The efficiency of RNA 3'-end formation is determined by the distance between the cap site and the poly(A) site in spleen necrosis virus

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The efficiency of RNA 3'-end formation of spleen necrosis virus (SNV) is determined by the distance between the cap site and the poly(A) site. When the distance between the cap site and the poly(A) site was shorter than 500 bases, only 3–9% of the RNA was polyadenylated at the SNV poly(A) site. However, when the distance between the cap site and the poly(A) site was 1400 bases or more, 70% of the total RNA was polyadenylated at the SNV poly(A) site. In contrast, the poly(A) signal sequences of the thymidine kinase (tk) and SV40 late genes functioned at high efficiency, even with a distance between the cap site and the poly(A) site that was short enough to inactivate the SNV poly(A) signal. Therefore, this distance-dependent inactivation of RNA 3'-end formation is specific for SNV sequences and perhaps for related retroviruses. This finding explains the difference between the 5' and 3' poly(A) sites in many retrovirus RNAs.

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Eukaryotic mRNA undergoes post-transcriptional processing. One of the post-transcriptional steps is the 3'-end formation of primary transcripts. The 3'-end formation involves two reactions: cleavage of the transcript and addition of the 200- to 300-base polyadenylation chain (for review, see Birnstiel et al. 1985; Humphrey and Proudfoot 1988; Manley 1988). At least two sequence elements are known to be necessary for 3'-end formation in cells and in cell-free systems: the AAUAAA sequence and the downstream element (for review, see Birnstiel et al. 1985; Humphrey and Proudfoot 1988; Manley 1988). The AAUAAA sequence is located 10–30 bases upstream from the cleavage/polyadenylation site [poly(A) site] and is well-conserved among genes. The downstream element is located 10–50 bases downstream from the poly(A) site and consists of a GU-rich sequence. It is less conserved among genes.

In retroviral replication, viral RNA is expressed from proviral DNA with cellular enzymes and undergoes post-transcriptional processing in a manner similar to the mRNA of cellular genes. The 3'-end formation of retroviral RNA is also carried out by cellular enzymes and requires the AAUAAA and downstream poly(A) signals [Bohnlein et al. 1989]. However, the AAUAAA and downstream poly(A) signals appear twice in the viral RNA of some species of retroviruses. In these retroviruses, it is necessary to transcribe through the first poly(A) site to express full-length viral RNA. This group of retroviruses includes murine leukemia viruses, avian reticuloendotheliosis virus/spleen necrosis virus (SNV), murine mammary tumor virus, and human immunodeficiency virus (HIV) [Weiss et al. 1985], as well as retrotransposons, such as Ty elements in yeast [Elder et al. 1983; Boeke et al. 1985; Clare and Farabaugh 1985].

In viruses of this group, the AAUAAA sequence is located in the R region, the poly(A) site is at the junction between the R and U5 regions; and the downstream element is located in the U5 region [see Fig. 1A] [Weiss et al. 1985; Bohnlein et al. 1989]. The U3 region contains enhancer and promoter elements. Therefore, viral RNA is initiated at the 5' end of the R region in the 5' long terminal repeat (LTR) and is polyadenylated at the 3' end of the R region transcribed from the 3' LTR; consequently, a poly(A) site near the 5' end of the RNA remains unprocessed. The question we approached was how these retroviruses differentiate between the 5' and 3' poly(A) sites.

It was proposed that the U3 region, which is transcribed from the 3' LTR and is present only at the 3' end of the RNA [see Fig. 1A], leads to the functional difference between the retroviral 5' and 3' poly(A) sites [Benz et al. 1980; Dougherty and Temin 1987]. However, we have shown that this hypothesis is unlikely [Iwasaki and Temin 1990]. Therefore, to understand why retroviral RNA is polyadenylated at the 3' poly(A) site and not at the 5' poly(A) site, we proposed three alternative hypotheses: (1) The poly(A) signal sequence in retroviral RNA is inefficient and allows readthrough at both
poly[A] sites, [2] viral genomic sequences outside of the U3, R, and U5 regions [designated non-LTR regions] are responsible for the functional difference between the 5’ and 3’ poly[A] sites; and [3] the short distance between the cap site and poly[A] site prevents RNA 3’-end formation at the 5’ poly[A] site.

Here, we report experiments to test each hypothesis. Our experiments support the third hypothesis. The efficiency of RNA 3’-end formation of SNV is determined by the distance between the cap site and the poly[A] site. In contrast, the poly[A] signal sequences of the thymidine kinase (tk) and SV40 late genes function even with a distance between the cap site and the poly(A) site that is short enough to inactivate the SNV poly(A) signal. Therefore, this distance-dependent inactivation of RNA 3’-end formation is specific for the SNV poly(A) signal sequences and perhaps for related retroviral poly(A) signal sequences.

Results

The poly(A) site of SNV is used efficiently

To test the first hypothesis, that the poly(A) signal sequence in retroviral RNA is inefficient and allows readthrough at both poly(A) sites, and also to measure the efficiency of RNA 3’-end formation at the 3’ poly(A)
site of SNV, we constructed plasmids pSNV(wt) and pSNV(SV40) [plasmids 2 and 8 in Fig. 1A]. pSNV(wt) contains the 5’ LTR, the primer binding site (pbs), the encapsidation sequence (E), the hygromycin phosphotransferase B gene (hygro), the polypurine tract (ppt), the 3’ LTR, and the herpes simplex virus thymidine kinase gene (tk). The region from the 5’ LTR to the 3’ LTR is identical to an SNV vector, JD214Hy [plasmid 1 in Fig. 1A], which replicates efficiently in the presence of SNV proteins [Dougherty and Temin 1986]. For convenience, an XhoI linker was inserted at the U3/R boundary in the 3’ LTR of pSNV(wt), which pJD214Hy does not contain; insertion of this linker had no effect on RNA production [Iwasaki and Temin 1990]. This sequence is shown in Figure 1B. pSNV(SV40) is identical to pSNV(wt), except for replacement of the R and U5 regions in the 3’ LTR [15–166 bases in Fig. 1B] by the SV40 late poly(A) signal sequence. With these two constructs, RNA transcribed through the 3’ poly(A) site of SNV or through the SV40 poly(A) site can be detected. D17 (a dog osteosarcoma cell line) and chicken embryo fibroblast (CEF) cells were transfected with these constructs. Both cells are permissive for replication of SNV [Watanabe and Temin 1983]. Forty-eight hours after transfection, RNA was isolated, digested with DNase I to remove contaminating plasmid DNA, and subjected to Northern blot hybridization. The percentage of processed RNA at the SNV 3’ poly(A) site (full-length RNA) compared with total RNA (full-length plus readthrough RNAs) was 96% in D17 cells and 90% in CEF cells [lane 2, SNV(wt) in Fig. 1C; data of CEF not shown]. The percentage for the SV40 poly(A) site was >97% in D17 cells [Fig. 1C, lane 8, SNV(SV40)]. The percentage for the SV40 poly(A) site in SNV(SV40) was not determined in CEF cells. Thus, in the context of this vector, the poly(A) signal sequence of SNV is efficient.

Deletions of the viral genome do not decrease the level of full-length RNA

The second hypothesis, that viral genomic sequences outside the U3, R, and U5 regions (designated non-LTR regions) are responsible for the functional difference between the 5’ and 3’ poly(A) sites, can be divided into two possibilities: [1] the non-LTR regions inhibit RNA 3’-end formation at the 5’ poly(A) site, and [2] the non-LTR regions enhance RNA 3’-end formation at the 3’ poly(A) site. To test these hypotheses, we deleted various regions of the SNV genome from pSNV(wt) [plasmids 3–7 in Fig. 1A] and measured the level of RNA transcribed from these vectors by transfection and Northern blot analysis.

If the non-LTR regions inhibit the RNA 3’-end formation of the 5’ poly(A) site, these deletions should decrease the level of total RNA by activating the function of the 5’ poly(A) site. However, the relative levels of total RNA [full-length and readthrough RNAs] transcribed from these vectors were approximately the same as that of total RNA from SNV(wt) [Fig. 1C, lanes 2–7]. This result suggested that these deletions in the SNV genome did not significantly decrease the level of total RNA in D17 and CEF cells (data of CEF not shown). Therefore, it is unlikely that the non-LTR regions inactivated the RNA 3’-end formation function at the 5’ poly(A) site to allow expression of full-length RNA.

Deletions of the U3 region slightly decreased the percentage of full-length RNA over total RNA from 96% to 85% in D17 cells [Fig. 1C, lane 3]. The deletion of the polypurine tract [ppt] and U3 regions decreased the percentage of full-length RNA only 2% more [Fig. 1C, lane 4]. The deletion of the encapsidation sequence [E] region alone decreased the percentage of full-length RNA from 96% to 92% [Fig. 1C, lane 5]. The percentage of full-length RNA containing deletions of the E and U3 regions [SNV(ΔE/ΔU3)] and of the E, ppt, and U3 regions [SNV(ΔE/ppt/ΔU3)] were 81% and 83%, respectively [Fig. 1C, lanes 6 and 7]. Therefore, it is possible that the non-LTR and/or U3 regions enhance RNA 3’-end formation at the 3’ poly(A) site to some limited extent.

Lengthening the distance between the cap site and the poly(A) site activates 3’-end formation of SNV RNA

We then constructed plasmids to test the third hypothesis, that the short distance between the cap site and the poly(A) site prevents RNA 3’-end formation at the 5’ poly(A) site. The length of the R region was increased by insertion of the neo gene fragment [Fig. 2A]. The neo gene fragment was inserted into the XhoI site, as shown in Figure 1B. These insertions did not seem to change the levels of transcription initiation, because the levels of total RNA (5’ RNA and full-length RNA) transcribed from all the constructs used in our experiments were similar to one another [see Figs. 2B and 5B, below]. pR/US[1423], in which the poly(A) site was located 1423 bases downstream of the cap site, gave two transcripts, 5’ RNA and full-length RNA, detected with the 3’ neo probe [Fig. 2B, lane 14 in the 3’ neo probe panel]. The percentage of 5’ RNA relative to total RNA [5’ RNA and full-length RNA] was 71% in this construct. When the distance from the cap site to the poly(A) site was 1042 bases [R/US[1042]], the ratio of the RNAs was changed slightly [Fig. 2B, 61%, lane 13]. In R/US[471], the percentage of 5’ RNA relative to total RNA was 8% [Fig. 2B, lane 10].

To detect full-length RNA for all of the constructs, a TK probe was used [Fig. 2B, TK probe panel]. The level of full-length RNA of R/US[90] was almost the same as that of R/US[471] [Fig. 2B, lanes 9 and 10].

Similar deletions were made in the neo gene fragment from the 3’ end instead of the 5’ end [Fig. 2A, 11 and 12]. Transfection and Northern analyses of these 3’-deletion constructs were performed. When the distance between the cap site and the poly(A) site was 1023 bases, the percentage of 5’ RNA relative to total RNA was 51% [Fig. 2A, plasmid 12; Northern hybridization data not shown]. When the distance between the cap site and the poly(A) site was 497 bases, the percentage of 5’ RNA relative to total RNA was <3% [Fig. 2A, plasmid 11; Northern hybridization data not shown].
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Figure 2. A short distance between the cap site and the poly(A) site inactivates RNA 3'-end formation function of SNV. [A] Constructs used in the experiments. The numbers in parentheses in the names of the constructs represent the numbers of bases between the cap site and the SNV poly(A) site. pr/U5(2586) contained both the neo' and hygro' fragments. (Right-hatched box) neo' gene fragment; (crosshatched boxes) probes used for Northern blot analyses. (Left-hatched box) hygro' gene fragment; (right-hatched box) tk gene; (crosshatched boxes) probes used for Northern blot analyses. D17 cells were transfected with the constructs shown [see the legend to Fig. 1(C)].

To eliminate the possibility that this distance-dependent RNA 3'-end formation depended on the specific nature of the fragment inserted into the R region, we inserted the hygro' gene fragment instead of the neo' gene fragment to extend the length of the R region. The hygro' gene fragment was deleted from the 5' and 3' ends of the hygro' fragment [the structures of these constructs are not shown]. The relative ratios of 5' RNA to total RNA with these constructs were determined by Northern hybridization analysis as described above. The percentages of 5' RNA over total RNA were 58% for 1123 bases from the cap site to the poly(A) site, 54% for 936 bases, 44% for 858 bases, <3% for 377 bases, and 9% for 265 bases [20, 19, 18, 17, and 16, respectively, in Fig. 6, below; Northern hybridization data not shown]. Therefore, the efficiency of 3'-end formation was determined by the distance between the cap site and the poly(A) site and was independent of the sequence inserted into the R region.

pr/U5(2586) was constructed to extend the length of the R region to 2586 bases, by insertion of both the hygro' and neo' gene fragments [Fig. 2A, plasmid 15]. The insertion of the hygro' and neo' gene fragments gave a percentage of 5' RNA over total RNA of 66% [plasmid 15 in Figs. 2A and 6, below; Northern hybridization data not shown]. Therefore, extending the length of the R region >14000 bases [Fig. 2A and 6, below, plasmid 14] did not further enhance RNA 3'-end formation at the SNV poly(A) site.

Because the Northern analyses described above did not reveal the level of 5' RNA from R/U5(90), which contains an intact LTR [Fig. 2A, plasmid 9], we decided to use the polymerase chain reaction (PCR) to measure the level of 5' RNA of R/U5(90) [Fig. 3A]. It has been reported that PCR can be used to measure DNA or RNA levels in a quantitative manner [Delidow et al. 1989; Wang et al. 1989]. The same preparation of RNA used in the experiment of Figure 2B was subjected to reverse transcription in the presence of a d[T]25-30 primer to copy the RNA into cDNA. Immediately after mixing all components, one-fifth of the reverse transcription mixture was removed and combined with 2 μCi of [32P]dCTP. The incorporation of 32P was assessed by TCA precipitation to estimate the amount of cDNA synthesized in the reaction. The same amount of cDNA from each sample was then subjected to PCR with each set of primers [Fig. 3A].

To measure the levels of full-length RNA, the TK-2 and d[T] PCR primers and the TK-3 probe were used [Fig. 3A]. The sizes of the PCR products were determined by agarose gel analysis. Because of the poly(A) tail of mRNA, the agarase gel analysis showed a smearing pattern from 100 to 1000 bases, in which a peak of the signal was ~300-500 bases [data not shown]. In Figure 3B, panel b (hybridization with the TK-3 probe), the one-fifth and one-twenty-fifth dilutions of R/U5(1423) cDNA prior to PCR indicated that the levels of the full-length PCR products were proportional to the levels of the original cDNA. When the level of the full-length product of R/U5(1423) was taken as 1.00, the levels of the full-length PCR products were 0.72 for R/U5(1042), 2.35 for R/U5(471), and 0.72 for R/U5(90). We found some variation between PCR and Northern analyses. The intensities of the full-length bands in the Northern analysis were 1.0 for R/U5(1423), 1.5 for R/U5(1042), 4.0 for R/U5(471), and 3.0 for R/U5(90) [Fig. 2B, lanes 14, 13, 10, and 9, respectively, in the TK probe panel]. In the PCR analysis, the full-length RNAs from all of the constructs were detected at a range between 0.72 and 2.35.

To measure the levels of 5' RNA, we used the R21-1 and d[T] PCR primers and the R51 probe [Fig. 3A]. The sizes of the PCR products were determined by agarase gel analysis. The agarase gel analysis showed a smearing pattern from 100 to 1000 bases, in which a peak of the signal was ~300-500 bases [data not shown]. In Figure 3B, panel a (hybridization with the R51 probe), the one-
biphasic (curves not shown). One phase was seen for up to 8-15 hr and had a steep slope; the other phase was seen after 15 hr and had a gradual slope. The half-lives of the full-length RNA and 5' RNA were plotted, and the decay curves for the RNAs were estimated from the slopes of the first fifth and one-twenty-fifth dilutions of R/U5(1423) cDNA prior to the PCR again verified that the levels of the 5' RNA PCR products were proportional to the levels of original cDNA. When the signal of the 5' RNA of R/U5(1423) was taken as 1.00, the signal of the 5' RNA of R/U5(1042) was 1.00, that of R/U5(471) was 0.10, and that of R/U5(90) was 0.02. In the Northern analysis, the relative levels of the 5' RNA were 1.00 for R/U5(1423), 2.20 for R/U5(1042), and 0.28 for R/U5(471) [Fig. 2B, 5' RNAs in lanes 14, 13, and 10, respectively, in the 3' neo probe panel]. This PCR experiment showed that the 5' RNA of R/U5(90) was expressed at a level as low as the 5' RNA of R/U5(471).

One possible explanation for the observation that the levels of shorter 5' RNAs were lower than the levels of longer 5' RNAs was that the shorter RNAs were less stable than the longer 5' RNAs. To check the stability of these RNAs, we performed an actinomycin D chase experiment [Fig. 4]. D17 cells were transfected with pR/U5(1423) and pR/U5(471) [Fig. 2A, plasmids 14 and 10, respectively]. Thirty-six hours after transfection, actinomycin D was added to the medium of the cells. After different times of incubation in the presence of actinomycin D, RNA was isolated and subjected to Northern analysis (Fig. 4). The data from this Northern analysis were plotted, and the decay curves for the RNAs were biphasic (curves not shown). One phase was seen for up to 8-15 hr and had a steep slope, the other phase was seen after 15 hr and had a gradual slope. The half-lives of the RNAs were estimated from the slopes of the first phase. The half-lives of the full-length RNA and 5' RNA of R/U5(1423) were 7 and 8 hr, respectively. The half-life of the 5' RNA of R/U5(471) was 10 hr, and the half-life of the full-length RNA of R/U5(471) was 11 hr. Therefore, no significant differences in half-lives were found between the 5' RNA and the full-length RNA of R/U5(1423) and between the 5' RNA and the full-length RNA of R/U5(471). This observation indicated that the low levels of the 5' RNAs from the constructs having short R regions were not the result of instability of these RNAs.

**Short distance between the cap site and the poly(A) site in the tk and SV40 poly(A) signals did not inactivate RNA 3'-end formation**

We then tested whether the distance-dependent function of RNA 3'-end formation is a general phenomenon or is specific to the SNV poly(A) signal. To test these hypotheses, we made constructs containing the tk (Wagner et al. 1981) and SV40 late poly(A) signal sequences [Fig. 5A, Wickens and Stephenson 1984]. When the distance from the cap site to the poly(A) site of the tk gene was 1421 bases, the percentage of 5' RNA relative to total RNA (5' RNA and full-length RNA) was 85% [lane 28 in the 3' neo probe panel of Fig. 5B]. When the distance from the cap site to the poly(A) site was 1031 bases, the percentage of 5' RNA relative to total RNA was 87% [Fig. 5B, lane 27]. When the distance from the cap site to the poly(A) site was 460 bases, the percentage of 5' RNA relative to total RNA was still 81% [Fig. 5B, lane 26]. When the distance from the cap site to the
poly(A) site of the tk gene was only 83 bases, the level of full-length RNA of tk[83] was not significantly increased compared with the three other tk constructs (Fig. 5B, lane 25 in the TK probe panel). Therefore, shortening the distance between the cap site and the poly(A) site had little effect on the RNA 3'-end formation function of the tk sequence (Fig. 5B).

A similar phenomenon was observed with constructs that contained the SV40 late poly(A) signal sequence (Fig. 5A, 21–24; Northern hybridization data of these constructs not shown). The only difference observed between the tk and SV40 poly(A) signal sequences was the percentage of RNA processed at these poly(A) sites. The SV40 poly(A) signal was stronger than the tk signal sequence. With the SV40 poly(A) signal, the percentage of RNA processed at the SV40 poly(A) site was 92–97% in all of the constructs (Figs. 5A and 6, plasmids 22–24).

**Discussion**

We tested three hypotheses to explain why retroviral RNA is polyadenylated at the 3' poly(A) site and not at the 5' poly(A) site: [1] The poly(A) signal sequence in retroviral RNA is inefficient and allows readthrough at both poly(A) sites; [2] non-LTR regions in the viral genome are responsible for the functional difference between the 5' and 3' poly(A) sites; and [3] the short distance between the cap site and the poly(A) site prevents RNA 3'-end formation at the 5' poly(A) site.

To test the first hypothesis, that the poly(A) signal sequence in retroviral RNA is inefficient and allows readthrough at both poly(A) sites, (1) the level and half-life of the RNA processed at the 3' poly(A) site compared with the full-length RNA was measured by transfection and Northern analysis and by actinomycin D chase experiments; and [2] the level of the 5' RNA from an intact LTR construct was measured by using PCR. Our results were [1] the poly(A) signal of SNV was efficient (Fig. 1C); [2] the lower level of the shorter 5' RNA was not the result of RNA instability (Fig. 4); and [3] a PCR experiment showed that the 5' RNA of the intact LTR construct [pR/US[90]] was expressed at a low level (Fig. 3B). Therefore, we conclude that this hypothesis is unlikely.

The second hypothesis, that the non-LTR regions are responsible for the functional difference between the 5' and 3' poly(A) sites, was tested with constructs containing deletions of various sequences of the SNV genome (Fig. 1A). The deletion of the SNV genomic sequences did not decrease the level of total RNA compared with the parental vector [SNV[wt]] in DI7 and CEF cells (Fig. 1C; CEF data not shown). This deletion decreased the levels of RNA processed at the SNV 3' poly(A) site slightly (Fig. 1C; CEF data not shown). However, if this phenomenon completely explained the functional difference between the 5' and 3' poly(A) sites in SNV, the relative amounts of RNA that are processed at the 5' poly(A) site and that pass through the 5' poly(A) site would be 80% and 20%, respectively. However, we found the RNA that was processed at the 5' poly(A) site and the RNA that passed through the 5' poly(A) site were <10% and >90%, respectively (Fig. 3). Therefore, even though the non-LTR regions and/or the U3 region change the percentage of RNA processed at the 3'
Distance-dependent polyadenylation of SNV RNA

Figure 6. Summary of Northern analyses. Percentage of processed RNA was defined as the amount of 5' RNA divided by the amount of total RNA (5' RNA and full-length RNA) in each construct (for designation of RNAs, see Figs. 2A and 5A). The data plotted in this graph were the mean values of at least two experiments. Probes for constructs containing the 5' deletions of the neo gene (plasmids 10, 13, 14, and 26–28), the 3' deletions of the neo gene (plasmids 11, 12, 14, and 22–24), the 5' deletions of the hygro gene (plasmids 15, 17, 19, and 20), and the 3' deletions of the hygro gene (plasmids 15, 16, 18, and 20) were the 3' neo, 5' neo, 3' hygro, and 5' hygro probes, respectively (see Figs. 2A and 5A). [O, □, and △] Data from experiments with D17 cells; [●] data from CEF cells. The number near each point on this graph represents the number of the construct in Figs. 2A and 5A. The structures of the pR/U5 series containing the hygro gene instead of the neo gene to extend the length of the R region are not shown (16–20). The results of some of the pR/U5 and ptk series in D17 cells are shown in Figs. 2B and 5B, but those in CEF and the results of the other constructs in D17 cells are not shown. The data from pR/U5(90) (plasmid number 9) were obtained from the PCR experiments (Fig. 3B).

The results of Northern hybridization and PCR analyses supported the third hypothesis, that the short distance between the cap site and the poly(A) site prevents RNA 3'-end formation at the 5' poly(A) site. When the distance between the cap site and the poly(A) site was shorter than 500 bases, 3–9% of the RNA was processed at the 5' SNV poly(A) site. As the distance became larger, the amount of RNA processed at the 5' SNV poly(A) site increased. At 1423 bases between the cap site and the poly(A) site, the percentage of RNA processed at the 5' SNV poly(A) site was 70% compared with total RNA. Extending the length of the R region >1423 bases did not further enhance RNA 3'-end formation at the 5' SNV poly(A) site. In contrast, the poly(A) signals of the tk and SV40 late genes functioned at high efficiency even with a distance between the cap site and the poly(A) site that was short enough to inactivate the SNV poly(A) signal. Therefore, this distance-dependent inactivation of RNA 3'-end formation seemed to be specific for the SNV poly(A) signal sequence.

In the PCR analysis, we expected that a PCR product extending from the R21-1 primer to the poly(A) site of the tk gene would be generated along with the PCR product between the R21-1 primer and the poly(A) site at the 3' end of the R region [Fig. 3A]. However, the product from the R21-1 primer to the poly(A) site of the tk gene was not detected (Fig. 3B, slot blot of TK-3 probe in panel a). This observation could be explained by the low reverse transcription efficiency of long sequences by reverse transcription and PCR. It has been reported that the reverse transcription reaction is inefficient for long mRNA (>500 bases) when total RNA is used (Williams and Mason 1985). To avoid this problem of elongation efficiency, we used another set of primers, TK-2 and d(T)15–30, to detect full-length RNA (Fig. 3A).

Recently, Russnak and Ganem (1990) reported that sequences upstream from the AAUAAA poly(A) signal are responsible for increasing the level of RNA processed at the 3' poly(A) site of hepatitis B virus (HBV) and that the upstream sequences can, to some extent, be replaced by the U3 sequences of SNV and HIV. They reported that the U3 sequences of SNV and HIV increased RNA processed at the HBV poly(A) site from 5% to 40–50% relative to total RNA (processed RNA and readthrough RNA), although the HBV sequence itself gave >95% of RNA processed at the HBV poly(A) site.

The percentage of RNA processed at the 3' poly(A) site of SNV(wt) was 96% in D17 cells [Fig. 1C, lane 2]. The deletion of the U3 region decreased the percentage of full-length RNA from 96% to 85% in D17 cells (lane 3 in Fig. 1). The observation by Russnak and Ganem (1990) might reflect the difference of the levels of processed RNA between SNV(wt) and SNV[ΔU3] in the case of the SNV sequence.

During preparation of this manuscript, Sanfacon and Hohn (1990) published data suggesting that proximity to the promoter inhibits recognition of the cauliflower mosaic virus poly(A) signal. These results are consistent with ours, indicating that a pararetrovirus and a retrovirus utilize similar mechanisms to avoid RNA 3'-end formation at the 5' poly(A) site. However, our data suggested that the poly(A) signals of the tk and SV40 genes behaved differently from the poly(A) signal of SNV.

We propose that the R and U5 regions of SNV RNA contain signals for RNA 3'-end formation and an additional signal for the distance-dependent function. This additional signal may be a binding sequence for a protein that blocks RNA 3'-end formation.

Materials and methods

Nomenclature

Plasmid names are preceded by the letter p [e.g., pR/U5(90)], whereas transcripts and viruses made from these plasmids are not [e.g., R/U5(90)].

RNAs that are polyadenylated at the first poly(A) site [e.g., polyadenylated at the poly(A) site of the 5' LTR] are designated 5' RNAs. RNAs that are transcribed through the first poly(A) site and polyadenylated at the second poly(A) site are designated full-length RNAs. RNAs that are transcribed through the first and second poly(A) sites and are polyadenylated at the third poly(A) site are designated readthrough RNAs.
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Cells

D17 cells and CEF cells were grown as described previously (Watanabe and Temin 1983). For tests of RNA stability, cells were treated with 10 μg/ml of actinomycin D (Sigma) (Temin 1963). Transfection of cells was performed as described (Kawai and Nishizawa 1984).

Plasmids

Recombinant DNA techniques were as described (Maniatis et al. 1982). All constructs in Figure 1A were cloned into the PstI–EcoRI sites of pUC19, except pID214Hy, pSNV[wtk] containing an XhoI linker at the U3/R boundary in the 3' LTR (see Fig. 1B), which pID214Hy did not contain, this linker had no effect on RNA production [Iwasaki and Temin 1990]. The sequences used for cloning are defined as follows: (E) Bases 576–915 [BamHI–BamHI fragment]; (p) bases 2017–2077 [Clal–SacI fragment]; (U3 in the 3' LTR) bases 2078–2511 [SacI–XhoI fragment]; (R/U5 in the 3' LTR) bases 2512–2663; [the hygromycin gene] bases 912–2017 [XhoI–Clal fragment] in pID214Hy (Dougherty and Temin 1986). The tk fragment comprises bases 1200–3455 in pFG5 (Colbere-Garapin et al. 1979); pJD214Hy did not contain; this linker had no effect on the gene was defined as 942–2267 bp of XhoI site at (Zhang and Cole 1987). The 5' and 3' neo probes are fragments from the 5' end to the EcoRI site and from the HindIII site to the 3' end of the neo gene. The 5' and 3' hygromycin probes are fragments from the 5' end to the EcoRI site and from the HindIII site to the 3' end of the neo gene. The 5' and 3' neo probes are fragments from the 5' end to the EcoRI site and from the HindIII site to the 3' end of the neo gene fragment, respectively. The 5' and 3' hygromycin probes are fragments from the 5' end to the EcoRI site and from the HindIII site to the 3' end of the neo gene fragment.

PCR

Ten micrograms of total RNA was subjected to the reverse transcriptase reaction, as described (Maniatis et al. 1982). One-fifth of the reverse transcriptase mixture was separated from the rest of the mixture and incubated with [32P]dCTP (2 μCi) (Delidow et al. 1989). Incorporation of [32P]P was monitored by TCA precipitation. Two to 10 ng of cDNA, as calculated from the incorporation of [32P], was subjected to PCR. For each PCR, the same amount of cDNA from each sample was used. PCR was performed for up to 22 cycles with the TK-2 and d(T) primers or 25 cycles with the R21-1 and d(T) primers. At this number of PCR cycles, the levels of products were proportional to the levels of original cDNA (see Fig. 3B). The concentration of MgCl2 was 0.5 mM for R21-1 and d(T)25–30 primers, and 1.0 mM for TK-2 and d(T)25–30 primers. The temperatures for denaturation, annealing, and extension for both sets of primers were 94°C, 45°C, and 72°C, respectively (Ohara et al. 1989). Six microliters of the PCR products was denatured and blotted to nitrocellulose, as described (Maniatis et al. 1982). The probe oligonucleotides were end-labeled by T4 polynucleotide kinase, as described (Maniatis et al. 1982). Hybridization of the nitrocellulose with [32P] oligonucleotides was performed at 47°C, as described (Maniatis et al. 1982). pR/U5[1423] DNA was linearized with EcoRI, and HphI DNA was linearized with HindIII. These plasmids were included in each slot blot hybridization as positive and negative controls, respectively. The intensities of the signals were measured by microdensitometry.

R21-1, CCTACACATTGTGTTGGTGAC, is located 9 bases downstream from the 5' end of the R region. R51 is 51 bases long and contains 48 bases complementary to the minus strand of the R region. R51 does not overlap the R21-1 and d(T) primers. For details about the locations of the primers and probes in the PCR analysis, see Figure 3A. TK-2, GAACACGGAG-GAGACAATA, is located 58 bases upstream from the major poly[A] site of the tk gene. TK-3 is 21 bases long and is located 11 bases upstream from the major poly[A] site of the tk gene. TK-3 is complementary to the plus strand of the tk region and does not overlap the TK-2 and d(T) primers.

In Figure 3B, panel a, hybridization with the TK-3 probe did not detect any signals. In panel b, hybridization with the R51 probe detected only very weak signals.

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References

Benz, E.W., R.M. Wygro, B. Nadal-Ginard, and D. Dino. 1980. Moloney murine sarcoma proviral DNA is a transcriptional...
Distance-dependent polyadenylation of SNV RNA

Stinski, M.F. and T.J. Roehr. 1985. Activation of the major immediate early gene of human cytomegalovirus by cis-acting elements in the promoter-regulatory sequence and by virus-specific trans-acting components. J. Virol. 55:431–441.

Temin, H.M. 1963. The effects of actinomycin D on growth of Rous sarcoma virus in vitro. Virology 20:577–582.

Wagner, M.I., J.A. Sharp, and W.C. Summers. 1981. Nucleotide sequence of the thymidine kinase gene of herpes simplex virus type 1. Proc. Natl. Acad. Sci. 78:1441–1445.

Wang, A.M., M.V. Doyle, and D.F. Mark. 1989. Quantitation of mRNA by the polymerase chain reaction. Proc. Natl. Acad. Sci. 86:9717–9721.

Watanabe, S. and H.M. Temin. 1983. Construction of a helper cell line for avian reticuloendotheliosis virus cloning vectors. Mol. Cell. Biol. 3:2241–2249.

Weiss, R., N. Teich, H. Varmus, and J. Coffin. 1985. RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Wickens, M. and P. Stephenson. 1984. Role of the conserved AUAAA sequence: Four AUAAA point mutations prevent messenger RNA 3′ end formation. Science 226:1045–1051.

Williams, J.G. and P.J. Mason. 1985. Primer extension in hybridization in the analysis of RNA. In Nucleic acid hybridization: A practical approach (ed. B.D. Hames and S.J. Higgins), pp. 152–160. IRS Press, Oxford.

Zhang, F. and C.N. Cole. 1987. Identification of a complex associated with processing and polyadenylation in vitro of herpes simplex virus type 1 thymidine kinase precursor RNA. Mol. Cell. Biol. 7:3277–3286.
The efficiency of RNA 3'-end formation is determined by the distance between the cap site and the poly(A) site in spleen necrosis virus.

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Genes Dev. 1990, 4:1990
Access the most recent version at doi:10.1101/gad.4.12b.2299

References
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