sequences involved in glucocorticoid induction of gene expression (12, 14). Finally, endogenous MuMTV proviruses have integrated at many different chromosomal sites (6, 10, 16, 34), and the manner in which these genes are regulated varies among different mouse strains. Some of the endogenous MuMTVs are expressed as infectious and tumorigenic virions (19), whereas others remain unexpressed. Mechanisms underlying differential control over the various endogenous MuMTV proviruses can provide insights into the general strategies used by eucaryotic cells for regulation of gene expression.

Two strains of mice, BALB/c and C57BL, are particularly interesting in this regard. Their endogenous MuMTV proviruses are usually not expressed in the form of detectable virions; but, as first described by Varmus et al. (39), BALB/c mice have an extremely small amount of MuMTV RNA in their lactating mammary glands (LMG), whereas C57BL mice contain a substantial amount of MuMTV RNA in their LMG. It would appear that in BALB/c mice the control is at the transcriptional level, whereas in C57BL mice it is at some post-transcriptional stage. This paper describes our investigation of this phenomenon. There appear to be three unlinked genetic loci in C57BL mice involved in the moderately high transcription of MuMTV proviruses. MuMTV RNA in C57BL LMG is processed and transported to the cytoplasm, where it cosediments with EDTA-sensitive polyosomes. Cell-free translation in a heterologous system revealed that MuMTV RNA from C57BL LMG is capable of directing the synthesis of immunoprecipitable viral polypeptides. In vivo, however, no viral proteins are detectable by highly sensitive radioimmunoassays (RIAs). We propose that MuMTV mRNA in C57BL LMG may belong to a class of messengers that are potentially functional, but for unknown reasons, these are blocked at the translational level, even though they have been transported to the cytoplasm.

MATERIALS AND METHODS

Mice, tissues, and viruses. All of the mice were from our colony. C57BL mice were mated with BALB/c mice to obtain F_1 progeny, and these F_1 mice were mated with BALB/c mice to obtain the backcross progeny. The female parental, F_1 , and backcross mice were mated with their littermate males. Four to 6 days after parturition, their LMG and livers were removed and frozen at -70°C . For isolation of polyosomes, LMG from C57BL females were processed immediately after removal. We have empirically determined that in C57BL LMG, the highest levels of MuMTV RNA are observed around day 5 postpartum; hence, all LMG were removed at this period. Sucrose gradient-purified MuMTV (C3H) was obtained through the courtesy of the Division of Research Resources, Biological Carcinogenesis Branch, National Cancer Institute, Bethesda, Md.

Extraction of nucleic acids and cDNA synthesis. RNA was extracted from LMG by homogenization in guanidinium thiocyanate followed by either centrifugation through 5.7 M CsCl or differential precipitations with ethanol, as described previously by Chirgwin et al. (4). DNA was extracted from livers by procedures described elsewhere (16). MuMTV 70S RNA was purified by sodium dodecyl sulfate (SDS) disruption, proteinase-K digestion, phenol extraction, and rate-zonal centrifugation, as published previously (36, 37).

Radioactive DNA complementary to MuMTV RNA (cDNA) labeled with either ^3H or ^{32}P was synthesized by using avian myeloblastosis virus reverse transcrip-

tase in a reaction containing MuMTV RNA as a template and random oligonucleotide primers derived from DNase I-treated calf thymus DNA. The reaction conditions have been published previously (38). For some experiments, ^{32}P -labeled MuMTV probe was obtained by avian myeloblastosis viral DNA polymerase-directed DNA synthesis in a reaction containing cloned MuMTV DNA insert (from p7-1a, a generous gift of J. Majors) with calf thymus DNA oligonucleotides as primers. The plasmid p7-1a contains most of the MuMTV information, except for a deletion covering the so-called "poison sequence" present near the U5 region (Majors, personal communication).

Hybridizations. Liquid hybridization reactions were carried out with various amounts of purified RNA and ^3H -labeled single-stranded MuMTV cDNA in 10- μl volumes, as previously described (35, 37). Quantitations of MuMTV-specific RNA were obtained from the ratio of $C_{r,t_{1/2}}$ for the purified viral RNA and the $C_{r,t_{1/2}}$ for the sample RNA. $C_{r,t_{1/2}}$ is the product of the concentration of RNA and the time of incubation required for 50% of the cDNA to form hybrids.

For Southern blot analysis (31), about 5 μg of each of the DNA samples was digested to completion with *EcoRI* (Boehringer-Mannheim Corp.) under the reaction conditions recommended by the manufacturer, except that a 5- to 10-fold excess of the enzyme was used. Agarose gel electrophoresis, transfer to nitrocellulose sheets, hybridization with ^{32}P -labeled MuMTV cDNA, and radioautography were performed as described previously (16).

To locate MuMTV RNA in sucrose gradients of polyosomes (see below), dot hybridizations were performed as suggested by Thomas (33). Nitrocellulose sheets were soaked in $20\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and dried under a heat lamp. Samples (5 μl) of sucrose gradient fractions were spotted on the nitrocellulose sheet and dried under a lamp. After incubation at 80°C for 2 h in a vacuum oven, prehybridization, hybridization, washing, and radioautography were done as for the Southern blots (16).

Isolation of polyosomes and sucrose gradient analyses. Freshly excised LMG were finely minced and Dounce homogenized in cold buffer A (0.25 M sucrose, 20 mM Tris-hydrochloride [pH 7.6], 0.1 M ammonium acetate, 5 mM magnesium acetate, 1 mM dithiothreitol, 1 mg of heparin per ml). After centrifugation at 15,000 rpm for 15 min in an SW27.1 rotor, the supernatant was made 1% with respect to deoxycholate and layered over 7 ml each of 1.8 M sucrose and 1.0 M sucrose in buffer A. Polysomal pellets obtained by centrifugation at 25,000 rpm for 16 h in an SW27 rotor were suspended in buffer B (20 mM Tris-hydrochloride [pH 7.6], 0.25 M KCl, 5 mM magnesium acetate, 1 mg of heparin per ml). The suspension was divided into two parts, and EDTA was added to one half to a final concentration of 10 mM. The two samples were layered on 15 to 40% (wt/wt) sucrose gradients made either in buffer B (for polyosomes) or in buffer B with 10 mM EDTA but no Mg^{2+} (for dissociated polyosomes). After centrifugation for 3.5 h at 25,000 rpm in an SW27.1 rotor, fractions were collected from the bottom of the tubes while absorbance was monitored at 260 nm with a flow cell.

RIAs. The major glycoprotein of MuMTV, gp50, and the major core protein, p28, were purified by ion-

exchange and gel filtration chromatography as described previously (27). These proteins were labeled with ^{125}I , using the Bolton-Hunter reagent (3). Competition RIAs with monospecific sera were carried out as described earlier (16, 30). These assays could detect 1-ng quantities of the viral proteins.

Isolation of mRNAs and cell-free translation. About 50 mg of total RNA from LMG, purified as described above, was passed through an oligodeoxythymidylic acid-cellulose column in a buffer containing 0.5 M NaCl, 0.02 M Tris-hydrochloride (pH 7.4), 0.005 M EDTA, and 0.2% SDS and washed extensively with the same buffer. The bound RNA was eluted with 0.02 M Tris-hydrochloride with 0.2% SDS, and this eluted RNA was again passed through the oligodeoxythymidylic acid-cellulose column after adjusting to the loading buffer salt concentrations. Polyadenylate-containing RNA (about 500 μg) eluted from this second passage through oligodeoxythymidylic acid-cellulose was ethanol precipitated, suspended in water, heated at 68°C for 5 min, and loaded over a 36-ml gradient of 15 to 30% sucrose in 0.02 M Tris-hydrochloride (pH 7.4)–0.05 M NaCl–0.01 M EDTA–0.2% SDS. After centrifugation at 24,000 rpm in an SW27 rotor for 18 h, fractions were collected from the bottom of the tube. In a parallel gradient, non-polyadenylated (poly A[−]) RNA was centrifuged to obtain the positions of 28 and 18S rRNA. Fractions corresponding to 30 to 40S and 18 to 25S were pooled separately and diluted with water, and SDS was removed by precipitation with 0.2 M potassium acetate. The RNA in the supernatants was precipitated by addition of 2.5 volumes of ethanol in the presence of mouse tRNA.

Cell-free translations were carried out with a micrococcal nuclease-treated (24) rabbit reticulocyte lysate (Amersham Corp.), with [^{35}S]methionine (ca. 1,000 Ci/mmol) as the labeled amino acid. The reaction conditions were as specified by the manufacturer, and incubation was carried out at 30°C for 75 min. Protein synthesis was monitored by incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material. Immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and radiofluorography of in vitro-synthesized proteins was carried out by techniques described earlier (38).

Polysomal run-off reactions. For run-off reactions, polysomes were isolated in the manner described above, except that no heparin was used and the polysomal pellet was resuspended in buffer A minus

sucrose. The reaction conditions, adopted from Schreier and Staehelin (28), for a 100- μl reaction mixture were the following: 20 μl of polysomes (about 4 optical density units) in buffer A (minus sucrose); 20 μl of pH 5 enzymes from mouse liver (9); 2.5 μmol of HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], pH 7.6; 4 μmol of KCl; 1 μmol of creatin phosphate; 5 μg of creatin kinase; 9 μmol of ammonium acetate; 0.2 μmol of magnesium acetate; 0.6 μmol of dithiothreitol; 0.1 μmol of ATP; 40 nmol of GTP; 5 nmol each of 19 amino acids except methionine; and 100 μCi of [^{35}S]methionine (specific activity, $\approx 1,000$ Ci/mmol). Incubation was carried out at 30°C , and at 0, 15, and 60 min, 3- μl samples were withdrawn to monitor incorporation of [^{35}S]methionine into acid-precipitable material. After 60 min, the reaction was stopped by quick-freezing in liquid N₂, and immunoprecipitations with various antisera were carried out by published methods (38). [^{35}S]methionine-labeled polypeptides were displayed by SDS-polyacrylamide gel electrophoresis and visualized by radiofluorography.

RESULTS

Endogenous MuMTV expression and mammary tumor incidence in some mouse strains. Even though all inbred strains of mice carry endogenous MuMTV proviruses, expression of these genes in LMG varies considerably among different strains. The manner in which proviral gene expression is controlled correlates with the incidence of mammary tumors in several strains carrying only endogenous MuMTV (Table 1). Steady-state levels of MuMTV RNA in LMG from primiparous and multiparous females were measured by RNA-cDNA hybridization kinetics, whereas levels of the major MuMTV envelope protein, gp50, and core protein, p28, were determined by RIAs. BALB/c mice had an extremely small amount of MuMTV RNA and no detectable viral proteins in their LMG throughout their lives, and they did not develop spontaneous mammary tumors. C57BL LMG had substantial levels of MuMTV RNA at all lactations, but no viral proteins can be detected, and the tumor incidence was zero. On the other

TABLE 1. Expression of endogenous MuMTV in LMG and mammary tumor incidence in three mouse strains

Strain	MuMTV RNA (% of total) ^a		gp50 (ng/mg of total protein) ^b		p28 (ng/mg of total protein) ^b		Mammary tumor incidence
	Primiparous	Multiparous	Primiparous	Multiparous	Primiparous	Multiparous	
BALB/c	<0.00005	<0.00005	Undetectable	Undetectable	Undetectable	Undetectable	0
C57BL	0.002	0.002	Undetectable	Undetectable	Undetectable	Undetectable	0
C3HfC57BL	0.00005	0.002	5.0	>625	ND ^c	ND	40

^a Concentrations of MuMTV RNA were determined by kinetics of hybridization between MuMTV cDNA and total LMG RNA: % viral RNA = $(C_{\text{v}}t_{1/2} \text{ for MuMTV RNA} / C_{\text{t}}t_{1/2} \text{ for total LMG RNA}) \times 100$.

^b Concentrations of gp50 and p28 proteins were determined by group-specific radioimmunoassays (sensitivity of detection, 1 ng/mg).

^c ND, Not determined.

hand, the C3HfC57BL strain (a substrain of C3H in which milk-transmitted MuMTV has been removed by foster-nursing on C57BL mothers) had very small amounts of MuMTV RNA and proteins in LMG at the first parity. In multiparous mice, MuMTV expression was very high, and about 40% of the female C3HfC57BL mice in our mouse colony developed mammary tumors at an average age of about 19 months.

These observations suggest that the endogenous MuMTV genes are regulated in ways that eventually affect the incidence of mammary tumors. If in earlier pregnancies MuMTV expression is blocked at either the transcriptional or post-transcriptional stage (as in BALB/c and C57BL mice, respectively), subsequent virus replication and spread are prevented, which results in the low tumor incidence. In contrast, even a low level of MuMTV expression (as in C3HfC57BL) may permit virus spread. These new proviruses will probably be integrated at different chromosomal sites, and this may lead to the emergence of a transformed cell.

Genetic loci responsible for high MuMTV RNA levels in C57BL LMG. The regulation of MuMTV gene expression in C57BL mice appears unusual; hence, we have studied this strain in some detail. Using crosses between C57BL and BALB/c mice, we were interested in determining the dominance-recessiveness of MuMTV expression and the number of loci responsible for the relatively high levels of MuMTV RNA in C57BL LMG. From molecular hybridization kinetics, it was very easy to distinguish the levels of MuMTV RNA in BALB/c LMG from that in C57BL LMG (Fig. 1). We have denoted the levels of viral RNA in BALB/c mice as the L phenotype, whereas that in C57BL mice is called the H phenotype. We bred (BALB/c × C57BL)_F₁ hybrids and BALB/c × (C57BL × BALB/c) _F₁ backcrosses for segregation analysis of genes involved in the expression of MuMTV RNA in C57BL LMG. Hybrid and

FIG. 1. Representative profiles of kinetics of hybridization between MuMTV cDNA and total RNA from LMG of mice. Total RNAs from LMG of BALB/c (○), C57BL (●), (BALB/c × C57BL)_F₁ (▲), or two different BALB/c × (BALB/c × C57BL)_F₁ mice (◇, ◆) were hybridized with a limiting amount of single-stranded, ³H-labeled MuMTV cDNA. Percent cDNA hybridization at different C_t values was determined by digestion with S1 nuclease. Open symbols represent the L phenotype and closed symbols the H phenotype of MuMTV expression (see text).

backcross females were mated at maturity, and between days 4 to 6 postpartum, they were sacrificed for the removal of LMG and livers (for Southern blot analysis, see below). The amount of MuMTV RNA in LMG from individual mice was determined by the kinetics of hybridization between total LMG RNA and ³H-labeled MuMTV cDNA. All (C57BL × BALB/c)_F₁ and (BALB/c × C57BL)_F₁ hybrids and the H phenotype (Table 2), indicating the dominance of the H phenotype with no maternal influence over the expression of endogenous MuMTV in the hybrids. Out of 32 individual BALB/c × (C57BL × BALB/c)_F₁ backcross LMG that were tested for the level of MuMTV RNA, 27 had the H phenotype, whereas 5 had the L phenotype.

TABLE 2. MuMTV-specific RNA in LMG of hybrid and backcross progeny of BALB/c and C57BL mice^a

Strain	No. tested	No. of mice with given phenotype (%)	
		H ^b	L ^c
C57BL	3	3 (100)	0 (0)
BALB/c	3	0 (0)	3 (100)
(C57BL × BALB/c) _F ₁	3	3 (100)	0 (0)
(BALB/c × C57BL) _F ₁	3	3 (100)	0 (0)
BALB/c × (C57BL × BALB/c) _F ₁	32	27 (84.4)	5 (15.6)

^a Female mice were mated at maturity, and their LMG were removed on day 4 to 6 postpartum. Total RNA was extracted from LMG of individual mice, and the amount of MuMTV RNA was determined by molecular hybridization kinetics as described in the text.

^b H phenotype refers to those mice in which 0.001 to 0.004% of the total LMG RNA was MuMTV specific.

^c L phenotype refers to those mice in which <0.00005% of the total LMG RNA was MuMTV specific.

These results agree ($\chi^2 = 1.5$; $P < 0.05$) with the hypothesis that there are three unlinked, independently segregating loci in C57BL mice which are responsible for the H phenotype; the presence of any one of these loci would lead to moderately high levels of MuMTV RNA in these backcrosses.

LMG from all of the hybrid and backcross females were also examined by RIA for the presence of the major MuMTV glycoprotein, gp50, and the major internal protein, p28; no viral proteins were detectable at a sensitivity level of 1 ng of viral proteins. This indicates that the strategy used by C57BL LMG to control translation of MuMTV RNA is still active in hybrids and backcrosses, and it may be *cis* dominant. It is also possible that BALB/c mice may possess the same elements that block translation of endogenous MuMTV mRNA, and for that reason, segregation of translational control is not observed. It should be pointed out that both BALB/c and C57BL LMG will allow translation and virion synthesis when infected with exogenous MuMTV.

MuMTV proviruses of BALB/c and C57BL strains and their segregation in backcross mice. Since MuMTV proviruses derived from the C57BL parent will be segregating in BALB/c $\times$ (C57BL $\times$ BALB/c) F_1 mice, we examined DNA from livers of the same mice from which LMG were removed for the presence and absence of various MuMTV proviral elements. *Eco*RI cleaves once within the proviral DNA of most MuMTVs and has been used in conjunction with the Southern blot technique (31) to identify and classify endogenous MuMTV proviruses of vari-

ous inbred mouse strains (6, 34). *Eco*RI cleavage of BALB/c DNA reveals five hybridizing bands (Fig. 2A), and Cohen et al. (5) have examined these in some detail; we shall use the nomenclature suggested by Cohen and colleagues (5, 6, 34) to describe various proviral units. The 16.7-kilobase (kb) band has been identified as being derived from an incomplete provirus and is called unit I; the 8.5- and 6.7-kb bands are from a genomic-length provirus designated unit II; and the 10- and 7.8-kb bands are from another full-length provirus called unit III. In the C57BL strain, units II and III were observed, but unit I was absent; in addition, we observed 13- and 5.3-kb bands in C57BL mice (Fig. 2A), and these are not observed in the very closely related strains C57BL/6 and C57BL/10 (16). Apparently, during the divergence of the C57BL/6 and C57BL/10 strains from C57BL, this C57BL-specific provirus (which we have tentatively called unit B, for Black) was lost. To differentiate between units II and III derived from the C57BL parent and those from BALB/c mice, we carried out Southern blot analyses with several other restriction endonucleases but were unable to detect any differences (data not shown).

Our inability to determine the parental origin of two of the proviruses in backcross mice made it difficult to correlate MuMTV RNA levels with the presence or the absence of these two proviruses. However, all of the backcross mice with the L phenotype had units I, II, and III and lacked unit B (in some instances, the 16.7-kb unit I has not transferred well in these particular blots; however, it was observed in other gels as expected, since it was derived from the BALB/c

FIG. 2. Southern blot analyses of liver DNA from parental and backcross mice. DNA samples were digested with *Eco*RI, electrophoresed in agarose gel, transferred to nitrocellulose sheets, and hybridized with 32 P-labeled MuMTV cDNA. Representative radioautograms of the Southern blot hybridization for either parental mice (C57BL and BALB/c) or BALB/c $\times$ (C57BL $\times$ BALB/c) F_1 backcrosses with either L or H phenotypes are shown. kbp, kilobase pairs.

parent) (Fig. 2B). On the other hand, unit B was not always present in mice with the H phenotype (Fig. 2C). This is not surprising, since the C57BL/6 and C57BL/10 strains lacking unit B still express MuMTV RNA in their LMG (16). There are two possible ways to interpret these data. (i) There are two independently segregating *trans* regulatory elements in C57BL mice that turn on the transcription of endogenous unit II or III (or both) derived from either BALB/c or C57BL mice, whereas unit B is *cis* controlled; or, (ii) each one of the three proviruses of C57BL is *cis* controlled, and the presence of any one could lead to the H phenotype. We favor the second possibility as being the most direct, but cannot rule out the first interpretation without further experimentation.

Processing and location of MuMTV RNA in C57BL LMG. The amount of MuMTV RNA in C57BL LMG is substantial: about 0.002% of the total RNA is MuMTV-specific, numbering about 200 copies per cell, including the stromal cells; the actual amount in mammary epithelia may be even higher. But even with a highly sensitive RIA that can detect MuMTV proteins present at as low a level as 0.0001% of the total protein, no viral proteins could be detected in these tissues. Hence, we entertained the possibility that MuMTV RNA in C57BL LMG may be either not appropriately processed or not transported, or both. Most of the MuMTV RNA was found to be poly A⁺, as judged by its binding to oligodeoxythymidylic acid-cellulose. Poly A⁺ RNA from C57BL LMG was size separated by electrophoresis in a denaturing formaldehyde-agarose gel (15) and transferred to a nitrocellulose sheet (33). Hybridization with ³²P-labeled MuMTV DNA revealed three size classes of MuMTV RNA, corresponding to 35, 24, and 20S (Fig. 3). In MuMTV-producing cells, 35S RNA codes for *gag* and probably *gag-pol* precursor proteins, whereas 24S codes for the *env* precursor protein (8, 29). The constitutive expression of a 20S MuMTV RNA in C57BL LMG seen here has also been reported by Robertson and Varmus (26), but it is unclear what protein(s) that mRNA codes for, if any. These authors have also shown that the subgenomic size classes of MuMTV RNA in C57BL LMG contain sequences derived from the 5' end of the genomic RNA through a splicing event.

To localize MuMTV RNA within the cell, polysomes were isolated from C57BL LMG, as described above, and centrifuged in sucrose gradients in the presence of either 5 mM Mg²⁺ or 10 mM EDTA. The position of MuMTV RNA in gradient fractions was determined by spotting a 5- μ l sample of each fraction on a nitrocellulose sheet, followed by hybridization with ³²P-labeled MuMTV cDNA and radioautography. In

FIG. 3. Size classes of MuMTV mRNA in C57BL LMG. Poly A⁺ RNA from C57BL LMG was displayed by formaldehyde-agarose gel electrophoresis, transferred to a nitrocellulose sheet, and hybridized with a ³²P-labeled MuMTV probe prepared from a cloned viral DNA insert (p7-1a, a gift from J. Majors). Three size classes of MuMTV mRNA—35, 24, and 20S—are visible by radioautography.

the presence of Mg²⁺ MuMTV RNA cosedimented with very fast-sedimenting polysomal elements, but treatment with EDTA released these RNA molecules as the polysomes dissociated (Fig. 4). After treatment with EDTA, MuMTV RNA still appeared to be present in a complex that sedimented faster than 80S. In none of these fractions were MuMTV proteins detectable by RIA (not shown). From these experiments, we conclude that MuMTV RNA in C57BL LMG is poly A⁺, spliced, and transported to the cytoplasm where it copurifies with polysomes; yet, viral proteins are undetectable.

MuMTV mRNAs from C57BL LMG in a cell-free translation system. One obvious reason for our inability to detect MuMTV proteins in C57BL LMG could be that the viral RNA is somehow defective and incapable of directing viral protein synthesis. To examine this possibility, we fractionated poly A⁺ RNA from C57BL LMG on a sucrose gradient and pooled fractions corresponding to either 30 to 40S or 18 to 25S. RNA from these two regions of the gradient was added to an mRNA-dependent translation system (24) from rabbit reticulocyte lysates. Protein synthesis was carried out in the presence of [³⁵S]labeled methionine, and the products were

FIG. 4. MuMTV RNA in C57BL LMG cosedimenting with EDTA-sensitive polysomes. Polysomes from C57BL LMG were isolated and subjected to sucrose gradient analysis either in the presence of Mg^{2+} (upper panel) or EDTA (lower panel), as described in the text. Thirty-three fractions were collected from each of the gradients, and 5- μ l samples were spotted on a nitrocellulose sheet. The location of MuMTV RNA was determined by hybridization with ^{32}P -labeled MuMTV cDNA and radioautography.

immunoprecipitated with normal goat serum, goat anti-gp50, and goat anti-p28. After electrophoresis in an SDS-polyacrylamide gel, immunoprecipitated proteins were visualized by radiofluorography. RNA from the 30 to 40S region (Fig. 5A) directed synthesis of immunoprecipitable *gag* precursor polypeptides, but no *env* precursor was detectable. On the other hand, RNA from the 18 to 25S region (Fig. 5B) directed synthesis of immunoprecipitable *env* precursor protein. Synthesis of *env* precursor is probably directed by the 24S mRNA (Fig. 3) by analogy with studies by other workers (8, 29). Successful cell-free translation of endogenous MuMTV RNA from C57BL LMG was reproducible in several independent experiments. These experiments show that MuMTV RNA from C57BL LMG when presented in a purified form is able to initiate, elongate, and terminate viral protein synthesis even though it is not translated *in vivo*.

MuMTV protein synthesis by C57BL LMG polysomes under run-off conditions. One explanation for our inability to detect MuMTV proteins in C57BL LMG in spite of the presence of MuMTV RNA in association with polysomes could be the following: viral proteins are synthesized in LMG but are rapidly degraded by some highly specific proteases so as to make the detection of these polypeptides impossible. To test this possibility, we isolated polysomes in the absence of heparin from LMG of C57BL, as well as from C57BLfRIII mice. The latter substrain is derived by foster nursing C57BL newborns on RIII mothers, resulting in an exogenous

MuMTV infection; these mice have a very high mammary tumor incidence, and their LMG produce infectious MuMTV. Polysomes from the LMG of these two strains were incubated under run-off conditions (9, 28) to complete the synthesis of associated nascent polypeptides in the presence of [^{35}S]methionine. After 60 min, the run-off reactions were stopped and subjected to immunoprecipitation. The C57BLfRIII polysomes had more MuMTV RNA than the C57BL polysomes. By dot blot hybridization analysis, we estimated that the C57BLfRIII polysomes had about an eightfold higher concentration of MuMTV RNA than the C57BL polysomes. Hence, to compensate for these differences, 1×10^7 cpm of C57BLfRIII and 8×10^7 cpm of C57BL run-off proteins were used for immunoprecipitations. This contributed to the higher backgrounds observed in the C57BL lanes in Fig. 6. It can be seen in Fig. 6 that both *env* and *gag* precursor proteins were synthesized in run-off reactions of C57BLfRIII LMG polysomes but that these polypeptides were not detectable in C57BL LMG polysomal run-offs. To rule out the possibility that our inability to detect MuMTV proteins in C57BL run-offs was due to a generalized inefficiency of run-off reactions directed by C57BL polysomes, we carried out immunoprecipitations with anticasein antibody. Run-off products of both C57BLfRIII and C57BL polysomes (8×10^6 cpm of each) were subjected to immunoprecipitation by anticasein, and similar quantities of various casein polypeptides were immunoprecipitated from both of these polysomal run-off products (Fig. 6, lower

FIG. 5. MuMTV mRNA in C57BL LMG directs both *gag* and *env* polypeptide synthesis in rabbit reticulocyte lysate translation system. Poly A⁺ RNA from C57BL LMG was centrifuged in sucrose velocity gradients, and fractions corresponding to either 30 to 40S (A) or 18 to 25S (B) were pooled. After ethanol precipitation in the presence of mouse tRNA as a carrier, the fractionated RNA was dissolved in water and used to direct protein synthesis in a micrococcal nuclease-treated, mRNA-dependent rabbit reticulocyte lysate translation system containing [³⁵S]methionine. The reaction mixtures were subjected to immunoprecipitation by normal goat serum (NS), goat anti-gp50 antiserum (a gp50), or goat anti-p28 antiserum (a p28), and the precipitated labeled polypeptides were visualized by SDS-polyacrylamide gel electrophoresis followed by radiofluorography. Precursor polypeptides of MuMTV are identified. Increased background in immunoprecipitates in B is due to a large number of different mRNAs present in these fractions which code for proteins that are non-specifically precipitated.

lanes). These results suggest that MuMTV mRNAs cosedimenting with polysomes of C57BL LMG are not engaged in protein synthesis in spite of their ability to direct viral protein synthesis in a heterologous cell-free translation system.

DISCUSSION

We used classical genetic techniques in combination with molecular hybridization assays to define the genetic loci responsible for transcription of endogenous MuMTV proviruses in C57BL mice. C57BL mice have three unlinked, independently segregating genes involved in the synthesis of MuMTV RNA in LMG. Since two of the three MuMTV proviruses of C57BL are

indistinguishable by restriction endonuclease digestions from two of the BALB/c proviruses, we could not monitor the segregation of these proviruses in BALB/c × (C57BL × BALB/c)_{F1} backcrosses. The additional C57BL provirus, however, was not detected in any of the backcrosses with the L phenotype for MuMTV expression. Although our data do not rule out the possibility that genes responsible for MuMTV transcription in C57BL LMG may be *trans*-acting regulatory elements, we favor the interpretation that all three of the endogenous MuMTV proviruses of C57BL are transcriptionally active, that this phenomenon is *cis* dominant, and that these are the three segregating unlinked genes involved in MuMTV RNA expression. Since the endogenous proviruses of BALB/c mice are indistinguishable by restriction endonuclease digestions from two of the C57BL proviruses yet they are not transcribed, the BALB/c proviruses may be different in some undetected structural aspects from their C57BL counterparts so that they remain unexpressed. If our interpretation that all three proviruses are transcribed in C57BL LMG is correct, then each one of them must be expressed at the transcriptional level but blocked at the translational level. This block at the translational level is specific only for endogenous C57BL MuMTV proviruses, since expression of exogenously introduced MuMTVs from a variety of sources (RIII, GR, C3H/c57BL) in C57BL LMG is not controlled at the translational level; such infected C57BL mice secrete MuMTV particles in their milk and develop mammary tumors (19). To further investigate these phenomena, it will be essential to clone proviral DNA from BALB/c and C57BL strains which can then be used for detailed comparative analysis as well as for transfection experiments. Such studies are currently being done in our laboratory.

We have shown that the endogenous MuMTV RNA in C57BL LMG is synthesized, processed, and transported to the cytoplasm, where it remains in association with EDTA-sensitive polysomes. The association with polysomes is intriguing since viral proteins were not detected either *in vivo* or in polysomal run-off reactions. In the absence of any detectable nascent or completed MuMTV proteins, it is difficult to imagine that MuMTV RNA in C57BL LMG carries with it normally initiated and elongated 80S ribosomes. Perhaps these RNA molecules are present as ribonucleoprotein complexes, and, in the presence of a divalent cation, they happen to cosediment with polysomes.

In contrast with the lack of detectable translation of MuMTV RNA *in vivo*, these molecules are capable of initiating, elongating, and terminating synthesis of MuMTV *gag* and *env* pro-

FIG. 6. MuMTV polypeptides in run-off reactions of polysomes isolated from C57BL LMG. Polysomes from LMG of either C57BL or C57BLfRIII mice were isolated and incubated under conditions that permit run-off protein synthesis (9, 28) as described in the text. Reactions were carried out in the presence of [35 S]methionine, and 1×10^7 cpm of C57fRIII and 8×10^7 cpm of C57BL polysomal run-off products were immunoprecipitated with normal rabbit serum (nr), rabbit anti-gp50 antiserum ($\bar{a}$ 50), normal goat serum (ng), or goat anti-p28 antiserum ($\bar{a}$ 28). In addition, 8×10^6 cpm of the run-off products were subjected to immunoprecipitation with either normal rabbit (nr) or rabbit anticasein ($\bar{a}$ cas.) sera as depicted in the lower lanes. Immunoprecipitates were displayed by SDS-polyacrylamide gel electrophoresis and radiofluorography. The *env* and *gag* precursors are identified in the C57fRIII lanes, and the expected positions of these polypeptides are indicated by arrowheads in the C57BL lanes, whereas various casein polypeptides are identified in lower lanes. Radiofluorography was carried out for 5 days in the case of the upper lanes and for 20 h in the case of the lower lanes.

teins in a heterologous cell-free translation system. Apparently, the primary sequence of endogenous MuMTV RNA in C57BL LMG is not defective. In support of this, Nusse et al. (21) have observed that hormone treatment of C57BL females can lead to synthesis of MuMTV proteins in LMG, whereas untreated females do not have any detectable viral proteins. It has also been noticed that some C57BL females transiently express MuMTV proteins in LMG (18). Furthermore, Michalides et al. (17) found that in one out of three hormonally induced mammary tumors of C57BL mice examined, additional proviral DNA (presumably acquired from reinfection by an induced endogenous provirus) was detectable by the Southern blot technique. Taken together, all of these results suggest that C57BL LMG, by unknown mechanisms, exert a block over apparently functional endogenous MuMTV mRNA and that certain stimuli, like hormones, can release these mRNAs from such a control, leading to the synthesis of viral proteins and possibly infectious virions. We speculate that had translational control not been active in C57BL LMG, this mouse strain would have had a high incidence of mammary tumors.

Why would a cell go to the trouble of synthesizing a transcript, processing it, and transporting it to the cytoplasm, only to keep the mRNA unexpressed at the translational stage? It is conceivable that a cell may need, on occasion, to respond very quickly to a stimulus by synthesizing a new set of proteins and that the time required after the transmission of the signal for the synthesis, processing, and transport of mRNA coding for such proteins may be too long. In such cases, expression of these proteins can be controlled by having the mRNAs in a holding pattern in the cytoplasm ready to respond to the stimulus by releasing the mRNAs for translation. Of course, we cannot assign any such role for the endogenous MuMTV mRNA in C57BL LMG. We can only speculate that owing either to unique structural aspects of the mRNAs or to the position at which endogenous MuMTV proviruses are integrated, these mRNAs are inadvertently caught in the holding pattern.

Sequestering of mRNAs as a means for gene regulation is not a new concept. It has been known for some years now that RNA transcripts from maternal nuclei accumulate in sea urchin egg cytoplasm without being translated and that a burst of protein synthesis follows fertilization (7). Another situation in which mRNAs are blocked at the translational level exists in heat-shocked *Drosophila melanogaster* cells. When these cells are exposed to elevated temperature, transcription and translation of normal genes

ceases, with concomitant increases in transcription and translation of a set of genes coding for the so-called heat-shock proteins (1). Pre-heat-shock mRNAs, however, are not degraded and are still found in association with the polysomes (13). These mRNAs are translated efficiently in heterologous or homologous (from unshocked *Drosophila* cells) translation systems; a translation system derived from heat-shocked *Drosophila* cells does not permit pre-heat-shock mRNA translation, whereas mRNAs for heat-shock proteins are efficiently translated (13, 32). Mechanisms behind such discriminatory translational control are not understood. If such strategies do exist in eucaryotic cells to cope with drastic changes in environment such as elevated temperature, it is conceivable that these cells may use similar mechanisms to control gene expression under normal physiological conditions. Translational control over endogenous MuMTV gene expression in C57BL LMG may turn out to be an example of other genes which are controlled in a similar manner.

ACKNOWLEDGMENTS

We thank John E. Majors for providing MuMTV plasmid p7-1a, Tom Daly for purifying caseins and anticasin antibody, and Alice Smith for typing the manuscript.

This work was supported by Public Health Service grants CA-22413 (A.B.V.) and CA-22720 (C.A.L.) from the National Cancer Institute.

LITERATURE CITED

1. Ashburner, M., and J. J. Bonner. 1979. The induction of gene activity in *Drosophila* by heat shock. *Cell* 17:241-254.
2. Bentvelzen, P., and J. Hilgers. 1980. Murine mammary tumor virus, p. 311-355. In G. Klein (ed.), *Viral oncology*. Raven Press, New York.
3. Bolton, A. E., and W. M. Hunter. 1975. The labeling of proteins to high specific radioactivities by conjugation to a ¹²⁵I-containing acylating agent. *Biochem. J.* 133:529-538.
4. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active RNA from sources enriched in ribonuclease. *Biochemistry* 18:5294-5299.
5. Cohen, J. C., J. E. Majors, and H. E. Varmus. 1979. Organization of mouse mammary tumor virus-specific DNA endogenous to BALB/c mice. *J. Virol.* 32:483-496.
6. Cohen, J. C., and H. E. Varmus. 1979. Endogenous mammary tumour virus DNA varies among wild mice and segregates during inbreeding. *Nature (London)* 275:418-423.
7. Davidson, E. H. 1976. *Gene activity in early development*. Academic Press, Inc., New York.
8. Dudley, J. P., and H. E. Varmus. 1981. Purification and translation of murine mammary tumor virus mRNA's. *J. Virol.* 39:207-218.
9. Falvey, A. K., and T. Staehelin. 1970. Structure and function of mammalian ribosomes. I. Isolation and characterization of active liver ribosomal subunits. *J. Mol. Biol.* 53:1-19.
10. Groner, B., and N. E. Hynes. 1980. Number and location of mouse mammary tumor virus proviral DNA in mouse DNA of normal tissue and of mammary tumors. *J. Virol.* 33:1013-1025.
11. Hayward, W. S., B. G. Neel, and S. M. Astrin. 1981.

- Activation of a cellular *onc* gene by promoter insertion in ALV-induced lymphoid leukemia. *Nature* (London) **290**:475-480.
12. Huang, A. L., M. C. Ostrowski, J. Berard, and G. L. Hager. 1981. Glucocorticoid regulation of the Ha-MuSV p21 gene conferred by sequences from mouse mammary tumor virus. *Cell* **27**:245-255.
 13. Krüger, C., and B.-J. Benecke. 1981. In vitro translation of *Drosophila* heat-shock and non-heat-shock mRNAs in heterologous and homologous cell-free systems. *Cell* **23**:595-603.
 14. Lee, F., R. Mulligan, P. Berg, and G. Ringold. 1981. Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumor virus chimeric plasmids. *Nature* (London) **294**:228-232.
 15. Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker. 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry* **16**:4743-4751.
 16. Long, C. A., U. J. Dumaswala, S. L. Tancin, and A. B. Vaidya. 1980. Organization and expression of endogenous murine mammary tumor virus genes in mice congenic at the H-2 complex. *Virology* **103**:167-177.
 17. Michalides, R., E. Wagenaar, B. Groner, and N. E. Hynes. 1981. Mammary tumor virus proviral DNA in normal murine tissue and non-virally induced mammary tumors. *J. Virol.* **39**:367-376.
 18. Moore, D. H., and J. A. Holben. 1978. Observations on the question of horizontal transmission of mouse mammary tumor virus. *Cancer Res.* **38**:2455-2457.
 19. Moore, D. H., J. A. Holben, and J. Charney. 1976. Biologic characteristics of some mouse mammary tumor viruses. *J. Natl. Cancer Inst.* **57**:889-896.
 20. Moore, D. H., C. A. Long, A. B. Vaidya, J. B. Sheffield, A. S. Dion, and E. Y. Lasfargues. 1979. Mammary tumor viruses, p. 347-418. In G. Klein and S. Weinhouse (ed.), *Advances in cancer research*, vol. 29. Academic Press, Inc., New York.
 21. Nüsse, R., R. Michalides, L. M. Boot, and G. Röpcke. 1980. Quantification of mouse mammary tumor virus structural proteins in hormone-induced mammary tumors of low mammary tumor mouse strains. *Int. J. Cancer* **25**:377-383.
 22. Nüsse, R., and H. E. Varmus. 1982. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* **31**:99-109.
 23. Payne, G. S., J. M. Bishop, and H. E. Varmus. 1982. Multiple arrangements of viral DNA and an activated host oncogene in bursal lymphomas. *Nature* (London) **295**:209-214.
 24. Pelham, H. R. B., and R. J. Jackson. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* **67**:247-256.
 25. Ringold, G. M. 1979. Glucocorticoid regulation of mouse mammary tumor virus gene expression. *Biochim. Biophys. Acta* **560**:487-508.
 26. Robertson, D. L., and H. E. Varmus. 1981. Dexamethasone induction of the intracellular RNAs of mouse mammary tumor virus. *J. Virol.* **40**:673-682.
 27. Sarkar, N. H., and A. S. Dion. 1975. Polypeptides of the mouse mammary tumor virus. I. Characterization of two group-specific antigens. *Virology* **64**:471-491.
 28. Schreier, M. H., and T. Staehelin. Initiation of mammalian protein synthesis: the importance of ribosome and initiation factor quality for the efficiency of in vitro systems. *J. Mol. Biol.* **73**:329-349.
 29. Sen, G. C., S. W. Smith, S. L. Marcus, and N. H. Sarkar. 1979. Identification of the messenger RNAs coding for the *gag* and *env* gene products of the murine mammary tumor virus. *Proc. Natl. Acad. Sci. U.S.A.* **76**:1736-1740.
 30. Sheffield, J. B., T. Daly, A. S. Dion, and N. Taraschi. 1977. Procedures for radioimmunoassay of the mouse mammary tumor virus. *Cancer Res.* **37**:1480-1485.
 31. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503-517.
 32. Storti, R. V., M. P. Scott, A. Rich, and M. C. Pardue. 1980. Translational control of protein synthesis in response to heat shock in *D. melanogaster* cells. *Cell* **22**:825-834.
 33. Thomas, P. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. U.S.A.* **77**:5201-5205.
 34. Traina, V. L., B. A. Taylor, and J. C. Cohen. 1981. Genetic mapping of endogenous mouse mammary tumor viruses: locus characterization, segregation, and chromosomal distribution. *J. Virol.* **40**:735-744.
 35. Vaidya, A. B., M. M. Black, A. S. Dion, and D. H. Moore. 1974. Homology between human breast tumour RNA and mouse mammary tumour virus genome. *Nature* (London) **249**:565-567.
 36. Vaidya, A. B., E. Y. Lasfargues, G. Heubel, J. C. Lasfargues, and D. H. Moore. 1976. Murine mammary tumor virus: characterization of infection of nonmurine cells. *J. Virol.* **18**:911-917.
 37. Vaidya, A. B., E. Y. Lasfargues, J. B. Sheffield, and W. G. Coutinho. 1978. Murine mammary tumor virus (MuMTV) infection of an epithelial cell line established from C57BL/6 mouse mammary glands. *Virology* **90**:12-22.
 38. Vaidya, A. B., C. A. Long, J. B. Sheffield, A. Tamura, and H. Tanaka. 1980. Murine mammary tumor virus deficient in the major glycoprotein: biochemical and biological studies on virions produced by a lymphoma cell line. *Virology* **104**:279-293.
 39. Varmus, H. E., N. Quintrell, E. Medeiros, J. M. Bishop, R. C. Nowinski, and N. H. Sarkar. 1973. Transcription of mouse mammary tumor virus genes in tissues from high and low tumor incidence mouse strains. *J. Mol. Biol.* **79**:663-679.