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Purification and Translation of Murine Mammary Tumor Virus mRNA's

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We have studied the functions of the intracellular RNAs of mouse mammary tumor virus (MMTV) by purification and translation in vitro. Two major size classes of MMTV RNA, 35S and 24S RNA, were isolated from MMTV-infected rat (XC) cells and cultured mammary tumor cells by preparative hybridization of whole cell or polyadenylated RNA to cloned MMTV DNA covalently bound to chemically activated paper disks (diazobenzyloxymethyl paper). Genomic-length (35S) RNA was prepared free of 24S RNA by annealing to a 4-kilobase *Pst*I fragment apparently deficient in sequences homologous to 24S RNA; the 24S species was separated from 35S RNA by rate zonal sedimentation in sucrose gradients. Experiments using [^{3}H]uridine-labeled cellular RNA indicated that the preparative annealing method was highly specific and capable of effecting a 300-fold enrichment for viral RNA; the recovered RNA appeared to be intact under denaturing conditions and directed synthesis of full-length *gag* and *env* polypeptides in vitro. The products of in vitro translation were identified by gel mobility, immunoprecipitation tests with antisera against *gag* and *env* products, and partial digestion with *Staphylococcus* V8 protease. The 35S RNA species directed synthesis of several *gag*-related polypeptides, including three previously reported in extracts of infected cells; 24S RNA directed synthesis of two polypeptides closely related to *env* proteins from infected cells. Therefore, 35S RNA includes mRNA's for *gag* and *gag-pol*, whereas 24S RNA is the mRNA for *env*. These results help establish the position of *env* on the physical map of the MMTV genome and bear upon the coding potential of the genome.

The unusual features of the mouse mammary tumor virus (MMTV), its regulation by glucocorticoid hormones (19, 42) and its capacity to induce mammary carcinomas (19), command a detailed understanding of the organization and mode of expression of the viral genome. However, compared with other retroviruses, MMTV is difficult to propagate in culture, and there is no convenient biological assay for infection of cultured cells. Since the definition of genes by classical methods has not been possible, investigators have relied principally upon more recent biochemical methods to obtain a tentative picture of the viral genome and of the strategy for its expression. Evidence to date, gathered from analysis of MMTV proteins synthesized in vivo and in vitro (5, 7, 26, 41) and of intracellular species of viral RNA (16, 38, 43), conforms to the view that the genome of MMTV is similar to that of other replication-competent retroviruses, with three essential genes, *gag*, *pol*, and *env*, positioned from 5′ to 3′ on the viral 35S RNA subunit. Furthermore, the mechanism of MMTV gene expression appears to resemble that of other retroviruses, with the *gag* and *pol* genes being expressed via cleavable polyproteins translated from messenger RNAs of subunit size and the *env* gene being expressed via a polyprotein, later modified, translated from a spliced, subgenomic mRNA (24S RNA). Several additional RNA species have been described in MMTV-producing cells (16, 38, 43), but no function has been assigned to them.

To test rigorously these prevailing views in the absence of genetic analysis, we have purified intracellular MMTV RNAs by preparative hybridization techniques and subjected them to functional analysis by translation in vitro. By using cloned MMTV DNA linked to chemically activated diazobenzyloxymethyl (DBM) paper disks (1, 48, 55) to select virus-specific RNA, the 35S and 24S species, in apparently purified and intact form, were shown to program the synthesis of *gag*-related and *env*-related polypeptides, respectively, in an in vitro translational system. These results support the current model for organization and expression of the MMTV ge-

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nome. In addition, by using subgenomic restriction fragments of MMTV DNA, we have placed constraints upon the position of *env* within the viral genome. Our inability to find other virus-specific RNAs in this study argues against the existence of heretofore unidentified genes (e.g., for nonstructural proteins) in the MMTV genome.

MATERIALS AND METHODS

Cell lines and virus. The rat cell line XC, derived from a tumor induced in a Wistar rat by the Prague strain of Rous sarcoma virus (49) was obtained from J. Levy (University of California, San Francisco). MMTV-infected XC cells (960) and a derivative clonal line (D108) were obtained from D. Robertson (University of California, San Francisco). C3H (line H-1) (42) and BALB/cfC3H (39) mouse mammary tumor cells were kindly provided by J. S. Butel (Baylor College of Medicine) and N. H. Sarkar (Sloan-Kettering Cancer Center), respectively. Cell lines were propagated at 37°C in Dulbecco minimal essential medium (GIBCO Laboratories) containing 10% fetal calf serum or 2% fetal calf serum and 8% calf serum (GIBCO or Irving Scientific Co.), 50 μg of gentamicin (Schering Corp.) per ml, and 125 ng of amphotericin B (E. R. Squibb & Sons) per ml.

XC cells were infected with MMTV as described previously for mink lung cells (38). Virus used for infections and virion RNA preparations was purified from the C3H mouse mammary tumor cell line Mm5mt/cl by the Frederick Cancer Research Center (12).

Isolation of RNA. Total virion RNA was prepared as described previously (9), using a sodium dodecyl sulfate (SDS)-proteinase K digestion, followed by extraction with phenol-chloroform. MMTV 70S RNA was isolated by sedimentation in 15 to 30% (wt/wt) sucrose gradients containing 10 mM Tris-hydrochloride, pH 7.5, 0.1 M NaCl, 1 mM EDTA, and 0.2% SDS. Gradients were centrifuged at 40,000 rpm for 3 h at 20°C in an SW41 rotor. The 70S fraction was subsequently heat-denatured in 1 mM EDTA at 100°C for 1 to 2 min and layered onto a 15 to 30% (wt/wt) SW41 gradient containing 10 mM Tris-hydrochloride, pH 7.5, and 10 mM EDTA. Gradients were centrifuged at 35,000 rpm for 17 h at 4°C, and the RNA sedimenting at values greater than 16S (relative to rRNA markers) was pooled and used for complementary DNA (cDNA) synthesis and in vitro translations.

Isolation of total cellular RNA and oligodeoxythymidylate [oligo(dT)]-cellulose chromatography were performed as described previously (9), with the following exceptions. Total RNA was heated to 80 to 90°C in 1 mM EDTA before it was loaded onto oligo(dT)-cellulose columns; the columns were then washed with three sequential Tris buffers containing either 0.5 M, 0.1 M, or no KCl, in addition to 1 mM EDTA.

Isolation and purification of cloned DNAs. Unintegrated MMTV DNA from XC cells infected with the C3H strain of MMTV was isolated by the Hirt procedure as described previously (44), cleaved with the restriction enzyme *Pst*I, and cloned into the single *Pst*I site of pBR322 (J. Majors, unpublished data). The p7-1A and p2-1A clones were derived by cleaving MMTV supercoiled DNA at the single *Eco*RI site and cloning in the vector λgtWESλB. DNA from recombinant phage was extracted, cleaved with *Eco*RI, and subcloned into the RI site of pBR322 to generate p7-1A and p2-1A. By endonuclease restriction mapping, p7-1A appeared to lack sequences corresponding to a portion of the *Pst*I-C and D fragments and most of the *Pst*I-E fragment as described on a physical map of unintegrated MMTV DNA by Shank et al. (44) (Fig. 1). p2-1A has a more extensive deletion than p7-1A, including part of the *Pst*I-A fragment.

Recombinant plasmids grown in *Escherichia coli* HB101 were amplified in the presence of 170 μg of chloramphenicol (Sigma Chemical Co.) per ml, and the DNA was purified as described previously (18). After chromatography on Bio-Rad A50, the excluded material was pooled, and the supercoiled DNA was recovered after equilibrium sedimentation on CsCl-PI_2 gradients (37).

Synthesis of cDNA. MMTV cDNA's which represent the 3′ terminus of viral RNA ($cDNA_{3'}$) or the entire genome ($cDNA_{rep}$) were synthesized in reactions using MMTV virion RNA and oligo$(dT)_{12-18}$ and calf thymus oligonucleotides as primers, respectively, as described previously (38, 44).

Labeled DNAs complementary to cloned MMTV DNA were synthesized as follows. Supercoiled MMTV DNA cloned in pBR322 was digested with the restriction enzyme *Pst*I (obtained from J. Majors) in 20 mM Tris-hydrochloride, pH 7.5, 10 mM $MgCl_2$, and 50 mM $(NH_4)_2SO_4$ at 37°C for 1 h, extracted twice with phenol-chloroform, and ethanol precipitated. The DNA was diluted to 100 μg/ml, heated to 100°C for 5

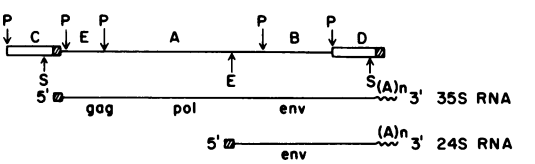

FIG. 1. *Physical and genetic maps of MMTV DNA and RNA. The positions of restriction endonuclease sites for Pst (P), EcoRI (E), and SacI (S) on the linear, unintegrated form of DNA from the C3H strain of MMTV are illustrated in relation to the probable positions of the accepted coding domains in the MMTV genome, gag, pol, and env. Sequences from the 3′ end (open boxes) and the 5′ end (cross-hatched boxes) of viral RNA are present at both ends of viral DNA in a structure referred to as the long terminal repeat. The major species of MMTV RNA, 35S and 24S RNA, are drawn below the DNA, with the box at the 5′ end of both RNAs, indicative of sequences from the 5′ terminus of viral RNA joined to sequences on the 5′ side of the env gene in 24S RNA, as suggested by work presented here and elsewhere (38). Hybridization reagents used in this paper include $cDNA_{rep}$, transcribed randomly from genomic (35S) RNA; cloned PstI fragments A (4.0 kb), B (1.8 kb), and C (1.3 kb); and cloned incomplete MMTV DNA molecules which include all of the DNA on the right side of the map from at least the EcoRI site to the SacI site (p7-1A and p2-1A; J. Majors, unpublished data).*

min, and quickly cooled on ice. Two hundred nanograms of this DNA was added to a mixture containing 250 μCi of [α-^{32}P]dCTP (300 Ci/mmol; New England Nuclear Corp.), 120 μM each of dGTP, dATP, and dTTP, 100 μg of calf thymus oligonucleotide primers, 62.5 mM Tris-hydrochloride, pH 8.1, 10 mM $MgCl_2$, 50 mM KCl, 2.5 mM dithiothreitol, and 200 U of avian myeloblastosis virus DNA polymerase (provided by J. Beard, Life Sciences, Inc.) per ml in a volume of 200 μl. The reaction was incubated at 37°C for 1 h, adjusted to 20 mM EDTA, 1% SDS, and 100 μg of proteinase K per ml, and the incubation was continued for 30 min. Approximately 40 μg of single-stranded salmon sperm DNA was added, and the mixture was extracted with phenol-chloroform. Subsequently, the reaction was adjusted to 0.3 N NaOH, incubated for 1 h at 50°C, and neutralized with 1.2 N HCl. The unincorporated radioisotope was removed by G-50 column chromatography. Specific activities were approximately 10^8 to 2×10^8 cpm/μg.

Agarose gel electrophoresis of RNA and transfer to diazotized paper. Total cellular RNA was subjected to electrophoresis on 1.2% agarose gels containing 10 mM methylmercury hydroxide (Alfa Division, Ventron Chemical) as described previously (2, 38). Preparation and activation of DBM paper and alkali treatment of methylmercury gels were performed as described by Alwine et al. (1). After RNA transfer, DBM paper was prehybridized for at least 5 h at 41°C in a solution containing 50% formamide, 0.1% polyvinyl pyrrolidone (PVP-360; Sigma Chemical Co.), 0.1% Ficoll (Sigma), 0.1% bovine serum albumin, 0.1% SDS, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, pH 7.4, 1 mg of yeast RNA per ml, 0.45 M NaCl, 45 mM trisodium citrate, 100 μg of single-stranded salmon sperm DNA per ml, and 1% glycine. The RNA-DBM paper was then hybridized to 2×10^6 to 5×10^6 cpm of [^{32}P]cDNA in the above solution lacking glycine with the addition of 10% (wt/vol) dextran sulfate (Pharmacia Fine Chemicals, Inc.) (52) for 6 to 18 h at 41°C. Hybridization was terminated by washing the paper several times in 0.1× SSC (0.15 mM NaCl and 1.5 mM trisodium citrate) at room temperature and in 0.1× SSC and 0.1% SDS for 1 h at 53°C. The paper was then dried and subjected to autoradiography as described by Robertson and Varmus (38).

Preparation of DNA-DBM paper disks. Purification of viral mRNA's was achieved by using DBM paper essentially as described by Alwine et al. (1) and modified by others (35, 48, 55). Purified supercoiled recombinant plasmids were linearized with an appropriate restriction endonuclease (either *Pst*I or *Eco*RI), extracted with phenol-chloroform, and ethanol precipitated. In the case of p7-1A, plasmid DNA was separated from MMTV sequences by centrifugation on 10 to 40% (wt/vol) sucrose gradients containing 10 mM Tris-hydrochloride, pH 7.5, 0.1 M NaCl, and 1 mM EDTA in a Beckman SW27 rotor at 25,000 rpm at 20°C for 30 h. The DNA preparations (including approximately 10,000 to 20,000 cpm of MMTV [^{32}P] $cDNA_{rep}$) were resuspended in 25 mM sodium phosphate buffer, pH 6.0, at a concentration of 5 mg/ml and heated for 2 min at 80 to 100°C. Subsequently, the DNA was cooled on ice, diluted to 1 mg/ml with dimethyl sulfoxide, and added to 1-cm^2 activated DBM paper disks. The disks were allowed to stand overnight at room temperature and then washed with 1 liter of water per cm^2, four times with 0.4 N NaOH (1 ml/disk) at 37°C for 10 min, and again with three changes of water. The paper disks were subsequently monitored for DNA binding by Cerenkov counting; the percentage of DNA bound ranged between 15 and 25% of the cDNA added, and 6 to 11 μg of MMTV DNA was bound to each disk. Disks were stored at 4°C in 50% formamide containing 20 mM 1,4-piperazine diethanesulfonic acid (PIPES) buffer, pH 6.4. Disks have been used for up to 17 consecutive preparative annealings without apparent deterioration.

DNA-filter hybridizations. Approximately 10 to 50 μg of polyadenylic acid [poly(A)]-containing RNA was suspended in 50 μl of a buffer containing 50% deionized formamide, 20 mM PIPES buffer, pH 6.4, 0.4 M NaCl, 5 mM EDTA, 0.4% SDS, 250 μg of polyriboadenylic acid [poly(rA)], 2× Denhardt's buffer (6) (diethylpyrocarbonate treated), and 170 μg of chicken liver tRNA and subsequently hybridized to DBM-DNA-containing disks at 41°C for 5 to 10 h. DNA disks were briefly alkali-treated with 0.4 N NaOH and then neutralized with 20 mM PIPES buffer, pH 6.4, before hybridization.

Hybridization was terminated by washing the disks four times in 0.1× SSC at room temperature and five times in 0.1× SSC and 0.1% SDS for 5 min each at 53°C. Purified RNA was eluted in 100 μl of buffer containing 95% deionized formamide, 10 mM HEPES, pH 7.4, 3 mM EDTA, and 0.1% SDS at 68°C for 1 to 2 min. The elution was repeated once with 50 μl of formamide buffer; the eluates were pooled and ethanol precipitated.

RNA labeling. Cell cultures were exposed to 10^{-5} M dexamethasone in growth medium for 24 to 48 h, to labeling medium (growth medium containing 10^{-5} M dexamethasone and 2% dialyzed fetal calf serum as a substitute for other sera) for 12 h, and to labeling medium containing 0.5 mCi of [^{3}H]uridine (25 to 30 Ci/mmol) per ml for 24 h. Cells were then extracted for RNA as described above.

In vitro translation and polyacrylamide gel electrophoresis. Translations were performed as described by Weiss et al. (53). Reactions contained poly(A)-containing RNA at concentrations of 50 μg/ml or less, 75 mM KCl, 1.7 mM $MgCl_2$, 50 μM amino acids except methionine, 20 mM HEPES, pH 7.8, 10 mM creatine phosphate, 1 mM ATP, 2 mM GTP, 0.3 mM spermidine, 50 μg of creatine phosphokinase per ml, approximately 500 μCi of [^{35}S]methionine (500 to 1,200 Ci/mmol) per ml, and 76% micrococcal nuclease-treated rabbit reticulocyte lysates in a total volume of 20 μl. Reticulocyte lysates were nuclease-treated by the method of Pelham and Jackson (33). RNA samples purified by hybridization to DNA-DBM paper were washed once in 66% ethanol and 67 mM sodium acetate, pH 5.0, before translation. Translations were incubated at 28°C for 90 to 120 min and then either used for immunoprecipitation or diluted in 2× gel sample buffer to final concentrations of 62.5 mM Tris-hydrochloride, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromophenol blue (sample buffer).

Polyacrylamide gel electrophoresis was performed according to Laemmli (22), with modifications by Oppermann et al. (27). After fixation, gels were soaked in 1 M sodium salicylate for 30 min, rinsed briefly in water, dried, and exposed to preflashed Kodak Royal X-Omat film at −70°C.

Antisera and immunoprecipitations. Antisera against the MMTV structural proteins were prepared by multiple injections of purified proteins into rabbits and were kindly supplied by D. Robertson. Immunoprecipitations were performed essentially as described by Oppermann et al. (28), using Formalin-fixed *Staphylococcus aureus* to precipitate immune complexes (20).

Immunoprecipitates of MMTV structural polypeptide precursors were obtained by incubating dexamethasone-treated H-1 cells with 500 μCi of [^{35}S]methionine per ml for 45 min before lysis of the cells. D. Robertson supplied immunoprecipitates of proteins from MMTV-infected rat cells treated with 1 μg of tunicamycin per ml.

Protease mapping. Gel slices for analysis by partial digestion with protease (4) were cut from the gel, washed in water for 30 min, and then washed in 0.1× gel sample buffer containing 10% glycerol for 10 min. Slices were placed in sample wells and overlaid with 50 to 75 μl of 4 μg of *Staphylococcus* V8 protease per ml in the sample buffer mentioned above. Samples were analyzed by polyacrylamide gel electrophoresis at 15 mA through a 4.5% stacking gel and a 14% separating gel. Gels were subjected to fluorography, dried, and exposed to film as previously described.

RESULTS

Identification and mapping of MMTV RNA species by hybridization to RNA bound to DBM paper. Before attempting to purify putative mRNA's for MMTV proteins by preparative hybridization, the major species of virus-specific intracellular RNA were defined by using analytical techniques. Whole cell RNA was isolated from MMTV-infected rat cells or mouse mammary tumor cells after growth in the presence of dexamethasone, fractionated by agarose gel electrophoresis under denaturing conditions, transferred to chemically activated cellulose (DBM paper), and detected with various ^{32}P-labeled reagents derived from MMTV DNA cloned in procaryotic hosts (Fig. 2 and above). Two species of MMTV RNA were observed in these experiments. The larger species, ca. 8.0 kilobases (kb) (referred to as 35S RNA), was the same length as a subunit of the viral genome and, as expected, annealed to all of the probes tested (*Pst*I-A [4.0 kb], *Pst*I-B [1.8 kb], *Pst*I-C [1.3 kb], p7-1A, and $cDNA_{rep}$). For several reasons, RNA of this size is predicted to direct synthesis of *gag* and *pol* proteins (5, 25, 27). The smaller species, ca. 3.1 kb (referred to as 24S RNA), annealed to probes representing the 3′ one-third of viral RNA, *Pst*I-B (1.8 kb), and

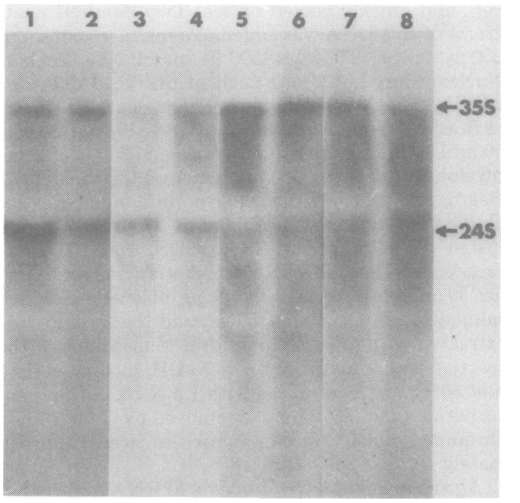

FIG. 2. *Characterization of intracellular MMTV RNA by hybridization with cloned PstI fragments. Total cellular RNA was isolated from MMTV-infected XC cells (line D108) or mouse mammary tumor cell lines after growth for 24 to 72 h in the presence of dexamethasone. RNA was fractionated by electrophoresis in 1.2% agarose gels containing methylmercury hydroxide, transferred to DBM paper, and detected by annealing with 5 × 10^6 cpm of [^{32}P]PstI-B (1.8 kb) (lanes 1 through 4) or PstI-A (4.0 kb) (lanes 5 through 8). Autoradiographic exposures were for 12 h (lanes 3, 4, 7 and 8) or 13 days (lanes 1, 2, 5 and 6). The arrows indicate the positions of viral species of 35S and 24S RNA; the blanched areas of the films correspond to the positions of 28S and 18S rRNA's in the gel. Lanes 1 and 5: 960 cellular RNA; lanes 2 and 6: GR cellular RNA; lanes 3 and 7: BALB/cfC3H cellular RNA; lanes 4 and 8: H-1 (C3H) cellular RNA.*

*Pst*I-C (1.3 kb), but not to the *Pst*I-A (4.0 kb) fragment derived from the central portion of the viral genome (see Fig. 2 and unpublished data). Identical results were obtained by using RNA derived from GR, C3H, or BALB/cfC3H tumor cell lines or from MMTV-infected XC cells. These results conform to previous suggestions that 24S RNA is a subgenomic species responsible for synthesis of proteins encoded near the 3′ end of viral RNA (16, 38, 43). However, due to high backgrounds observed with the *Pst*I-A probe and its probable content of 24S RNA sequences (see below), low levels of hybridization to 24S RNA may have been overlooked. None of the other previously described species of MMTV RNA (16, 38, 43) were detected in these and subsequent analyses; the reasons for this are considered below.

The results presented in Fig. 2 indicated that most if not all of the *env* gene may lie on the 3′ side of the *Pst*I site which divides *Pst*I-A (4.0

kb) from *Pst*I-B (1.8 kb) and that it might be possible to separate 35S RNA from 24S RNA by preparative hybridization to the *Pst*I-A (4.0 kb) fragment immobilized on DBM paper.

Characterization of the preparative hybridization method for purification of MMTV RNA. Other laboratories have reported the use of cloned DNA linked to DBM paper for the preparative isolation of defined species of RNA (46, 55). The specificity of this procedure was examined for our purposes by determining the proportion of RNA which binds adventitiously to filters containing nonhomologous DNA and by measuring the enrichment achieved by annealing RNA to filters containing viral DNA. Approximately 3.3% of [^{3}H]uridine-labeled, polyadenylated [poly(A)$^+$]RNA and 0.4% of poly(A)$^-$ RNA [RNA not binding to oligo(dT)-cellulose] from a line of MMTV-infected XC cells (line D108) annealed to filters bearing DNA from a hybrid plasmid composed of pBR322 sequences and most of the MMTV genome (p7-1A) (Table 1). These results are consistent with previous observations that about 0.1 to 0.5% of total cellular RNA is virus specific in dexamethasone-treated cells of this lineage and that a 10- to 20-fold enrichment for viral RNA can be effected by selection of poly(A)$^+$ RNA (38). Only 0.01% of the same RNA preparation bound to filters containing pBR313 DNA alone, indicating that the annealing step can provide a ca. 300-fold enrichment for viral sequences. [^{3}H]uridine-labeled poly(A)$^+$ [and poly(A)$^-$] RNA from uninfected XC cells also bound inefficiently (0.01 to 0.02%) to filters carrying either pBR313 or p7-1A DNA. Similar values for "background" adherence to DBM paper have been reported by Stark and Williams (48).

TABLE 1. *Quantitation of [^{3}H]RNA hybridization to DNA-DBM paper disks*[a]

DNA on DBM disk[b]	Source of labeled RNA[c]	Poly(A) fraction	cpm added ($\times 10^{-6}$)	cpm hybridized ($\times 10^{-3}$)	% RNA hybridized
p7-1A	D108	+	1.49	48.60	3.26
p7-1A	D108	−	3.05	11.60	0.38
p7-1A	XC	+	1.49	0.30	0.02
p7-1A	XC	−	1.59	0.07	0.01
pBR313	D108	+	1.43	0.17	0.01
pBR313	XC	+	1.49	0.13	0.01

[a] Labeled RNA was hybridized to DNA disks in a 50% formamide hybridization buffer (see the text) for 6.5 h at 41°C. Disks were washed, and the hybridized RNA was eluted and counted in 5 ml of Tritosol scintillation cocktail (13).

[b] DNA (9 to 11 μg) from p7-1A (a pBR322 hybrid plasmid containing most of the MMTV genome) or from pBR313 was bound to DBM paper disks as described in the text.

[c] MMTV-infected XC cells (line D108) or uninfected XC cells were labeled for 24 h with [^{3}H]uridine (500 μCi/ml) before preparation of whole cell RNA and fractionation by oligo(dT)-cellulose chromatography (see the text).

Results shown in Table 1 were confirmed by analysis of the size and sequence specificity of the selected species of RNA in experiments also designed to determine whether intact MMTV RNA survived the selection procedure (Fig. 3). [^{3}H]uridine-labeled RNA from D108 cells was divided into poly(A)$^+$ and poly(A)$^-$ fractions, and each was annealed preparatively to cloned MMTV DNA (p2-1A) bound to DBM paper.

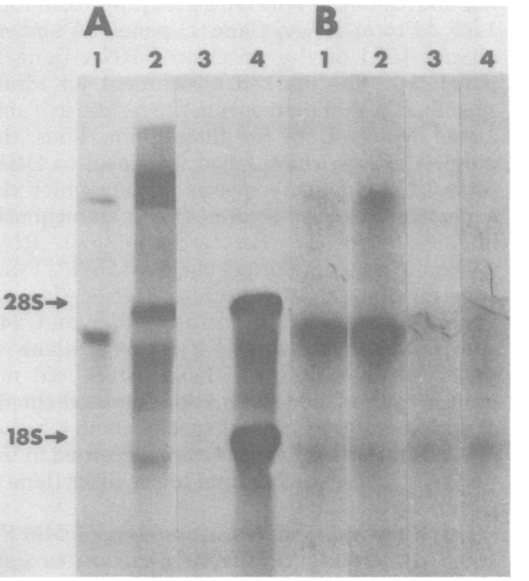

FIG. 3. *Analysis of RNA from MMTV-infected XC cells after hybridization to MMTV DNA-containing DBM paper. MMTV-infected XC cells (line D108) were labeled for 24 h with [^{3}H]uridine (500 μCi/ml), and poly(A)$^+$ and poly(A)$^-$ RNA fractions were prepared by oligo(dT)-cellulose chromatography. After incubation of each fraction with MMTV p2-1A DBM paper disks, unbound and bound components were concentrated and subjected to electrophoresis in a 1.2% agarose gel containing methylmercury hydroxide. [Approximately 1.52% (3.0 × 10^4 cpm) of the poly(A)$^+$ RNA and 0.06% (7.6 × 10^3 cpm) of the poly(A)$^-$ RNA were bound to the DBM disks in this experiment, and only 20 to 50% of each was recovered after ethanol precipitation.] After electrophoresis, one-half of the gel (panel A) was prepared for fluorography and exposed to film for 24 days; RNA in the other half (panel B) was transferred to a sheet of DBM paper and hybridized with [^{32}P]cDNA$_{rep}$ (2 × 10^6 cpm). Panel B shows an autoradiogram exposed for 19 days. Lane 1: 25% of the poly(A)$^+$ RNA which annealed to DBM disks; lane 2: 0.3% (panel A) and 5% (panel B) of poly(A)$^+$ RNA not annealed to DBM disks; lane 3: 25% of the poly(A)$^-$ RNA which annealed to DBM disks; lane 4: 0.15% (panel A) and 5% (panel B) of poly(A)$^-$ RNA not annealed to DBM disks.*

Portions of RNA which hybridized or failed to hybridize in this step were then subjected to agarose gel electrophoresis and examined either by fluorography (panel A) or by hybridization with [^{32}P]MMTV cDNA$_{rep}$ and autoradiography (panel B). The tests with cDNA$_{rep}$ showed that most (if not all) of the intact MMTV 35S and 24S RNA was in the poly(A)$^+$ fractions (lanes 1 and 2) and that at least 20% of the viral RNA had been selected by annealing to DBM filters under these conditions. (This was deduced from the similar intensities of bands obtained with one-fourth of the selected RNA [approximately 1.5% of total RNA] [lane 1, panel B] and an aliquot [5%] of the unselected RNA [lane 2, panel B]). The marked enrichment for virus-specific RNA predicted from the results in Table 1 was evident from the fluorogram. Thus, the poly(A)$^+$ RNA which failed to anneal to DBM filters contains many species, among which the expected viral species cannot be unambiguously identified (lane 2), whereas the poly(A)$^+$ RNA which did anneal to filters bearing MMTV DNA consists of only two discrete species (lane 1, panel A) comigrating with the 35S and 24S species detected with [^{32}P]cDNA$_{rep}$ (lane 1, panel B). The poly(A)$^-$ RNA which did not adhere to the DBM disks was composed chiefly of 28S and 18S ribosomal species (lane 4, panel A), and no discrete species were discerned in the poly(A)$^-$ RNA which bound to the disks (lane 3, panel A).

Purification and fractionation of MMTV RNA by preparative hybridization to PstI fragments. Preliminary tests of the preparative hybridization method (Table 1, Fig. 3) employed cloned MMTV DNA representing most of the viral genome. Based upon results presented in Fig. 2, it appeared that preparative annealing with cloned *Pst*I fragments would allow separation of the two major species of viral RNA as well as marked enrichment for viral RNA. This prediction was confirmed in the experiments shown in Fig. 4. [^{3}H]poly(A)$^+$ RNA was isolated from MMTV-infected XC cells (line 960) or from cultured C3H mammary tumor cells (line H-1) and annealed preparatively to DBM disks bearing p7-1A, *Pst*I-B (1.8 kb), or *Pst*I-A (4.0 kb) DNA. Hybridization to the p7-1A or *Pst*I-B (1.8 kb) resulted in selection of both major species of viral RNA (lanes 1 and 2, panels A and B), whereas hybridization to the larger *Pst* fragment selected only the 35S RNA. This result confirmed earlier evidence (Fig. 2) that 24S RNA contains little or no sequence homology with the *Pst*I-A (4.0 kb) fragment and also indicated that the 35S species could be separated from 24S RNA without fractionation by size.

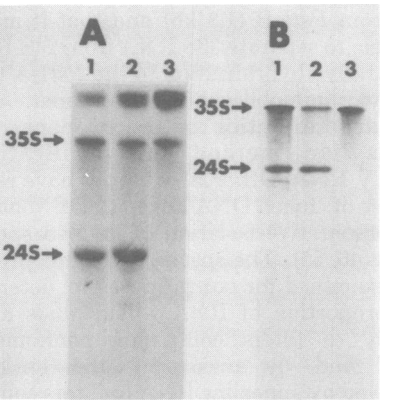

FIG. 4. *Selection of MMTV intracellular RNAs by preparative annealing to cloned PstI fragments. MMTV-infected XC cells (line 960; panel A) or virus-producing C3H mammary tumor cells (panel B) were grown for ca. 72 h with dexamethasone and labeled with [^{3}H]uridine for 24 h at 500 μCi/ml. Poly(A)$^+$ RNA was prepared from whole cells and annealed to DBM paper disks bearing either p7-1A DNA (lane 1), PstI-B (1.8 kb) DNA (lane 2), or PstI-A (4.0 kb) DNA (lane 3) for 9 h at 41°C. After elution of the hybridized RNA, samples were analyzed by electrophoresis in 1.2% agarose gels containing methylmercury hydroxide before fluorography. The bands of slowly migrating material in panel A were shown to represent labeled cellular DNA and could be eliminated by pretreatment of the sample with 3 M sodium acetate, pH 6.0 (9) (cf. panel B) or DNase (data not shown). The gels were calibrated by inclusion of 28S and 18S rRNA in parallel lanes. Autoradiograms were exposed to film for 2 days (panel B) or 3 days (panel A).*

Products of translation of MMTV RNA selected by preparative hybridization. To assess the coding potential of the 35S and 24S RNA species obtained by preparative annealing to DNA bound to DBM paper disks, C3H tumor cell RNA selected by this procedure was translated in an in vitro protein-synthesizing system prepared from rabbit reticulocytes. Products were examined in polyacrylamide gels and compared with products of translation of subunit-sized virion RNA (Fig. 5, panel A); in addition, the products were analyzed by immunoprecipitation with antisera directed against products of the *gag* and *env* genes (p27 and gp52, respectively) (Fig. 5, panel B).

Virion RNA and RNA selected by annealing to either *Pst*I-A (4.0 kb) or *Pst*I-B (1.8 kb) directed synthesis of a series of polypeptides immunoprecipitable with anti-p27 antisera, as well as several smaller polypeptides not precipitable with this antiserum. Although these *gag*-related products migrated as doublets for unknown reasons, most of them appeared to be similar in size (ca. 75,000, 110,000, and 160,000 M_r) to previously described *gag* and *gag-pol*

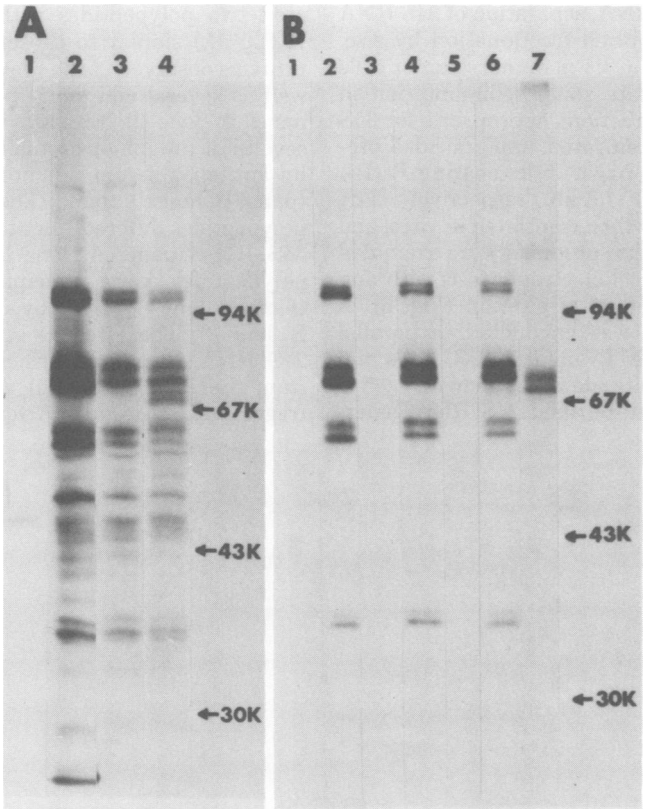

FIG. 5. *Translation products of MMTV RNA selected by hybridization to PstI fragments. [^{35}S]methionine-labeled polypeptides synthesized in a nuclease-treated rabbit reticulocyte system (see the text) were analyzed by electrophoresis in 7.5% discontinuous polyacrylamide gels before (panel A) or after (panel B) precipitation with indicated sera. Panel A: Products of synthesis with no added RNA (lane 1); with 0.5 μg of 26 to 40S MMTV virion RNA (lane 2); with poly(A)$^+$ RNA from C3H mammary tumor cells after selection by annealing to DBM disks containing PstI-A (4.0 kb) DNA (lane 3); and with poly(A)$^+$ RNA from C3H tumor cells after selection by annealing to PstI-B (1.8 kb) DNA (lane 4). Panel B: Portions of the translation products shown in panel A were precipitated with normal rabbit serum (lane 1), anti-p27^{gag} serum (lanes 2, 4, and 6), or anti-gp52^{env} serum (lanes 3, 5, and 7). Polypeptides in lanes 1 through 3 were from the sample analyzed in lane 2 of panel A; polypeptides in lanes 4 and 5 were from the sample analyzed in lane 3 of panel A; and polypeptides in lanes 6 and 7 were from the sample analyzed in lane 4 of panel A. Phosphorylase B (94,000 M_r), bovine serum albumin (67,000 M_r), ovalbumin (43,000 M_r), and carbonic anhydrase (30,000 M_r) served as molecular weight markers.*

translation products of genomic and intracellular 35S RNA (5, 26, 43). In addition, we observed *gag*-related products of ca. 60,000 and 35,000 M_r in all three samples but have not investigated these further. These results confirm earlier contentions that the 35S RNA species includes the mRNA's for *gag* and *gag-pol* proteins (38).

Experiments presented in previous sections demonstrated that RNA selected by annealing to the *Pst*I-B (1.8 kb) fragment contains the 24S species, but RNA annealed to the *Pst*I-A (4.0 kb) fragment does not. Translation products of the RNA selected with the *Pst*I-B (1.8 kb) fragment included polypeptides of 68,000 and 70,000 M_r (panel A, lane 4) not evident among products of the other translations (panel A, lanes 1–3). These polypeptides were precipitated by anti-gp52 serum (panel B, lane 7), whereas little or no protein was precipitated from the other translation mixtures by this serum (panel B, lanes 3 and 5). Such results indicate that the 24S species of MMTV RNA directs the synthesis of *env* polypeptides, confirming the predictions outlined in Fig. 1.

Translation of 24S RNA purified after separation from 35S RNA. Evidence in the preceding section for the coding function of 24S RNA was based upon translation of 24S RNA copurified with 35S RNA. Since the sequences in 24S RNA appear to overlap completely with

the content of 35S RNA, separation of 24S RNA from 35S RNA required fractionation by size. RNA from cultured C3H mammary tumor cells was subjected to rate zonal sedimentation in sucrose, pooled in fractions appropriate for 35S and 24S RNA, concentrated, and annealed preparatively to DBM paper disks bearing *Pst*I-A (4.0 kb) and *Pst*I-B (1.8 kb), respectively. The recovered RNA was then translated in vitro, and the labeled translation products were examined by polyacrylamide gel electrophoresis with and without prior immunoprecipitation (Fig. 6). A second experiment was conducted in parallel with RNA from MMTV-infected XC cells and yielded similar results (data not shown).

Translation of the purified 24S RNA generated two polypeptides with sizes (68,000 and 70,000 M_r) similar to those of the *env*-specific proteins observed in the previous experiment, as well as several additional polypeptides (Fig. 6, panel A, lane 2); the two major proteins and several of the minor products were precipitated by anti-gp52 serum but not by p27 antiserum (panel B, lanes 1 and 2). The mobilities of these polypeptides differed from those synthesized by 35S RNA (panel A, lane 3); moreover, these polypeptides were precipitated by anti-p27 (panel B, lane 4) but not by anti-gp52 sera (panel B, lane 3). These results strongly suggest that 24S RNA, composed principally of sequences from the *Pst*I-B (1.8 kb) and *Pst*I-C (1.3 kb) fragments, is the mRNA for envelope proteins

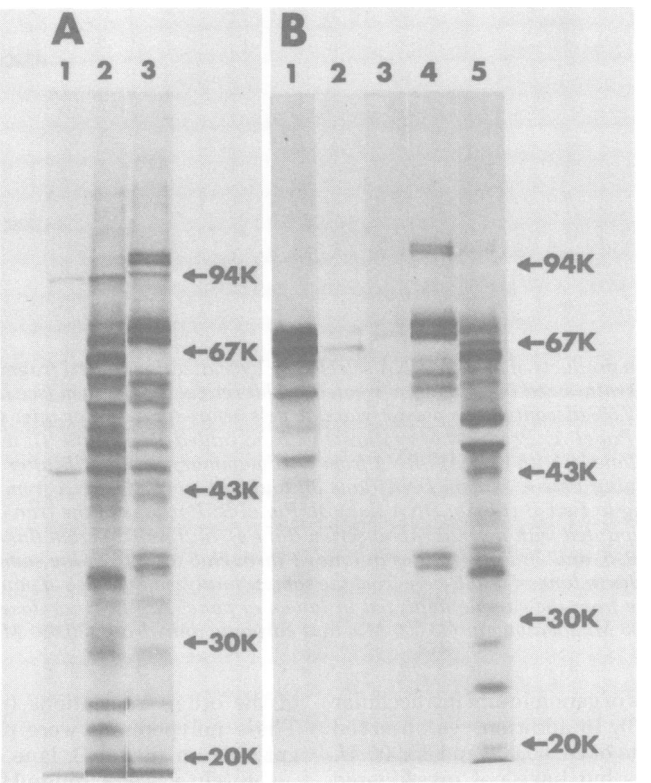

FIG. 6. *Translation products of size-fractionated 24S and 35S RNA. Whole cell RNA was prepared from dexamethasone-treated C3H mammary tumor cells, selected twice on oligo(dT)-cellulose, and sedimented in rate zonal sucrose gradients as described in the text. Fractions containing 24S and 35S RNA were concentrated by ethanol precipitation, and the RNA was annealed to DBM paper disks bearing either PstI-B (1.8 kb) (for 24S RNA) or PstI-A (4.0 kb) (for 35S RNA). After elution, the hybridized RNAs were translated in vitro (cf. Fig. 5 and the text) and the [^{35}S]methionine-labeled products were subjected directly to electrophoresis in 7.5% polyacrylamide gels (panel A) or immunoprecipitated with the indicated antiserum before electrophoresis (panel B). Panel A. Products of translation with: no added RNA (lane 1); with 24S RNA (lane 2); and with 35S RNA (lane 3). Film exposure was for 6 days for all lanes. Panel B. Products of translation with 24S RNA after immunoprecipitation with anti-gp52 serum (lane 1) or anti-p27^{gag} serum (lane 2); with 35S RNA after precipitation with anti-gp52 serum (lane 3) or anti-p27 serum (lane 4); with 16-26S virion RNA after precipitation with anti-gp52 serum (lane 5). Film exposure was for 6 days for all lanes.*

and that the *env* gene must lie within ca. 3 kb of the 3′ terminus of viral RNA (see Fig. 1). In agreement with this prediction, the synthesis of *env* proteins was also observed from poly(A)$^+$ 16 to 26S RNA prepared from virions (panel B, lane 5).

Identification of translation products by mapping with *Staphylococcus* V8 protease. To document the identity of putative *env* polypeptides synthesized in vitro from purified MMTV 24S mRNA, translation products were subjected to partial protease digestion (Fig. 7). Current evidence suggests that the primary product of *env* is processed in cells by removal of amino-terminal amino acids, presumably a "signal peptide" (7; D. Robertson and H. E. Varmus, submitted for publication), and by glycosylation at multiple sites (7). For this reason, the *Staphylococcus* V8 protease products of both the 68,000 and 70,000 M_r products of intracellular 24S RNA (lanes 3 and 4) were compared with the products of a 63,000-M_r *env* protein synthesized in MMTV-infected XC cells in the presence of tunicamycin, an inhibitor of glycosylation (50) (lane 1), with the products of a 68,000-M_r *env* polypeptide synthesized in vitro by 16 to 26S virion RNA (lane 2), and with the products of a 73,000-M_r *env* polypeptide (gPr73^{env}) synthesized in infected cells (lane 5). In addition, the products were compared to digestion products of a 77,000-M_r *gag* polypeptide synthesized in vitro from virion 35S RNA (lane 6) or purified intracellular 35S RNA (lane 7).

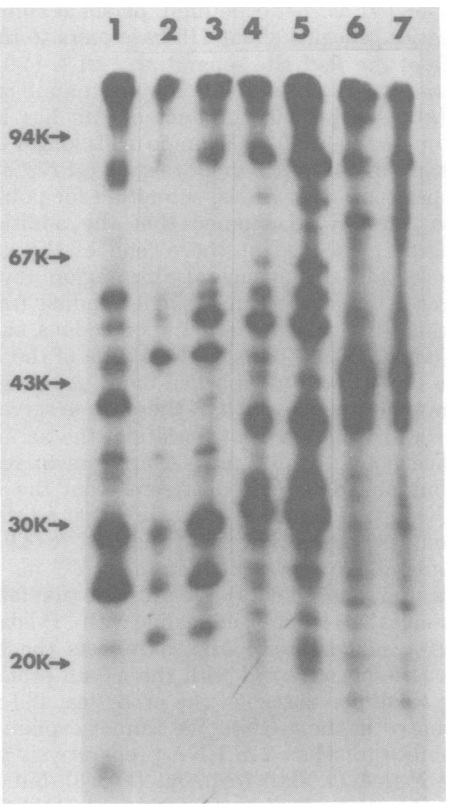

FIG. 7. *Partial protease digestion of polypeptides synthesized from purified mRNA. Polypeptides were excised from 7.5% polyacrylamide gels and digested with Staphylococcus V8 protease (see the text). Products were separated on a 14% polyacrylamide gel. Lane 1, 63,000-M_r polypeptide immunoprecipitated with gp52 antiserum from tunicamycin-treated MMTV-infected XC cells; lane 2, 68,000-M_r polypeptide immunoprecipitated with anti-gp52 serum from translates of 16 to 26S virion RNA; lane 3, 68,000-M_r polypeptide from translates of purified 24S mammary tumor cell RNA; lane 4, 70,000-M_r polypeptides from purified 24S RNA translates; lane 5, 73,000-M_r polypeptide immunoprecipitated from mammary tumor cells with gp52 antiserum; lane 6, 77,000-M_r gag polypeptide from translates of purified 35S mammary tumor cell RNA; lane 7, 77,000-M_r gag polypeptide from translates of 26 to 40S virion RNA. Film exposure was for 22 days for all lanes.*

Digestion of the 68,000-M_r polypeptides programmed by 24S mRNA and subgenomic-length virion RNA yielded identical patterns which were very similar to the pattern of the product of the 63,000-M_r polypeptide from tunicamycin-treated cells; we assume the difference reflects the presence of the uncleaved "signal" sequence in the in vitro products. The 70,000-M_r product of 24S RNA produced a pattern more closely related to that seen with gPr73^{env}, suggesting that the more slowly migrating of the *env* proteins synthesized in vitro may have been partially glycosylated. Although glycosylation has not generally been observed during translation in the rabbit reticulocyte system (34), this interpretation was supported by the elimination of the more slowly migrating *env* species after digestion with endoglycosidase H (data not shown). The V8 protease products of the *gag* protein appeared unrelated in size to the products of *env* proteins, whereas the V8 digestion products of virion and intracellular 35S RNA translates were indistinguishable.

DISCUSSION

We have used preparative molecular hybridization to cloned viral DNA fixed to DBM paper disks to isolate the mRNA's of MMTV and assess their functions by in vitro translation. The products of translation were then identified by electrophoretic mobility, antigenic reactivity, and size of protease digestion products. Two size classes of intracellular MMTV RNA have been studied with these procedures. The 35S RNA

programs the synthesis of *gag* (and presumably *pol*) polypeptides, whereas 24S RNA directs synthesis of *env* polypeptides, indicating that the three known genes involved in the replication of MMTV are expressed in the manner illustrated for other retroviruses (15, 20, 22, 25, 27, 29, 31, 53) and previously proposed for MMTV (16, 38, 43).

This study is unique in that both species of retroviral RNA (35S and 24S) detected previously by hybridization were purified and translated in a cell-free system. In the single instance in which retroviral RNAs have been both purified and translated (3), only two viral polypeptides of 76,000 and 60,000 M_r were synthesized. Although it was suggested that these products were *gag* and *src* specific, no definite identification was made, and no envelope-specific products were detected.

The use of preparative hybridization for mRNA purification has been facilitated by the availability of abundant amounts of viral DNA cloned in procaryotic host-vector systems and by the development of an efficient method for fixing DNA to filter paper (1, 36). The procedure adopted here has an acceptably low background (Table 1), permits up to 300-fold enrichment in a single cycle of annealing (Table 1), yields RNA which appears physically intact, even under denaturing conditions (Fig. 3 and 4), and can be translated into large polypeptides in vitro (Fig. 5 and 6). This solid-phase system also offers the advantage of filter reuse (up to 17 times in our hands without apparent loss of quality). In combination with other simple isolation methods, oligo(dT)-cellulose chromatography and rate zonal sedimentation, the preparative annealing step appears to permit virtually complete purification of the virus-specific species studied here, as judged by gel electrophoresis of labeled RNA (Fig. 3 and 4) or by products of in vitro translation (Fig. 5 and 6). These methods may prove useful in the analysis of other species of virus-related RNAs which are present at much lower concentrations.

By using restriction endonuclease fragments to select viral RNA it is possible to separate various species and to locate their sequences on the physical map of the genome. In this study, the largest *Pst*I fragment failed to anneal with MMTV 24S RNA (Fig. 2 to 4) in either analytical or preparative procedures. Since isolated 24S RNA programs the synthesis of *env* proteins of normal size and complexity (Fig. 5 to 7), most or all of *env* must reside on the 3′ side of the *Pst*I site which separates *Pst*I-A (4.0 kb) from *Pst*I-B (1.8 kb), and few of the sequences (coding or noncoding) from *Pst*I-A (4.0 kb) can be present in 24S RNA (see Fig. 1). We have shown elsewhere that 24S RNA also contains sequences from within 135 bases of the 5′ terminus of 35S RNA and from the region adjacent to the poly(A) tract at the 3′ end (38). The position of *env*, as deduced here from hybridization experiments (see Fig. 1), is in reasonable accord with the position as deduced from nucleotide and amino acid sequencing studies. The nucleotide sequence which encodes the amino terminus of gp52 (the major virion glycoprotein, derived from the amino-terminal portion of the *env* precursor [40; G. Schochetman, personal communication]) begins about 115 base pairs to the 3′ side of the *Pst*I site separating *Pst*I-A (4.0 kb) from *Pst*I-B (1.8 kb) (J. Majors, unpublished data). The primary product of *env* has been estimated to be 50 to 90 amino acids longer than the glycosylated precursor to gp52 ($gPr73^{env}$) (7; Robertson and Varmus, submitted for publication), and it is assumed that the additional length is encoded at the 5′ end of the gene. Nucleotide sequencing of this region reveals three AUG codons in a single reading frame, without interrupting termination codons, ca. 40, 75, and 180 base pairs to the 5′ side of the *Pst*I site (J. Majors, unpublished data). Although it is not yet known which of these three serves as the initiation site for translation, the acceptor splice point for *env* mRNA appears to reside about one base pair upstream from the first AUG in this sequence, indicating that a total of about 180 nucleotides from the *Pst*I-A (4.0 kb) fragment are represented in $mRNA^{env}$ (J. Majors, unpublished data). We presume that failure to detect 24S RNA by annealing with ^{32}P-labeled *Pst*I-A (4.0 kb) (see Fig. 2) reflects the high background observed with the 4.0-kb probe or the small percentage of the probe (ca. 4%) represented in the mRNA. We cannot explain our inability to select 24S RNA preparatively with the *Pst*I-A (4.0-kb) fragment (Fig. 3), but it is possible that selection of mRNA with DNA fixed to DBM paper requires a greater region of homology than does DNA fixed to nitrocellulose paper. In the latter case, 50 to 100 base pairs were claimed to suffice for mRNA selection (36).

Our data do not directly identify the 3′ boundary of *env*, but extrapolation from the size of the primary gene product suggests that the gene ends about 100 to 200 base pairs to the 5′ side of the *Pst*I site which separates the *Pst*I-B and D fragments. This implies that the long terminal redundancy sequence, which begins ca. 10 base pairs to the 5′ side of this *Pst*I site, does not contribute to *env*, although there is an extensive open reading frame in the long terminal redundancy (8; Dickson and Peters, personal communication; J. Majors, unpublished data).

In this study, only two size classes of MMTV

RNA, 35S and 24S RNA, the functional and structural correlates of the *gag* (and *gag-pol*) and *env* mRNA's described for other retroviruses (3, 10, 11, 14, 17, 23, 30–32, 45, 47, 51, 54), have been observed. However, we and others have previously reported a variety of other species not seen in the present studies. These other species include a discrete 13S RNA detectable only with MMTV $cDNA_{3'}$ (38); a heterogeneous group of 13 to 15S RNAs (43); a species slightly smaller than genomic length (16); and a 20S species annealing to probes from both the 3′ and 5′ termini of viral RNA (Robertson and Varmus, submitted for publication). Although we cannot explain our failure to detect some of these species, we have recently found that the 13S RNA is nonviral in origin, hence not capable of annealing to the cloned viral DNAs used here; previous detection of this species apparently depended upon the presence of an abundant species of cellular poly(A)$^+$ RNA in the virions used as a source of template for synthesis of $cDNA_{3'}$ (Robertson and Varmus, submitted for publication). The 20S RNA has been found transiently, generally 4 to 8 h after dexamethasone treatment of cultured cells, and was not likely to be present in appreciable amounts under the growth conditions used here. Current findings tentatively indicate that the MMTV genome may direct synthesis only of structural proteins, encoded in *gag*, *pol*, and *env*, in either infected heterologous cells or mouse mammary tumor cells. This conclusion has obvious implications for mechanisms of oncogenesis by this virus.

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