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Characterization of an RNP complex that assembles on the Rous sarcoma virus negative regulator of splicing element

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ABSTRACT

We have characterized an RNP complex that assembles in nuclear extracts on the negative regulator of splicing (NRS) element from Rous sarcoma virus. While no complex was detected by native gel electrophoresis under conditions that supported spliceosome assembly, gel filtration revealed a specific ATP-independent complex that rapidly assembled on NRS RNA. No complexes were formed on non-specific RNA. Unlike the non-specific H complex, factors required for NRS complex assembly are limiting in nuclear extract. The NRS complex was not detected in reactions containing ATP and pre-formed complexes were dissociated in the presence of ATP. In addition, the assembly process was sensitive to high salt but NRS complexes were salt stable once formed. Assembly of the NRS complex appears functionally significant since mutated NRS RNAs that fail to inhibit splicing *in vivo* are defective for NRS complex assembly in nuclear extract. The probable relationship of the NRS complex to spliceosomal complexes is discussed.

INTRODUCTION

Splicing of precursor mRNA (pre-mRNA) *in vitro* takes place in a macromolecular complex called the spliceosome that assembles in a stepwise pathway through the binding of small nuclear ribonucleoprotein particles (snRNPs) and a large number of non-snRNP factors (1–3). The pathway for mammalian spliceosome assembly was initially investigated by native gel electrophoresis which defined a non-specific complex (H complex) and two ATP-dependent complexes that were specific to splice site containing substrates, the prespliceosome (A complex) and mature spliceosome (B complex) (4,5). Subsequently, gel filtration proved to be a powerful tool for studying spliceosome assembly and identifying components of complexes in the assembly pathway (6–8). Using this approach, an early forming, ATP-independent complex (E complex) was identified that is not detected with native gels (6,7). Many of the protein components of prespliceosome and spliceosome complexes were determined with a scaled-up gel filtration protocol (8–11). The column procedure was also used to identify and characterize complexes formed on general RNA splicing enhancers (12) and a regulated splicing enhancer involved in alternative splicing of the *Drosophila dsx* pre-mRNA (13). The gel filtration approach was very recently used to demonstrate the requirement of cap-binding proteins for assembly of E5′, a complex formed on an isolated 5′ splice site (5′ ss) (14).

While most cellular pre-mRNAs are spliced to completion, retrovirus replication requires that a large amount of unspliced viral RNA be preserved to serve as gag–pol mRNA and as genomic RNA for progeny virions (15,16). In Rous sarcoma virus (RSV), only ~25% of the primary transcripts are spliced to sub-genomic RNAs. One *cis*-acting RNA sequence involved in splicing suppression in RSV, the negative regulator of splicing (NRS), is novel in that it does not reside near any of the splice sites (17,18). The element inhibits splicing of heterologous introns *in vivo* (17,19) and *in vitro* (20) and seems to function through the binding of factors that are actually required for splicing. Sequences within the ~230 nucleotide (nt) minimal NRS are similar to the 5′ and 3′ splice site consensus sequences, and one study showed that U1 and U2 snRNPs, which bind to 5′ and 3′ splice sites, can interact with the NRS (20). In addition, pre-mRNA substrates harboring the NRS in the intron fail to splice *in vitro* and form abnormally large RNP complexes that contain not only the complete complement of spliceosomal snRNPs but two additional snRNPs, U11 and U12, which are not associated with conventional spliceosomes (20). U11 snRNP appears to play a significant role in NRS function since mutations that abolish NRS binding *in vitro* severely affect splicing inhibition *in vivo* (20). Interestingly, U12 snRNP was recently shown to functionally replace U2 in a novel spliceosome that removes a minor class of intron containing non-canonical consensus sequences (21,22); the evidence that U11 replaces U1 snRNP is compelling but less certain. To date, the relative roles of the different snRNPs in NRS splicing control are unknown. In addition to snRNPs, it was recently shown that SR protein splicing factors (primarily SF2/ASF) associate with the 5′ half of the NRS *in vitro*, and SR protein binding correlated with *in vivo* inhibitory activity (23). Consistent with their proposed function (reviewed in 3,24), it is plausible that the SR proteins assist snRNP binding to the NRS. These observations are compatible with models in which the NRS serves as a pseudo splice site that interacts with authentic splice sites to block splicing (20,23).

The findings that components of the splicing machinery functionally interact with the NRS suggested the possibility that

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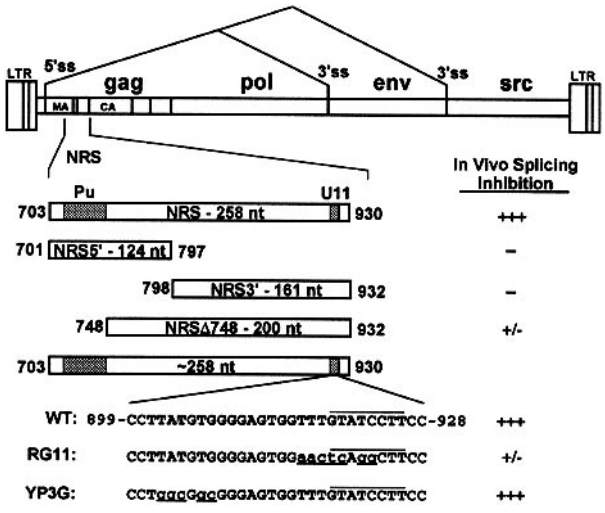

Figure 1. Schematic representation of NRS substrates used in this study. Indicated on the structure of the RSV provirus are the long terminal repeats (LTR), the 5′ and 3′ splice sites (5′ss and 3′ss), the gag, pol, env and src genes, and the location of the NRS relative to the matrix (MA) and capsid (CA) genes. The NRS fragments are depicted as boxes with the endpoints of the RSV sequence indicated outside the box and the transcript length, including polylinker, inside the box. The stippled areas represent the purine-rich 5′ region required for SR protein binding (Pu) and the site of homology to the minor class 5′ splice site that is required for U11 binding (U11). The sequence of wild-type RSV and the RG11 (247 nt) and YP3G/VP2G (YP3G, 250 nt) mutants between nt 899 and 928 is shown with the mutations underlined and in lowercase, and the U11 site overlined. The previously reported abilities of the fragments to block splicing of a heterologous intron *in vivo* are shown on the right. The data for YP3G/VP2G were generated in this study. The steady state unspliced RNA levels determined by PhosphorImager analysis are as follows: –, 0–10% (percent unspliced with no insert): +/–, 11–20%; +, 21–30%; ++, 31–50%; +++, 51–80%.

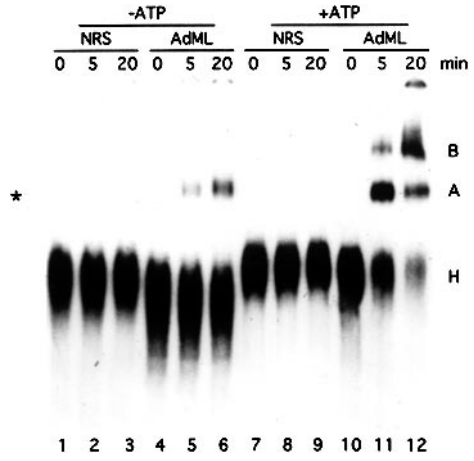

Figure 2. Native gel electrophoresis fails to detect assembly of specific complexes on NRS RNA. Radiolabeled NRS RNA (lanes 1–3 and 7–9) or AdML pre-mRNA (lanes 4–6 and 10–12) was incubated under splicing conditions in nuclear extract in the absence (lanes 1–6) or presence (lanes 7–12) of ATP for the indicated times, heparin was added to 0.25 mg/ml, and samples were subjected to native gel electrophoresis in a 4% polyacrylamide gel (acrylamide:bisacrylamide 80:1) for 3.5 h at 200 V/cm. The gel was dried and autoradiography performed. The positions of the H, A and B complexes are indicated on the right. The asterisk to the left of the –ATP lanes indicates the small amount of –ATP complex formed on AdML.

the element itself might support the assembly of spliceosome-like complexes. We show here that while no complexes were detected with the native gel system developed for the study of spliceosomes, gel filtration chromatography revealed a distinct NRS complex whose formation was dependent on factors that are limiting in nuclear extract. NRS complex assembly was ATP-independent, and pre-formed complexes were dissociated by ATP. Significantly, the complex did not form on mutant NRS RNAs that fail to inhibit splicing *in vivo*, establishing a correlation between assembly and activity and indicating that the complex is relevant to NRS function.

MATERIALS AND METHODS

Plasmids, RNA synthesis and *in vitro* splicing reactions

Plasmid pAdHS was produced by inserting a *Hin*dIII–*Sca*I fragment of pAd2H/B (25) into the *Hin*dIII/*Sma*I digested pGEM-4Z (Promega Biotech) such that the adenovirus major late splicing substrate could be generated with T7 RNA polymerase. The DNA linearized with *Eco*RI generated an RNA with a 66 nt first exon, a 113 nt intron, and a 56 nt second exon. Plasmids p3ZBB, p3ZBB5′, p3ZBB3′ and pKS-XSΔ748 and RNA synthesis from them (NRS, NRS5′, NRS3′ and NRSΔ748) were described previously (23). To make p3Z-RG11, PCR amplification (26) of an RSV clone harboring the RG11 mutation (20; pAPrC-RG11, gift of K. Beemon) was done to generate a fragment containing nt 701–1006 [numbering system of Schwartz *et al.* (27)], the fragment was digested with *Mro*I and blunt-ended, re-digested with *Sph*I, and inserted into *Sma*I/*Sph*I cut pGEM-3Z. Plasmid p3Z-YP3G/VP2G contains five base changes from nt 902 to 907 (28) and was generated by PCR from a proviral clone harboring the mutations (gift of K. Beemon) with primers containing engineered *Kpn*I and *Xba*I sites. PCR products were digested with *Kpn*I and *Xba*I and cloned into the same sites in pGEM-3Z. Both plasmids were linearized with *Bst*NI for transcription to generate 247 nt (RG11) and 250 nt (YP3G) RNAs. The splicing inhibition activity of YP3G/VP2G was determined as described (19) after insertion into the myc intron of pRSVNeo-int and transfection into 293 cells. *In vitro* transcripts were synthesized in standard reactions (29) as described (23) with [^{32}P]UTP and T7 RNA polymerase, and a description of the NRS substrates is shown in Figure 1. Similar results were obtained with capped and uncapped transcripts. *In vitro* splicing reactions were essentially as described (30) except that polyvinyl alcohol was omitted. Reactions for native gel analysis contained 40% nuclear extract and the gel conditions were as described (4), except that heparin was added to a final concentration of 0.25 mg/ml prior to electrophoresis.

Gel filtration chromatography of RNP complexes

In vitro splicing reactions (100 µl) contained 60% nuclear extract and the amount of RNA indicated in the figure legends. Reactions performed in the absence of ATP also lacked $MgCl_2$ and creatine phosphate, and the nuclear extract was pre-incubated 20 min at room temperature to deplete any endogenous ATP. The chromatography conditions were exactly as described by Michaud and Reed (7) with a flow rate of 6 ml/h. Samples were applied directly onto a 1.5 × 50 cm Sephacryl S-500 column equilibrated in FSP buffer (20 mM Tris–HCl pH 7.8, 0.1% Triton X-100, 60 mM KCl, 2.5 mM EDTA) and 1 ml fractions were collected. In some instances, ~25 ml of the void volume was discarded and 0.66 ml

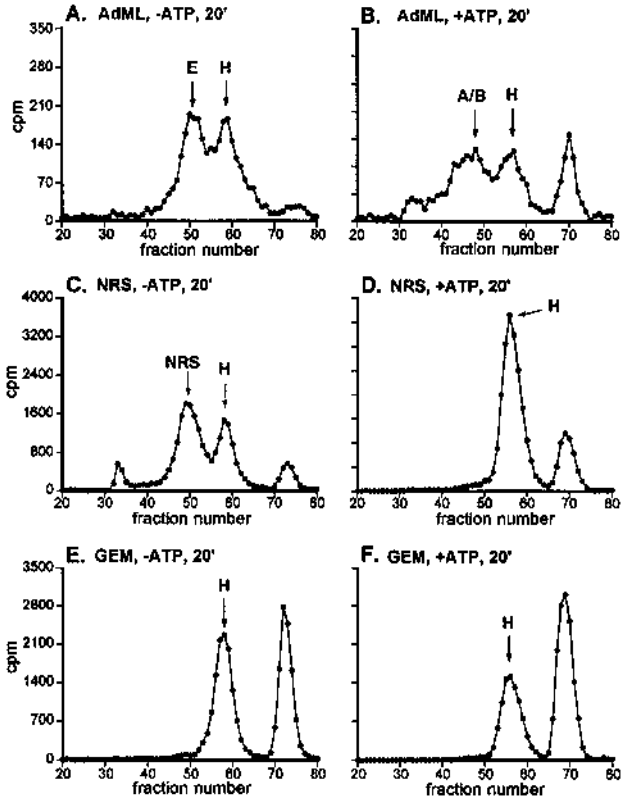

Figure 3. Detection of NRS complexes by gel filtration chromatography. Splicing reactions (100 µl) containing 15 ng of ^{32}P-labeled AdML pre-mRNA (**A** and **B**), or ~30 ng of labeled NRS RNA (**C** and **D**) or pGEM sequences (**E** and **F**) were incubated for 20 min in the absence (–ATP) or presence (+ATP) of ATP, and fractionated by gel filtration on Sephacryl S500 columns. The –ATP reactions also lacked $MgCl_2$ and creatine phosphate. Peaks containing non-specific H complexes, the spliceosomal E and A/B complexes, and the NRS complex are indicated. The peak eluting between fractions 65 and 80 contains degraded RNA, while the void volume (containing variable amounts of aggregated material) eluted between fractions 25 and 35.

fractions were collected. For 0 min time points, RNA was added to extract and immediately applied to the column. To test NRS complexes for ATP stability, a minimal amount of buffer or an ATP/$MgCl_2$/creatine phosphate mixture was added after a 20 min incubation without ATP, and the incubation continued 15 min further. The non-hydrolyzable ATP analog adenylyl-(β-γ-methylene)-diphosphonate (AMP-PCP; Boehringer-Mannheim) was used at 10 mM in the absence of $MgCl_2$ and creatine phosphate. Column profiles were generated by counting 40 µl of each fraction in a Packard microwell plate scintillation counter, or entire fractions were counted by Cerenkov counting. Due to variability among columns, the precise fractions in which the different complexes elute can be slightly different between experiments.

RESULTS

The observation that a number of splicing factors bind the NRS suggested the possibility that a complex similar to those described in the splicing pathway (E, A, B or C complexes) might form on the NRS element itself. To detect such complexes, we initially employed a native gel electrophoresis approach that is commonly used to assess spliceosome assembly (4), using HeLa cell nuclear extracts and an adenovirus major late transcript as an assembly control. The NRS transcripts used here and in subsequent experiments are described in Figure 1. Since assembly of the A, B and C complexes is ATP-dependent but the E complex is ATP-independent, reactions were done in the presence and absence of ATP. As shown in Figure 2, only the non-specific H complex was detected on NRS and AdML RNA at 0 min in the absence or presence of ATP (lanes 1, 4, 7 and 10). As expected, AdML RNA formed spliceosomal complexes only in the presence of ATP, with the A complex appearing at 5 min and B complex predominating at 20 min (Fig. 2, lanes 4–6 and 10–12). A small amount of an –ATP complex (31) was also detected on AdML in the absence of ATP (denoted by asterisk). The role of the –ATP complex in splicing is unknown (9). In contrast with AdML, under no condition was a specific complex detected on NRS RNA (Fig. 2, lanes 1–3 and 7–9) over a range of heparin concentrations (data

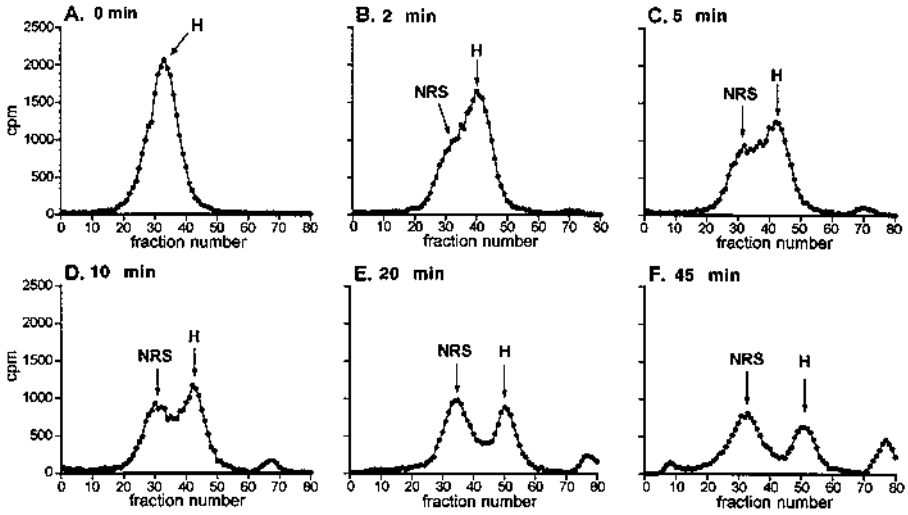

Figure 4. Time course of NRS complex assembly. Radiolabeled NRS RNA (30 ng) was incubated in 100µl splicing reactions in the absence of ATP, creatine phosphate and $MgCl_2$ for the indicated times, and samples were resolved by gel filtration. Peaks corresponding to the H and NRS complexes are indicated. The peak eluting after the H complex is degraded RNA. Approximately 25 ml of the void volume was discarded before fractions were collected.

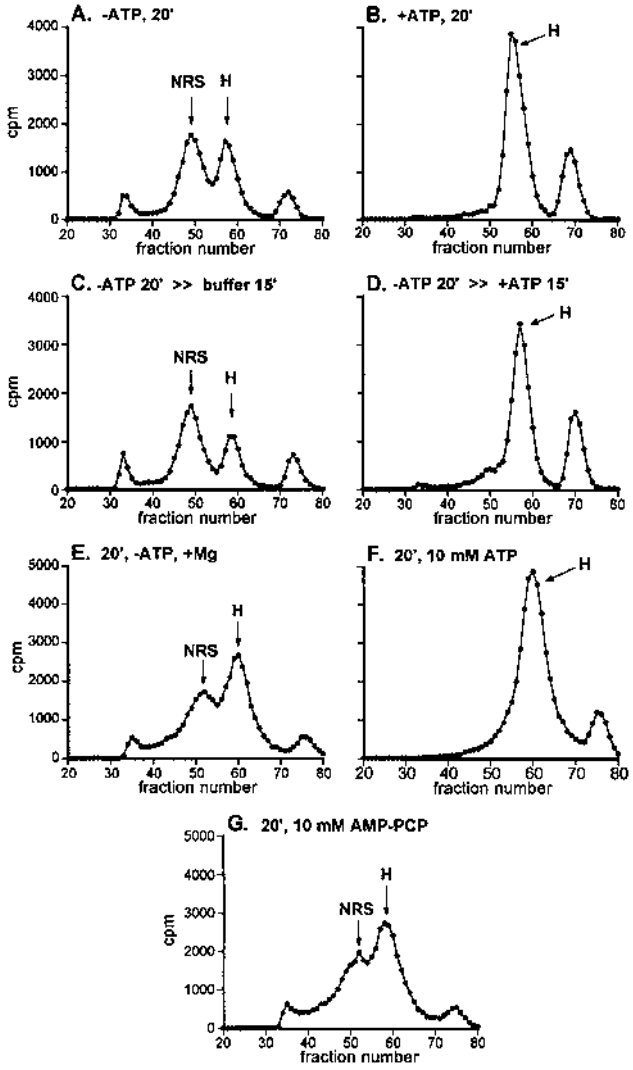

Figure 5. Pre-formed NRS complexes are dissociated by ATP. Radiolabeled NRS RNA (30 ng) was incubated in standard splicing reactions in the presence (**B**) or absence (**A**) of ATP for 20 min and applied to the gel filtration column. In addition, samples incubated for 20 min in the absence of ATP had either buffer [(**C**) >> buffer] or a mixture of ATP, $MgCl_2$ and creatine phosphate [(**D**) >> +ATP] added and the incubation was continued for an additional 15 min. Samples were then resolved by gel filtration. A control assembly reaction containing $MgCl_2$ but lacking ATP and creatine phosphate is shown in (**E**). To address the role of ATP hydrolysis in complex dissociation, reactions were done at 10 mM ATP in the absence of $MgCl_2$, which alleviated the requirement for creatine phosphate (**F**), or with 10 mM AMP–PCP (**G**). The NRS and H complexes are indicated. Peaks eluting in fractions 25–35 are the void volume, and peaks eluting after fraction 65 contain degraded RNA.

not shown). In addition to the possibility that a complex does not assemble on the NRS, the failure to detect an NRS complex would be explained if the complexes were not stable under these gel conditions or if they were obscured by the H complex.

Since the earliest forming splicing complex, the ATP-independent E complex, can be detected by gel filtration chromatography but not by native gel electrophoresis (7), we next used Sephacryl S500 gel filtration in an attempt to detect an NRS complex. Again, AdML RNA was used to monitor E complex assembly in the absence of ATP, and formation of A/B complexes in the presence of ATP. Only the non-specific H complex was detected at 0 min with all the substrates (data not shown). Consistent with the observations of Michaud and Reed (7) and as shown in Figure 3, the E and A/B complexes were efficiently assembled on AdML RNA by 20 min in the absence and presence of ATP, respectively (Fig. 3A and B). When NRS RNA was used, a second distinct peak in addition to H was observed upon incubation for 20 min in extract lacking ATP (Fig. 3C); we refer to this as the NRS complex. Interestingly, no NRS complex was observed when ATP was included in the reactions (Fig. 3D). The ATP-independent complex formed on NRS RNA was specific since only the H complex was detected with a non-specific RNA derived from vector sequences, irrespective of ATP addition (Fig. 3E and F).

The kinetics of NRS complex assembly was addressed with a time course experiment and reactions lacking ATP. As shown in Figure 4, the NRS complex was detected as early as 2 min, assembly peaked between 10 and 20 min, and the complexes were stable for at least 45 min (Fig. 4A–F). These kinetics are reminiscent of those reported for the pre-spliceosomal E complex (7). Since it was possible that the kinetics of NRS complex assembly differed in reactions containing ATP such that complexes were missed because the 20 min time point in Figure 3 was too long or short, a time course was also performed with reactions containing ATP. The NRS complex was not observed at time points as short as 2 min or up to 1 h (data not shown).

To further explore the apparent ATP sensitivity, NRS complexes were assembled in reactions lacking ATP, and then buffer or ATP was added and the incubation was continued for an additional 15 min. As shown in Figure 5, addition of buffer alone had little effect on the complex compared with the control reaction (Fig. 5A and C) whereas the profile generated after ATP addition was indistinguishable from the one produced by reactions containing ATP (Fig. 5B and D). In the experiments described above, $MgCl_2$ and creatine phosphate were added with the ATP. $MgCl_2$ was not responsible for NRS complex lability since its inclusion alone in an assembly reaction resulted in only a small diminution of the NRS peak (Fig. 5E). The addition of creatine phosphate did cause disassembly, presumably by regenerating a small amount of ATP endogenous to the extract (data not shown). The requirement for ATP hydrolysis in NRS complex dissociation was addressed using the non-hydrolyzable ATP analog, AMP-PCP. In control reactions lacking creatine phosphate, the NRS complex persisted with 0.5 mM ATP (data not shown), which was likely due to rapid ATP hydrolysis, but this problem was circumvented and disassociation was observed with 10 mM ATP (Fig. 6F). In contrast, the NRS complex was largely unaffected by 10 mM AMP-PCP (Fig. 5G). Thus, the data show that NRS complexes do not form in and are dissociated by ATP in an energy requiring process.

To characterize NRS complex stability further, complexes were assembled in the absence of ATP but with increasing KCl concentrations, and then loaded onto columns equilibrated to the salt concentration of the reaction. In contrast with the standard reactions containing 60 mM KCl (Fig. 6A), complexes were not observed in reactions containing 150 or 250 mM KCl (Fig. 6B and C). To determine if elevated salt concentrations could dissociate pre-formed NRS complexes, 20 min assembly reactions containing 60 mM KCl were applied to columns pre-equilibrated to 150 or 250 mM KCl. The results show that once formed, NRS complexes are stable to high salt, although the amount decreased slightly in 250 mM KCl (Fig. 6E and F). Similar results were

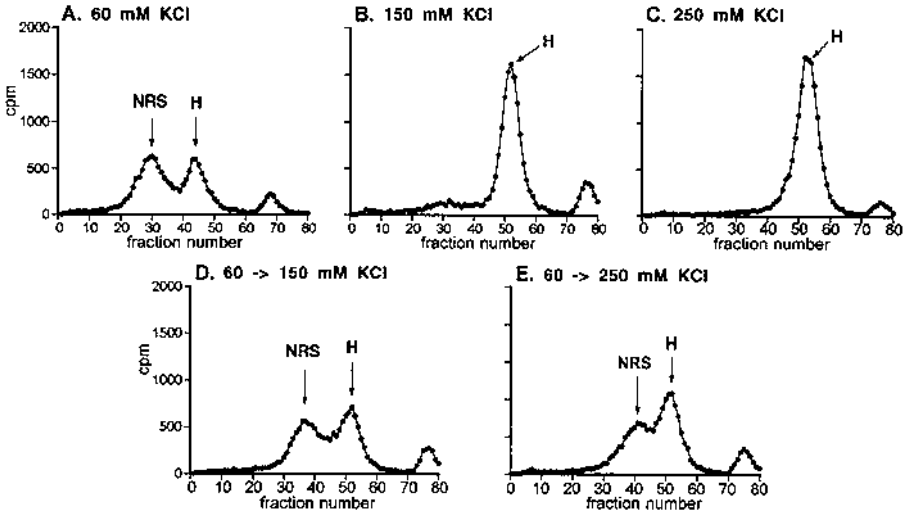

Figure 6. NRS complex assembly is salt sensitive, but pre-formed complexes are stable to high salt. Radiolabeled NRS RNA was incubated in standard splicing reactions lacking ATP [(**A**) 60 mM KCl], or in reactions containing either 150 mM (**B**) or 250 mM (**C**) KCl, and samples were resolved by gel filtration in columns equilibrated to the salt concentration of the reaction. NRS complex stability was tested by applying NRS complexes assembled in the absence of ATP at 60 mM KCl to gel filtration columns equilibrated to either 150 mM (**D**) or 250 mM (**E**) KCl. The NRS and H complexes are indicated. The peak eluting after fraction 65 is degraded RNA. Approximately 25 ml of the void volume was discarded before fractions were collected.

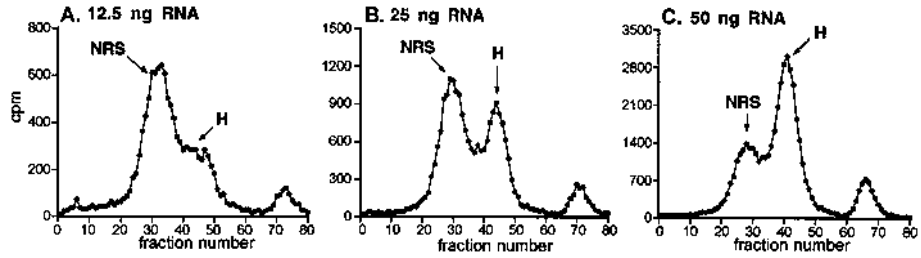

Figure 7. Factors required for NRS complex formation are limiting in nuclear extract. The indicated amounts of radiolabeled NRS RNA were incubated for 20 min in standard splicing reactions (100 µl) in the absence of ATP, creatine phosphate and $MgCl_2$, and the samples were subjected to gel filtration. The NRS and H complexes are indicated. The peak following H complex is degraded RNA. Approximately 25 ml of the void volume was discarded before fractions were collected.

obtained when reactions were incubated an additional 5 min at the higher salt concentrations prior to applying samples to the columns (data not shown). These results are reminiscent of the effect of high KCl concentrations on spliceosome assembly; high salt prevents spliceosome assembly but spliceosomes are stable and active once formed (32).

To assess the abundance of extract factors involved in NRS complex assembly relative to the H complex, various amounts of NRS RNA were incubated in extract and the samples were applied to gel filtration columns. When a low amount of NRS RNA was used, the NRS complex was approximately twice as abundant as the H complex (Fig. 7A). With an intermediate amount of RNA, roughly equal levels of NRS and H complex were observed (Fig. 7B), whereas the level of H complex was about double that of the NRS complex with 50 ng of RNA (Fig. 7C). Thus, the addition of increasing amounts of NRS RNA did not result in a corresponding increase in NRS complex whereas this was roughly true for the H complex. The H complex contains a large number of proteins, many of which belong to the highly abundant hnRNP protein family (8). Therefore, it was not unexpected that the level of H complex increased proportionately when additional RNA was added. The results show that, unlike for the H complex, the factors involved in NRS complex assembly are limiting in nuclear extract. Attractive candidates for the limiting factors are SR proteins and/or U11 snRNP rather than the abundant U1 or U2 snRNPs.

It was previously shown that the 5′ and 3′ halves of the NRS are both required for splicing inhibition and completely nonfunctional in isolation (19), and a number of deletion and site-directed NRS mutants have been constructed that are severely impaired for splicing inhibition *in vivo* (19,20,23). If NRS complex formation *in vitro* is related to its function *in vivo*, then one would predict that NRS complex assembly should correlate with activity and that the mutants would assemble complexes less efficiently. To test this prediction, complex assembly was examined with a number of mutant NRS RNAs (Fig. 1), including the inactive NRS 5′ and 3′ halves, a 5′ end deletion (BBΔ748) that fails to bind SR proteins (23) and RG11, a mutant with seven base changes that disrupt U11 snRNP binding (20), the latter two having severely compromised activity *in vivo*. A control substrate for RG11, YP3G/VP2G (28), contains five base changes just upstream of RG11, binds U11 *in vitro* and is fully functional (Fig. 1). In each case, molar amounts of RNA were used that produced similar levels of NRS and H complex on the authentic

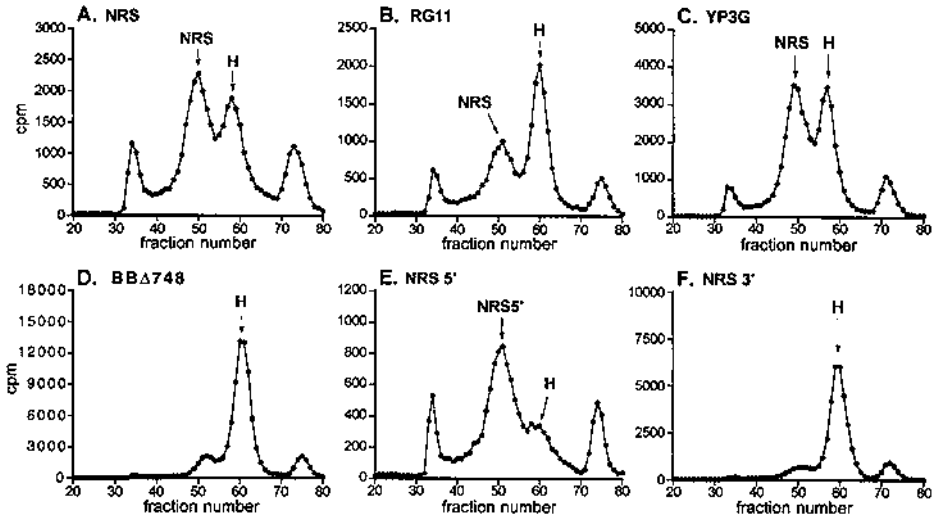

Figure 8. Mutant NRS RNAs are impaired for NRS complex assembly. Equimolar amounts of NRS RNA (25 ng) or mutant substrates (Fig. 1) were incubated in reactions lacking ATP, creatine phosphate and $MgCl_2$ for 20 min and reactions were resolved by gel filtration. RG11 harbors base changes in the presumptive U11 snRNP binding site, and the control mutant YP3G contains five changes immediately upstream which do not affect activity or U11 binding. BBΔ748 has a 5′ terminal deletion to nt 748 and is only marginally active. NRS5′ and NRS3′ are the 5′ and 3′ halves of the NRS, respectively, and are completely inactive. The NRS, H and NRS5′complexes are indicated. Peaks eluting before fraction 40 are the void volume, and fractions 70–80 contain degraded RNA.

NRS (Fig. 8A). Complexes formed efficiently on YP3G/VP2G (Fig. 8C) but were barely detectable with the 3′ half (Fig. 8F) or BBΔ748 (Fig. 8D) substrates, while the level observed with RG11 was reduced by a factor of two (Fig. 8B). Interestingly, a complex did assemble on the NRS5′ substrate (Fig. 8E) although it is completely non-functional for splicing inhibition (19). We surmise that this represents an RNA enhancer-like complex; the purine-rich NRS 5′ half was recently shown to bind SR proteins (23), a general property of RNA splicing enhancers (33–37), and to function as a splicing enhancer *in vitro* and *in vivo* (unpublished results). Two other authentic RNA splicing enhancers were shown by Staknis and Reed (12) to form ATP-independent complexes as assessed by gel filtration chromatography. Knowing that the NRS complex was unstable in ATP, ATP stability was used to gauge its relationship to the NRS5′ complex, an enhancer complex (Enh) formed on the bovine growth hormone enhancer (FP element; 37), and E5′ assembled on the isolated adenovirus 5′ ss. Surprisingly, the NRS5′ complex was relatively stable, while the E5′ and FP enhancer complexes were dissociated by ATP (data not shown). Consequently, it was not possible to differentiate the NRS complex from E5′ and Enh complexes; determining the nature of the NRS5′ complex also requires further study. We conclude from the correlation between NRS complex assembly and activity that the NRS complex is relevant to function. The gel filtration approach should be useful in efforts to determine components of the NRS complex and its relationship to NRS5′, E, and enhancer complexes.

DISCUSSION

SR proteins and a number of snRNPs known to be involved in splicing interact with the negative regulator of splicing element from RSV *in vitro*, prompting the prediction that a spliceosome-like complex might form on the NRS. While no specific complex was observed by native gel analysis, we have shown by gel filtration chromatography that the NRS rapidly assembles into a large, specific complex in HeLa nuclear extracts, which was not the case for non-specific RNA. There was a general correlation between NRS complex assembly *in vitro* and splicing inhibition *in vivo*, indicating that the NRS complex formed *in vitro* represents a functionally relevant complex whose dissection should yield important mechanistic insights into NRS-mediated splicing inhibition.

A gel filtration approach has been used to characterize a number of complexes in the spliceosome assembly pathway (E, A, B and C; 7,11), a complex specific to a regulated splicing enhancer in the *Drosophila dsx* gene (dsxRC; 13), and a complex that assembles on RNA splicing enhancers (Enh; 10). The NRS complex is unlikely to be related to the spliceosomal A, B or C complexes, which are ATP-dependent, since reactions containing ATP do not support NRS complex assembly. The well-studied spliceosomal E complex and the enhancer complex represent the two ATP-independent complexes so far described. The E complex forms on pre-mRNA as well as substrates containing only 5′ or 3′ splice sites (E5′ and E3′; 9) and contains U1 snRNP and SR proteins, which promote its assembly (12). The enhancer complexes formed on the bovine growth hormone and ASLV enhancers share these characteristics except that splice sites are not required for assembly (12). Our results indicate that the NRS complex has a number of characteristics in common with the E and Enh complexes. First, specific NRS complexes are seen by gel filtration but not in native gels. Second, assembly is ATP-independent and pre-formed NRS complexes are dissociated by ATP. Third, U1 snRNP and SR proteins bind the NRS *in vitro*, and while a functional role of U1 snRNP binding has not been demonstrated, SR protein binding does correlate with splicing inhibition activity (20,23). Fourth, factors required for NRS complex assembly are limiting in nuclear extract since addition of increasing amounts of NRS RNA did not result in increased NRS complex levels but rather decreased the ratio of NRS complex to H complex. The NRS complex may be related to splicing enhancer complexes since splicing enhancer activity is associated

with the purine-rich NRS 5′ half, which itself formed a complex. However, the RG11 mutation that abolishes U11 snRNP binding and affects complex formation does not influence enhancer activity associated with the 5′ half of the NRS (unpublished results), making the argument that the NRS complex is simply an enhancer complex unlikely. An attempt to exploit ATP sensitivity to compare the NRS complex with E and Enh complexes was uninformative since all were ATP labile. In this regard, the NRS5′ complex seems less similar to the NRS complex than the other two. The presence of splice site-like sequences (for U1, U2 and U11) indicate that the NRS complex is more closely related to the E complex. Resolution of these issues awaits direct analysis of the components in each complex.

Of the snRNPs that have been shown to interact with the NRS (U1, U2 and U11/U12), evidence for functionally significant binding exists only for U11. Point mutations in a sequence near the NRS 3′ end that closely resembles the 5′ ss of minor class introns eliminate U11 binding *in vitro* and NRS activity *in vivo* (20), whereas many mutations throughout the NRS, including sites predicted to bind U1 or U2 snRNP, have shown little effect on splicing inhibition (19; unpublished results). The role of U2 snRNP in the assembly process is questionable since binding to conventional splicing substrates requires ATP, and the NRS complex is only observed in the absence of ATP. Perhaps U1 and U2 snRNP binding is to cryptic sites and reflects their abundance in extract rather than any functional role in inhibition. Because of the functionally significant U11 binding, an intriguing possibility is that the NRS complex represents a U11 snRNP-type E5′ complex. Another issue that needs to be addressed is whether the NRS complex is a single complex containing all the snRNPs, or if there are separate complexes containing only one snRNP that co-elute in gel filtration. The presence of multiple complexes would explain why the crippling RG11 mutation causes only a 50% diminution in NRS complex assembly. These issues cannot be addressed until the actual binding sites of U1 and U2 snRNPs within the NRS are mapped and mutated. It is hoped that more detailed analyses of the NRS complex will illuminate the roles of SR proteins and the various snRNPs in the assembly process, facilitate a more complete comparison with spliceosomal complexes, and sharpen our concept of how factors bound to the NRS elicit splicing inhibition.

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