doi:10.1016/S0022-2836(02)00542-9 available online at http://www.idealibrary.com on IDEAL® J. Mol. Biol. (2002) 321, 423–432

JMB

Shared Protein Components of SINE RNPs

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The heterogeneous, short RNAs produced from the high, copy, short mobile elements (SINEs) interact with proteins to form RNA–protein (RNP) complexes. In particular, the BC1 RNA, which is transcribed to high levels specifically in brain and testis from one locus of the ID SINE family, exists as a discrete RNP complex. We expressed a series of altered BC1, and other SINE-related RNAs, in several cell lines and tested for the mobility of the resulting RNP complexes in a native PAGE assay to determine which portions of these SINE RNAs contribute to protein binding. When different SINE RNAs were substituted for the BC1 ID sequence, the resulting RNPs exhibited the same mobility as BC1. This indicates that the protein(s) binding to the ID portion of BC1 is not sequence specific and may be more dependent upon the secondary structure of the RNA. It also suggests that all SINE RNAs may bind a similar set of cellular proteins. Deletion of the A-rich region of BC1 RNA has a marked effect on the mobility of the RNP. Rodent cell lines exhibit a slightly different mobility for this shifted complex when compared to human cell lines, reflecting evolutionary differences in one or more of the protein components. On the basis of mobility change observed in RNP complexes when the A-rich region is removed, we decided to examine poly(A) binding protein (PABP) as a candidate member of the RNP. An antibody against the C terminus of PABP is able to immunoprecipitate BC1 RNA, confirming PABP's presence in the BC1 RNP. Given the ubiquitous role of poly(A) regions in the retrotransposition process, these data suggest that PABP may contribute to the SINE retrotransposition process.

Keywords: PABP; RNA binding proteins; ribonucleoprotein complexes; short interspersed element; retroelements

Introduction

Short interspersed elements (SINEs) belong to a class of DNA repetitive mobile elements that undergo amplification through a retroposition process. SINEs are transcribed by RNA polymerase III (pol III), followed by the reverse transcription of the RNA, and subsequent integration of the cDNA into a new site in the genome.[1–3] SINEs are set apart from other retroelements in that they do not have any protein coding capacity, and yet they have amplified to extremely high copy numbers. Because of their lack of coding capacity, they are considered non-autonomous elements, which probably rely on activities from the L1, or other, retrotransposons. Copy numbers of SINEs range anywhere from tens of thousands of copies to over a million in the case of the human SINE, *Alu*. However, only a very limited number of the elements within a SINE family, termed master or source elements, appear to be capable of actively undergoing retroposition.[4–6] Although a variety of factors influence the retroposition process,[7] details of what makes some SINEs retropositionally active are still poorly understood. Because SINEs do not code for any proteins, it has been proposed that SINEs depend on the gene products from another group of mobile elements, termed the long interspersed elements (LINEs), for the retrotransposition process. It also seems likely that both SINEs and LINEs depend on endogenous cellular proteins for some aspects of the retrotransposition

Abbreviations used: RNP, RNA–protein complex; PABP, poly(A) binding protein; SINE, short interspersed element; Ab, antibody; pol III, polymerase III; LINE, long interspersed element; TB-RBP, testis-brain RNA binding protein.

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0022-2836/02/$ - see front matter

Figure 1. Schematic of transfection construct RNAs. These represent the RNAs produced from a series of chimaeric constructs. $p^{7SL}BC1^{BC1}$ produces authentic BC1 RNA with a 75 bp ID element region followed by an A-rich region (53 bp) and then a 23 bp unique region. $p^{7SL}BC1^{\delta U}$ makes the same RNA, but with a randomly varied unique region. $p^{7SL}B2^{BC1}$ has the ID element region replaced with the B2 element, and $p^{7SL}Ya5^{BC1}$ with the consensus Ya5 *Alu* element. $p^{7SL}BC1^{XAUNT}$ represents the BC1 transcript without the A-rich and unique region. $p^{7SL}B2t^{XAUNT}$ and $p^{7SL}Ya5t^{XAUNT}$ represent the shortened portions of B2 and Ya5 elements (the left half), respectively, also without the A-rich and unique regions. Sequences can be obtained from our website (http://129.81.225.52/). All the constructs containing the BC1 unique region were probed with the unique-1 oligo. All other constructs used different oligo probes complementary to their specific sequence as indicated in Materials and Methods.

process. For instance, most cellular RNAs, including SINEs[8,9] are associated with cellular proteins to form ribonucleoprotein complexes (RNPs). It is likely that the proteins in the SINE RNP influence the retrotransposition process.

The brain cytoplasmic 1 (BC1) RNA gene belongs to the rodent-specific family of SINEs, termed ID.[10] Like other SINEs, it is transcribed by RNA pol III.[11] BC1 is an unusual pol III transcript because it has both developmental and tissue-specific regulation being expressed only in brain and to a lesser extent in testis.[12–14] Although, there are 200–120,000 copies of ID elements in a given rodent genome, only one ID element locus, the BC1 RNA gene, contributes to the very high level of neuronal expression.[15] While other ID-containing loci make low levels of RNAs with heterogeneous 3′ ends,[15] the BC1 locus makes large quantities of a discrete RNA species. It is likely that the upstream sequences of the BC1 locus influence its extremely high level of transcription relative to the other ID copies.[14,16,17] The BC1 locus is the only SINE locus to date that has been demonstrated to serve as a "master" locus for SINE amplification.[15] This fact along with the other unique properties of the BC1 transcript prompted us to use it as a model system to study portions of SINE RNAs and their interactions with cellular proteins. While the upstream region of BC1 contributes significantly to regulation and tissue specificity, other data indicate that post-transcriptional regulation also plays a role.[17] One possible post-transcriptional variable may be the proteins that bind to BC1 RNA to form the endogenous RNP.[9,10] The RNP has been shown to form in all rodent tissues when BC1 is artificially expressed in transgenic animals. In addition, the RNP is able to form at the earliest developmental time points at which BC1 RNA is expressed.[17] These data indicate that BC1 RNP proteins should be abundant and ubiquitously expressed. There may be different proteins involved at different stages of BC1 RNA expression, with some involved in nuclear stability and transport, as well as others involved in the cytoplasmic complex. BC1 must be transiently present in the nucleus, but because most of the mass of BC1 RNA exists as a cytoplasmic RNP, our studies address only this final complex.

BC1 RNA consists of three sequence domains.[16] The first 75 nucleotides is the portion derived from $tRNA^{Ala}$ that gives rise to ID elements[18] and forms a fairly stable hairpin structure.[19] An A-rich region (53 bases in BC1) is also present in all SINE transcripts, but varies in length (from zero to greater than 100 bases) and in A content. This A-rich region is the one feature that is common to almost all of the non-LTR retrotransposons, and is thought to play a critical role in the priming of reverse transcription.[20] The unique portion of the 3′ end is different for each SINE transcript and is therefore the only portion of the transcript that varies greatly between loci. The 3′ end of BC1 RNA is a unique sequence which has been highly conserved at that locus throughout rodent evolution.[16]

The proteins that comprise the BC1 RNP are still unknown, although several candidates have been proposed.[21–23] Studies have characterized the nuclear complex,[22,24] or have used *in vitro* assays with co-purification correlations[23,25] to suggest candidate BC1 RNP components. No direct manipulation of BC1 RNA itself has been tested for impact on the cytoplasmic RNP formation. Here we have created constructs expressing transcripts where one or more of the three sequence domains have been removed or altered. We then characterized the cytoplasmic RNP complexes

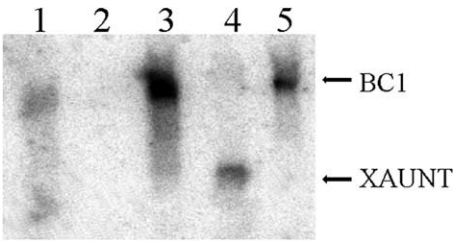

Figure 2. RNP mobility differences between $BC1^{BC1}$ and $BC1^{XAUNT}$. Northern blot analysis to detect complexed BC1 RNA was performed on a native mobility shift gel of cells expressing various SINE chimaeric RNAs. C6 glioma cells were transiently transfected with no DNA (lane 2), $p^{7SL}BC1^{BC1}$ (lane 3), $p^{7SL}BC1^{XAUNT}$ (lane 4) or $p^{7SL}B2^{BC1}$ (lane 5). Brain extract (lane 1) is shown to indicate the mobility of the endogenous BC1 complex. Arrows indicate the mobility of the endogenous BC1 complex and the $BC1^{XAUNT}$ RNP. The mobility of the free BC1 RNA would be located just off the bottom of this gel. A significant shift in the RNP mobility is seen when the A-rich and unique regions are deleted.

made with these various altered SINE RNAs to determine which sequence(s) is important for the formation of the endogenous BC1 RNP complex.

Results

Native mobility shift assay and construct design

Our approach to studying the RNPs formed with BC1 and other SINEs was to perform electrophoretic mobility shift analysis by neutral PAGE using crude cytoplasmic extracts to look at the endogenous RNPs. In order to study mutant or altered RNA structures, we used transfection experiments in cultured cells to introduce plasmids that express variant RNAs into the cells. Although in some situations the complexes may change after cell lysis, we believe that this assay is best for detecting authentic RNP complexes. Figure 1 illustrates the schematic for each of the RNAs expressed. Each of these RNAs was expressed from a transient transfection into various cell types and Northern blots of the RNAs produced in the cells were carried out to confirm that the dominant RNA species produced was of the appropriate size (data not shown). Nomenclature for the RNA molecules only includes the name of the element transcribed in the RNA with the 3′ portion of the transcript in superscript. The 5′ end of each RNA consists of sequences from three different repetitive elements, BC1, B2 or the consensus Ya5 *Alu*. Both B2 and Ya5 *Alu* are considerably longer than BC1. Therefore, constructs were also made that generate truncated B2 and Ya5 RNA transcripts (shortened to the length of BC1) and called B2t or Ya5t, respectively. The RNA structure was evaluated by the mfold program†. The truncated versions of BC1 and B2 RNAs are predicted to form smaller versions of the hairpin structure characteristic of the full length RNA. Ya5t RNA is predicted to form the same structure formed by the left half of an *Alu* RNA. Using the truncated versions will control for RNA length and structural changes that may result in mobility shifts in the RNP complex, leaving the RNA sequence as the main variable. The 3′ end of the BC1 RNA gene was manipulated in two ways. The BC1 wild-type A-rich and unique regions were either present (BC1) or deleted (XAU) or the unique region of BC1 was changed to an unrelated sequence (δU). All constructs contain the RNA pol III normal terminator (NT) sequence for BC1, including the five T residues used for termination and their immediate downstream sequence.

† http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html

RNP mobility differences between $BC1^{BC1}$ and $BC1^{XAUNT}$

The different RNP mobilities from transiently transfected cells were compared to the endogenous BC1 RNP complex present in the rodent brain by Northern blot analysis of a native gel shift. No significant difference was observed between the mobility of endogenous brain BC1 RNP (lane 1) and the RNP detected from C6 glioma cells transfected with the $p^{7SL}BC1^{BC1}$ (lane 3) or $p^{7SL}B2^{BC1}$ (lane 5) construct (Figure 2). However, the $BC1^{XAUNT}$ (lane 4) RNP is both faster in mobility and less diffuse (Figure 2). This experiment has been reproduced numerous times, including experiments where isolated RNA was included (as in Figure 4, below) to demonstrate that all of the complexes have significantly slower mobilities than the free RNA. In numerous other studies, we found that the mobility of complexes in the neutral mobility shift gels is dominated by the mobility of the proteins involved and relatively insensitive to the size of the nucleic acid binding to a given protein. Thus, we believe that the change in mobility of the complexes for the RNAs with and without the A-rich regions suggests the loss of protein(s) from the RNP complex when the A-rich and unique sequence of the BC1 3′ end is absent from the RNA. There are various reasons why we only focused on the mobilities, and not the obvious differences in expression between the various RNAs used in these studies. First, there are numerous factors, including differences in promoter strength, transport and RNA stability that might cause differences between different RNA-expressing constructs. Because of some heterogeneity in the bands present, these native PAGE blots are also not the best quantitative approach. Additionally, the different RNAs were detected using different hybridization probes, which may have varying efficiencies. Thus, our analysis

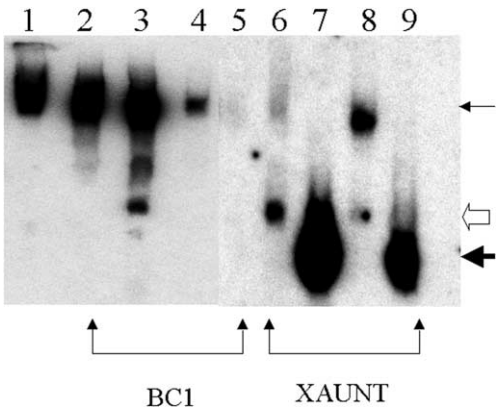

Figure 3. $BC1^{BC1}$ or $BC1^{XAUNT}$ RNP formation in rodent and human cells. Transfected cell lines were analyzed by the native gel shift mobility assay. Lane 1, brain extract. Different cell lines were transfected with $p^{7SL}BC1^{BC1}$ (lanes 2–5) or $p^{7SL}BC1^{XAUNT}$ (lanes 6–9) as follows: lanes 2 and 6, C6 cells; lanes 3 and 7, 293 cells; lanes 4 and 8, NIH3T3 cells; and lanes 5 and 9, HeLa cells. Lanes 1–4 were exposed to X-ray film for a total of six hours and lanes 5–9 for 24 hours. The mobility of endogenous BC1 (small arrow), rodent $BC1^{XAUNT}$ (open arrow) and human $BC1^{XAUNT}$ (large arrow) are indicated. Note that endogenous BC1 RNP can be seen in rodent cells (lanes 6 and 8) transfected with the $p^{7SL}BC1^{XAUNT}$ plasmid. The $BC1^{XAUNT}$ RNP faster mobility shift is seen in all cell types, but the human and rodent $BC1^{XAUNT}$ RNPs differ slightly in their mobilities.

focused solely on the information provided by the gel mobilities.

Species specificity of RNP formation

The C6 glioma cell line has some neuronal properties and is rodent in origin. Because BC1 is normally both neuronal and rodent-specific, we wanted to test for tissue or species-specific changes in the BC1 RNP using alternate cell lines. To test this possibility, we used rodent (NIH 3T3) and human (HeLa and HEK 293) cell lines of non-neural origin to evaluate the RNP complexes formed by the different RNAs. The authentic BC1 RNP in the rodent brain extract has the same mobility as all the extracts from $p^{7SL}BC1^{BC1}$ transfected cells (Figure 3). The $BC1^{XAUNT}$ RNP exhibits the same faster mobility in both rodent cell lines as previously observed (Figure 2). However, the $BC1^{XAUNT}$ RNP complex in rodent cells exhibits a slower mobility than the RNP observed in human cells with the same construct. This suggests a difference between the rodent and human forms of the protein(s) binding the double-strand (ID sequence) region of BC1.

RNP complexes formed with different SINE transcripts

Because the $BC1^{XAUNT}$ RNP exhibits different electrophoretic mobilities in different species, further investigation with other SINEs was carried

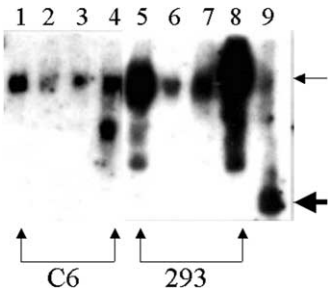

Figure 4. RNP formation in C6 or 293 cell lines transfected with full-length SINE constructs. Northern blot analysis was performed on a native mobility shift gel where $p^{7SL}BC1^{BC1}$, $p^{7SL}BC1^{\delta U}$, $p^{7SL}B2^{BC1}$, and $p^{7SL}Ya5^{BC1}$ were transfected into C6 (lanes 1–4) or 293 cells (lanes 5–8), respectively. Lane 9 shows the mobility of both the endogenous BC1 RNP complex and free BC1 RNA. We believe the weak transcripts seen at higher molecular masses probably represent spurious high molecular mass transcripts generated from the transfection plasmid. The mobility of endogenous BC1 (small arrow) and free BC1 RNA (large arrow) are indicated. Both cells types contain a similar RNP complex with all SINE constructs.

out in one cell line of each species. Different full-length SINE RNAs (B2 and *Alu* Ya5) containing the 3′ end of BC1 were expressed in C6 glioma and HEK 293 cells (Figure 4). No significant difference in mobility was seen between any full-length RNA complex and the endogenous brain BC1 RNP, even if there are significant differences between the lengths of $Ya5^{BC1}$ (367 nt; lanes 4 and 8) and $B2^{BC1}$ (255 nt; lanes 3 and 7) relative to $BC1^{BC1}$ (190 nt; lanes 1 and 5) RNA. $Ya5^{BC1}$ RNA shows some band heterogeneity (Figure 4, lanes 4 and 8). This may be due to the previously described interaction between the signal recognition particle (SRP) proteins 9 and 14 and the *Alu* sequence,[26] or RNA degradation intermediates as they can also be observed in the $BC1^{BC1}$ (lane 5). In addition, the full length $B2^{XAUNT}$, and $Ya5^{XAUNT}$ presented complexes with faster mobilities that were not significantly different from the complex observed for the $BC1^{XAUNT}$ (data not shown).

Replacing the unique sequence of BC1 with a random sequence also has no effect on the mobility of the complex (Figure 4, lanes 2 and 6). Because of the reasons described earlier, we do not feel that we can make any conclusions regarding the differences in expression level. Although we cannot rule out some heterogeneity in the complexes, these mobility data indicate that different SINE element RNAs are able to bind similar proteins to form a complex with the same mobility as the BC1 RNP.

Species specificity of $SINE^{XAUNT}$ RNPs

We wished to determine whether the apparent commonality between the RNP complexes of different SINEs was primarily due to their common A-rich 3′-end sequence, or whether the main body

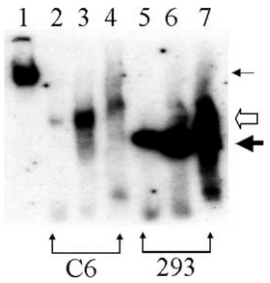

Figure 5. RNP formation with SINE RNAs lacking A-rich and unique regions in C6 and 293 cells. Northern blot analysis was performed on a native mobility shift gel of C6 (lanes 2–4) or 293 (lanes 5–7) transfected cell lines. Lane 1 is brain extract. The cells were transfected with the following constructs: lanes 2 and 5, $p^{7SL}BC1^{XAUNT}$; lanes 3 and 6, $p^{7SL}B2t^{XAUNT}$; and lanes 4 and 7, $p^{7SL}Ya5t^{XAUNT}$. The mobility of endogenous BC1 (small arrow), rodent $BC1^{XAUNT}$ (open arrow) and human $BC1^{XAUNT}$ (large arrow) are indicated. All constructs which lack an A-rich and unique region show a faster mobility than the endogenous BC1 complex. Within each cell type, all constructs exhibit similar mobilities, suggesting that similar proteins are able to bind each SINE sequence. Lanes 4 and 7 would be expected to show some difference because of the expected binding of SRP9/14 to the left end of *Alu*. Between the rodent and human cell line, there is a difference between $SINE^{XAUNT}$ RNP mobilities.

of the SINE RNA (which forms extensive secondary structure) also forms a similar complex. Therefore, we expressed SINE sequences of approximately equal length to BC1, with no A-rich or unique sequence at the 3′ end. No change in RNP mobility is seen between $BC1^{XAUNT}$ (lanes 2 or 5) and $B2t^{XAUNT}$ RNA (lanes 3 or 6) within a given cell line (Figure 5). The $Ya5t^{XAUNT}$ RNA (lanes 4 and 7) shows a slightly retarded and more heterogeneous mobility of the RNP complex when compared to the other constructs. This could be due to the larger size of the RNA or binding of SRP 9/14 causing a more heterogeneous complex. The same change in RNP mobility is shared between the different SINEs in the rodent and human cell lines as observed in Figure 4.

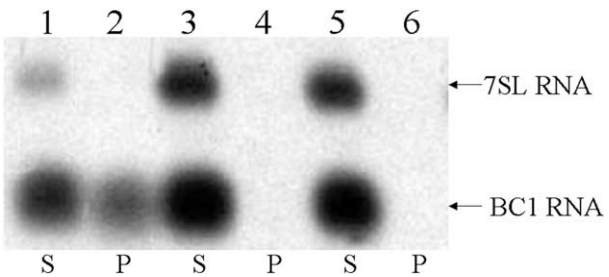

Figure 6. BC1 RNA immunoprecipitation by PABP C-terminal antibody. The immunoprecipitation pellets (P) and supernatants (S) from brain extracts of BC1 over-expressing transgenic mice were evaluated for BC1 and 7SL RNA presence by Northern blot analysis. Immunoprecipitations were performed with: lanes 1 and 2, anti-PABP; lanes 3 and 4, no antibody control; lanes 5 and 6, anti-Hsc70 control.

Poly(A) binding protein

The change in RNP mobility upon deletion of the A-rich region of BC1 suggests the possibility that one of the BC1-binding proteins might be the poly(A) binding protein (PABP). Therefore, we utilized antibodies to PABP to test this hypothesis. An immunoprecipitation experiment with a polyclonal antibody to the C-terminal portion of PABP was able to co-immunoprecipitate BC1 RNA (Figure 6). Controls either without any primary antibody or using anti-Hsc70 Ab did not immunoprecipitate any BC1 RNA. None of the antibodies precipitated 7SL RNA, a non-polyadenylated, pol III-transcribed, small RNA used as a control. Incomplete precipitation of BC1 RNA (detection of the RNA in both the pellet and supernatant) may reflect the presence of some BC1 RNA in a PABP-free RNP, partial protein dissociation due to sample manipulation, saturation of the amount of antibody used, or alternatively represent poor accessibility of the PABP epitopes in the BC1 RNP complex making the immunoprecipitation inefficient. However, this experiment demonstrates that a minimum of a third of the BC1 RNA in the cell is complexed with PABP.

Discussion

SINE elements are small retroposons present within all eukaryotes. All SINEs are ancestrally derived from either the 7SL RNA gene or tRNA genes. Primate and rodent SINEs are the most intensely studied and most abundant of the SINE elements. The function of SINEs, if any, is unproven but their effects are well documented.[27,28] SINEs affect the host genome in a number of ways. By replicating and re-inserting into the genome, the site in which they land can be dramatically altered and its properties changed. SINEs can have positive, neutral, or most often, deleterious effects upon insertion. In addition, SINEs can carry with them regulatory elements that alter expression of genes near their insertion site.[29] SINEs also serve as templates to encourage non-homologous recombination in the host genome.[27,30] Most SINE elements are thought to be unable to retropose and multiple factors have been suggested to influence the retroposition capability of the few active elements.[7] Because SINEs have no coding capacity, they must interact with endogenous cellular factors to achieve this retroposition. Among these factors, the proteins present in the SINE RNP complex are likely to play an important role in modulating this retrotransposition as well as any other possible functions that may be associated with SINE RNAs.[31,32]

Several proteins have been suggested as candidates to form specific complexes with BC1 RNA.

These include translin/testis-brain RNA binding protein (TB-RBP),[23,25] La/SS-B,[22] and Purα.[23,25] TB-RBP is the rodent homologue of human translin.[33] TB-RBP is an attractive candidate because, like BC1, it is localized in the testis and brain.[34] It has also been shown that translin may attach certain transported RNAs to microtubules.[24] Purα has also been shown to play a role in microtubule association of BC1[25] and is able to bind BC1 in the nucleus.[24] While TB-RBP is an excellent and interesting candidate, it is implicated only by correlative evidence. TB-RBP co-purifies with the BC1 RNA in a modest purification protocol, but there is no direct evidence that TB-RBP is a component of the cytoplasmic BC1 RNP. We have been unable to co-immunoprecipitate BC1 RNA or super shift the RNP using an anti-TB-RBP antibody (the kind gift from Dr N. Hecht). However, we cannot be sure that the antibody has appropriate access to the epitopes against which it was raised if the protein was complexed in the BC1 RNP. The ubiquity of the BC1 RNP complex in the $BC1^{BC1}$ transgenic mouse[17] is also inconsistent with the TB-RBP tissue expression pattern.[24]

La is a 50 kDa protein found in both the nucleus and cytoplasm of cells. It binds the 3′-terminal U stretch at the end of pol III transcribed RNAs.[35] La binding has several effects on pol III transcribed RNAs. It can increase the amount of transcript produced by increasing the recycling of the polymerase and template complex[25,36] and by removing the RNA from the DNA template through its helicase activity.[37] La is also able to interact with the 3′ and 5′ end of RNAs to block processing.[35,38] It is highly likely, given La's function in the cell that it does interact with BC1, although this interaction is likely to be transient and nuclear. We were unable to co-immunoprecipitate BC1 RNA with several anti-La antisera (a gift from Dr J. Keene). In contrast to these previous studies, we present here an alternate experimental method designed for the study of the endogenous SINE RNP complexes formed *in vivo*.

First, our studies confirm that the ID portion of the BC1 RNA does bind protein(s), but cannot determine the number or nature of those proteins. RNA-folding analysis of the sequences of the truncated SINEs (B2t and Ya5t) predicts their RNAs to fold into a hairpin structure. Finding RNP complexes of the same mobility does not definitely demonstrate that they have the same protein components. However, as the mobility of the complexes in native PAGE gels is dominated by the mobility of the protein components, common mobility is strongly suggestive of similar or identical components. Therefore, finding that B2t is able to assemble a complex with the same mobility as BC1 suggests that this particular complex involves proteins that recognize the RNA structure more than a specific sequence. The ID portion of BC1 exhibits a high level of secondary structure and both BC1 and B2t are derived ancestrally from tRNA sequences. Thus, the proteins may have affinity for double-stranded RNAs, or even be ubiquitous parts of the tRNA RNPs.

Our data on the 3′ truncated SINE RNAs demonstrate that there is protein binding to the A-rich region (Figures 2, 3 and 5) and that this protein component is a major contributor to the large mobility shift of the SINE RNPs in neutral PAGE. Co-immunoprecipitation with anti-PABP antibodies suggests that the major protein component binding to the A-rich region is PABP. Changing the unique sequence of the RNA did not alter the mobility of the RNP complex. Similar studies by the Brosius laboratory have confirmed the presence of PABP in several SINE RNPs (R. Muddashetty, T. Khanam, A. Kondrashov, M. Bundman, A Iacoangeli, J. Kremerskothen, K. Duning, A. Barnekow, A. Huttenhoffer, H. Tiedge & J. Brosius, personal communication). However, our studies are not at sufficient resolution that we can unambiguously rule out a specific protein binding to the unique region if it were small and did not alter the mobility of the complex sufficiently to be detected (Figure 4).

Although there may be small differences in the RNPs formed with different SINEs, our data suggest that most of the components of the SINE RNPs are both ubiquitous[17] and in common between different SINE complexes. It has been proposed that much of the selection for active elements may occur at the level of the RNA.[15,26,39] RNAs from different *Alu* subfamilies have been shown to have different affinities for the SRP9/14 protein.[26] Thus, small changes in SINE sequence may have subtle effects on the stability or nature of the SINE RNP complexes that are not measured in our assay. However, the A-rich and unique 3′ sequences are the most variable parts of SINE transcripts. The A-rich region can vary in length on different copies of the elements, from no A-tail to greater than 100 bp long A-tails. The typical A-tail is 10 to 40 bp long with an average of about 20 bp. In addition, the A-tails in the different elements vary with respect to the presence of other bases and simple-sequence repeats within this A-rich region. This could influence the number of molecules of PABP that bind to each RNA as well as providing the possibility of unique protein binding regions. PABP has been reported to stabilize RNAs when it binds internal A sequences,[40] which may offer selective advantage to elements with longer poly(A) regions. In addition, the A-tail is the only evident feature that SINE sequence shares with the LINE elements, making it a potential connection for the SINE to be able to share the LINE proteins for retroposition.[20] We have noted that the most recent *Alu* inserts generally have A-rich regions that are much longer than average (>40 bases)[30,41–44] and therefore it may be that binding of more PABP in a long A-rich region gives some advantage to amplification of a SINE. There are very few elements of the human genome with A-tails over 40 bp long, making it possible that A-tail length is a critical

factor in determining whether an element is capable of serving as a source or master element for retroposition.[4,6]

It has been proposed that there are three requirements for SINE retroposition,[25] germline transcription, availability of the transcribed RNA and access to reverse transcriptase. It has also been suggested that SINEs are able to amplify because they can effectively compete with L1 elements for the reverse transcriptase and other enzyme activities they produce for retrotransposition.[20] It is possible that a long A-rich region, capable of greater PABP binding, could help in concentrating the RNAs in the vicinity of the ribosome and may contribute to more successful competition for the L1 retrotransposition apparatus. This would be reasonable considering PABP's ability to bind proteins that are associated with translation, including PAIP1 (PABP-interacting protein-1),[45] eIF4G (eukaryotic initiation factor 4G)[34] and eRF3 (eukaryotic release factor).[46] However, we must also note that a long A-rich region by itself is not sufficient to allow high levels of retrotransposition, as mRNAs are only very inefficiently subjected to retroposition.[47,48] At this point we also cannot rule out the possibility that the unique region at the 3′ end of specific SINE RNAs may modulate this process, either through protein binding or some other mechanism, to allow selection of the "master" elements for amplification. It may also be significant that testis-specific PABPs exist[49,50] and total PABP is five to ten times higher in testis than other tissues.[51] Germ-line expression is a key feature of retroposable elements and the high level of BC1 transcript in testis may take advantage of the elevated PABP levels in this tissue or interact with tissue-specific isoforms differentially to increase retroposition efficiency. It is interesting to note that the testis-specific PABP was formed as a processed pseudo-gene through retroposition.[49,51]

Dendritically localized vasopressin mRNA was recently shown to retain a longer A-tail than axonally localized species.[52] This study also reports that dendritic vasopressin mRNAs co-localize with ribosomal machinery and that *in vitro* the RNA binds PABP. These same studies also map internal regions of the vasopressin mRNA responsible for the transport, but it is possible that BC1 RNA exhibits similar dendritic localization in part because it has a longer than average A-tail which also binds PABP effectively.

While definition of the SINE RNP structures is in its infancy, the observation of PABP as a component of an endogenous SINE RNP will be a valuable tool in elucidating other proteins that may also interact with these sequences. In light of the results outlined here, it seems likely that there are ubiquitous proteins that are perhaps secondary-structure-specific SINE RNA-binding proteins. These proteins are likely to have important influences on most of the properties of SINE RNAs, including transport and retrotransposition.

Materials and Methods

Construction of plasmids

The variants of the $p^{7SL}BC1^{BC1}$ construct[17] were derived by PCR amplification using primers that incorporated the specific nucleotide changes desired. Nomenclature of the vectors follows our previously described format[17] where the upstream region is superscripted, followed by the SINE body, and then the unique 3′ end of the transcript (including the terminator) is again in superscript. The final PCR product of the complete construct was cloned using the pGEM-Teasy vector system kit (Promega) following the manufacturer's instructions. Evaluation of both strands by sequencing confirmed the integrity of the constructs. The individual sequence can be obtained from the following accession numbers: 7SL-M20910 (positions 4–125); $BC1^{BC1}$-M16113 nt 1–152; $BC1^{XAUNT}$-M16113 nt 1–75 and 147–152; $BC1^{\delta U}$-M16113 (positions 1–152) with substitution at positions 127–146 to 5′-GACCTATGCTTGAATCGT-3′; $B2^{BC1}$-AC011194 (gil11024922: 17,988–18,180) (positions 17,988–18,163) plus BC1 sequence from positions 75–152 of M16113; $B2t^{XAUNT}$-AC011194 (gil11024922:17,988–18,180) (positions 17,988–18,065) plus BC1 sequence from positions 147–152 of M16113; $Ya5^{BC1}$-AC002347 (gil2828783: c55,820–55,520) (positions 55,823–55,938) plus BC1 sequence from positions 75–152 of M16113 (small mutation in 3′ end); $Ya5t^{XAUNT}$-AC002347 (gil2828783: c55,820–55,520) (positions 55,823–55,938) plus BC1 sequence from positions 147–152 of M16113.

The constructs $p^{7SL}BC1^{BC1}$ and $p^{7SL}B2^{BC1}$ have been described[17] and $p^{7SL}Ya5^{BC1}$ is described by Alemán *et al.*[53] The upstream primers are the same for all constructs and the 3′ end primers for unpublished constructs are as follows: $BC1^{XAUNT}$ (5′-TTGAAAATGAAAAAGGTCG-GAAGCTGAGGACC-3′); $Ya5t^{XAUNT}$ (5′-TTGAAAATGA-AAAAGGTAGTAGAGACGGGGTTTCA-3′); and $B2t^{XAUNT}$ (5′-TTGAAATGAAAAGGTGTGGTTGCTGGAATTTG-3′). $BC1^{\delta U}$ required two separate subsequent amplifications with two different 3′ end primers: primer 1 (5′-GGTACG-ATTCAAGCATAGGTCCATT-3′) and primer 2 (5′-GGT-CTTTGAAAATGAAAAAGGTACGAT-3′). Sequences of the constructs can be obtained from our website†.

Transient transfection in cell lines

Transient transfections were carried out in the rodent cell lines, C6 glioma (ATCC CCL107) and NIH3T3 (ATCC CRL1658), and the human cell lines, HeLa (ATCC CCL2) and HEK 293 (ATCC CRL1573). Monolayers were grown to 60–70% confluence in T75 flasks and transfected with 5 μg of the construct-containing plasmid using LipofectAminePlus (Gibco BRL) following the manufacturer's recommended protocol. Cytoplasmic extracts were isolated 16–20 hours post-transfection as described below.

Native mobility shift assay

Cytoplasmic extracts were obtained as described[8] with minor modifications as indicated below. Cells were scraped from the flask in the presence of chilled PBS. The collected suspension was then briefly centrifuged to pellet the cells. The cell pellet was resuspended in low-ionic strength buffer with the addition of NP-40 to aid

† http://129.81.225.52/

in cell lysis: 50 mM KCl, 15 mM $MgCl_2$, 10 mM Tris (pH 7.5), 0.32 M sucrose, 0.05% (v/v) NP-40, 0.2 mM PMSF, 1 mM DTT, 0.1% (w/v) aprotinin and protease inhibitor cocktail at 1 ml/20 g cell weight (SIGMA). Samples were centrifuged at 2000*g* for five minutes and the supernatant collected. Samples were fractionated in high-ionic, non-denaturing polyacrylamide gels.[54] The fractionated samples were transferred to zeta-probe blotting membrane (Bio-Rad) in Tris-glycine buffer (0.05 M Tris, 0.375 M glycine, 2 mM EDTA (pH 8.5)) for one hour at 350 mA. For RNA detection, UV-crosslinked membranes were hybridized with end-labeled oligonucleotides complementary to the 3′ unique portion of $BC1^{BC1}$ (5′-TGTGTGTGCCAGTTACCTTG-3′), $BC1^{\delta U}$ (5′-ACGATTCAAGCATAGGTCCA-3′), $BC1^{XAUNT}$ (5′-GGTCGGAGCTGAGGACC-3′), $Ya5t^{XAUNT}$ (5′-ACCGTTTTAGCCGGGATGGTC-3′), $B2t^{XAUNT}$ (5′-AACTCTGGACCTTCGGAAGAG-3′) or 7SL (5′-CCGATCGGCATAGCGCACTA-3′) in 5 × SSC, 5 × Denhardt's, 1% (w/v) SDS and 100 μg/ml herring sperm DNA. Oligonucleotides were end-labeled with [γ-^{32}P]ATP (Amersham) using T_4 polynucleotide kinase (NEB) and purified by filtration through a Sephadex G-50 column (Amersham). Blots were washed three times at 42°C with a low stringency buffer (2 × SSC and 1% SDS) and analyzed *via* autoradiography. (SSC is 0.15 M sodium chloride, 0.015 M trisodium citrate (pH 7.0).)

Immunoprecipitation

Brain samples were harvested and homogenized in immunoprecipitation buffer previously adapted[34] with added protease inhibitor cocktail (SIGMA). A buffer-to-tissue ratio of 1 ml: 2 g was used. Fifty microliters of rabbit polyclonal Ab raised to the C-terminal portion of PAPB per 500 μl of Sepharose G beads (Amersham Pharmacia Biotech) was added in tissue collection buffer plus 1 mM DTT. The bead/Ab mixture was incubated overnight at 4 °C with shaking. The 100 μl of brain extract was incubated with 300 μl Ab/bead suspension at 4 °C for four hours. Supernatant was collected and beads were washed four times in buffer with DTT. RNA was extracted from each sample using Trizol Reagent (Gibco BRL) and hybridizations were performed as described above.

Acknowledgments

A.M.R. was supported by a Brown Foundation fellowship from the Tulane Cancer Center. This research was supported by National Institutes of Health RO1 GM45668 and by the Department of the Army DAMD17-98-1-8119 (to P.L.D.). We thank Dr N. Hecht at the University of Pennsylvania Medical Center for generously supplying the anti-TB-RBP antibody and Dr J. Keene at Duke University Medical Center, Durham, North Carolina for kindly supplying the several anti-La antisera used in this study.

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Edited by J. Karn

(Received 13 March 2002; received in revised form 20 May 2002; accepted 23 May 2002)