A Spliceosomal Recycling Factor That Reanneals U4 and U6 Small Nuclear Ribonucleoprotein Particles

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The spliceosome removes introns from pre–messenger RNAs by a mechanism that entails extensive remodeling of RNA structure. The most conspicuous rearrangement involves disruption of 24 base pairs between U4 and U6 small nuclear RNAs (snRNAs). Here, the yeast RNA binding protein Prp24 is shown to reanneal these snRNAs. When Prp24 is absent, unpaired U4 and U6 small nuclear ribonucleoprotein particles (snRNPs) accumulate; with time, splicing becomes inhibited. Addition of purified Prp24 protein regenerates duplex U4/U6 snRNPs for new rounds of splicing. The reannealing reaction catalyzed by Prp24 proceeds more efficiently with snRNPs than with deproteinized snRNAs.

Pre–messenger RNA (pre-mRNA) splicing is a dynamic process that promotes intron removal. The pre-mRNA substrate and snRNAs (U1, U2, U4/U6, U5) undergo a series of helical exchanges within the spliceosome, a large RNP complex (*1, 2*). The most conspicuous rearrangement involves disruption of base pairing between U4 and U6 snRNAs (*3*). U4/U6 duplex is packaged with proteins in a snRNP particle and joins the spliceosome in a U4/U6.U5 triple snRNP (*4*). With each round of splicing, the adenosine triphosphate (ATP)–dependent displacement of U4 from the spliceosome allows U6 snRNA to refold, creating the active site for splicing catalysis (*5, 6*). In contrast, U4 snRNA is not required for the chemical steps of splicing (*7*). Because snRNPs must be reused (*1*), a recycling process must exist to reanneal the RNAs within the free U4 and U6 snRNPs and thus reverse their previous unwinding on the spliceosome.

Genetic suppression studies initially suggested that the yeast U6 snRNP protein Prp24 annealed free U6 and U4 (*8*). Prp24 harbors three RNA recognition motifs (RRMs) (*8*). In many proteins, RRMs contact RNA directly (*9*), and some RRM proteins promote annealing of complementary RNAs in vitro (*10, 11*). Recombinant Prp24 was similarly shown to anneal synthetic U4 and U6 RNAs in vitro, albeit inefficiently (*12*).

To test the hypothesis that Prp24 reanneals U4 and U6 snRNPs in its role as a spliceosomal recycling factor, we asked if removal of Prp24 from a splicing reaction mixture resulted in the accumulation of free U4 and free U6 snRNPs. We immunodepleted >97% of epitope-tagged Prp24 (*13, 14*) from yeast cell extract (ΔPrp24 extract) (*15*). The snRNP populations in mock-depleted and ΔPrp24 extracts were visualized by native gel electrophoresis (*16*). Splicing in ΔPrp24 extract generated large amounts of free U4 snRNP and U6*, a fast-migrating precursor to free U6 snRNP that apparently lacks Prp24 (Fig. 1A, lanes 10 versus lanes 4 and 6). Virtually all U4/U6.U5 and U4/U6 snRNPs disappeared (Fig. 1A, lanes 10 versus lanes 4). This snRNP imbalance resulted from splicing because depletion of U4/U6.U5 required intact 5′ splice site and branchpoint signals in pre-mRNA, incubation at 23°C (*17*), and ATP (Fig. 1A, lanes 10 versus lanes 9). A normal snRNP profile was recovered with the addition of Prp24 protein purified from yeast (*18, 19*): free U4 was diminished, U6* was abolished, and U4/U6 and U4/U6.U5 snRNPs were restored (Fig. 1A, lanes 14 versus lanes 10). Splicing in wild-type extract also produced some free U4 and U6*, which decreased in the presence of purified Prp24 (Fig. 1A, lanes 4 and 6). Thus, splicing consumes U4/U6.U5 and liberates free U4 and U6* snRNPs. Prp24 is necessary and sufficient to convert free U4 and U6* to U4/U6 snRNPs, facilitating the regeneration of U4/U6.U5 snRNPs.

Adding ATP to extract caused snRNP rearrangements even in the absence of exogenous pre-mRNA. Addition of ATP to ΔPrp24 extract resulted in the appearance of free U4 snRNP and U6*; mock-depleted extract also produced U4/U6 (Fig. 1A, lanes 7 and 8 versus lanes 1 and 2). Purified Prp24 protein restored U4/U6 formation in ΔPrp24 extract (Fig. 1A, lanes 7 and 8 versus lanes 11 and 12 and lanes 1 and 2). Thus, without added pre-mRNA, ATP disrupts some U4/U6.U5 snRNPs into free U4, U6*, and U5 snRNPs, and indirectly induces Prp24-dependent snRNP recycling.

If snRNP recycling does not directly require ATP, then Prp24 should reanneal U4 and U6 snRNPs even when ATP has been

Fig. 1. Splicing in ΔPrp24 extract causes aberrant snRNP distributions that can be rescued by the addition of purified Prp24 protein. Epitope-tagged Prp24-3HA extract (*13, 15*) was subjected to mock- and immunodepletion (ΔPrp24) (*14*). Western blotting revealed that ΔPrp24 extract was >97% depleted of Prp24-3HA (*17*). Polyoma-tagged Prp24 was overexpressed in yeast (*18*) and immunoaffinity-purified (*19*). A single 53-kD polypeptide was detected by silver staining (*17*). (**A**) Native gel analysis of snRNPs. Mock-depleted and ΔPrp24 extracts were incubated with or without ATP, pre-mRNA, and Prp24 purified from yeast and electrophoresed on a native gel (*16*) to resolve snRNPs. After Northern blotting (*33*), the individual snRNAs were sequentially detected by stripping and reprobing (*34*). Only blots probed for U4 and U6 snRNAs showed significant differences with and without Prp24. (**B**) Prp24 reforms U4/U6 snRNPs and facilitates U4/U6.U5 snRNP regeneration in an ATP-independent manner. ΔPrp24 extract was sequentially incubated with ATP and pre-mRNA (incubation 1); 0 or 5 mM glucose (incubation 2); and finally with either buffer (−), Prp24 purified from *E. coli* (r24), or Prp24 purified from yeast (y24) (incubation 3). The snRNPs were resolved by electrophoresis on a native gel, which was blotted and probed for U4 snRNA as in (A). [α-^{32}P]ATP added to these extract samples was reduced to undetectable levels by glucose treatment, as determined by thin-layer chromatography (*17*).

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removed. We incubated ΔPrp24 extract with ATP and pre-mRNA (Fig. 1B, lanes 1 to 7) so that free U4 and free U6 snRNPs would accumulate. Then, we depleted ATP in some reaction mixtures by adding glucose (Fig. 1B, lanes 5 to 7) (*20*). In a third incubation, we included either buffer or Prp24 purified from *Escherichia coli* or from yeast. Prp24 protein from either *E. coli* or yeast was capable of converting free U4 snRNP to U4/U6 and U4/U6.U5 snRNPs independent of ATP (Fig. 1B, lanes 5 to 7 versus lanes 2 to 4). Thus Prp24, and not any contaminating adenosine triphosphatase, is required to reassemble U4/U6 and U4/U6.U5 snRNPs.

If the sole function of Prp24 is to replenish U4/U6.U5 snRNPs for spliceosome assembly, then Prp24 should be unnecessary for in vitro splicing as long as U4/U6.U5 levels are adequate. In a typical reaction, the molar ratio of active U4/U6.U5 snRNPs to input pre-mRNA molecules may be large (*21*), and mRNA may be produced even when snRNPs are not regenerated. The primary recycling function of Prp24 may thus be obscured in the standard assay. Indeed, in vitro splicing reactions (*22*) proceeded normally in ΔPrp24 extract (Fig. 2A). In contrast, all other snRNP proteins that are essential for splicing in vivo are essential for splicing in vitro (*23*). For in vitro splicing efficiency to reflect snRNP recycling capability (and thus Prp24 function), U4/U6.U5 snRNPs in the extract must be exhausted.

We devised an in vitro splicing assay that would be sensitive to a recycling defect (Fig. 2B). To force most U4/U6.U5 snRNPs through a round of splicing, we added unlabeled pre-mRNA to splicing reaction mixtures. Subsequent splicing events were monitored by adding labeled pre-mRNA in a second incubation. When Prp24 was present, even a 10-fold excess of unlabeled pre-mRNA did not fully impede later rounds of splicing (Fig. 2B, lane 4). In contrast, prior incubation of ΔPrp24 extract with twofold excess of unlabeled pre-mRNA largely blocked subsequent rounds of splicing (Fig. 2B, lane 7). Prp24 was the critical component because purified Prp24 partially complemented the ΔPrp24 splicing defect (Fig. 2C). Although Prp24 is not required for conventional in vitro splicing, it is necessary when splicing relies on snRNP recycling.

To analyze associations of Prp24 with snRNPs, we used antibodies against epitope-tagged Prp24. We observed two specific supershifts of free U6 snRNP (*24, 25*), corroborating previous data (*8*). Under splicing conditions (in the presence of ATP and pre-mRNA), these supershifted species were diminished, suggesting that the free U6-Prp24 complex was depleted by splicing (*24*). We also monitored snRNAs that coimmunoprecipitated with tagged Prp24 from whole cell extract (*26*). Consistent with its presence in the free U6 snRNP, tagged Prp24 associated predominantly with unpaired U6 snRNA in the absence of ATP (Fig. 3, lane 3) (*24*). When ATP was added, Prp24 coimmunoprecipitated base-paired U4/U6 as well as free U6 and small amounts of free U4 RNAs (Fig. 3, lane 4) (*24*). Inclusion of pre-mRNA in the reaction mixture did not change the spectrum of snRNAs that coprecipitated with Prp24, suggesting that Prp24 does not assemble onto the spliceosome (Fig. 3, lanes 5 and 6 versus lanes 3 and 4).

U4/U6.U5 previously has been observed to dissociate in the presence of ATP (Fig. 1A) (*5*). We determined whether Prp24 reanneals native U4 and U6 snRNPs released by ATP from higher order snRNP complexes. We used antibodies against epitope-tagged Brr2/Snu246, a reported U4/U6.U5 snRNP component (*27*), to immu-

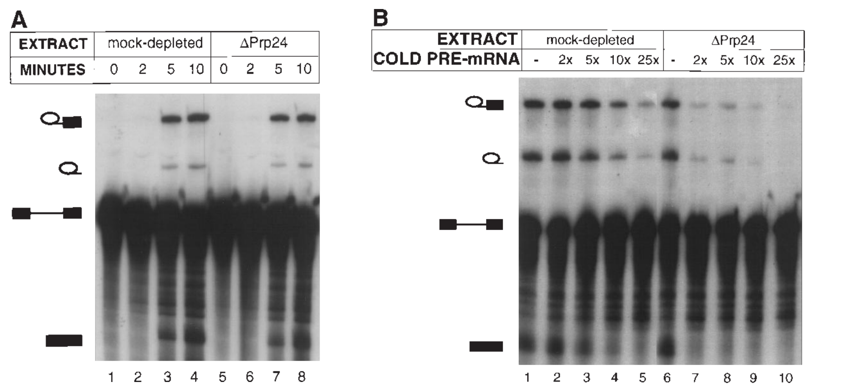

Fig. 2. Immunodepletion of Prp24 does not inhibit conventional in vitro splicing but does impede later rounds of splicing. (**A**) Time course of standard in vitro splicing reactions in mock-depleted (lanes 1 to 4) and ΔPrp24 extract (lanes 5 to 8). In vitro splicing was performed and analyzed as described (*22*), except that reaction mixtures contained 50% extract. Intermediates and products of splicing reaction from top to bottom are as follows: lariat 3′-exon, excised lariat intron, pre-mRNA, mature mRNA. (**B**) Sensitized in vitro splicing in mock-depleted (lanes 1 to 5) and ΔPrp24 extract (lanes 6 to 10). In vitro splicing assay sensitized to snRNP recycling defects: splicing reaction mixtures as in (A) contained various amounts of very low specific activity (unlabeled) actin pre-mRNA in the first incubation (10 min) and were supplemented with 0.3 nM high specific activity actin pre-mRNA in the second incubation (15 min). Reactions were analyzed as in (A). Concentrations of unlabeled pre-mRNA in reaction mixtures are indicated as fold excess cold pre-mRNA over 0.3 nM labeled pre-mRNA. When labeled and unlabeled pre-mRNAs were added simultaneously, splicing efficiency in mock and ΔPrp24 extracts was similar, indicating that preincubation is necessary for the ΔPrp24 splicing defect (*17*). Moreover, the pre-mRNA in the initial incubation mixture required intact intron consensus sequences to inhibit later rounds of splicing in ΔPrp24 extract (*17*). (**C**) Complementation of ΔPrp24 extract splicing defect with 4 nM Prp24 protein purified from yeast (lanes 6 to 10). Concentrations of unlabeled (cold) pre-mRNA in reaction mixtures are as in (B).

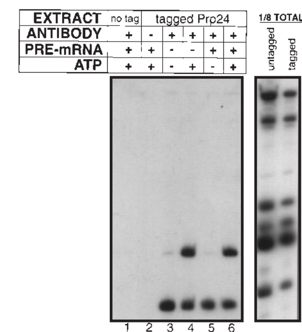

Fig. 3. Prp24 associates dynamically with free U6, free U4, and duplex U4/U6 snRNAs. Denaturing gel analysis of snRNAs coimmunoprecipitated with Prp24. Epitope-tagged Prp24-3HA was immunoprecipitated (*26*) from samples of whole cell extract incubated with or without ATP and pre-mRNA. The coprecipitated RNAs from one-half of each sample were subjected to Northern analysis on a denaturing gel (*33*). U2, U1, U5 long (U5L), U5 short (U5S), U4, and U6 snRNAs migrate at the positions indicated on the right. In untagged extract (lane 1) or in tagged extract without antibody (lane 2), no snRNAs are coimmunoprecipitated. Lanes marked "1/8 total" contain deproteinized extract equivalent to one-eighth of each immunoprecipitation sample from lane 1 (untagged) or from lanes 2 to 6 (tagged). Additional figures described in text (native gel analysis of supershifted snRNPs and nondenaturing gel analysis of snRNAs coimmunoprecipitated with Prp24) are presented online (*24*).

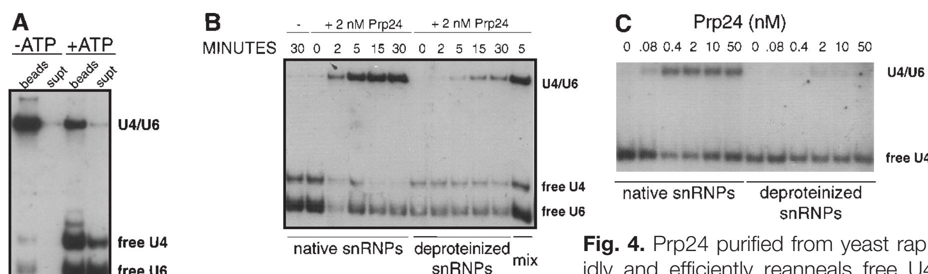

Fig. 4. Prp24 purified from yeast rapidly and efficiently reanneals free U4 and free U6 snRNPs released from duplex U4/U6. Deproteinized RNAs were analyzed on nondenaturing gels (*35*). (**A**) Dissociation of U4/U6. Brr2 snRNP complexes bound to beads contain U4/U6 duplex (−ATP); when ATP is added (+ATP), the U4/U6 helices are disrupted, and free U4 and free U6 are released into the supernatant (supt) (*36*). (**B**) Time course of annealing. Prp24 (2 nM) was added to supernatants containing ~0.4 nM free U4 and free U6 from (A) and incubated at 23°C for the indicated times. Alternatively, supernatants were treated with proteinase K beads before annealing (deproteinized snRNPs). Deproteinized snRNPs do not inhibit annealing of native snRNPs (mix), indicating that no proteinase K remains in the annealing reaction mixtures. (**C**) Titration of Prp24 protein. Annealing reaction mixtures as in (B) were incubated for 2 min at 23°C with the indicated amount of Prp24. Prp24 protein purified from *E. coli* also annealed free U4 and free U6 snRNPs (*17*).

nopurify complexes that contained U4/U6 on beads (*28*). The U4/U6 duplex bound to the beads was disrupted when ATP was added, and unpaired U4 and U6 were released into the supernatant (Fig. 4A). Prp24 rapidly converted freed U4 and U6 to a duplex U4/U6 species with a melting temperature of ~55°C, identical to native U4/U6 (*17*) (Fig. 4B). Reannealing of native snRNPs by Prp24 was complete within 5 min; however, when the released U4 and U6 snRNPs were deproteinized before incubation with Prp24, annealing was markedly slowed (Fig. 4B). Moreover, efficient reannealing of native U4 and U6 snRNPs occurred with 0.4 to 2 nM Prp24, whereas deproteinized snRNPs were poorly reannealed by 2 to 50 nM Prp24 (Fig. 4C). Thus, other proteins in the supernatants contribute to the rate of annealing. The absence of these proteins may explain why lengthy incubations (1 to 3 hours) with higher concentrations of Prp24 (80 nM) are necessary to anneal synthetic U4 and U6 RNAs (*12*). A plausible hypothesis is that the U4 and U6 RNAs are uniquely structured in the released snRNP particles. Indeed, properly packaged RNPs may be the optimal substrates for hnRNP A1 and other proteins with only modest RNA annealing activity in vitro (*11*).

The following model of snRNP recycling is consistent with our observations (Fig. 5). The U4/U6 helices are disrupted on spliceosomes (or in U4/U6.U5 snRNPs) through the action of ATP, but subsequent snRNP reassembly is ATP-independent. Preexisting Prp24 with free U6 snRNP captures the released free U4 snRNP, and Prp24 reanneals the RNAs to convert free snRNPs into duplex U4/U6 snRNPs. U4/U6 snRNPs are then reincorporated into U4/U6.U5 snRNPs, which are used in new rounds of spliceosome assembly. Because neither Prp24 protein nor U4/U6 annealing activity has been detected in the U4/U6.U5 snRNP or on spliceosomes (*29*), Prp24 is likely to leave U4/U6 when U5 joins. Spliceosome disassembly and U4/U6.U5 dissociation liberate U6*, which may then bind Prp24 to replenish the Prp24 with free U6 snRNP. In summary, Prp24 catalyzes a specific RNA annealing step necessary for spliceosomal recycling. Other dynamic RNPs in the spliceosome (*30*) and ribosome (*31*) may use similar annealing proteins to assist their structural transitions.

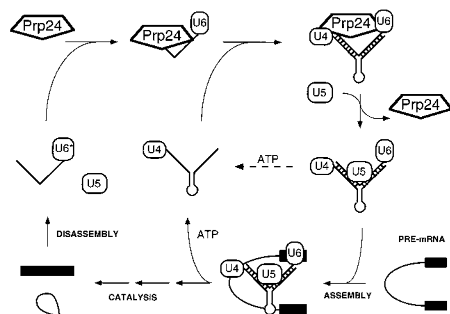

Fig. 5. Model depicting the proposed recycling role of Prp24 in pre-mRNA splicing. For simplicity, only U4, U6, and U5 snRNPs are shown. Prp24 reanneals U6* and free U4 released from spliceosomes, facilitating the regeneration of U4/U6.U5 snRNP. Some U4/U6.U5 snRNP dissociates in the presence of ATP; the resulting free U4 and U6* are similarly recycled by Prp24.

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13. A Not I site was introduced immediately upstream of the stop codon of the *PRP24* gene by polymerase chain reaction (PCR); no mutations were found in the amplified region. The 121–base pair (bp) triple hemagglutinin (3HA) tag [M. Tyers and B. Futcher, *Mol. Cell. Biol.* **13**, 5659 (1993)] and 54-bp polyoma (Pya) tag (*32*) were separately inserted into the Not I site, and the resulting *PRP24-3HA* and *PRP24-Pya* fragments were subcloned into pUN90 [S. J. Elledge and R. W. Davis, *Gene* **70**, 303]. These plasmids were transformed into PRY98 (*MAT*α *his3*Δ *trp1*Δ *lys2-801 ura3-52 ade2-101 PRP24::LYS2 pUN50-PRP24*) and were shown to complement the *PRP24::LYS2* deletion by plasmid shuffling on 5-fluoroorotic acid [C. Guthrie and G. R. Fink, Eds., *Guide to Yeast Genetics and Molecular Biology* (Academic Press, San Diego, CA, 1991)]. The resulting *PRP24-3HA* strain (PRY112, or PRY98 with *pUN90-PRP24-3HA*) was used for biochemical analysis.
14. Aliquots (100 μl) of Prp24-3HA extract were incubated with 5 μg of 12CA5 antibody (ΔPrp24) or 5 μl of phosphate-buffered saline (mock-depleted) on ice for 1 hour and then nutated for 1 hour with ~20 μl of protein A–Sepharose beads (Pharmacia) washed with buffer D [20 mM Hepes (pH 7.9), 0.2 mM EDTA, 50 mM KCl, 20% (v/v) glycerol, 0.5 mM dithiothreitol (DTT)]. The depleted supernatants lacked Prp24 and free U6 snRNP (Fig. 1A, lanes 7 to 10).
15. Yeast whole cell extracts were prepared by the liquid nitrogen method [J. G. Umen and C. Guthrie, *Genes Dev.* **9**, 855 (1995)] with modifications [A. Ansari and B. Schwer, *EMBO J.* **14**, 4001 (1995)].
16. Native gel analysis (without heparin) was modified from Konarska and Sharp [M. M. Konarska and P. A. Sharp, *Cell* **49**, 763 (1987)]. Samples (5 μl) contained 40% extract, 2 mM ATP, 2.5 mM $MgCl_2$, 3% polyethylene glycol (PEG) 8000, 60 mM potassium phosphate (pH 7), 1 mM spermidine, 4 nM actin pre-mRNA unless otherwise stated. Reaction mixtures were incubated 30 min at 23° to 25°C and loaded on a pre-run, 4% polyacrylamide (80:1) gel (15 × 15 × 0.15 cm) made up in TGM buffer (50 mM tris base, 50 mM glycine, 2 mM $MgCl_2$). Electrophoresis was for 6 to 7 hours at 160 V in TGM buffer at 4°C. Northern analysis was conducted as described (*33*). To supershift snRNP complexes formed during 23°C incubation, samples were incubated with 1 μg of 12CA5 antibody for 1 hour on ice before loading.
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18. The first 220 bp of the *PRP24* gene were PCR amplified as a Bam HI–Apa I fragment (using an internal Apa I site and a primer with a Bam HI site just upstream of the start codon). This fragment was inserted downstream of the GPD (glyceraldehyde-3-phosphate dehydrogenase) promoter of pG1 [M. Schena, D. Picard, K. R. Yamamoto, *Methods Enzymol.* **194**, 389 (1991)] along with either a 1.6-kb Apa I–Sal I

PRP24 fragment or a 1.4-kb Apa I–Sal I *PRP24-Pya* fragment (*13*). Both constructs overexpressed Prp24p when transformed into PRY98 as determined by Western blotting with anti-Prp24 polyclonal antibodies.
19. Three liters of each strain, PRY115 (PRY98 with *pG1-PRP24* as sole *PRP24* gene) and PRY116 (PRY98 with *pG1-PRP24-Pya* as sole *PRP24* gene), were harvested in late logarithmic phase. Whole cell extract was prepared (*15*), and 150 mg of each was subjected to 30 to 55% ammonium sulfate precipitation. The precipitates were resuspended in 40 ml of AGK 200 [10 mM Hepes (pH 7.9), 200 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, pepstatin A (1 μg/ml), leupeptin (1 μg/ml)] and incubated with 150 μl of protein G–Sepharose (washed in AGK 200) on a nutator at 4°C for 30 min. (This step removed proteins that bound nonspecifically to the resin.) The supernatants were added to 1 ml of protein G–Sepharose coupled to anti-polyoma antibodies (beads: AGK 200 = 1:1) as in (*32*). After 1.5 hours nutating at 4°C, the supernatants were removed, and the beads were washed 5 × 20 ml of AGK 700 (AGK buffer with 700 mM KCl), 1 × 15 ml of AGK 200, and 1 × 15 ml of AGK 50 (AGK buffer with 50 mM KCl). The washed beads were eluted twice by nutating at 23°C for 10 min with 2 × 400 μl (1 mg/ml) of peptide EYMPME (Glu-Tyr-Met-Pro-Met-Glu) (*32*) in AGK 50. Each eluate was dialyzed against 2 × 2 liters of buffer D at 4°C for 3.5 hours. The dialyzates were microcentrifuged 10 min, and the supernatants were collected. Approximately 15 μg of Prp24 was recovered, and no snRNAs copurified under these conditions (with a sensitivity to ~1 fmol of U6, no U6 was detected in 1 pmol of Prp24 protein).
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21. A typical splicing reaction mixture may contain ~3 nM U4 and ~6 nM U6 snRNAs, compared with 0.2 to 0.5 nM pre-mRNA. (It is not known what fraction of the snRNAs is competent for splicing.) Levels of U6 snRNA are constitutively lower in PRY112 (*13*) (Fig. 3), so sensitivity to snRNP recycling defects may be enhanced in this extract.
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25. Supershifted free U6 snRNP did not hybridize to U4, appeared in Prp24-3HA extract and not in untagged Prp24 extract, and increased with antibody concentration. Only the faster migrating, diffuse supershift was observed at lower antibody concentrations, suggesting that multiple antibody molecules may bind the 3HA epitope (*17*).
26. Splicing reaction mixtures (80 μl) (*22*) containing extract, ATP, and unlabeled actin pre-mRNA as noted were incubated 30 min at 25°C. After nutating for 1 hour at 4°C with 3.2 μg of 12CA5 antibody and 400 μl of NET 50 [50 mM tris-HCl (pH 7.4), 50 mM NaCl, 0.05% NP-40], antibody complexes were mixed with protein A–Sepharose (Pharmacia) for 1 hour at 4°C. The bound complexes were washed three times with 800 μl of NET 50, extracted on ice twice with phenol chloroform, and ethanol-precipitated to isolate RNAs.
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29. On a glycerol gradient, the vast majority of Prp24-3HA comigrates with U6 and U4/U6 snRNPs and away from triple snRNPs and spliceosomes (*17*). Also, Prp24 is not detected in Prp8-3HA immunoprecipitates that contain U4, U6, and U5 snRNAs (*17*). Thus, we have no evidence indicating that Prp24 is a component of U4/U6.U5 snRNPs or of the spliceosome.
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34. Blots were stripped for 1 hour at 65°C in 0.9 M NaCl and 0.09 M sodium citrate containing 0.2% SDS to remove probes. Subsequent probings were performed as described (*33*).
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36. U4/U6 snRNPs were coimmunoprecipitated with polyoma-tagged Brr2 (*28*). The bound complexes were washed with 3 × 500 μl of NET 50 and then incubated for 10 min at 23°C with 150 μl of buffer (40% buffer D, 2.5 mM $MgCl_2$, 3% PEG 8000, 60 mM potassium phosphate, with or without 2 mM ATP). The supernatants were separated from the beads, and the beads were washed with 1 × 500 μl of NET 50. For Fig. 4A, the samples were directly deproteinized for nondenaturing gel analysis of U4 and U6 snRNAs. For Fig. 4, B and C, 1 μl of purified Prp24 was added to 11 μl of supernatant containing ATP (~0.4 nM each U4 and U6) and incubated for 2 min at 23°C unless otherwise stated. Reactions were stopped by the addition of 200 μl of 0.3 M sodium acetate, 6 mM EDTA, and 0.5% SDS on ice. RNAs were isolated by proteinase K digestion (*3*) or by phenol chloroform extraction and ethanol precipitation. To deproteinize U4 and U6 snRNPs before annealing, 100 μl of supernatant containing ATP was incubated at 23°C for 30 min with 10 mg of proteinase K beads (Sigma) washed in buffer D. The deproteinized supernatant was carefully collected for annealing reactions.
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Fishing Down Marine Food Webs

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The mean trophic level of the species groups reported in Food and Agricultural Organization global fisheries statistics declined from 1950 to 1994. This reflects a gradual transition in landings from long-lived, high trophic level, piscivorous bottom fish toward short-lived, low trophic level invertebrates and planktivorous pelagic fish. This effect, also found to be occurring in inland fisheries, is most pronounced in the Northern Hemisphere. Fishing down food webs (that is, at lower trophic levels) leads at first to increasing catches, then to a phase transition associated with stagnating or declining catches. These results indicate that present exploitation patterns are unsustainable.

Exploitation of the ocean for fish and marine invertebrates, both wholesome and valuable products, ought to be a prosperous sector, given that capture fisheries—in contrast to agriculture and aquaculture—reap harvests that did not need to be sown. Yet marine fisheries are in a global crisis, mainly due to open access policies and subsidy-driven over-capitalization (*1*). It may be argued, however, that the global crisis is mainly one of economics or of governance, whereas the global resource base itself fluctuates naturally. Contradicting this more optimistic view, we show here that landings from global fisheries have shifted in the last 45 years from large piscivorous fishes toward smaller invertebrates and planktivorous fishes, especially in the Northern Hemisphere. This may imply major changes in the structure of marine food webs.

Two data sets were used. The first has estimates of trophic levels for 220 different species or groups of fish and invertebrates, covering all statistical categories included in the official Food and Agricultural Organization (FAO) landings statistics (*2*). We obtained these estimates from 60 published mass-balance trophic models that covered all major aquatic ecosystem types (*3, 4*). The models were constructed with the Ecopath software (*5*) and local data that included detailed diet compositions (*6*). In such models, fractional trophic levels (*7*) are estimated values, based on the diet compositions of all ecosystem components rather than assumed values; hence, their precision and accuracy are much higher than for the integer trophic level values used in

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