that is a previously known transcription-associated kinase, Snf1, and demonstrate that histone H3 Ser-10 phosphorylation by Snf1 leads to Gcn5-mediated acetylation at the *INO1* promoter. The linked and sequential modifications are required for full *INO1* transcriptional induction. The finding that Snf1 functions as a histone kinase suggests that other transcription-linked kinases may target histones for phosphorylation. The data support a targeting model where Snf1 is recruited to certain promoters, perhaps through the function of associated subunits. We have shown that Snf1 cofractionates in an apparent multisubunit complex, which may include the previously identified activating component Snf4 and substrate targeting subunits Sip2, Sip4, and Gal83 (*27*). These latter proteins may promote association of Snf1 with activators to direct catalysis to promoter-bound histones.

The experiments reported here demonstrate an interdependent pattern of histone modifications during gene activation. There is also evidence for multiple linked modifications (deacetylation leading to methylation) occurring during heterochromatic silencing in *Schizosaccharomyces pombe* and mammalian cells (*28*, *29*). The relation between histone modifications for silencing and those required for gene activation remains to be determined, although the balance between Lys-9 methylation and Ser-10 phosphorylation could represent a critical switch.

Why are there multiple modifications? Two general mechanisms are possible for the role of modifications. In the first, the electrostatic charge alterations may serve to directly alter nucleosome structure (*30*), and dual acetylation/phosphorylation would increase negative charge beyond either modification alone. In the second model, modifications provide an interaction surface for binding of proteins, an idea advanced as the "histone code" hypothesis (*2*, *31*). Phosphorylation of serine may provide an intermediate step, as discussed above, for binding of proteins in a sequence, or in tandem with acetylation, could provide a stronger or more selective binding surface. In either mechanism, multiple modifications could provide increased combinatorial and synergistic control during gene regulation.

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32. We thank F. Winston, M. Grunstein, C. D. Allis, H. Goto, and M. Inagaki for plasmids, strains, and antibodies, and N. Keiser for assistance with construction of the K14A yeast strain. We thank P. Lieberman, G. Moore, D. Sterner, K. Wang, and members of the Berger laboratory for discussions. Supported by National Institute of General Medical Sciences (S.L.B. and R.S.) and NSF (S.L.B.).

7 May 2001; accepted 11 July 2001

Argonaute2, a Link Between Genetic and Biochemical Analyses of RNAi

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Double-stranded RNA induces potent and specific gene silencing through a process referred to as RNA interference (RNAi) or posttranscriptional gene silencing (PTGS). RNAi is mediated by RNA-induced silencing complex (RISC), a sequence-specific, multicomponent nuclease that destroys messenger RNAs homologous to the silencing trigger. RISC is known to contain short RNAs (~22 nucleotides) derived from the double-stranded RNA trigger, but the protein components of this activity are unknown. Here, we report the biochemical purification of the RNAi effector nuclease from cultured *Drosophila* cells. The active fraction contains a ribonucleoprotein complex of ~500 kilodaltons. Protein microsequencing reveals that one constituent of this complex is a member of the Argonaute family of proteins, which are essential for gene silencing in *Caenorhabditis elegans*, *Neurospora*, and *Arabidopsis*. This observation begins the process of forging links between genetic analysis of RNAi from diverse organisms and the biochemical model of RNAi that is emerging from *Drosophila* in vitro systems.

RNA interference is a process whereby double-stranded RNA (dsRNA) induces the silencing of cognate genes (*1*). Posttranscriptional silencing phenomena have also been observed in plants (e.g., PTGS) and fungi (e.g., quelling), and genetic studies indicate that these are likely to be mechanistically related to RNAi. Moreover, RNAi per se has been demonstrated in a variety of experimental systems, including insects, protozoans, and mammals (*2*).

A synthesis of in vivo and in vitro experiments has led to a mechanistic model for RNAi/PTGS. Silencing is initiated by exposure of a cell to dsRNA. This "trigger" may be introduced experimentally or may derive from endogenous sources such as viruses, transgenes, or cellular genes (e.g., transposons). Double-stranded RNAs are processed into discrete ~21- to 25-nucleotide (nt) RNA fragments known as siRNAs (small interfering RNAs) (*3*, *4*). These small RNAs join a multicomponent nuclease complex, RISC, and guide that enzyme to its substrates through conventional base-pairing interactions (*5*). Recognition of mRNAs by RISC leads to their destruction.

To date, mechanistic studies have approached RNAi/PTGS from two standpoints. Genetic studies have identified nearly a dozen genes that affect the dsRNA response. These include genes that encode putative nucleases [*mut-7* (*6*), helicases [*qde-3* (*7*), *SDE3* (*8*), *mut-6* (*9*)], RNA-dependent RNA polymerases [e.g., *ego-1* (*10*), *qde-1* (*11*), *SDE1* (*12*)/*SGS2* (*13*)], and members of the Argonaute family [*rde-1* (*14*), *qde-2* (*15*),

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AGO1 (*16*)]. Biochemical studies, carried out exclusively in extracts from *Drosophila* embryos and cultured cells, have identified enzymatic activities that are proposed to contribute to the interference process (*4, 5, 17, 18*). However, links between biochemical and genetic studies of RNAi have yet to be made.

We have sought to identify the proteins and RNAs that carry out RNAi in vitro as a step toward unifying biochemical and genetic data into a single mechanistic model. Previously, we identified a ribonuclease III family enzyme, Dicer, as a candidate for processing long dsRNA silencing triggers into ~22-nt siRNAs (*18*). Recently, a requirement for Dicer in RNAi in vivo has been demonstrated in *C. elegans* (*19*). Here we report the biochemical purification of RISC, the effector nuclease of RNAi, and the identification of one subunit of this enzyme. This protein is a member of the Argonaute family, which has been linked to RNAi through genetic studies in several experimental systems.

RNA interference can be provoked in cultured *Drosophila* S2 cells by transfection with dsRNA, or indeed by simply adding dsRNA to the culture media (*5, 20*). Extracts from such cells contain a nuclease complex, RISC, that specifically degrades mRNAs that are homologous to the dsRNA trigger. The hypothesis that this nuclease constitutes the effector activity of RNAi is strengthened by the observation that RISC cofractionates with ~22-nt RNAs that are derived directly from the silencing trigger (*5*). Furthermore, this nuclease contains an essential nucleic acid subunit, which is presumably a siRNA.

We have developed a biochemical fractionation protocol that permits the purification of RISC to near-homogeneity. Our previous studies have shown that RISC is bound to ribosomes in cell-free extracts; however, the biological relevance of this association remains to be established (*5*). Ribosomes can be concentrated from S2 lysates by high-speed centrifugation, and soluble RISC can be recovered from the ribosome pellet by extraction with high concentrations of salt.

Size fractionation of soluble RISC yielded a single peak of sequence-specific nuclease activity (Fig. 1A) (*21*). Thus, a single complex contains all the activities and information needed to identify and degrade cognate mRNAs. The large size of this complex (~500 kD) is consistent with its being composed of several subunits, which, according to our previous studies, comprise both RNA and protein. We developed a series of additional chromatographic steps that yielded a fraction with a sequence-specific nuclease activity, which was purified ~1:10,000 from the crude extract. These are outlined in Fig. 1B, and representative activity profiles from several columns are shown in Fig. 1A and Fig. 2, A to C (*21*).

Analysis of fractions from the hydroxyapatite column by SDS–polyacrylamide gel electrophoresis (PAGE) indicated that the complex had not been purified to complete homogeneity; however, several proteins clearly cofractionated with the active RISC fraction. Candidate proteins were excised from the gel and microsequenced using tandem mass spectroscopy (*22*). Two of four bands failed to produce protein sequence. However, numerous peptides were obtained from bands of ~87 and ~130 kD, which matched a single *Drosophila* gene. Database and domain searches identified this as

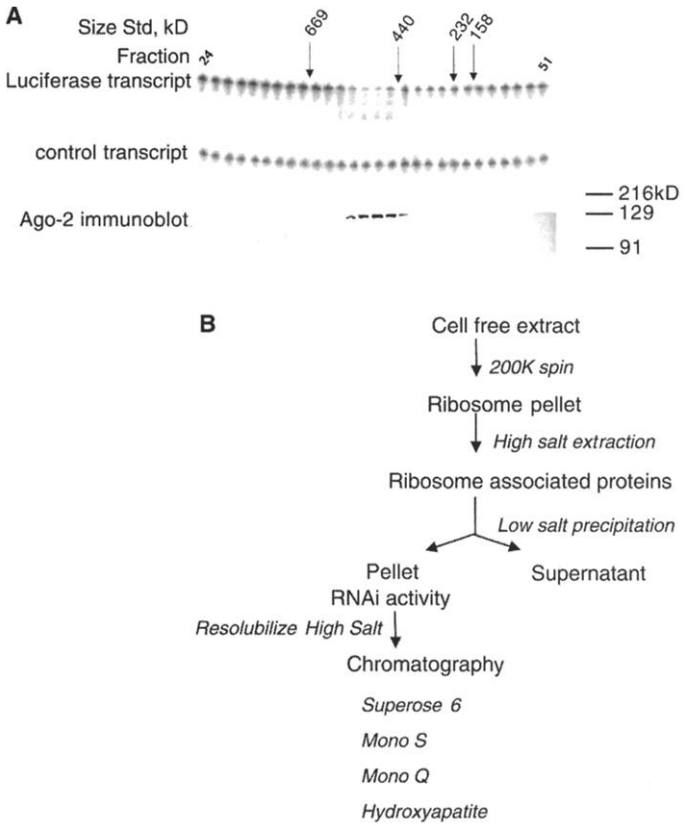

Fig. 1. (**A**) Soluble extracts were prepared from luciferase dsRNA–transfected S2 cells and fractionated on a Superose-6 sizing column. Fractions were assayed for RISC activity toward cognate luciferase mRNA and a control (cyclin E) mRNA. Fraction numbers are indicated. Size standards were used to calibrate the column; peaks of standards are indicated. Fractions were subjected to Western blotting using anti-AGO2. Western blot standards are indicated at right. (**B**) Strategy used for purification of RISC.

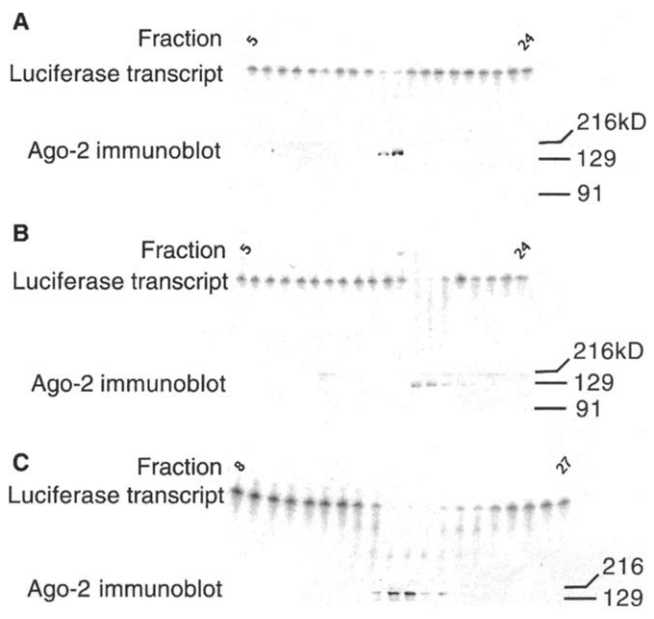

Fig. 2. (**A**) The fraction with peak activity from the Superose-6 column (Fig. 1A) was loaded on a Mono S column and eluted with a linear KCl gradient. Fractions were assayed for RISC activity and subjected to Western blotting for AGO2 protein. Western size standards are indicated on the right. (**B**) The fraction with peak activity from the Mono S column in (A) was loaded on a Mono Q column and eluted with a linear KCl gradient. Fractions were assayed for RISC activity and subjected to Western blotting for AGO2 protein. Western size standards are indicated on the right. (**C**) The activity peak from the Superose 6 column (Fig. 1A) was loaded on a hydroxyapatite column and eluted with a linear K_2HPO_4 gradient. Fractions were assayed for RISC activity and subjected to Western blotting for AGO2 protein. Western size standards are indicated on the right.

a homolog of *rde-1*, a member of the Argonaute gene family, which is essential for RNAi in *C. elegans* (*14*). We have named this gene *Argonaute2* (*AGO2*, Flybase annotation number CG7439) because of the prior assignment of *Argonaute1* to another gene in the *Drosophila* genome. Although the *Drosophila* genome contains at least four Argonaute family members—*AGO1*, *AGO2*, *Piwi*, and *Sting*—we identified only AGO2 as a component of RISC in S2 cells. However, we cannot exclude the possibility that other *Drosophila* Argonaute family members join the RISC complex in specific tissues or at specific times during development.

The sequence of AGO2 is shown in Fig. 3A, with peptides obtained from protein sequencing highlighted in red. In the sequence predicted from the *Drosophila* genome database, homology to existing Argonaute family members extended beyond the putative initiating methionine. This strongly suggested that the 5′ end of the gene was missing from the predicted sequence. To address this possibility, we constructed a λZap library from a mixture of RNAs from *Drosophila* embryos and S2 cells and identified numerous *AGO2* cDNA clones (*23*).

Sequencing revealed several discrepancies with the predicted *AGO2* gene. Sequences that had been predicted to be intronic appeared instead as part of the mature mRNA. This altered the presumptive reading frame and added a kilobase of additional coding sequence. The presence of this predicted intron in the mature *AGO2* mRNA was confirmed by Northern blotting using an oligonucleotide probe (Fig. 3B) (*24*). The major constituent of this 5′ extension is a series of 63-nt direct repeats (Fig. 3C). The precise number of copies of this repeat was variable in different, individual cDNA clones, possibly as a result of recombination during the propagation of these clones in bacteria. To the best of our ability, we resolved this problem by means of polymerase chain reaction (PCR) analysis of genomic DNA and reverse-transcription PCR of S2 cell mRNA. These experiments consistently indicated the presence of 11 repeats (Fig. 3D) (*25*). Ultimately, the analysis of the *AGO2* coding sequence predicts a protein of ~130 kD. Thus, we hypothesize that the ~87-kD band, which also yielded numerous AGO2 peptides, may have been generated by proteolysis during purification.

The 63-nt direct repeats translate into a region composed largely of glutamine residues (Fig. 3A). Similar glutamine-rich repeats are found in a number of proteins, including an *Arabidopsis* member of the Argonaute family, *AGO1* (Fig. 3E). The functional importance of this glutamine-rich domain is currently unclear. In addition, AGO2 also contains the PAZ and PIWI domains, which are recognized signatures of this gene family. Like the polyglutamine domain, the functions of the PAZ and PIWI domains are unknown.

To verify the presence of AGO2 in RISC, we generated AGO2-specific antibodies (*26*). Western blotting of chromatography column fractions with affinity-purified anti-AGO2 showed precise cofractionation of a ~130-kD AGO2 protein and the active RISC fraction through each purification step (Fig. 1A and Fig. 2, A to C). In addition, we tested the association between AGO2 and another component of RISC, the siRNAs. We constructed a version of AGO2 that was tagged at its NH_2-terminus with both a T7 epitope and polyhistidine. This was expressed in cells in which RNAi had been induced against firefly luciferase (*27*). Tagged AGO2 protein cofractionated with endogenous AGO2, and with the active RISC fraction, in the 500-kD size range (*28*). RISC was affinity-purified from cell extracts on a polyhistidine-binding resin (Talon, Clontech). Analysis of the imidazole elution profile from this column

Fig. 3. (**A**) Peptides that were obtained from microsequencing are indicated in red on the AGO2 protein sequence. (**B**) Total RNA from *Drosophila* embryos (0 to 12 hours), whole adult flies, and S2 cells was fractionated and probed with a 50-base oligonucleotide corresponding to the predicted intron. Some cross-hybridization was observed with rRNA (lower band) as a result of the low stringency necessitated by use of a short probe. (**C**) The domain structure of Argonaute2 is shown. The position of the intron, predicted in the *Drosophila* genome database, is indicated. (**D**) PCR amplification of the polyglutamine repeats was done using primers flanking the repeat region. The templates used are indicated. The size of the product indicates 11 repeats of 63 nt each, plus the primer sequence. The ladder seen in the pIZ lane was observed only in some PCR reactions and may be due to polymerase skipping. (**E**) Domain structure of Argonaute family members from several organisms. The boundaries of the piwi and PAZ domains are based on (*36*). Accession numbers: A.t. Argonaute1 (*Arabidopsis thaliana*), U91995; H.s. eIF2C (*Homo sapiens*), NM_012199; N.c. QDE-2 (*Neurospora crassa*), AF217760; C.e. RDE-1 (*Caenorhabditis elegans*), AF180730; D.m. Piwi (*Drosophila melanogaster*), AF104355; D.m. Sting, AF145680; D.m. Argonaute1, AB035447; D.m. Argonaute2, AE003530.

A

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QCVEVVLASPCHNKAIRVGR**SFFK**MSDPNNRHELDDGYEALVGLYQAFMLGDRPFLNV
DISHKSFPISMPMIEYLERFSLKAK**INNTTNLDYSR**RFLEPFLRGINVVYTPPQSFQS
APRVYRVNGLSR**APASSETFEHDGK**KVTIASYFHSRNYPLKFPQLHCLNVGSSIKSIL
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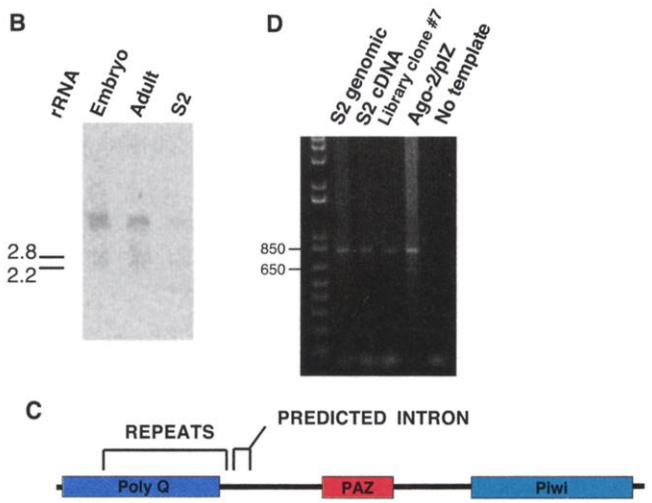

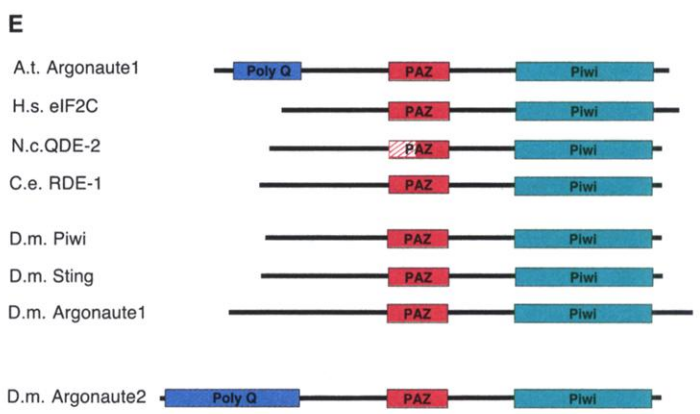

by Western blotting with a T7 antiserum and by Northern blotting with a luciferase probe indicated cofractionation of the tagged AGO2 and 22-nt siRNAs (Fig. 4A) (*27*). Considered together, our data strongly support the hypothesis that AGO2 is a component of RISC.

To test whether AGO2 is essential for RNAi in *Drosophila* S2 cells, we used RNA interference to suppress endogenous AGO2, much as we had previously done to establish a role for Dicer in RNAi (*18*). Treatment of S2 cells with either of two different ~1000-nt dsRNAs homologous to *AGO2* reduced the levels of this protein by a factor of >10 (Fig. 4B) (*29*). We assessed the ability of these cells to carry out RNAi by transfection with a mixture of firefly and *Renilla* luciferase (as an internal control) expression plasmids in combination with either a control dsRNA (green fluorescent protein, GFP) or a firefly luciferase dsRNA (Fig. 4C) (*29*). Suppression of *AGO2* expression correlated with a pronounced reduction in the ability of cells to silence an exogenous reporter by RNAi.

The biochemical function of Argonaute family members is completely unknown. However, one domain of this protein, the PAZ domain, is shared with Dicer, which initiates RNAi by processing dsRNA silencing triggers into siRNAs (*18*). We therefore considered the possibility that Dicer and AGO2 might physically interact, perhaps through their shared PAZ domains. Indeed, endogenous AGO2 can be coimmunoprecipitated with an epitope-tagged version of Dicer protein from transfected S2 cells (Fig. 4D) (*30*). We have previously shown that Dicer and RISC are biochemically separable, and none of our purified RISC fractions is able to process dsRNA into 22-nt fragments. One possibility is that Dicer is indeed a component of RISC but fails to process dsRNA when present in this complex. However, our current model is that the interaction between AGO2 and Dicer facilitates the incorporation of siRNAs into RISC complexes, which ultimately dissociate from Dicer and target cognate mRNAs for destruction.

Previous genetic studies in three organisms have indicated that Argonaute family members are essential for RNAi/PTGS. The first link between Argonaute proteins and RNAi was shown by the isolation of *C. elegans rde-1* in a screen for RNAi-deficient mutants (*14*). In *Neurospora*, another member of the Argonaute family, QDE-2, emerged from a selection of mutants that were defective in a transgene cosuppression phenomenon, termed quelling (*15*). The founding member of this family (AGO1) was first identified in *Arabidopsis* in a screen for mutants with aberrant leaf morphology (*31*). Subsequently, *ago1* was re-isolated in a screen for plants that were defective in transgene cosuppression (*16*).

Argonaute proteins are typically members of multigene families. In *Drosophila* there are four annotated genes: *Sting*, *Piwi*, *AGO1*, and *AGO2*. Mutations in three family members (*ago1*, *piwi*, and *sting*) have previously been studied. *Piwi* is required for maintenance of cell proliferation in both the male and female germ line, and *sting* mutations lead to spermatid defects and male sterility. *Ago1* was identified in a screen for mutations in the *wingless* pathway, and null mutations in this gene cause defects in neurological development (*32*).

Thus, Argonaute family members have been linked both to gene silencing phenomena and to the control of development in diverse species. The critical question is whether these two roles of Argonaute proteins are mechanistically related. It is already clear that RNAi-related silencing pathways can control the activity of endogenous genetic elements (e.g., transposons). The possibility also exists that these same pathways may control the expression of endogenous protein-coding genes that regulate development. An answer to this question is likely to emerge both from further genetic studies of RNAi pathways and from a search for endogenous targets of RISC that may be identified via its internal RNA guides to substrate selection.

Note added in proof: Recent data from Mello and colleagues (*33*) have also demonstrated a role for Dicer in RNAi. Furthermore, these investigators and Zamore and colleagues (*34*) have implicated Dicer and other components of the RNAi machinery in the regulation of developmental timing via the processing of small temporal RNAs. A role for RNAi, and in particular the Argonaute family member Sting, in control of the Stellate locus has also been described since the submission of this report (*35*).

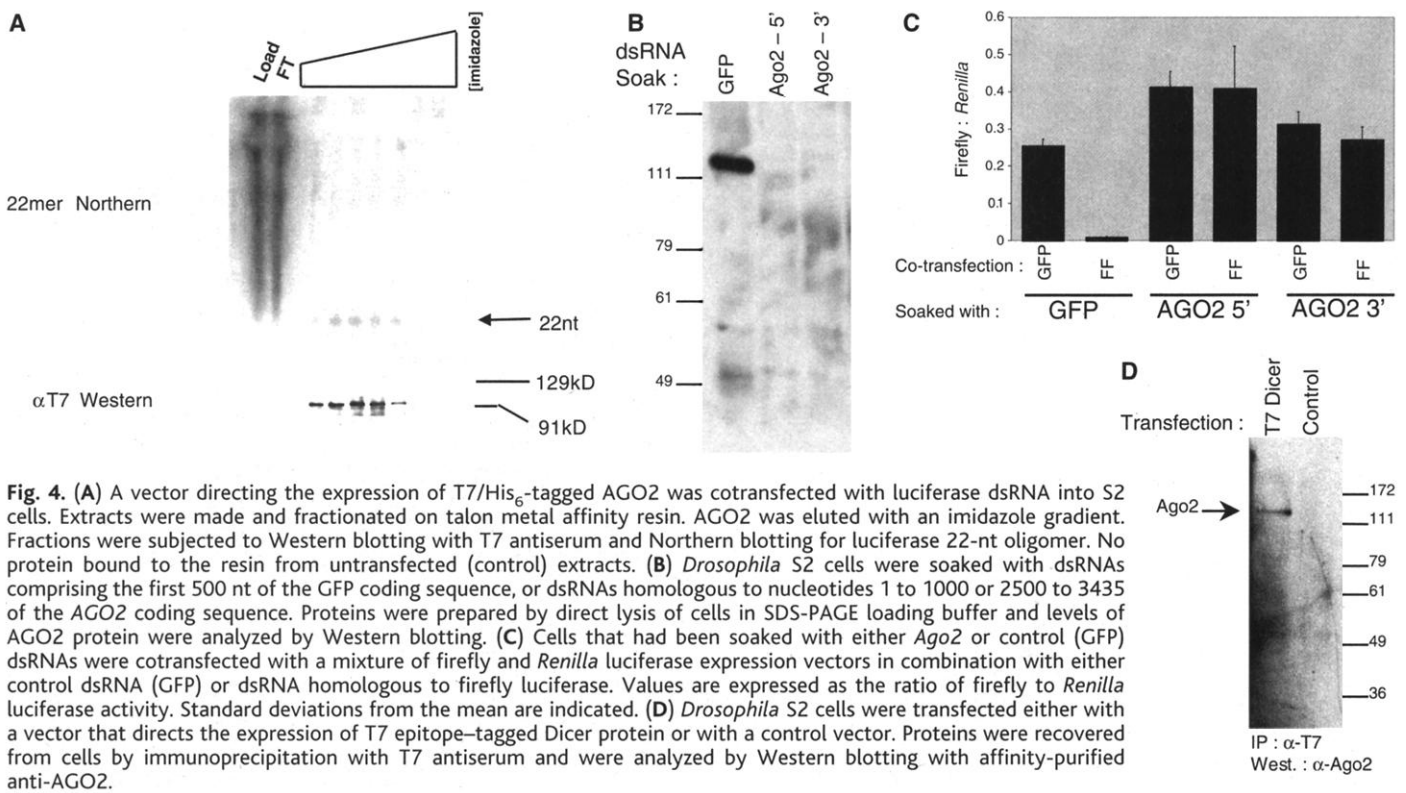

Fig. 4. (**A**) A vector directing the expression of T7/His_6-tagged AGO2 was cotransfected with luciferase dsRNA into S2 cells. Extracts were made and fractionated on talon metal affinity resin. AGO2 was eluted with an imidazole gradient. Fractions were subjected to Western blotting with T7 antiserum and Northern blotting for luciferase 22-nt oligomer. No protein bound to the resin from untransfected (control) extracts. (**B**) *Drosophila* S2 cells were soaked with dsRNAs comprising the first 500 nt of the GFP coding sequence, or dsRNAs homologous to nucleotides 1 to 1000 or 2500 to 3435 of the *AGO2* coding sequence. Proteins were prepared by direct lysis of cells in SDS-PAGE loading buffer and levels of AGO2 protein were analyzed by Western blotting. (**C**) Cells that had been soaked with either *Ago2* or control (GFP) dsRNAs were cotransfected with a mixture of firefly and *Renilla* luciferase expression vectors in combination with either control dsRNA (GFP) or dsRNA homologous to firefly luciferase. Values are expressed as the ratio of firefly to *Renilla* luciferase activity. Standard deviations from the mean are indicated. (**D**) *Drosophila* S2 cells were transfected either with a vector that directs the expression of T7 epitope–tagged Dicer protein or with a control vector. Proteins were recovered from cells by immunoprecipitation with T7 antiserum and were analyzed by Western blotting with affinity-purified anti-AGO2.

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21. S2 cells were soaked with luciferase dsRNA at 3 mg/liter and harvested after 7 days. Hypotonic extracts were spun at 200,000*g* for 3 hours to pellet ribosomes, which were resuspended and extracted in 400 mM potassium acetate (KOAc). Resultant soluble protein was precipitated by 1:4 dilution in hypotonic buffer and redissolved in buffer A [20 mM Hepes (pH 7.0), 2 mM $MgCl_2$, 2 mM dithiothreitol, and 0.5% octyl glucoside] with 400 mM KOAc. This protein was then fractionated on Superose-6 HR10/10 (Pharmacia). Fractions were assayed for RISC activity as described (*5*). The fraction showing peak activity was diluted 1:5 in buffer A and fractionated over a Mono S HR5/5 column (Pharmacia) with a linear gradient elution of buffer A with 0 to 500 mM KCl. The RISC fraction with peak activity from the Mono S column was then fractionated on a Mono Q HR5/5 column (Pharmacia); in turn, the active RISC fraction from this column was fractionated on hydroxyapatite HT (Bio-Rad) in a HR5/5 column (Pharmacia). Elution was in buffer A with 400 mM KCl, with a gradient of KH_2PO_4 from 0 to 500 mM. Fractions were analyzed on a 10% polyacrylamide gel. Four bands appeared to cofractionate with the active RISC fraction and were excised for microsequencing. Total protein was reduced by a factor of 10^6 during the entire purification. The estimated purification was 1:10,000.
22. Protein bands were digested with modified trypsin (Roche) and analyzed by liquid chromatography–mass spectrometry (LCQ MS/MS; Hewlett-Packard HP1100 connected to a Thermo-Finnigan LCQdeca electrospray ionization ion trap mass spectrometer). The acquired MS data were analyzed by SEQUEST software (Thermo-Finnigan) against the National Center for Biotechnology Information (NCBI) database.
23. Polyadenylated RNA was obtained from S2 cells and *Drosophila* 0- to 12-hour embryos using Trizol (Gibco) followed by magnetic oligo(dT) bead selection (Dynal); 2.5 μg of RNA from each source was used to construct a λZAP cDNA library using a commercial kit (Stratagene). This library was screened using a PCR probe corresponding to nucleotides 811 to 1069 in the GenBank predicted coding sequence. Two of four clones were full length; however, one had four missing NH_2-terminal polyglutamine repeats.
24. Total RNA from S2 cells, *Drosophila* 0- to 12-hour embryos, and adult whole body was fractionated on 1% agarose formaldehyde gel and transferred to Hybond N+ (Pharmacia). The membrane was hybridized with a ^{32}P-labeled oligonucleotide, CTGAGGCGGTAATGGTAATGGGGCAGCCTGCTGTTGCTGAGGTGGTCCAC.
25. PCR was done using 1 μg of S2 genomic DNA, first-strand cDNA prepared using the *C. thermus* kit (Roche), 0.001 ng of the full-length λZAP clone, or 0.001 ng of ago2/pIZ expression construct as templates. Primers were AACAGCAGGTACAAGGGTGGA and GATTGGTATTGGCCTTGGCTC. Roche taq conditions were used for 45 cycles.
26. A peptide corresponding to the eight COOH-terminal amino acids of AGO2 was conjugated to KLH and used for inoculation into rabbits for polyclonal antibody production (Covance). Antibodies were affinity-purified on a peptide-conjugated resin (Sulfolink, Pierce Biochemicals).
27. Ago2 was amplified with the primers ACCGATATCACCATGGCTAGTATGACTGGTGGTCAACAAATGGGTCACCATCACCATCATCACATGCCTTCTGTGGCATACCAC and ACCCTCGAGTCAGACAAAGTACATGGGGGT. This initiates at the second methionine codon, truncating the polyglutamine tract. The product was cloned into the expression vector pIZ (Invitrogen). S2 cells were transfected with 15 μg of luciferase dsRNA and 45 μg of ago2/pIZ per 15-cm plate, as described. Hypotonic extracts were made, adjusted to 400 mM KOAc, 0.5% octyl glucoside, and loaded on 1 ml of talon resin (Clontech) in a hi trap column (Pharmacia). Protein was eluted with an imidazole gradient, 0 to 100 mM, 10 column volumes. Protein was analyzed by Western blotting using T7 antiserum (Novagen). RNA was extracted with trizol and run on 15% acrylamide/urea/tris-acetate-EDTA gel. RNA was electroblotted as described and membranes probed with luciferase sense riboprobe (*5*).
28. S. M. Hammond, S. Boettcher, A. A. Caudy, R. Kobayashi, G. J. Hannon, data not shown.
29. *Drosophila* S2 cells were incubated with dsRNA (30 mg/liter) corresponding to either the first 500 nt of GFP or the first or last ~1000 nt of *AGO2* for 30 min in the absence of serum (*20*). Serum was added to a final concentration of 10%, and cells were cultured in suspension for 48 hours at 27°C. Cells were then divided into six-well dishes and cotransfected with a combination of *Renilla* and firefly luciferase expression vectors (3 μg total) and with dsRNA corresponding either to a control (GFP) or to firefly luciferase (75 ng). Assays for Firefly and *Renilla* luciferase were performed 36 hours after transfection. All analyses were performed on triplicate transfections. Identical experiments were also performed with *Renilla* dsRNA with a qualitatively similar outcome. For Western blotting, aliquots of soaked cells were lysed directly in SDS sample loading buffer.
30. S2 cells were transfected with a vector that directs the expression of a T7 epitope–tagged Dicer protein (*18*) or with a control vector. Cells were lysed and Dicer protein was immunoprecipitated as described (*18*). Proteins were released from Protein A–Sepharose beads by boiling in SDS sample loading buffer and were analyzed by Western blotting with the affinity-purified AGO2 antibody.
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37. We thank E. Bernstein, A. Denli, and D. Conklin for critical reading of the manuscript. S.M.H. is a visiting scientist at CSHL from Genetica Inc. A.A.C. is a George A. and Marjorie H. Anderson Fellow of the Watson School of Biological Sciences and is a predoctoral fellow of the Howard Hughes Medical Institute. Supported in part by NIH grant RO1-GM62534 (G.J.H.) and by a grant from Genetica Inc. G.J.H. is a Rita Allen Foundation scholar.

3 July 2001; accepted 11 July 2001

Transitions in Distinct Histone H3 Methylation Patterns at the Heterochromatin Domain Boundaries

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Eukaryotic genomes are organized into discrete structural and functional chromatin domains. Here, we show that distinct site-specific histone H3 methylation patterns define euchromatic and heterochromatic chromosomal domains within a 47-kilobase region of the mating-type locus in fission yeast. H3 methylated at lysine 9 (H3 Lys^9), and its interacting Swi6 protein, are strictly localized to a 20-kilobase silent heterochromatic interval. In contrast, H3 methylated at lysine 4 (H3 Lys^4) is specific to the surrounding euchromatic regions. Two inverted repeats flanking the silent interval serve as boundary elements to mark the borders between heterochromatin and euchromatin. Deletions of these boundary elements lead to spreading of H3 Lys^9 methylation and Swi6 into neighboring sequences. Furthermore, the H3 Lys^9 methylation and corresponding heterochromatin-associated complexes prevent H3 Lys^4 methylation in the silent domain.

In eukaryotes, chromosomes are partitioned into structurally and functionally distinct regions that help to separate independently regulated parts of the genome (*1*, *2*). Specialized DNA elements, known as insulators or boundary elements, have been suggested to mark the borders between adjacent chromatin domains and to act as barriers against the effects of enhancer and silencer elements from neighboring regions (*3*–*5*). The best characterized example of such long-range chromatin effects comes from studies of po-