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Evidence for a Stem Cell-Specific Repressor of Moloney Murine Leukemia Virus Expression in Embryonal Carcinoma Cells

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A negative regulatory element (NRE) spanning the tRNA primer-binding site (PBS) of Moloney murine leukemia virus (M-MuLV) mediates repression of M-MuLV expression specifically in embryonal carcinoma (EC) cells. We precisely defined the element by base-pair mutagenesis to an 18-base-pair segment of the tRNA PBS and showed that the element also restricted expression when moved upstream of the long terminal repeat. A DNA-binding activity specific for the M-MuLV NRE was detected in vitro by using crude EC nuclear extracts in exonuclease III protection assays. Binding was strongly correlated with repression in EC cells. Mutations within the NRE that relieved repression disrupted binding activity. Also, nuclear extracts prepared from permissive, differentiated EC cell cultures showed reduced binding activity for the NRE. These results indicate the presence of a stem cell-specific repressor that extinguishes M-MuLV expression via the NRE at the tRNA PBS.

Moloney murine leukemia virus (M-MuLV) does not productively infect early embryonic stem cells (22) or murine embryonal carcinoma (EC) cells (17, 40, 42). EC cells share many properties with early embryonic stem cells (24) and are a convenient cell culture system with which to study M-MuLV restriction. The block in the viral life cycle occurs at the stage of viral RNA accumulation, subsequent to proviral DNA integration (11, 41). At least two *cis*-acting elements are responsible for the block in RNA expression: the M-MuLV enhancer and an M-MuLV intragenic element. The M-MuLV enhancer is inactive in EC cells (29, 30), possibly due to an enhancer-specific repressor (19) or the lack of specific positive enhancer-binding factors (36). The intragenic element spans the M-MuLV proline tRNA primer-binding site (PBS) and inhibits RNA expression from M-MuLV recombinants specifically in EC cells (13, 30, 47). Competition assays indicate that the inhibition is mediated by a *trans*-acting factor. The mechanism of action is probably at the transcriptional level, since inhibition is independent of the orientation of the intragenic element (31). In addition, a point mutation in the tRNA PBS of an M-MuLV host range mutant, termed B2, allows virus replication (4) and expression (47) in EC cells.

In this report, we precisely define the borders of the intragenic inhibitory element, identify the element as a transcriptional silencer sequence, and correlate in vitro factor-binding activity at the tRNA PBS to EC-specific repression.

MATERIALS AND METHODS

Cell lines. The PC13 (2), F9 (5), and C2 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics. The F9 and PC13 EC cells were grown on gelatin-coated plasticware.

Recombinant constructions. The mutagenized recombinants used to map the negative regulatory element (NRE) were derived by synthesis of the indicated oligonucleotides with a *Sal*I 4-base-pair (bp) overhang and a *Bgl*II restriction site at the 5′ and 3′ termini, respectively. The annealed oligonucleotides were ligated with MLV.4Δ44 (Fig. 1) that had been cleaved with *Sal*I. The ligation products were digested with *Bgl*II and religated to circularize the plasmids. The resultant recombinants have the M-MuLV long terminal repeat (LTR) followed by a 29-bp or 59-bp fragment, derived from the synthetic oligonucleotides, linked to the *neo* reporter gene at the *Bgl*II restriction site. Each recombinant has a disrupted *Sal*I restriction site and an intact *Bgl*II site. The identity of the recombinants was confirmed by sequence analysis.

RNA isolation. Cytoplasmic RNA was isolated from trypsinized cells exactly as described previously (30). Some RNA preparations were enriched for polyadenylated [poly(A)$^+$] species by oligo(dT)-cellulose chromatography (3).

DNA transfections. The transient expression assays were done in the presence of $Ca_3(PO_4)_2$ (48) as described previously (30). The amounts of plasmid DNA used in the transfections are indicated in the figure legends. Transformation frequencies to G418 resistance were done by the addition of G418 (400 μg/ml for PC13 cells and 500 μg/ml for C2 cells) 36 h after DNA addition. Selection was maintained for 10 to 14 days, and drug-resistant colonies were counted after staining with 0.25% methylene blue in 50% methanol. Stable cotransformants were obtained by cotransfection of two unlinked plasmids, the selectable marker pSV_2hygro (6) and the M-MuLV-*neo* (MLVneo) recombinant. Selection procedures were done as previously described (31). The amounts of DNA used in the cotransfections are indicated in the figure legends, and the total DNA concentrations were brought to 20 μg per 10^6 cells with carrier pGem1 (Promega Biotec) DNA.

Nucleic acid electrophoresis, transfer, and hybridization. (i) RNA. RNA samples were denatured at 55°C for 15 min in 60% formamide–6% formaldehyde–1× MOPS (morpholinepropanesulfonic acid) buffer, pH 7.0 (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA), and resolved on a 6% formaldehyde–1% agarose gel in 1× MOPS buffer. The RNA was partially depurinated with 50 mM NaOH, neutralized,

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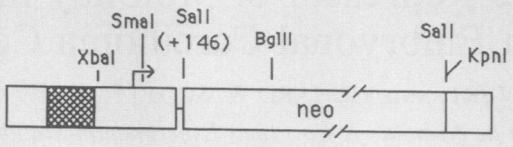
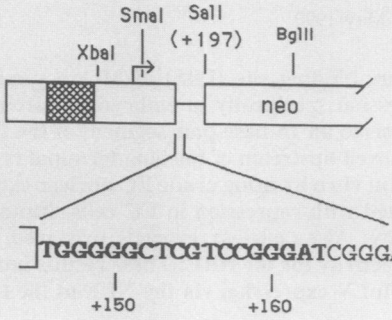
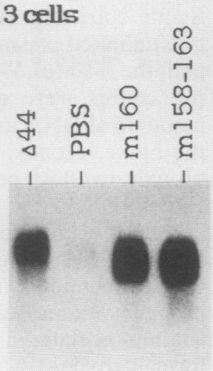
A.





		Ratio of G418-resistance transformation frequencies (n/MLV.4Δ44)	
		PC13	C2
	MLV.4Δ44	1.0	1.0
TGGTCGATTGGGGGCTCGTCCGGGATCGGGAG.....neo(+197)	MLV.4Δ22	0.007	0.5
TGGTCGATTGGGGGCTCGTCCGGGATCGGGAG.....neo(+197)	MLV.PBS-197	0.008	1.1
TGGTCGATTGGGGGCTCGTCCGGGATCGG...neo(+167)	MLV.PBS	0.002	0.7
TGGTCGATgGGctagggcctgctcggCGGGAG.....neo(+197)	MLVmPBS	1.2	1.1
TGGTCGATTGGGGGCTCGTCgcGGtgCGGGAG.....neo(+197)	MLVm158-163	1.5	0.8
TGGTCGATTGGGGGCTCGTCCGaGATCGGGAG.....neo(+197)	MLVm160	1.6	2.0
TGGTCGATTGGGGGCTCGTCCttcATCGGGAG.....neo(+197)	MLVm159-161	0.9	0.7
TGGTCGATTGGGGGCTCGTCCGGGATatt...neo(+167)	MLVm164-166	0.000	0.7
TGGTCGATaccccgaCGTCCGGGATCGGGAG.....neo(+197)	MLVm146-154	1.2	0.9
TGGTCGATTGGcGcCcgaaCaGGGAcCGGGAG.....neo(+197)	MLVmLys	1.7	1.0

B. Stable RNA Expression - PC13 cells



and transferred to a nylon membrane in 20× SSC (1× SSC is 0.15 M NaCl, 15 mM sodium citrate) overnight (30). Filters were hybridized with single-stranded RNA probes at 62°C in a 60% formamide buffer for 18 h (25) and washed in 30% formamide–0.1× SSC–0.1% sodium dodecyl sulfate (SDS) at 60°C.

(ii) DNA. DNA samples were digested with a threefold excess of restriction enzyme and resolved on a 1.5% agarose gel. The DNA was denatured in NaOH, neutralized, and transferred to a nylon membrane in 10× SSC overnight. The filters were hybridized with single-stranded RNA probes at 52°C in a 50% formamide buffer (25) and washed in 0.1× SSC–0.1% SDS at 55°C. Following the washes, the filters were treated with 50 μg of RNase A per ml in 2× SSC at 42°C for 30 min to reduce background signal.

Single-stranded, *neo*-specific RNA probe. The 1.3-kilobase (kb) *Hin*dIII-*Sma*I fragment from pSV2neo (38) was subcloned into pGem2 (Promega Biotec). Uniformly labeled, single-stranded RNA was synthesized from a *Hin*dIII-linearized template according to the supplier's conditions.

Radioactively labeled templates. To generate a template radioactively labeled on the coding strand, plasmid DNA was digested with *Xba*I, at position −150, treated with phosphatase, and end-labeled with [γ-^{32}P]ATP and polynucleotide kinase. The template was then digested with *Bgl*II, position +197, and the appropriate fragment was isolated from a 5% native polyacrylamide gel. The final DNA fragment was suspended to a concentration of 20,000 cpm/μl (approximately 1 ng/μl). To generate a template labeled on the noncoding strand, the plasmid was first digested with *Bgl*II, treated with kinase, and then digested with *Xba*I.

Preparation of nuclear extracts. PC13 stem and differentiated nuclear extracts were prepared from 10^9 cells by a modification of the procedure of Parker and Topol (34) as described by Shapiro et al. (37). The final protein concentration was approximately 10 mg/ml. Fractionation over heparin-agarose was done by passing 2 to 3 ml of extract over a 2-ml column of Affi-Gel-heparin (Bio-Rad Laboratories) in column buffer (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.2], 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol) plus 50 mM KCl and eluting the bound material with column buffer plus 0.5 M KCl. The flowthrough and 0.5 M KCl fractions were concentrated to approximately 3 mg/ml by filtration through a Centricon 30 microconcentrator (Amicon).

Exonuclease III protection assay. Exonuclease III protection assays were done by the procedure of Wu (49). For each binding reaction, 10,000 Cerenkov counts, representing about 0.5 ng of DNA template, was incubated for 15 min at room temperature in 50 μl of binding buffer (75 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EGTA [ethylene glycol tetraacetic acid], 15 mM Tris hydrochloride [Tris-HCl, pH 7.4], 0.5 mM dithiothreitol, 2 mM sodium phosphate [pH 7.0], 5% glycerol) with 1 μg of φX174 DNA cut with *Hae*III, 10 μg of yeast tRNA, 1 μg of mixed deoxynucleotide [p(dN)5; Pharmacia-PL], and 25 μg of nuclear protein. After binding, exonuclease III (Boehringer-Mannheim), freshly diluted (at 4°C) in 50 mM Tris-HCl (pH 8.0)–10 mM 2-mercaptoethanol–500 μg of bovine serum albumin per ml, was added to final concentrations of 80 to 16,000 U/ml and incubated with the protein-DNA complex or DNA alone, as a control, for 10 min at 30°C. The reaction was stopped with an equal volume of 20 mM disodium EDTA–1% SDS. The samples were extracted with phenol-chloroform, ethanol precipitated, denatured in a formamide dye buffer, and run on a 7% polyacrylamide–8 M urea sequencing gel.

RESULTS

Fine-mapping of the EC-specific inhibitory domain. Previously, we identified and mapped an NRE within an M-MuLV recombinant that inhibited RNA expression 20- to 50-fold specifically in EC cells (30, 31). DNA transfection assays localized the *cis*-acting sequences to a 29-bp region that spanned the tRNA PBS. To precisely define the borders of the NRE, recombinant templates were constructed with deletions and base-pair alterations in this 29-bp region (Fig. 1). By using DNA transfection techniques, each DNA recombinant was assayed in EC cells as well as permissive C2 cells. Initially, the recombinants were screened for G418 resistance. Since the degree of restriction imposed by the NRE is so great, this assay is adequate for rapidly scoring restricted and unrestricted templates (30). Transformation frequencies were standardized to that of an unrestricted reference recombinant, MLV.4Δ44, measured in parallel transformation assays. The M-MuLV portion of the recombinant MLV.4Δ22 extends to +199, contains the entire 29-bp region of inhibitory sequences, and is fully restricted in EC cells as reflected by a greater than 100-fold difference in the frequency of G418 resistance compared with MLV.4Δ44. In C2 cells, both templates have an equivalent transformation potential.

To construct recombinants with alterations in the NRE of MLV.4Δ22, MLV.4Δ44 was digested with *Sal*I and a synthetic oligonucleotide was ligated at the +146 position. After further digestion with *Bgl*II, the 3′ end of the oligonucleotide was ligated to the *neo* reporter gene.

MLV.PBS-197 was constructed to test whether a recombinant analogous to MLV.4Δ22 made with this cloning procedure was restricted in EC cells. It contains the M-MuLV sequences from +146 to +197 as an oligonucleotide insert and is nearly identical in this region to MLV.4Δ22 except for the 8-bp remnant of the *Sal*I restriction site between the 3′ border of the LTR and the tRNA PBS. Like MLV.4Δ22, MLV.PBS-197 was restricted in EC cells but

FIG. 1. Fine-mapping of the EC-specific NRE. (A) DNA transformation assays. Transformation frequencies to G418 resistance were determined for each recombinant in PC13 and C2 cells. Values are expressed relative to the transformation frequencies obtained for the unrestricted reference plasmid MLV.4Δ44, done in parallel. The DNA transfection procedures with PC13 cells were done with 5 μg of plasmid DNA per 10^6 cells in the presence of $Ca_3(PO_4)_2$. Plasmid DNA (1 μg) with 5 μg of carrier pGem1 DNA per 10^6 cells was used for C2 cells. G418 was added 36 h after DNA addition at concentrations of 400 and 500 μg/ml for PC13 and C2 cells, respectively. Selection was maintained for 10 to 14 days, at which time transformation frequencies were determined from an average of three plates. The boldface letters indicate the tRNA PBS domain, and the lowercase letters represent the base-pair alterations. The cross-hatched area represents the enhancer region in the M-MuLV LTR. (B) RNA expression in stable cotransformants. Cytoplasmic RNA from hygromycin-resistant PC13 cells cotransformed with either MLV.4Δ44, MLV.PBS, MLVm160, or MLVm158-163 was isolated from expanded pools of 50 to 100 colonies. The pools for each cell line were established from cotransfection experiments with 2 μg of pSV_2hygro and 5, 10, or 20 μg of MLV.4Δ44, MLV.PBS, MLVm160, or MLVm158-163 DNA. Pools containing comparable amounts of MLVneo DNA were selected and expanded for analysis. Each lane contains 10 μg of poly(A)$^+$ RNA.

not C2 cells. Since the *SalI* linker was inserted between the LTR and tRNA PBS, the U5 LTR domain is probably not involved in the EC-specific restriction. This was supported by the results with an internal deletion mutant in which virtually all of the U5 domain was removed, leaving an intact PBS linked to the LTR sequences at position +74. Expression from this recombinant was also restricted in EC cells but not in C2 or L cells (data not shown).

The NRE was further defined with MLVmPBS, in which virtually the entire tRNA PBS is disrupted but the 3′-flanking sequences between +164 and +197 are left intact. The frequency of G418-resistant colonies from MLVmPBS was nearly identical to that from MLV.4Δ44, showing that EC-specific repression has been lost. The role of the tRNA PBS in restriction was clearly seen with MLV.PBS. This recombinant has an intact tRNA PBS plus an additional 3 bp of 3′-flanking DNA. Expression was comparable to that of MLV.4Δ44 in C2 cells but fully restricted in the PC13 EC cells. The involvement of the three 3′-flanking nucleotides in restriction was eliminated with MLVm164-166. A change from CGG to ATT at positions 164 to 166 had no effect on the restriction of expression in EC cells. Conversely, it was found that nucleotide changes within the tRNA PBS relieved restriction. Changing four of the last six nucleotides at the 3′ terminus (MLVm158-163) or the first eight nucleotides at the 5′ terminus of the tRNA PBS (MLVm146-154) completely restored transformation frequencies to values comparable to that with MLV.4Δ44.

Since the NRE mapped to the proline tRNA PBS, it was tested whether the recombinant MLVmlys, which has the lysine tRNA PBS sequence from mouse mammary tumor virus (MMTV) substituted for the proline sequence, was also restricted in EC cells. The MMTV PBS sequence was 56% homologous to the M-MuLV sequence (Fig. 1A). The transformation frequency with MLVmlys showed that the MMTV PBS was not inhibitory in PC13 EC cells and indicated that restriction was not caused by any tRNA PBS but rather was specific for the M-MuLV sequence.

A viral mutant, termed B2, has an increased capacity for replication in F9 EC cells (4). Sequence analysis of this mutant identified a single-base-pair change within the tRNA PBS that mapped to position +160. We constructed a DNA recombinant with this point mutation and tested it in our transformation system (Fig. 1A). We found that the change from G to A at +160 completely relieved EC-specific repression. Another recombinant, MLVm159-161, had the GGG nucleotides at positions 159 to 161 changed to TCC. As predicted from the results with MLVm160, MLVm159-161 relieved restriction in EC cells.

To substantiate the results shown in Fig. 1A, RNA expression of selected recombinants was assayed from pools of stable cotransformants. The pools of PC13 EC cells were established by cotransfection of the recombinant DNA with pSV_2hygro, a dominant marker that confers resistance to hygromycin (6). An average of 100 hygromycin-resistant colonies were pooled and expanded for RNA and DNA analyses. Expression was only compared between pools that incorporated comparable levels of MLVneo DNA, as described previously (31) (data not shown). The RNA was resolved on a denaturing agarose gel, transferred to a nylon support membrane, and hybridized with a *neo*-specific RNA probe (Fig. 1B). In agreement with the transformation data in Fig. 1A, RNA expression from MLVm160 and MLVm159-160 was comparable to that from MLV.4Δ44, while expression from MLV.PBS was reduced over 20-fold versus that from MLV.4Δ44. The integrity of the RNA samples was substantiated with a β-actin-specific probe (data not shown). The quantitative differences between the two assays have been observed previously (30) and apparently reflect the levels of expression required to confer G418 resistance in the stable transformation assay.

Position dependence of repression. We have shown that the NRE at the tRNA PBS domain inhibits expression independently of its orientation (31). This suggests that repression occurs, at least in part, at the DNA level. We were interested in the position dependence of inhibition and constructed a series of recombinants in which the NRE was moved outside of the transcription unit, 5′ to the LTR, in two orientations (Fig. 2). The control plasmids were MLV.6 and MLV.4. In MLV.6 the M-MuLV LTR, truncated at +30, is linked directly to the *neo* reporter gene. MLV.4 includes an additional 750-bp DNA fragment between the LTR and the *neo* reporter gene. This fragment, from the M-MuLV recombinant provirus FVXMneo, encodes leader sequences and carries the NRE. Steady-state *neo* transcript levels in transient expression assays (Fig. 3A) demonstrated that expression from MLV.4 relative to that from MLV.6 was severely restricted in PC13 EC cells.

The recombinant MLV.7 has the 750-bp leader fragment placed 5′ to the LTR (Fig. 2). This positions the NRE in its original orientation approximately 1.2 kbp upstream from the M-MuLV cap site. MLV.7A has a 167-bp subfragment of the leader sequence. This fragment contains the NRE, and in MLV.7A it is in the correct orientation, while in the companion recombinant, MLV.7AR, it is in the reverse orientation. In these constructs, the inhibitory sequences are approximately 600 bp from the M-MuLV cap site. To control for the effects of DNA insertion, we made the recombinant MLV.7B, which has 570 bp of noninhibitory leader sequence placed 5′ to the LTR.

In transient assays, equivalent amounts of steady-state MLVneo transcripts accumulated with MLV.6, MLV.7A, and MLV.7AR in PC13 EC cells (Fig. 3A). This indicates that repression mediated by the NRE is position dependent, since the inhibitory domain placed 600 bp 5′ to the M-MuLV cap site, in either orientation, did not restrict RNA expression. In stable cotransformation assays, contradictory results were obtained (Fig. 3B). Placement of the NRE 1.2 kb (MLV.7) or 600 bp (MLV.7A and MLV.7AR) upstream of the M-MuLV cap site restricted accumulation of MLVneo transcripts in the hygromycin-resistant PC13 cotransformant pools by greater than 20-fold compared with MLV.6. Position-independent repression in stable cotransformant pools was also observed in F9 EC cells.

The degree of restriction in EC cells did not appear to be as great when the NRE was placed in the 5′ context (MLV.7) compared with its normal 3′ position (MLV.4). Possibly, restriction is not as stringent when the inhibitory element is moved outside of the transcription unit, altering the spatial relationship between the regulatory elements within the LTR and the NRE.

Compared with MLV.6, little or no effect on RNA expression was seen when the noninhibitory leader fragment was inserted upstream from the LTR (MLV.7B). In agreement with the EC specificity of restriction, stable cotransformant pools of L and C2 cells showed comparable expression from MLV.7A, MLV.7AR, and the template lacking the NRE, MLV.6 (data not shown).

The reason for the difference in the position dependence of restriction between the transient and stable cotransformation assays is not known (see Discussion). However, identical results were obtained with the heterologous brain

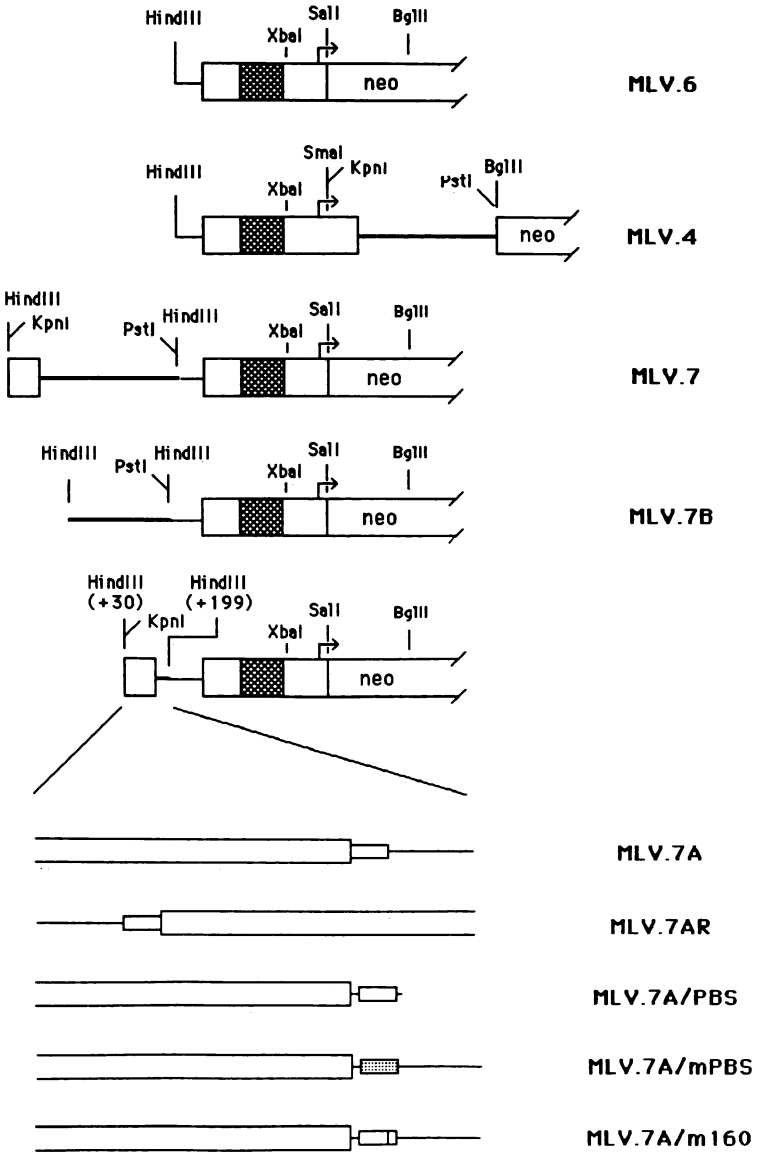


FIG. 2. Structure of recombinants for analysis of position dependence of inhibition. The derivation of MLV.6 and MLV.4 has been previously described (30). For MLV.7, the 750-bp *Sma*I-*Bgl*II leader fragment from MLV.4 was inserted 5′ to the M-MuLV LTR in MLV.6 at the *Hin*dIII restriction site, placing the tRNA PBS domain 1.2 kb upstream of the transcription start site. For MLV.7A and MLV.7AR, the 167-bp *Sma*I-*Sal*I leader fragment from MLV.4Δ22 was inserted at the *Hin*dIII restriction site of MLV.6 in both orientations, placing the tRNA PBS approximately 600 bp upstream of the transcription start site. For recombinants carrying truncated and altered tRNA PBS fragments in the upstream configuration, the *Sma*I-*Bgl*II fragment that spans the tRNA PBS was isolated from MLV.PBS, MLVmPBS, and MLVm160. Following conversion of the termini to *Hin*dIII restriction sites, the fragments were inserted 5′ to the LTR of MLV.6 at the *Hin*dIII restriction site, creating MLV.7A/PBS, MLV.7A/mPBS, and MLV.7A/m160, respectively. A 570-bp fragment, isolated from MLV.4Δ23 (30), that contains noninhibitory leader sequences was inserted at the *Hin*dIII restriction site of MLV.6 to create MLV.7B.

creatine kinase promoter (data not shown). Since transcription of the proviral DNA during the retroviral lifecycle occurs after integration into the host chromosome, we believe that the stable cotransformation assay results are the most reliable.

Mapping the inhibitory activity in the upstream configuration. To verify that it was the NRE at the tRNA PBS that restricted expression in the upstream configuration, several of the mutated segments in Fig. 1 were placed 5′ of the LTR (Fig. 2). Levels of steady-state RNA were measured from stable cotransformant pools with comparable levels of MLVneo DNA and compared with those of MLV.6. The 137-bp fragment with an intact tRNA PBS that conferred EC-specific restriction in the normal downstream configuration (MLV.PBS, Fig. 1) also conferred restriction in the upstream configuration (MLV.7A/PBS, Fig. 3B). Mutation of the entire tRNA PBS or a single-nucleotide change at +160 not only relieved repression in the downstream configuration (MLVmPBS and MLVm160, respectively, Fig. 1) but also relieved repression in the upstream configuration (MLV.7A/mPBS and MLV.7A/m160, Fig. 3B). Thus, it is the NRE at the tRNA PBS that causes restriction in both the 3′ and 5′ positions, and the properties of position and orientation independence characterize the NRE as a si-

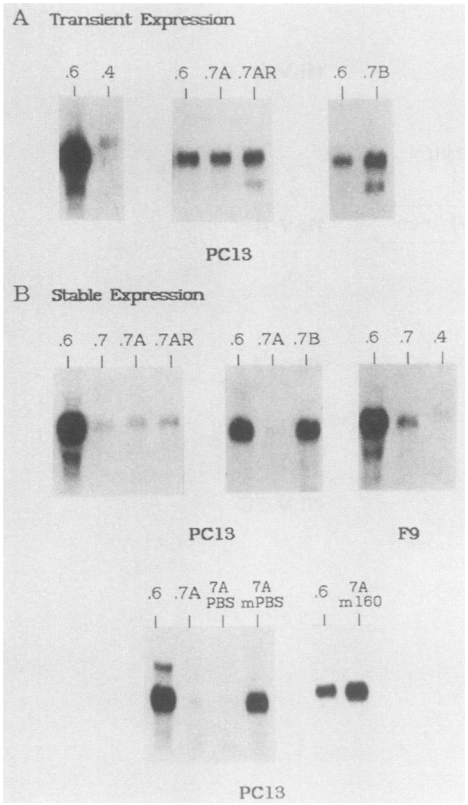


FIG. 3. Effect of position of the tRNA PBS domain on EC-specific repression. (A) Steady-state MLVneo transcripts in transient expression assays. The DNA transfections were done in the presence of $Ca_3(PO_4)_2$ with 1 μg of MLVneo DNA plus 5 μg of pGem1 carrier DNA per 10^6 cells. Cytoplasmic RNA was prepared from a total of 10^7 cells 36 h after DNA addition and was resolved by Northern (RNA) blot analysis. Each lane contains 10 μg of poly(A)$^+$ RNA. (B) Stable cotransformation. Cytoplasmic RNA samples were prepared from hygromycin-resistant pools of PC13 and F9 cells cotransformed with the indicated plasmid DNA. Transfections were done in the presence of $Ca_3(PO_4)_2$ with 2.5 μg of pSV_2hygro DNA and 2.5, 5, or 15 μg of MLVneo DNA per 10^6 cells. From 50 to 100 hygromycin-resistant colonies were pooled, expanded, and analyzed for DNA content. Transformants containing comparable MLVneo DNA levels were selected for subsequent RNA analysis. MLVneo transcripts were identified in 10-μg poly(A)$^+$ RNA samples.

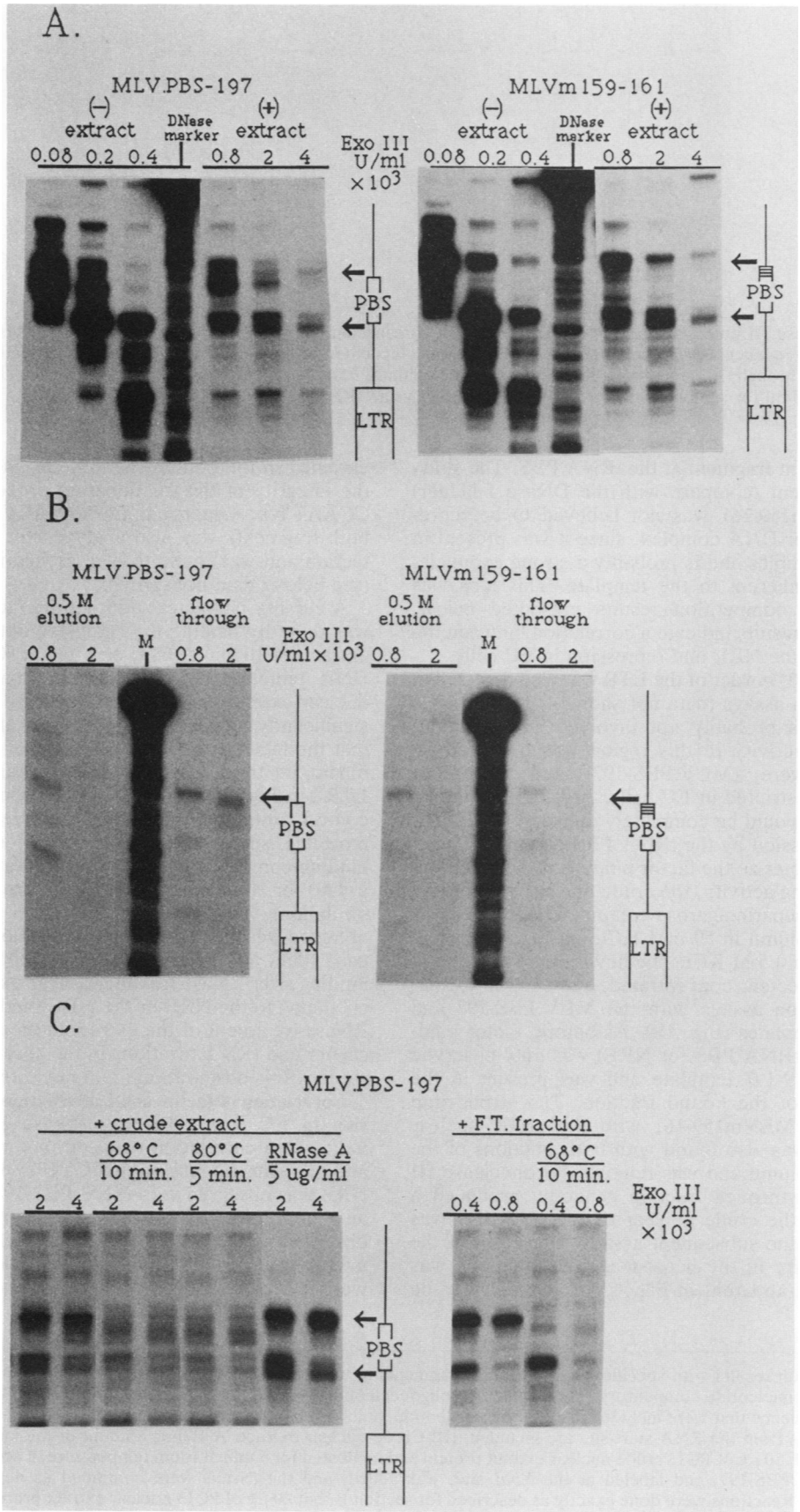
lencerlike element similar to that described for the HML and HMR silent mating type loci in *Saccharomyces cerevisiae* (1, 8, 12).

In vitro DNA-factor binding assays: exonuclease III protection assays detect specific factor-DNA interactions at the NRE. Having mapped the *cis*-acting NRE to the tRNA PBS, in vitro protection assays were used to visualize cellular factor-binding activity at this sequence. Initially, DNase I protection assays were done. Hypersensitive sites were observed on both DNA strands at the NRE that were absent on an unrestricted template bearing point mutations in the tRNA PBS (data not shown). However, the appearance of the hypersensitive sites on the restricted template was subtle and not a reliable indication of factor-binding activity at the NRE. With crude nuclear extracts, a more sensitive technique than DNase I protection is the exonuclease III protection method (49). Factors bound to the DNA will block or impede the 3′ to 5′ progression of the exonuclease, and DNA-factor complexes, even if they are only a small fraction of the DNA population, can be visualized when separated from unbound templates on denaturing acrylamide gels. The assays were done with double-stranded DNA fragments containing the tRNA PBS from MLV.PBS-197 and MLVm159-161. MLV.PBS-197 contains an intact tRNA PBS and is restricted in EC cells, while MLVm159-161 has point mutations in the 3′ portion of the tRNA PBS and is efficiently expressed in EC cells (Fig. 1). The fragments were end labeled on the coding strand at the *Xba*I site (−150), incubated in the presence or absence of crude nuclear extract from PC13 EC cells, and treated with increasing amounts of exonuclease III. In the extract-free samples, strong exonuclease III stop fragments are normally seen that are inherent to the DNA template, and they disappear with increasing amounts of enzyme. In the extract-containing samples, bound complexes generate exonuclease III-resistant fragments that are either unique or enriched compared with extract-free samples. By using templates end labeled on the coding strand, the 3′ border of a DNA-factor complex can be determined from the size of the exonuclease III-resistant fragment.

In the presence of PC13 nuclear extract, the MLV.PBS-197 fragment generated two unique exonuclease III-resistant fragments (Fig. 4A). The 3′ terminus of one fragment was at +167, just 3′ to the tRNA PBS, and apparently corresponded to factor binding at the NRE. The 3′ terminus of the second fragment was at the *Sal*I linker site between the LTR and tRNA PBS sequences and probably represented a factor-DNA complex at the LTR border. These two exonuclease III-resistant fragments persisted through a range of increasing enzyme concentrations and were not found in the extract-free samples.

The exonuclease III-resistant fragment at the LTR border was also observed with the MLVm159-161 template, and its signal was comparable to that detected with MLV.PBS-197. However, in contrast to MLV.PBS-197, there was no exo-

FIG. 4. Exonuclease III protection analysis at the NRE. All assays were done with the *Xba*I-*Bgl*II fragment of MLV.PBS-197 and MLVm159-161, end labeled at the *Xba*I site. Arrows indicate the positions of exonuclease III (Exo III)-resistant fragments. The DNase marker is the DNase I digestion profile of the fragment in the absence of extract that was included to mark the positions of the various elements. The DNase I digestion profiles were previously mapped next to Maxam-Gilbert sequencing reactions (9, 32) of the same fragment (data not shown). (A) Crude extract. The end-labeled fragment (0.5 ng) was incubated either in the absence of extract (−) and digested with 80 to 400 U of exonuclease III per ml or in the presence of 20 μg of extract protein (+) and digested with 800 to 4,000 U of exonuclease III per ml. (B) Fractionated extract. The crude nuclear extract was passed over a heparin-agarose column in 50 mM KCl. The flowthrough and 0.5 M KCl batch-eluted fractions were assayed for binding activity. Approximately 20 μg of protein was assayed for both fractions with 0.5 ng of template, and the samples were digested for 10 min with 800 to 2,000 U of exonuclease III per ml. Lane M, Partial DNase I digestion profile of the fragment that is used as a size marker ladder. (C) Heat and RNase A treatment. Before addition of the end-labeled fragments, samples with 10 μg of crude extract protein were heat-treated for 10 min at 68°C or 5 min at 80°C. Alternatively, the samples were treated with RNase A (5 μg/ml) for 10 min at room temperature. Exonuclease III concentrations were 2,000 to 4,000 U/ml for the crude extract samples and 400 to 800 U/ml for the flowthrough (F.T.) samples.


A.
MLV.PBS-197
(-) extract
DNase marker
(+) extract
0.08 0.2 0.4
0.8 2 4
Exo III U/ml ×10³
PBS
LTR
MLVm159-161
(-) extract
DNase marker
(+) extract
0.08 0.2 0.4
0.8 2 4
PBS
LTR
B.
MLV.PBS-197
0.5 M elution
flow through
0.8 2
M
0.8 2
Exo III U/ml×10³
PBS
LTR
MLVm159-161
0.5 M elution
flow through
0.8 2
M
0.8 2
PBS
LTR
C.
MLV.PBS-197
+ crude extract
68°C 10 min.
80°C 5 min.
RNase A 5 ug/ml
2 4 2 4 2 4 2 4
+ F.T. fraction
68°C 10 min.
0.4 0.8 0.4 0.8
Exo III U/ml ×10³
PBS
LTR

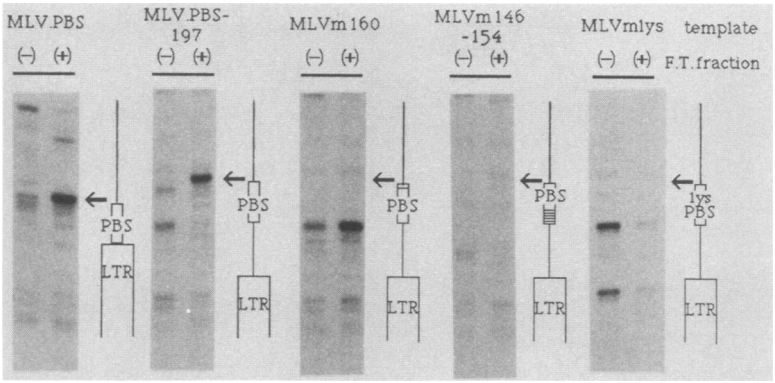


FIG. 5. Exonuclease III protection analysis at the NRE of EC-restricted and unrestricted templates. Templates (0.5 ng) with intact or mutated tRNA PBS sequences, end labeled at the *Xba*I site, were incubated in the absence or presence of 20 μg of PC13 nuclear extract protein from the flowthrough (F.T.) fraction of a heparin-agarose column. Exonuclease III concentrations were 400 U/ml for samples without extract (−) and 800 U/ml for samples with extract (+). lys PBS, Lysine tRNA PBS sequence from MMTV.

nuclease III-resistant fragment at the tRNA PBS. The slower-migrating fragment (compare with the DNase I ladder) found with MLVm159-161 was not believed to be representative of a factor-DNA complex, since it was present in the extract-free samples and is probably a strong exonuclease III stop site inherent to the template. This idea was supported by the competition results presented below. These preliminary results indicate a correlation between the binding activity at the NRE and repression in EC cells.

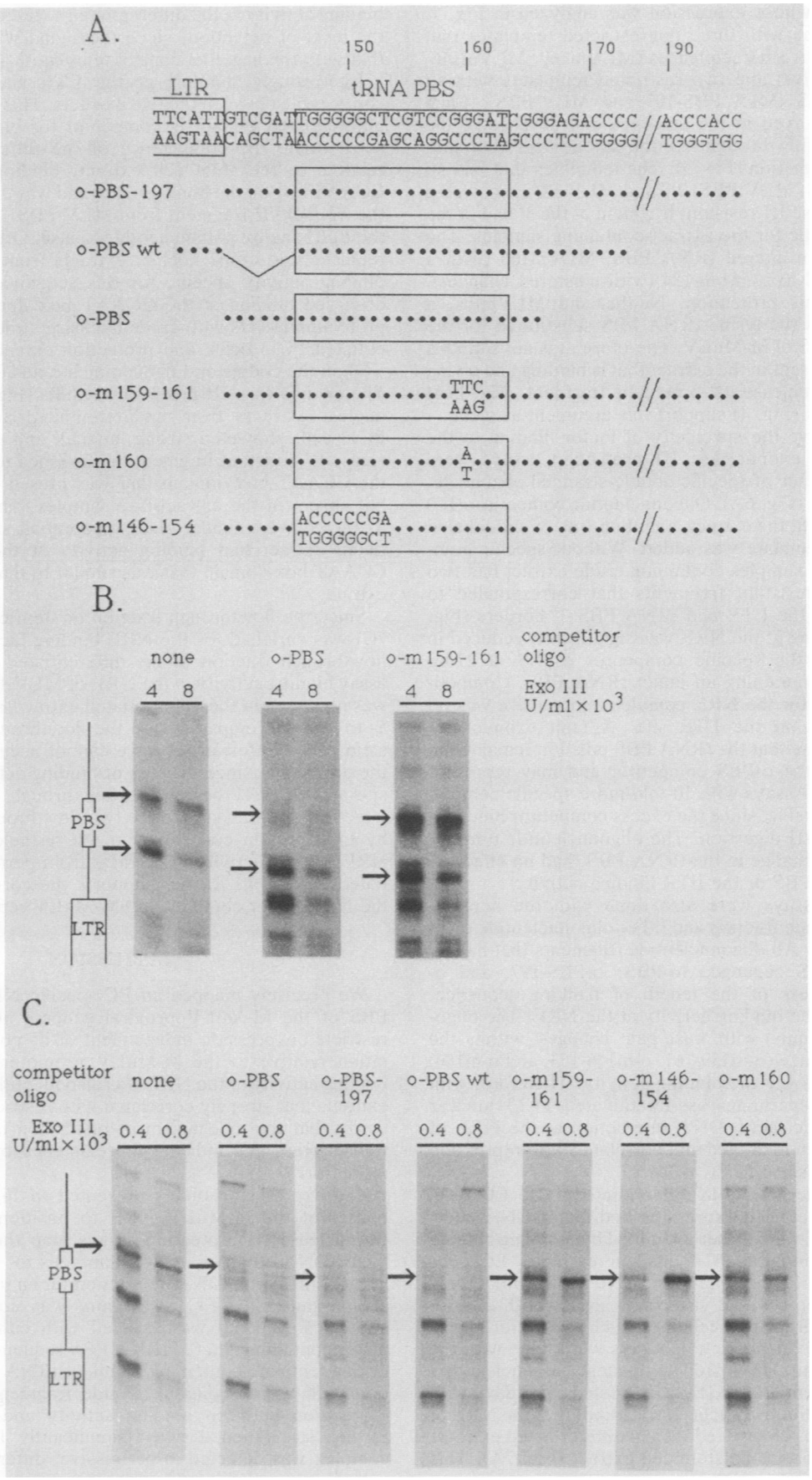
The factor at the 3′ border of the LTR was also detected in DNase I protection assays (data not shown). Its identity is unknown, but it is probably not involved in restriction. Identical binding activity in this region was observed on templates that were (MLV.PBS-197) and were not (MLVm159-161) restricted in EC cells. Also, as mentioned earlier, this region could be completely deleted without any effect on full repression by the tRNA PBS domain.

To further characterize the factor binding at the NRE and enrich for its binding activity, the crude nuclear extract was fractionated on a heparin-agarose column. The extract was passed over the column in 50 mM KCl, and bound protein was batch-eluted in 0.5 M KCl. The flowthrough and bound fractions were collected, concentrated, and used in exonuclease III protection assays with the MLV.PBS-197 and MLVm159-161 templates (Fig. 4B). As before, factor binding activity at the tRNA PBS (or NRE) was only observed with the MLV.PBS-197 template and was present in the flowthrough but not the bound fraction. The strong-stop fragment seen in MLVm159-161 with the crude nuclear extract (Fig. 4A) was also found with both fractions of the heparin-agarose column and was relatively exonuclease III sensitive. The flowthrough fraction generally produced a better signal than the crude nuclear extract and thus was used in several of the subsequent assays. Detection of the LTR-binding activity in the heparin-agarose fractions was variable and is not apparent in Fig. 4, yet it seemed to be enriched in the bound fraction (data not shown). To verify the integrity of the fractionation procedure, binding at the CCAAT box sequence in the M-MuLV promoter present on both fragments was also analyzed by exonuclease III protection and was found to be enriched in the bound fraction (see below; data not shown).

A cursory characterization of the factor-DNA interaction was done by heating the samples containing crude nuclear extract for 10 min at 68°C or 5 min at 80°C before adding the DNA template (Fig. 4C). Heat treatment of the crude nuclear extract or the heparin-agarose flowthrough fraction significantly reduced binding activity at the NRE, indicating that the factor is a protein. The effect of heat treatment on binding at the LTR site was not clear because of the low LTR-binding activity with the crude nuclear extract in the control, untreated samples. To address whether an RNA molecule, such as the proline tRNA, is a component of the binding complex, the extract was treated with RNase A (5 μg/ml) for 10 min at room temperature. There was no effect on binding activity (Fig. 4C), and similar results were obtained when RNase A concentrations of 25 μg/ml were used (data not shown). The slight enhancement in protein binding after RNase treatment suggests that an RNA species is binding to the NRE or the NRE-binding factor. However, RNase treatment of the extract in the absence of the tRNA carrier and tRNA titrations in the absence of RNase did not support this observation (data not shown).

Correlation of factor-DNA interaction with template repression in EC cells. The exonuclease III protection assays indicated a correlation between factor binding at the NRE and template repression in EC cells: binding activity at the NRE was observed with MLV.PBS-197, a template that has an intact tRNA PBS and is repressed in EC cells, but was not observed with MLVm159-161, a template that bears a 3-bp alteration in the PBS and is not restricted in EC cells. We were interested in whether this correlation would extend to

FIG. 6. Competition results with specific oligonucleotide fragments in exonuclease III (Exo III) protection assays. (A) Structure of the double-stranded oligonucleotide competitors. The competitor oligonucleotides span the tRNA PBS. Solid circles represent the nucleotides from the proviral sequence that were included in each oligonucleotide, and base-pair changes are indicated. The box encloses the tRNA PBS domain, and positions from the RNA start site are included. (B) Crude nuclear extract. A 100-ng amount of the indicated oligonucleotides (oligo) was mixed with 10 μg of PC13 crude nuclear extract protein and incubated for 5 min at room temperature. Then, 1 ng of the *Xba*I-*Bgl*II fragment from MLV.PBS-197, end labeled at the *Xba*I site, was added, and the assays were continued as described in the text. (C) Fractionated extract. Reactions were done exactly as described for section B, but 20 μg of PC13 nuclear extract protein from the flowthrough fraction of a heparin-agarose column was used.


A.
150
160
170
190
LTR
tRNA PBS
TTCATTGTCGATTGGGGGCTCGTCCGGGATCGGGAGACCCC//ACCCACC
AAGTAACAGCTAACCCCCGAGCAGGCCCTAGCCCTCTGGGG//TGGGTGG
o-PBS-197
o-PBS wt
o-PBS
o-m159-161
TTC
AAG
o-m160
A
T
o-m146-154
ACCCCCGA
TGGGGGCT
B.
none
o-PBS
o-m159-161
competitor oligo
4 8
4 8
4 8
Exo III U/ml×10³
PBS
LTR
C.
competitor oligo
none
o-PBS
o-PBS-197
o-PBS wt
o-m159-161
o-m146-154
o-m160
Exo III U/ml×10³
0.4 0.8
PBS
LTR

other templates whose expression was analyzed in Fig. 1. Assays were done with three nonrestricted templates that have altered tRNA PBS sequences (MLVm160, MLVm146-154, and MLVmlys) and two restricted templates with an intact tRNA PBS (MLV.PBS-197 and MLV.PBS). Each template was assayed in the presence and absence of the heparin-agarose flowthrough fraction and compared at similar extents of digestion (Fig. 5). The templates that had an intact tRNA PBS, MLV.PBS-197 and MLV.PBS, showed a strong exonuclease III-resistant fragment at the 3′ end of the tRNA PBS specific for the extract-containing samples. The templates with an altered tRNA PBS, MLVm160 (with a 1-bp change) and MLVm146-154 (with clustered changes), did not show this protection. Neither did MLVmlys, a template that has the lysine tRNA PBS substituted for the proline tRNA PBS of M-MuLV. Therefore, it is not simply a tRNA moiety present in the extract that is binding and giving rise to the exonuclease III-protected fragment. The heat sensitivity results (Fig. 4) support this argument as well.

To further prove the specificity of factor binding to the M-MuLV NRE, exonuclease III protection assays were done in the presence of specific double-stranded oligonucleotide competitors (Fig. 6A). Oligonucleotide competitor (100 ng) was mixed with the extract, and then 1 ng of end-labeled MLV.PBS-197 template was added. Without specific competitor DNA, the samples containing crude extract had two exonuclease III-resistant fragments that corresponded to factor binding at the LTR and tRNA PBS 3′ borders (Fig. 6B). Factor binding at the NRE was significantly reduced in the presence of the specific competitor o-PBS, a 27-bp oligonucleotide containing an intact tRNA PBS. Competition was specific for the NRE complex, since there was no effect on binding at the LTR site. A faint exonuclease III-resistant fragment at the tRNA PBS position remained in the presence of the o-PBS competitor and may represent residual binding. Assays with 10-fold-more specific competitor were inconclusive, since the excess competitor hindered the exonuclease III digestion. The oligonucleotide o-m159-161, with a 3-bp change in the tRNA PBS, had no effect on either the tRNA PBS or the LTR-binding activity.

Competition assays were also done with the heparin-agarose flowthrough fraction and other oligonucleotide competitors (Fig. 6C). All oligonucleotide fragments that had an intact tRNA PBS sequence (o-PBS, o-PBS-197, and o-PBS-wt), regardless of the length of flanking sequence, competed away the binding activity at the NRE. The oligonucleotide fragments with base-pair changes within the tRNA PBS sequence (o-m159-161, o-m146-153, and o-m160) did not compete away the binding activity. Therefore, with the crude and heparin-agarose-fractionated PC13 nuclear extracts, a specific factor-DNA interaction at the NRE of M-MuLV has been identified and correlated with repression of template expression in EC cells.

Factor-DNA interactions in differentiated PC13 EC cells. The correlation between expression and factor-DNA interaction at the NRE was further examined by assaying binding activity in differentiated PC13 nuclear extracts. EC cells differentiated in vitro become permissive for M-MuLV replication, although recovered virus titers are lower than those from infected differentiated cell lines, such as 3T3 fibroblasts (33). Exonuclease III protection assays were done with the MLV.PBS-197 and MLVm160 templates and nuclear extracts from differentiated PC13 cells treated for 7 days with retinoic acid and dibutryl cyclic AMP. Binding at the NRE of MLV.PBS-197 was clearly reduced in differentiated extracts compared with that in the stem cell extract (Fig. 7A). This binding activity in the differentiated extracts was at or below the level of detection, since the signal was comparable to that with the negative control template MLVm160.

Interestingly, binding at the LTR site (Fig. 7A) was comparable between the two extracts. This indicates that the differentiated extract is competent for binding assays. To substantiate the competence of the differentiated extract relative to the stem cell extract, binding activity at the CCAAT box on the noncoding strand was examined by using the *Xba*I-*Bgl*II fragment from MLV.PBS-197 end labeled at the *Bgl*II site by polynucleotide kinase. Others (14, 44) have reported that crude nuclear extracts from EC cells have a binding activity specific for this sequence. We have also observed binding at the CCAAT box domain in DNase I protection assays with crude nuclear extracts from undifferentiated PC13 cells, and protection extended from −63 to −88 on the coding and noncoding strands (data not shown). The exonuclease III protection results (Fig. 7B) with crude nuclear extracts from undifferentiated and differentiated PC13 cells showed a strong, unique, and persistent exonuclease III-resistant fragment that mapped to the 5′ border of the CCAAT box domain that was present in both extracts but absent in the extract-free samples. Therefore, the two extracts have a similar binding potential, since the differentiated extract had binding activity at the LTR site and CCAAT box domain that was similar to that of the stem cell extract.

Since the flowthrough fraction of stem cell extracts (Fig. 7C) was enriched for the NRE-binding factor, we used the flowthrough fraction of the differentiated extract to again assay binding activity at the NRE of MLV.PBS-197. Binding was now seen in the differentiated extract, although reduced 5- to 10-fold compared with the flowthrough fraction from stem cells. Unfortunately, we cannot accurately quantitate the difference, since the loss of binding activity at the LTR site and CCAAT box in the flowthrough fraction necessitated that we standardize the two flowthrough extracts only by total protein concentration. Nevertheless, the reduced NRE-binding activity in extracts from permissive, differentiated PC13 cells further supports the correlation between the NRE-factor complex and M-MuLV repression.

DISCUSSION

We precisely mapped an EC-specific NRE to the tRNA PBS of the M-MuLV proviral genome and found that it restricted expression independent of its position and orientation relative to the M-MuLV promoter. A factor-DNA binding activity at the NRE was also identified in EC nuclear extracts that strongly correlated with restriction. These data verify that the mechanism of restriction is at the level of transcription and indicate the existence of an EC-specific repressor.

The expression studies presented in Fig. 1 map the 3′ border of the M-MuLV NRE to position +163, and the exonuclease III protection assays map the 3′ border of a factor-DNA complex in EC stem cells to +167. The specificity of the factor-DNA interaction for an intact tRNA PBS, also required for EC restriction, was confirmed by two strategies. Binding was assayed with either various templates containing altered tRNA PBS sequences (Fig. 4 and 5) or one template containing an intact tRNA PBS in competition with various oligonucleotide fragments (Fig. 6). The correlation between binding activity and restriction was further strengthened when significantly less factor-DNA complex was detected in permissive, differentiated EC cell

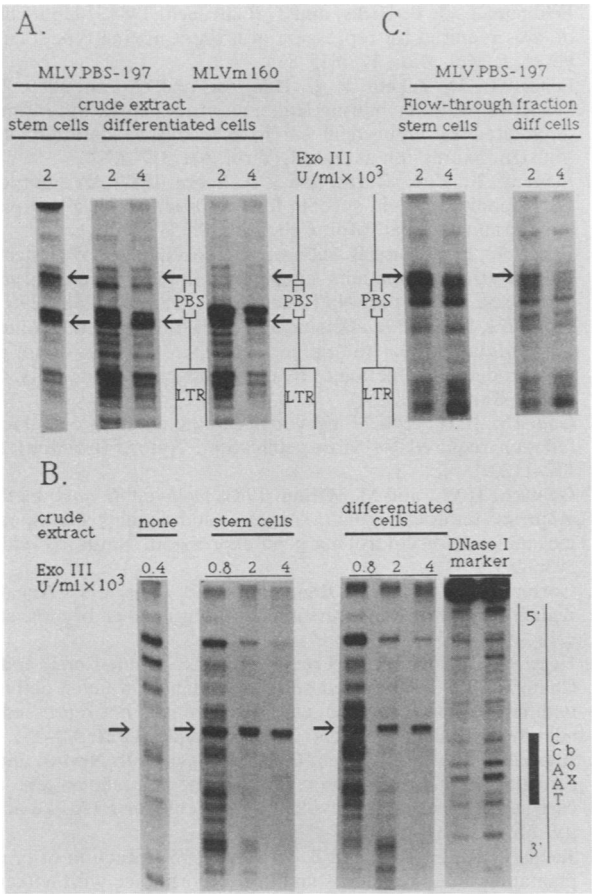


FIG. 7. Exonuclease III (Exo III) protection assays with nuclear extracts from differentiated PC13 cells. (A) Protection at the NRE with crude nuclear extracts. The *Xba*I-*Bgl*II fragment (0.5 ng) from MLV.PBS-197 or MLVm160, end labeled at the *Xba*I site, was incubated with 10 μg of crude nuclear extract protein prepared from undifferentiated or differentiated cultures of PC13 cells. (B) Protection at the CCAAT box with crude nuclear extracts. The *Xba*I-*Bgl*II fragment (10 ng) from MLV.PBS-197, end labeled at the *Bgl*II site, was incubated in the absence or presence of 10 μg of crude nuclear extract protein prepared from undifferentiated and differentiated PC13 cells. The position of the CCAAT box was determined from the DNase I digestion profile of the same fragment, and the solid bar represents the protected region observed in DNase I protection assays (data not shown). Arrows indicate the position of the exonuclease III-resistant fragment corresponding to protection of the CCAAT box. (C) Protection at the NRE in fractionated extracts. Assays were done exactly as described for section A, except 20 μg of protein from the heparin-agarose flowthrough fraction prepared from nuclear extracts of undifferentiated and differentiated PC13 cells was used.

extracts than in stem cell nuclear extracts (Fig. 7). These results strongly argue that a *trans*-acting, cellular repressor restricts M-MuLV expression in EC cells through an interaction with the NRE at the tRNA PBS of the proviral DNA. One possibility that cannot be excluded at this time is that factor binding at the NRE facilitates binding or action of a second factor that in turn is responsible for repression.

The NRE-binding activity in the stem and differentiated cell nuclear extracts was standardized to the binding activities at the 3′ end of the LTR and the CCAAT box domain, present on the M-MuLV templates. Equivalent CCAAT box-binding activity was observed in nuclear extracts from stem and differentiated cells. This finding differs from the results of Flamant et al. (14) but agrees with the report from Tsukiyama et al. (44) and is consistent with our previous results that showed that the M-MuLV promoter is functional in EC stem cells (30). Factor binding at the 3′ border of the LTR has been found in a variety of differentiated cell types (data not shown), but its identity is unknown. This region has no apparent function in the regulation of RNA expression, since its presence or absence has no effect on levels of RNA accumulation in EC or differentiated cell types (30, 31). Sequences at the 3′ border of the M-MuLV LTR have been shown to affect integration of the proviral DNA (10), although to our knowledge no cellular factors have been directly implicated in this process.

The coincidence of the NRE with the tRNA PBS suggests that a tRNA molecule is responsible for the binding activity and restriction. This is not likely, since heat treatment virtually eliminated binding activity at the NRE and binding was resistant to treatment of the extract with RNase A (Fig. 4). This indicates that the factor is a protein.

It is surprising that the NRE maps precisely to the tRNA PBS. Barklis et al. (4) identified a host range mutant with a base-pair mutation at +160 which implied a role for the tRNA PBS domain in EC restriction. Yet it seemed that sequences outside of the tRNA PBS must also be involved. Myeloproliferative sarcoma virus can replicate more efficiently in EC cells than M-MuLV (15, 36). It has the same tRNA PBS sequences as M-MuLV, but the sequences immediately 3′ to the PBS are different, and the myeloproliferative sarcoma virus tRNA PBS domain was not found to be highly restrictive in EC cells in viral infection assays (47). This implies that the NRE should extend beyond the tRNA PBS into the 3′-flanking region. In contrast, the deletion analyses in Fig. 1 demonstrated that the restrictive element is within the tRNA PBS and that sequence alterations immediately 3′ or 5′ to the PBS do not affect repression. We conclude that sequences flanking the PBS may influence repression in EC cells but are not directly involved in NRE function.

The reason for the contradictory results between the transient expression and stable cotransformation assays when the NRE was placed 5′ to the LTR is not known. One possibility is that episomal DNA is not stringently restricted during the transient expression period, and placement of the NRE outside of the transcription unit results in the loss of the inhibitory effect. The inhibitory function is regained when the template integrates into the chromosome and obtains a nucleosomal and higher-order chromatin structure.

Another possibility is that there are two steps in the restriction mechanism. The initial restriction, identified in the transient expression assays, is orientation independent but position dependent; restriction was observed with the NRE downstream but not upstream of the M-MuLV LTR. The second mechanism occurs with either time or integration of the template into the chromosome. It is independent of the position of the NRE; it silences expression from recombinants that have the restrictive element in the upstream configuration. The mapping data presented in Fig. 1 and 3 show that if the two mechanisms are operative, they are mediated by the same DNA element or DNA-factor complex. One candidate for the second mechanism is DNA methylation. Others have proposed a two-step mechanism of retroviral inactivation involving methylation in mouse EC cells (18, 33). We have found that integrated templates carrying the M-MuLV NRE in either the upstream or downstream configuration are hypermethylated in EC cells relative to templates without this element (data not shown).

However, we have no evidence for a direct role of the NRE in directing methylation of the M-MuLV templates.

Negative regulation of other genes in EC cells has been proposed. Viral E1a represses enhancer-dependent transcription (7, 45), and the relative inactivity of several viral enhancers in EC cells may be due to a cellular E1a-like activity identified in F9 and PCC4 EC stem cells (21, 26). A point mutation that increases the activity of the polyomavirus B enhancer in EC cells (16, 23) may allow the binding of a positive activator (43) and relieve the repressor effects (20, 35). Regulation of the polyomavirus A enhancer in EC and other differentiated cell types may be modulated by the interaction of positive and negative factors that bind to neighboring and overlapping sites (46). The immunoglobulin heavy-chain enhancer is also inactive in EC cells, and its repression has been attributed to an EC-specific octamer-binding factor, termed NF-A3 (27).

In contrast to the examples above, the NRE at the M-MuLV tRNA PBS is not part of any previously identified promoter or enhancer sequence. Rather, it is more than 150 bp downstream from them. The exact mechanism of restriction by the NRE is not known. Based on our data, M-MuLV repression is mediated by the binding of an EC-specific repressor that presumably does not act by competing for binding sites with positive activators. More likely, the repressor interferes with the function of an activator protein or the basal transcription complex in a quenching or direct-repression mechanism (for review, see reference 28).

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