Asymmetric Transcription of Mouse Mammary Tumor Virus Genes In Vivo and In Vitro

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Mouse mammary tumor virus (MMTV) DNA fragments were cloned into M13 bacteriophage, and the single-stranded recombinant phage DNAs were used as strand-specific nucleic acid hybridization probes to measure synthesis of plus (genomic) and minus strands of MMTV RNA in cultured cell lines and in cell-free preparations of nuclei. Pulse-labeling studies showed that synthesis of MMTV RNA in three different cell lines was highly asymmetric. In nuclear preparations from a cloned line of MMTV-infected rat hepatoma cells, elongation of nascent MMTV RNA chains and initiation of new MMTV RNA chains with nucleoside (β -S)triphosphates were also highly asymmetric.

In many eucaryotic viruses, both strands of viral DNA serve as templates for transcription of specific mRNAs. For example, early and late mRNAs in SV40 and polyomavirus are transcribed from opposite strands (9). In the adenovirus genome, the late genes are oriented in the rightward direction, whereas early genes are oriented in both directions (2, 4). For most viruses, leftward and rightward transcriptions occur in separate regions of the genome; however, in some cases (e.g., adenovirus), a specific region is transcribed in both directions at different stages of infection.

In contrast, stable retroviral transcripts are plus-stranded RNA, having the same orientation as that of the single-stranded RNA genomes (31). As with other retroviruses, mouse mammary tumor virus (MMTV) RNA is synthesized by host RNA polymerase II (27), using the integrated proviral DNA as the template for transcription. MMTV is unique, however, in that its transcription is stimulated by glucocorticoid hormones (20). Two major species of MMTV RNA (35S and 24S) have been observed in a variety of stably infected mammalian cell lines grown either in the presence or absence of glucocorticoids (24). All of the known viral genes are oriented to give rise to plus-stranded mRNAs. The 35S RNA is the mRNA for the *gag* and *pol* proteins, and the 24S species is the mRNA for the *env* proteins (24 and references cited therein). An open reading frame in the long terminal repeat (LTR), oriented in the same direction, may code for an additional protein that has not yet been detected in vivo (7, 8, 25). Another short open reading frame in the LTR of an endogenous MMTV provirus is oriented in the opposite direction (14). It is not yet clear whether these latter potential coding sequences are functional, cryptic, or fortuitous.

At present there is no evidence that minus-stranded RNA plays any role in retroviral infections. Stavnezer et al. (29), using a solution hybridization procedure, detected very small amounts of minus-stranded MMTV RNA and avian sarcoma virus RNA in infected cells. These experiments, which measured the steady-state levels of viral RNA, did not eliminate the possibility that short-lived minus-stranded RNA might be synthesized at appreciable rates. In other biological systems, some RNA species found in the cell nucleus do not appear in the cytoplasm (5). Furthermore, there is abundant evidence for selective degradation of

specific RNA sequences, including the introns that interrupt the coding sequences of most eucaryotic genes (1).

In this study, we have used single-stranded M13 clones of various regions of the MMTV genome in nucleic acid filter hybridization assays to measure directly the rates of synthesis of plus- and minus-stranded MMTV RNAs in three different infected cell lines. We have used the same assay to evaluate the degree of asymmetry of transcription in preparations of nuclei. The cell-free system may provide a closer look at the selectivity of transcription for two reasons. First, normal RNA processing and selective RNA degradation events may not be preserved in the cell-free system; this would allow us to observe transcription in the absence of posttranscriptional events. Second, unlike pulse-labeling in whole cells, the use of nucleoside (β -S)triphosphates in the cell-free system (28) allows us to examine separately the elongation of nascent MMTV transcripts and the initiation of new MMTV transcripts.

MATERIALS AND METHODS

Chemicals and enzymes. Restriction endonucleases and *Escherichia coli* RNA polymerase holoenzyme were purchased from New England Biolabs; DNase I and RNase A were from Worthington Diagnostics; pronase was from Calbiochem-Behring Corp.; dexamethasone was from Sigma Chemical Co.; [3 H]uridine (50 Ci/mmol) and [3 H]CTP (25 Ci/mmol) were from ICN; [3 H]dTTP (50 Ci/mmol) and [α - 32 P]UTP (600 Ci/mmol) were from New England Nuclear Corp.

Bacterial strains, plasmids, and viruses. Plasmids pP0.9, pP1.1, and pP2.5 are pBR322 vectors containing *Pst*I inserts representing regions I, II, and V, respectively (see Fig. 1) of the MMTV genome (16). Plasmids pA'P and pPA contain *Pst*I-*Sau*3A fragments of the MMTV LTR (regions III and IV, respectively) cloned into the *Bam*HI site of pBR322 with linkers (3). These plasmids were propagated in *E. coli* HB101 or JA221.

M13 bacteriophage strains mp7 (18) and mp8 (19) and host bacterial strain *E. coli* K-12 JM103 were kindly provided by Bethesda Research Laboratories.

Labeled MMTV-specific hybridization reagents. 32 P-labeled 70S RNA from MMTV virions was prepared as described before (28). MMTV [3 H]cDNA complementary to the entire viral genome was prepared as described by Ringold et al. (22), using reverse transcriptase and random calf thymus DNA primers. [32 P]RNA complementary to

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specific fragments of MMTV DNA (cRNA) was prepared as described before (17), using *E. coli* RNA polymerase and the appropriate purified *Pst*I insert from plasmid pP0.9, pP1.1, or pP2.5. Inserts were separated from the vector DNA by *Pst*I digestion and agarose gel electrophoresis and then purified by disrupting the agarose gel with sodium periodate and adsorbing the DNA onto glass powder (33).

Mammalian cell lines. M1.54 is a clonally derived line of MMTV-infected rat hepatoma cells (22). GR cells (21) are derived from a spontaneous mammary carcinoma in the GR strain of mouse. S49A2 is a mouse T-lymphosarcoma cell line (12). M1.54 and GR cells grown in monolayer cultures in Dulbecco-modified essential medium supplemented with 5% horse serum (Flow Laboratories) were harvested before confluency was reached. S49 cells grown in suspension culture in the same medium with 10% horse serum were harvested in log phase.

Transfer of cloned MMTV DNA fragments from pBR322 to M13 vectors. Recombinant pBR322 plasmids and M13 replicative-form DNAs were prepared as described by Lawther et al. (15) and further purified by pronase digestion (0.2 mg of pronase per ml for 1 h at 37°C in a solution of 10 mM Tris-hydrochloride [pH 8.0], 100 mM NaCl, 1 mM EDTA, and 0.5% SDS), phenol-chloroform extraction, and ethanol precipitation. The inserts were excised from pP0.9, pP1.1, and pP2.5 with *Pst*I and from pA'P and pPA with *Bam*HI. The digestion products were ligated with M13mp7 or M13mp8 replicative-form DNA linearized with the same enzyme. The ligation products were transfected into *E. coli* JM103, and bacteria producing recombinant phage were selected on X-gal plates (10, 18, 19). Recombinant phage were plaque purified, and the insert size of the recombinant replicative form was determined by the miniscreen procedure described below. The identity of the insert of each recombinant M13 phage was further verified by nucleic acid hybridization experiments described below.

Miniscreen procedure for determining size of insert in recombinant M13 replicative-form DNA. The miniscreen procedure used was a modification of the one described by Davis et al. (6). The frozen cell pellet from a 2-ml culture was thawed and suspended in 75 μ l of cold 25% sucrose–50 mM Tris-hydrochloride, pH 8.0. After addition of 15 μ l of 5-mg/ml lysozyme in 50 mM Tris-hydrochloride, pH 8.0, the suspension was incubated for 15 min on ice. Next, the mixture was adjusted to 0.1 M in Tris-hydrochloride (pH 8.0), 50 mM in EDTA, and 0.1 mg/ml in RNase A (DNase-free) and incubated for 5 min more on ice. An equal volume

of 2% Triton X-100–10 mM EDTA–50 mM Tris-hydrochloride (pH 8.0) was added, and, after a further incubation of 10 min on ice, the lysate was centrifuged for 10 min at 4°C in a microfuge (13,000 $\times$ g). The supernatant was carefully removed and dialyzed against three changes of 20 mM Tris-hydrochloride (pH 8.0)–20 mM NaCl–1 mM EDTA for a total of 9 h. The dialyzed lysate was extracted twice with phenol-chloroform (1:1), ethanol precipitated, and dissolved in 10 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA. Insert size was determined by digestion with the appropriate restriction endonuclease followed by electrophoresis on 0.8% neutral agarose gels with size markers.

Pulse-labeling of cell lines with [³H]uridine and purification of RNA. Subconfluent M1.54 or GR cells or log-phase S49 cells were grown in the presence of 1 μ M dexamethasone for 60 min and then incubated with the hormone and 0.2 mCi of [³H]uridine per ml (50 Ci/mmol) in growth medium for 15 min. RNA was purified from the pulse-labeled cells by pronase digestion and phenol extraction (23) and analyzed by nucleic acid filter hybridization as described below.

Transcription by isolated nuclei in vitro with nucleoside [β -S]triphosphates and purification of RNA initiated in vitro. The transcription procedure has been described previously (28). Briefly, whole nuclei were prepared from M1.54 cells grown with 1 μ M dexamethasone for 30 min before harvest. The nuclei were incubated in a transcription reaction mixture containing [³H]CTP, UTP, ATP, and GTP β S [guanosine 5'-O-(2-thiotriphosphate)]. RNA was purified from the reaction mixture, and the RNA chains initiated with GTP β S were separated from the remaining RNA by mercury-Sepharose column chromatography. The initiated and noninitiated fractions were analyzed by nucleic acid filter hybridization as described below.

Nucleic acid filter hybridization. Single-stranded M13 phage DNA was prepared as described before (11). Single-stranded DNA filters were prepared and used for nucleic acid filter hybridization as described by McKnight and Palmiter (17) and modified by Ucker et al. (30). Liquid scintillation counting was performed at 15°C in a Packard model 300C counter. Each sample was counted for a total of 30 min.

RESULTS

Characterization of M13/MMTV recombinant DNA clones. Each of the five MMTV DNA regions illustrated in Fig. 1 was excised from the plasmid pBR322 vector and ligated into the replicative form of M13 mp7 or mp8 DNA. The orienta-

TABLE 1. Characterization of M13/MMTV phage DNAs by hybridization with strand- and region-specific MMTV probes

M13/MMTV DNA on filter				% hybridization for hybridization probes:			
Clone name	Genomic ^a region	DNA strand	% of genome	MMTV ^b [³² P]RNA (+)	MMTV ^c [³ H]cDNA (–)	Region I ^d [³² P]cRNA ($\pm$)	Region V ^d [³² P]cRNA ($\pm$)
mp7.93	I	+	18	0.1	2.6	7.6	<0.3 ^e
mp7.95	I	–	18	2.3	<0.5 ^e	12.7	<0.3 ^e
mp71.14	II	+	22	0.1	2.0	<0.2 ^e	ND ^f
mp71.15	II	–	22	3.6	<0.5 ^e	<0.2 ^e	ND ^f
mp82.56	V	+	49	<0.1 ^e	11.6	ND ^f	14.8
mp82.55	V	–	49	7.7	2.4	<0.2 ^e	14.8

^a See Fig. 1.

^b Purified 70S RNA from virus particles.

^c Synthesized by avian myeloblastosis virus reverse transcriptase with random calf thymus DNA primers.

^d Transcribed by *E. coli* RNA polymerase from the purified double-stranded DNA fragment.

^e Zero cpm detected. Calculations are based upon 3 cpm.

^f ND, Not done.

FIG. 1. MMTV DNA fragments used for nucleic acid filter hybridization. The MMTV genome is shown integrated into host cell DNA. The virion RNA is aligned colinearly with the corresponding DNA sequences. The vertical arrow at the 5' end of the RNA represents the presumptive site of transcription initiation. Restriction endonuclease cleavage sites are represented by the following symbols: O, *Pst*I, X, *Sau*3A (only those *Sau*3A sites within region I are shown). The bar (1 kilobase) represents the length of 1,000 nucleotides. Clone I is a 1,400-nucleotide *Pst*I fragment that contains one complete copy of the LTR and a short nonredundant sequence. Clones II (1,700 nucleotides) and V (4,000 nucleotides) are *Pst*I fragments from the nonredundant region of the provirus. Clones III (380 nucleotides) and IV (740 nucleotides) are *Sau*3A-*Pst*I subfragments of clone I.

tion of the inserted MMTV sequence with respect to the M13 DNA determines which MMTV DNA strand is represented in the single-stranded recombinant phage DNA produced by *E. coli* cells transfected with the recombinant replicative form. Phage clones representing the plus and minus strands of all five MMTV DNA regions depicted in Fig. 1 were isolated.

The identity of each insert was verified by determining its size and its nucleic acid hybridization specificity. For each recombinant clone, the miniscreen procedure was used to show that the size of the double-stranded MMTV insert in the recombinant replicative form was the same as that of the insert from the corresponding pBR322 recombinant plasmid. Nitrocellulose filters containing the single-stranded recombinant M13/MMTV DNAs were hybridized to [³²P]RNA (plus strand) from MMTV virions, [³H]cDNA (minus strand) from MMTV RNA templates, and [³²P]cRNA (plus and minus strands) that had been synthesized by transcribing nonspecifically the purified double-stranded inserts with *E. coli* RNA polymerase. Each type of M13/MMTV single-stranded DNA showed a preference for hybridization to plus- or minus-stranded probes and also hybridized only to cRNA representing the appropriate region of the genome (Table 1).

Rate of synthesis of plus- and minus-stranded MMTV RNAs in whole cells. GR cells (a mouse mammary tumor line), S49 cells (a mouse T-lymphosarcoma line), and M1.54 cells (an MMTV-infected rat hepatoma line) all produce large amounts of MMTV RNA (21, 22). Growing cultures were exposed to 1 μ M dexamethasone for 1 h to induce the maximum rate of MMTV RNA synthesis; the cultures were then pulse-labeled with [³H]uridine for 15 min and harvested. RNA from each culture was hybridized to a set of M13/MMTV DNA filters representing the plus and minus strands of MMTV genome regions I and V (Fig. 1). MMTV RNA synthesis in M1.54 and S49 cells was highly asymmetric (Table 2), with no detectable minus-stranded RNA synthesis (<3 cpm above background). In GR cells, MMTV transcription was also asymmetric, but a small amount of minus-stranded [³H]RNA was detected. Specifically, the plus-strand region V filter bound 19 cpm above background; the combined scintillation counter background and nonspecific RNA binding was 12 cpm for this reaction. Similar results were obtained with the GR cells in an independent repetition of this experiment (data not shown). With the low backgrounds obtained in these analyses, we have generally

found that as few as 3 to 4 cpm represents a reproducible positive hybridization result; however, at least 10 cpm above background is generally required to obtain reproducible quantitations.

Asymmetry of MMTV RNA synthesis in vitro. We have previously demonstrated that MMTV RNA synthesis in isolated nuclei is accomplished by RNA polymerase II (27) and that initiation of MMTV RNA synthesis in isolated nuclei occurs in the same region of the proviral DNA (the LTR) as it does in whole cells (28). To measure initiation, transcription was performed in the presence of [³H]CTP and nucleoside (β -S)triphosphates; ³H-labeled RNA chains initiated with the thiol analogs retain the thiol group at the 5' end and can be separated from thiol-free RNA by mercury-Sepharose column chromatography.

In the present study, the M13/MMTV single-stranded DNA filters were used to quantitate plus- and minus-stranded MMTV [³H]RNAs in the thiol-containing and thiol-free RNA fractions synthesized by M1.54 nuclei in vitro in two independent transcription experiments (Table 3). MMTV [³H]RNA in the thiol-free fraction, which contains RNA elongated but not initiated in vitro, was almost exclusively plus stranded. MMTV RNA initiated in vitro (the thiol-containing fraction) represented primarily the plus strand of region I, which contains the transcription start site used in vivo (13, 30). Very little, if any, of the thiol-containing RNA represented the minus strand of region I or either strand of region V. Essentially identical results were obtained in both transcription experiments (Table 3). Thus, both elongation of nascent MMTV RNA chains and initiation of new MMTV RNA chains in the nuclear preparations exhibited a high degree of strand specificity or asymmetry similar to that of synthesis of MMTV RNA in whole cells.

DISCUSSION

Transcription of MMTV RNA in three different cultured cell lines was highly asymmetric. No minus-stranded RNA synthesis was detected in S49 mouse lymphoma and M1.54 rat hepatoma cells; the ratio of plus-strand synthesis to minus-strand synthesis was at least 100 in the unique region of the genome (region V) and at least 10 in the LTR (region I). The apparent difference in asymmetry ratios for regions I and V was due to the difference in the sizes of the two regions. The amounts of minus-stranded RNA synthesized were at or below the limits of detection of our assay and

TABLE 2. Rate of synthesis of plus- and minus-stranded MMTV RNAs in three cell lines

Source ^a of pulse-labeled [³ H]RNA	M13/MMTV DNA ^b on filter		cpm bound to filter ^c		% cpm bound to filter ^d		Corrected % ^e [³ H]RNA bound	Transcriptional ^f asymmetry ratio (+/-)
	Region	Strand	[³² P]cRNA	[³ H]RNA	[³² P]cRNA	[³ H]RNA		
M1.54	I	+	68	0	5.6	<0.0006	<0.005 (-)	
	I	-	150	79	12.5	0.015	0.060 (+)	>12
	V	+	117	0	13.0	<0.0006	<0.002 (-)	
	V	-	147	345	16.1	0.062	0.193 (+)	>96
GR	I	+	139	3	4.2	0.0005	0.005 (-)	
	I	-	289	48	8.8	0.0071	0.040 (+)	8
	V	+	531	19	9.6	0.0031	0.016 (-)	
	V	-	586	143	10.6	0.023	0.109 (+)	7
S49	I	+	138	0	4.0	<0.0002	<0.003 (-)	
	I	-	292	210	8.5	0.014	0.082 (+)	>27
	V	+	179	3	3.8	0.0002	0.003 (-)	
	V	-	1,069	2,041	22.4	0.142	0.317 (+)	106

^a Cells were incubated with 1 μ M dexamethasone for 1 h and then pulse-labeled with [³H]uridine for 15 min in the presence of dexamethasone. RNA was purified from the cells and analyzed by filter hybridization. Each M1.54 datum is the average from two filter hybridization assays on the same RNA preparation.

^b See Fig. 1.

^c [³²P]cRNA representing the appropriate MMTV region was added to monitor hybridization efficiency. Scintillation counter backgrounds (12 cpm for ³²P, 6 cpm for ³H) and nonspecific RNA binding to control DNA filters (0 to 5 cpm for ³²P, 5 to 15 cpm for ³H) have been subtracted. ³H counts per minute have been corrected for ³²P spillover (1 to 2%).

^d The counts per minute in the previous column were divided by the total counts per minute in the assay. Approximate total ³H counts per minute in each hybridization reaction were: M1.54, 500,000; GR, 650,000; S49, 1,500,000. When <3 cpm was detected, calculations were based upon 3 cpm as the limit of sensitivity, and the results are preceded by the symbol < (less than).

^e The percent ³H counts per minute bound was divided by the hybridization efficiency, which is twice the fraction of ³²P counts per minute bound (because the DNA on the filter is single stranded, whereas the [³²P]cRNA represents both strands of MMTV). The plus or minus in parentheses indicates which strand of RNA is represented.

^f The ratio of plus-stranded to minus-stranded RNA synthesis.

TABLE 3. Initiation and elongation of plus- and minus-stranded MMTV RNAs in nuclear preparations from M1.54 cells

Expt ^a	M13/MMTV DNA ^b on filter		cpm bound to filter ^c		% cpm bound to filter ^d		Corrected % ^e [³ H]RNA bound	Transcriptional/ asymmetry ratio (+/-)
	Region	Strand	[³² P]cRNA	[³ H]RNA	[³² P]cRNA	[³ H]RNA		
I								
Thiol-containing RNA (initiated with GTPβS)	I	+	65	4	5.6	0.0025	0.022	
	I	-	80	43	6.0	0.0269	0.195	9
	V	+	98	5	11.9	0.0031	0.013	
	V	-	101	9	12.2	0.0056	0.023	2
Thiol-free RNA from the GTPβS reaction	I	+	63	1.5	4.5	<0.0007	<0.008	
	I	-	140	68	10.4	0.0173	0.083	>10
	V	+	202	9	13.4	0.0022	0.008	
	V	-	229	480	14.8	0.104	0.351	44
II								
Thiol-containing RNA (initiated with GTPβS)	I	+	58	1	5.0	0.0011	0.011	
	I	-	105	18	9.1	0.0193	0.106	10
	V	+	128	2	10.1	0.0021	0.010	
	V	-	105	5	8.3	0.0052	0.031	3
Thiol-free RNA from the GTPβS reaction	I	+	48	4	3.3	0.0008	0.012	
	I	-	177	101	12.8	0.0259	0.101	8
	V	+	133	1	9.1	<0.0006	<0.003	
	V	-	322	572	23.0	0.1270	0.276	>92

^a [³H]RNA from two independent in vitro transcription experiments was fractionated by mercury-Sepharose column chromatography and analyzed by filter hybridization. Approximate total ³H counts per minute in each hybridization reaction were: experiment I, 160,000 cpm of thiol-containing RNA and 430,000 cpm of thiol-free RNA; experiment II, 95,000 cpm of thiol-containing RNA and 460,000 cpm of thiol-free RNA. The data given for thiol-free RNA are the average from two hybridization reactions.

^{b-f} See Table 2, footnotes b to f.

could not be accurately measured; region V, being larger than region I, hybridized a larger amount of plus-stranded [³H]RNA than did region I and thereby yielded a higher apparent asymmetry ratio. In the GR mouse mammary tumor cells, the amounts of minus-stranded MMTV RNA synthesized were small but well above the limits of sensitivity of our assay. Nevertheless, the plus/minus ratio of 7 to 8 indicates highly asymmetric transcription. Since these GR cells contain ca. 40 copies of the MMTV genome per cell (32), it is conceivable that minus-stranded RNA could be synthesized by read-through from a flanking host promoter instead of a virus-encoded minus-stranded promoter. It is curious that S49 cells, which contain comparably large amounts of integrated MMTV DNA (26), did not synthesize detectable amounts of minus-stranded MMTV RNA. M1.54 cells contain 5 to 10 copies of the MMTV genome (22). The lack of minus-stranded MMTV RNA synthesis in S49 and M1.54 cells suggests that there are no strong minus-stranded promoters in the viral genome.

The asymmetry of MMTV transcription *in vivo* was used as a criterion for evaluating the fidelity of MMTV transcription *in vitro*. Both elongation of nascent MMTV RNA chains and initiation of new MMTV RNA chains were highly asymmetric in our preparations of isolated nuclei. In addition, the initiation of MMTV RNA *in vitro* occurred in the same region of the genome that is used for initiation *in vivo* (Table 3 and see reference 28). By these two criteria, it appears that faithful initiation of MMTV RNA occurred in these preparations of whole nuclei. S1 nuclease mapping studies are under way to compare the precise locations of initiation *in vitro* and *in vivo*.

The use of single-stranded DNA instead of double-stranded DNA as the filter-bound species for filter hybridization has several distinct advantages. First, milligram quantities of M13 phage DNA can be produced and purified much more easily than similar quantities of most plasmids. Second, the strand-specific M13 DNAs allow quantitation of plus- and minus-stranded RNAs independently as shown here. Third, the absence of the cDNA strand eliminates potential hybridization competition. Fourth, with single-stranded DNA filters, hybridization efficiency can be correctly monitored by using ³²P-labeled cRNA; these RNAs are conveniently prepared with *E. coli* RNA polymerase from double-stranded DNA templates that represent the insert of the recombinant DNA on the filter. The commonly used practice of hybridizing these [³²P]cRNAs to double-stranded DNA filters is not actually appropriate because it measures the combined hybridization efficiency of both plus- and minus-stranded RNAs. In contrast, hybridization of the [³²P]cRNAs to filters with single-stranded M13 DNA containing a minus-stranded insert measures the hybridization efficiency of plus-stranded RNA only, which is the desired result in most cases.

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