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The *Schizosaccharomyces pombe* Proteins that Bind to the Human HnRNPA1 Winner RNA

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Although extensively characterized in human cells, no heterogeneous nuclear ribonucleoprotein (hnRNP) has been found in the fission yeast *Schizosaccharomyces pombe* which is amenable to genetic studies and more similar to mammals than *Saccharomyces cerevisiae* is in terms of RNA processing. As a first step to characterize hnRNPs from *S. pombe*, attempt was made to find human hnRNP A1 homologs from *S. pombe*. The RNA molecule (A1 winner) containing the consensus high-affinity hnRNP A1 binding site (UAGGGA/U) was synthesized *in vitro* and used in an ultravilolet (UV) light-induced protein-RNA cross-linking assay. A number of *S. pombe* proteins bound to the A1 winner RNA. An approximately 50-kDa protein (p50) cross-linked more efficiently to the A1 winner RNA than other proteins. The p50 protein did not cross-link to a nonspecific RNA, but rather to the A1-5'SS RNA in which the consensus 5' splice junction sites of S. pombe introns were abolished. This suggests that the p50 protein binds specifically to the consensus high affinity hnRNP A1 binding site. The p50 protein, however, did not bind to the single-stranded DNA to which the human hnRNP A1 could bind and be eluted with 0. 5 M NaCl. Further analysis should reveal more features of this RNA-binding protein.

Key words: hnRNP A1, RNA-binding protein, *Schizosaccharomyces pombe*, UV light-induced cross-linking

Gene expression can be regulated at the transcriptional and/or posttranscriptional levels. The control at the level of transcription is determined primarily by the interaction between cis-acting elements and trans-acting DNA-binding proteins (23). Likewise, RNA-binding proteins play important roles in posttranscriptional control (3). Proteins that are directly associated with RNA polymerase II transcripts include heterogeneous nuclear ribonucleoproteins (hnRNPs) and cytoplasmic mRNA-binding proteins (mRNPs) (1, 9). HnRNPs bind pre-mRNAs as nascent transcripts and are associated with them during the processing events required for the formation of mature mRNA (9). In human HeLa cells, hnRNP complexes contain more than 20 abundant proteins designated A1 (35 kDa) through U (120 kDa) (8, 17). HnRNPs have been suggested to be involved in several important steps in the biogenesis of mRNA, including pre-mRNA packaging, constitutive and alternative pre-mRNA splicing, and polyadenylation (4, 7, 9, 15). In addition, a subset of hnRNPs have beer found to shuttle between the nucleus and the cytoplasm, suggesting that these proteins may function both in cellular compartments and in nucleocytoplasmic mRNA transport (18).

To better understand how RNA-binding proteins influence the posttranscriptional regulation of gene expression, these proteins have been studied in *Drosophilar melanogaster* (14) and *Saccharomyces cerevisiae* (2, 11, 13, 20), organisms which are amenable to genetic studies. Although extensively characterized in human cells, relatively few hnRNPs have been characterized in *S. cerevisiae* (2, 13). Three major proteins are cross-linked by UV light to poly $(A)^+$ RNA in living *S. cerevisiae* cells (13). These are the 72-kDa poly $(A)^+$ binding protein and proteins of 60 and 50 kDa (13). Although the fission yeast *Schizosaccharomyces pombe* provides another excellent experimental tool for genetic study, no hnRNP in this organism has yet been characterized. *S. pombe* is also thought to be more similar to mammals than *S. cerevisiae* is in terms of RNA metabolism because at least one intron from the SV40 small T antigen transcript is accurately spliced in both mammalian cells and *S. pombe*. Furthermore, the U2 snRNA of *S. pombe* is of the same length and general structure as the vertebrate's (5, 12)

In this study, an attempt was made to search for hnRNPs from *S. pombe*. As a first step toward the attempt, the *S. pombe* homolog of the human hnRNP A1 was probed for using an UV light-induced cross-linking assay with A1 winner RNA to

which the hnRNP A1 binds with high affinity (6).

Materials and Methods

Oligonucleotides and *in vitro* transcription

The RNAs for the UV light-induced cross-linking experiments were produced from synthetic DNA oligonucleotide templates and contained an additional two guanosine residues at their 5' ends to increase the efficiency of the transcription (16) (Table 1). To make the synthetic templates, the following oligonucleotides were used:

T7TOP: 5'-TAATACGACTCACTATA-3'
A1WINNER: 5'-ATTATGCTGAGTGATATCCATACTATCCCTGAATCCCAC-3'
βGLO/INT: 5'-ATTATGCTGAGTGATATCCCTAGTGAACACAGTTGTGTC-3'
A1-5'SS: 5'-ATTATGCTGAGTGATATCCAGACTATCCCTGAATCCCAG-3'

The T7TOP oligonucleotide and one of the other three oligonucleotides were annealed by heating for 5 min at 65°C in 40 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 50 mM NaCl, and slowly to room temperature. 750 pmoles of the synthetic template were used for T7 run off transcription. The T7 transcrition reaction contained 40 mM Tris-HCl (pH 8.0), 1 mM spermidine, 9.5 mM $MgCl_2$, 5 mM DTT, 0.01% TritonX-100, 1 mM GTP, 0.2 mM ATP, 0.2 mM CTP, 20 μM UTP, 150 μCi of α-^{32}P-UTP, 0.3 μM synthetic template, and 100 units of T7 RNA polymerase (USB). The reaction was incubated for 2 hr at 37°C. The DNA template was destroyed by adding 50 units of RNase-free DNase (Boehringer Mannheim) and incubating at 37°C for 15 minutes. The labelled RNA was purified on a 18% polyacrylamide-8 M urea gel according to Sambrook *et al.* (21).

Single-stranded DNA affinity chromatography

S. pombe cell lysate (a kind gift from Qing Leu) was prepared using the D18 (972 *leu1.32 ura4*) strain. The D18 strain was grown at 30°C in YPD medium (1% yeast extract, 2% Bacto Peptone, 2% dextrose) to an optical density at 595 nm of 0.2 to 0.6, pelleted by centrifugation at 4,500×*g* for 10 min at 4°C, resuspended in ice-cold phosphate-buffered saline, and repelleted as described above. Each gram of the yeast cells was resuspended in 2 ml of lysis buffer (10 mM Tris-HCl [pH 7.4], 100 mM NaCl, 2.5 mM $MgCl_2$, 5% aprotinin, 2 μg of leupeptin per ml, 2 μg of pepstatin A per ml, 1 mM phenylmethylsulfonyl fluoride) and passed three times through a French press (2×10^4 lb/in^2). The lysate was immediately diluted with three volumes of the lysis buffer and pelleted for 10 min at 4,500×*g*. The supernatant (*S. pombe* cell lysate) was frozen in liquid nitrogen and stored at -80°C.

A previously described protocol (17) was applied for the ss DNA affinity chromatography. The S. *pombe* cell lysate was bound to ssDNA-cellulose (USB), the column was washed with 1 mg of heparin per ml (in 50 mM sodium phosphate [pH 7.4] and 100 mM NaCl) and bound proteins were eluted with 0.5 M NaCl (in 50 mM sodium phosphate [pH 7.4]). Proteins remaining bound to the column were eluted by denaturation with 4 M guanidine HCl. The elutes were frozen in liquid nitrogen and stored at -80°C until needed.

Ultraviolet light-induced cross-linking assay

For the cross-linking assay, approximately 1.5×10^5 cpm RNA was included in 10 μl of reaction mixture containing various amount of the protein extracts, incubated on ice for 5 minutes, and then irradiated for 8 minutes with a 15-W germicidal lamp as previously described (6). Irradiated samples were treated with a cocktail of RNAse A (10 μg/sample) and micrococcal nuclease (10 units/sample), and were subject to SDS-polyacrylamide gel electrophoresis. The gel was dried and exposed to a X-ray film.

Results

S. pombe proteins cross-linked by ultraviolet (UV) light to A1 winner RNA

In order to examine *S. pombe* proteins that associate with A1 winner RNA, oligonucleotides were designed to produce *in vitro* transcripts as shown in Table 1. A1 winner RNA contains two consensus high-affinity hnRNP A1 binding sites (UAGGGA/U) (6). This RNA is the highest-affinity sequence for the hnRNP A1, but has sequences similar to the consensus 5' splice junction sites of S. pombe introns at both ends (19). Therefore, A1-5'SS RNA

Table 1. The sequences of *in vitro* transcripts used in this experiment

Designation	Sequences[a]
	* *
Al winner	5'-GGUAUGAUAGGGACUUAGGGUG-3'
Al-5'SS	5'-GGUCUGAUAGGGACUUAGGGUC-3'
βglo/int	5'-GGGAUCACUUGUGUCAACACAG-3'

aFirst two G's at the 5'-end of the RNAs were added in order that T7 RNA polymerase could make *in vitro* transcripts efficiently. The consensus high-affinity hnRN PA1 binding site were underlined. The consensus 5' splice junction sites of *S. pombe* introns were lined above. The bases changed to destroy the consensus 5' splice sites of *S. pombe* introns were indicated with asterisks.

was included in the UV cross-linking experiment in order to distinguish true A1-winner-RNA binding proteins from the 5' splice-junction-site-binding proteins. It has been known that mRNA splicing does not occur when the G/GUA or G/GUG of the 5' splice junction sites of S. pombe introns are changed to G/GUC (10). Assuming that the sequence change from G/GUA or G/GUG to G/GUC abolishes mRNA splicing by inhibiting the interaction of 5' splice-junction-site binding proteins with RNA, both G/GUA and G/GUG of the A1 winner RNA were changed to G/GUC in the A1-5'SS RNA. As a control for nonspecific binding, β glo/int RNA was used. Its sequence is derived from the first intron of the human β globin gene (6) and it does not contain any sequence that resembles consensus hnRNP A1 binding site or the consensus splice sites of S. pombe introns (6). The hnRNP A1 shows the highest affinity (Kd=1×10^{-9} M) for A1 winner RNA, but an approximately 300-fold reduced affinity (Kd=3×10^{-7} M) for β glo/int RNA (6).

To examine if any *S. pombe* protein associates with A1 winner RNA, UV light-induced cross-linking experiments were carried out with different amounts of *S. pombe* cell lysate (Fig. 1). A number of proteins cross-linked much better to the A1 winner RNA than to the β glo/int RNA, indicating that these proteins associate with the A1 winner RNA with much higher affinity. In general, signals for cross-linked proteins weakened as the amount of cell lysate decreased. However, the signal for an approximately 50-kDa protein (p50) increased as the amount of the cell lysate decreased. This indicates that the p50 protein associates with the A1 winner RNA with higher affinity, as compared to other proteins.

As a control, HeLa cell nuclear extracts were also used for the UV light-induced cross-linking experiments (Fig. 2). The proteins cross-linked with HeLa cell nuclear extracts were detected as previously shown (6). Again, the signal for the p50 protein was prominent with the *S. pombe* cell lysate. Many proteins in HeLa cell nuclear extracts also associated well with the β glo/int RNA, although the profile of proteins cross-linked to the β glo/int RNA was different from those to the A1 winner RNA (6). Meanwhile, the cross-linking experiment with the *S. pombe* cell lysate showed that *S. pombe* proteins barely associate with the β glo/int RNA (Fig. 1). This suggests that *S. pombe* has quite a different hnRNP constitution than do HeLa cells. The cross-linked product of the hnRNP A1 in the HeLa cell nuclear extract migrates as an approkimately 35 kDa molecule (Fig. 2) (6). *S. pombe* cell lysate did not contain a

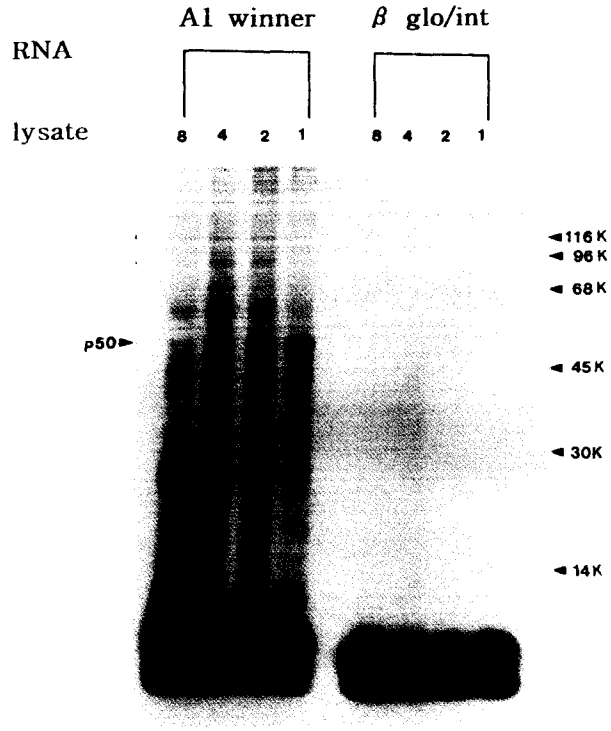

Fig. 1. *Schizosaccharomyces pombe* proteins cross-linked by ultraviolet (UV) light to the A1 winner RNA sequence. Approximately 1.5×10^{5} cpm of internally ^{32}P-labelled RNA sequences (β glo/int and A1 winner) were incubated with various amounts (8, 4, 2, 1 μl) of *S. pombe* cell lysate and cross-linked with UV light. Cross-linked products were then resolved by SDS-polyacrylamide gel electrophoresis and autoradiography. The amount of *S. pombe* cell lysate is indicated at the top of each lane and the migration of molecular weight standards (in kilodaltons) is also indicated. The p50 indicates an approximately 50-kDa protein that was cross-linked to the A1 winner RNA more efficiently than other proteins.

protein whose cross-linked product migrated at a similar molecular weight when the A1 winner RNA was used (Fig. 1).

To examine whether the p50 protein binds to the consensus 5' splice junction site (G/GUA) of the A1 winner RNA, the cross-linking experiment was performed with the A1-5'SS RNA in which the G/GUA sequence was changed to G/GUC (Fig. 3). The p50 protein still cross-linked to the A1-5'SS RNA, suggesting that the p50 protein is a true binder to A1 winner RNA.

The p50 protein does not bind to single-stranded DNA.

Since many hnRNPs bind to single-stranded (ss) DNA, ss DNA column chromatography has been used to purify them (17). Most hnRNP proteins remain bound to ss DNA column and elute from the

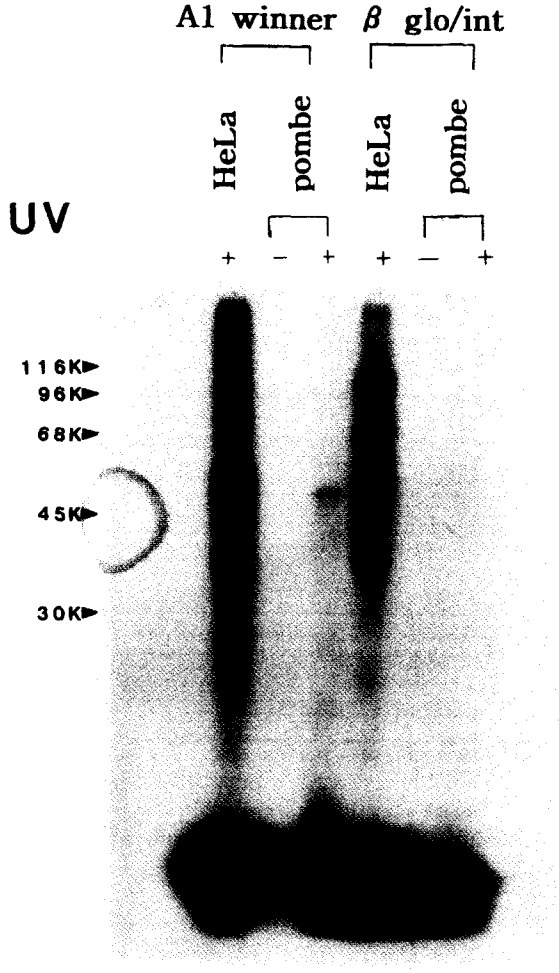

Fig. 2. Comparison of UV light-induced cross-linking experiments with HeLa cell nuclear extract (HeLa) and *S. pombe* cell lysate (pombe). The presence (+) and absence (-) of UV light treatment are indicated. One microliter of *S. pombe* cell lysate was used in each pombe lane. Due to the lighter exposure of autoradiogram, the approximately 50-kDa protein (p50 protein) indicated in Fig. 1 is the only one shown in the pombe lane. The UV cross-linking experiment with HeLa cell nuclear extract was done as described previously (6).

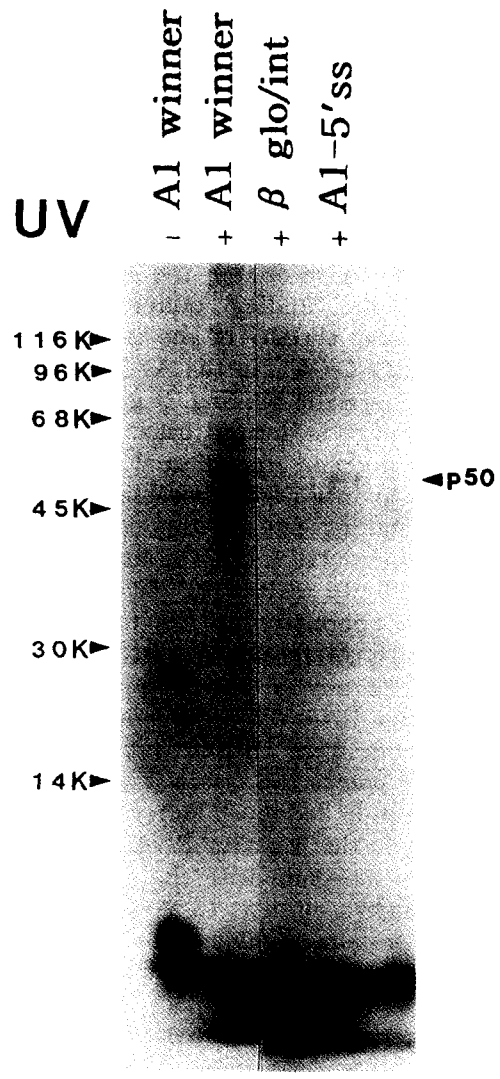

Fig. 3. The p50 protein cross-linked specifically to the A1 winner RNA. Three different RNAs (A1 winner, β glo/int, and A1-5'SS) were incubated with 1 μl of *S. pombe* cell lysate, respectively.

column at different salt concentrations (17). A heparin-wash step is also included in the ss DNA column chromatography, because some hnRNP proteins elute from the column in the presense of heparin. The column is finally eluted with 4 M guanidine HCl to remove any bound protein from the ss DNA. These elution steps provide us with criteria to classify the hnRNPs and a tool to purify them (17). The bulk of human hnRNP A1 protein remains bound to the ss DNA column after the heparin wash and elutes from the column at 0.5 M NaCl (17). This characteristic of the hnRNP A1 protein was used to examine if the p50 protein was the *S. pombe* homolog of the hnRNP A1 protein. The *S. pombe* cell lysate was applied to the ss DNA column. The column was first washed with heparin and subsequently eluted with 0.5 M NaCl and 4 M guanidine HCl. Each elute underwent the cross-linking experiment with the A1 winner RNA (Fig. 4). With the flow-through fraction, the p50 and an approximately 90-kDa protein (p90) cross-linked to the A1 winner RNA. With the heparin-wash fraction, only the p90 protein was detected. With the 0.5 M NaCl elute fraction, no protein was detected. With the 4 M-guanidine-HCl elute fraction, an approximately 60-kDa, major protein cross-linked to the A1 winner RNA. These results indicate that the p50 protein does not show a similar ss DNA-binding profile to that of the human

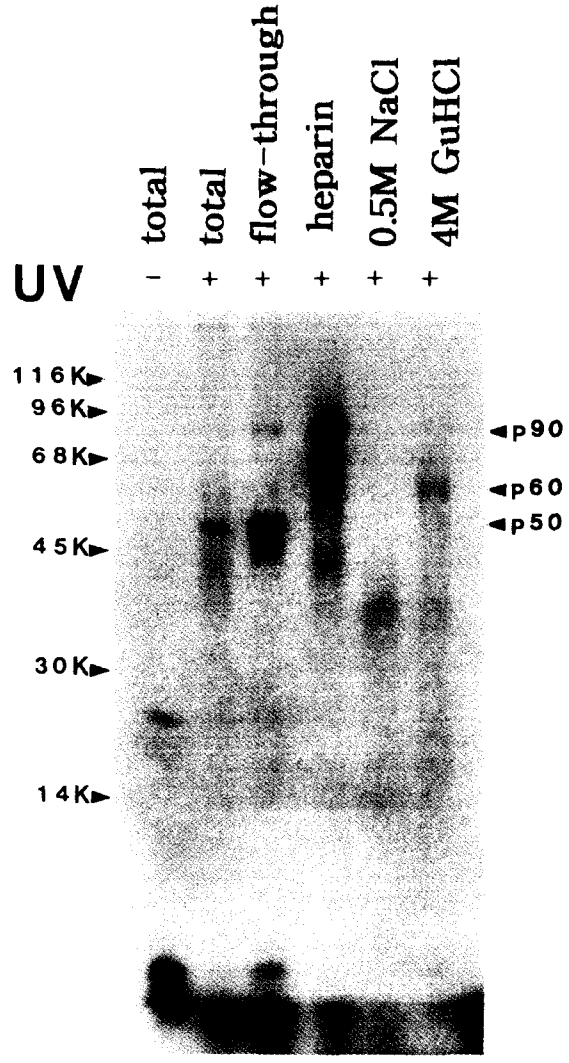

Fig. 4. UV light-induced cross-linking experiments with candidate RNA binding proteins separated by single-stranded (ss) DNA chromatography. *S. pombe* cell lysate (total) was bound to ssDNA-cellulose at 0.1 M NaCl. The column was washed with 1 mg of heparin per ml (heparin), eluted with 0.5 M NaCl (0.5 M NaCl), and then with 4 M guanidine HCl (4M GuHCl). One microliter of each fraction protein was incubated with the A1 winner RNA.

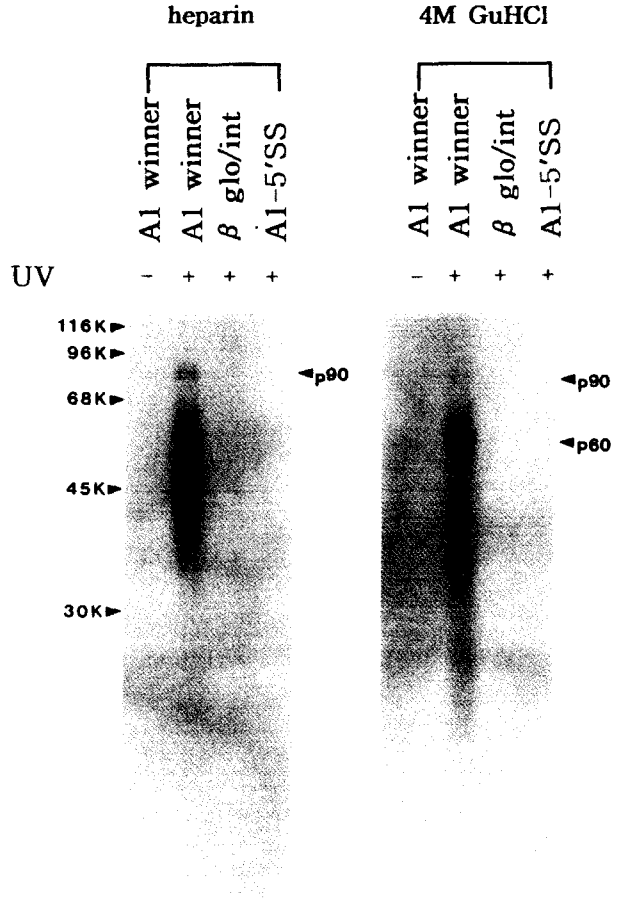

Fig. 5. The cross-linked products of the heparin-eluted proteins and the 4 M-guanidine-HCl-eluted proteins bind specifically to the consensus 5' splice junction sites of *S. pombe* introns. Three different RNAs (A1 winner, β glo/int, and A1-5'SS) were incubated with 1 μl of either the heparin-eluted proteins (heparin) or the 4 M-guanidine-HCl-eluted proteins (4 M GuHCl).

hnRNP A1 protein. Meanwhile, the p60 protein is a strong ss DNA binder, as it was removed only by the 4 M guanidine-HCl wash.

Most of the p90 protein was removed from the ss DNA column with the heparin wash, but some still remained bound to the column and was removed with 4 M guanidine HCl (Fig. 5). These characteristics of the p90 protein are similar to that of the human hnRNP U protein (17). The p60 and p90 proteins could be candidates for the *S. pombe* homologs of the hnRNP A1 protein in two respects. First, these proteins both cross-linked to the A1 winner RNA. Second, they both baund to ss DNA, although they showed different characteristics from those of the human hnRNP A1 protein. Therefore, it was examined if these proteins cross-link specifically to A1 winner RNA (Fig. 5). Both proteins did not cross-linked to the A1-5'SS RNA, indicating that they bound the putative 5' splice junction sites of *S. pombe* introns. These results told us that both the p60 and p90 proteins are not *S. pombe* homologs of the human hnRNP A1 protein, but rather might be splicing factors bound to the 5' splice junction sites of *S. pombe* introns.

Discussion

Human hnRNP A1 binds to the A1 winner RNA in HeLa cell nuclear extract much better than to the unrelated β glo/int RNA (6). Several other proteins

cross-link to A1 winner RNA and most of them cross-link to nearly all RNA tested, suggesting that these interactions are relatively nonspecific (6). These results indicate the possibility that A1 winner RNA could be used as a reagent to find hnRNP A1 homologs from other organisms similarly to that antibodies are being used to screen for homologous proteins from different organisms. In this study, a number of *S. pombe* proteins in *S. pombe* cell lysate were found to cross-link to A1 winner RNA much better than to the unrelated β glo/int RNA. The S. pombe cell lysate did not contain any protein which cross-linked to the β glo/int RNA to the extent seen in the HeLa cell nuclear extract. Among the proteins that cross-linked to the A1 winner RNA, an approximately 50-kDa protein (p50 protein) cross-linked more efficiently to the A1 winner RNA as the amount of the *S. pombe* cell lysate decreases, indicating that the p50 protein associated with a the A1 winner RNA with higher affinity as compared to other proteins. Therefore, the p50 protein could be a good candidate for being the S. pombe homolog of the human hnRNP A1, although the cross-linked product of the hnRNP A1 migrates as an 35-kDa molecule (6). The *D. melanogaster* homolog (14) of the human hnRNP A1 has a Somewhat higher molecular weight than the human hnRNP A1's and so the human hnRNP A1 homologs from lower eukaryotes such as *S. pombe* may also have different molecular weights.

To further examine if the p50 protein is the S. pombe homolog of human hnRNP A1, single stranded DNA column chromatography was performed to fractionate the *S. pombe* cell lysate. Each fraction was then tested to see which fraction contains the p 50 protein cross-linked to the A1 winner RNA. The human hnRNP A1 binds to the ss DNA column and can be eluted by 0.5 M NaCl (17). p50 protein did not bind to the ss DNA. Therefore, the p50 protein is not the *S. pombe* homolog of human hnRNP A1 in terms of ss DNA-binding ability. Cloning the gene for the p50 protein would tell us if the p50 protein is homologous with the human hnRNP A1 despite differences in its ss DNA-binding ability. A genomic clone which show sequence similarity to metazoan hnRNP proteins has recently been identified from *S. pombe* (22). This clone contains an open reading frame for a protein of about 40 kDa. It would be interesting to see if this protein shows the functional characteristics of the hnRNP proteins as well as binds to human hnRNPA1 winner RNA.

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