RNA (2000), *6*:861–879. Cambridge University Press. Printed in the USA.

Box C/D snoRNA-associated proteins: Two pairs of evolutionarily ancient proteins and possible links to replication and transcription

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ABSTRACT

The eukaryotic nucleolus contains a diverse population of small nucleolar RNAs (snoRNAs) essential for ribosome biogenesis. The box C/D snoRNA family possesses conserved nucleotide boxes C and D that are multifunctional elements required for snoRNA processing, snoRNA transport to the nucleolus, and 2′-*O*-methylation of ribosomal RNA. We have previously demonstrated that the assembly of an snoRNP complex is essential for processing the intronic box C/D snoRNAs and that specific nuclear proteins associate with the box C/D core motif in vitro. Using a box C/D motif derived from mouse U14 snoRNA, we have now affinity purified and defined four mouse proteins that associate with this minimal RNA substrate. These four proteins consist of two protein pairs: members of each pair are highly related in sequence. One protein pair corresponds to the essential yeast nucleolar proteins Nop56p and Nop58p. Affinity purification of mouse Nop58 confirms observations made in yeast that Nop58 is a core protein of the box C/D snoRNP complex. Isolation of Nop56 using this RNA motif defines an additional snoRNP core protein. The second pair of mouse proteins, designated p50 and p55, are also highly conserved among eukaryotes. Antibody probing of nuclear fractions revealed a predominance of p55 and p50 in the nucleoplasm, suggesting a possible role for the p50/p55 pair in snoRNA production and/or nucleolar transport. The reported interaction of p55 with TATA-binding protein (TBP) and replication A protein as well as the DNA helicase activity of p55 and p50 may suggest the coordination of snoRNA processing and snoRNP assembly with replication and/or transcriptional events in the nucleus. Homologs for both snoRNA-associated protein pairs occur in Archaea, strengthening the hypothesis that the box C/D RNA elements and their interacting proteins are of ancient evolutionary origin.

Keywords: intronic snoRNA; snoRNA processing; snoRNP proteins

INTRODUCTION

The nucleolus is the site of eukaryotic ribosome biogenesis where preribosomal RNA is transcribed, processed, and ultimately assembled into ribosomal subunits. A multitude of small nucleolar RNAs (snoRNAs) interact with the nascent pre-rRNA and facilitate the folding, nucleotide modification, and cleavage of this RNA precursor (Maxwell & Fournier, 1995; Smith & Steitz, 1997; Bachellerie & Cavaille, 1998). A few snoRNAs have been shown to participate in pre-rRNA cleavage reactions that process and trim 18S,5.8S, and 28S rRNAs (Li et al., 1990; Savino & Gerbi, 1990; Morrissey & Tollervey, 1993; Peculis & Steitz, 1993; Tycowski et al., 1994; Enright et al., 1996). However, the primary function of most snoRNAs is to serve as guide RNAs for base-specific nucleotide modification of rRNA (Tollervey, 1996; Lafontaine & Tollervey, 1998). The box C/D snoRNAs direct the 2′-*O*-methylation of pre-rRNA ribose sugars by base pairing with the pre-rRNA at selected sites of nucleotide modification (Bachellerie et al., 1995; Cavaille et al., 1996; Kiss-Laszlo et al., 1996). The second major family, the H/ACA snoRNAs, guides the conversion of selected uridine residues to pseudouridine, again via snoRNA base pairing with the rRNA precursor (Ganot et al., 1997; Ni et al., 1997).

The box C/D snoRNAs are characterized by conserved boxes C and D that are essential for snoRNA biogenesis and function. The terminal box C and D consensus sequences are multifunctional elements, found in a folded RNA motif defined as the box C/D core motif (Watkins et al., 1996, 1998). Boxes C and/

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or D have been shown to be important for processing intronic snoRNAs from the host pre-mRNA intron (Caffarelli et al., 1996; Cavaille & Bachellerie, 1996; Watkins et al., 1996; Xia et al., 1997; Kiss-Laszlo et al., 1998), for snoRNA transport into the nucleolus (Lange et al., 1998; Samarsky et al., 1998; Narayanan et al., 1999), for 2′-*O*-methylation of rRNA nucleotides (Cavaille et al., 1996; Kiss-Laszlo et al., 1996), and for trimethyl cap formation of nonintronic snoRNAs (Terns & Dahlberg, 1994). The box C/D snoRNAs occur in the nucleolus as small, stable ribonucleoprotein (snoRNP) complexes. Members of the box C/D snoRNA family are associated with the conserved nucleolar protein fibrillarin (Schimmang et al., 1989; Tyc & Steitz, 1989; Watkins et al. 1996). Recently, two additional yeast proteins designated Nop56p and Nop58p have been shown to associate with fibrillarin, suggesting that they are core proteins of the box C/D snoRNP complex (Gautier et al., 1997; Wu et al., 1998). The ability to immunoprecipitate the box C/D snoRNAs using antibody to Nop58 demonstrates that this nucleolar protein is indeed a core protein of the box C/D snoRNP complex (Lafontaine & Tollervey, 1999; Lyman et al., 1999).

We have been investigating the proteins associated with the U14 box C/D core motif and recently reported the biochemical fractionation of an in vitro-assembled mouse U14 snoRNP complex (Watkins et al., 1998). Three proteins of apparent molecular weights of 50, 55, and 65 kDa were shown to bind the U14 box C/D core motif, and their binding was dependent upon boxes C and D. In this work, we report the affinity chromatographic isolation of these same proteins and the identification, through peptide sequence analysis and gene cloning, of four mouse U14 snoRNA-associated proteins. Two of the mouse proteins are homologs of the essential yeast nucleolar proteins Nop56p and Nop58p (Gautier et al., 1997; Lafontaine & Tollervey, 1999). Two additional proteins designated p50 and p55 constitute a second pair of nucleoplasmic proteins. The reported DNA helicase activity of the nucleoplasmic p55/p50 protein pair as well as p55's interaction with TATA-binding protein (TBP) and repA may suggest the coordination of snoRNP biogenesis with replication and/or transcriptional events. Homologs for both protein pairs in Archaea indicate that the box C/D motif and associated proteins are of ancient origin.

RESULTS

Affinity chromatographic isolation of the U14 box C/D snoRNP proteins

Biochemical fractionation of an in vitro-assembled mouse U14 snoRNP complex has previously identified three nuclear proteins of 65, 55, and 50 kDa associated with the U14 box C/D core motif (Watkins

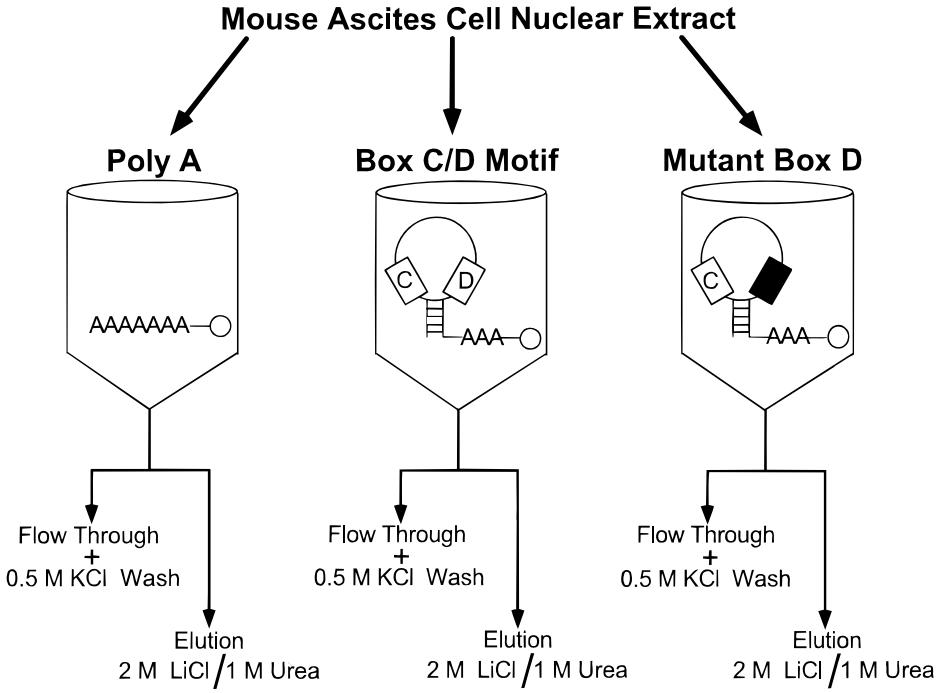

FIGURE 1. Affinity chromatographic isolation of box C/D snoRNA-associated proteins. Schematic presentation of the affinity chromatographic isolation procedure for the preparation of mouse box C/D snoRNA-associated proteins. In vitro transcribed RNAs possessing or lacking a box C/D snoRNA core motif were covalently attached to CNBr-activated Sepharose beads via a 3′ polyadenosine tail. A mouse Taper ascites cell nuclear extract (Watkins et al., 1998) was then passed over the affinity columns and, after a high salt wash, bound proteins eluted in buffer containing 2 M LiCl and 1 M urea.

et al., 1998). To further assess the binding capabilities of these proteins, affinity columns were prepared by covalently attaching various snoRNAs to CNBr-activated Sepharose 4B via a 3′-terminal poly(A) tail. RNAs attached to the Sepharose matrix included not only full-length and truncated versions of the box C/D snoRNAs U14 and U15, but also nonbox C/D RNAs such as the snoRNAs U17 and MRP and the homopolymer poly(A) RNA. Taper ascites cell nuclear extract was then passed over each affinity column and, after extensive washing with high salt buffer, bound proteins were eluted from the individual columns (Fig. 1).

SDS-polyacrylamide gel electrophoresis of proteins remaining bound to the various columns after high salt washes revealed that those column-bound RNAs that possessed an intact box C/D core motif retained the three previously identified snoRNP proteins (Fig. 2). The 65, 55, and 50 kDa snoRNP proteins were retained by the U14 and truncated U14 affinity columns as well as by the full-length and truncated U15 affinity columns. These same three proteins were also retained by full-length and truncated U3 affinity columns, although at lower efficiency (data not shown). When box D in any of the full-length or truncated box C/D snoRNAs was mutated, these three proteins did not associate with the snoRNA. They were also absent from the eluate fractions of the nonbox C/D snoRNA columns (U17 and MRP) and the poly(A) RNA column. These results indicated that the 65, 55, and 50 kDa snoRNP proteins require an intact box C/D element for binding and are common to all box C/D snoRNAs.

To isolate these snoRNP proteins in sufficient quantity for peptide sequencing and protein identification, a U14 box C/D core motif (ΔAV RNA) large-scale affinity column was prepared and used for protein isolation. The three snoRNP proteins were excised from preparative polyacrylamide gels and selected peptides generated by trypsin digestion were sequenced. At least two peptide sequences were obtained for each of the three isolated proteins. The NCBI GenBank protein database was then searched to identify these proteins. From this sequence analysis, two pairs of highly related proteins that are well conserved in eukaryotes were identified.

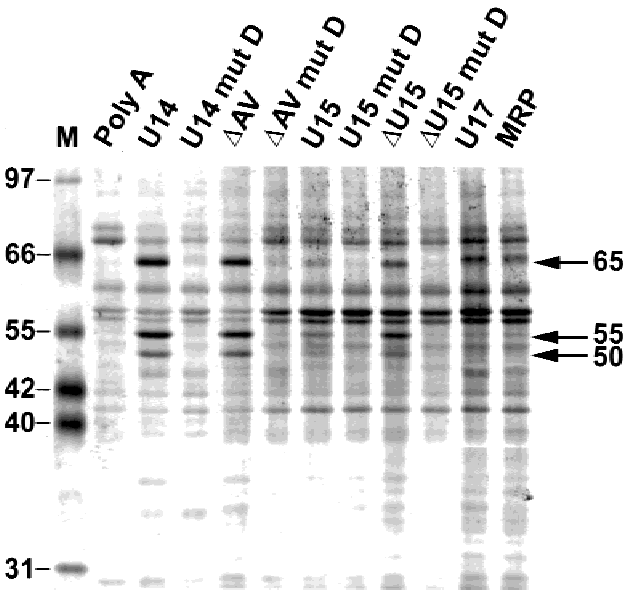

FIGURE 2. Nuclear proteins of 65, 55, and 50 kDa are associated with the box C/D snoRNA core motif. Proteins of the mouse Taper ascites cell nuclear extract retained on individual snoRNA affinity columns were analyzed by SDS-polyacrylamide gel electrophoresis. Specific RNAs covalently attached to the individual affinity columns are indicated at the top of each lane of the SDS-polyacrylamide gel. Those lanes designated "Δ" are snoRNAs that have been truncated, such that little more than the box C/D core motif remains in the snoRNA. Those lanes designated "mut D" are snoRNAs in which box D has been replaced with the sequence CCCC. Lane M: protein molecular weight markers. Poly A: polyadenosine (negative control). U14: full-length mouse U14 snoRNA. U14 mut D: full-length U14 snoRNA with mutated box D. ΔAV: mouse U14 snoRNA with regions A and V deleted. ΔAV mut D: mouse U14 ΔAV snoRNA with mutated box D. U15: full-length human U15 snoRNA (a box C/D snoRNA). U15 mut D: full-length human U15 snoRNA with mutated box D. ΔU15: human U15 snoRNA with an internal 110 nt removed, but still retaining the box C/D motif. ΔU15 mut D: internally deleted human U15 snoRNA with mutated box D. U17: human U17 snoRNA (an H/ACA snoRNA). MRP: human MRP RNA (a nonbox C/D, nonH/ACA snoRNA). Arrows at the side indicate molecular weights of the three proteins specifically retained by RNAs possessing a box C/D core motif.

Mouse Nop58p and mouse Nop56p are a pair of evolutionarily conserved nucleolar proteins

Three sequenced peptides of the 65-kDa snoRNP protein identified a pair of highly homologous but distinct nucleolar proteins (Fig. 3) previously identified in yeast and designated Nop58p (alternatively Nop5p) and Nop56p (Gautier et al., 1997; Wu et al., 1998). A partial sequence of mouse Nop58p was present in the data base, having been designated "Sik-similar protein" because of its relatedness to the "Suppressor of 1 kappa B" protein (SIK1p). However, complete sequencing of our mouse Nop58 clone revealed that this reported sequence lacked 63 N-terminal amino acid residues. The complete length of the mouse Nop58 protein is therefore 536 amino acids with a calculated molecular weight of 60.35 kDa (Fig. 3A). Yeast Nop58p is one of several nucleolar proteins that exhibits a characteristic C-terminal KKE/D repeat motif. Although mouse Nop58p lacks this triplet-repeat motif, the C-terminus of the mouse protein is also highly positively charged with an abundance of lysine amino acid residues. Nop58p is highly conserved in eukaryotes, exhibiting 47% identity and 71% similarity between mouse and yeast. Searches of the NCBI database using the BLASTP program identified additional homologs for the Nop58 protein that are also presented in Figure 3A.

Mouse Nop56p is highly related to mouse Nop58 (36% identical and 58% similar) with a total length of

A. Nop58

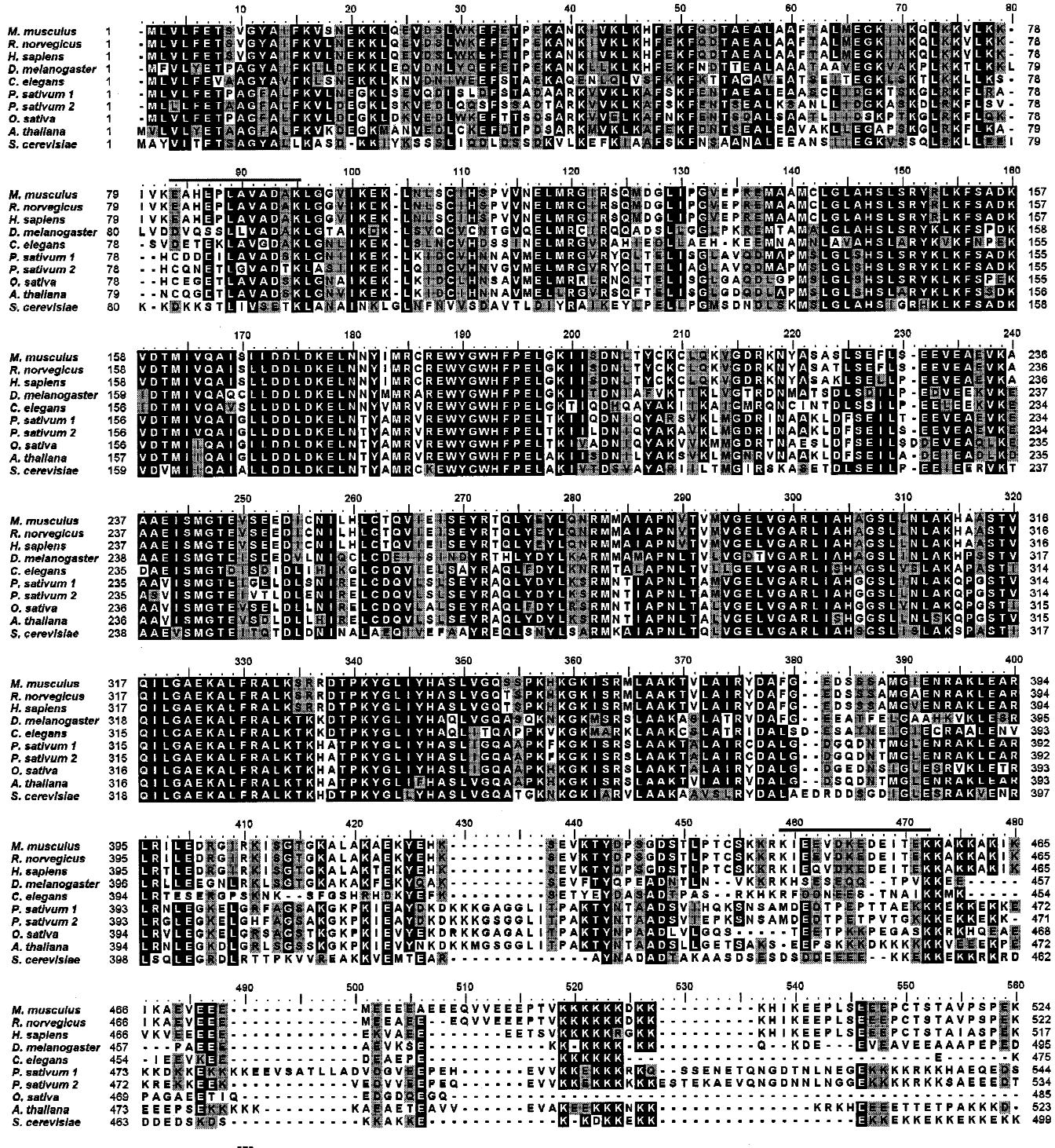

FIGURE 3. (*Figure continues on facing page.*)

B. Nop56

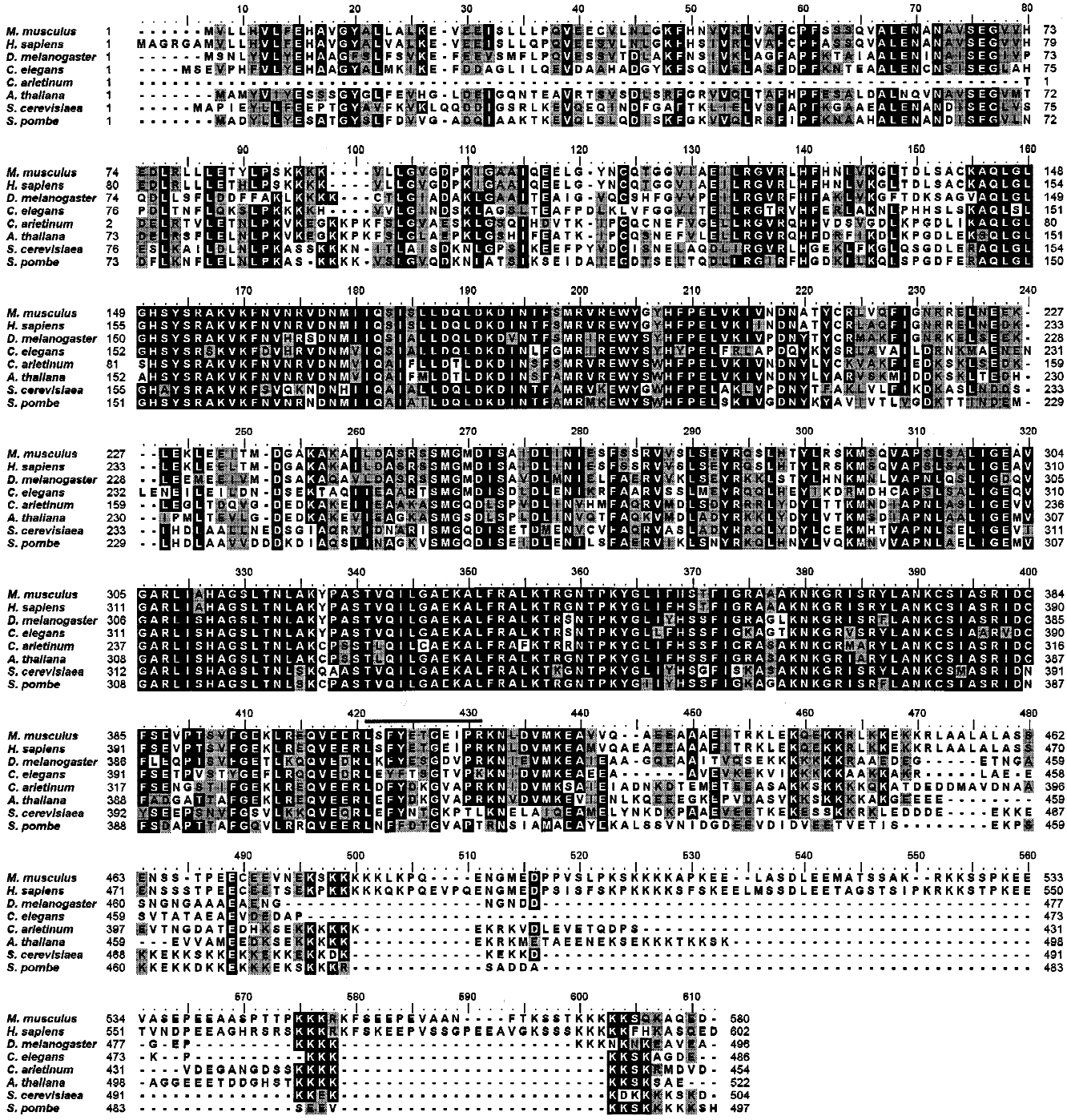

FIGURE 3. Alignment and sequence comparison of mouse Nop58 and Nop56 proteins with their eukaryotic homologs. The determined mouse (*Mus musculus*) Nop58 (**A**) and Nop56 (**B**) sequences are aligned and compared with corresponding homologs from other eukaryotes. Amino acid sequences determined from peptide sequencing of the isolated p65 protein are overlined. Identical amino acid residues are indicated by white letters on black blocks, whereas conserved residues are designated by gray shading. Dashes indicate spaces inserted to aid in the amino acid sequence alignments.

580 amino acids and a calculated molecular weight of 64.5 kDa (Fig. 3B). Like mouse Nop58, it also possesses a lysine-rich C-terminus. Although mouse Nop56 has a higher molecular weight than mouse Nop58, they comigrate on SDS-polyacrylamide gels and appear as a single band despite a 4-kDa difference in molecular weight. Anomalous migration of yeast Nop56p and yeast Nop58p on SDS-polyacrylamide gels has also been

A. Nop56/Nop58

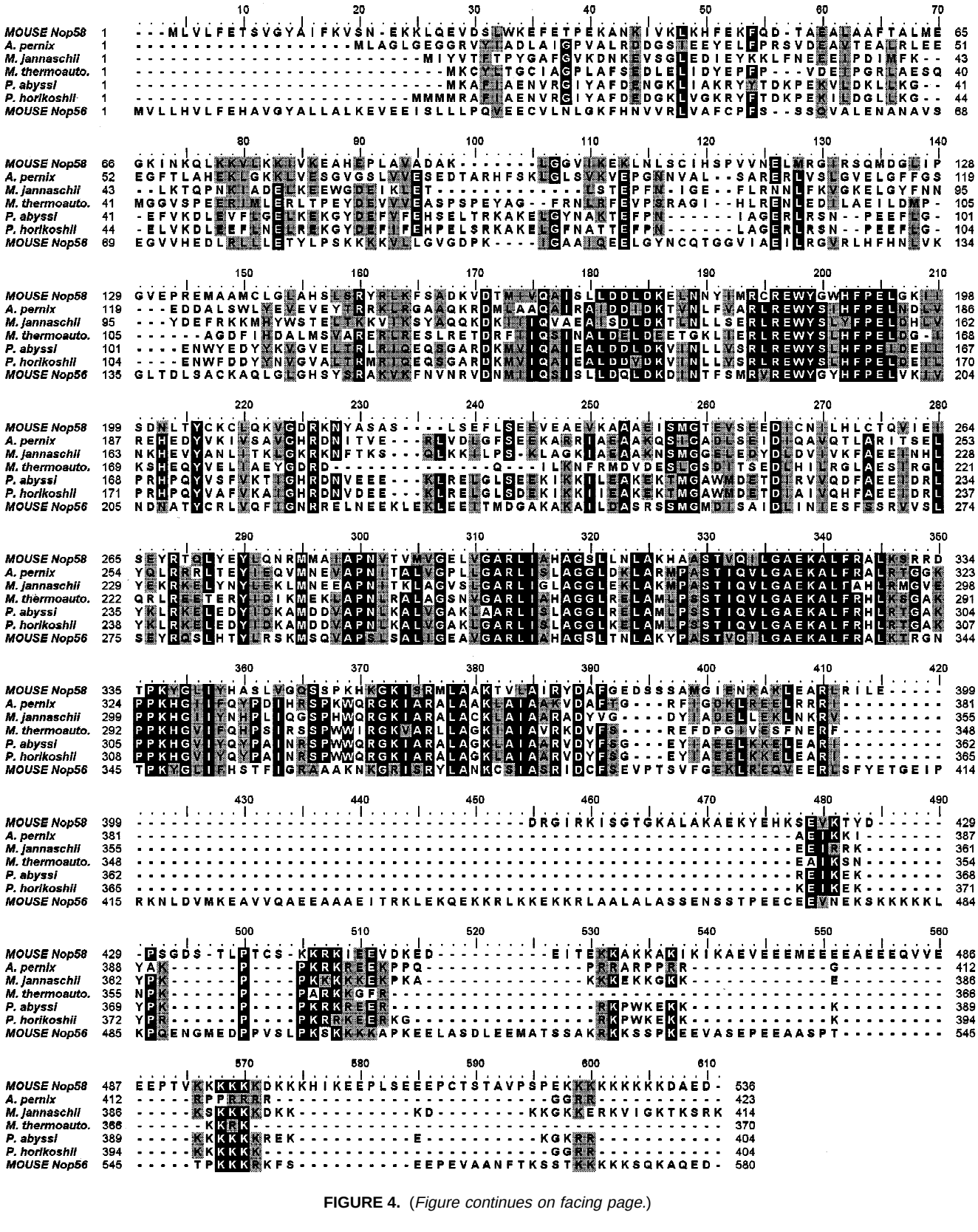

FIGURE 4. (*Figure continues on facing page.*)

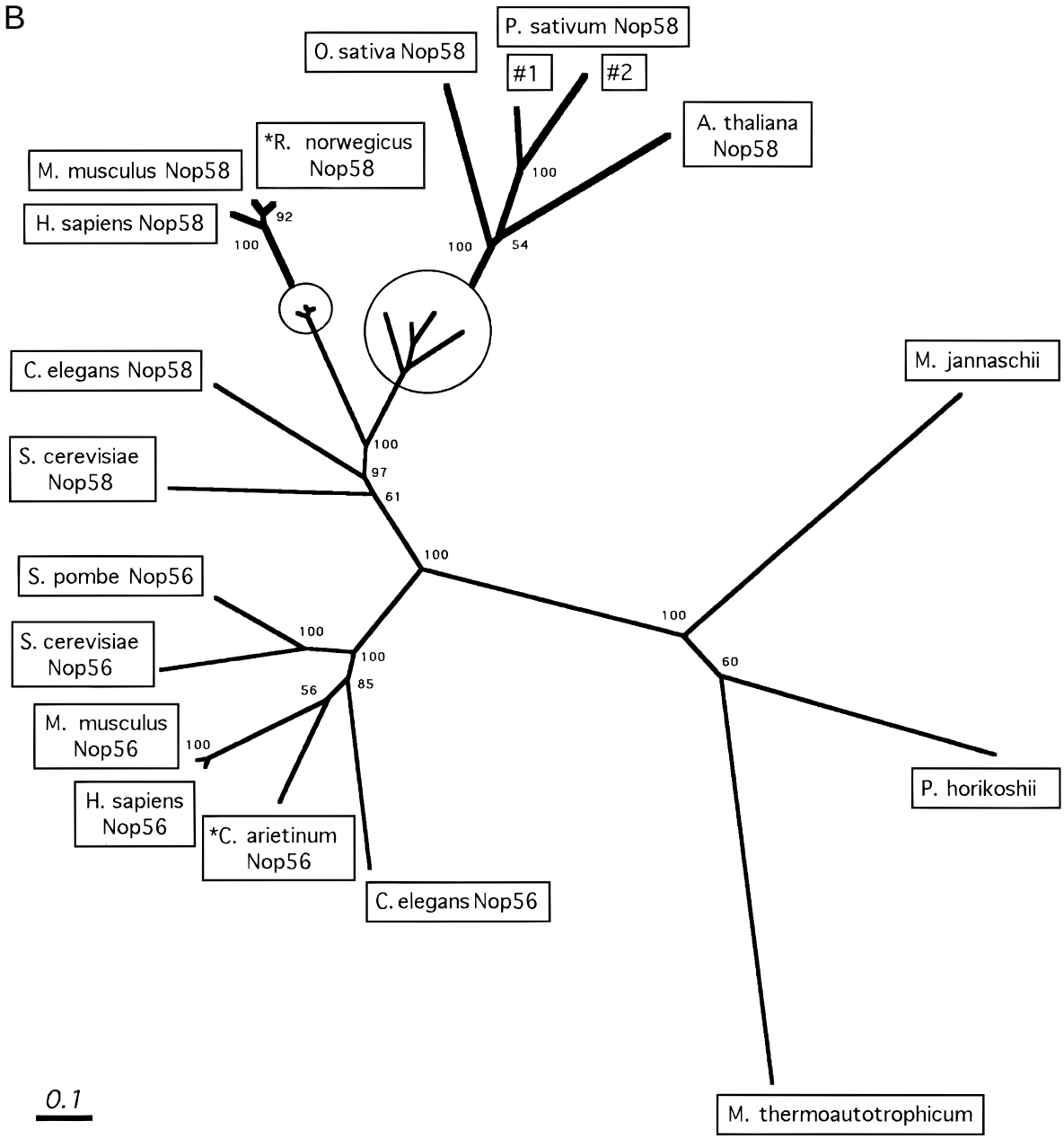

FIGURE 4. Alignment and sequence comparison of mouse Nop58 and mouse Nop56 proteins with the archaeal homologs and the phylogenetic relationship of eukaryotic and archaeal Nop56/Nop58 proteins. **A**: Mouse Nop58 and mouse Nop56 are aligned with archaeal homologs. Identical amino acid residues are indicated by white letters on black blocks and conserved residues are designated by gray shading. Spaces inserted to aid the amino acid sequence alignments are indicated by dashes. **B**: An unrooted, neighbor-joining, phylogenetic tree for selected Nop56/Nop58 protein homologs is presented. To assess the likelihood of these phylogenetic relationships, bootstrap resampling of the data set was performed 100 times to produce 100 possible neighbor-joining data sets. The percentage of neighbor-joining bootstraps for species distal to a given fork is indicated numerically at the fork. Values below 50 are not indicated. Circles designate those areas of a branch expanded for clarity. *: sequences that may be incomplete. The scale bar corresponds to 0.1 changes per nucleotide site.

FIGURE 5. Alignment and sequence comparison of mouse p55 and p50 proteins with their eukaryotic homologs. The determined mouse (*M. musculus*) p55 (**A**) and p50 (**B**) sequences are aligned and compared with corresponding homologs from other eukaryotes. Amino acid sequences determined from peptide sequencing of the isolated p55 and p50 proteins are overlined. Identical amino acid residues are indicated by white letters on black blocks and conserved residues are designated by gray shading. Dashes indicate spaces inserted to aid in the amino acid sequence alignments. The conserved Walker A and Walker B motifs are indicated by boxes. (*Figure on following two pages.*)

A. p55

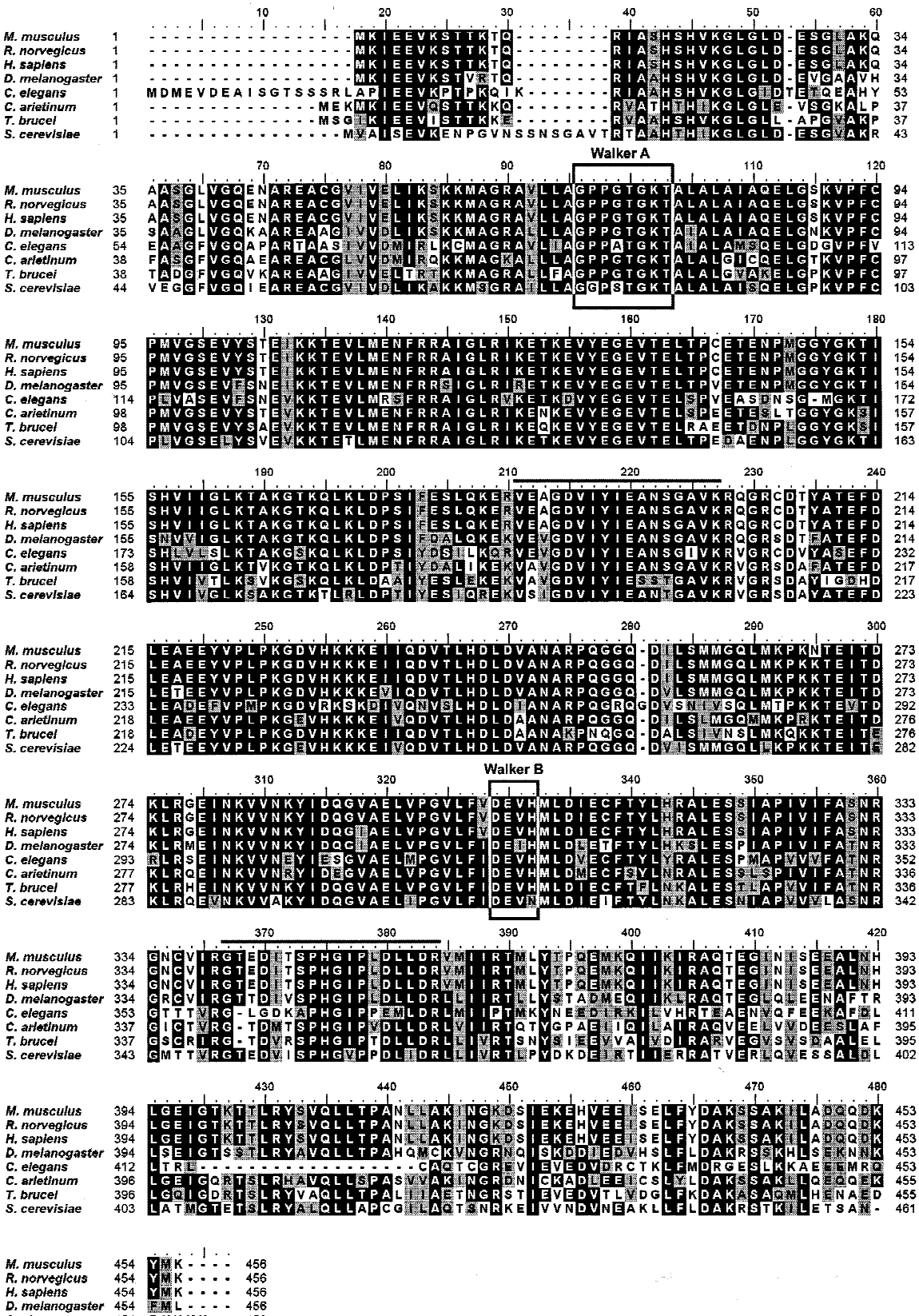

FIGURE 5A. (*Legend on page 867.*)

B. p50

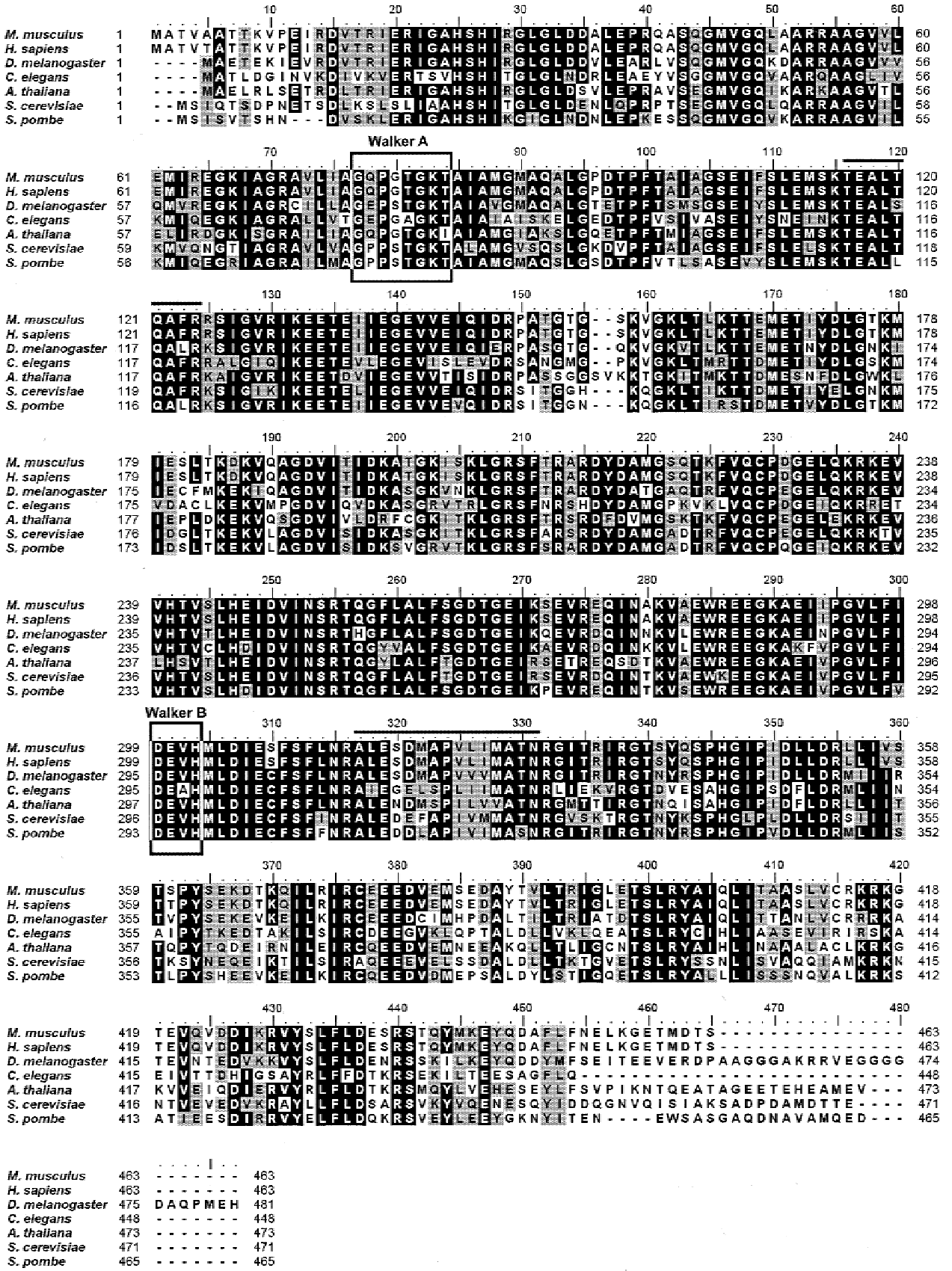

FIGURE 5B. (*Legend on page 867.*)

A. p55/p50

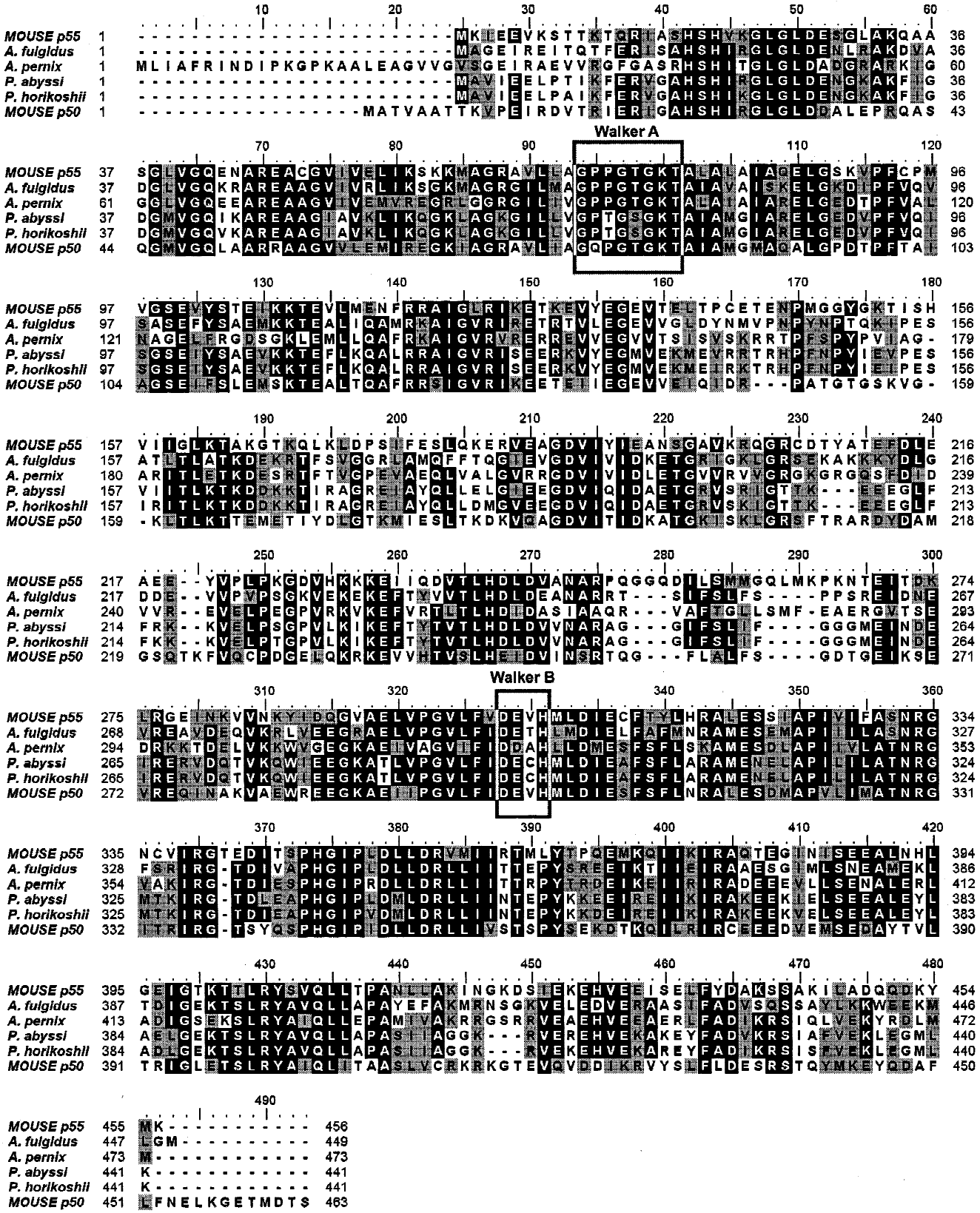

FIGURE 6. (*Legend on facing page.*)

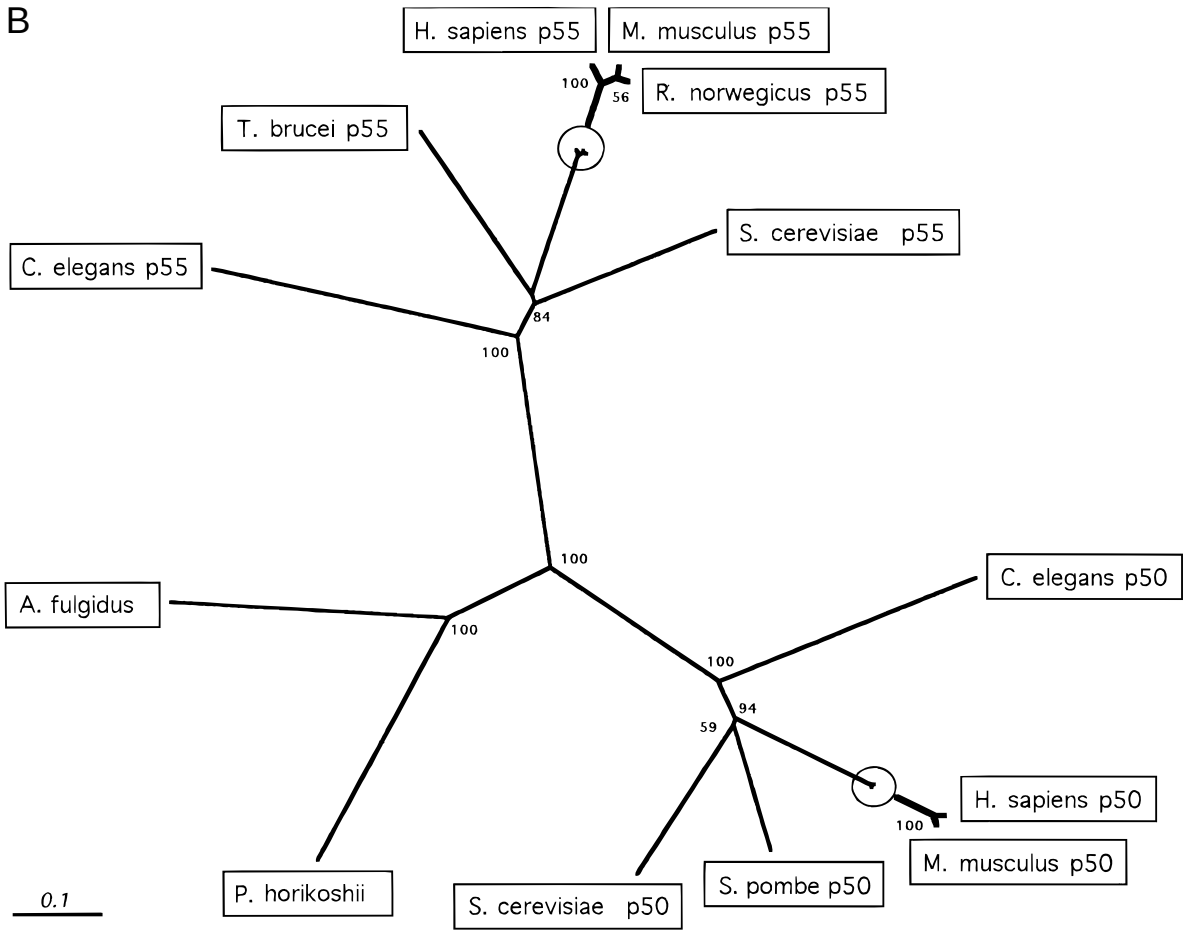

FIGURE 6. Alignment and sequence comparison of mouse p55 and p50 proteins with the archaeal homologs and the phylogenetic relationship of eukaryotic and archaeal p55/p50 proteins. **A**: Mouse p55 and mouse p50 proteins are aligned with archaeal homologs. Identical amino acid residues are indicated by white letters on black blocks and conserved residues are designated by gray shading. Spaces inserted to aid the amino acid sequence alignments are indicated by dashes. The conserved Walker A and B motifs are indicated by boxes. **B**: An unrooted neighbor-joining phylogenetic tree for the p55/p50 protein homologs is presented. To assess the likelihood of these phylogenetic relationships, bootstrap resampling of the data set was performed 100 times to produce 100 possible neighbor-joining data sets. The percentage of neighbor-joining bootstraps for species distal to a given fork is indicated numerically at the fork. Values below 50 are not indicated. Circles designate those areas of a branch expanded for clarity. *: sequences that may be incomplete. The scale bar corresponds to 0.1 changes per nucleotide site.

noted and is the result of the highly charged C-terminal ends of these proteins (Wu et al., 1998). Nop56 is highly conserved in eukaryotes, exhibiting 49% identity and 71% similarity between mouse and yeast.

Homologs for Nop58/Nop56 have been noted in Archaea (Gautier et al., 1997), and searches of the database using both mouse Nop58p and Nop56p sequences revealed homologs in several organisms (Fig. 4A). Interestingly, whereas eukaryotic species have two distinct members of this nucleolar protein pair, each archaeal species appears to possess only a single homolog. Phylogenetic analysis of selected Nop56/Nop58 homologs grouped these proteins into three separate evolutionary branches of an unrooted tree (Fig. 4B). Archaeal homologs occupy one major branch, distinct from the major branches of the eukaryotic Nop58 and Nop56 homologs.

p55 and p50 constitute a second pair of highly conserved nuclear proteins

Two sequenced peptides of p55 identified a protein recently designated Tip49 (TBP-interacting protein of 49 kDa) in rat (Kanemaki et al., 1997). This protein was originally named as such because of its ability to interact with TBP. From the EST database, the mouse homolog was identified and cloned. The mouse protein is 456 amino acids in length with a calculated molecular weight of 50.2 kDa (Fig. 5A). A yeast homolog, YDR190c (463 amino acids, 50.5 kDa), was also identified by searching the yeast genome database. The mouse and yeast genes exhibit remarkable conservation with 70% identity and 88% similarity in amino acid sequences. From its initial designation as Tip49, this protein has been reported using various nomenclatures. A human

homolog has been designated RUVBL1 [helicase RuvB-like 1 (Qiu et al., 1998)], NMP238 [nuclear matrix protein 238 (Holzmann et al., 1998)], and Pontin52 [pons for a "bridge" protein linking β-catenin to TBP (Bauer et al., 1998)] (see Discussion). Our database searches have also identified additional p55 protein homologs. The high level of amino acid sequence conservation for this protein is illustrated in Figure 5A. As noted previously, p55 possesses both Walker A [ATP/GTP-binding site motif or "P-loop" (GPPGTGKT)] and Walker B [ATP hydrolysis motif (DEVH or modified DEAD box motif)] sequences. These motifs are often characteristic of DNA/RNA helicases that are found in Eukarya, Archaea, and Eubacteria (see Discussion).

Sequencing of two p50 peptides identified the open reading frame YPL235w in the yeast genome, and that sequence was used in turn to identify the mouse homolog in the EST database. The cloned mouse p50 protein is 463 amino acids, with a calculated molecular weight of 51.1 kDa and is 69% identical and 87% similar to the yeast homolog (Fig. 5B). Strikingly, p50 exhibits extensive homology with p55, establishing these two proteins as a highly related pair. Mouse p55 and p50 are 42% identical and 68% similar. Not surprisingly, p50 also possesses Walker A and B motifs that are well conserved in all the homologs of this nuclear protein.

Similar to the Nop56/Nop56 protein pair, homologs for the p55/p50 pair have been identified in several Archaea (Fig. 6A). As with the Nop56/Nop58 pair, only a single homolog for the p55/p50 protein pair is found in each archaeal organism. The archaeal homologs of p50/p55 exhibit similar homologies to their eukaryotic p50 and p55 counterparts. Construction of an unrooted phylogenetic tree of the p50/p55 proteins establishes three separate branches occupied by eukaryotic p50, p55, and the archaeal homologs, respectively (Fig. 6B).

Mouse p55 and p50 are nucleoplasmic proteins

The mouse p55 and p50 genes were cloned into the pET15b expression plasmids, recombinant proteins expressed in *Escherichia coli*, and polyclonal antibodies for each protein generated in rabbits. These antibodies specifically recognized p55 or p50, not only for mouse, but also for HeLa cells and *Xenopus laevis* (Fig. 7A).

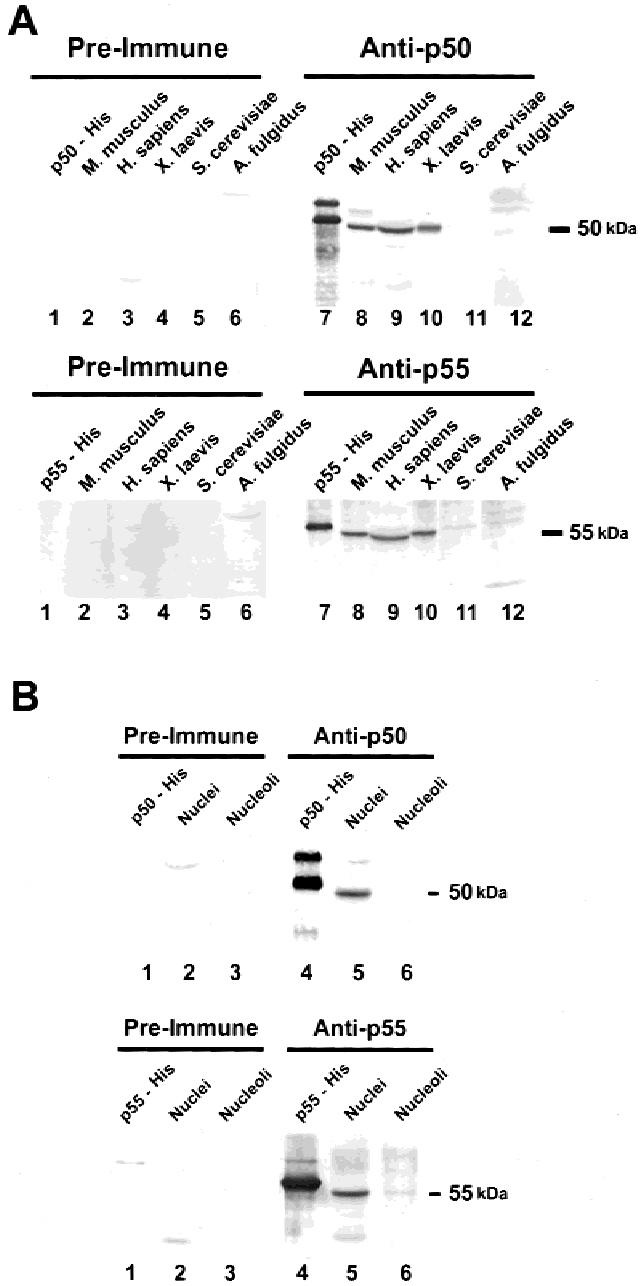

FIGURE 7. Nuclear distribution of mouse p55 and p50 proteins. Polyclonal antibodies generated against mouse p55 and mouse p50 proteins were used to assess the nuclear/nucleolar distribution of these proteins in mouse Taper ascites cells nuclei. **A**: Total cellular proteins prepared from *A. fulgidus*, *S. cerevisiae*, *X. laevis* oocytes, HeLa cells, and mouse Taper ascites cells were resolved on an SDS-polyacrylamide gel, blotted to membranes, and then probed with either the p55 or p50 antibody as designated. Resolved protein fractions are indicated above the respective western blot lanes. Proteins resolved in lanes 1–6 were probed with preimmune serum. Proteins resolved in lanes 7–12 were probed with either p50 or p55 antibodies. **B**: Nucleoplasmic/nucleolar distribution of mouse p55/p50. Isolated total nuclear and nucleolar protein fractions from equivalent numbers of mouse Taper ascites cells were resolved on an SDS-polyacrylamide gel and the distribution of p55/p50 assessed by western blotting. Proteins resolved in gel lanes 1–3 were probed with preimmune serum. Proteins resolved in gel lanes 4–6 were probed with either p55 or p50 antibody as designated. Resolved protein fractions are indicated above the respective lanes of the western blot. p55-His: recombinant mouse p55 protein bearing an N-terminal six-histidine tag. Nuclei: isolated mouse Taper ascites cell total nuclear proteins. Nucleoli: isolated mouse Taper ascites cell nucleolar proteins. The migration position of the mouse p55 and p50 proteins are indicated at the side.

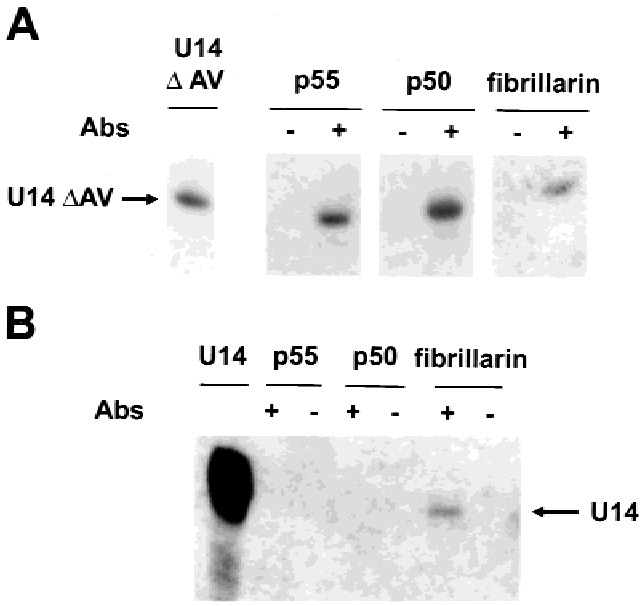

FIGURE 8. Immunoprecipitation of the assembled U14 snoRNP complex with p50 and p55 antibodies. Antibodies directed against p50, p55, and fibrillarin were used to assess the association of these proteins with U14 snoRNA. **A**: Antibodies to p50, p55, and fibrillarin were incubated with the in vitro assembled U14 snoRNP (complex assembled with radiolabeled U14 ΔAV snoRNA) and then coupled with Protein(A)-Sepharose for subsequent immunoprecipitation. After extensively washing the Protein(A)-Sepharose beads, bound snoRNP was eluted with 0.1 M glycine, pH 3.0, phenol-extracted, and the ethanol-precipitated RNA analyzed on a 10% polyacrylamide-7 M urea gel. Incubation of the Protein(A)-Sepharose beads with the in vitro-assembled U14 snoRNP complex in the presence (+) or absence (−) of the designated antibody is indicated. The marker lane containing radiolabeled U14 ΔAV is designated. **B**: Antibodies to p50, p55, and fibrillarin were incubated with a mouse ascites cell nuclear sonicate and then coupled with Protein(A)-Sepharose for subsequent immunoprecipitation. After extensively washing the Protein(A)-Sepharose beads, bound snoRNP was eluted with 0.1 M glycine, pH 3.0, and phenol extracted. The ethanol precipitated RNA was resolved on a 10% polyacrylamide-7 M urea gel and electroblotted to nylon membrane before hybridization with a ^{32}P-radiolabeled DNA oligonucleotide complementary to the 3′-terminal region of mouse U14 snoRNA. Incubation of the Protein(A)-Sepharose beads with the nuclear sonicate in the presence (+) or absence (−) of designated antibody is indicated. The lane designated U14 is a full-length U14 snoRNA marker.

These antibodies were then used in western blot analysis to assess the nuclear/nucleolar distribution of both the proteins in mouse Taper ascites cells. Ascites cell nuclei were isolated and from purified nuclei, nucleoli were also prepared. Isolated nucleoli contained few unbroken nuclei as judged by microscopy, and Northern analysis has shown that the vast majority of U14 snoRNA is contained in this nucleolar preparation (data not shown). The results shown in Figure 7B demonstrate that both p55 and p50 are found predominantly, if not exclusively, in the nucleoplasm. These results are consistent with previous immunofluorescence data indicating a nucleoplasmic localization for the p55 protein (Bauer et al., 1998; Holzmann et al., 1998).

Immunoprecipitation of the assembled U14 snoRNP complex with p55 and p50 antibodies

The association of p50 and p55 with the U14 snoRNP complex was examined by immunoprecipitating both native and in vitro-assembled U14 snoRNP complexes with antibodies directed against both snoRNP proteins. Antibodies directed against both p50 and p55 proteins immunoprecipitated the assembled U14 snoRNP, demonstrating the binding of these nucleoplasmic proteins to the complex in vitro (Fig. 8A). The in vitro-assembled snoRNP complex was also precipitated with fibrillarin monoclonal antibody (72B9), demonstrating the association of this nucleolar box C/D snoRNP protein with the complex, under the low salt conditions of in vitro snoRNP assembly. Fibrillarin binding was at reduced levels, but this was not unexpected as this core snoRNP protein binds weakly to the U14 complex when the snoRNP complex is assembled with truncated U14 constructs (Watkins et al., 1996). These same three antibodies were also utilized for immunoprecipitation analysis of a mouse ascites cell nuclear sonicate containing native U14 snoRNP complexes (this nuclear sonicate is distinct from the nuclear protein extract used for in vitro snoRNP assembly studies). Fibrillarin antibody immunoprecipitated the native U14 snoRNP containing full-length, fully processed U14 snoRNA (Fig. 8B). In contrast, immunoprecipitation of U14 with either the p50 or p55 antibody was not observed. This was not unexpected as we believe that p50 and p55 are snoRNP accessory proteins transiently associated with the box C/D snoRNP during snoRNA processing or snoRNA transport to the nucleolus (see Discussion). Previous work in *Xenopus* oocytes has demonstrated that this fibrillarin antibody immunoprecipitates not only the mature U14 snoRNP complex, but also radiolabeled snoRNA:pre-mRNA precursor and snoRNA precessing intermediates as fibrillarin-bound snoRNP (Watkins et al., 1996). However, the immunoprecipitation of non-radioactive steady-state U14 snoRNP complexes from the nuclear sonicate will yield only processed U14 as the snoRNA/snoRNP precursors would be present in this sonicate in very small amounts.

DISCUSSION

Previous analysis of an in vitro-assembled mouse U14 snoRNP complex identified three nuclear proteins (Watkins et al., 1998). These same proteins have now been isolated via affinity chromatography using the U14 box C/D core motif as ligand, and their binding to other box C/D snoRNAs indicates they are proteins common to all members of this snoRNA family. Sequence analysis of these mouse snoRNP proteins and subsequent cloning of the corresponding mouse genes have identified two pairs of proteins, with the individual members of each pair highly related to the other in sequence. Pre-

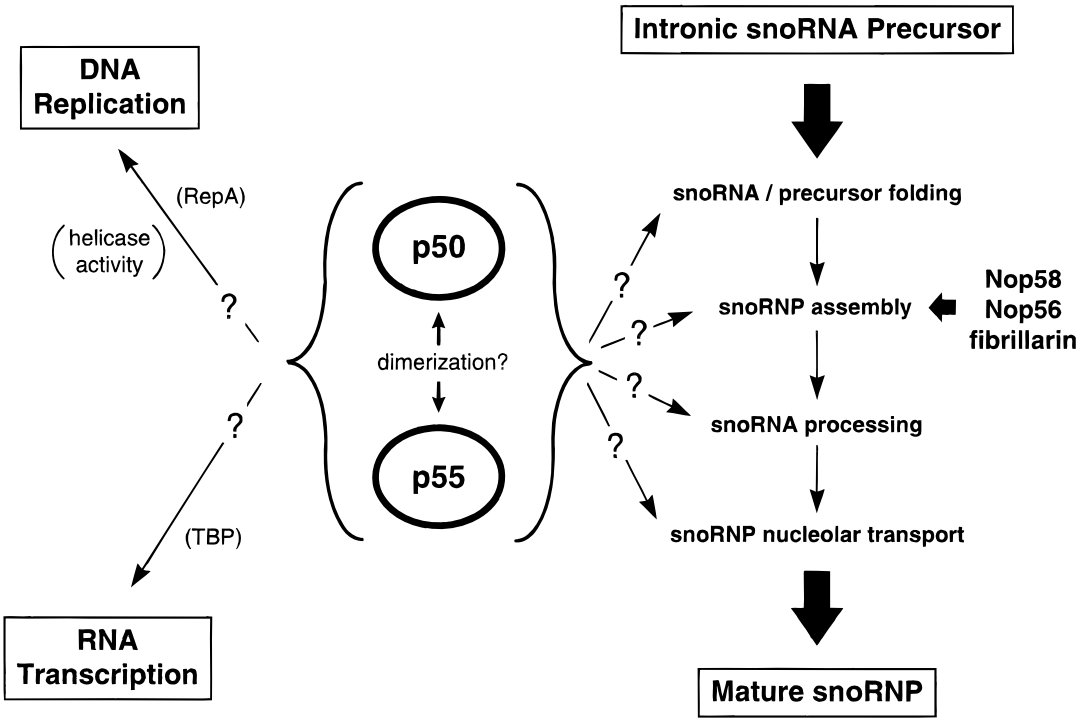

FIGURE 9. Possible roles for the p55/p50 proteins in the eukaryotic nucleus.

viously reported mouse protein p65 is actually a pair of nucleolar proteins whose homologs in yeast have been designated Nop56p and Nop58p. Investigations have demonstrated that Nop58 is associated with the box C/D snoRNAs (Wu et al., 1998; Lafontaine & Tollervey, 1999). The binding of mouse Nop58 and mouse Nop56 to the U14 box C/D core motif confirms the interaction of Nop58 with the box C/D core motif and demonstrates that the closely related Nop56 is also a core protein of the box C/D snoRNP complex. This is consistent with the recent work demonstrating that fibrillarin, Nop58, and Nop56 are three proteins common to all box C/D snoRNAs (Lafontaine & Tollervey, 2000). The finding that Nop58 and Nop56 are bound to this RNA motif in vitro attests to the validity of this assembly system for the biochemical/structural study of the box C/D snoRNP core complex. Previously defined proteins p55 and p50 represent a second, novel pair of highly related nucleoplasmic box C/D snoRNA-associated proteins that have been recently reported with varying nomenclatures (Kanemaki et al., 1997; Bauer et al., 1998; Holzmann et al., 1998; Qiu et al., 1998).

During the course of our studies, yeast NOP56 and NOP58 were identified through a genetic analysis as interacting with fibrillarin/Nop1p, a well conserved, box C/D snoRNP protein (Gautier et al., 1997). Immunoprecipitation analysis has since shown that yeast Nop58p is associated with all yeast box C/D snoRNAs (Wu et al., 1998) as well as Nop58 with vertebrate box C/D snoRNAs (Lyman et al., 1999). Both Nop56p and Nop58p are essential for yeast viability, consistent with a role for these two proteins in box C/D snoRNA biogenesis and stability. Overexpression of either Nop56p or Nop58p in yeast does not compensate for disruption of the other protein's gene (Gautier et al., 1997), thus indicating that each has a unique function despite exhibiting such sequence similarity. It is likely that the lethal phenotype of both proteins is a result of the inability to assemble a core snoRNP complex and thus stabilize essential box C/D snoRNAs required for rRNA processing. Consistent with this scenario, recent work has shown that yeast Nop58p is required for box C/D snoRNA accumulation and rRNA processing (Lafontaine & Tollervey, 1999).

Yeast Nop56 and Nop58 are members of a yeast protein family characterized by KKE/D triplet repeats located at the C-terminal end of the protein (Gautier et al., 1997). Additional members of this group include Cbf5p (the putative pseudouridyl synthase associated with the box H/ACA snoRNAs) and Dbp3p (a putative RNA helicase required for pre-rRNA cleavage at A_3). Interestingly, this distinct triplet repeat is unique to the yeast, as the KKE/D motif of the Nop56/Nop58 homologs in other eukaryotes has been replaced with a highly positively charged C-terminal sequence containing numerous lysine residues. The function of this highly charged C-terminal region is currently unknown, but deletion analysis in yeast has demonstrated that it is dispensable for Nop56p/Nop58p

function and nucleolar localization (Gautier et al., 1997; Wu et al., 1998).

Mouse p55 and p50 represent a second and novel pair of box C/D snoRNA-associated proteins. Like the Nop56/Nop58 pair, they are related to each other in sequence, highly conserved in eukaryotes with homologs found in Archaea (Kanemaki et al., 1999; Makino et al., 1999; this work). The observation that p55 and p50 are primarily, if not exclusively, localized to the nucleoplasm (Bauer et al., 1998; this work) suggests a transient association of these proteins with the box C/D snoRNAs and potential roles in snoRNA biogenesis. Indeed, recent gene-disruption experiments in yeast have shown that both p50 and p55 are encoded by essential genes that are required for snoRNA accumulation and rRNA processing (King et al., in prep.). The presence of Walker A and B motifs in both members of this protein pair indicate ATP/GTP binding ability and potential helicase activity. Several reports have suggested that p55 is a potential eukaryotic homolog of RuvB-like helicase of prokaryotes (Qiu et al., 1998; Gohshi et al., 1999; Kanemaki et al., 1999; Kikuchi et al., 1999). Our sequence analysis indicates little homology between p55/p50 homologs and RuvB helicase beyond those regions containing the Walker A and B motifs. However, recent work has indeed indicated that both p55 and p50 do exhibit DNA helicase activity in vitro (Kanemaki et al., 1999; Makino et al., 1999). These same Walker A and B motifs are also characteristic of the DEAD/H-box family of RNA-dependent ATPases (Gorbalenya et al., 1989; Koonin, 1993), whose members have been found in the spliceosomal RNP complex and are believed to be important for conformational rearrangement of the spliceosomal RNAs during splicing (Guthrie, 1991; Madhani & Guthrie, 1994). A similar functional role could be envisioned for p50 and/or p55 during the processing of the intronic snoRNAs from their pre-mRNA precursors. Interestingly, observations indicate that p55 interacts with p50 in vivo, suggesting that these two proteins, whatever their function, may act as a heterodimer in the nucleus (Kanemaki et al., 1999).

The p55 homolog of the p55/p50 protein pair has been reported in the literature under various nomenclatures, depending upon the observed characteristics or postulated roles for this protein. These designations include TIP49 (TBP-interacting protein of 49 kDa) (Kanemaki et al., 1997), RUVBLI (RuvB-like protein) (Qiu et al., 1998), NMP238 (nuclear matrix protein 238) (Holzmann et al., 1998), and Pontin52 (pons or bridging protein of 52 kDa) (Bauer et al., 1998). A striking characteristic of p55 is its observed interaction with other proteins. P55 was originally defined as a protein that interacts with TBP, thus suggesting a role in RNA transcription (Kanemaki et al., 1997). A similar interaction with TBP was noted when p55's interaction with β-catenin was observed (Bauer et al., 1998). The involvement of β-catenin in the Wnt signaling pathway again links p55 with transcriptional events. Interestingly, human p55 has been observed to cofractionate with RNA polymerase II (Qiu et al., 1998). Several other reports have suggested possible roles for p55 in DNA-replication events. P55 was isolated using a yeast two-hybrid screen with the RPA3 subunit of human replication A protein (eukaryotic homolog of prokaryotic SSB protein) serving as bait (Qiu et al., 1998). The finding of DNA helicase activity for both p55 and p50 is also consistent with a possible role in replication (Kanemaki et al., 1999; Makino et al., 1999). Collectively, these observations suggest potentially multifunctional roles for p55/p50 in the eukaryotic nucleus (see Fig. 9).

All four box C/D snoRNA-associated proteins are highly conserved in Eukarya, and their occurrence in Archaea is striking. An archaeal homolog for fibrillarin was noted several years ago (Amiri, 1994), and more recently homologs for the Nop56/Nop58 and the p50/p55 protein pairs have been defined (Lafontaine & Tollervey, 1998; Kanemaki et al., 1999; Makino et al., 1999; this work). The presence of these proteins in Archaea raises the question as to their function in organisms that do not possess nuclei/nucleoli. However, recent investigations in Archaea have demonstrated the presence of small RNAs possessing box C/D core motifs that direct the 2′-*O*-methylation of rRNA (Omer et al., 2000). Therefore, the mechanism of utilizing small RNAs as guides for nucleotide modification has been conserved across kingdoms, and the conservation of proteins that are associated with the box C/D motif is therefore not unexpected. Interestingly, only a single archaeal homolog/gene for each of these box C/D snoRNA-associated protein pairs has been found. This may suggest that if Nop56/Nop58 and p50/p55 do indeed function as heterodimers in eukaryotes, then the single archaeal protein for each of these pairs might act as a homodimer in archaeal organisms.

In vitro assembly of the U14 snoRNP complex using the box C/D snoRNA core motif as a minimal RNA substrate has defined these two protein pairs. Nucleolar proteins Nop56 and Nop58, along with fibrillarin, constitute three core proteins of the box C/D snoRNP complex. Interestingly, fibrillarin is not a protein component of the biochemically fractionated, in vitro-assembled mouse U14 snoRNP complex (Watkins et al., 1998) nor is it isolated as a box C/D core motif-associated protein using the affinity chromatographic approach described in this investigation. This is not surprising, as fibrillarin binding to U14 snoRNA is salt sensitive (Watkins et al., 1996). The high salt conditions used for snoRNP fractionation (Watkins et al., 1998) or for washing the box C/D affinity column (500 mM) before snoRNP protein elution would be expected to dissociate fibrillarin from the assembled complex. However, it is clear from the immunoprecipitation analysis of the in vitro-assembled U14

snoRNP complex with fibrillarin antibodies under low salt conditions (this work) that fibrillarin is initially bound to the snoRNP complex. Isolation of nucleolar proteins Nop56 and Nop58, but not fibrillarin, using the box C/D affinity column indicates that both Nop56 and Nop58 can bind the box C/D snoRNA core motif in the absence of fibrillarin. However, it is possible that fibrillarin is required for the initial, in vitro assembly of the core snoRNP complex, but is ultimately removed during the high salt wash. Current studies examining U14 snoRNP assembly are underway.

The nucleoplasmic distribution of p50/p55 implies a transient association of these proteins with the snoRNA/snoRNP complex, whereas the intronic snoRNA precursor is assembled into an snoRNP complex, the snoRNA processed, and finally transported to the nucleolus. The inability to immunoprecipitate the steady-state U14 snoRNP complex from ascites cells with antibodies directed against p50 and p55 is consistent with this scenario. Thus, these two nucleoplasmic proteins could be considered accessory snoRNP proteins that are not part of the mature core complex. Potential nucleoplasmic functions for such accessory proteins include: (1) guiding snoRNA precursor folding, (2) assisting in binding of the core snoRNP proteins on the snoRNA during snoRNP assembly, (3) functioning in specific snoRNA processing events and the stabilization of a mature snoRNP complex, (4) assisting in the transport of the mature snoRNP complex into the nucleolus, and/or (5) coordinating snoRNA biogenesis and nucleolar transport with other nucleoplasmic events such as DNA replication and/or RNA transcription (see Fig. 9). We are continuing our investigation of these box C/D snoRNA-associated proteins using both structural and functional studies to elucidate their roles in snoRNA processing, snoRNP biogenesis, and snoRNP function in the eukaryotic nucleus.

MATERIALS AND METHODS

Generation of poly(A)-tailed snoRNAs

DNA templates for T7 transcription of poly(A)-tailed snoRNAs were synthesized via PCR amplification using previously constructed plasmids as templates, except where noted. DNA oligonucleotides (listed below) possessing a T7 prefix were used as upstream primers and encoded a T7 RNA polymerase promoter sequence, followed by the 5′ region of the designated snoRNA-coding sequence. DNA oligonucleotides possessing a poly(T) prefix were used as downstream primers and encoded 20 or 25 deoxythymidines, followed by sequence complementary to the 3′ terminal region of the designated snoRNA. Oligos used to construct templates for deletion mutant snoRNAs are designated by a "Δ"; those designated "mutD" were used to construct transcription templates of mutant snoRNAs where the box D (CUGA) was changed to CCCC.

1. SP64SEQ: 5′-ACACAGGAAACAGCTATGACC-3′;
2. poly(T)U14: 5′-TTTTTTTTTTTTTTTTTTTTTTTTCATTCGCTCAGACATCCAAGG-3′;
3. poly(T)U14 mutD: 5′-TTTTTTTTTTTTTTTTTTTTTTTTTTCATTCGCGGGGACATCCAAGG-3′;
4. T7humanU15: 5′-CGCTAATACGACTCACTATAGGCCTTCGATGAAGATGATGACG-3′;
5. poly(T)human: 5′-TTTTTTTTTTTTTTTTTTTTCCTTCTCAGACAAATGCC-3′;
6. poly(T)human mutD: 5′-TTTTTTTTTTTTTTTTTTTTCCTTCGGGGACAAATGCC-3′;
7. humanΔU15 UP: 5′-CCTTCGATGAAGAGATGATGACGAGTAGGCA-3′;
8. humanΔU15 DN: 5′-GCGGATCCCTTCTCAGACAAATGCCTACTCGTCATC-3′;
9. T7humanU17: 5′-CGCTAATACGACTCACTATAGGTCCAACGTGGATACACCC-3′;
10. poly(T)humanU17: 5′-TTTTTTTTTTTTTTTTTTTTGGCTGTTTCCTGCATGG-3′;
11. T7humanMRP: 5′-CGCTAATACGACTCACTATAGGTTCGTGCTGAAGGCCTG-3′; and
12. poly(T)humanMRP: 5′-TTTTTTTTTTTTTTTTTTTTAGCCGCGCTGAGAATGAGCCC-3′.

Plasmids, template construction, and transcription

Plasmid pSP64T7U14 (Watkins et al., 1998), that contains a T7 polymerase promoter downstream from the primer SP64SEQ annealing site, was used as the template for PCR amplification of the poly(A)-tailed mouse U14 DNA and the poly(A)-tailed mouse U14 mutD DNA constructs using oligo combinations 1 + 2 and 1 + 3, respectively; transcription of these PCR products resulted in poly(A)U14 and poly(A)U14 mutD RNAs. Plasmid pSP64T7U14ΔAV (Watkins et al., 1998), with regions A (one of two regions of U14 with complementarity to rRNA) and V (variable) deleted from wild-type mouse U14, was the template for the poly(A)-tailed U14ΔAV and poly(A)-tailed U14ΔAV mutD DNA constructs using oligo combinations 1 + 2 and 1 + 3, respectively; transcription resulted in poly(A)ΔAV and poly(A)ΔAV mutD RNAs. Similarly, plasmid pBS+HU15 (Watkins et al., 1996) was the template for PCR amplification of poly(A)-tailed human U15 DNA constructs using oligo combinations 4 + 5 and 4 + 6; transcription of these constructs resulted in poly(A)HU15 and poly(A)HU15 mutD RNAs, respectively. The template for PCR amplification of the transcription templates for poly(A)ΔHU15 and poly(A)ΔHU15 mutD RNAs was ΔHU15, itself a PCR-amplified product of two oligonucleotides (7 + 8) annealed to each other. The ΔHU15 DNA construct removed nt 26–135 from the coding sequence of human U15, but retained the natural termini with the included box C/D core motif and some internal U15 sequence flanking the box C/D core motif. PCR amplification of the ΔHU15 construct using oligo pairs 4 + 5 and 4 + 6 then resulted in the templates for T7 transcription. Plasmid pBS+HU17 (Watkins et al., 1996) was the template for the PCR amplification of the poly(A)-tailed human U17 DNA construct using oligos 9 + 10; transcription resulted in poly(A)HU17 RNA. Plasmid pSP6MRP (Watkins et al., 1996) was the template for the PCR amplification of the poly(A)-tailed human MRP DNA construct using oligos 11 + 12; transcription produced poly(A)HMRP RNA. Resultant DNA

templates containing snoRNA coding sequences were transcribed using the RiboMAX System (Promega) according to the manufacturer's protocol.

Affinity chromatographic isolation of snoRNP proteins

Poly(A)-tailed snoRNAs were covalently linked to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's protocol. Briefly, activated Sepharose 4B was incubated overnight with poly(A)-tailed RNAs in 50 mM K_2HPO_4 buffer at pH 8.0 at 4 °C. RNA incubation with activated beads at this pH strongly favors attachment of the RNA through the aromatic amines of the single-stranded 3′ poly(A) tail (Poonian et al., 1971), leaving the folded snoRNA ligand unattached and available for RNP formation. Following RNA attachment, unreacted sites on the Sepharose beads were blocked by adding solid glycine to a final concentration of 0.2 M and incubating for 4 h at 22 °C. Beads were collected by centrifugation and resuspended in an equal volume of 1 M triethanolamine (TEA, pH 8.0) for a further 1-h incubation at 22 °C. After extensive washing with 50 mM K_2HPO_4 (pH 8.0), the prepared Sepharose was packed in columns of 2–3 mL (small scale) or 15–20 mL (large scale). Columns were equilibrated in 15 mM HEPES (pH 7.0), 150 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.6 mM DTT, and 10% glycerol. A mouse Taper ascites cell nuclear extract (Watkins et al., 1998) in buffer of 20 mM HEPES (pH 7.0), 100 mM KCl, 3 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 10% glycerol was mixed with an equal volume of dilution buffer [40 mM HEPES (pH 7.0), 400 mM KCl, 6 mM $MgCl_2$, 1.5 mM DTT, 0.2 mg/mL tRNA, and 20 μg/mL poly(A) RNA]. Following incubation at 30 °C for 30 min to absorb poly(A)-binding as well as nonspecific RNA-binding proteins, the mixture was applied to the equilibrated affinity columns at room temperature. Columns were extensively washed with 20–40 column volumes of buffer DLG 200 [20 mM HEPES (pH 7.0), 200 mM KCl, 3 mM $MgCl_2$, 0.5 mM DTT, and 10% glycerol] and then with 25–30 column volumes of buffer DLG 500 (same as buffer DLG 200 but containing 500 mM KCl). Box C/D snoRNP proteins were eluted in 20 mM HEPES (pH 7.0) buffer containing 2 M LiCl and 1 M urea. Peak fractions of the LiCl/urea eluate were pooled and dialyzed overnight at 4 °C against 10 mM HEPES (pH 7.0), 10 mM KCl, and 1 mM EDTA. Proteins were subsequently precipitated with trichloroacetic acid (final concentration of 30%). Protein pellets were collected by centrifugation, washed with ice-cold acetone, dried under vacuum, and resuspended in SDS sample buffer. Isolated proteins were resolved by SDS-polyacrylamide gel electrophoresis and revealed by silver staining.

Peptide sequencing, protein identification, gene cloning, and protein analysis

Isolated snoRNP core proteins were resolved on preparative 10% SDS-polyacrylamide gels, fixed and stained with Coomassie blue, and then destained in acetic acid/methanol. Excised gel slices containing the desired proteins were sent to the Harvard Microchemistry Facility for peptide analysis. Peptide sequences obtained for individual snoRNP proteins were screened in the NCBI nonredundant GenBank database for identity/similarity with known proteins using the BLASTP program (Altschul et al., 1997). Mouse cDNA EST clones were identified by TBLASTN; archaeal homologs were identified by BLASTP and PSI-BLAST. I.M.A.G.E. Consortium cDNA clones (Lennon et al., 1996) were obtained from the American Type Culture Collection (ATCC). Two EST clones (ATCC #1416459 and ATCC #971387) together contained the complete gene for mouse Nop58. The correct 5′ end of the 3′-truncated clone (EST AA065550, ATCC #1416459) was excised by digestion with *Bam*HI and *Sap*I; the correct 3′ end of the 5′-truncated clone (EST AA655644, ATCC #971387) was excised by digestion with *Sap*I and *Xho*I; and the two fragments were ligated at the unique *Sap*I site (T4 DNA Ligase, Promega) and cloned into pGEM-3z (Promega) at the *Bam*HI and *Xho*I sites. The complete mouse Nop56 gene was obtained by RACE-PCR using a Marathon Ready cDNA mouse liver library kit (Clontech), adapter primer 1, and gene-specific primers to generate overlapping 5′ and 3′ RACE products. The RACE-PCR products were annealed to each other and extended with Vent polymerase (NEB) to produce the full-length coding sequence. I.M.A.G.E. clones containing cDNAs for mouse p55 (EST AA869108, ATCC #1361131) and for mouse p50 (EST AA726368, ATCC #1476884) were obtained from the ATCC, sequenced, and found to contain the entire protein coding sequences. Protein sequences were aligned using the GCG Pileup program. Unrooted, neighbor-joining trees were constructed using Phylip 3.572, bootstrapped with 100 resampling iterations, and drawn by the Treeview program. Parsimony trees, bootstrapped 100 times, agreed well with the neighbor-joining trees (data not shown). PAM residue weights tables and ClustalW were used in the phylogenetic analysis.

In vivo expression of cloned p50 and p55 proteins, antibody production, and western blotting

The coding sequences for the p55 and p50 proteins were PCR amplified from the I.M.A.G.E. clones using gene-specific primers that added an *Nde*I site at the start codon and a *Sal*I site downstream from the stop codon. The p55/p50 PCR products were digested with *Nde*I and *Sal*I, gel-purified, and ligated into the expression vector pET15b (Novagen) at the *Nde*I and *Xho*I sites. Selected clones were sequenced to confirm that the encoded p55/p50 sequences were correct and in frame with the N-terminal histidine ($\times$6) tag. Cloned p55/p50 proteins were expressed in *E. coli* [strain BL21(DE3)] and his-tagged protein purified by nickel chelation chromatography. Purified p55 and p50 proteins were used to produce polyclonal antibodies in New Zealand rabbits (Cocalico). Harvested *Archaeoglobus fulgidus*, *Saccharomyces cerevisiae*, and HeLa whole cells, isolated *X. laevis* oocyte germinal vesicles (nuclei), and isolated mouse Taper ascites cell nuclei (Maxwell & Martin, 1986) and nucleoli (Muramatsu & Onishi, 1977) were resuspended in SDS sample buffer, boiled for 5 min, and then stored at −80 °C. For western blots, whole cell, nuclear, and nucleolar protein preparations were resolved by SDS-polyacrylamide gel electrophoresis and blotted to PVDF membranes (BioRad) by electrophoretic transfer. After membrane washing and incubation with p55 or p50 antibodies, the secondary antibody [alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega)] was incubated with the membranes,

and the presence of p55/p50 proteins assessed by colorimetric detection of alkaline phosphatase activity.

Immunoprecipitation analysis of the U14 snoRNP complex

Mouse Taper ascites cell nuclear sonicate was prepared according to the following protocol. Isolated nuclei were resuspended in 20 mM HEPES buffer, pH 7.4, containing 150 mM KCl and 3 mM $MgCl_2$ and sonicated for six 30-s intervals interspersed with 1-min cooling periods on ice. Disrupted nuclei were centrifuged for 10 min at 10,000 $\times$ *g*, and the supernatant collected for immunoprecipitation analysis. Immunoprecipitation analysis of U14 snoRNP complexes was accomplished by first swelling Protein(A)-Sepharose in 10 mM Tris, pH 7.4, buffer containing 100 mM KCl and 3 mM $MgCl_2$. Antibodies against p50, p55, or fibrillarin were incubated with the mouse nuclear sonicate or an ascites cell nuclear extract in which radiolabeled U14 snoRNA had been added and the U14 snoRNP complex assembled in vitro (Watkins et al., 1998). After incubation at 4 °C for 45 min on ice, prepared Protein(A)-Sepharose was added and the incubation continued with gentle mixing for an additional 45 min. The Protein(A)-Sepharose coupled with antibody:snoRNP complex was then washed with 10 mM Tris, pH 7.4, buffer containing 100 mM KCl, 3 mM $MgCl_2$, and 0.1% NP-40. Bound snoRNP complexes were subsequently eluted in 0.1 M glycine, pH 3.0, phenol extracted, and the RNA precipitated with ethanol. Isolated RNA fractions were resolved on 10% polyacrylamide-7 M urea gels and electroblotted to nylon membranes. The presence of U14 was revealed by autoradiography (in vitro-assembled U14 snoRNP complex) or with Northern blot analysis (immunoprecipitation of the ascites cell nuclear sonicate) using a ^{32}P-radiolabeled DNA oligonucleotide complementary to the 3′ region of mouse U14 snoRNA.

ACKNOWLEDGMENTS

We wish to thank Joyce Liu for preparation of the mouse nuclear extract, Diana Borton for help in cloning the p55 and p50 protein genes into expression vectors, James Brown for sharing his expertise in constructing phylogenetic trees, Marybeth Pysz for providing *A. fulgidus* cells, and Michael Pollard for fibrillarin antibodies. This work was supported by National Science Foundation grant MCB9727945 (ESM).

Received November 16, 1999; returned for revision December 3, 1999; revised manuscript received April 10, 2000

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