[17] Labeling and Characterization of Small RNAs Associated with the RNA Interference Effector Complex RITS

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Abstract

RNA interference (RNAi) is a gene silencing mechanism that acts at both the posttranscriptional and transcriptional levels. We have recently identified an RNA-containing complex, named RNA-induced transcriptional silencing (RITS), that directly links RNAi to transcriptional gene silencing in *Schizosaccharomyces pombe*. Here we review the affinity purification methods we use to isolate RITS and describe how to purify, detect, and analyze RNAs associated with this complex.

Introduction

RNA interference (RNAi) is a general silencing mechanism that is triggered by double-stranded RNA (dsRNA; Fire *et al.*, 1998; Hannon, 2002; Zamore, 2001). RNAi-based silencing mechanisms appear to be conserved in a wide variety of eukaryotes, ranging from the fission yeast *Schizosaccharomyces pombe* to mammals. RNAi is initiated by Dicer, an RNase III-like enzyme, which cleaves large dsRNAs into small RNA duplexes of ~21 nt, called small interfering RNAs (siRNAs; Bernstein *et al.*, 2001; Elbashir *et al.*, 2001; Hamilton *et al.*, 1999; Zamore *et al.*, 2000). siRNAs load onto effector complexes, the RNA-induced silencing complexes (RISCs), which contain a member of the Argonaute family of proteins. Argonaute proteins are believed to interact directly with siRNAs (Caudy *et al.*, 2002; Ishizuka *et al.*, 2002). Recognition of a target mRNA by RISC involves direct base pairing interactions between siRNAs and target mRNAs and leads to mRNA degradation or translational inactivation, depending on the degree of homology between the siRNA and the target mRNA (Doench *et al.*, 2003; Hutvagner and Zamore, 2002). In either case, protein expression is inhibited, and this process has been defined as posttranscriptional gene silencing (PTGS). In some organisms, an RNA-directed RNA polymerase (RdRp) activity has been proposed to be involved in amplifying the RNAi response by using siRNAs as primers to produce more dsRNAs (Lipardi *et al.*, 2001; Sijen *et al.*, 2001).

In addition to its role in PTGS, several studies suggest that the RNAi pathway also acts at the level of DNA and transcription (Matzke *et al.*, 2004).

0076-6879/05 $35.00

Several components of the RNAi pathway are conserved in *S. pombe*, and deletion of the genes that encode these components results in a loss of transcriptional gene silencing (TGS) at heterochromatic DNA regions (Volpe *et al.*, 2002). Moreover, the RNAi pathway is required for initiating the assembly of heterochromatin (Hall *et al.*, 2002) and can also initiate transcriptional gene silencing at an ectopic locus in response to production of a complementary hairpin RNA (Schramke and Allshire, 2003). Using *S. pombe*, we have recently identified a new type of RNAi effector complex that contains siRNAs and an Argonaute protein but induces silencing at the level of transcription (Verdel *et al.*, 2004). We have named this complex RNA-induced transcriptional silencing (RITS; Verdel *et al.*, 2004) complex. TGS induced by RITS requires Dicer and other RNAi components as well as the presence of siRNAs, suggesting that, in a similar fashion to RISC, RITS uses siRNAs to recognize genomic DNA regions that should be silenced.

The key components of the RNAi pathway are conserved in *S. pombe*. But unlike most eukaryotes, *S. pombe* possess only one copy of each Dicer, RdRp, and Argonaute (*dcr1*$^+$, *rdp1*$^+$, and *ago1*$^+$, respectively). Moreover, studies of RNAi in *S. pombe* offer the possibility of combining rapid genetic manipulations with extensive biochemical analysis, making this organism an ideal system for dissecting the mechanism of RNAi-dependent gene silencing.

Tandem affinity purification (TAP) is a powerful strategy developed by Seraphin and coworkers (Puig *et al.*, 2001), which employs two successive affinity purification steps that greatly reduce the levels of nonspecifically associated proteins. The most popular double tag used in yeast is composed of a calmodulin-binding peptide (CBP) and two protein A repeats (Gould *et al.*, 2004). We have used this strategy extensively in our laboratory to purify yeast silencing complexes (Hoppe *et al.*, 2002; Tanny *et al.*, 2004; Verdel *et al.*, 2004). Here we describe its use in purification of the RITS complex, which also contains small RNA molecules. We describe our protocols for both isolation of the RITS complex and for labeling its associated small RNAs.

Purification of the RNA-Containing Complex RITS

Strain Constructions

To obtain *S. pombe* strains expressing a protein fused to a tag, we used a PCR-based lithium acetate transformation procedure described by Bahler *et al.* (1998). Fission yeast can efficiently integrate foreign DNA in its genome by homologous recombination. Therefore, a sequence coding for a tag can be inserted in the genome together with a selective marker such as KanMX4 to obtain a strain that expresses a fusion protein with the tag at its N- or C-terminal end (Fig. 1). When the tag is inserted at the C terminus, expression

of the gene is under its own promoter (Fig. 1A). Conversely, when the tag is integrated at the N terminus, expression is under the control of the inducible *nmt1* promoter (Fig. 1B). If necessary, an N-terminally tagged protein can be expressed under the control of its own promoter (Storici *et al.*, 2001).

The purification of RITS was performed by adding the TAP tag at the C terminus of Chp1, as described in Verdel *et al.* (2004). To obtain *S. pombe* strains expressing Chp1 fused to the TAP tag, we used a PCR-based lithium acetate transformation procedure described by Bahler *et al.* (1998). The DNA cassette encoding the TAP tag and a selective marker was amplified by PCR from a pFA6 vector and used for transformation of *S. pombe*, as described previously (Bahler *et al.*, 1998).

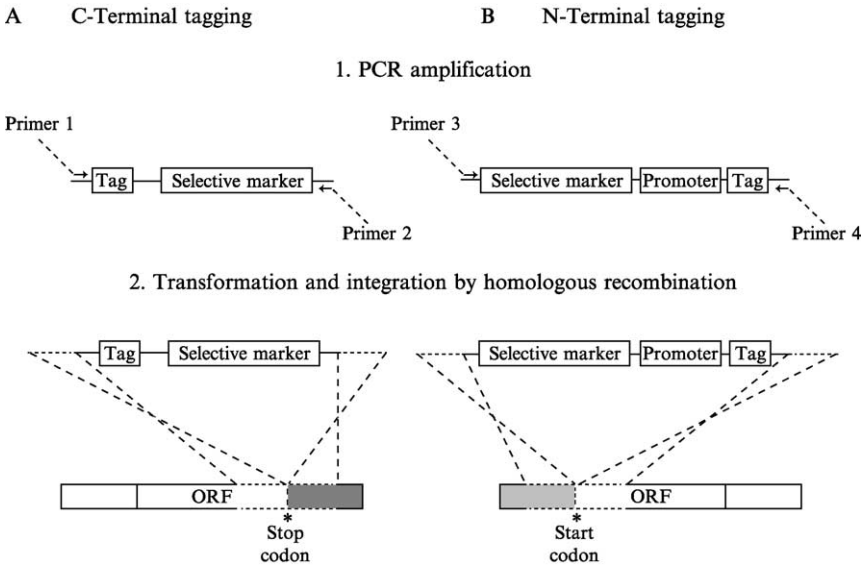

FIG. 1. PCR-based construction of yeast strains that express tagged proteins. We use gel-purified primers approximately 100 nt in length, containing 20 nt at their 3′ end required for amplification of the cassette and about 80 nt identical to the sequence present on each side of the site of integration (dashed portion of the arrows) to allow the integration of the cassette by homologous recombination. (The crossed dashed lines indicate the DNA regions involved in the homologous recombination.) After PCR and purification, the DNA product is introduced into yeast by a lithium acetate method described by Bahler *et al.* (1998) and integrated in the genome. This transformation strategy on average results in 50% positive transformants, but the proportion of positive transformants can greatly vary (from ~5 to 100%), depending on the locus. (A) C-terminal tagging. The DNA cassette that contains the tag and a selective marker is integrated downstream of the open reading frame (ORF) after the last codon and before the stop codon at the 3′ end of the gene (dark gray). (B) N-terminal tagging. The integration of the DNA cassette leads to separation of the endogenous promoter (light gray) from the ORF. Expression of the N-terminal-tagged protein is then under the control of an ectopic promoter.

Growth Conditions

We grow 3- to 12-L cultures of yeast cells, depending on the abundance of the protein (and on the amount of purified material desired). Usually, for 3- to 6-L cultures, we use 2.8-L baffled flasks containing 1.5 L of rich medium (5 mg/ml yeast extract; 30 mg/ml dextrose; and 225 mg/ml each of adenine, leucine, lysine, histidine, and uracil). Each 1.5-L culture is inoculated with 2–5 ml of an overnight preculture and allowed to grow for approximately 16 h at 30–32° with constant agitation at 225 rpm to an optical density at 600 nm (OD_{600}) of 2.0–3.0. These cultures produce typically 3–4 g of cells per liter.

For 12-L cultures, we use a fermenter. The growth medium is inoculated with 10 ml of a saturated preculture, and cells are grown at 32° with agitation at 400 rpm for approximately 20 h until they reach an OD_{600} of approximately 5. To maintain logarithmic growth, 1% dextrose is added to the culture when the OD_{600} is around 0.5. Under these growth conditions, 6–8 g of cells are produced per liter of culture. We have noticed that by using the fermenter, we can harvest *S. pombe* cells at a higher OD_{600} without affecting the subsequent lysis efficiency.

Cells are harvested by centrifugation at 7000 rpm for 8 min at room temperature (Beckman Coulter, Avanti J-20XP; rotor JLA 8.1000), washed once in approximately 10 volumes of water and harvested by centrifugation at 3000 rpm for 5 min (Sorvall RC 5C plus; rotor SLA-1500). Cell pellets are flash frozen in liquid nitrogen and stored at −80° until use.

Tandem Affinity Purification (TAP) of RITS

Cell Lysis and Cell Extract Preparation. We use two scales of TAPs. For the smaller scale, 6–12 g of cells are lysed. Frozen cell pellets are first broken to obtain a fine powder by grinding for approximately 10–15 min in a prechilled mortar and pestle. The cell powder is transferred to a beaker and thawed at room temperature until the edges start to melt. One volume of room-temperature lysis and wash buffer (LW buffer; 50 m*M* HEPES-KOH, pH 7.6, 300 m*M* potassium acetate, 10% glycerol, 1 m*M* EGTA, 1 m*M* EDTA, 0.1% NP-40, 5 m*M* magnesium acetate, 1 m*M* NaF, 20 m*M* sodium β-glycerophosphate, 1 m*M* DTT, 1 m*M* PMSF and benzamidine, 1 μg/ml leupeptin, aprotinin, bestatin, and pepstatin; add DTT and protease inhibitors immediately before use) is added to resuspend the cells. This procedure appears to minimize proteolysis. The cell suspension is then divided in six cold polypropylene round-bottom Falcon™ tubes containing 2.5 ml of cold glass beads. Cells are lysed at 4° by vortexing 12 times for 30 sec with 90-sec rest intervals on ice.

When working with 40–100 g of cells, we use the Bead-Beater™ (BioSpec, Bartlesville, OK) to lyse the cells. As described previously, the frozen cell

pellets are first ground in a prechilled mortar. After filling about half the precooled 380-ml bead-beating chamber with cold glass beads, cell powder and two volumes of room-temperature LW buffer (see previous paragraph) are added. The beads are mixed to eliminate trapped air, the chamber completely filled by adding more beads if necessary, and the chamber assembled with an outside ice-water jacket. Cells are then lysed by bead beating for 8–12 cycles of 20 sec each with 40–60 sec of rest interval on ice between every cycle. The cells are transferred to cold 30-ml centrifuge tubes, and the beads are washed with one volume of cold LW buffer, which is pooled with the broken cells. The lysate is centrifuged at 40,000*g* for 25 min at 4° to eliminate cell debris (SA600, 16500 rpm), and the supernatant is used for affinity purification.

Two-Step Affinity Purification. We use a modified version of the purification method described by Seraphin and colleagues. The first affinity purification step is mixing 400 μl of IgG Sepharose™ beads (1:1 slurry with lysis buffer, Amersham Biosciences, England) with the supernatant from the previous step and incubating on a rotating platform at 4° for 1–2 h. IgG beads are prewashed three times in 1 ml of cold LW buffer (without any protease inhibitors). After incubation with extract, the beads are recovered by centrifugation at 500*g* for 3 min at 4°. Approximately 90% of the supernatant is discarded, and the rest is used to resuspend the beads to facilitate their transfer into a 10-ml PolyPrep column (Bio-Rad, Hercules, CA). The column is washed successively with 30 ml of LW buffer and 10 ml of TEV-C buffer (10 m*M* Tris-HCl, pH 8.0, 150 m*M* potassium acetate, 0.1% NP-40, 0.5 m*M* EDTA, 1 m*M* DTT; add DTT immediately before use). The tagged protein is eluted from the IgG Sepharose by incubation with 1 ml TEV-C buffer (added directly to column) containing 30 units of recombinant TEV (Invitrogen, Carlsbad, CA) or GST-TEV protease at room temperature for 1 h. Every 20 min, the IgG Sepharose is resuspended by pipetting. The eluate is then collected in a 15-ml conical Falcon tube, and the column is washed with 500 μl of TEV-C buffer. The eluate is diluted with two volumes of CAM-B buffer (10 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, 1 m*M* magnesium acetate, 1 m*M* imidazole, 2 m*M* $CaCl_2$, 10 m*M* β-mercaptoethanol; add β-mercaptoethanol immediately before use), and the final concentration of calcium is adjusted to 2 m*M*. The second affinity purification step is performed by adding 300 μl of washed Calmodulin Sepharose™ (Amersham Biosciences; 1:1 slurry, prewashed three times with CAM-B buffer) and incubating on a rotating platform at 4° for 1 h. The beads are poured into a PolyPrep column (Bio-Rad), and the column is washed three times with 10 ml of CAM-B buffer containing 0.1% NP-40. We have found that these washes can be done with much lower volumes of CAM-B (1–2 ml) with little or no increase in background of nonspecific proteins. To elute the bound protein from the Calmodulin Sepharose column, 200 μl of CAM-E buffer (10 m*M* Tris-HCl,

pH 8.0, 150 m*M* NaCl, 1 m*M* magnesium acetate, 1 m*M* imidazole, 10 m*M* EGTA, 10 m*M* β-mercaptoethanol; add β-mercaptoethanol immediately before use) is pipetted to the top of the column bed, this step repeated four times, and fractions 2 and 3 and 4 and 5 pooled. About 10–50% of fractions 2 and 3 (which usually contain most of the purified material) are TCA precipitated and analyzed by SDS-PAGE and silver staining (Fig. 2A) or mass spectrometry. The remainder is used for *in vitro* activity assays and can be stored at -80°.

Identification of the Purified Proteins

Two types of mass spectrometry analysis are combined to identify proteins present in our final eluate: band and mixture analysis (Peng and Gygi, 2001; Shevchenko *et al.*, 1996). For both analyses, 20–50% of the final

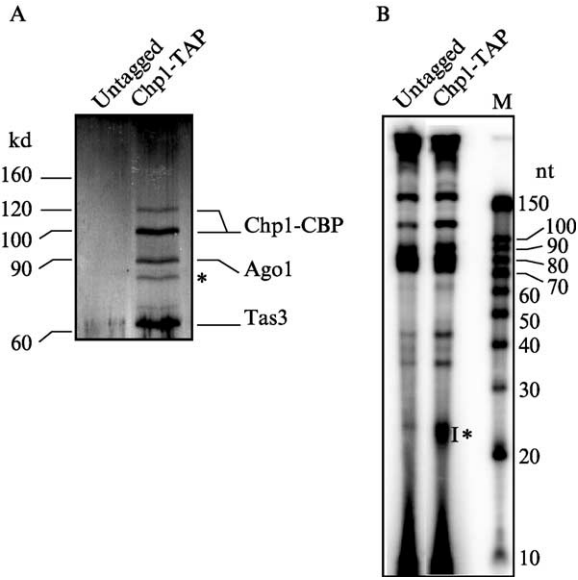

FIG. 2. Purification of the RNA-induced transcriptional silencing (RITS) complex and labeling of its associated RNAs (see Verdel *et al.*, 2004). (A) Purifications were conducted using 12 g of cells of a control wild-type strain (untagged) and a strain expressing Chp1-TAP. A total of 2.5% of pooled fractions 2 and 3 (see text for details) was run on an 8.5% SDS-PAGE. The proteins were detected by silver staining. The asterisk indicates a degradation product of Chp1. (B) Detection of RNAs present in the final eluate after the tandem affinity purification. RITS was purified from 7 g of wild-type (untagged) or Chp1-TAP cells. RNAs were then isolated from 50% of pooled fractions 2 and 3, and 3′ end-labeled with [^{32}P]-pCp as described in the text and in Verdel *et al.* (2004). Small RNAs specifically associated with Chp1-TAP are indicated by the asterisk.

eluate is TCA precipitated. TCA is added to the purified sample to a final concentration of 20%, and the mixture is incubated 20 min on ice. After spinning at 4° for 20 min at 12,000*g*, the pellet is washed with 100% cold (−20°) acetone. For mixture analysis, the pellet is then used for trypsin digestion followed by liquid chromatography and tandem mass spectrometry (LC-MS/MS; Peng and Gygi, 2001). For gel analysis, the protein pellet is resuspended in 10 μl of sample buffer, heated for 3 min at 100°, and loaded on a 4–12% acrylamide-SDS gel (Invitrogen). Proteins are then detected by silver or colloidal Coomassie staining. Protein bands are excised from the gel, digested with trypsin, and identified by mass spectrometry (LC-MS/MS; Hoppe *et al*., 2002; Shevchenko *et al*., 1996).

Detection of Small RNAs Associated with the TAP-Purified Material

Nucleic Acid Purification from the Final Eluate

To isolate any RNAs that may be specifically associated with the final eluate from the TAP purification, the purified proteins are first digested by adding proteinase K to 0.2 mg/ml and SDS to 0.1% and incubated at 37° for 45 min. The resultant peptides are eliminated by two successive extractions: the first with one volume of phenol:chloroform:isoamyl alcohol and the second with one volume of chloroform. RNAs are then precipitated by adding one-tenth volume of 3 *M* sodium acetate (pH 5.2), 20 μg glycogen, and 2.5 volumes of 100% ethanol. After incubation at −80° for 30 min, RNAs are pelleted at 4° for 20 min at 12,000*g*. The RNA pellets are resuspended in ~5 μl RNAase-free water and used for either direct detection by 3′-end labeling or analysis by Northern or Southern blotting. The RNA mixture can also be used as the starting material for constructing a library to identify specific RNAs that may be associated with a complex of interest.

3′-End Labeling and Detection of RNA

About 10–50% of the purified material is used for RNA-labeling experiments. Typically, RNAs are labeled at their 3′ end with [5′ ^{32}P]-pCp (England *et al*., 1980; Fig. 2B). The [5′ ^{32}P]-pCp labeling reaction is carried out at 4° for 16 h by incubating the purified RNAs in a 10-μl reaction containing 10% DMSO, 10 μg/ml BSA, 10 units RNase inhibitor (Roche, Switzerland), 10 μCi [5′ ^{32}P]-pCp (3000 Ci/mmol, NEN Life Sciences, Boston, MA), 1 μl T4 RNA ligase buffer (NEB) and 20 units T4 RNA ligase (NEB; Bruce and Uhlenbeck, 1978). The reaction is quenched by adding 90 μl water and precipitating the RNAs as described previously.

Alternatively, the 3′ end of the RNAs can be labeled with cordycepin (Fig. 3A; Lingner and Keller, 1993). First, the RNAs are added to a 10-μl reaction containing 2 μl Poly(A) polymerase reaction buffer (USB), 50 μCi [^{32}P]-cordycepin (5000 Ci/mmol, NEN Life Sciences), and 1000 units poly(A) polymerase. The reaction is incubated at 30° for 30 min. The reaction is then stopped by adding 90 μl water and run through a G-25 MircroSpin column (Amersham Biosciences) to eliminate nonincorporated nucleotides. The flow-through is precipitated as described previously.

Labeled small RNAs are separated on a 15% denaturing urea-acrylamide gel, and the dried gels are exposed to either a Kodak film (X-Omat AR film) or to a PhosphorImager™ (Bio-Rad) screen. In general, we observe more robust labeling with poly(A) polymerase and [^{32}P]-cordycepin than that with [5′ ^{32}P]-pCp and T4 RNA ligase (Fig. 3A).

Analyzing RNAs Associated with the Purified Complex

Northern Blot

Purified RNAs are separated on a 15% denaturing urea-acrylamide gel and electrophoretically transferred to Zeta-Probe GT membranes (Bio-Rad). RNAs are crosslinked to the membrane by UV radiation (1200 mJ/cm) and by baking the membrane at 80° for 1 h.

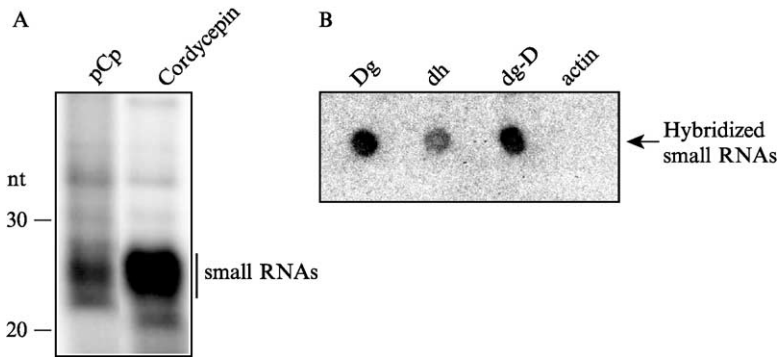

FIG. 3. Analysis of RITS siRNAs. (A) Comparison of the 3′-end labeling of siRNAs with [^{32}P]-pCp or [^{32}P]-cordycepin. RNAs associated with the purified RITS complex were labeled by [^{32}P]-pCp or [^{32}P]-cordycepin as described in the text. (B) Use of dot blot to determine whether siRNAs present in the RITS complex hybridize with selected DNA regions (Verdel *et al.*, 2004). DNA fragments from different regions of the *S. pombe* genome were bound to a nylon membrane as described in the text. Dg, dg-D, and dh DNAs represent repetitive DNA sequences present in heterochromatic regions at centromeres, and actin represents an active euchromatic DNA region.

DNA oligonucleotides to be used as probes are 5′ labeled in a 15-μl reaction containing 20 pmol oligonucleotide, 1.5 μl T4 polynucleotide kinase buffer (NEB), 75 μCi γ-[^{32}P]-ATP (6000 Ci/mmol, NEN Life Sciences), and 10 units T4 polynucleotide kinase (NEB) and incubated for 1 h at 37°. The reaction is stopped by adding 2 μl of 0.5 *M* EDTA and 33 μl of 1 *M* Tris-HCl (pH 7.5) and incubating at 68° for 15 min. Labeled oligonucleotides are separated from free label by filtering the reaction mix through a G-25 MicroSpin™ column (Amersham Biosciences).

The membrane is prehybridized for 15 min at 45° with constant rotation in 30 ml hybridization buffer (0.5 *M* sodium phosphate, pH 7.2, 1 m*M* EDTA, and 7% SDS). The hybridization step is conducted for 3 h at 45° by incubating the membrane in 10 ml hybridization buffer containing one oligonucleotide-labeling reaction. The membrane is washed once in hybridization buffer for 1 min at 25° and again for 1 min at 55° and exposed to X-ray film or a PhosphorImager screen as described previously.

Purification of Labeled RNAs and Their Use as Probes for Southern Blots

Affinity-purified RITS preparations often contain both specific and nonspecific RNAs (Fig. 2B). We use gel purification to isolate RNAs that are specifically associated with RITS. After 3′-end labeling of the RNAs present in the final elution, labeled RNAs are separated on a 15% urea-acrylamide gel. The gel is exposed to an X-ray film to locate the region of the gel containing RITS-specific RNAs. RNAs are eluted by excising the regions of interest, slicing the excised gel into smaller pieces, and agitating them at room temperature for 16 h in 300 μl of elution buffer (0.3 *M* sodium acetate, pH 5.2, 0.2% SDS and 0.05 mg/ml tRNA). RNAs are then precipitated by adding 30 μl of 0.3 *M* sodium acetate, 1 μl glycogen (20 μg/μl), and 825 μl of 100% ethanol, followed by incubation at −80° for 20–30 min. The RNA is pelleted by centrifugation at 12,000*g* for 20 min at 4° and resuspended in hybridization buffer.

For Southern blots, purified, PCR-amplified DNA is separated on an agarose gel and capillary-transferred to nylon membranes (Zeta-Probe GT, Bio-Rad) according to the manufacturer's instructions. The membrane is rinsed briefly in 2× SSC buffer, and DNA is crosslinked to the membrane as described for the Northern blot (Verdel *et al.*, 2004). For dot blots, 5.5 μg of DNA is incubated for 1 h at 60° in 200 μl of a solution containing 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, and 0.3 *M* NaOH. One volume of 6× SSC buffer is added to the DNA solution, and 2-μl aliquots are deposited on a prewetted membrane with 6× SSC. The membrane is dried at room temperature for 20 min, and the DNA is crosslinked to the membrane as

described previously. The prehybridization, hybridization, and exposure of Southern and dot blots are conducted as described previously (Fig. 3B).

Concluding Remarks

We have described strategies used in our laboratory to purify and characterize a fission yeast RNA-containing complex, RITS, which directly links the RNAi pathway to heterochromatin assembly. The strategies described here are generally applicable and have been used to study other RNA-containing complexes. The conservation of RNAi in fission yeast provides an opportunity to combine rapid genetic manipulations with affinity purification and biochemical analysis of proteins and complexes to understand how RNA-dependent mechanisms direct heterochromatin formation.

Acknowledgments

We thank Julie Huang for her comments on the manuscript and other members of the Moazed laboratory for their help and support. A. V. is a fellow of the International Human Frontier Science Program. This work is supported by grants from the NIH and a Carolyn and Peter S. Lynch Award in Cell Biology and Pathology. D. M. is a scholar of the Leukemia and Lymphoma Society.

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