JOURNAL OF VIROLOGY, Aug. 1986, p. 518–521
0022-538X/86/080518-04$02.00/0

Vol. 51, No. 2

Differential Expression of Endogenous Mouse Mammary Tumor Virus Genes during Development of the BALB/c Mammary Gland

JANICE E. KNEPPER,[1] DANIEL MEDINA,[2] AND JANET S. BUTEL[1]*

Departments of Virology and Epidemiology[1] and Cell Biology,[2] Baylor College of Medicine, Houston, Texas 77030

Received 3 February 1986/Accepted 29 April 1986

Expression of endogenous mouse mammary tumor virus sequences varied over the course of development of the mammary gland during primary pregnancy and lactation in virus-free BALB/c mice. Although RNA from all regions of the genome was detected, both the level and temporal regulation of expression were different for long terminal repeat-, *env*-, and *gag-pol*-specific RNAs. Analysis of the methylation status of proviral DNA indicated differential accessibility of the three endogenous units during development. The results demonstrated noncoordinate regulation of mouse mammary tumor virus expression with respect to provirus template utilized and specific transcripts accumulated.

Expression of mouse mammary tumor virus (MMTV) genes is tissue specific and stimulated by steroid hormones (21, 24, 25). In addition, the incidence of mammary tumors in mice is increased by parity and may be influenced by artificial manipulation of hormone levels (9). Because of the association of MMTV with mammary neoplasia, we wished to determine the range of expression of endogenous viral genes in normal mammary gland cells of virus-free mice.

Female BALB/cCrl/Med mice (2 to 4 months old) used in this study were sacrificed at the indicated times of their first pregnancies. At the time of sacrifice, the uterine contents were examined to confirm the stage of pregnancy. The incidence of spontaneous mammary tumors in breeding females of this BALB/c substrain between 1979 and 1984 was 0.3%.

Endogenous MMTV-specific RNAs are expressed differentially during pregnancy in BALB/c mice. RNA was extracted from tissues by solubilization in guanidine hydrochloride as previously described (6) and purified by sedimentation in CsCl (4). Total cellular RNA prepared from mammary glands of mice at various stages of pregnancy and lactation was dotted onto nitrocellulose as described by Thomas (22) and hybridized (26) to probes derived from defined regions of the MMTV genome (11).

Transcripts representative of each of the regions were detectable during this developmental process (Table 1). The kinetics of expression from the different regions of the viral genome varied. Very low levels of virus-specific transcripts were expressed in glands taken from virgin animals or early in pregnancy. RNA complementary to the long terminal repeat (LTR) probe appeared at elevated levels in the middle of pregnancy, whereas *env*- and *gag-pol*-specific messages rose abruptly in concentration only at lactation. Levels of the *env* and *gag-pol* RNAs declined to baseline levels after weaning of the pups, whereas levels of LTR-specific transcripts remained somewhat elevated. The highest levels of each of the RNAs were present in mammary glands of lactating animals, but even those maximal amounts represented less than 10% of the MMTV-specific RNAs detected in virus-positive tumors. Differential expression of MMTV-specific RNAs was confirmed with samples taken from mice during precisely timed pregnancies (Table 2).

LTR-specific message was always detected in much greater amounts than *env* or *gag-pol* hybridizing sequences. Whereas the *gag-pol*-specific probe can detect only genome-length message (8.9 kilobases [kb]), the envelope probe can hybridize to both 8.9- and 3.8-kb RNAs. The LTR probe would detect a 1.6-kb transcript in addition to the larger two (23, 26). RNAs from various developmental stages of the mouse mammary gland were sized on formaldehyde denaturing gels and transferred to nitrocellulose. The pattern produced when such a blot was hybridized against an LTR probe is shown in Fig. 1. MMTV-specific RNAs were detected at 1.6 and 3.8 kb at all stages of development, but the 1.6-kb LTR message was increased in concentration relative to the envelope-specific 3.8-kb message in mammary glands of mice from the middle of pregnancy onward. The lane containing the sample from lactating animals (N) is vastly overexposed and illustrates the relative intensities of reactivity when equivalent amounts of RNA from glands at different stages of development were analyzed; a shorter exposure time showed that levels of 1.6-kb message were high relative to the 3.8-kb RNA in lactating tissue (lane N'). The 8.9-kb genome-length message was not detected in these blots. Concentrations of *gag-pol*-specific RNA as determined in the dot-blot assay were quite low relative to LTR

TABLE 1. MMTV gene expression during BALB/c mammary gland development

Gene probe[a]	Stage of mammary gland development[b]				
	Virgin	Early pregnancy	Mid pregnancy	Lactating	Regressing
LTR	3.7 (0.24)	3.8 (0.25)	66 (4.36)	100 (6.60)	22 (1.45)
env	12.3 (0.27)	20.9 (0.46)	19.5 (0.43)	100 (2.2)	9.5 (0.21)
gag-pol	8.7 (0.09)	14.8 (0.16)	7.3 (0.08)	100 (1.1)	11.5 (0.13)

[a] Probes for the LTR, *env*, and *gag-pol* regions of (C3H) MMTV were prepared from plasmids generously supplied by J. Majors and H. Varmus (11). Inserts were excised from the vectors by digestion with *Pst*I and isolated on agarose gels.

[b] Total cellular RNA was prepared from mammary glands of primiparous BALB/c mice (4 to 6 mice per determination) as described in the text. RNA (5 μg) was reacted in dot-blot hybridization assays with the indicated MMTV probe. Early pregnancy - 6 to 8 days of gestation; mid pregnancy - 12 to 14 days of gestation; lactating - 1 week after delivery; regressing - >30 days after weaning. Numbers represent relative detection of transcripts normalized to maximum detection (100) at lactation for each probe. Numbers in parentheses indicate the absolute values from the densitometer scans on which the normalized values are based.

* Corresponding author.

TABLE 2. MMTV gene expression during BALB/c mammary gland development in carefully timed pregnancies

Gene probe	Stage of mammary gland development[a]							
	Virgin	Day 6	Day 9	Day 12	Day 16	Day 19	Lactating (3 wk)	Regressing
LTR	12	11	37	33	65	45	100	50
env	22	15	30	11	29	19	100	14
gag-pol[b]	3	12	12	4	5	7	100	1

[a] The approach was the same as that detailed in the footnotes to Table 1, except that the days of pregnancy were precisely timed. The gestation period for the mouse is 21 days. The mammary glands of 4 to 6 animals were pooled for each point. The numbers represent relative detection of transcripts normalized to maximum detection at lactation (100) for each probe. The ratio of actual levels of the three transcripts at lactation was approximately 100:20:<5 for LTR:*env*:*gag-pol*, although differences in specific activities and sizes of probes were not considered.

[b] Levels of *gag-pol* RNAs are lower than those shown in Table 1. Contaminating envelope sequences were removed from the *gag-pol* probe (11) by digestion with *Eco*RI and purification from agarose gels.

and *env* hybridizing sequences (Table 1); as transfer of large-molecular-weight RNA species from the separating gel may not be quantitative, small amounts of the 8.9-kb message might be present but not observable in these assays. The kinetics of appearance and disappearance of LTR-specific RNA differed from that of *env* and *gag-pol* messages (Tables 1 and 2; Fig. 1). This difference suggests that the LTR message arises from transcription of a different template from that yielding *env* or *gag-pol* RNA.

Some studies have failed to detect any but LTR-specific sequences in BALB/c mice (1, 16, 23); this may reflect differences in colony history. However, *env*- and *gag*-related proteins can be detected by immunoblotting (E. Durban, D. Medina, and J. S. Butel, manuscript in preparation) and by competition radioimmunoassay (21), so limited transcription of more extensive regions of the MMTV genome must occur under certain physiological conditions.

BALB/c mice are not infected with exogenous virus. To ensure that MMTV-specific RNAs detected in the mammary glands of BALB/c mice were transcribed from endogenous proviruses, it was necessary to establish that the mice did not harbor an infection with exogenous virus. Infected mice exhibit extensive viral replication in the mammary glands of lactating animals, accompanied by frequent new proviral insertions in the chromosomal DNA of those cells. It is possible to distinguish exogenous proviral sequences from endogenous ones with enzymes that cleave MMTV DNA into subviral fragments. DNA isolated from pooled mammary glands from five lactating BALB/c mice was digested with *Pst*I or *Sst*I and analyzed by Southern hybridization (Fig. 2A) by using a *gag-pol*-specific probe. Digestion with *Pst*I yielded only two *gag-pol*-specific bands 5.9 and 5.4 kb in size. These fragments are those predicted from endogenous units III and II, respectively. Confirmatory results were obtained when lactating-gland DNA was digested with *Sst*I. This enzyme cleaves each intact BALB/c endogenous provirus only once. Unit I was not detected in these blots because it does not contain *gag-pol*-specific sequences.

BALB/c proviruses are differentially hypomethylated during mammary gland development. We wished to determine whether the response of the endogenous units to the hormonal stimulation of pregnancy was governed more by their presumed sequence similarities or by their obvious differences in chromosomal domain. Because the proviral units of MMTV in BALB/c mice are well characterized (and defined by *Eco*RI fragments) (5), it is possible to assess the extent of methylation of each unit at different stages of development and identify which units might be candidates to serve as templates of the observed transcription. If DNA is digested with *Eco*RI alone and compared with the same preparation digested with *Eco*RI and *Hpa*II, the diagnostic *Eco*RI fragment(s) is diminished in the double digest in samples with undermethylation of certain MMTV sequences (8). Results of such an experiment in which the LTR probe was used for hybridization are shown in Fig. 2B. Five MMTV-specific *Eco*RI fragments were detected in DNA from mammary glands of virgin and lactating mice. Partial demethylation of the 16-kb band was apparent in virgin glands, as evidenced by decreased reactivity of that band in the double digest for hybridization to the LTR probe, as well as by generation of slightly lower-molecular-weight fragments. This 16-kb band represents unit I, a subgenomic provirus consisting largely of LTR-related sequences (5). Other fragments containing MMTV sequences appeared relatively hypermethylated in virgin animals. DNA from lactating mammary glands, however, exhibited significant hypomethylation of the 16- and 10-kb bands, the latter representing the 3′ half of unit III (Mtv-9), which is known to encode envelope-specific information. The remaining *Eco*RI fragments appeared to be relatively unaffected by digestion with *Hpa*II, indicating a higher degree of methylation of those sequences. The extent of methylation of each *Eco*RI fragment in virgin and lactating animals is compared in Fig. 2C, which emphasizes that unit I and the 3′ end of unit III are undermethylated in lactating mammary tissue of BALB/c mice.

The methylation data tend to support the possibility of transcription of the 1.6-kb LTR message from the unit I template. The fact that unit I is hypomethylated relative to the other proviruses at all stages of development may be indicative of increased transcription from that region.

Noncoordinate regulation of expression of endogenous

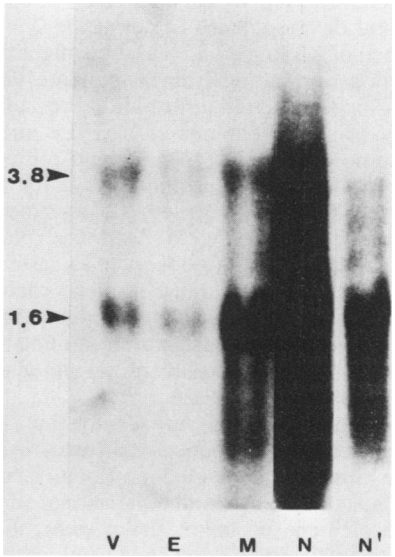

FIG. 1. Northern analysis of MMTV-specific RNAs. Total cellular RNAs from pooled mammary glands of 4 to 6 primiparous BALB/c mice were sized on a formaldehyde denaturing gel (12), blotted onto nitrocellulose, and hybridized (26) against the LTR probe (11). RNA preparations (20 μg) from virgin (V), early pregnant (E), midpregnant (M), and nursing (N,N′) mice were analyzed. Lane N′ represents a shorter exposure of lane N. The positions of the 3.8- and 1.6-kb transcripts are indicated on the left.

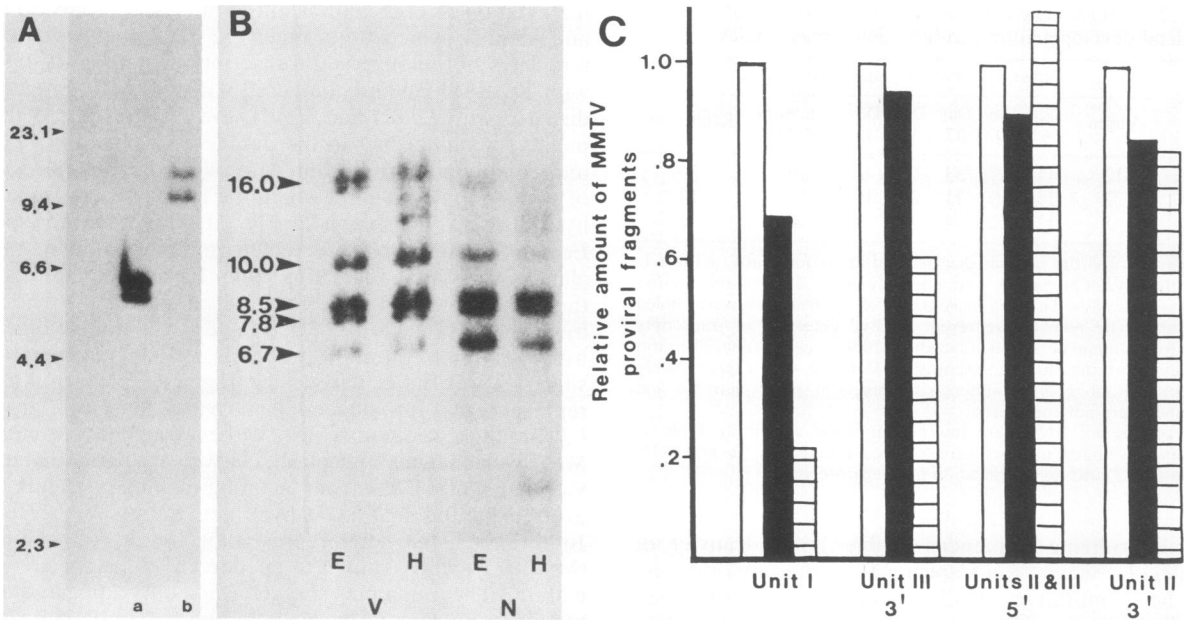

FIG. 2. MMTV proviruses and their methylation patterns in BALB/cCrl/Med mice. DNA (10 μg) isolated (7) from pooled mammary glands of 4 to 6 primiparous animals was digested with restriction enzyme, separated on a 0.8% agarose gel (12), blotted onto nitrocellulose (20), and analyzed by hybridization (12) with an MMTV probe. Molecular weight markers (in kilobases) are indicated on the left. (A) Absence of exogenous MMTV infection. DNA from lactating (1 week) mice was digested with *Pst*I (lane a) or *Sst*I (lane b) and detected with a *gag-pol* probe (11). (B) Methylation of endogenous MMTV proviruses. DNA from mammary glands of virgin (V) or nursing (N) (1 week) animals was digested with *Eco*RI alone (E) or *Eco*RI and *Hpa*II (H). Hybridization was against the LTR probe (11). (C) Autoradiographs of blots described in B were scanned with a Kontes scanning densitometer. The average area of peaks (two experiments) is given, normalized to the detection of *Eco*RI fragments (open bars). Closed bars represent detection of *Eco*RI fragments after digestion of mammary gland DNA from virgin animals with *Eco*RI and *Hpa*II; striped bars represent DNA from glands of lactating animals after digestion with *Eco*RI and *Hpa*II. Unit I (Mtv-6) = 16.0 kb; unit III (Mtv-9) 3′ = 10.0 kb; unit II (Mtv-8) 3′ = 6.7 kb; units II and III 5′ = 8.5 and 7.8 kb.

MMTV sequences was observed and probably reflects different underlying mechanisms. Specific regions of the genome were expressed to very different extents over the course of gland development (Tables 1 and 2). The relative overabundance of LTR-specific message might be explained by transcription originating from a template unable to encode *gag-pol*- or *env*-related information (i.e., unit I). As the three MMTV proviral genomes of BALB/c mice reside on separate chromosomes (3, 18), this could represent regulation of transcription at the chromosomal level, a possibility supported by examination of methylation states of the endogenous units of MMTV (Fig. 2). Another explanation must be invoked, however, for the observed excess of *env* RNA over *gag-pol* transcripts. As units II and III each contain the appropriate sequences, it might be supposed that a preferential splicing event results in greater amounts of *env* message. Altered stability of certain transcripts also cannot be ruled out at this time.

MMTV is unique among viruses in that it has well-characterized genomic sequences that are responsible for interaction with the cellular glucocorticoid receptor (2, 10, 13–15, 19). Thus, it may respond to hormonal stimulation not only as one element of many under generalized cellular growth control mechanisms but as an individual gene with specific positive transcriptional regulation. A similar interaction with hormone receptors presumably is possible at specific sequences within each proviral LTR. However, it appears that this interaction, if it occurs, plays a secondary role in regulation of RNA accumulation. Otherwise, one would have expected to detect the three transcripts regulated in parallel, as well as the utilization of both intact templates. Hormonal effects may be accomplished within the context of an accessible gene (17) that might, in turn, be determined by more extensive chromosomal conformations.

This study was supported in part by Public Health Service grants CA33369 and CA25215 from the National Cancer Institute.

LITERATURE CITED

1. **Breznik, T., V. Traina-Dorge, M. Gama-Sosa, C. W. Gehrke, M. Ehrlich, D. Medina, J. S. Butel, and J. C. Cohen.** 1984. Mouse mammary tumor virus DNA methylation: tissue-specific variation. Virology **136**:69–77.
2. **Buetti, E., and H. Diggelmann.** 1983. Glucocorticoid regulation of mouse mammary tumor virus: identification of a short essential DNA region. EMBO J. **2**:1423–1429.
3. **Callahan, R., D. Gallahan, and C. Kozak.** 1984. Two genetically transmitted BALB/c mouse mammary tumor virus genomes located on chromosomes 12 and 16. J. Virol. **49**:1005–1008.
4. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid 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. **Deeley, R. G., J. I. Gordon, A. T. H. Burns, K. P. Mullinix, M. Bina-Stein, and R. F. Goldberger.** 1977. Primary activation of the vitellogenin gene in the rooster. J. Biol. Chem. **252**: 8310–8319.
7. **Frisby, D. P., R. A. Weiss, M. Roussel, and D. Stehelin.** 1979. The distribution of endogenous chicken retrovirus sequences in the DNA of Galliform birds does not coincide with avian phylogenetic relationships. Cell **17**:623–634.
8. **Gunzburg, W. H., and B. Groner.** 1984. The chromosomal

integration site determines the tissue-specific methylation of mouse mammary tumor virus proviral genes. EMBO J. **3**:1129–1135.
9. **Hilgers, J., and M. Sluyser (ed.).** 1981. Mammary tumors in the mouse. Elsevier/North-Holland Publishing Co., Amsterdam.
10. **Majors, J., and H. E. Varmus.** 1983. A small region of the mouse mammary tumor virus long terminal repeat confers glucocorticoid hormone regulation on a linked heterologous gene. Proc. Natl. Acad. Sci. USA **80**:5866–5870.
11. **Majors, J. E., and H. E. Varmus.** 1981. Nucleotide sequences at host-proviral junctions for mouse mammary tumor virus. Nature (London) **289**:253–258.
12. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Miller, P. A., M. C. Ostrowski, G. L. Hager, and S. S. Simons, Jr.** 1984. Convalent and non-covalent receptor-glucocorticoid complexes preferentially bind to the same regions of the long terminal repeat of murine mammary tumor virus proviral DNA. Biochemistry **23**:6883–6889.
14. **Pfahl, M., D. McGinnis, M. Hendricks, B. Groner, and N. E. Hynes.** 1983. Correlation of glucocorticoid receptor binding sites on MMTV proviral DNA with hormone inducible transcription. Science **222**:1341–1343.
15. **Ponta, H., N. Kennedy, P. Skroch, N. E. Hynes, and B. Groner.** 1985. Hormonal response region in the mouse mammary tumor virus long terminal repeat can be dissociated from the proviral promoter and has enhancer properties. Proc. Natl. Acad. Sci. USA **82**:1020–1024.
16. **Popko, B. J., and R. J. Pauley.** 1984. Organization and expression of mouse mammary tumor virus sequences in normal and neoplastic C3Hf/HeSed mouse tissues. J. Virol. **52**:328–335.
17. **Ringold, G. M.** 1983. Regulation of mouse mammary tumor virus gene expression by glucocorticoid hormones. Curr. Top. Microbiol. Immunol. **106**:79–103.
18. **Robbins, J. M., D. Gallahan, E. Hogg, C. Kozak, and R. Callahan.** 1986. An endogenous mouse mammary tumor virus genome common in inbred mouse strains is located on chromosome 6. J. Virol. **57**:709–713.
19. **Scheiderert, C., and M. Beato.** 1984. Contacts between hormone receptor and DNA double helix within a glucocorticoid regulatory element of mouse mammary tumor virus. Proc. Natl. Acad. Sci. USA **81**:3029–3033.
20. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
21. **Teramoto, Y. A., D. Medina, C. McGrath, and J. Schlom.** 1980. Noncoordinate expression of murine mammary tumor virus gene products. Virology **107**:345–353.
22. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77**:5201–5205.
23. **Van Ooyen, A. J. J., R. J. A. M. Michalides, and R. Nusse.** 1983. Structural analysis of a 1.7-kilobase mouse mammary tumor virus-specific RNA. J. Virol. **46**:362–370.
24. **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.
25. **Varmus, H. E., G. Ringold, and K. R. Yamamoto.** 1979. Regulation of mouse mammary tumor virus gene expression by glucocorticoid hormones, p. 253–278. *In* J. Baxter and G. Rousseau (ed.), Glucocorticoid hormone action. Springer-Verlag KG, Berlin.
26. **Wheeler, D. A., J. S. Butel, D. Medina, R. D. Cardiff, and G. L. Hager.** 1983. Transcription of mouse mammary tumor virus: identification of a candidate mRNA for the long terminal repeat gene product. J. Virol. **46**:42–49.