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SLIPPED DNA STRUCTURES WITHIN THE ENHANCER REGION OF THE MOLONEY MURINE LEUKEMIA VIRUS

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We have examined the S1 nuclease sensitivity of supercoiled plasmids harboring the Moloney Murine Leukemia Virus (MoMuLV) long terminal repeat (LTR). S1 sensitivity was found within the LTR enhancer direct repeats. Transformation of E. coli DH5 cells with a construct containing most of the MoMuLV LTR yielded the precise deletion of one direct repeat and loss of S1 sensitivity. The dependence of S1 sensitivity on the presence of both direct repeats, together with the exact excision of one direct repeat by E. coli, suggests the presence of slipped DNA within the enhancer. Such structures may represent targets for effector proteins which mediate vital functions during viral propagation.

Increasing evidence suggests that DNA conformational microheterogeneity due to the presence of unusual structures plays a central role in cellular processes such as replication, recombination, transcription, mutagenesis and DNA repair (1,2). Such unusual DNA structures include left handed Z DNA (3,4), H DNA (5), cruciforms (6), Pur-Pyr structures (7,8,9), anisomorphic DNA (10), bent DNA (11) and slipped DNA structures (12,13). Although the biological significance of such DNA structures is not understood, they may constitute targets for specific regulatory DNA binding proteins. Many unusual DNA structures are sensitive to the single-strand specific S1 nuclease. Pur-Pyr structures in the major late promoters of adenovirus (7), cruciform structures of Col E1 plasmids (14), and B to Z transitions have been found to be cleaved by S1 nuclease in a site-specific manner (4). We have analyzed the DNA conformational organization of the enhancer region of the Moloney Murine Leukemia Virus (MoMuLV) long terminal repeat (LTR) by S1 nuclease analysis, and we present evidence for the presence of slipped DNA structures within the enhancer region of this LTR.

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MATERIALS AND METHODS

Bacterial Strains: Escherichia coli strains DH5 (endA1, recA1, hsdR17 (r_K^-, m_K^+), sup E44, thi-1,λ-, gyrA96, relA1, F^-) and HB101 (F^-, hsdS20 (r_B^-, m_B^-), supE44, ara14, galK2, lacY1, proA2, rpsL20(str^R), x_Y15, leu, mtl1,λ-, recA13) were obtained from Bethesda Research Laboratories (Gaithersburg, MD).

Plasmid Constructions: Plasmid pLTRM6 was prepared by subcloning a 2.4 kB EcoRI fragment harboring the complete MoMuLV 3'LTR from pZIPneoSV(x)1 (15) into a 1.4 kB EcoRI-AvaI fragment from pBR322 harboring the tetracycline resistance gene, after converting the AvaI site to an EcoRI site. Following propagation in E. coli HB101 cells, pLTRM6 DNA was partially digested with NheI. The plasmid was then end-repaired with T_4 DNA polymerase in the presence of all four deoxyribonucleoside triphosphates, ligated to PstI linkers with T_4 DNA ligase, and digested with PstI. The DNA was self-ligated with T_4 DNA ligase, and the resulting plasmid pLTRM6ΔPst was used to transform E. coli HB101 cells (Fig. 1). Plasmid pLTRM6ΔXho was prepared by double-digestion of pLTRM6ΔPst DNA with XhoI and PstI under standard conditions, end-repaired as described above, ligated with XhoI linkers, XhoI digested, and self-ligated (Fig. 1). The ligated pLTRM6ΔXho DNA was propagated in E. coli DH5 cells. Plasmids pLTRM6ΔPst and pLTRM6ΔXho were extensively characterized by restriction enzyme analysis including EcoRV, whose recognition sites lie within the 75bp direct repeats of the MoMuLV enhancer (Fig. 2). Plasmid pMCAT was prepared by subcloning the 0.4 kB XhoI-HindIII fragment from pLTRM6ΔXho upstream from the chloramphenicol acetyl transferase (CAT) gene sequences of pSVIXCAT (Fig. 1). pMCAT was propagated in E. coli DH5 cells. pACAT was obtained from Dr. W. Haseltine (16).

S1 Nuclease Analysis: Approximately 10 µl of each pLTRM6ΔPst and pLTRM6ΔXho DNAs were digested with 1200 units of S1 nuclease (Boehringer Mannheim,IN) in a 500 µl reaction mixture containing 200mM sodium chloride, 50mM sodium acetate, pH 4.5, 1 mM $ZnSO_4$, 0.5% glycerol, and 10 µg/ml yeast

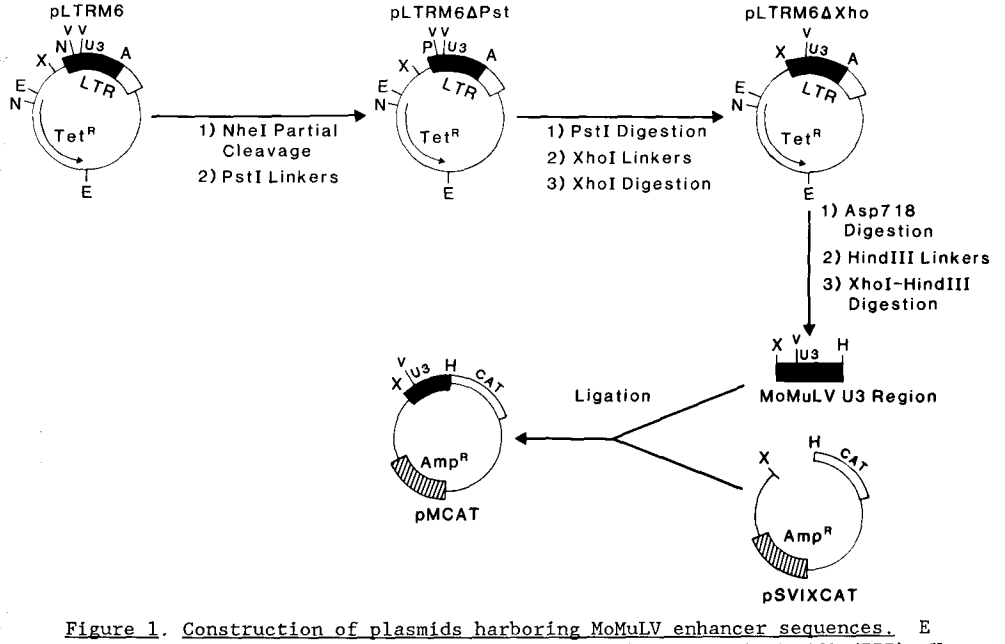

Figure 1. Construction of plasmids harboring MoMuLV enhancer sequences. E (EcoRI), N (NheI), X (XhoI), A (Asp718), P (PstI), H (HindIII), V (EcoRV).

Figure 2. Nucleotide sequence of the MoMuLV enhancer region. D.R. (direct repeat).

tRNA, for 10 min at 10° C. The reaction was stopped by the addition of 6 µl of 0.5 M EDTA, and the S1 nuclease-digested DNA was purified by chromatography through Elutip columns (Schleicher and Schuell, NH) followed by ethanol precipitation. After centrifugation, each DNA pellet was resuspended in 20 µl of 10 mM Tris-HCl, pH 7.6, 1 mM EDTA. Aliquots of 1 µl of pLTRM6ΔPst and pLRTM6ΔXho DNAs treated with S1 nuclease were further digested with 10 units of NcoI, EcoRI or NheI under standard conditions. The products were analyzed by 0.7 or 1.4% agarose gel electrophoresis in the presence of lambda DNA digested with HindIII and OX174 RF DNA digested with HaeIII as molecular weight markers.

DNA Sequence Analysis: Plasmid pLTRM6ΔXho was digested with Asp718, end-repaired as previously described, ligated with HindIII linkers, and digested with both XhoI and HindIII. A DNA fragment of approximately 0.4 kB containing most of the MoMuLV U3 region was purified by agarose gel electrophoresis followed by electrolution, Elutip chromatography, and ethanol precipitation. The purified fragment was subcloned into the plasmid vector Bluescript M13 (Strategene, CA) which had been previously digested with HindIII and XhoI, and dephosphorylated with calf intestine alkaline phosphatase. This construct was propagated in DH5 cells. Sequencing of the XhoI-HindIII insert was performed by the double-stranded sequencing method (17,18,19).

Chloramphenicol Acetyl Transferase (CAT) Activity Directed By pMCAT and pACAT. Murine cell lines M12 (B-cell leukemia) and L691-6 (T-cell lymphoma) (16) were grown in RPMI 1640 medium supplemented with L-glutamine, penicillin, streptomycin, 10% fetal calf serum and $5x10^{-5}$M β-mercaptoethanol. Each cell line was transfected with equivalent molar amounts (1 µg) of pMCAT and pACAT as previously described (16). After 72h, the cells were washed with cold phosphate buffered saline, pH 7.2, and resuspended in 150 µl of 0.25M Tris-HCl, pH 8.0. Cell extracts were prepared by 3 cycles of freezing (-70°C) and thawing (37°C), centrifuged, and the supernatants were assayed for protein concentration and CAT activity. Results are expressed as relative percentage conversion of ^{14}C-chloramphenicol to ^{14}C-chloramphenicol acetate by extracts standardized for the same amount of protein.

RESULTS AND DISCUSSION

Restriction Endonuclease and S1 Nuclease Analysis of pLTRM6ΔPst and pLTRM6ΔXho DNAs: Both pLTRM6ΔPst and pLTRM6ΔXho contained the predicted restriction endonuclease sites. However, upon EcoRV digestion, the 75bp DNA

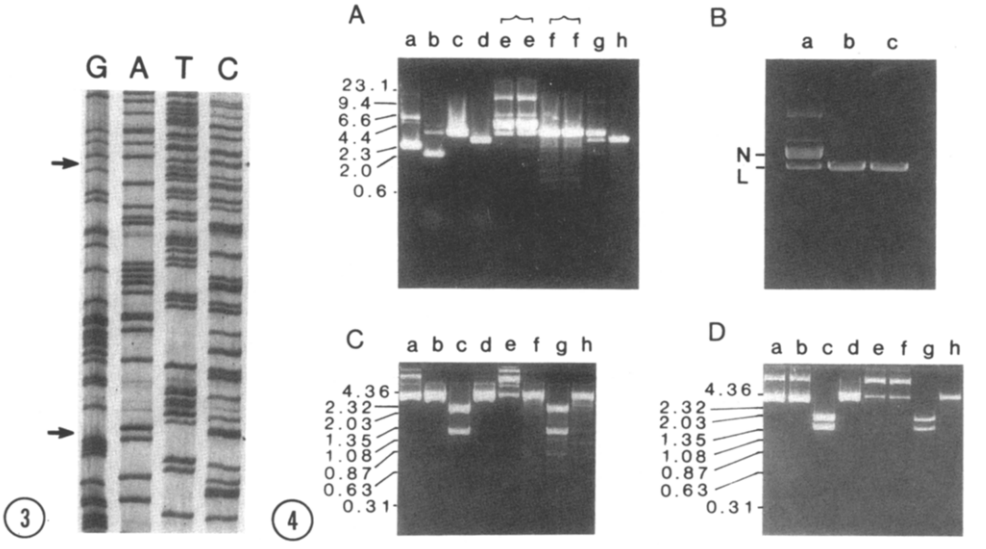

Figure 3. Nucleotide sequence analysis of the enhancer region of pLTRM6ΔXho. Arrows denote the sequence boundaries of the unique enhancer direct repeat found within this plasmid.

Figure 4. A. S1 nuclease sensitivity of pLTRM6ΔPst and pLTRM6ΔXho. 0.7% Agarose gel electrophoresis. a) pLTRM6ΔPst uncut, b) pLTRM6ΔXho uncut, c) pLTRM6ΔPst + NheI, d) pLTRM6ΔXho + NheI, e) pLTRM6ΔPst + S1 nuclease, f) pLTRM6ΔPst + S1 nuclease + NheI, g) pLTRM6ΔXho + S1 nuclease, h) pLTRM6ΔXho + S1 nuclease + NheI. B. S1 nuclease sensitivity of linearized pLTRM6ΔPst. a) pLTRM6ΔPst + S1 nuclease, b) pLTRM6ΔPst + NheI, c) pLTRM6ΔPst + NheI followed by S1 nuclease. C. and D. Mapping of S1 nuclease hypersensitive sites in pLTRM6ΔPst and pLTRM6ΔXho respectively. 1.4% Agarose gel electrophoresis. a) uncut plasmids, b) NcoI, c) EcoRI, d) NheI, e) S1 nuclease, f) S1 nuclease + NcoI, g) S1 nuclease + Eco RI, h) S1 nuclease + NheI .

fragment spanning 38 nucleotides of the enhancer's first and 37 nucleotides of its second direct repeat was present only in pLTRM6ΔPst but not in pLTRM6ΔXho. This suggests that a small deletion of enhancer sequences occurred in pLTRM6ΔXho. DNA sequence analysis of the U3 region of pLTRM6ΔXho showed a deletion within the enhancer region of the MoMuLV LTR. One of the two 75 bp direct repeat units contained within the enhancer had been deleted precisely (Fig. 3) during propagation in E. coli DH5 cells. The same phenomenon has been observed previously when MoMuLV proviruses were cloned into lambda Charon 21A and propagated in E. coli DP50 sup F (20,21).

Specific S1 nuclease sensitive sites were observed preferentially in pLTRM6ΔPst as compared to pLTRM6ΔXho (Figs. 4A,4C,4D). Mapping of these sites in pLTRM6ΔPst showed an S1 sensitive region within the LTR enhancer element (Fig. 5). In contrast, no specific S1 nuclease hypersensitivity was observed within the pLTRM6ΔXho LTR enhancer region (Figs. 4A,4D). S1 sensitivity was observed only in supercoiled and not in previously linearized plasmid of either construct (Fig. 4B).

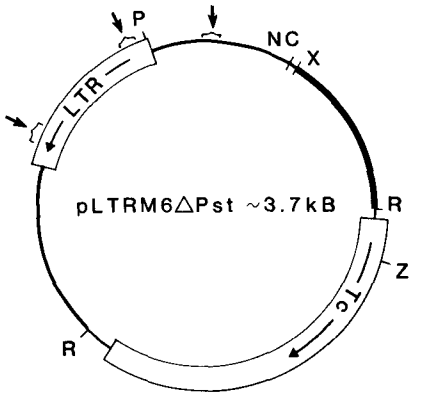

Figure 5. S1 nuclease hypersensitive regions of pLTRM6ΔPst. P(PstI), NC(NcoI), R (EcoRI), N (NheI), X (XhoI). Arrows denote the location of the main S1 hypersensitive regions.

The presence of an S1 nuclease sensitive region within the enhancer LTR region in pLTRM6ΔPst, together with the deletion of a complete enhancer direct repeat in pLTRM6ΔXho, suggests the existence of unusual DNA structures within the MoMuLV LTR enhancer element in supercoiled pLTRM6Δ Pst. Sequence analysis of the MoMuLV LTR enhancer direct repeats does not reveal consensus sequences capable of adopting Z-DNA configuration, such as alternating purine-pyrimidine sequences. In addition, the lack of inverted repeats within the enhancer element argues against the presence of cruciform structures. However, the precise deletion of one direct repeat could have arisen from a slipped DNA structure (12,13). Such a model predicts the formation of single-stranded structures within direct repeats, contained within supercoiled DNA. Slipped DNA structures have been proposed for the chicken and mouseα2 (1) collagen promoter (13). We propose a similar phenomenon in the MoMuLV enhancer region of supercoiled pLTRM6ΔPst DNA (Fig. 6). The first repeat on one strand base-pairs with the second repeat on the other strand, or vice versa, producing two staggered single-stranded loops.

Partially single-stranded regions are known to occur in supercoiled DNA plasmids. The energy requirements for stabilizing partially single stranded sequences are offset by relieving stress introduced by DNA supercoiling. As in the extrusion of cruciforms in supercoiled plasmid DNA, where the disruption of the plasmid double helix is partially compensated by basepairing of the stem and thus leads to stabilization of the partially single-stranded DNA structures, the basepairing occurring between direct repeat 1 and direct repeat 2 can stabilize the slipped DNA structure. Therefore, the formation of the staggered single-stranded loops could account for the S1 nuclease sensitivity of the MoMuLV LTR enhancer region in protein-free plasmid preparations.

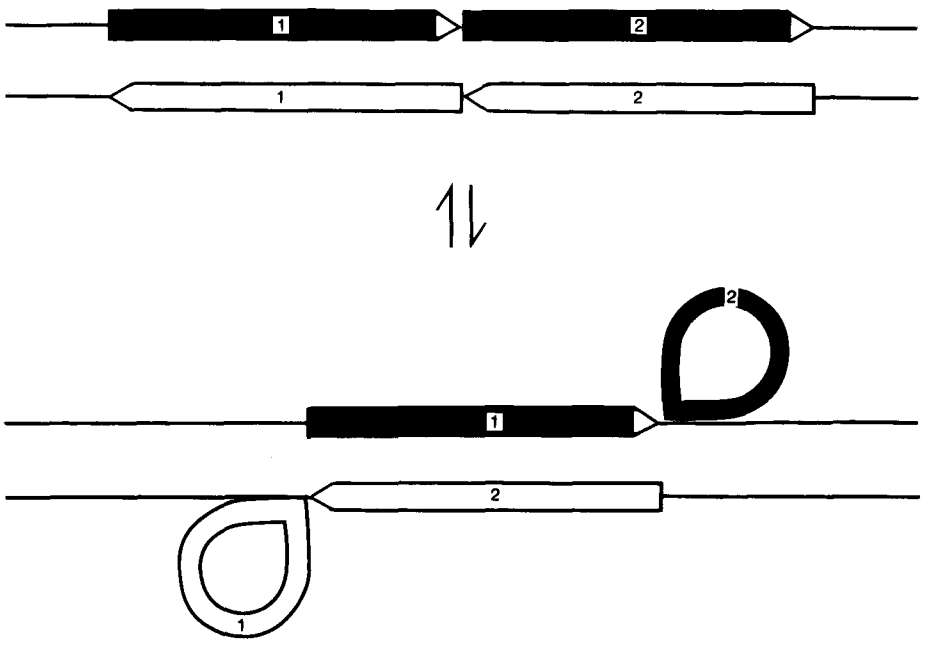

Figure 6. Schematic model representing slipped DNA structures within the enhancer region of MoMuLV LTR.

It has been suggested that slipped DNA structures may be important in the regulation of transcription (1,13). Transfection of pMCAT into murine cell lines resulted in the appearance of chloramphenicol acetyl transferase activity which was expressed in a cell lineage-specific manner (Table 1). Higher activity was observed in the T-cell derived L691-6 cell line and lower activities in the B-cell derived M12 cell line compared to the activities showed by pACAT (harboring the Akv virus U3 region with both enhancer 99bp direct repeats) transformed cell lines. These results confirm a previous report that one direct repeat within the enhancer element is sufficient for cell-lineage specific enhancer activity (22). In addition, MoMuLV with a single enhancer direct repeat has been shown to be infectious; however, the onset of the virus-induced disease was delayed significantly (23).

TABLE 1

Expression of chloramphenicol acetyl transferase (CAT) directed by pMCAT and pACAT promoter-enhancer regions

Plasmid	Parental Virus for U3 Region	% Relative CAT Activity	
		T Cells (L691-6)	B Cells (M12)
MCAT	MoMuLV	100	100
ACAT	Akv	62	753

The single-stranded loops may have been excised by enzymes of the E. coli DNA repair system such as those carrying out mismatch repair (24). In eukaryotes, slipped DNA structures may be stabilized by specific DNA binding proteins, and such structures may be targets for other effector regulatory proteins.

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