Restricted chromosomal silencing in nucleolar dominance

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Failure of one parent's chromosomes to organize nucleoli in an interspecific hybrid is an epigenetic phenomenon known as nucleolar dominance. Selective gene silencing on a scale of millions of bp is known to be involved, but the full extent to which nucleolus organizer region (NOR)-bearing chromosomes are inactivated beyond the NORs is unknown. Aided by genome sequence data for *Arabidopsis thaliana*, we have mapped the extent of nucleolar dominance-induced silencing in *Arabidopsis suecica*, the allotetraploid hybrid of *A. thaliana* and *Arabidopsis arenosa*. Using a sensitive reverse transcription PCR assay, we show that the four *A. thaliana* NORs, each ≈4 Mbp in size, are ≈99.5% silenced in *A. suecica* vegetative leaves, whereas the NORs inherited from *A. arenosa* remain fully active. The two *A. thaliana* NORs, *NOR2* and *NOR4,* abut the telomeres on chromosomes 2 and 4, thus there are no genes distal to the NORs. The three protein-coding genes nearest *NOR4* on its centromere-proximal side, the closest of which is only 3.1 kb from rRNA gene sequences, are shown to be transcribed in the hybrid despite the silencing of the adjacent ≈4-Mbp NOR. These data argue against hypotheses in which NOR inactivation is attributed to the spread of silencing from adjacent chromosomal regions, but favor models in which NORs or rRNA genes are the targets of regulation.

epigenetic phenomena | ribosomal RNA gene | nucleolus organizer | polyploidy | genetic hybrid

Nucleolus organizer regions (NORs) are genetic loci (1) in eukaryotes at which the genes encoding the precursor of the three largest ribosomal RNAs (rRNAs) are clustered in hundreds to thousands of copies, collectively spanning millions of bp along the chromosome (2–4). Transcription of the rRNA genes by RNA polymerase I initiates the formation of a nucleolus (5), the subnuclear region where ribosomes are assembled (6–8). In interspecies (interspecific) hybrids, the NORs of one progenitor species are often inactivated, regardless of whether that species served as the maternal or paternal parent, whereas NORs inherited from the other progenitor species remain fully active (9–12). Silenced rRNA genes are efficiently derepressed by chemical inhibitors of DNA methylation or histone deacetylation, implicating chromatin modifications in the enforcement (maintenance) of nucleolar dominance (13–15). The same chromatin-mediated repression mechanisms may be responsible for controlling the effective dosage of rRNA genes in "pure" (nonhybrid) species in which not all rRNA genes are expressed at any one time (see ref. 11 for an expanded discussion). However, the mechanisms that discriminate among parental rRNA genes and that lead to the establishment of nucleolar dominance in hybrids remain obscure.

Studies of nucleolar dominance in hybrid frogs and cultured mammalian cells have suggested that parental rRNA gene types might compete for one or more RNA polymerase I transcription factors, with the dominant genes being those that can best recruit the factor(s) (12, 16). However, in plants, transient expression and *in vitro* transcription studies have failed to detect any preferential expression of dominant rRNA genes as would be predicted if dominant genes have a higher binding affinity for transcription factors (17). The latter studies have also shown that plasmid-borne rRNA genes can be efficiently transcribed in hybrid cells in which their endogenous chromosomal counterparts are repressed, indicating that nucleolar dominance is strictly a chromosomal phenomenon (17). Chromosome rearrangements that move NORs to new chromosomal environments, or that delete sequences adjacent to NORs without altering rRNA gene sequences, can also induce or negate nucleolar dominance (18–21). Collectively, these data suggest that the chromosomal context of an NOR is an important determinant of nucleolar dominance, for reasons that are currently unknown.

One hypothesis to explain the effects of chromosomal context on NOR function could be that NORs are passively inactivated as a byproduct of silencing much larger chromosome segments, with silencing then spreading to the NOR. A prediction is that genes adjacent to the NORs should also be silenced when nucleolar dominance occurs. A model system ideally suited to address this possibility is *Arabidopsis suecica*, an allotetraploid species that arose by combining diploid genome equivalents from *Arabidopsis arenosa* (also known as *Cardaminopsis arenosa*) and *Arabidopsis thaliana*. Ribosomal RNA genes of both progenitors are maintained in *A. suecica*, but S1 nuclease protection assays detect only *A. arenosa* rRNA gene transcripts; *A. thaliana* rRNA transcripts are typically not detected (14). There are two NORs in *A. thaliana*, *NOR2* and *NOR4*, located at the distal northern ends of chromosomes 2 and 4, respectively (22, 23). The NORs are capped directly by consensus telomere repeats, thus there are no genes distal to the NORs (23). Each NOR is composed of ≈350–400 rRNA genes (each ≈10–10.5 kb) organized in uninterrupted head-to-tail arrays; thus, no sequences other than rRNA genes are thought to be embedded within the NORs (24). On the centromere-proximal side of the *A. thaliana* NORs, the flanking chromosome sequences have been determined by the whole-genome sequencing effort of the *Arabidopsis* Genome Initiative (25–27). A complex jumble of incomplete retrotransposable element sequences spans the ≈50-kb region between *NOR2* and the first predicted protein-coding gene on chromosome 2 (25). However, the analogous region flanking *NOR4* is comparatively simple and amenable to study, with predicted protein coding genes located within 3.1 kb of rRNA gene sequences (Fig. 1). In this paper, we show that transcripts can be detected from the three predicted protein-coding genes adjacent to NOR4 and that these genes remain transcriptionally active in *A. suecica* despite the inactivation of the adjacent ≈4-Mbp NOR. These data argue against the possibility that silencing spreads to the NOR from adjacent regions. Instead, the data favor models in which the rRNA genes or some other feature of an NOR are the specific targets of silencing in nucleolar dominance. Furthermore, the fact that silencing is

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Abbreviations: NOR, nucleolus organizer region; RT, reverse transcription; ITS1, internal transcribed spacer 1; rRNA, ribosomal RNA.

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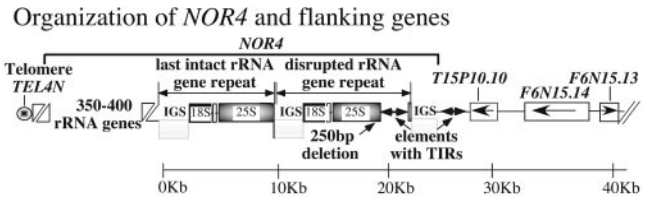

Fig. 1. Organization of the northern end of *A. thaliana* chromosome 4. Consensus telomere repeats at locus *TEL4N* are added directly to the first of ≈350–400 rRNA genes, each of which consists of coding sequences for 18S, 5.8S, and 25S rRNA and is separated from the next rRNA gene by an intergenic spacer (23). The most centromere-proximal 25S rRNA gene coding sequence is disrupted by a 2.2-kb sequence bearing 141-bp terminal inverted repeats (TIRs); we predict this to be a nonautonomous retrotransposon. The disrupted rRNA gene is followed by several kilobases of repetitive intergenic spacer (IGS) sequences. The nearest predicted protein-coding gene, *T15P10.10* is located ≈3.1 kb downstream of the final IGS repeats. A predicted 1.6-kb nonautonomous retrotransposon, characterized by discontinuous 221-bp TIRs, is present in the ≈3.1-kb interval between the IGS repeats and T15P10.10. Arrows denote the direction of transcription for loci *T15P10.10*, *F6N15.14*, and *F6N15.13*.

restricted to the NOR suggests that a barrier may exist to prevent repressive chromatin structures from being propagated from silenced rRNA genes to flanking genes.

Materials and Methods

Plant Material and Nucleic Acid Isolation. *A. suecica* accession LC1, *A. arenosa* accessions 3651 and 9810 (28, 29), and *A. thaliana* accession Col-0 (*Arabidopsis* Biological Resource Center, Ohio State University, Columbus) were grown in growth chambers or a glasshouse. Nucleic acids were typically isolated from leaf tissue pooled from 5–10 plants. Tissues were frozen in liquid nitrogen, ground to a powder, and mixed vigorously with 3 vol (wt/vol) of extraction buffer (250 mM Tris·HCl, pH 8.5/375 mM NaCl/25 mM EDTA, pH 8.0/1% SDS/1% β-mercaptoethanol/0.5 mg/ml heparin). The homogenate was extracted twice with phenol/chloroform, and total nucleic acids were precipitated from the aqeous phase with 2 vol of ethanol. After centrifugation, pellets were resuspended in sterile water and total RNA was precipitated with 3 M LiCl. Genomic DNA in the supernatant was recovered by ethanol precipitation.

PCR Assays. PCR of genomic DNA used ≈100 ng of DNA in a 50 μl reaction with 20 pmols of each primer. Reverse transcription (RT)-PCR was performed by using RNA that had been treated with RQ1 DNase I (Promega) to eliminate any contaminating genomic DNA. Total RNA was used in all cases except in the case of *F16N15.13*, for which poly(A)$^+$ RNA was isolated on oligo(dT) paramagnetic beads according to the manufacturer's instructions (Dynal, Great Neck, NY). RT reactions (20 μl) typically contained 4 μg RNA and 200 units of reverse transcriptase (GIBCO Superscript II) by using conditions recommended by the supplier. Subsequent PCR used RT product resulting from 100–400 ng of input RNA. For PCR amplification of the rRNA gene ITS1 region, 29 cycles (94°, 30 s; 59°, 30 s; 72°, 60 s) were performed with 5′-GCGCTACACTGATGTATTCAACGAG-3′ as the forward primer and 5′-CGCACCTTGCGTTCAAAGACTCGA-3′ as the reverse primer. For *F6N15.13* PCR amplification, 35 cycles were performed (94°, 15 s; 57°, 30 s; 72°, 90 s) with 5′-TCGAACAAGCTGCTCGATCTTCGCG-3′ as the forward primer and 5′-GGCGGTCGTCCCAAACAAGCCTGCC3-′ as the reverse primer. For *F6N15.14* PCR amplification, 35 cycles were performed (94°, 30 s; 62°, 30 s; 72°, 60 s) with 5′-CTCAGGAAGCACATTGTTCTGATAG-3′ as the forward primer and 5′-TTGTACTTGAAATCACTGCCTCTAG-3′ as the reverse primer. A single nucleotide polymorphism at the 3′ terminus of the *F6N15.14* forward primer prevented amplification of a paralogous gene on *A. thaliana* chromosome V. Primer specificity was verified by using the primer for genomic cycle sequencing. Two forward primers were used to amplify the *T15P10.10* gene, one specifically designed to amplify only the *A. thaliana* gene copy of interest on chromosome IV: 5′-GATTCAGAAATACTTTACTTACATGCG-3′ (A.t. primer in Fig. 5). A second forward primer amplified the *A. arenosa* gene and a paralogous gene on *A. thaliana* chromosome V: 5′-GATTCAGAAACACTTTACTTACATGCC-3′ (A.a. primer in Fig. 5). Single-nucleotide polymorphisms at the very 3′ end of the primers were exploited to achieve these specificities, which were verified by using the primers for genomic cycle sequencing. The reverse primer, used with both forward primers, was 5′-GGATATGGGCACTGCTGGTTTTG3-′). A total of 31 cycles of PCR were performed in each case (94°, 30 s; 60°, 30 s; 72°, 60 s).

PCR amplification products were resolved on 2% agarose gels in TBE (50 mM Tris·HCl/50 mM boric acid/1 mM EDTA, pH 8.0) buffer and gels were stained with ethidium bromide by using standard procedures (28). Images of stained gels were obtained by digital photography.

Accession Numbers. Overlapping annotated bacterial artificial chromosome clones that represent the junction region where NOR4 meets the centromere-proximal arm of chromosome IV are F6N15 (GenBank accession no. AF069299) and T15P10 (GenBank accession no. AF167571). Additional sequence data obtained by the *Arabidopsis* Genome Initiative but not released in GenBank (at the time of this writing) are available on request from C.S.P.

Results and Discussion

Quantitation of rRNA Silencing in Nucleolar Dominance. In a previous study (14), we showed that *A. arenosa* rRNA genes are highly expressed in *A. suecica* but that *A. thaliana* rRNA transcripts are not detectable by using S1 nuclease protection assays with end-labeled, species-specific probes. To quantify the degree to which *A. thaliana* rRNA genes are repressed in *A. suecica*, a sensitive RT-PCR assay was devised (Fig. 2). This assay exploits a polymorphic *Hha*I site in the internal transcribed spacer 1 (ITS1) region that allows *A. thaliana* and *A. arenosa* rRNA genes and their transcripts to be discriminated in their allotetraploid hybrid, *A. suecica* (Fig. 2*a*, lanes 1 and 2). Both *A. thaliana* and *A. arenosa* rRNA genes are detected in *A. suecica* after PCR of genomic DNA and digestion with *Hha*I (Fig. 2*a*, lane 3). Using the same primers for RT-PCR followed by *Hha*I digestion, rRNA transcripts from the *A. arenosa* genes are readily detected in *A. suecica* (Fig. 2*a*, compare lanes 5 and 6). By contrast, *A. thaliana* rRNA genes are repressed in *A. suecica* (Fig. 2*a*, compare lanes 4 and 6), as expected based on previous results (14). However, *A. thaliana* transcripts can be detected in trace amounts by using RT-PCR (Fig. 2*a* lane 6). In control reactions (Fig. 2*a* lanes 7–9), purified RNA not reverse transcribed into cDNA, because of the omission of reverse transcriptase from the reaction, yielded no PCR products. These control reactions show that contaminating genomic DNA is absent from the purified RNA samples and is not contributing to the signals in Fig. 2*a*, lanes 4–6.

To determine the sensitivity of detection using the PCR assay for nucleolar dominance and to obtain a quantitative estimate for the trace amounts of *A. thaliana* rRNA gene transcripts detected in *A. suecica* (Fig. 2*a*, lane 6), a titration experiment was performed (Fig. 2*b*). In this experiment, nucleic acid concentrations and all other reaction conditions were identical to those used in Fig. 2*a*, but the ratio of *A. thaliana* to *A. arenosa* RT product was varied systematically. Based on this titration experiment, the limit of detection using these PCR conditions is approximately 1 *A. thaliana* transcript per 525 *A. arenosa* rRNA

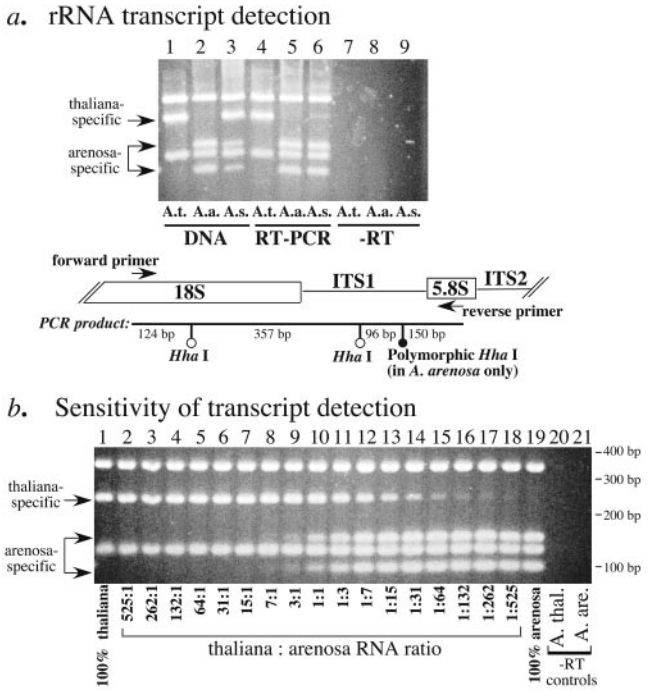

Fig. 2. *A. thaliana* rRNA genes are >99% repressed relative to *A. arenosa* rRNA genes in the allotetraploid hybrid, *A. suecica.* (*a*) Ethidium-bromide stained agarose gel showing genomic DNA (lanes 1–3) or reverse-transcribed (RT) total RNA (lanes 4–6) amplified by PCR using primers flanking internal transcribed spacer 1 (ITS1; see diagram) and then subjected to digestion with *Hha*I. An extra *Hha*I site in ITS1 of *A. arenosa* rRNA genes allows *A. arenosa* (A.a.) and *A. thaliana* (A.t.) genes and their transcripts to be discriminated in *A. suecica* (A.s). Both progenitors' rRNA genes are present in *A. suecica* (lane 3; note that *A. arenosa*-specific bands are under-represented when in competition with *A. thaliana*), but only the transcripts from the *A. arenosa* rRNA genes are abundant in the hybrid (lane 6). RNA samples from which reverse transcriptase was omitted before PCR show that RNA samples are free of contaminating DNA (lanes 7–9). (*b*) Titration experiment to determine the sensitivity of the RT-PCR assay for detection of under-dominant *A. thaliana* rRNA gene transcripts. RT product resulting from 100 ng of input total RNA was subjected to PCR in each reaction, but the ratio of *A. thaliana* to *A. arenosa* RT product was changed by symmetrical serial dilution. Conditions were identical to those used in *a*. Note that *A. thaliana* transcripts are detectable at levels as low as 1:525 relative to *A. arenosa* rRNA transcripts.

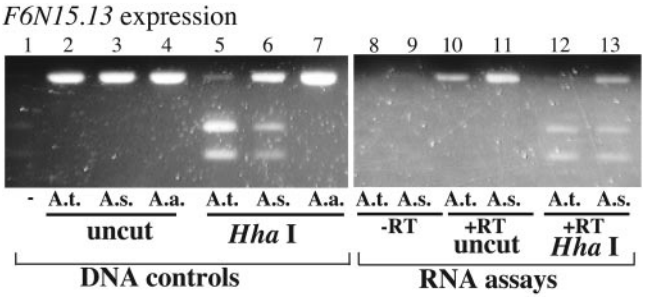

Fig. 3. *A. thaliana* locus *F6N15.13* is expressed in *A. suecica*. PCR using genomic DNA (lanes 1–7) or reverse-transcribed poly(A)$^+$ RNA (lanes 8–13) was performed by using a primer pair that amplifies a single exon. *Hha*I cuts the *A. thaliana* (A.t.) PCR products but not the *A. arenosa* (A.a) products, allowing the progenitors' genes and transcripts to be discriminated in *A. suecica* (A.s) (lanes 5–7). Note that *A. thaliana* and *A. arenosa* transcripts are both detected in *A. suecica* (lane 12). RNAs not subjected to RT served as negative control in lanes 8 and 9.

transcripts (Fig. 2*b*, lane 18). Note that PCR favors the amplification of *A. thaliana* PCR products (also true in Fig. 2*a*, lane 3) when *A. thaliana* and *A. arenosa* rRNA gene sequences are both present in the reaction. The reasons for this bias are not clear given that the primer-annealing sites are of identical sequence in the two progenitors and the PCR products are the same length. Regardless, the increased sensitivity of detection for *A. thaliana* rRNA genes is advantageous in this case given that these genes are the under-dominant (repressed) class of rRNA gene in *A. suecica.* The trace amount of the *A. thaliana*-specific band in lane 18 of Fig. 2*b* is equivalent to the trace amount detected in *A. suecica* (Fig. 2*a*, lane 6), suggesting an ≈500-fold repression of *A. thaliana* rRNA gene transcription relative to *A. arenosa* rRNA gene transcription in the hybrid. This level of repression is equivalent to expressing only ≈3 of the ≈1,500 *A. thaliana* rRNA genes (24) that are present in *A. suecica*. Note that there are four *A. thaliana*-derived NORs in *A. suecica*: two each of *NOR2* and *NOR4*, suggesting that, on average, there is less than one rRNA gene active per NOR. At present, we have no method to determine whether the small number of active *A. thaliana* rRNA genes are located at a single NOR or are dispersed among the four NORs. Considering both possibilities, we estimate that each of the ≈4-Mbp *A. thaliana* NORs in *A. suecica* are 99.2%–99.8% repressed in the vegetative leaves from which the RNA tested in Fig. 2*a* was purified.

Expression Analysis of Genes Flanking NOR4. *F6N15.13* is a predicted gene locus for which a corresponding cDNA clone (expressed sequence tag W43412) has been partially sequenced. The gene is located ≈12.3 kb from the 3′ end of *NOR4* (see Fig. 1). To determine whether *F6N15.13* is silenced in *A. suecica*, coincident with the silencing of the NOR, a primer pair that amplifies a single exon was used (Fig. 3). The primer pair amplifies *A. thaliana*, *A. arenosa*, and *A. suecica* genomic DNA sequences with similar efficiency, yielding a PCR product of identical size (Fig. 3, lanes 2–4). *Hha*I cleaves the *A. thaliana* PCR product (lane 5) but not the *A. arenosa* product (Fig. 3, lane 7), allowing transcripts of both progenitor species to be discriminated and detected in the hybrid. Using genomic DNA for conventional PCR, both progenitors' genes are present in *A. suecica*, as expected (Fig. 3, lane 6). To determine whether RNA transcripts from the *A. arenosa* and *A. thaliana* orthologs are made in *A. suecica*, RT-PCR followed by *Hha*I digestion was used. Purified RNA not reverse-transcribed into cDNA yielded no PCR products (Fig. 3, lanes 8 and 9) whereas reverse-transcribed RNA yielded the expected products (Fig. 3, lanes 10 and 11; note that RT-PCR and genomic DNA PCR products are identical in size because a single exon has been amplified). Digestion of RT-PCR products with *Hha*I showed that *A. thaliana F6N15.13* transcripts are readily detected in *A. suecica* (Fig. 3, compare lanes 12 and 13; also compare these lanes to the genomic DNA controls in lanes 5 and 6) as are transcripts from the *A. arenosa* orthologs (top band in Fig. 3, lane 13; see genomic DNA controls in lanes 6 and 7). These data indicate that silencing in nucleolar dominance does not include silencing of locus *F6N15.13*.

F6N15.14 is a predicted gene with similarity to the human breast cancer susceptibility gene *BRCA2*; it is located ≈10 kb from the NOR (see Fig. 1). A forward primer was designed that could amplify both *F6N15.14* and its *A. arenosa* homolog(s) but not a paralogous gene located on *A. thaliana* chromosome 5. The primer was made paralog-specific by having the 3′ terminal deoxynucleotide of the primer correspond to a position at which a single nucleotide polymorphism is located, such that perfect complementarity is achieved only with F6N15.14 on chromosome 4. Specificity was verified by using the oligonucleotide to prime PCR-based sequencing reactions (cycle sequencing), yielding only the sequence of the chromosome 4 paralog (data not shown). The reverse primer used for PCR spanned an exon–exon junction such that only spliced mRNA could be

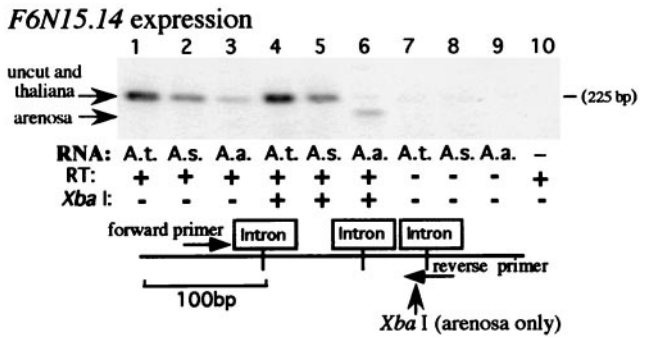

Fig. 4. Expression of *A. thaliana* locus *F6N15.14* in *A. suecica*. Using the primer pair shown in the diagram, an *Xba*I site is created in the *A. arenosa* RT-PCR product, allowing the *A. thaliana* (lanes 1 and 4) and *A. arenosa* (lanes 3 and 6) transcripts to be discriminated in *A. suecica.* Note that *A. thaliana* transcripts are detected in *A. suecica* (lane 5) but the *A. arenosa*-derived gene's transcripts are not detected (compare lanes 5 and 6). Negative control reactions are shown in lanes 7–10. Black and white were reversed in the digital image to improve the visibility of the weak bands in lanes 3 and 6.

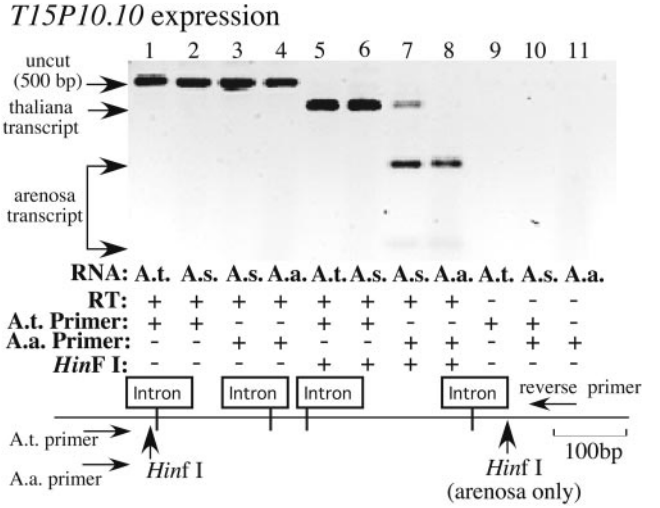

Fig. 5. *A. thaliana* locus *T15P10.10*, located only 3.1 kb from rRNA gene sequences, is expressed in *A. suecica*. A specific forward primer (A.t. primer) was designed to amplify *A. thaliana T15P10.10* transcripts but not homologous *A. arenosa* transcripts. A second primer (A.a primer) was designed to amplify the *A. arenosa* homolog. The A.a primer also amplifies an *A. thaliana* paralog located on chromosome V. A common reverse primer was used for all PCR amplifications. Lanes 1–4 show uncut RT-PCR products using the two primer combinations. Lanes 5–8 show *Hin*fI-digested RT-PCR products. *A. thaliana* products are cut once by *Hin*fI (lanes 5 and 6) whereas *A. arenosa* RT-PCR products are cut twice (lanes 7 and 8), allowing them to be discriminated in *A. suecica* (lanes 6 and 7). Note that *T15P10.10* is expressed in *A. suecica* (lane 6) as is its paralog on *A. thaliana* chromosome V (lane 7, upper-most band) and its *A. arenosa* homolog (lane 7, bottom 2 bands). Negative control reactions from which reverse transcriptase was omitted was run in lanes 9–11. Black and white were reversed in the digital image to improve the visibility of the weak bands at the bottom of lanes 7 and 8.

amplified by RT-PCR. A derived cleaved amplified polymorphic sequence (29) marker was engineered by taking advantage of a single nucleotide polymorphism between *A. thaliana* and *A. arenosa* to create an *Xba*I site only in the *A. arenosa* PCR product (Fig. 4). In the absence of *Xba*I digestion, RT-PCR using the forward and reverse primers yielded products that were the same size in the progenitor species and in the hybrid (Fig. 4, lanes 1–3). After *Xba*I digestion, the uncut *A. thaliana* product was readily detected in *A. suecica* (compare Fig. 4, lanes 4 and 5). Interestingly, the expected *A. arenosa* product that is clearly detected in the *A. arenosa* control (Fig. 4, lane 6) was not detected in the hybrid (Fig. 4, lane 5), suggesting that the *arenosa* gene(s) is not expressed in *A. suecica*. This observation might be explained by the results of a recent study showing that in *A. suecica* many orthologous genes are expressed from one or the other progenitor's genomes, but not both (30).

The predicted gene nearest to *NOR4*, *T15P10.10,* is located only 3.1 kb from the most centromere-proximal rRNA gene sequences. A specific forward primer (A.t. primer) was designed to specifically amplify *T15P10.10* but not an *A. thaliana* chromosome 5 paralog, nor the *A. arenosa* homolog(s) (Fig. 5). Primer specificity was again based on a single nucleotide polymorphism that could be exploited at a site complementary to the 3′ terminal position of the oligonucleotides and was verified by DNA cycle sequencing (data not shown). A second forward primer (A.a. primer) was designed to amplify the *A. arenosa* gene copy. This second primer also amplifies the *A. thaliana* paralog on chromosome 5 (verified by sequencing; data not shown). Fortunately, after PCR with a common reverse primer, PCR products from the *A. arenosa* and *A. thaliana* chromosome-5 homologs can be distinguished because the *A. arenosa* PCR product contains a distinctive *Hin*fI site missing in *A. thaliana* homologs. In the absence of *Hin*fI digestion, PCR products of the same size were generated by using the two specific forward primers and reverse transcribed RNA of *A. thaliana* (Fig. 5, lane 1), *A. suecica* (Fig. 5, lanes 2 and 3), or *A. arenosa* (Fig. 5, lane 4) as template. When the primer specific for *A. thaliana* T15P10.10 was used on chromosome 4, a *Hin*fI-trimmed band was detected in both *A. thaliana* and *A. suecica*, indicating that T15P10.10 is expressed in the hybrid (Fig. 5, lanes 5 and 6). When the *A. arenosa* primer was used, *A. arenosa* transcripts were also detected in *A. suecica* by virtue of their distinctive *Hin*fI digestion pattern (compare Fig. 5, lanes 7 and 8). The *A. thaliana* paralog located on chromosome V was also expressed in *A. suecica*, and its transcripts were detected by using the *A. arenosa* primer (upper-most band in Fig. 5, lane 7).

Mechanistic Implications of NOR-Restricted Chromosome Silencing. Collectively, our data show that genes located as near as 3.1 kb from a repressed NOR remain active, despite the complete, or almost complete, silencing of the adjacent ≈4 Mbp of chromosomal DNA. We conclude that silencing in nucleolar dominance is restricted to the NORs and is not part of a larger chromosome-silencing phenomenon, an example of which is the inactivation of one X chromosome in somatic cells of female mammals (31). Thus, NORs are unlikely to be repressed because of the propagation of repressive chromatin structures or silencing signals that are initiated in chromosomal regions adjacent to the NORs. Likewise, the data suggest that the nucleolar dominance-induced assembly of rRNA genes into repressive chromatin structures, as implicated by their decreased DNase sensitivity (32, 33) and by their derepression if cytosine methylation or histone deacetylation are inhibited (13), does not spread from the NOR to adjacent genes. The latter finding invites the speculation that a chromatin boundary element or insulator (34) might be located at or near the junction between the NOR and adjacent genes, thus allowing their independent regulation as separate chromatin domains. Examples of such boundary elements include sequences flanking heat-shock loci, clustered homeotic genes, and *gypsy* transposable elements in *Drosophila*, silent mating type loci in yeast, and globin and lysozyme genes in chicken (for reviews see refs. 35–38). An intriguing possibility is that the retrotransposon sequences found in numerous copies flanking *NOR2* and in two copies at the 3′ end of *NOR4* (see Fig. 1) may have insulator functions in plants analogous to the properties of *gypsy* elements in flies. Intergenic spacer sequences of the rRNA

genes themselves may also have the properties of boundary elements or insulators based on studies of *Xenopus* rRNA genes (39). These speculations will need to be tested experimentally in transgenic plants.

Several possibilities concerning the establishment of nucleolar dominance are suggested by the finding that silencing is restricted to the NORs. One hypothesis is that rRNA genes themselves are the targets of regulation and that sequence differences, presumably in the rapidly evolving intergenic spacer regions, allow dominant and under-dominant genes to be discriminated from one another. Such sequence differences in the rRNA gene spacers, which include the promoter and other regulatory sequences, could influence RNA polymerase I transcription factor binding affinities. Consistent with this hypothesis, nucleolar dominance in *Xenopus* can be mimicked in oocytes by coinjecting dominant and under-dominant rRNA genes, and spacer sequences have been shown to be responsible for this competitive effect (16). However, competitive transient expression or *in vitro* transcription experiments have thus far failed to reveal any differences in competitive strengths of rRNA gene promoters in *Arabidopsis* or its relatives in the genus *Brassica* (14, 17), making it unclear whether transcription-factor competition can explain nucleolar dominance in plants. It is possible that other properties of NORs are influenced by rRNA gene sequences in ways that affect nucleolar dominance even if dominant and under-dominant genes have identical transcription factor binding affinities. For instance, in yeast, every rRNA gene has a potential origin of replication in its intergenic spacer, although only a fraction of these origins are used in any given S phase of the cell cycle (40). Hence, a possibility is that rRNA gene sequence differences might influence replication timing such that dominant genes have first access to the transcription machinery.

A caveat to all speculations that invoke rRNA gene sequence differences as the basis for discriminating parental rRNA gene types in nucleolar dominance is that one would predict that rRNA genes would remain dominant or under-dominant independent of their chromosomal context. However, in *Drosophila* (18) and cereals (20, 21, 41), chromosome rearrangements that are not thought to alter rRNA gene sequences can have dramatic consequences, suggesting that rRNA gene sequences alone are not sufficient to explain nucleolar dominance. These considerations lead us to think that a combination of factors is likely to influence the establishment of nucleolar dominance. These might include intrinsic structural features of the NORs defined by rRNA gene sequences, sequences immediately flanking the rRNA genes (e.g., telomeres, retrotransposons, sequences between NORs and flanking genes), and broader chromosomal effects that might include signals for positioning within the three-dimensional space of the nucleus (42).

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1. McClintock, B. (1934) *Zeit. Zellforsch. Mik. Anat.* **21,** 294–328.
2. Ritossa, F. M. & Spiegelman, S. (1965) *Proc. Natl. Acad. Sci. USA* **53,** 737–745.
3. Wallace, H. & Birnstiel, M. L. (1966) *Biochem. Biophys. Acta* **114,** 296–310.
4. Phillips, R. L., Kleese, R. A. & Wang, S. S. (1971) *Chromosoma* **36,** 79–88.
5. Karpen, G. H., Schaefer, J. E. & Laird, C. D. (1988) *Genes Dev.* **2,** 1745–1763.
6. Birnstiel, M. L. (1967) *Annu. Rev. Plant Physiol.* **18,** 25–58.
7. Scheer, U. & Weisenberger, D. (1994) *Curr. Opin. Cell Biol.* **6,** 354–359.
8. Shaw, P. J. & Jordan, E. G. (1995) *Annu. Rev. Cell. Dev. Biol.* **11,** 93–121.
9. Navashin, M. (1934) *Cytologia* **5,** 169–203.
10. Pikaard, C. S. (2000) *Plant Mol. Biol.* **43,** 163–177.
11. Pikaard, C. S. (2000) *Trends Genet.* **16,** 495–500.
12. Reeder, R. H. (1985) *J. Cell Biol.* **101,** 2013–2016.
13. Chen, Z. J. & Pikaard, C. S. (1997) *Genes Dev.* **11,** 2124–2136.
14. Chen, Z. J., Comai, L. & Pikaard, C. S. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 14891–14896.
15. Viera, A., Morais, L., Barao, A., Mello-Sampayo, T. & Viegas, W. S. (1990) *Genome* **33,** 707–712.
16. Reeder, R. H. & Roan, J. G. (1984) *Cell* **38,** 39–44.
17. Frieman, M., Chen, Z. J., Saez-Vasquez, J., Shen, L. A. & Pikaard, C. S. (1999) *Genetics* **152,** 451–460.
18. Durica, D. S. & Krider, H. M. (1978) *Genetics* **89,** 37–64.
19. Neves, N., Silva, M., Heslop-Harrison, J. S. & Viegas, W. (1997) *Chromosome Res.* **5,** 125–131.
20. Schubert, I. & Kunzel, G. (1990) *Chromosoma* **99,** 352–359.
21. Nicoloff, H. (1979) *Theor. Appl. Genet.* **55,** 247–251.
22. Copenhaver, G. P., Doelling, J. H., Gens, J. S. & Pikaard, C. S. (1995) *Plant J.* **7,** 273–286.
23. Copenhaver, G. P. & Pikaard, C. S. (1996) *Plant J.* **9,** 259–272.
24. Copenhaver, G. P. & Pikaard, C. S. (1996) *Plant J.* **9,** 273–282.
25. Lin, X., Kaul, S., Rounsley, S., Shea, T. P., Benito, M. I., Town, C. D., Fujii, C. Y., Mason, T., Bowman, C. L., Barnstead, M., *et al.* (1999) *Nature (London)* **402,** 761–768.
26. Mayer, K., Schuller, C., Wambutt, R., Murphy, G., Volckaert, G., Pohl, T., Dusterhoft, A., Stiekema, W., Entian, K. D., Terryn, N., *et al.* (1999) *Nature (London)* **402,** 769–777.
27. The *Arabidopsis* Genome Initiative (2000) *Nature (London)* **408,** 796–815.
28. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
29. Neff, M. M., Neff, J. D., Chory, J. & Pepper, A. E. (1998) *Plant J.* **14,** 387–392.
30. Lee, H. S. & Chen, Z. J. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 6753–6758. (First Published May 22, 2001; 10.1073/pnas.121064698)
31. Avner, P. & Heard, E. (2001) *Nat. Rev. Genet.* **2,** 59–67.
32. Macleod, D. & Bird, A. (1982) *Cell* **29,** 211–218.
33. Thompson, W. F. & Flavell, R. B. (1988) *J. Mol. Biol.* **204,** 535–548.
34. Kellum, R. & Schedl, P. (1991) *Cell* **64,** 941–950.
35. Eissenberg, J. C. & Elgin, S. C. R. (1991) *Trends Genet.* **7,** 335–340.
36. Udvardy, A. (1999) *EMBO J.* **18,** 1–8.
37. Geyer, P. K. (1997) *Curr. Opin. Genet. Dev.* **7,** 242–248.
38. Gerasimova, T. I. & Corces, V. G. (1996) *Curr. Opin. Genet. Dev.* **6,** 185–192.
39. Robinett, C. C., O'Connor, A. & Dunaway, M. (1997) *Mol. Cell. Biol.* **17,** 2866–2875.
40. Linskens, M. H. K. & Huberman, J. A. (1988) *Mol. Cell. Biol.* **8,** 1210–1213.
41. Viera, R., Mello-Sampayo, T. & Viegas, W. (1990) *Genome* **33,** 713–718.
42. Csink, A. K. & Henikoff, S. (1996) *Nature (London)* **381,** 529–531.