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The Long Terminal Repeat of Moloney Sarcoma Provirus Enhances Transformation

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A. Introduction

Transforming retroviruses are generally thought to arise from a recombination between nontransforming viruses and sequences of cellular origin. Until recently, it has not been possible to test whether the acquired cell sequences *(onc)*[1] of transforming retroviruses act in concert with the conserved retrovirus sequences to establish transformation or whether the *onc* sequences alone are sufficient to cause transformation. For example, if retrovirus sequences are not essential, a finite number of naturally occurring malignancies would be expected to be caused by expression of *C-onc* sequences. Alternatively, if a region(s) of the retrovirus genome is (are) required for expression of the malignant phenotype, then the properties of this viral sequence may reveal how a normal cell sequence is modified to activate its transformation potential. We have cloned from normal mouse cell genomic DNA a sequence, *C-mos* that is homologous to the Moloney sarcoma virus (MSV) acquired *V-mos* sequence (Oskarsson et al. 1980). This cloned fragment and cloned integrated MSV proviral DNA have provided a direct means for addressing these questions and for testing the transforming activity of various combinations between the *V-mos, C-mos,* and MSV sequences derived from Moloney murine leukemia virus (M-MuLV).

The biologic activity of both in vitro synthesized and molecularly cloned MSV proviral DNA has been demonstrated (Oskarsson et al. 1980; Andersson et al. 1979; Vande Woude et al., to be published; Canaani et al. 1979; Aaronson et al., to be published; Blair et al. 1980). These studies showed that subgenomic DNA fragments of MSV could transform cells in a direct DNA transfection assay and that the entire MSV genome was not essential for efficient transformation. Here we review evidence (Oskarsson et al. 1980; Vande Woude et al., to be published; Blair et al. 1980) demonstrating that the long terminal repeat (LTR) of the MSV retrovirus enhances cell transformation by an internal MSV fragment containing *V-mos*. Furthermore we show that a single LTR placed covalently 5′ to *C-mos* activates the transforming potential of the otherwise inactive *C-mos*.

The LTR sequences of MSV are direct repeats of 588 base pairs (bp) in length which bracket the MSV provirus (Vande Woude et al. 1979, to be published; Dhar et al. 1980; McClements et al., to be published b). The LTR is derived from the 5′ and 3′ ends of the genomic viral RNA during proviral DNA synthesis and for MSV are conserved from the parental M-MuLV (Dhar et al. 1980; Shoemaker et al., to be published). The nucleotide sequence of the MSV LTR reveals putative transcription control sequences (Dhar et al. 1980) and bears several striking parallels to the prokaryotic insertion sequence (IS) elements (Dhar et al. 1980; McClements et al., to be published a,b).

[1] *Onc* is a general term for all cellular sequences with malignant transforming potential. These are designated *C-onc* when part of the cell genome and *V-onc* in the retrovirus genome. Mos has been adopted as the name for the acquired sequence in MSV. Thus *mos* is a member of the set of *onc* sequences present in mice. This nomenclature supersedes utilization of *src* and *sarc* for viral and cellular transforming sequences

B. Results

I. LTR Enhances Transformation by V-mos

Figure 1 shows physical maps of integrated HT1 MSV provirus cloned in phage λ(λHT1) (Vande Woude, to be published, 1979) together with clones of subgenomic portions of the provirus cloned in pBR322. The biologic activity of the complete provirus in the DNA transfection assay is compared to the activities of the cloned subgenomic fragments (Table 1) (Blair et al. 1980). A cloned internal MSV fragment, pHT10, containing approximately 800 bp of M-MuLV derived sequences and 1200 bp of *V-mos*, transforms NIH 3T3 cells with a specific infectivity ~10,000-fold lower than the intact HT1 MSV DNA (λHT1). Two clones of external MSV fragments, pHT15 and pHT13, containing respectively the 5′ or 3′ LTR, lack *V-mos* and are inactive in this assay. When sequences in pHT13 are added to the MSV sequences in pHT10, reconstructing the 3′ end of HT1 MSV provirus as in pHT21 and pHT22, the efficiency of transformation is enhanced 1000-fold over pHT10 (Table 1) (Oskarsson et al. 1980; Blair et al. 1980). Also shown in Table 1 is clone pHT25 which contains the entire 5′ portion of HT1 MSV through *V-mos* and transforms with the same efficiency as pHT21 and pHT22. The only sequence in common among these three subclones, besides the MSV sequences in pHT10, is the LTR.

II. Cotransfection-Transformation by V-mos plus LTR

We have used deletion mutants of the hybrid λMSV phages to further demonstrate that transformation enhancement is due to the LTR. When either λHT1 or λml [a hybrid phage containing the integrated provirus of the ml strain of MSV (Vande Woude et al. 1979, to

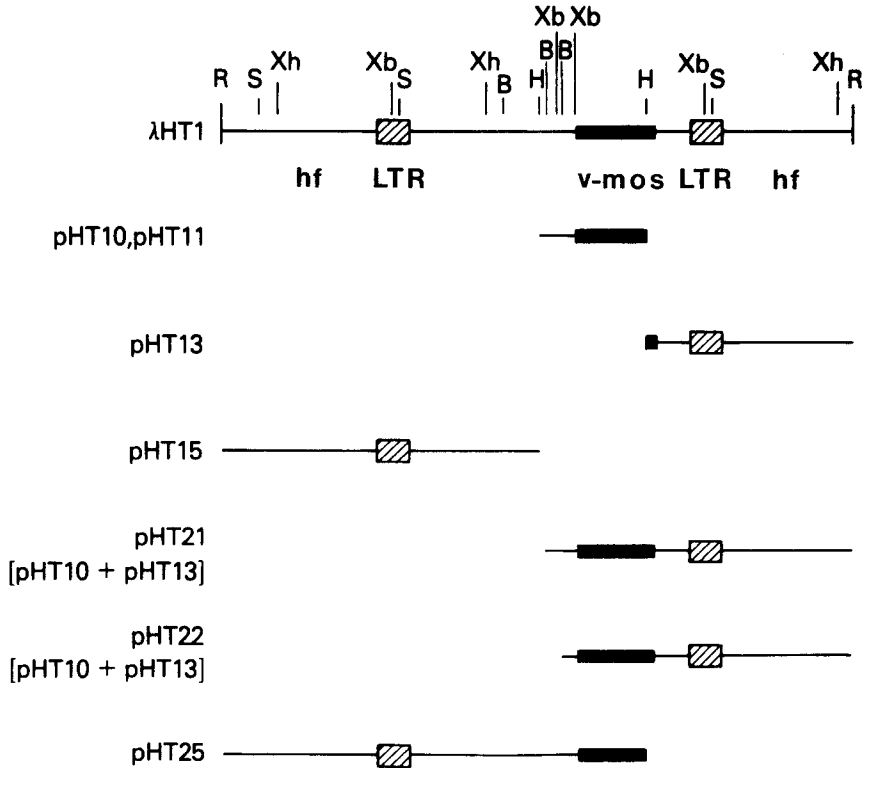


Fig. 1. Physical maps of HT1 MSV and subclones. The simplified map of the integrated provirus is shown indicating the *V-mos (heavy line)*, LTR *(cross hatch)*, and hf *(host flank)* sequences. Pertinent restriction endonuclease sites are shown: *R, Eco* RI; *Xb, Xba* I; *S, Sac* I; *B, Bgl* II; *Xh, Xho* I; *H, Hind* III. The specific portions of λHT1 subcloned in pBR322 are indicated. Plasmids pHT10 and pHT11 are identical, independently derived clones of the 2.1 kb *Hind* III *V-mos* fragment of λHT1. Plasmids pHT15 and pHT13 were generated by *Hind* III digestion of the purified *Eco* RI insert of λHT1 and cloned into the *Hind* III and *Eco* RI sites of pBR322. Partial *Hind* III digestion of the same *Eco* R1 fragment allowed construction of pHT25. Plasmids pHT21 and pHT22 were made in vitro by cloning the *Bgl* II-*Hind* III MSV fragment of pHT10 into pHT13. All subclones have been characterized by restriction mapping

Table 1. Transforming activity of cloned MSV fragments

Fragment Tested[a]	ffu/pmole[b]
λHT1	37,000
pHT10	7
pHT15	ND
pHT13	ND
pHT21	6,900
pHT22	8,100
pHT25	7,800

[a] DNA was transfected onto NIH 3T3 cells as previously described (Andersson et al. 1979; Blair et al. 1980; Graham and van der Eb 1973; Lowy et al. 1977)

[b] Focus-forming units (ffu) per picomole. ND = no foci were detected. Data from Blair et al. (1980)

Table 2. MSV LTR enhancement of transformation by *V-mos*

Plasmid	ffu/pmole
pHT10[a]	7
pmlsp	ND
pHT1sp	ND
pmlsp+pHT10	2,100
pHT1sp+pHT10	850
pBR322+pHT10	ND

[a] The concentration of each plasmid was 0.25 μg/ml applied to each plate containing 3×10^5 NIH 3T3 cells/dish using the Ca^{++} precipitation procedure (Graham and van der Eb 1973; Lowy et al. 1977). ND = no foci were detected

be published)] is propagated in *E.coli* a specific deletion in the inserted fragment is readily detected in a percentage of the phage progeny (Vande Woude et al., to be published; McClements et al., to be published b). The inserts of these deletion mutants retain one LTR and the bracketing host sequences, while one LTR and the unique MSV sequences are lost. The generation and physical characterization of these has been described in detail elsewhere (Vande Woulde et al., to be published; McClements et al., to be published b). The retained sequences have been cloned in pBR322 and designated "pmlsp" and "pHT1sp" (Fig. 2B); they are compared to the λTH1 and λml maps in Fig. 2A (Vande Woude et al. 1979, to be published).

We have shown that pmlsp enhances transformation by the *V-mos* sequences in pHT10 when a mixture of both is transfected onto NIH 3T3 cells (Blair et al. 1980; McClements et al. 1980, to be published a). In experiments summarized in Table 2 neither pmlsp nor pHT1sp produces foci of transformation. However, when either is cotransfected with pHT10, the number of foci produced is between 100- and 300-fold higher than pHT10 alone. No stimulatory effect is observed when the vector, pBR322, is cotransfected with pHT10. The only MSV sequences in common between pHT1sp and pmlsp is the single LTR (Vande Woude et al., to be published; McClements et al., to be published b). These data suggest that the LTR is responsible for enhancement of transformation.

III. The Effect of MSV Sequences of Transformation by C-mos

We have cloned and characterized the C-mos sequences from normal Balb/c mouse genomic DNA and have shown that C-mos is inactive in the transfection-transformation assay (Oskarsson et al. 1980; McClements et al., to be published a). Moreover, when pHT13 sequences are linked 3′ to *C-mos*, this new clone (λLS_1) is still inactive. This data, from Oskarsson, et al. (1980), is shown in Table 3. Recall that in the analogous procedure linking pHT13 sequences 3′ to *V-mos* enhanced transformation ~1000-fold (cf. pHT21 and pHT22, Table 2 to λLS_1, Table 3). From these results, we have proposed that the M-MuLV sequences immediately 5′ to *V-mos* in pHT10 contribute to its transforming activity. To test this hypothesis a hybrid, λLS_2, was constructed by covalently linking 5′ MSV sequences from λml to *C-mos*. This hybrid is structurally equivalent to the 5′ end of mlMSV thru *V-mos* (Fig. 2, 0–4.2-kb map units) and analogous to the pHT25 subclone of HT1 MSV (Table 2) (Blair et al. 1980). Like the latter, λLS_2 transforms NIH 3T3 cells efficiently (Table 3). This result demonstrated that the *C-mos* sequences can transform cells if the normal Balb/c sequences 5′ to *C-mos* are replaced with MSV sequences of M-MuLV origin (Oskarsson et al. 1980). These M-MuLV-derived sequences include the LTR and portions of the *gag*, *pol*, and *env* genes (Vande Woude et al., to be published; Sherr et al. 1980). This result

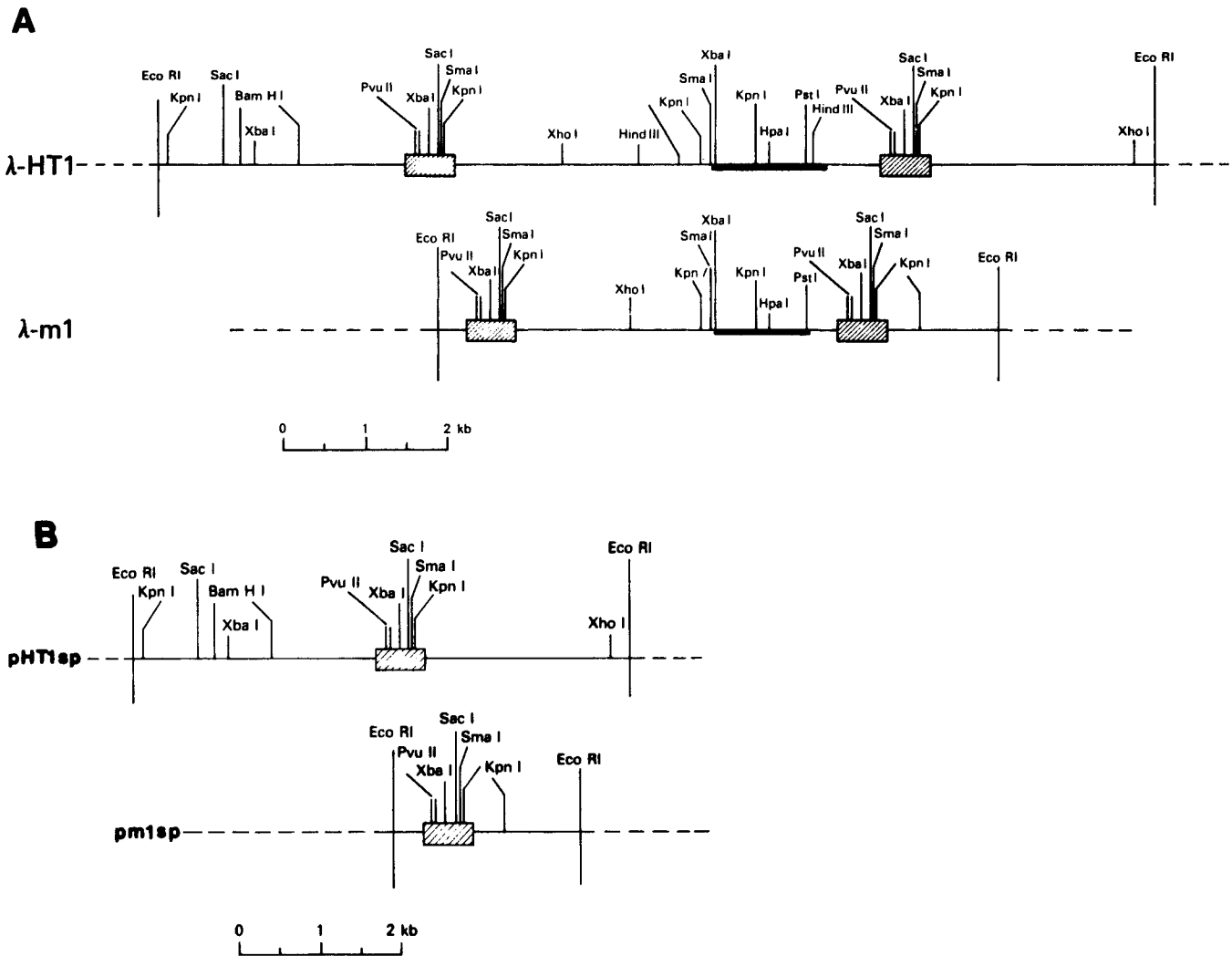


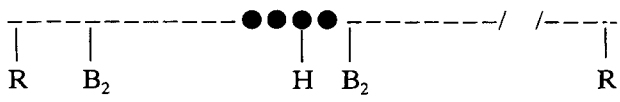
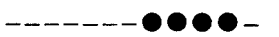
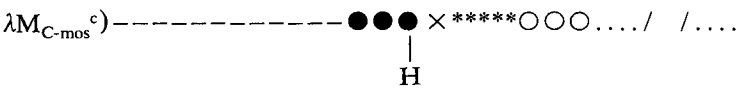
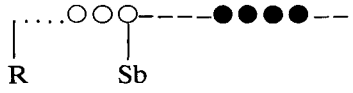
Fig. 2. Physical maps of cloned integrated MSV and their deletion mutants. **A** The maps show some of the restriction endonuclease sites in the inserted Eco R1 DNA fragments of λHT1 and λml (Vande Woude et al. 1979, to be published; McClements et al., to be published b). Additional *Pst 1, Bg 1* II and *Pvu*II sites in MSV and mink flanking sequences are not shown. The *solid horizontal lines* represent insert DNA, the *heavy lines* represent *V-mos,* the *crossed-hatched rectangles* are the long terminal repeats (LTR), and the *dashed lines* represent the prokaryotic vector sequences. **B** The deleted inserts pHT1sp and pm1sp were subcloned in pBR322. The characters and symbols are the same as in A above

taken with the pHT10 result suggests a contribution of the *env* gene sequences that immediately preceed *V-mos* in pHT10 or *C-mos* in λLS_2 to the activation of the transforming potential (Oskarsson et al. 1980; McClements et al. 1980). We have proposed that an *env* gene splice acceptor site could facilitate activation of *V-mos* expression by an upstream promoter, perhaps the one in the LTR (Dhar et al. 1980). To test whether *env* sequences are absolutely required for *C-mos* expression, we covalently linked the mlMSV LTR to cellular sequences ~600 bp from the 5′ end of *C-mos* generating the hybrid pTS_1 (Table 3). This hybrid transformed with an efficiency equivalent to that of a subgenomic fragment of MSV such as pHT25, pHT22, or pHT21 but aside from the LTR lacked all other M-MuLV-derived sequences.

C. Discussion

We have demonstrated that the normal cell sequence *C-mos* does not transform cells in the DNA transfection assay (Table 3) (Oskarsson et al. 1980). In contrast, as part of the viral genome *V-mos* does transform. Using recombinant DNA techniques, we have identified portions of the viral genome that are essential

Table 3. Transformation activity of *C-mos*

Clone designation	Schematic of fragment tested[a]	DNA ng	Foci (No.)	Activity (ffu/pmole)
$\lambda M_{C\text{-}mos}$		570	0	ND[b]
		1000	0	ND
		3200	0	ND
pMS_1		420	0	ND
		2100	0	ND
λLS_1(pHT13+$\lambda M_{C\text{-}mos}$[c])		90	0	ND
		810	0	ND
		1350	1	4
		1800	1	3
λLS_2(λml+pMS_1)		141	78	2600
		30	13	2400
		15	12	4000
pTS_1(pm1sp+pMS_1)		100	136	5900
		50	36	3300
		25	17	3000

[a] To approximate scale ..... indicates normal mink sequences flanking the integrated proviral DNA; OOO=the 588-bp-long terminal repeat sequences (LTR) of the provirus (Dhar et al. 1980); *****=MSV sequences of M-MuLV origin (Vande Woude et al., to be published; ×××× *V-mos* sequences (Vande Woude et al., to be published); −−−−−=normal Balb/c sequences flanking *C-mos;* ●●●●=*C-mos* sequences. The approximate location of restriction endonuclease sites used in the construction of λLS_1, λLS_2 and pTS_1 are indicated; R=*Eco* R1; H=*Hind* III; B_1=*Bgl I;* B_2=*Bgl II;* and S=*Sma* I. Sb indicates that Ba1 31 exonuclease digested pMS_1 (~600 bp 5′ to *C-mos*) was ligated to the *Sma* I site in the MSV LTR to generate the plasmid pTS_1

[b] ND=no foci detected

[c] See schematic of pHT13 in Fig. 1 and its biologic activity given in Table 1. λLS_1 was generated by joining pHT13 sequences to the 3′ end of *C-mos* at their common *Hind* III site. λLS_2 was generated from λml MSV by joining the entire 5′ MSV portion of ml MSV at the *Bgl* I site in *V-mos* to the 3′ portion of *C-mos* at its *Bgl* I site. pTS_1 was generated by blunt-end ligation of a *Ba1* 31 digested pMS_1 to the *Sma* I site of the MSV LTR

for activating the transforming potential of *C-mos*.

In experiments with subgenomic portions of the MSV provirus we demonstrated that an MSV fragment containing an LTR placed covalently either 3′ or 5′ to *V-mos* enhanced transformation. Also, simply cotransfecting a plasmid containing *V-mos* sequences with a second plasmid containing a single LTR enhanced transformation. Clearly, the enhancement of transformation could be associated with one or several biologic functions of the LTR. It could be that in this assay the LTR is providing a maintainance function (e.g., an origin of replication) or an integration function by facilitating a stable association of *mos* with the genome of the transfected cell. However, the LTR possesses putative transcription control elements (Dhar et al. 1980), and it is likely that at least one of its transformation enhancement activities is due to the promotion of *mos* transcription. We have shown that the MSV LTR has several structural and at least one functional feature in common with bacterial IS elements (Blair et al. 1980; Dhar et al. 1980; McClements et al., to be published a,b). It is well known that IS elements are involved in the transposition (and integration) of DNA sequences, and some IS elements are believed to contain transcriptional control signals that affect expression of adjacent genes (see review in Bukhari et al. 1977). The LTR may be acting in a manner analogous to IS elements.

Our results with λLS_2 in which the cellular sequences preceeding *C-mos* were replaced by M-MuLV-derived MSV sequences suggested

that in addition to the LTR, MSV sequences immediatly preceeding *V-mos* are required for transformation (McClements et al. 1980). However, the data do not rule out the possibility that the mere removal of these cellular sequences allows transformation by *C-mos*. There may be controlling cellular sequences preceeding *C-mos* that block its expression. The activation of *C-mos* in clone pTS_1 is consistent with this interpretation if (1) the LTR sequences override the cellular control elements or (2) the putative controlling cellular sequences are more than approximately 600 bp before *C-mos*. The latter possibility suggests that removing normal cell sequences 5′ to *C-mos* should activate its malignant potential in the transfection assay. Indeed, this is consistent with the model proposed by Cooper et al. (1980) to explain the transforming activity of normal cellular DNA after shearing.

Because the LTR is directly repeated at both ends of the provirus it could be expected to allow transcription to occur from the 3′ LTR into the cell genome. Thus, random integration of retroviruses into multiple sites in the host cell genome (see Weinberg 1980 for review) could result in the activation of normally quiescent cell genes. For example, 17 out of 19 tumors isolated from birds infected with avian leukosis virus show evidence for activation of expression of normal cell sequences by LTR sequences (Hayward et al. this volume). The loss of almost all of the retrovirus genome in some of the tumors, except for a residual LTR, could mean that LTR-promoted expression of normal cell sequences is responsible for the disease. Certainly in this case all other avian leukosis viral genes can be excluded. We have shown with pTS_1 (Table 3) that a single LTR which is linked 5′ to *C-mos* activates transformation. This in vitro construction is analogous to a retrovirus integrating 5′ (upstream) to a normal cell sequence (e.g., *C-mos*) in vivo, whereby the 3′ LTR could activate expression of this sequence and cause transformation. This may be a useful model for other (nonviral) forms of transformation. Normal cellular sequences with functional properties of an LTR (e.g., an eukaryotic IS element) could become juxtaposed to quiescent cellular genes with transformation potential as a result of either chemically induced, radiation induced, or simply spontaneous genomic arrangements.

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