JOURNAL OF VIROLOGY, Nov. 1988, p. 4410–4413
0022-538X/88/114410-04$02.00/0

Vol. 62, No. 11

Transactivating Potential of the 3′ Open Reading Frame of Murine Mammary Tumor Virus

P. VAN KLAVEREN* AND P. BENTVELZEN

Department of Retrovirology, Radiobiological Institute TNO, P.O. Box 5815, 2280 HV Rijswijk, The Netherlands

Received 18 April 1988/Accepted 8 August 1988

The procaryotic chloramphenicol acetyltransferase (CAT) gene controlled by the murine mammary tumor virus (MMTV) promoter shows reduced activity in a rat mammary tumor cell line after infection with MMTV but to a considerably lesser extent than the CAT gene controlled by a heterologous promoter, indicating *trans*-acting regulation of promoter activity by MMTV. Cotransfection of vectors capable of expressing RNA from the 3′ open reading frame (*orf*) of MMTV with the CAT-MMTV construct resulted in enhanced CAT activity, suggesting that *orf* carries a transactivating potential. Since transactivation was also found with a vector containing only *orf* and part of the viral *env* gene, it was concluded that a separate transcriptional unit exists for the *orf* message.

The murine mammary tumor virus (MMTV) genome contains at its 3′ terminus an open reading frame of 960 nucleotides in addition to the common retroviral genes *gag*, *pol*, and *env*. This *orf* sequence is part of the proviral long terminal repeat (LTR) (6, 7, 10, 14, 16). In vitro it can code for four polypeptides with apparent molecular weights of 36,000, 24,000, 21,000, and 18,000, respectively (4, 5, 23, 24). The *orf* sequence may be responsible for enhanced lobuloalveolar development of the mammary gland (2, 3), preceding neoplastic conversion associated with the activation of otherwise dormant cellular *int* oncogenes by MMTV provirus integrated in their vicinity (11, 19, 20, 22). By analogy to human T-cell leukemia virus types I and II (26), the bovine leukemia virus (25), and the human immunodeficiency virus (1), we assumed that the *orf* sequence of MMTV could code for a *trans*-acting protein, which would stimulate transcription directed by the MMTV promoter and presumably also stimulate transcription of some cellular genes.

If transactivation occurs in the MMTV replication process, it should be detectable as a difference between infected and uninfected cells. At least one report describes the lack of a difference in MMTV promoter activity in the murine mammary tumor cell line 34i cl-101 as compared with the murine fibroblastic L-cell line (21). To our knowledge, however, there are no reports that deal with MMTV-infected and uninfected cells of the same origin. To compare MMTV-infected and uninfected cells, we turned to a rat mammary tumor cell line, SDMT/cl3. This cell line was derived from a spontaneous carcinosarcoma occurring in a 2-year-old SD/Rij breeding female in our animal facility (M. A. Dubbeld, personal communication). Infection with a host-range variant of MMTV (13) was checked by Northern blot analysis using a nick-translated probe of the MMTV LTR. All three virus-specific messages of 8.4, 4.0, and (faintly) 1.6 kilobases were found to be present in the infected cell line and absent in the uninfected control. Both infected and uninfected SDMT/cl3 cells were transfected with constructs with the chloramphenicol acetyltransferase (CAT) gene fused to either the simian virus 40 (SV40) promoter or the MMTV promoter (Fig. 1A). Cells were grown for 48 h in the presence of 5×10^{-6} M dexamethasone before being lysed. CAT assays were performed as described before (12). The protein content of all of the preparations was determined. Total amounts of protein in the samples for the CAT assays varied from 60 to 150 μg. Each value was matched to the equivalent of 100 μg of total protein.

The time course of the CAT assays of uninfected (Fig. 2A) and infected (Fig. 2B) SDMT/cl3 cells transfected with the plasmids containing the CAT gene under control of the SV40 promoter (pSV2CAT) or the MMTV promoter (pSupL) was determined. Overall CAT activities in the infected cells were lower than those in the uninfected cells. This is not due to differences in growth characteristics or cellular metabolism. Most likely it represents some infection-associated repression factor. The inactivation due to MMTV infection as seen in Fig. 2 is not exerted specifically on the MMTV LTR, since the SV40 promoter activity was also markedly decreased in the infected cells. The same holds true for a construct, pADA-CAT, containing the CAT gene under control of the promoter of the human adenosine deaminase gene (courtesy of D. Valerio). After 90 min of incubation in the CAT assay, uninfected cells showed 4.0% acetylation, which was reduced to 1.4% in the infected cells. This points to the existence of an aspecific down-regulation of certain promoters as a result of MMTV infection.

In the uninfected SDMT/cl3 cell line both the SV40 and the MMTV promoter were of approximately equal strength, but in the infected SDMT/cl3 cells the MMTV promoter was clearly the stronger one. After 90 min of incubation the relative strength of the MMTV promoter was 0.97 in the uninfected and 4.89 in the infected cells (a three- to fivefold difference was routinely observed). This difference could be interpreted as partial inactivation of both promoters due to MMTV infection, counteracted by specific transactivation of the MMTV promoter.

To examine whether MMTV *orf* could exert this possible transactivating potential, we used various plasmids containing the MMTV *orf* sequence (see Fig. 1B) as donors for transactivation. As determined by slot-blot analysis (Fig. 3), all of these plasmids were capable of synthesizing *orf*-specific RNA at levels of 0.3 to 1% of total cellular RNA, except pTorf. Digestion of pEnorf with *Bam*HI, which separates *env* sequences from bacterial sequences, did not influence RNA production, whereas digestion with *Pst*I, which dissociates the LTR from *env*, completely abolished RNA production. Transcription of *orf* could also be evoked

* Corresponding author.

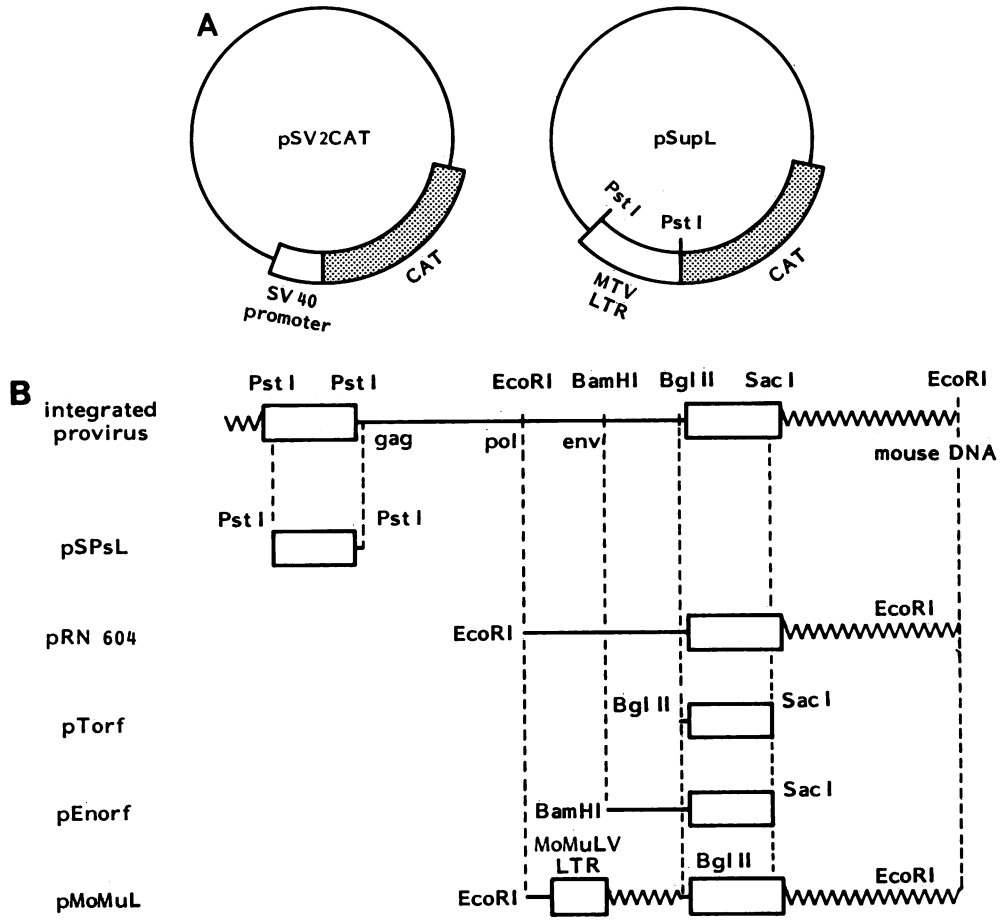

FIG. 1. Schematic representation of the constructs used either as targets (A) or donors (B) for transactivation of promoters directing the CAT gene. Donors include the following. pSPsL is a plasmid in which the first of the two tandem SV40 transcriptional units of pLO (8) was used to drive the 5′ MMTV LTR (open box) *Pst*I fragment. This sequence lacks the first initiation codon of *orf*. pRN604 is a pAT153-derived plasmid inserted with the junction of an MMTV provirus and the *int-1* gene isolated from mouse mammary tumor 604 DNA (a gift from R. Nusse from the Netherlands Cancer Institute). This plasmid contains the entire proviral *env* gene, the LTR, and flanking mouse DNA. pTorf is a pGEM-1 plasmid (Promega Inc., Leiden, The Netherlands), inserted with the entire LTR *orf*. The insert was cloned from the *Bgl*II site, 43 base pairs (bp) before the first initiation codon of *orf*, and the *Sac*I site, 27 bp beyond the termination codon. Plasmid pEnorf is as for pTorf, inserted with the complete *orf* sequence and the 3′ part of the *env* gene (size, 1 kbp). Plasmid pMoMuL was made by replacing in pRN604 the *env* gene by the LTR of Mo-MuLV. This LTR is flanked by approximately 200 bp of mouse DNA at the 3′ side and by approximately 200 bp of Mo-MuLV *env* sequences at the 5′ side.

by fusion to a heterologous eucaryotic promoter such as the SV40 promoter (pSPsL) or the Moloney murine leukemia virus (Mo-MuLV) LTR (pMoMuL).

These expression vectors were cotransfected with either the transactivation-susceptible target plasmid (pSupL) or the inert target plasmid (pSV2CAT) to NIH 3T3 cells. The highest relative promoter strength of the MMTV promoter, as compared with the SV40 one (which remained constant throughout the experiment, ruling out inactivation), was obtained with the plasmid pMoMuL (Table 1). This plasmid contains MMTV *orf*, whose expression is controlled by the promoter present in the LTR of Mo-MuLV.

The pSPsL construct, which contains the widely used *Pst*I fragment of the 5′ MMTV LTR fused to the SV40 promoter, had little stimulatory effect on the expression of the CAT gene directed by the MMTV promoter. This is not due to lack of transcriptional activity (Fig. 3 and Table 1). The *Pst*I fragment of the LTR lacks only a few nucleotides at the beginning of the *orf* sequence, including the first AUG codon. All four sequenced MMTV strains contain four internal AUG codons within *orf*. Initiation of protein synthesis on either of these starting codons would lead to proteins with molecular weights of 32,000, 24,000, 21,000 or 18,000 (6). Racevskis and Prakash (24) have argued, using the postulations put forth by Kozak (15), that the relative efficiency of the initiation codon of the 32,000-molecular-weight protein is far less than that of the 24,000-molecular-weight protein. They found an excellent correlation with the amount of in vitro translation products encoded by hybrid-selected RNAs isolated from mammary glands of lactating GR mice. Therefore, initiation at the next probable AUG codon could result in the loss of as much as one third of the coding potential. The presence of a complete *orf* sequence seems to be required for efficient stimulation of the CAT gene directed by the MMTV promoter.

High ratios for the MMTV promoter were also obtained by cotransfecting the plasmids pEnorf or pRN604, containing MMTV *orf* and a large part of the adjacent *env* gene. The pTorf plasmid, which contains the complete *orf* sequence of MMTV but only a small part of *env*, had no positive effect on

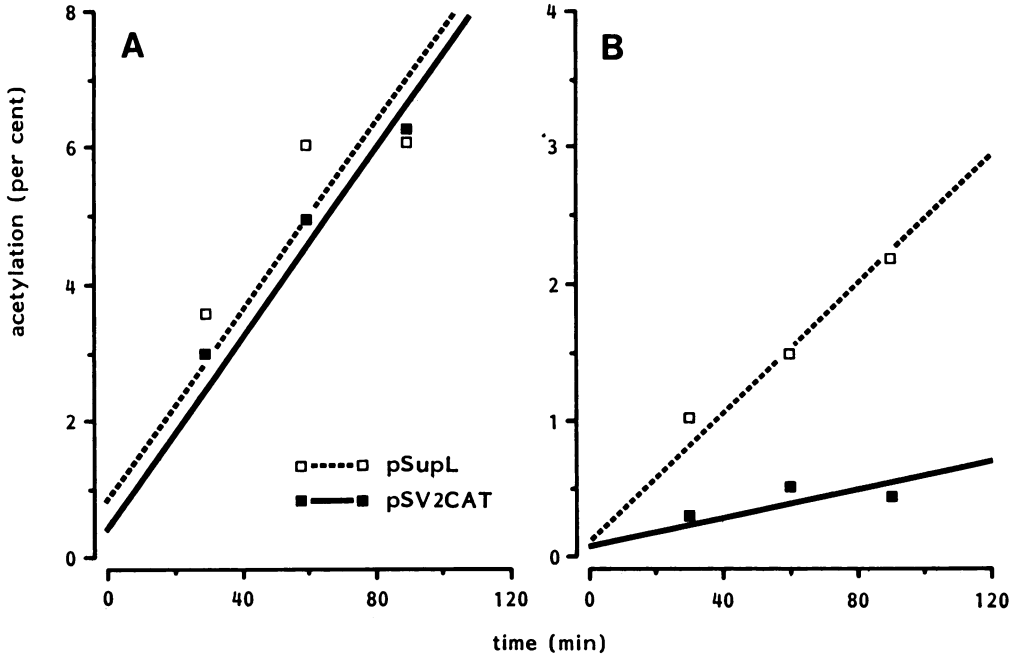

FIG. 2. Time course of the CAT assays performed on rat mammary tumor cells of line SDMT/cl3 (A) and on SDMT/cl3 cells infected with MMTV (B). Cells were transfected with 10 μg of a plasmid with the CAT gene under the direction of either the SV40 promoter (pSV2CAT, ■) or the MMTV LTR (pSupL, □) and were grown for 48 h in the presence of 5×10^{-6} M dexamethasone. CAT assays were performed as described (12). Percentage acetylation is shown as a function of the incubation time in the assay. Lines are best-fitted straights including the origin.

the CAT gene driven by the MMTV promoter. The MMTV LTR contains a region responsive to a negative regulatory factor (18, 21), probably a labile cellular protein. This could counteract transactivation to some extent, but could also result in an increase in CAT activity by dilution of this factor after cotransfection with an LTR-containing expression plasmid. The fact that pTorf and pEnorf contain the same part of the LTR, but have different effects on CAT activity, rules out this latter possibility. The lack of MMTV-specific RNA after transfection of pTorf in contrast to pEnorf indicates that transcription of *orf* is needed for transactivation.

These results also suggest that the *env* gene contains a promoter that may control the expression of *orf*. The *env* gene contains several TATA boxes (17), one of which is thought to be the inducible promoter present in *env* from which an LTR-specific RNA is synthesized in EL4.E1 cells (9). The transcription of this RNA does not stem from the 5′ LTR. The hypothesis of a separate transcription unit for the *orf* message is (at least for endogenous MMTV) supported by the identification of a 1.7-kilobase RNA molecule containing the *orf* sequence in lactating mammary glands of uninfected BALB/c mice (27).

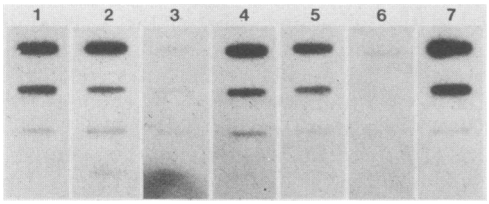

FIG. 3. RNA production of the various expression vectors as depicted in Fig. 1A, produced after transfection and incubation under conditions identical to the CAT assay. Total cellular RNA (amounts ranging from 2 to 20 μg) was spotted in one to five serial dilutions on nitrocellulose and hybridized to an *orf*-specific (1.1 kb *Bgl*II-*Sst*I LTR fragment) probe. Lanes: 1, pSPsL; 2, pRN604; 3, pTorf; 4, pEnorf; 5, pMoMuL; 6, expression vector pLO without LTR sequences; 7, MMTV RNA reference starting at 20 ng.

We thank A. L. J. Kneppers for excellent technical assistance. Plasmid pRN604 was kindly provided by R. Nusse, and pLO was provided by L. Dorssers. We are grateful to R. Michalides and D. Valerio (who also provided plasmid pADA-CAT) for reading the manuscript.

TABLE 1. Influence of cotransfection with activator donor plasmids on target plasmid CAT activity[a]

Activator plasmid	% RNA production[b]	Target promoter	% Acetylation	Relative promoter strength (MMTV/SV40)
pSPsL	0.3	MMTV LTR	4.6	1.8
		SV40	2.5	
pRN604	1.0	MMTV LTR	11.5	4.3
		SV40	2.7	
pTorf	<0.01	MMTV LTR	1.8	0.9
		SV40	2.0	
pEnorf	0.5	MMTV LTR	12.0	6.3
		SV40	1.9	
pMoMuL	0.6	MMTV LTR	23.5	7.3
		SV40	3.2	

[a] CAT assays were performed after the donor plasmids (Fig. 1B), together with either pSupL or pSV2CAT (Fig. 1A) at 10 μg each, were cotransfected to NIH 3T3 cells grown on a 10-cm dish. Lysates were incubated for 1 h. Every experiment was repeated at least once to test reproducibility of the observations.

[b] RNA production is expressed as a percentage of total cellular RNA determined by slot-blot hybridization (see Fig. 3).

LITERATURE CITED

1. **Arya, S. K., C. Guo, and F. Wong-Staal.** 1985. *trans*-Activator gene of human T-lymphotropic virus type III (HTLV III). Science **229**:69–73.
2. **Bentvelzen, P.** 1982. The putative mam gene of the murine mammary tumor virus. Adv. Viral Oncol. **1**:141–152.
3. **Bentvelzen, P., and J. Hilgers.** 1980. Murine mammary tumor virus, p. 311–355. *In* G. Klein (ed.), Viral oncology. Raven Press, Publishers, New York.
4. **Dickson, C., and G. Peters.** 1981. Protein-encoding potential of mouse mammary tumor virus genome RNA as examined by in vitro translation. J. Virol. **37**:36–47.
5. **Dickson, C., R. Smith, and G. Peters.** 1981. *In vitro* synthesis of polypeptides encoded by the long terminal repeat region of mouse mammary tumor virus DNA. Nature (London) **291**:511–513.
6. **Donehower, L. A., B. Fleurdelys, and G. L. Hager.** 1983. Further evidence of the protein-coding potential of the mouse mammary tumor virus long terminal repeat: nucleotide sequence of an endogenous proviral long terminal repeat. J. Virol. **45**:941–949.
7. **Donehower, L. A., A. L. Huang, and G. L. Hager.** 1981. Regulatory and coding potential of the mouse mammary tumor virus long terminal repeat redundancy. J. Virol. **37**:226–238.
8. **Dorssers, L., H. Burger, F. Bot, R. Delwel, A. H. M. Geurts van Kessel, B. Löwenberg, and G. Wagemaker.** 1987. Characterisation of a human multilineage-colony-stimulating factor cDNA clone identified by a conserved noncoding sequence in mouse interleukin-3. Gene **55**:115–124.
9. **Elliot, J. F., B. Pohajdak, D. J. Talbot, J. Shaw, and V. Paetkau.** 1988. Phorbol diester-inducible, cyclosporin-suppressible transcription from a novel promoter within the mouse mammary tumor virus *env* gene. J. Virol. **62**:1373–1380.
10. **Fasel, N., K. Pearson, E. Buetti, and H. Diggelmann.** 1982. The region of mouse mammary tumor virus DNA containing the long terminal repeat includes a long coding sequence and signals for hormonally regulated transcription. EMBO J. **1**:3–7.
11. **Gallahan, D., and R. Callahan.** 1987. Mammary tumorigenesis in feral mice: identification of a new *int* locus in mouse mammary tumor virus (Czech II)-induced mammary tumors. J. Virol. **61**:66–74.
12. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
13. **Howard, D. K., and J. Schlom.** 1978. Isolation of host-range variants of mouse mammary tumour viruses that efficiently infect cells *in vitro*. Proc. Natl. Acad. Sci. USA **75**:5718–5722.
14. **Kennedy, N., G. Knedlitschek, B. Groner, N. E. Hynes, P. Herrlick, R. Michalides, and A. J. J. van Ooyen.** 1982. Long terminal repeat of endogenous mouse mammary tumor virus contains a long open reading frame which extends into adjacent sequences. Nature (London) **295**:622–624.
15. **Kozak, M.** 1981. Possible roles of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosomes. Nucleic Acids Res. **9**:5233–5252.
16. **Majors, J. E., and H. E. Varmus.** 1981. Nucleotide sequence at host-proviral junctions for mouse mammary tumor virus. Nature (London) **289**:253–258.
17. **Majors, J. E., and H. E. Varmus.** 1983. Nucleotide sequencing of an apparent proviral copy of *env* mRNA defines determinants of expression of the mouse mammary tumor virus *env* gene. J. Virol. **47**:495–504.
18. **Morley, K. L., M. G. Toohey, and D. O. Peterson.** 1987. Transcriptional repression of a hormone-responsive promoter. Nucleic Acids Res. **15**:6973–6989.
19. **Nusse, R., A. van Ooyen, D. Cox, Y. K. T. Fung, and H. Varmus.** 1984. Mode of proviral activation of a putative mammary oncogene (*int*-1) on mouse chromosome 15. Nature (London) **307**:131–136.
20. **Nusse, R., and H. E. Varmus.** 1982. Many tumors induced by the mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell **31**:99–109.
21. **Ostrowski, M. C., A. L. Huang, M. Kessel, R. G. Wolford, and G. L. Hager.** 1984. Modulation of enhancer activity by the hormone responsive regulatory element from mouse mammary tumor virus. EMBO J. **3**:1891–1899.
22. **Peters, G., S. Broodes, R. Smith, and C. Dickson.** 1983. Tumorigenesis by mouse mammary tumor virus: evidence for a common region for provirus integration in mammary tumors. Cell **33**:369–377.
23. **Peters, G., R. Smith, S. Brookes, and G. Dickson.** 1982. Conservation of protein coding potential in the long terminal repeats of exogenous and endogenous mouse mammary tumor viruses. J. Virol. **42**:880–888.
24. **Racevskis, J., and O. Prakash.** 1984. Proteins encoded by the long terminal repeat region of mouse mammary tumor virus: identification by hybrid-selected translation. J. Virol. **51**:604–610.
25. **Rosen, C. A., J. G. Sodroski, L. Willems, R. Kettmann, K. Campbell, R. Zaya, A. Burny, and W. A. Haseltine.** 1986. The 3′ region of bovine leukemia virus genome encodes a *trans*-activator protein. EMBO J. **5**:2585–2589.
26. **Sodroski, J. G., C. A. Rosen, and W. A. Haseltine.** 1984. *trans*-Acting transcriptional activation of the long terminal repeat of human T lymphotropic viruses in infected cells. Science **225**:381–385.
27. **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.