8. M. Gottlieb, *J. Protozool.* **27**, 41A (1980).
9. C. De Duve, in *Lysosomes in Biology and Pathology*, J. T. Dingle and H. B. Fell, Eds. (North-Holland, Amsterdam, 1969), vol. 1, p. 3; *J. Cell Biol.* **50**, 20 (1971).
10. A. B. Novikoff, E. Essner, S. Goldfischer, M. Heus, in *Interpretation of Ultrastructure*, R. J. C. Harris, Ed. (Academic Press, New York, 1962), p. 149; A. B. Novikoff, in *Ciba Found. Symp.* **1963**, 36 (1963).
11. A. Solyom and E. G. Trams, *Enzyme* **13**, 329 (1972).
12. B. E. Brooker, *Z. Zellforsch.* **116**, 532 (1971); Y. Eeckhout, in *Comparative Biochemistry of Parasites*, B. Van Den, Ed. (Academic Press, New York, 1972); J. McLaughlin, H. A. Injeyan, E. Meerovitch, *Can. J. Biochem.* **54**, 365 (1976); B. E. Brooker and K. Vickerman, *Trans. R. Soc. Trop. Med. Hyg.* **58**, 293 (1964); S. G. Langreth and A. E. Balber, *J. Protozool.* **22**, 40 (1975); J. R. Seed, J. Byram, A. A. Gam, *ibid.* **14**, 117 (1967); S. Venkatesan, R. G. Bird, W. E. Ormerod, *Int. J. Parasitol.* **7**, 139 (1977); J. L. Avila, M. A. Casanova, A. Avila, A. Bretana, *J. Protozool.* **26**, 304 (1979); R. F. Steiger, F. Van Hoof, J. Bontemps, M. Nyssens-Jadin, J.-E. Druetz, *Acta Trop.* **36**, 335 (1979).
13. R. C. Hunt and D. J. Ellar, *Biochim. Biophys. Acta* **339**, 173 (1974); B. Zingales, C. Carniol, P. A. Abrahamson, W. Colli, *ibid.* **550**, 233 (1979); L. Rovis and S. Baekkekor, *Parasitology* **80**, 507 (1980).
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Activation of the Transforming Potential of a Normal Cell Sequence: A Molecular Model for Oncogenesis

Abstract. *The molecularly cloned, long terminal repeat (LTR) of the Moloney sarcoma virus (M-MSV) provirus has been covalently linked to* c-mos, *the cellular homolog of the M-MSV–specific sequence,* v-mos. *These newly constructed clones lack any M-MSV–derived sequences other than the LTR, but in DNA transfection assays they transform cells as efficiently as cloned subgenomic M-MSV fragments containing both* v-mos *and LTR. Cells transformed by LTR:*c-mos *hybrid molecules contain additional copies of* mos *DNA, and several size classes of polyadenylated RNA's with sequence homology to* mos. *The activation of the transforming potential of* c-mos *by the proviral LTR suggests a model whereby LTR-like elements could activate other normal cell sequences with oncogenic potential.*

The transforming retroviruses (that have short periods of latency) (*1–3*) provide an appropriate system for studying the activation of the oncogenicity of normal cellular genes. One such virus, Moloney murine sarcoma virus (M-MSV), is a recombinant between a cellular sequence, *mos*, and Moloney leukemia virus (M-MuLV) (*4–6*). The M-MSV–specific sequence is termed v-*mos* as part of the viral genome while the cellular homolog in normal mouse DNA is termed c-*mos*. We isolated biologically active recombinant DNA clones containing the entire M-MSV proviral genomes of the m1 and HT1 strains (*7, 8*) and used the cloned v-*mos* sequence to identify its cellular homolog and isolate it from normal mouse cell DNA (*9*).

The M-MSV long terminal repeat (LTR) enhances the transforming efficiency of M-MSV v-*mos* (*10, 11*). The proviral LTR contains putative transcriptional control elements (*12–15*), suggesting that the LTR may provide initiation and termination signals for v-*mos* messenger RNA (*10–12*). Cloned cellular DNA containing c-*mos* does not transform cells, although by restriction and heteroduplex analysis c-*mos* and v-*mos* are indistinguishable (*9*). However, c-*mos* was activated by replacing all sequences 5′ to c-*mos* with nontransforming sequences derived from the 5′ end of the m1 MSV provirus, including an LTR (*9, 16*). This result does not establish whether the LTR alone is sufficient to activate the transforming potential of c-*mos*. Therefore, we constructed recombinant DNA clones with an LTR inserted into mouse genomic DNA at various positions 5′ to c-*mos*, and tested their transforming activity. Some of these results have been described (*11*).

The recombinant clones used in our study (Fig. 1) were constructed with the use of LTR sequences from plasmid pm1sp (Fig. 1) (*7, 8, 13*) and c-*mos* sequences from plasmid pMSI (Fig. 1) (*9*). This plasmid is inactive in DNA transfection-transformation assays (*9*), while cloned intact m1 MSV proviral DNA (not shown) can induce 53,000 foci per picomole of DNA (*10*). Clones containing both c-*mos* and LTR sequences (pTS series) also transform. Their specific activities are lower than that of intact cloned M-MSV provirus but equivalent to M-MSV subgenomic fragments that contain v-*mos* and a single LTR (*10*). Clones lacking an LTR are either inactive (pMSB7), or exhibit very low activity (pMSB30; see below). Thus the LTR alone provides all the functions required for efficient activation of the transforming potential of c-*mos* and is capable of activating this sequence even if it is inserted at a distance of about 1500 base pairs (bp) in front of c-*mos* (Fig. 1, pTS101). Furthermore, the entire LTR is not required for c-*mos* activation since the pTS clones lack the 115 bp at the 3′ terminal of LTR, which includes a puta-

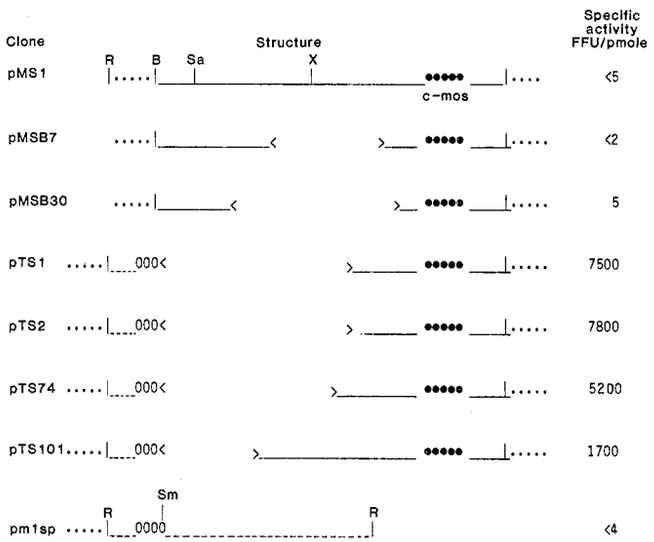

Fig. 1. Physical maps and specific activities of recombinant clones containing *c-mos*; (●●●) *c-mos* (*9*); (000) LTR (*12, 13*); (——) normal mouse DNA sequences (*9*); (····) plasmid pBR322 sequences; (- - -) normal mink sequences (*7, 8*). Restriction endonuclease sites are labeled *R*, Eco RI; B, Bgl II or Bam HI; Sa, Sac I; X, Xba I; Sm, Sma I. The pointed brackets (< >) approximately enclose the Bal 31 deletions. To generate the clones shown, plasmid pMSI was opened at either the Sac I or Xba I site and subjected to limited digestion with exonuclease Bal 31 to remove varying amounts of normal mouse cellular sequences. The resulting DNA was either closed again without further treatment (as in pMSB7 and pMSB30), or it was digested with Eco RI and linked to an Eco RI–Sma I fragment from plasmid pm1sp to generate the four pTS clones. The plasmid pMSB7 has ~ 1000 bp of normal mouse sequence removed by this procedure, whereas pMSB30 has ~ 1300 bp removed. In pTS1 and pTS2, the LTR is placed ~ 600 bp upstream to the 5′ end of c-*mos*, while in pTS74 and pTS101 the LTR is ~ 800 and ~ 1500 bp upstream, respectively. Cloned DNA was linearized by treatment with either Eco RI or Bam HI and then was used to transfect NIH 3T3 cells as described (*10*). Specific activities represent the number of foci induced per 2.5×10^5 cells transfected, and were calculated from at least four replicate determinations. The lower limits represent the limit of detectability (no foci observed) in these determinations.

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tive polyadenylation site (*12*). However, pTS recombinants do retain a putative promoter (*12*) and the sequence encoding the 5′ end of the genomic viral RNA.

Cellular DNA and polyadenylated RNA from cloned cell lines obtained from pTS1 DNA–induced foci were examined for the presence and expression of transfected c-*mos* sequences. Normal mouse DNA treated with Eco RI yields a single 15-kb DNA fragment containing c-*mos* (*9*), but additional *mos*-containing bands are detected in the Eco RI–digested DNA from pTS1 transformed cells (Fig. 2a, lane 1). Similarly, Sac I digestion of normal mouse DNA yields one band containing c-*mos* (not shown), while pTS1 transformed cell DNA digested with Sac I yields multiple bands (Fig. 2a, lane 2). Therefore, pTS1 transformed cells contain multiple copies of *mos* in contrast to the single copy observed in normal NIH 3T3 cells.

To determine whether the transfected *mos* sequences are actively transcribed in transformed cells, we compared polyadenylated RNA's from pTS1 transfected cells and NIH 3T3 cells. A *mos*-specific probe detects four bands in polyadenylated RNA from pTS1 transfected cells, while no bands are detected in RNA from NIH 3T3 cells (Fig. 2b, lanes 1 and 2). Similar results were obtained with the pm1sp plasmid clone as probe (Fig. 2b, lanes 3 and 4). Thus, the additional *mos* sequences in pTS1 transformed cells are transcribed into several RNA species. Further experiments (*17*) indicate both LTR and pBR322 sequences are also expressed in these cells. All of the bands observed are larger than the size expected on the basis of the LTR-*mos* separation in pTS1 (Fig. 1).

We can explain these results by proposing that the LTR provides a promoter sequence that allows the expression of *mos* and results in efficient transformation. This is consistent with our observation that v-*mos* transforms efficiently when the proviral LTR is covalently linked 5′ to the v-*mos* sequence (*10*). However, the precise mechanism for this activation is not known, and we cannot eliminate other roles for the LTR in c-*mos* activation. For example, it could act in some unknown way to assure the maintenance of the transfected DNA sequences.

These data demonstrate that it is possible to activate the transforming potential of a specific cellular gene, c-*mos*. They also suggest that the proviral LTR could be used in a cloning vehicle to activate various cellular sequences. Others have shown that transfection of genomic DNA from normal and chemically transformed mammalian cells will morphologically transform normal mouse cells (*18–20*). Transformation by sheared, normal genomic DNA is postulated to occur as a result of the activation of normal cell sequences with transforming potential (*19, 20*). Specific cellular sequences responsible for transformation and mechanisms by which they transform have not been identified. Cooper *et al.* (*19*) suggest that fragmentation of the cellular DNA removes sequences preventing the expression of transforming genes, and allows subsequent promotion of RNA synthesis from these genes by association with cellular promoters. Our data are consistent with this interpretation since plasmid pMSB30, from which most of the DNA sequences 5′ to c-*mos* have been removed, exhibits a low level of activity even though it lacks an LTR. The DNA rearrangements during transfection may occasionally position pMSB30 adjacent to carrier or host DNA sequences with promoter (LTR) -like functions. We have shown that the low transforming activity of v-*mos* is enhanced when cotransfected with equimolar amounts of LTR DNA (*10*). We believe that this increase is due to the more frequent association between LTR and the transforming v-*mos* sequence. Furthermore prior infection of recipient NIH 3T3 cells with either M-MuLV or Rauscher leukemia virus increases the efficiency of transformation by normal mouse DNA (*20*). In the absence of added LTR sequences (added either by cotransfection or prior infection), low-level transformation by sheared normal DNA may result by low-frequency association of transforming genes with cellular LTR-like sequences.

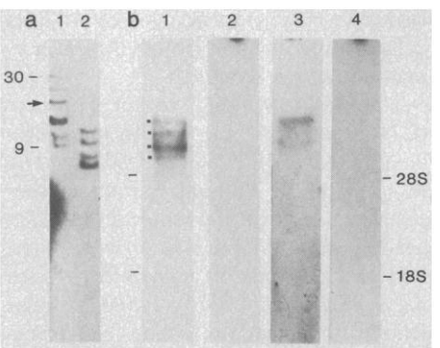

Fig. 2. Detection of pTS1 derived sequences in the genomic DNA and polyadenylated RNA of transformed cells. (a) High-molecular-weight DNA was prepared from NIH 3T3 cells and pTS1 DNA transformed NIH 3T3 cells (*7, 8*), was digested with either Eco RI (lane 1) or Sac I (lane 2) prior to electrophoresis on a 0.7 percent agarose gel, and analyzed by the Southern technique (*23*) with a *mos*-specific probe (*9*). The size of DNA bands detected is shown in kilobase pairs. The band corresponding to the 15-kb Eco RI normal mouse DNA fragment that contains c-*mos* sequences is indicated by an arrow. (b) Polyadenylated RNA was prepared from NIH 3T3 cells (lanes 2 and 4) and pTSI transformed NIH 3T3 cells (lanes 1 and 3). The RNA was fractionated by electrophoresis on 1.2 percent agarose gels containing 5 m*M* methylmercuric hydroxide (*24*), and analyzed (*25*) with the use of either a *mos*-specific probe (lanes 1 and 2) or plasmid pm1sp (containing LTR and pBR322 sequences) as probe (lanes 3 and 4). The relative mobilities of rabbit reticulocyte 18*S* and 28*S* RNA are shown.

Activation by the LTR-like sequences provides a useful model for both viral and nonviral oncogenesis (*11, 19*). The c-*mos* gene is just one of several cellular genes with oncogenic potential (*1–3*), and all may be capable of being activated in this fashion. Recently, it has been shown that some tumors induced by avian leukemia virus may result from proviral LTR-promoted expression of such a cellular gene c-*myc* (*21*). This could represent an in vivo equivalent of our in vitro activation of c-*mos*. Furthermore, endogenous LTR elements have striking structural parallels with both prokaryotic and eukaryotic transposable elements (*12–14, 22*). It is possible that LTR-like sequences activate the expression of quiescent cellular genes with transforming potential not only by insertion, but also by mediating DNA rearrangements. Any rearrangement which results in the juxtaposition of an active cellular promoter and a gene with oncogenic potential may result in cell transformation.

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References and Notes

1. P. J. Fischinger, in *Molecular Biology of RNA Tumor Viruses*, J. R. Stephenson, Ed. (Academic Press, New York, 1979), p. 163.
2. J. M. Bishop, *Annu. Rev. Biochem.* **47**, 35 (1978).
3. P. H. Duesberg, *Cold Spring Harbor Symp. Quant. Biol.* **44**, 13 (1979).
4. J. B. Moloney, *Natl. Cancer Inst. Monogr.* **22**, 139 (1966).
5. E. M. Scolnick, R. S. Howk, A. Anisowicz, P. Peebles, C. D. Sher, W. P. Parks, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4650 (1975).

6. A. E. Frankel, R. L. Neuebauer, P. J. Fischinger, *J. Virol.* **18**, 481 (1976).
7. G. F. Vande Woude, M. Oskarsson, L. W. Enquist, S. Nomura, M. Sullivan, P. J. Fischinger, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4464 (1979).
8. G. F. Vande Woude *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* **44**, 735 (1979).
9. M. Oskarsson, W. L. McClements, D. G. Blair, J. V. Maizel, G. F. Vande Woude, *Science* **207**, 1222 (1980).
10. D. G. Blair, W. McClements, M. Oskarsson, P. J. Fischinger, G. F. Vande Woude, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3504 (1980).
11. W. L. McClements, R. Dhar, D. G. Blair, L. Enquist, M. Oskarsson, G. F. Vande Woude, *Cold Spring Harbor Symp. Quant. Biol.*, in press.
12. R. Dhar, W. L. McClements, L. W. Enquist, G. F. Vande Woude, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3937 (1980).
13. W. L. McClements, L. W. Enquist, M. Oskarsson, M. Sullivan, G. F. Vande Woude, *J. Virol.* **35**, 488 (1980).
14. C. Shoemaker, S. Goff, E. Gilboa, M. Paskind, S. Mitra, D. Baltimore, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3932 (1980).
15. G. Ju and M. A. Skalka, *Cell* **22**, 379 (1980).
16. W. L. McClements, D. G. Blair, M. Oskarsson, G. F. Vande Woude, in *Animal Virus Genetics*, B. N. Fields, R. Jaenisch, C. F. Fox, Eds. (Academic Press, New York, 1980), pp. 455–460.
17. T. G. Wood, in preparation.
18. C. Shih, B. Z. Shilo, M. P. Goldfarb, A. Dannenberg, R. A. Weinberg, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5714 (1979).
19. G. M. Cooper, S. A. Okenquist, L. Silverman, *Nature (London)* **284**, 418 (1980).
20. V. Krup-Konvalinkova, and K. J. van den Berg, *ibid.* **287**, 353 (1980).
21. B. Neel, W. Hayward, H. Robinson, J. Fang, S. Astrin, *Cell* **23**, 323 (1981).
22. K. Shimotohno, S. Mizutani, H. M. Temin, *Nature (London)* **285**, 550 (1980); J. G. Sutcliffe, T. M. Schininck, I. M. Verma, R. A. Lerner, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3302 (1980); M. P. Carlos and J. H. Miller, *Cell* **20**, 579 (1980).
23. E. M. Southern, *J. Mol. Biol.* **38**, 503 (1975).
24. J. M. Bailey and N. Davidson, *Anal. Biochem.* **70**, 75 (1976).
25. J. C. Alwine, D. J. Kemp, G. R. Stark, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5350 (1977).
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Opiate Withdrawal in utero Increases Neonatal Morbidity in the Rat

Abstract. *Long-term oral administration of the long-acting opiate* l-α-*acetylmethadol (LAAM) to female rats beginning on the day of conception interfered with the dams' ability to carry litters to term. When treatment was initiated 3 weeks prior to mating this effect was not observed. Daily administration of the opiate antagonist naloxone from day 14 of gestation through term, to precipitate withdrawal in utero, resulted in increased stillbirths, decreased pup weight and size, and weight loss 24 hours after birth. These data question the validity of animal experiments which purport to be models for methadone maintenance programs but in which treatment is started immediately prior to or soon after conception. They also suggest that withdrawal in utero may be responsible for many of the adverse effects of opiates on human and animal development.*

In the United States methadone maintenance is the major form of therapy for heroin addiction. Considerable data have been gathered concerning the effects of methadone on the pregnant addict and her offspring. It is generally accepted that children born to methadone addicted women have a higher incidence of morbidity (for example, low birth weights) and mortality than do those born to nonaddicted women (*1*, *2*). These data, however, are difficult to interpret because many of the women use other drugs concurrently, few receive adequate prenatal care, and some may even experience withdrawal during pregnancy (*2*–*4*). When these factors are controlled, perinatal morbidity and mortality are significantly reduced (*2*).

Studies in the rat (*5*) have shown that offspring exposed to methadone in utero have low birth weights. The possibility that drug withdrawal in utero, rather than the direct effect of opiates on growth, may be responsible for the low birth weights has not been investigated. This is a reasonable alternative, since methadone is commonly administered once daily, even though the half-life of methadone in rat plasma after long-term administration of the drug is less than 2 hours (*6*). This schedule of administration might therefore result in opiate withdrawal prior to the subsequent injection, as has been demonstrated in rats injected twice daily with morphine (*7*). That the fetus may itself experience withdrawal has been shown in the lamb (*8*) and chick (*9*) by injection of the antagonist naloxone into the opiate-dependent fetus. Stryker (*3*) has recently presented clinical evidence for spontaneous fetal withdrawal from methadone, as shown by heart rate aberrations, increased fetal activity, and by meconium-stained amniotic fluid at birth.

The present study was undertaken to determine if withdrawal in utero, rather than opiate exposure per se, is responsible for at least some of the adverse effects of opiates in humans and other animals. We also examined the role of tolerance in the severity of so-called direct drug effects. One question was whether extended opiate treatment would attenuate any detrimental effects of the opiate that might occur as a result of the drug not being given until near the time of conception (*5*, *10*).

We administered *l*-α-acetylmethadol (LAAM), a long-acting derivative of methadone, to pregnant rats. In clinical trials for treatment of heroin addiction, LAAM is administered three times per week, whereas methadone is administered daily (*11*). Animal studies have shown that LAAM is more potent and longer acting than methadone (*12*), and results in a more stabilized state of dependence (*13*). We have shown (*14*) that operant behavior is suppressed for at least 24 hours after a single dose of LAAM, but completely recovers within 5 hours after relatively high doses of methadone. By using the long-acting opiate and by precipitating withdrawal with naloxone we hoped to distinguish between the direct effects of the opiate and those produced by withdrawal in utero. To study tolerance, we initiated treatment for one group of rats 3 weeks before they were mated and for a second group, at the time of conception.

The rats were Sprague-Dawley derived females (Holtzman) weighing approximately 280 g. LAAM (1 or 4 mg/kg) was administered orally in water, by gavage. For rats treated 3 weeks before being mated, the first three doses were spaced 3 days apart, the next two were 2 days apart, and all remaining doses were administered daily. After 21 days the females were randomly assigned to males of proved fertility (Holtzman). They were placed with the males for ten consecutive days or until mating had occurred, as determined by the presence of sperm in a vaginal lavage taken each morning. To study the effects of withdrawal in utero, naloxone (1 mg/kg) (Nx) was administered subcutaneously to half of the rats 4 and 2 hours before their dose of water (WNx) or LAAM (1LNx and 4LNx) from day 14 of gestation through parturition. The remaining rats received saline (WS, 1LS and 4LS). To study tolerance, a second group of females, as yet untreated, were mated as described. After conception (+C) they were assigned to treatment groups and given water (W+C) or LAAM (1 or 4 mg/kg) (1L+C or 4L+C) daily but were not injected with saline or naloxone during gestation.

Successful mating occurred in about 85 percent of all females, and was not affected by treatment. With the exception of the 4L+C group, 81 percent (47 of 58) of these matings resulted in the delivery of a litter. However, only 12.5 percent (one of eight) 4L+C dams delivered litters. Examination of the uteri of

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