Serine/Arginine-Rich Proteins Contribute to Negative Regulator of Splicing Element-Stimulated Polyadenylation in Rous Sarcoma Virus

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Rous sarcoma virus (RSV) requires large amounts of unspliced RNA for replication. Splicing and polyadenylation are coupled in the cells they infect, which raises the question of how viral RNA is efficiently polyadenylated in the absence of splicing. Optimal RSV polyadenylation requires a far-upstream splicing control element, the negative regulator of splicing (NRS), that binds SR proteins and U1/U11 snRNPs and functions as a pseudo-5′ splice site that interacts with and sequesters 3′ splice sites. We investigated a link between NRS-mediated splicing inhibition and efficient polyadenylation. In vitro, the NRS alone activated a model RSV polyadenylation substrate, and while the effect did not require the snRNP-binding sites or a downstream 3′ splice site, SR proteins were sufficient to stimulate polyadenylation. Consistent with this, SELEX-binding sites for the SR proteins ASF/SF2, 9G8, and SRp20 were able to stimulate polyadenylation when placed upstream of the RSV poly(A) site. In vivo, however, the SELEX sites improved polyadenylation in proviral clones only when the NRS-3′ splice site complex could form. Deletions that positioned the SR protein-binding sites closer to the poly(A) site eliminated the requirement for the NRS-3′ splice site interaction. This indicates a novel role for SR proteins in promoting RSV polyadenylation in the context of the NRS-3′ splice site complex, which is thought to bridge the long distance between the NRS and poly(A) site. The results further suggest a more general role for SR proteins in polyadenylation of cellular mRNAs.

Generation of mature mRNA in eukaryotes generally requires multiple processing steps, including capping, splicing, and polyadenylation, that are coupled to ensure proper processing (reviewed in reference 31). Retroviruses utilize the host transcription/RNA processing machinery to generate viral RNA, but due to peculiarities of their replication scheme, they often utilize the RNA processing machinery in unique ways. In the simple avian retrovirus Rous sarcoma virus (RSV), the env and src mRNAs are generated by RNA splicing from a common 5′ splice site (ss) to one of two alternative 3′ ss (10). However, unlike most host genes, retrovirus replication requires that a substantial portion of the primary viral transcripts remain completely unspliced to serve as gag-pol mRNA and as genomic RNA for progeny virions. RSV employs several mechanisms to preserve the pool of unspliced RNA, including the maintenance of suboptimal 3′ ss (25, 59) and the action of splicing repressor elements within the gag gene (the negative regulator of splicing, or NRS) (3, 39, 49) and upstream of the src 3′ ss (the suppressor of src splicing) (1, 39, 50). Generation of functional unspliced viral mRNA poses problems for the coupling of the splicing and polyadenylation reactions.

The NRS has been well characterized and is thought to act as a pseudo-5′ ss that sequesters viral 3′ ss in a nonproductive splicing complex (reviewed in reference 9). An upstream region of the ~230-nucleotide (nt) element binds SR proteins and hnRNP H (18, 35), whereas the 3′ region binds U1 and U11 snRNPs (presumably mutually exclusively, since the binding sites overlap) (7, 21, 23, 36). The SR proteins promote U1 snRNP binding to the NRS and an early interaction with a 3′ ss (11), which then matures into a noncatalytic splicing complex that prevents the 3′ ss from interacting with the authentic viral 5′ ss (20). U11 snRNP, the binding of which is mediated by hnRNP H interactions with downstream sites (37), antagonizes U1 binding and its inhibitory activity (23, 36, 44).

Use of the RSV poly(A) site is naturally inefficient, as ~15% of viral RNAs represent read-through transcripts (22), and RSV polyadenylation substrates are very poorly used in vitro (56 and N. L. Maciolek and M. T. McNally, unpublished data). The first suggestion that the NRS might play a role in polyadenylation came from Miller and Stoltzfus (41), who observed that deletions that encompassed the NRS led to increased read-through transcription. More specific NRS mutations have confirmed a role for the NRS in polyadenylation control; mutations in the SR protein-binding region and/or in the U1/U11-binding sites led to decreased polyadenylation efficiency, which suggested that the ability of the NRS to control splicing was integral to its role in polyadenylation control (17, 44). It is known that 3′ ss stimulate polyadenylation (13, 14, 43), and several direct interactions between splicing and polyadenylation factors have been described that contribute to the coupling of splicing and polyadenylation. These include interactions between the 3′ ss-binding factor U2AF65 and the polyadenylation factors poly(A) polymerase and cleavage factor I(m) (CFSIm), which acts early in poly(A) complex formation (42, 54), and between U2 snRNP components and cleavage and polyadenylation specificity factor (CPSF), which binds the

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poly(A) signal (27). Given the importance of 3′ ss in coupling splicing and polyadenylation, an attractive hypothesis was that the nonproductive splicing complex assembled on the NRS and viral 3′ ss stimulated polyadenylation in the absence of true splicing by stabilizing the binding of splicing factors to the weak viral 3′ ss (17).

In this study, in vitro and in vivo approaches were used to elucidate the mechanism of NRS-mediated polyadenylation control. The weak RSV poly(A) site could be activated in vitro to elucidate the mechanism of NRS-mediated polyadenylation sufficiently close to the 3′ polyadenylation, but they do so only when they are positioned sufficiently close to the 3′ end of the RNA via an interaction of the NRS with a downstream viral 3′ ss.

**MATERIALS AND METHODS**

Plasmid constructs. p3Z-SVL, to generate the positive control simian virus 40 (SV40) late (SVL) polyadenylation substrate was constructed by inserting an EcoRI fragment from pMXSVL (43) into the same sites of pGEM-ZZ and then deleting the SacI-BamHI fragment encompassing the adenovirus type 2 major late splicing cassette. p3Z-RSVPreul-PstI, to generate the RSV substrate, was made by inserting a 374-bp PvuII-PstI fragment (PvuII blunted with Klenow) into the Smal and PstI sites of pGEM-ZZ. The PvuII-PstI fragment was obtained from pBSKS + RSVSacl-SacI, which contains a 1,174-bp Sau3AI-SacI fragment from pAPc that surrounds the 5′ LTR. For p4Z-Ad5-22, an RSV sequence containing SacI, XhoI, BamHI, and EcoRI sites was inserted into the EcoRI and XhoI sites downstream of the adenovirus (Ad) 3′ exon in the previously described pAd(KX)BB (12), generating pAdBB(XB). A PCR fragment (with appended BamHI and XhoI sites) containing 123 nt of upstream and 87 nt of downstream sequence relative to the RSV poly(A) site (nt 9185 to 9311; coordinates are as described by Schwartz et al. [46]) was inserted into the BamHI and XhoI sites of pAdBB(XB) to make pAdBB-RSV. This was then digested with HindIII and KpnI, blunted, and recircularized. The Ad 3′ exon was similarly removed by digestion with XbaI from p3Z-src3 (34). The plasmid was digested with HindIII and XbaI, blunted, and recircularized to delete the Ad 5′ exon and NRS. p3Z-src3 was then used as a template for PCR reactions using oligos 41 nt of src exon and 80 nt of upstream intron (nt 6975 to 7096) with appended XbaI and EcoRI sites into the same sites of pGEM-ZZ. p3Zsrc3-RSV was created by replacing the Ad splicing cassette and NRS in pAdBB-RSV with a HindIII-EcoRI fragment from p3Z-src3. A construct harboring the NRS directly upstream of the RSV poly(A) site (pZ-BB[A]) was made by replacing the original KpnI-XbaI NRS fragment in pAdBB-RSV with a 285-nt KpnI-XbaI fragment (nt 703 to 930) harboring a deletion from nt 799 to 874 [pAd(KX)BB] (34). The plasmid was digested with HindIII and KpnI, blunted, and recircularized to remove the Ad 5′ ss (to make pZBB[A]-Ad3-RSV), and finally the Ad 3′ ss was similarly removed by XbaI and XhoI digestion followed by recircularization. For the construct containing the NRS fused to the src 5′ ss (pZBB[A]-src3-RSV), the Ad 3′ ss in pZBB[A]-Ad3-RSV was replaced with the HindIII-EcoRI src 3′ fragment from p3Z-src3.

To construct p4Z-RG11-RSV, p4Z-RSMMusR-RSV, pRS-SVSS-RSV, and pNRSS-RSV, the wild-type NRS in pAdBB-RSV was replaced with NRS PCR fragments containing the RG11 or mUtil mutation (see Fig. 3A) or the NRS 5′ and 3′ fragments (NRS5′ and NRS3′, respectively) (35). Subsequently, to remove the Ad 5′ exon, the plasmid was digested with HindIII and KpnI, blunted, and recircularized. The Ad 3′ exon was similarly removed by digestion with XbaI and XhoI as described above. Proviral clones were generated in p7TM14 (41), which contains the Prague A strain provirus upstream of a chloramphenicol acetyltransferase (CAT) gene and the SV40 early poly(A) signal. Constructs containing NRS deletions (ΔNRS and Δ712-798) (NRS3′ in Fig. 7) or the RG11 mutation have been described previously (17). Proviruses in which the NRS was replaced with SELEX consensus SR protein-binding sites (see Fig. 6) (8, 29, 51) were made in two steps. Mutations were inserted into the SacII fragment of pBSKS-RSVSacII, and the SacII fragments were used to replace the wild-type sequence in p7TM14. Oligonucleotides harboring binding sites for ASF/SF2, 9G8, or a control were annealed and used to replace the Mrolloph NRS fragment in pBSKS-RSVSacII (17). To replace just the NRS5′ (nt 703 to 798), PCR products containing control or SR protein-binding sites fused to the NRS 3′ end were inserted into the same sites of pBSKS-RSVSacII as those described above. For mutations in the downstream nRNPs H-binding sites, an Mrolloph NRS fragment from pSM-mg1 + 2 (38) was inserted into the same sites of pBSKS + RSV SacII. The SacII fragments were then shuttled into the same sites of p7TM14 to generate p7TM14-ASF, p7TM14-9G8, p7TM14-control, p7TM14-ASFR-RSS′, p7TM14-9G8-RSS′, p7TM14-control-RSS′, and p7TM14-mg1 + 2. The proviral deletion clones that bring the ASF/SF2 SELEX and control sequences closer to the poly(A) site, p7TM14-ASFΔpol-em3′, p7TM14-9G8Δpol-em3′, and p7TM14-controlΔpol-em3′, were made by deleting an FseI-HphI fragment from p7TM14-ASF, p7TM14-9G8, and p7TM14-control. Primer sequences are available upon request.

**In vitro transcription of substrates.** To make RNA substrates by in vitro transcription (40), p3Z-RSV was linearized with BstEII, p3Z-SVL was linearized with HindIII, and NRS-containing substrates were linearized with BamHI. Alternatively, template DNA was generated by PCR using primers to the upstream T7 promoter and RSV or SVL substrate (these substrates were used for experiments shown in Fig. 2A, 3C, and 4). Templates for the SELEX site-containing RNAs (8, 29, 51) were obtained by PCR of p3Z-RSV with a primer for RSV as the antisense primer and template-specific primers containing a T7 promoter, the appropriate SR-binding sequence, and the RSV sequence. RNA was transcribed in vitro with T7 or T3 polymerase and [γ-32P]ATP in a capped reaction and was gel purified on a 6% polyacrylamide (19:1) gel that contains 8 M urea as described previously (35). Primer sequences are available upon request.

Polyadenylation assays. Polyadenylation reactions were performed using 50,000 cpm of labeled substrate in 0.2% polyvinyl alcohol, 1 mM ATP, 20 μM creatine phosphate, 1 mM MgCl2, and 20 to 50% HeLa cell nuclear extract. Reaction mixtures were incubated at 30°C for the times specified in the figures. Substrates were then proteinase K treated, phenol extracted, and ethanol precipitated, and RNA was resolved on a 6% polyacrylamide gel that contains 8 M urea. For U1 snRNA inactivation, HeLa nuclear extract was preincubated for 10 min at 30°C with 100 μM 2′-O-methyl RNA oligonucleotide U11,14 (47) or U7,14,20 (48). Polyadenylation reaction mixtures with S100 contained 34% HeLa S100 extract supplemented with 16% HeLa nuclear extract alone or with 1 μg purified SR proteins (58). Images were obtained using a STORM 820 PhosphorImager (Amersham Biosciences) and/or autoradiography and were quantitated with ImageQuant 5.2 software. Since substrates were subject to degradation over time, polyadenylation efficiency was quantitated as the phosphorimager units in the polyadenylated product divided by the units in the substrate at time zero.

**Transfection and RNase protection assay.** Secondary chicken embryonic fibroblasts (CEF cells) were grown in medium 199 (Invitrogen) supplemented with 2% fetal calf serum (HyClone), 1% chicken serum (GIBCO), 1× antibiotic-antimycotic (Invitrogen). Cells were transfected with 2 μg DNA in medium 199 containing 200 μg/ml DEAE-dextran, and after 4 h cells were subjected to a 2-min 10% dimethyl sulfoxide shock. Total RNA was harvested using the QiAGEN RNasea kit 48 h later. RNase protection assays were carried out as previously described (17) using 2.5 μg of total RNA hybridized with 10 μg of riboprobe transcribed from p5′X11 (49) that spans the viral long terminal repeat and 5′ region of the gag gene (nt 218 to 630). Images were obtained and quantitated with a STORM 820 PhosphorImager (Amersham Biosciences) using ImageQuant 5.2 software. Bands were normalized for uridine content.

**RESULTS**

3′ ss do not support efficient RSV polyadenylation in vitro. The poor in vitro polyadenylation efficiency observed for RSV polyadenylation substrates (57 and Maciolek and McNally, unpublished) led to the hypothesis that there is an additional element(s) within the viral genome that contributes to poly(A) site recognition in vivo. Because 3′ ss promote polyadenylation of cellular and RSV RNAs and can do so in the absence of a 5′ ss in vitro (41, 43, 53), we considered a role for the 3′ ss

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in RSV polyadenylation control. The src 3' ss was chosen because it is proximal to the viral poly(A) signal, and the NRS suppresses src splicing more efficiently than env, suggesting a stronger interaction with this ss (44). Substrates were designed with the RSV poly(A) site in the context of an Ad 3' ss that is known to stimulate SV40 polyadenylation (43) or the weak src 3' ss (Fig. 1). The RSV poly(A) signal alone served as a negative control, since it is poorly polyadenylated in vitro, while the positive control was the efficiently used SVL substrate.

Substrates were uniformly radiolabeled and incubated in nuclear extract for the indicated times, and RNA was isolated and resolved in a denaturing polyacrylamide gel; polyadenylation manifests as an increase in size of the labeled substrate. After 30 min, the positive control SVL substrate was polyadenylated (30% in this experiment) (Fig. 2A, lanes 2 to 6) while, as observed previously (56 and Maciolek and McNally, unpublished), polyadenylation of the RSV substrate was barely detectable (~1%) (lanes 7 to 11). Placement of the Ad 3' ss upstream of the RSV poly(A) signal increased polyadenylation to 8% (lanes 13 to 17), whereas half this level was seen when the viral src 3' ss was used (lanes 18 to 22). Thus, neither ss stimulated RSV polyadenylation to the level seen with SVL substrate, and the src 3' ss was less effective than the Ad 3' ss. It was also reported recently that the env 3' ss does not stimulate efficient RSV polyadenylation (56). We conclude that the viral 3' ss alone do not enable efficient RSV polyadenylation in vitro and that an additional element(s) is required.

The NRS alone stimulates RSV polyadenylation in vitro. Previous observations suggested that a functional NRS plays a role in RSV polyadenylation in vivo (17, 41, 44) and led to a model in which the nonproductive splicing complex assembled on the NRS and downstream 3' ss promotes polyadenylation in the absence of true splicing. To explore this idea in vitro, substrates harboring the NRS alone or with the src 3' ss (Fig. 1) were tested in polyadenylation reactions. A low level of activity was detected with the NRS-src3'-RSV polyadenylation substrate (7%) (Fig. 2B, lanes 21 to 25) that was only slightly better than that seen with src3'-RSV (Fig. 2A). Surprisingly, the NRS alone strongly stimulated polyadenylation (63%) (Fig. 2B, lanes 14 to 19). These data indicate that the nonproductive NRS-3' ss complex is not required to stimulate RSV polyadenylation in vitro and that one or more NRS-binding factors are sufficient for this effect.

U1 and U11 snRNPs are not required for NRS-stimulated polyadenylation in vitro. The NRS harbors binding sites for multiple factors, including U1 snRNP, U11 snRNP, hnRNPH, and SR proteins (Fig. 3A). A role for U1 snRNP or U1-associated proteins in SV40 polyadenylation has been suggested previously (30, 55), and the U1 site within the NRS is required for optimal RSV polyadenylation (17, 44). Therefore, the requirement of U1 and/or U11 snRNPs for RSV-stimulated polyadenylation was tested with a substrate (RG11) in which both binding sites were mutated (21, 36) (Fig. 3A).
There does not appear to be an in vitro requirement for either snRNP, since there was no significant difference in polyadenylation activity between the wild-type (9%) and mutant NRS (8%) (Fig. 3B, lanes 2 to 5 and 6 to 9). To address the possibility that U1 might bind promiscuously to another site in the NRS-RSV substrate, polyadenylation assays were performed using nuclear extracts in which U1 snRNP was inactivated using 2'-O-methyl oligonucleotides. (B) NRS-RSV samples were run for 1 h longer to allow adequate separation of products. Polyadenylation appears as a slower-migrating smear. The results of quantitation are representative of at least three independent experiments. Polyadenylation activity between the wild-type (9%) and mutant NRS-RSV, 2%. (B) NRS-RSV and mutH-RSV, which contain the mutated hnRNP H-binding sites (Fig. 3A), were treated as described for panel A. The results of quantitation of polyadenylation at 30 min are the following: NRS-RSV, 13%; and NRSmutH-RSV, 13%. M, 32P-end-labeled pBR322/MspI markers, the sizes of which are indicated at left.

FIG. 3. U1 and U11 snRNPs are not required for NRS-stimulated RSV polyadenylation. (A) Schematic of the NRS (nt 703 to 932) indicating the NRS5'- (nt 703 to 798), NRS3'- (nt 798 to 932), and NRS-binding factors. Shown are the binding of SR proteins and hnRNP H to NRS5' and U1 and U11 snRNPs binding to NRS3'. Δ76 indicates a 76-nt deletion that does not markedly affect NRS function. The sequences of mutations that eliminate hnRNP H (mutH) and U1/U11 snRNP (RG11) binding are shown below the wild-type sequence. (B) RSV substrates harboring the wild-type NRS or the RG11 version contained nt 801 to 932. RNA was subjected to electrophoresis on a 6% polyacrylamide gel that contains 8 M urea, and the image was obtained with a PhosphorImager; the results are representative of at least three independent experiments. Polyadenylation appears as a slower-migrating smear. The results of quantitation of polyadenylation at 30 min are the following: NRS-RSV, 13%; NRS5'-RSV, 12%; and NRS3'-RSV, 2%. (B) NRS-RSV and mutH-RSV, which contain the mutated hnRNP H-binding sites (Fig. 3A), were treated as described for panel A. The results of quantitation of polyadenylation at 30 min are the following: NRS-RSV, 13%; and NRSmutH-RSV, 13%. M, 32P-end-labeled pBR322/MspI markers, the sizes of which are indicated at left.

FIG. 4. Polyadenylation stimulatory activity maps to NRS5', but hnRNP H-binding sites are not required. (A) RNA substrates were uniformly labeled with 32P and incubated in HeLa nuclear extract for the times (in minutes) indicated above each lane. The NRS5' and NRS3' regions are the same as those bracketed in Fig. 3A, except that this NRS5' version contained nt 801 to 932. RNA was subjected to electrophoresis on a 6% polyacrylamide gel that contains 8 M urea, and the image was obtained with a PhosphorImager; the results are representative of at least three independent experiments. Polyadenylation appears as a slower-migrating smear. The results of quantitation of polyadenylation at 30 min are the following: NRS-RSV, 13%; NRS5'-RSV, 12%; and NRS3'-RSV, 2%. (B) NRS-RSV and mutH-RSV, which contain the mutated hnRNP H-binding sites (Fig. 3A), were treated as described for panel A. The results of quantitation of polyadenylation at 30 min are the following: NRS-RSV, 13%; and NRSmutH-RSV, 13%. M, 32P-end-labeled pBR322/MspI markers, the sizes of which are indicated at left.
was explored using point mutations that eliminate hnRNP H binding to NRS (Fig. 3A) (17, 18). There was no difference in polyadenylation between the hnRNP H-binding mutant and the wild type (Fig. 4B). This suggests that hnRNP H is not required for NRS-stimulated polyadenylation, in agreement with previous data demonstrating that this hnRNP H-binding mutation had no effect on RSV polyadenylation in vivo (17).

SR proteins promote RSV polyadenylation in vitro. The 5′ region of the NRS contains a purine-rich region that binds SR proteins (35) and is required for optimal RSV polyadenylation (17). To test the hypothesis that SR proteins promote RSV polyadenylation, HeLa S100 extract that lacks SR proteins was used for polyadenylation assays, and purified total SR proteins were then added in an attempt to restore NRS-mediated polyadenylation activity. The S100 extract lacked a factor(s) required for polyadenylation, since it did not support polyadenylation, and the addition of SR proteins also had no effect (lanes 6 and 7). Polyadenylation was detected with NRS-RSV (2%), but importantly, addition of purified SR proteins stimulated polyadenylation significantly (8%) (lanes 9 and 10). These data strongly support a role for the SR proteins in NRS-mediated RSV polyadenylation.

The NRS binds the SR proteins ASF/SF2, 9G8, SRp20, and SRp40 (17, 35), and to further demonstrate a role for individual SR proteins in RSV polyadenylation, three copies of SELEX consensus binding sites for ASF/SF2, 9G8, SRp20, and SRp40 (8, 29, 51) were fused to the RSV poly(A) signal (Fig. 6A), and polyadenylation activity was determined in nuclear extract. Compared to a control substrate, the activity of which was slightly elevated relative to that of RSV alone (Fig. 6B, compare lanes 5 to 7 and 8 to 10), the binding sites for ASF/SF2, 9G8, SRp20 increased RSV polyadenylation (10, 7, and 9%, respectively) (lanes 11 to 19). The SRp40 sites were no more active than the control (lanes 20 to 22). One explanation is that the effect is specific to ASF/SF2, 9G8, and not shown). However, S100 was rendered polyadenylation competent when supplemented with a small amount of nuclear extract that, alone, was not active (data not shown). SVL polyadenylation was evident at 30 min (3% in this experiment), and addition of 1 μg of purified SR proteins had no effect (2%) (Fig. 5, lanes 3 and 4). RSV polyadenylation was barely detectable, and the addition of SR proteins also had no effect (~0.5%) (lanes 6 and 7). Polyadenylation was detected with NRS-RSV (2%), but importantly, addition of purified SR proteins stimulated polyadenylation significantly (8%) (lanes 9 and 10). These data strongly support a role for the SR proteins in NRS-mediated RSV polyadenylation.

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SRp20, or, alternatively, SRp40 is not as abundant as the other SR proteins in the nuclear extract. Regardless, the data support a role for the SR proteins ASF/SF2, 9G8, and SRp20 in NRS-stimulated RSV polyadenylation in vitro.

SR protein-binding sites stimulate RSV polyadenylation in vivo. In light of the above evidence that SR proteins mediate NRS-stimulated RSV polyadenylation in vitro, it was important to demonstrate their importance in a proviral setting in vivo. To address whether SR proteins alone could perform this function, three SELEX consensus binding sites for ASF/SF2 and 9G8 or a random sequence was substituted for the NRS in a proviral clone containing the CAT gene and SV40 early poly(A) signal at the 3′ end of the genome (41). Inclusion of the downstream SV40 signal ensures the polyadenylation and stabilization of read-through transcripts. Since previous data suggested a role for the NRS U1-binding site in polyadenylation control (17, 44), proviral clones also were made in which the SELEX sites were fused to NRS3′ (gray box). The positions and sizes of the RNase protection probe used for panel B and the protected products are shown below the lower schematic (B). RNase protection assays were performed using RNA from CEFs transfected with the indicated proviral clones lacking the entire NRS (ΔNRS) or containing only NRS3′ or with constructs having insertions of control, ASF/SF2, or 9G8 SELEX sites. On the right are constructs with mutations that eliminate U1/U11 snRNP binding (RG11) or hnRNP H binding to the downstream sites (mG1 + 2). Products were subjected to electrophoresis on a 6% polyacrylamide gel that contains 8 M urea and visualized with a PhosphorImager. Protected products are labeled at the right. P, probe (864 nt); US, unspliced RNA (630 nt); S, spliced RNA (398 nt); RT, read-through RNA (336 nt); PA, polyadenylated product (256 nt); WT, wild type. M, 32P-end-labeled pBR322/MspI markers, the sizes of which are indicated at the left. (C) Quantitation of the data from three replicate experiments for the percentages of spliced (top) and read-through (bottom) RNA.
alone did not support efficient splicing inhibition (~67% spliced) or polyadenylation (~33% read-through) (Fig. 7B, lane 8). Fusing the ASF/SF2 or 9G8 binding sites to NRS3 had variable effects on splicing inhibition but was sufficient to restore polyadenylation to near-wild-type levels (lanes 10 and 11). These effects were specific, since the level of splicing inhibition was unchanged by fusing the control sequence to NRS3 (~59% spliced), and polyadenylation was similar to that observed with the NRS3 deletion (~31% read-through) (lane 9). The ASF/SF2-binding site corrected the splicing defect when fused to NRS3 (lane 10), but the 9G8 sites appeared to cause oversplicing (~85% spliced) (lane 11). However, the level of unspliced 9G8 RNA appeared selectively reduced compared to that of other samples, such that the change in percent splicing may be due to destabilization of the unspliced RNA. Overexpression of SR proteins can result in reduced RNA stability (28, 60), so it is possible that 9G8 recruitment to the NRS3-containing unspliced RNA may target it for degradation. Collectively, the results indicate that RSV polyadenylation control in vivo requires SR proteins and the integrity of the NRS-3′ ss inhibitory complex, perhaps to position the SR proteins nearer to the poly(A) site.

NRS3′ harbors binding sites for U1 snRNP, U11 snRNP, and hnRNP H, of which U1 has a demonstrated role in RSV polyadenylation (17). To determine if mutations in strong hnRNP H-binding sites in NRS3′ affect RSV 3′-end formation in vivo, proviral clones with mutations that eliminate hnRNP H binding were examined (37). As in previous studies, eliminating U1/U11 binding with the RG11 mutation compromised NRS function and caused increased splicing and read-through transcripts (Fig. 7B, lane 17; quantitation of the data is shown in C). No change in splicing or polyadenylation was observed with the hnRNP H mutant provirus (mG1) (lane 18). These results confirm a previous report, which showed that the snRNP-binding sites are required for RSV splicing and polyadenylation control (17), and suggest that the strong, downstream hnRNP H-binding sites are not required for either activity.

SR protein-binding sites can stimulate RSV polyadenylation in vivo independent of the NRS-3′ ss complex. The observation that SR proteins are sufficient to stimulate RSV polyadenylation in vitro suggests that they perform the same function in vivo. While the requirement for the NRS-3′ ss complex for optimal RSV polyadenylation in vivo is consistent with positioning the SR proteins closer to the poly(A) site, it was also formally possible that an NRS-3′ ss complex factor other than SR proteins interfaces with the polyadenylation machinery (see Discussion). To determine if SR proteins alone could promote polyadenylation, a deletion was made in the ASF/SF2 and 9G8 SELEX and control proviral clones that moved the sites from ~8,300 nt to ~4,320 nt away from the poly(A) site (Fig. 8A); movement to this position is similar to what would occur if the NRS interacted with the env 3′ ss. As observed above, splicing was elevated with all the deletions (data not shown), and read-through transcription was elevated in the ∆NRS, control insert, and ASF/SF2 and 9G8 SELEX samples (Fig. 8B, lanes 4 to 7;
DISCUSSION

Previous work indicated that a functional NRS is required for optimal viral RSV polyadenylation (17, 44). In keeping with current models of coupled splicing and polyadenylation, it was proposed that the nonproductive splicing complex that forms on the NRS and a 3′ ss stimulate RSV polyadenylation by stabilizing the binding of splicing factors to the weak viral 3′ ss; these factors would then promote polyadenylation by conventional mechanisms (see below). In this model, NRS-binding factors are only indirectly involved in polyadenylation control. Surprisingly, we show here that the NRS alone promotes polyadenylation of an RSV substrate in vitro, and that U1 snRNP and a 3′ ss are not required for this effect. While this work was in progress, a report by Wilusz and Beemon (56) reached a similar conclusion. We further show that SR proteins mediate the polyadenylation stimulatory activity of the NRS in vitro, which has implications for potential polyadenylation control of cellular mRNAs. While the SR-related proteins have been shown to bind poly(A) factors and promote polyadenylation (32, 33, 42, 54), to our knowledge this is the first demonstration that SR proteins can promote polyadenylation. However, SR proteins alone were insufficient to stimulate polyadenylation in a proviral context in vivo. This is consistent with previous reports that the downstream snRNP-binding sites within the NRS are required for optimal viral polyadenylation (17, 44). One explanation for this discrepancy is that the distance between the SR protein-binding site and the poly(A) site is quite long in the provirus compared to those in the in vitro substrates, and there may be a distance constraint over which SR proteins can function. The NRS interaction with a viral 3′ ss would spatially bridge the long distance and position the SR proteins nearer to the poly(A) site (Fig. 9B).

Interestingly, SR proteins bind an exonic splicing enhancer located just downstream from the env 3′ ss (25), but these sites do not appear to be sufficient for poly(A) control, since elevated levels of read-through RNA are detected when these sites are present and the NRS is deleted (41). These sites also are present in our ΔNRS proviruses, for which considerable poly(A) read-through was observed. Perhaps the env exonic splicing enhancer does not bind SR proteins as well as the NRS, which represents an extensive SR protein-binding platform (17, 35), or, alternatively, other components of the NRS-3′ ss complex contribute to polyadenylation (see below).

It also was shown that deleting the region encompassing the env 3′ ss caused significant poly(A) read-through, but that a G-to-U mutation at the env 3′ ss was without effect (41). Our model would reconcile these observations; the env 3′ ss mutation would still allow the NRS-env complex to form, but deletion of the entire env 3′ ss region would disrupt the interaction with the NRS and repositioning of the SR proteins.

CFLIm,68, a large subunit of the CFLIm polyadenylation factor, has a domain rich in RD-, RE-, and RS-dipeptide repeats that can interact with SR proteins, presumably through the RS domain that is known to mediate SR protein interactions with other RS-domain-containing proteins (16, 26, 45, 57). An attractive possibility to be tested in the future is that the NRS and associated SR proteins recruit or stabilize CFLIm binding to the poorly utilized viral poly(A) site (Fig. 9A). A precedent for this idea comes from the observation that the RS domain within the 65-kDa subunit of U2AF stimulates polyadenylation in vitro via interaction with the alternating charged domain of CFLIm (42).

Proviral deletions that moved the ASF/SF2 and 9G8 SELEX sites alone to a position similar to that which would occur from an NRS-env 3′ ss interaction partially restored polyadenylation, indicating that SR proteins can directly promote polyadenylation independent of the NRS complex. The partial rescue may reflect a decreased capacity of the SELEX site relative to NRS to recruit SR proteins, or that other NRS complex components have a role in optimal RSV polyadenylation. For example, this could include U2AF65, whose binding to the viral 3′ ss might be stabilized within the NRS complex to recruit CFLIm through its respective RS-like domains (42); U2AF65 also interacts with poly(A) polymerase (54). In another scenario, U2 snRNP binding to the suboptimal env branch point (19, 25) may be stabilized within the stalled NRS complex, and U2 might assist in RSV poly(A) site recognition. This is based on the recent demonstration that the SF3b components of U2 snRNP and subunits of the CPSF interact, and that this interaction mediates coupling of splicing and 3′-end formation (27). Finally, two observations suggest the possibility that U1 snRNP within the NRS complex contributes to RSV
polyadenylation: U1A protein can interact with CPSF160 and stimulate polyadenylation (30), and U1 snRNP interacts with CFI\textsubscript{m} (4). Any of these interactions could collaborate with SR proteins to improve recognition of the RSV poly(A) site (Fig. 9B).

hnRNP H is an auxiliary polyadenylation factor for some viral and cellular poly(A) sites (2, 5), and hnRNP H/H\textsuperscript{+} sites have been found near numerous cellular poly(A) sites (24, 52). The NRS harbors two hnRNP H-binding regions, a weak upstream site that is embedded in the SR protein-binding region (17) and a strong downstream site(s) required to recruit U11 snRNP to the NRS (37). These observations suggested that hnRNP H might also contribute to RSV polyadenylation. This does not appear to be the case, since (i) a mutation in the upstream site had no effect on polyadenylation in vitro (the NRS substrate used in vitro lacked the downstream hnRNP H sites), (ii) mutation of the strong sites had no effect on RSV polyadenylation in vivo, and (iii) the ASF/SF2 SELEX sites alone promoted RSV polyadenylation in vivo. While evidence of a positive role for hnRNP H in RSV polyadenylation is lacking, a potential negative role was reported by Fogel et al. using proviral clones, but only when SR protein binding was compromised (17). This possibility was supported by a recent report in which, in vitro, sequestration of hnRNP H led to compromised (17). This possibility was supported by a recent report in which, in vitro, sequestration of hnRNP H led to compromised (17).

It has become clear that a large number of cellular poly(A) sites lack the canonical AAUAAA sequence, and these sites likely benefit from the action of a variety of auxiliary elements and binding factors (6, 24, 52). Our findings that SR proteins promote RSV polyadenylation suggest that SR proteins may be one such class of factors. Some SR proteins remain associated with spliced exons and may influence polyadenylation of cellular mRNAs more generally than has been appreciated. Alternatively, analogous to nonsplicing sites for U2AF that couple to polyadenylation (15), SR protein-binding sites that are distinct from those involved in splicing might act as auxiliary elements to influence recognition of suboptimal or alternative poly(A) sites. These elements would likely be in the 3’-terminal exon near the poly(A) site, unlike the distant sites in the NRS, which are thought to require the NRS-3’-ss complex for positioning SR proteins close to the poly(A) site.

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