The Secondary Structure of the R Region of a Murine Leukemia Virus Is Important for Stimulation of Long Terminal Repeat-Driven Gene Expression

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In addition to their role in reverse transcription, the R-region sequences of some retroviruses affect viral transcription. The first 28 nucleotides of the R region within the long terminal repeat (LTR) of the murine type C retrovirus SL3 were predicted to form a stem-loop structure. We tested whether this structure affected the transcriptional activity of the viral LTR. Mutations that altered either side of the stem and thus disrupted base pairing were generated. These decreased the level of expression of a reporter gene under the control of viral LTR sequences about 5-fold in transient expression assays and 10-fold in cells stably transformed with the LTR-reporter plasmids. We also generated a compensatory mutant in which both the ascending and descending sides of the stem were mutated such that the nucleotide sequence was different but the predicted secondary structure was maintained. Most of the activity of the wild-type SL3 element was restored in this mutant. Thus, the stem-loop structure was important for the maximum activity of the SL3 LTR. Primer extension analysis indicated that the stem-loop structure affected the levels of cytoplasmic RNA. Nuclear run-on assays indicated that deletion of the R region had a small effect on transcriptional initiation and no effect on RNA polymerase procesivity. Thus, the main effect of the R-region element was on one or more steps that occurred after the template was transcribed by RNA polymerase. This finding implied that the main function of the R-region element involved RNA processing. R-region sequences of human immunodeficiency virus type 1 or mouse mammary tumor virus could not replace the SL3 element. R-region sequences from an avian reticuloendotheliosis virus partially substituted for the SL3 sequences. R-region sequences from Moloney murine leukemia virus or feline leukemia virus did function in place of the SL3 element. Thus, the R region element appears to be a general feature of the mammalian type C genus of retroviruses.

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vation that the nucleotides predicted to be involved in base pairing were precisely conserved among all MuLVs, feline leukemia virus (FeLV), and type C primate viruses (13).

This study was undertaken to address whether the predicted secondary structure was important for activity of the MuLV R region. We also tested whether the MuLV R region functioned analogously to the TAR element of HIV in affecting the processivity of RNA polymerase. In addition, we examined whether the LTR R sequences from other retroviruses could substitute for the MuLV R region in driving the expression of a reporter gene.

**MATERIALS AND METHODS**

**Cell lines.** NIH 3T3 cells were maintained in Dulbecco’s modified Eagle medium with 10% bovine serum. K562 cells were maintained in RPMI 1640 with 10% fetal bovine serum. Media were supplemented with 100 U of penicillin and 100 μg of streptomycin per ml.

**Plasmids.** The SL3 and SL3 R-region deletion plasmids (Fig. 1) were previously described (13). We previously showed that the R region affected activity of LTR-reporter plasmids whether only part of the R region or a 400-nucleotide segment containing R, U5, and most of the 5′ untranslated sequence was present in the plasmids (13). The SL3-CAT (chloramphenicol acetyltransferase) plasmid containing only part of the R region was used in this study because the presence of convenient BssHII and HindIII restriction sites (Fig. 1C) facilitated the construction of the various plasmids. To generate each mutant, the BssHII-to-HindIII fragment of SL3-CAT was replaced with a fragment containing the appropriate mutation. To generate these fragments, complementary oligonucleotides containing the mutations were synthesized. The oligonucleotides were designed such that they contained BssHII and HindIII cohesive ends after the two strands were hybridized. The double-stranded fragments were inserted in the corresponding sites of SL3-CAT. The successful construction of each plasmid was confirmed by sequencing. The R region sequences of heterologous retroviruses were inserted into the SL3 LTR by a similar strategy using synthetic oligonucleotides. HIV-1 CAT and simian virus 40 Tat plasmids were described by Rosen et al. (41, 42).

**CAT assays.** Transient transfections and CAT assays were performed as previously described (13, 31, 34, 37, 53). Acetylation of chloramphenicol was quantitated by PhosphorImager analysis. Data are presented as means of multiple trials.

**Stable transformation of NIH 3T3 fibroblasts.** NIH 3T3 fibroblasts were transformed with 18 μg of LTR-CAT plasmid and 2 μg of pSV2neo as previously described (13). To minimize the effects of integration sites on LTR activity, G418-resistant clones were grown as pools. To minimize the possibility of single clones overgrowing the pools, they were used as soon as sufficient numbers of cells were obtained.

**CAT assays, primer extension analysis, and nuclear run-on assays with stably transformed cells.** CAT assays and primer extension analysis were performed as previously described (13). Nuclear run-on assays were performed as previously described (13), with the following changes. Before ethanol precipitation, the radiolabeled RNA was briefly fragmented by incubation in 0.2 M NaOH at 0°C for 10 min as described by Laspa et al. (25). Samples were neutralized by the addition of 1 M HEPES-KOH (pH 7.5) to a final concentration of 0.25 M and then ethanol precipitated. CAT and β-actin DNA probes were single-stranded fragments cloned in an M13 bacteriophage vector (25). The 5′ and 3′ probes were fragments H and V, respectively, of Laspa et al. (25).

**RESULTS**

Secondary structure is important for the activity of the MuLV R region. To test whether secondary structure is important for the activity of the R region, mutations were introduced into the 5′ end of the R region of the LTR of the MuLV SL3. The first 28 nucleotides of the 68-nucleotide-long R region of SL3 were predicted to form the stem-loop structure shown in Fig. 1A (13). The existence of this stem-loop was supported by the conservation of the nucleotides involved in base pairing among MuLVs and related retroviruses of cats and primates.

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**FIG. 1.** Structure of the predicted stem-loop at the 5′ end of the SL3 R region and the mutations that test its importance. (A) The predicted stem-loop at the 5′ end of the SL3 R region. Nucleotides are shown starting from the first transcribed nucleotide of R (+1). Base pairs are indicated by dashes between the nucleotides. (B) Sequences of mutations introduced into the R region of SL3. The top line shows the first 32 nucleotides transcribed from the SL3 R region. Nucleotides involved in base pairing are marked with dots. Sequences of the mutations are shown below the SL3 sequence. Altered nucleotides in each case are underlined. Names at the left identify the mutations: SΔR, SL3 R-region deletion; ASC, ascending side of the stem; DES, descending side of the stem; COMP, compensatory. Symbols at the right represent the expected effect of each mutation on the predicted secondary structure; squares indicate base substitutions, and stars indicate the substitution of five consecutive bases. (C) Organization of the LTR and CAT gene sequences in the reporter plasmids.
(13). With the exception of two nucleotides in the loop, the nucleotides that were implicated in base pairing are the only ones that are conserved among these viruses (13). Sixteen nucleotides were predicted to base pair to form the stem, and eight nucleotides were predicted to be present in the loop (Fig. 1A). On the descending side of the stem, three nucleotides were predicted to form a bulge. It is unclear whether the G in the middle of the bulge would base pair with the single C in the bulge that is present on the ascending side of the stem (Fig. 1A). Double-stranded oligonucleotides containing mutations in various parts of this predicted structure (Fig. 1B) were synthesized and substituted into a plasmid containing the SL3 LTR linked to the CAT reporter gene (Fig. 1C).

One mutation, SΔR, was a deletion of 28 nucleotides of the R region, from positions +4 to +31, inclusive (Fig. 1B). This deletion encompassed most of the stem-loop structure. As previously described (13), this mutation reduced LTR activity about 10-fold compared to wild-type SL3 in transient transfection assays (Fig. 2), thus confirming the importance of this portion of the R region.

Two nucleotides within the predicted loop structure, the U at position 11 and the A at position 15, are conserved among MuLVs and related viruses (13). To test the importance of these nucleotides, transversion mutations were introduced at these positions (Fig. 1B, Loop). The nucleotide at position 16 was also mutated as this resulted in the formation of a new XhoI site that facilitated screening for presence of the mutations. These substitutions had little effect on LTR activity (Fig. 2). Thus, the bases at these positions were not crucial for the activity of the R region.

To test whether the bulges in the stem were involved in the activity of the R region, we generated a mutant in which the A and U in the bulge on the descending side of the stem were deleted (Fig. 1B, Bulge). Presumably, this allowed the G between the A and the U (Fig. 1A) to base pair with the C at position 4 on the ascending side of the stem, thereby eliminating the bulge from the stem. This mutation had no effect on LTR activity (Fig. 2), indicating that the bulge structure was not important for the activity of the R region.

To test whether base pairing was important for the function of the R region, nucleotides involved in base pairing were mutated. Five nucleotides in either the ascending or the descending side of the stem were changed to disrupt base pairing (Fig. 1B, ASC and DES). In addition, we generated a compensatory mutation (Fig. 1B, COMP) in which both the ascending and descending sides of the stem were mutated such that the nucleotide sequence was different but the predicted secondary structure was maintained. Disruption of the secondary structure by mutation of the nucleotides on either side of the stem inhibited activity to levels approaching that of the R-region deletion (Fig. 2). However, when both mutations were combined to restore the predicted secondary structure, activity was restored to about 50% of the level of the intact LTR (Fig. 2). We interpret this result to mean that secondary structure is important for the full activity of the SL3 R region.

To distinguish whether the secondary structure of the R region affected the levels of mRNA or had an effect on translation of the R-region-containing transcripts, we performed primer extension analysis on cytoplasmic RNA. To facilitate this analysis, we generated pools of stably transformed cell lines by cotransfection of the LTR-CAT plasmids with a plasmid containing the neomycin resistance gene. First, we verified that the levels of LTR-driven CAT activity in these cells (Fig. 3) reflected what was observed in the transient expression assays. We previously observed that deletion of R-region sequences had a larger inhibitory effect in stably transformed cells (13). This was interpreted to mean that the R region had a greater effect on templates that were integrated into host chromosomes than on those in unintegrated templates. The same effect was observed here. The SΔR mutation decreased LTR activity to about 4% of that of the intact LTR sequences (Fig. 3). Mutations that disrupted secondary structure of the R region also had a greater effect when stably transformed cells were used compared to what was observed in transient expression assays (Fig. 3). Mutation of either the ascending or descending side of the stem inhibited LTR activity to about 1/10 of the level of the intact LTR. Restoration of the secondary structure in the compensatory mutant again restored activity to about half of that of the intact LTR (Fig. 3). The activity of the compensatory mutation was roughly five times as high as that of either of the mutations that disrupted base pairing. This finding confirmed the importance of secondary structure for the activity of the R region and showed that it is important in templates that are integrated into the host cell genome.
Cytoplasmic RNA from stably transformed cells was then isolated and used for primer extension assays. An 18-nucleotide primer in the CAT sequences that generated a single 137-nucleotide-long runoff product from the 5' end of the SL3-CAT transcript was used. The primer generated a single 18-nucleotide product from the SΔR mutant and 165-nucleotide products from the ASC, DES, and COMP mutants (Fig. 4). Thus, the secondary structure mutations did not alter the initiation site of transcription. The mutations did reduce the steady-state levels of cytoplasmic RNA (Fig. 4) to an extent similar to the reductions seen in the CAT assays (Fig. 3). The compensatory mutation restored the level of cytoplasmic RNA to about half of the level of the intact LTR (Fig. 4). Thus, the secondary structure of the R region was important for one or more processes that affected the steady-state levels of LTR-driven, cytoplasmic transcripts.

SL3 R-region sequences function differently from the HIV TAR element. Secondary structure is important for activity of both the SL3 R region and the HIV TAR element, and each element is positioned similarly in the viralLTRs. Therefore, we considered the possibility that the MuLV sequences function equivalently to HIV TAR. To test whether a cellular factor might recognize the SL3 R-region sequences and affect processivity of RNA polymerase II (7, 11, 21, 24, 25, 28, 29, 44, 50, 52, 54). To test whether a cellular factor might recognize the SL3 R-region sequences and affect processivity of RNA polymerase II, nuclei were prepared from the cells that were stably transformed with the SL3- and SΔR-CAT plasmids. Nuclear run-on assays were performed with probes from the 5' and 3' portions of the CAT genes as described by Laspia et al. (25). Deletion of the R region reduced the level of LTR-CAT transcripts produced to about 40% of the level of the intact LTR whether the probe used was from the 5' or the 3' end of the CAT gene (Fig. 5). Thus, deletion of the R region did not affect the fraction of transcripts that proceed from the 5' to the 3' part of the CAT template. Therefore, the SL3 R region had no effect on RNA polymerase II processivity.

We also tested whether the SL3 R sequences could functionally replace the HIV-1 TAR element. We generated a recombinant LTR in which the SL3 R region was linked to the U3 sequences from HIV type 1 (HIV-1) (Fig. 6A, HS). The activity of this construct was compared to that of the same construct containing the deletion of the SL3 R region from +4 to +31 (Fig. 6A, HSΔR). The chimericLTRs were tested in both human and mouse cells. The presence of the SL3 R region did not increase activity of the HIV-1 U3 (Fig. 6B) but did affect the activity of the SL3 LTR in parallel controls (Fig. 6B). As a control for the function of the HIV-1 LTR, cotransfection of a Tat expression plasmid was shown to stimulate its activity about 75-fold in human Jurkat cells (Fig. 6B). As reported by others (2, 15, 16, 32, 33, 47, 50), Tat was not active on the intact TAR element in mouse cells. We conclude that the MuLV R region cannot functionally substitute for the HIV TAR element. Thus, the MuLV and HIV-1 elements function by different mechanisms.

R-region sequences of other type C retroviruses can substitute for the SL3 element. R regions of other retroviruses were reported to affect LTR activity (39, 40). We tested whether several of these could functionally substitute for the SL3 element. LTR-CAT plasmids containing the heterologous sequences (Fig. 7A) were constructed and tested in transient expression assays.

As previously reported (13), the HIV R region yielded levels of activity about the same as those seen with the deletion of the SL3 R region element (Fig. 7B). Since the HIV-1 sequences can fold to form the well-characterized stem-loop structure that contains the TAR element, this result indicates that not every stem-loop can function equivalently to the SL3 element.

Moloney MuLV (Mo-MuLV) is another retrovirus that induces T-cell lymphomas in mice (9, 46). The first 32 nucleotides of the Mo-MuLV and SL3 R regions are identical except for a 1-bp substitution in the loop (Fig. 7A). As expected, the Mo-MuLV sequences functioned equivalently to the SL3 sequences (Fig. 7B).

FeLV is related to MuLVs (10). The FeLV R region contains extensive nucleotide substitutions within the loop and within the bulge on the descending side of the stem (Fig. 7A). In addition, the FeLV R region contains substitutions for two nucleotides of SL3 that are involved in base pairing of the stem.
(Fig. 7A). However, these changes appear to be compensatory in that they are predicted to maintain base pairing. When substituted into the SL3 LTR, the FeLV R region exhibited most of the activity of the SL3 sequences (Fig. 7B). We conclude that despite the nucleotide substitutions, the FeLV R region functions similarly to the SL3 element.

The R region of MMTV is only 15 nucleotides long. Curiously, 11 of these 15 nucleotides are identical to the first 15 nucleotides of the SL3 R region (Fig. 7A). The similarity between the two LTRs does not extend into the U5 region of MMTV (Fig. 7A). Sequences within the MMTV R region were reported to stimulate initiation of transcription from the MMTV LTR (39). To test whether the MMTV LTR could replace the SL3 element, two different chimeric constructs of the MuLV and MMTV LTRs were made. In one, MMTV R-U5, the SL3 R region sequences were replaced by the R region of MMTV and the first 18 nucleotides of the MMTV U5 (Fig. 7A). The sequences transcribed from this construct were predicted not to be able to fold into the stem-loop structure. In the other construct, MMTV R/MuLV R, only the first
was predicted based on evolutionary conservation of the nucleotides that base pair to form the structure. The mutagenesis studies presented here strongly support the argument that this structure is necessary for maximum activity of the SL3 LTR. Mutation of nucleotides involved in base pairing inhibited the activity of the R region. A compensatory mutation that formed the same predicted secondary structure but not the actual sequence restored most of the activity of the element. The equivalent sequences from Mo-MuLV and FeLV were able to substitute, at least partially, for the SL3 element. These results are consistent with the hypothesis that a similar element is a general feature of members of the mammalian type C genus (49) of retroviruses.

Previous results suggested that the SL3 R-element functions at two steps, initiation of transcription and a postinitiation step (13). The data presented here partially clarify the molecular mechanisms involved. Nuclear run-on assays indicated that the R-region element affected the loading of RNA polymerase onto the 5′ portion of the CAT template. However, the R region had no effect on whether the polymerase proceeded to the 3′ end of the template. We interpret these observations to mean that the R element has some effect on transcriptional initiation but no effect on polymerase processivity. Thus, the SL3 R region functions differently from the HIV Tat-TAR system. The quantitative effect of deletion of the R region in nuclear run-on assays was a decline of 2.5- to 3.5-fold (Fig. 5 and reference 13). This accounts for only a minor fraction of the roughly 20-fold effect of deletion of the R region seen in primer extension analysis and reporter gene expression assays. Therefore, the main effect is on one or more steps that occur after the template is transcribed by RNA polymerase. This implies that the main function of the R-element involves one or more steps in RNA processing.

We propose that the stem-loop structure of the first 28 nucleotides of the R region exists at the 5′ end of the LTR-driven transcripts. The nucleotide substitutions that reduced activity of the element were those that altered base pairing in the stem. None of the mutations that altered the loop had substantial effects on activity. Additional evidence that the sequence of the loop was not important was provided by the observation that the loop sequence varied among mammalian type C retroviruses (13). However, we did not test whether removal of the loop structure affected activity. Removal of the bulge in the stem had no effect on activity. On the other hand, the MMTV R/SL3 U5 construct had little activity. This mutant was predicted to have three differences in the bulge or the loop structure, one of which would be expected to affect activity. Our studies presented here strongly support the argument that this element is a general feature of members of the mammalian type C genus of retroviruses.

15 nucleotides of the SL3 R region were replaced by the MMTV R region (Fig. 7A). In the latter construct, only one of the MMTV nucleotide substitutions, the nucleotide at position +3, affected the predicted secondary structure. This substitution disrupted the base pair between the nucleotides at positions +3 and +26 in the SL3 structure. Both constructs exhibited activity similar to or even slightly lower than that of the deletion of the SL3 R region (Fig. 7B). Thus, the MMTV R region could not function in the context of the SL3 LTR. We conclude that the MMTV and MuLV R-region elements function by distinct mechanisms.

CSV is an avian retrovirus of the REV group. It was previously hypothesized that the first 37 nucleotides transcribed from its R region might form a stem-loop structure (40). The CSV sequences (Fig. 7A) were tested for the ability to substitute for the SL3 element (Fig. 7B). In K562 cells, the CSV R sequences had a level of activity similar to that of SΔR. In the murine fibroblast line NIH 3T3, the CSV sequences restored part of the activity (Fig. 7B). This finding suggests that the CSV and SL3 elements may function by similar mechanisms at least in this one cell line.

DISCUSSION

The existence of a stem-loop structure at the 5′ end of the R region of MuLVs and related feline and primate retroviruses...
shorter, with fewer base pairs to stabilize it. Curiously, the CSV sequence, which was also predicted to fold into a stem-loop structure (40), did give some activity in NIH 3T3 fibroblasts. It may be simply fortuitous that the CSV and SL3 elements fold into sufficiently similar structures that the CSV element can partially substitute for the SL3 element. However, it is also conceivable that the SL3 and CSV elements perform similar functions. The taxonomical position of the REV group within the Retroviridae is uncertain (10, 51). However, there is evidence that the REVs are related to mammalian retroviruses, all of which use tRNA as an internal ribosomal entry site in the genome 

The capsid and reverse transcriptase proteins share antigenic similarity (1, 3–5, 18, 36). The REV env-encoded proteins show sequence similarity with both the baboon endogenous virus and type D simian retroviruses (48). Spleen necrosis virus, a member of the REV group, shares a cellular receptor with type D simian retroviruses (22). MuLVs and REV may share a translational regulatory element (27). The ability of the CSV 5' end to substitute partially for the SL3 element may be an additional hint of similarity between these groups of retroviruses.

In summary, a small stem-loop structure of modest stability is important for the maximum expression of SL3 LTR-driven transcripts. The element appears to function mainly at a post-transcriptional step and presumably exists at the 5' end of LTR-driven transcripts. Other mammalian type C viruses appear to have similar elements.

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