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Transcription and tyranny in the nucleolus: the organization, activation, dominance and repression of ribosomal RNA genes.

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Introduction

In *Arabidopsis thaliana*, ~8 million of the genome's ~130 million basepairs are devoted to two chromosomal loci, NOR2 and NOR4. At these loci, the genes encoding the precursor transcripts of 18S, 5.8S and 25S ribosomal RNA are clustered. These gene clusters are remarkable in many ways. The ~375 ribosomal RNA (rRNA) genes at each NOR evolve rapidly, yet do so in almost perfect synchrony with one another, a phenomenon known as concerted evolution. The rRNA genes have their own transcription system, namely that of RNA polymerase I (pol I). Transcription factors of the pol I system co-evolve with the rapidly evolving promoter sequences in an evolutionary duet of molecular recognition. At times, the rRNA genes are the most active genes in the nucleus and at other times, their transcripts are virtually undetectable. When activated, rRNA genes are the driving force for the formation of one of the cell's most recognizable features, the nucleolus. Yet rRNA genes can be silenced by mysterious (epi)genetic forces of repression in response to inter-species genetic hybridization, a phenomenon known as nucleolar dominance. The wide range of biological questions for which rRNA genes are well-suited has made these genes the focus of studies in diverse eukaryotes. Research using *Arabidopsis* has contributed to the understanding of eukaryotic rRNA gene chromosomal organization, evolution, transcription and epigenetic regulation. Insights gleaned from studying *Arabidopsis* and its relatives, in the broader context of eukaryotic rRNA gene regulation, are the focus of this review.

Organization and expression of rRNA genes

rRNA genes and the nucleolus

When one peers at a cell nucleus through a microscope (or in a textbook) one's gaze is invariably drawn to its darkest, most dense feature - the nucleolus. The nucleolus is the place where ribosomes are assembled from four rRNAs, transcribed by RNA polymerase I (18S, 5.8S, 25S rRNAs) and RNA polymerase III (5S RNA), and approximately 85 proteins whose mRNAs are transcribed by RNA polymerase II (Scheer and Weisenberger, 1994; Shaw and Jordan, 1995). Ribosome assembly is orchestrated by a host of small nucleolar RNAs (snoRNAs), each associated with sets of specific proteins (Kiss, 2001; Ni et al., 1997; Tollervey and Kiss, 1997). These small nucleolar ribonucleoprotein complexes (snoRPs) mediate pre- rRNA cleavage events and specify sites of post-transcriptional modifications that include RNA methylation and pseudouridylation (Leary and Huang, 2001). It is increasingly clear that the nucleolus is a hub of intracellular trafficking and RNA processing, not only for ribosomal RNAs, but also for tRNAs and mRNAs (Pederson, 1998). The fact that the nucleolus is so dense with macromolecules, processing complexes, and ribosomal subunits in various stages of assembly is what accounts for its prominence under the microscope (Wachtler and Stahl, 1993).

Cell biologists have long been fascinated by the nucleolus, noting that its volume increases and decreases in growing and resting cells, respectively, and noting that it

forms and disperses once every cell division cycle. The cytogeneticist Heitz observed that nucleoli form at chromosomal features known as "secondary constrictions" (Heitz, 1931). These sites, seen at metaphase on chromosomes of a wide variety of eukaryotes in both the plant and animal kingdoms, get their name because they are places where chromosomes appear to be pinched, reminiscent of the way metaphase chromosomes become more narrow at centromeres (the primary constrictions). But unlike centromeres, where the pinching is due to a higher degree of chromatin compaction relative to surrounding chromatin, secondary constrictions are explained by the opposite phenomenon – they are places where the chromosomes do not fully condense at metaphase, and thus remain as thin strands of chromatin separating condensed chromosome segments (Wallace and Langridge, 1971). Barbara McClintock, studying maize, provided experimental evidence that the nucleolus is formed at the chromosomal loci where secondary constrictions occur (McClintock, 1934). Her evidence came from a X-ray-induced reciprocal chromosome translocation involving chromosomes 6 and 9. The break on chromosome 6 occurred at the place where the single nucleolus in maize normally associates with a secondary constriction. As a result of the translocation, two nucleoli were formed and these corresponded to two secondary constrictions, one on each translocated chromosome (Figure 1). These results demonstrated that the site of nucleolus formation on wild-type chromosome 6 is a specific locus made up of redundant genetic information that can be split into at least two functional segments (McClintock, 1934). McClintock's name for the locus, the "nucleolar organizer" is still in use today, though most researchers use the slightly modified term: nucleolus organizer region (abbreviated NOR).

Decades after McClintock's cytogenetic observations, biochemical fractionation procedures were devised to disrupt purified nuclei, isolate fractions enriched in nucleoli, and investigate their composition. The analyses revealed that nucleoli are rich in ribosomal RNAs, ribosomal proteins and pre-ribosomal particles, suggesting that NORs might be sites where the repetitive rRNA genes are located (Birnstiel, 1967). Supporting evidence came from the study of frogs (*Xenopus laevis*) bearing a mutation, known as *anucleolate*, which prevents the formation of nucleoli in homozygous mutants and decreases by half the number of nucleoli in heterozygotes. Molecular hybridization experiments using purified RNA and DNA showed that anucleolate frogs were deficient in rRNA production, suggesting that the mutation could be explained as a deletion of the rRNA genes (Brown and Gurdon, 1964; Wallace and Birnstiel, 1966). More direct proof awaited the advent of *in situ* hybridization. Using radioactive rRNAs hybridized to the chromosomal loci that encoded them, the NORs were indeed shown to be sites where rRNA genes are clustered (Phillips et al., 1971; Ritossa and Spiegelman, 1965).

The chromosomal organization of *Arabidopsis thaliana* rRNA genes

In all eukaryotes, the rRNA genes that comprise an NOR are arranged head-to-tail with each transcription unit separated by an intergenic spacer (Figure 2) (Brown and Dawid, 1969; Reeder, 1974). The primary transcript for the genes is a large RNA precursor that is then cleaved multiple times to release the 18S, 5.8S and 25-28S (the size depends on the species) structural rRNAs located at the heart of the ribosome. Unlike most other gene families that evolve independently, every rRNA gene remains virtually identical in sequence to every other rRNA gene within the individual and even within a population. However, when one compares the rRNA genes of different species, even species that are closely related, one finds that the rRNA genes can vary substantially, at least in the non-coding regions such as the intergenic spacer. This phenomenon of rRNA gene sequence uniformity within a species but rapid change across species boundaries is known as concerted evolution (Coen et al., 1982; Dover et al., 1982; Dover and Flavell, 1984; Flavell, 1986; Gerbi, 1985). Current thinking is that unequal crossing over and/or gene conversion events are the mechanisms that account for rRNA gene homogenization and concerted evolution (Dover, 1982), an idea for which there is some supporting evidence, especially in yeast (Klein and Petes, 1981; Petes, 1980; Szostak and Wu, 1980).

The concerted evolution of ribosomal RNA genes within a species creates a number of technical challenges that makes them difficult to study. In the case of *Arabidopsis thaliana*, cytogenetic studies had shown that there were two NORs located on chromosomes 2 and 4 (Albini, 1994; Ambros and Schweizer, 1976; Bauwens et al., 1991; Maluszynska and Heslop-Harrison, 1991; Sears and Lee-Chen, 1970) but there was a relatively long delay in placing the NORs on genetic maps due to the lack of informative RFLP markers that could discriminate ecotype-specific alleles in a mapping population. Though there are differences among rRNA genes both within an individual and within a population, these differences are mostly due to different numbers of repetitive elements in the intergenic spacers. As a result, rRNA genes can differ in overall length but have essentially identical sequence complexity.

A useful strategy for NOR mapping was ultimately devised by searching available sequences of cloned rRNA genes from the *A. thaliana* ecotype Columbia (Gruendler et al., 1991; Gruendler et al., 1989; Unfried and Gruendler, 1990; Unfried et al., 1989) for restriction endonucleases that were predicted to cut once or not at all. The logic was that some of the ~1,500 rRNA genes in a diploid might possess or lack sites predicted to be missing or unique, respectively, based on the analysis of a few cloned genes.

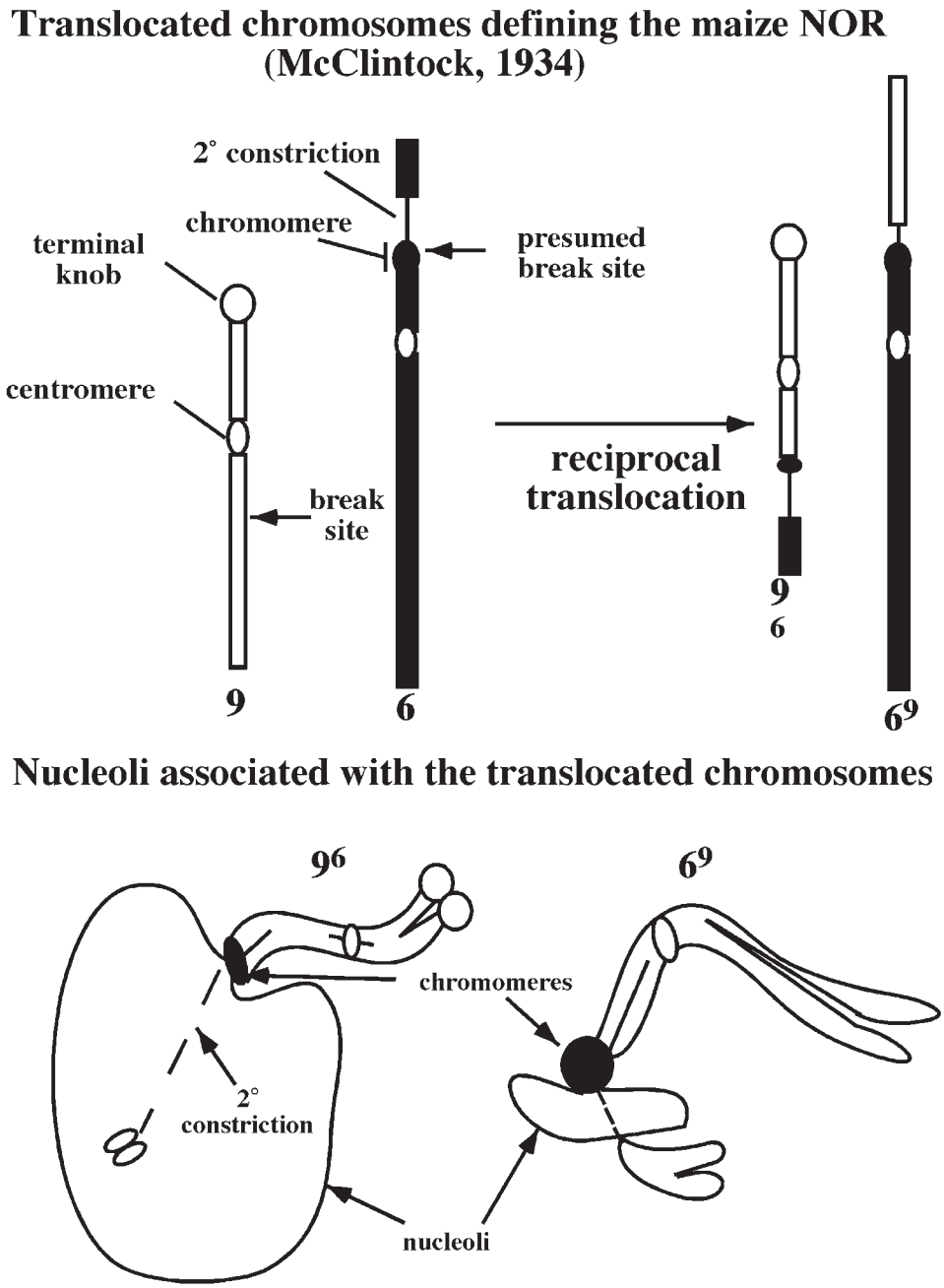

Figure 1. Diagrammatic representations of McClintock's observations that defined a specific locus on maize chromosome 6 as the nucleolus organizer region. **A.** Chromosomes 6 and 9 and their reciprocal translocation products. In wild type maize, a single nucleolus is associated with chromosome 6 on the distal side of a dark knob of heterochromatin known as a chromomere. A secondary constriction is adjacent to this chromomere at metaphase. A reciprocal translocation resulting from double-strand breaks in both chromosomes 6 and 9 produced chromosomes 9^6 and 6^9. Nucleoli are associated with both translocated chromosomes, which suggested to McClintock that the breakage site in chromosome 6 must have occurred within a nucleolar organizer whose genetic information was redundant. **B.** When together in the same microspore (shown at prophase), the 9^6 chromosome forms a larger nucleolus than does the 6^9 chromosome, which suggested to McClintock that the two NORs compete for a limiting substance. The graphics are adapted from McClintock's drawings (McClintock, 1934). This figure is reprinted, with permission from the publisher, from (Pikaard, 2000b).

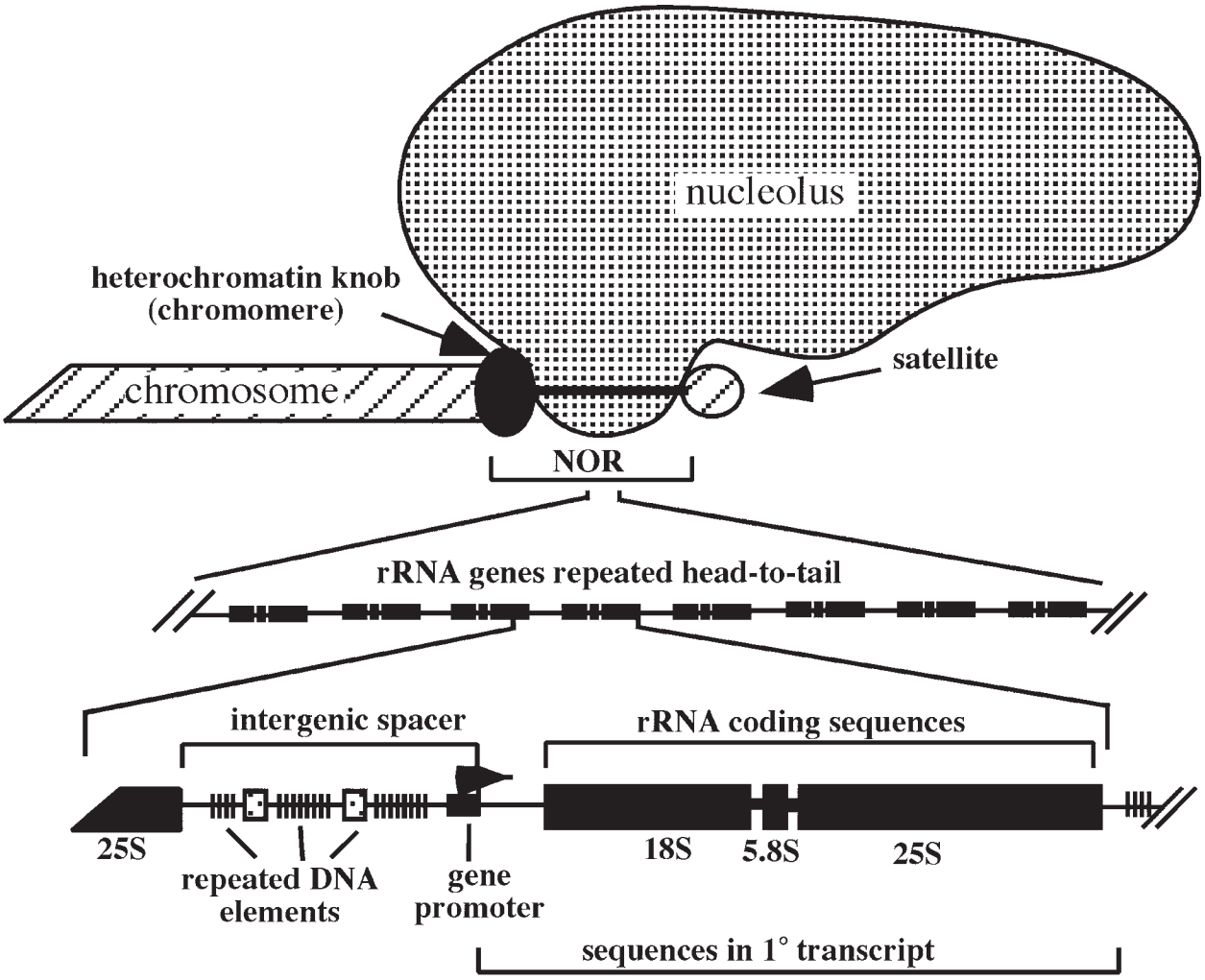

Figure 2. Organization of a generic nucleolus organizer region. NORs consist of long head-to-tail repeats of the genes encoding the precursor of the three largest ribosomal RNAs (18S, 5.8S and 25S). The NOR includes both transcriptionally active rRNA genes, which give rise to the secondary constriction on a metaphase chromosome, and silent rRNA genes which are sometimes packaged into dense heterochromatin (as in maize). At metaphase, the proteinaceous remnant of the nucleolus often remains associated with the NOR and is traversed by the secondary constriction. Within the NOR, each rRNA gene is nearly identical in sequence, though variation in the number of repeated DNA elements in the intergenic spacer is common. Intergenic spacer regions evolve rapidly whereas coding regions are highly conserved. This figure is reprinted, with minor modifications, from (Pikaard, 2000b) with permission from the publisher.

This strategy proved to be successful, ultimately identifying rare-cutting restriction endonucleases that yielded ecotype-specific restriction endonuclease fragments due to their cutting once every 10-100 genes (Copenhaver et al., 1995; Copenhaver and Pikaard, 1996a). For instance, *Hin*d III cut once in some but not all rRNA genes to yield strain-specific RFLPs of 100-700 kb (fragments 10-70 rRNA genes in size). These RFLPs were too big for conventional electrophoresis but could be visualized by pulsed-field gel electrophoresis and Southern blotting (Copenhaver et al., 1995). *Hin*d III RFLPs specific for the Columbia and Landsberg ecotypes segregated among a recombinant inbred mapping population (derived from a cross between these two ecotypes) and led to a map position for one of the NORs at the very top of chromosome 2 (Copenhaver et al., 1995) (Figure 3). This locus was named *NOR2*. Likewise, *Bst*E II polymorphisms led to the mapping of *NOR4* to the top of chromosome 4 (Copenhaver

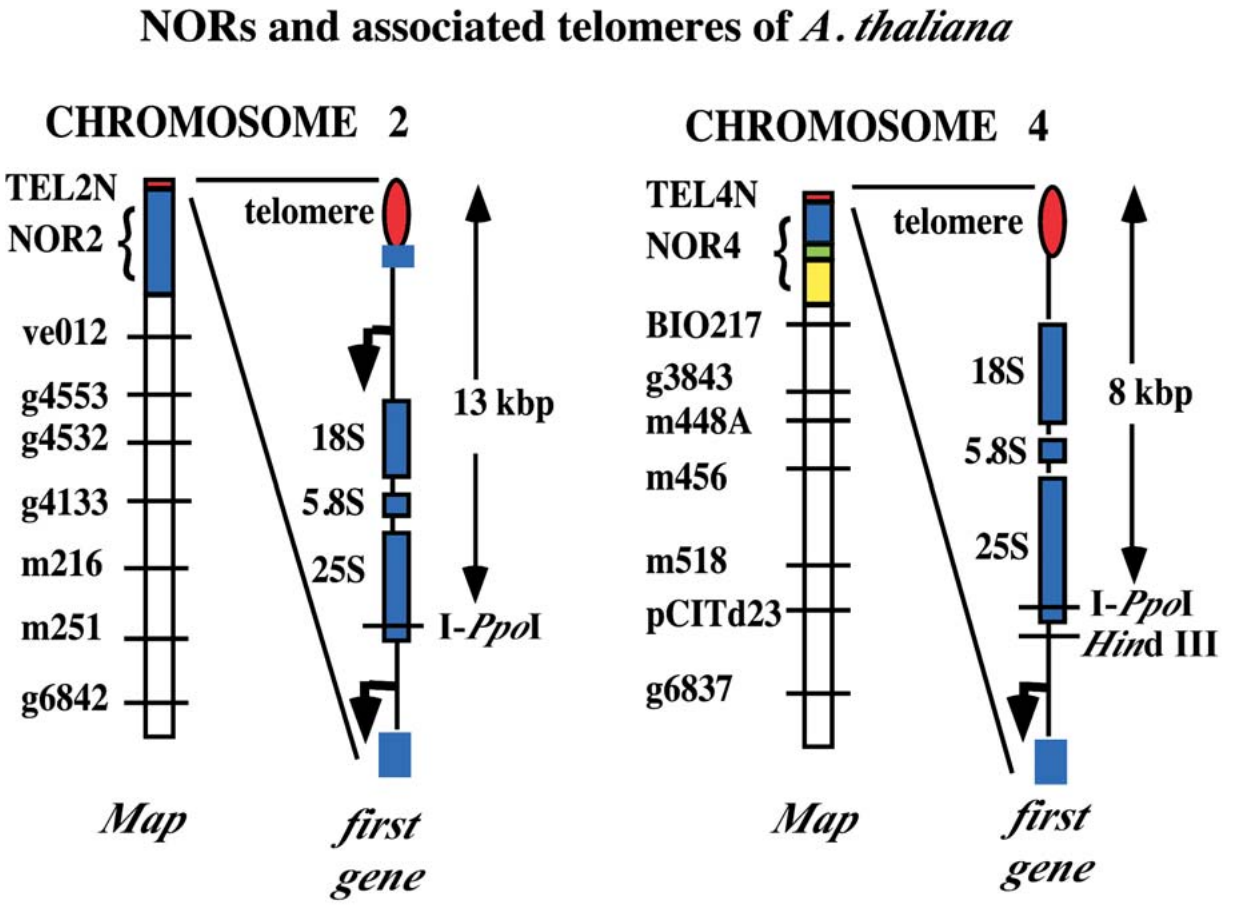

Figure 3. Organization of NORs and telomeres at the tips of *A. thaliana* chromosomes 2 and 4 (in the ecotype La-0). *NOR2* and *NOR4* are each ~4 Mbp in size, including ~350-400 rRNA genes at each locus (Copenhaver and Pikaard, 1996b). The locations of the NORs relative to other molecular markers used to map the NORs in 1996 are shown. Telomeres *TEL2N* and *TEL4N* directly abut the first (most distal) rRNA genes of *NOR2* and *NOR4.* Digestion with *I-PpoI,* an essentially rRNA-gene specific endonuclease, releases the ends of the chromosomes as 8 kb or 13 kb fragments (Copenhaver and Pikaard, 1996a). The telomere repeats join the first rRNA gene of NOR4 downstream of the gene promoter such that this gene is probably inactive. The rRNA genes at both NORs are oriented such that transcription proceeds toward the centromere. Whereas all rRNA genes at *NOR2* have relatively long intergenic spacers (blue), long, short (yellow) and intermediate-length (green) variants are present at *NOR4* (Copenhaver and Pikaard, 1996b). The rRNA gene length variants are not intermingled, but instead are highly clustered, suggesting local spreading of variants as the mode of gene homogenization.

and Pikaard, 1996a).

To map the NORs relative to the tips of the chromosomes, an essentially rRNA-gene-specific endonuclease, I-*Ppo*I, was exploited in combination with Southern blotting using a telomere probe (Copenhaver and Pikaard, 1996a). I-*Ppo*I is a remarkable enzyme from the slime-mold *Physarum polycephalum*. It recognizes and cleaves a 15 nucleotide sequence (CTCTCTTAAGGTAGC) in the 25S rRNA coding region to allow a self-splicing Group I intron that encodes the I-*Ppo*I enzyme to spread, by gene conversion, among rRNA genes that lack the intron (Ellison and Vogt, 1993; Muscarella et al., 1990). The I-*Ppo*I cleavage site is highly conserved in nature, and is present once per plant rRNA gene, located near the 3' end of the 25S coding sequences. I-P*po*I digestion released only two of the ten possible *A. thaliana* telomere fragments, and these fragments mapped to the tops of chromosomes 2 and 4, coincident with *NOR2* and *NOR4*, thus defining the telomere loci *TEL2N* and *TEL4N*, respectively (Copenhaver and Pikaard, 1996a). One of the telomere-rDNA junctions was cloned using PCR. Sequence analysis revealed consensus telomere repeats added directly to rRNA gene sequences, as if a chromosome break had been healed by telomere addition (Copenhaver and Pikaard, 1996a). The

degenerate telomere repeats and sub-telomeric repetitive sequences common at other chromosome ends (Richards et al., 1992) were absent at the cloned rRNA gene-associated telomere.

Only unit-length rRNA gene fragments (~10kb) were detected on Southern blots following digestion of *A. thaliana* genomic DNA with I-*Ppo*I and hybridization to an rRNA gene probe (Copenhaver and Pikaard, 1996a). This suggests that all rRNA genes are oriented in the same direction, strictly head-to-tail. By mapping the I-*Ppo*I site relative to a second restriction site at the telomere-proximal ends of the NORs (e.g. *Hin*d III at *NOR4*; see Figure 3), the rRNA genes could be oriented such that the direction of transcription is towards the centromere (Copenhaver and Pikaard, 1996a). The *Arabidopsis* genome sequencing effort subsequently showed that the rRNA genes at the centromere-proximal ends of NOR2 and NOR4 are oriented in this same direction (Lin et al., 1999; Mayer et al., 1999; The-Arabidopsis-Genome-Initiative, 2000), providing independent evidence that rRNA genes are arranged head-to-tail in only one orientation. Generation of only unit length rRNA gene fragments following I-*Ppo*I digestion suggests that there are no sequences other than rRNA genes located within the NORs. If there were, I-*Ppo*I sites would be pushed apart by the intervening DNA, yielding fragments larger than 10 kb.

With the knowledge of restriction enzymes that selectively cut NOR2 and NOR4 and the ability to resolve the large fragments by pulsed-field gel electrophoresis, it was possible to deduce the fine structure of the NORs and gain insights into how gene homogenization and concerted evolution must proceed (Copenhaver and Pikaard, 1996b). Copenhaver used two-dimensional gel techniques to determine the relative locations of four classes of rRNA gene variants in the ecotype Landsberg. These variants were defined by differences in the lengths of their intergenic spacers. He showed first that *NOR2* and *NOR4* are each approximately 3.5-4.0 Mbp in size. All the genes at

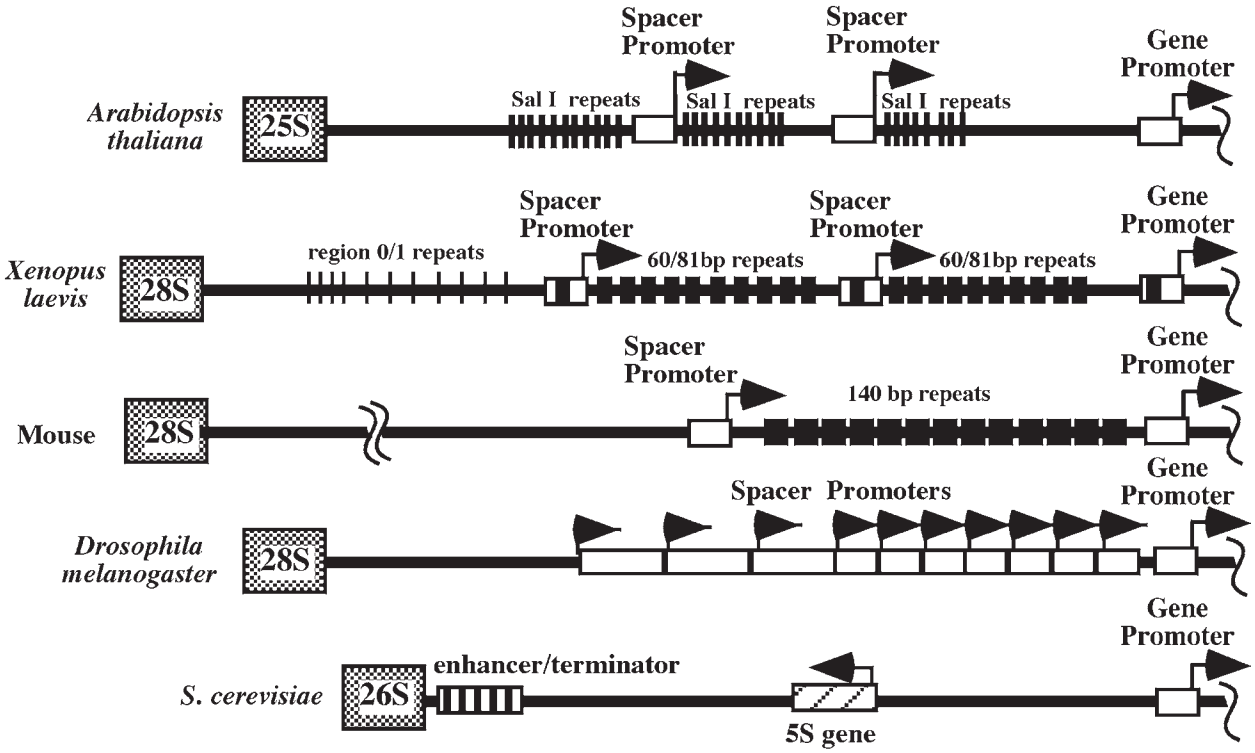

Figure 4. Comparison of eukaryotic rRNA gene intergenic spacers. The spacers of multicellular eukaryotes are typically dominated by one or more classes of repetitive elements. In *A. thaliana*, *X. laevis*, mouse and *D. melanogaster*, these include duplicated promoters known as spacer promoters. Repetitive elements that are located between the gene and spacer promoters are also found in *Arabidopsis*, *Xenopus* and mouse. *Xenopus* 60/81 bp repeats, *Arabidopsis* Sal repeats and mouse 140 bp repeats share no obvious sequence similarity, yet all display enhancer activity when attached to a *Xenopus* rRNA gene promoter and injected into *Xenopus* oocytes (Doelling et al., 1993; Pikaard et al., 1990). Note the very different organization of rRNA gene intergenic spacers in yeast, which lack prominent arrays of repetitive elements and which include a 5S RNA gene, transcribed by RNA polymerase III, positioned in opposite orientation relative to the direction of pol I transcription.

NOR2 were found to have a single intergenic spacer length whereas *NOR4* is composed of three spacer length variant classes. These different variants at *NOR4* are not intermingled or randomly distributed throughout the NOR. Instead, variants are clustered, with long variants at one end of the NOR, short variants at the other and intermediate-length variants in the middle (see NOR4 map in Figure 3). These observations suggest that the homogenization processes responsible for concerted evolution tend to act at short-range, leading to local spreading of rRNA gene variants (Copenhaver and Pikaard, 1996b). However, occasional gene conversion and/or unequal crossing over events between NORs must occur to allow for the concerted evolution of all rRNA gene sequences within the genome.

rRNA gene transcription

Ribosomal RNA genes have their own dedicated transcription system, namely that of RNA polymerase I. In actively growing cells, RNA polymerase I accounts for as much as 80% of the total transcription activity in the nucleus (Jacob, 1995; Warner, 1999). In non-growing cells, pol I transcription falls to undetectable levels. The molecular mechanisms that control the activity of pol I transcription are not fully understood, though the logic seems clear enough: actively growing cells require ribosomes to accomplish the massive amounts of protein synthesis required by the cell, whereas inactive cells are not synthesizing proteins at a rapid rate and have a lesser need for ribosomes. In prokaryotes, there is evidence that the rate of rRNA synthesis directly controls growth rate (Gourse et al., 1996). Thus pol I transcription regulation appears to be closely linked to the mechanisms that regulate cell proliferation.

Sequences that control transcription are present in the intergenic spacer

The intergenic spacers that separate adjacent rRNA coding sequences contain the crucial cis-acting DNA elements that regulate ribosomal RNA gene transcription, including the gene promoter. A comparison of eukaryotic rRNA gene intergenic spacers is shown in Figure 4. In eukaryotes other than yeast, rRNA gene intergenic spacers are typically dominated by the presence of repeated sequences. The best-studied intergenic spacer is that of *Xenopus laevis*. Functional elements that have been defined in *Xenopus* include the gene promoter, transcription terminators, 60 or 81 bp repetitive enhancer elements that stimulate transcription from the adjacent gene promoter, and duplications of the gene promoter that are known as “spacer promoters” (Reeder, 1989). The similar sizes and organizations of *Arabidopsis thaliana* and *Xenopus laevis* intergenic spacers is intriguing (Figure 4).

Promoters

Analysis of promoters directing pol I transcription is more laborious than for genes transcribed by pol II. Unlike mRNAs, pol I transcripts lack a 7-methylguanylate cap and thus are not recruited to ribosomes or translated (Rhoads, 1988). Consequently, one cannot use promoter fusions to reporter genes (e.g. GUS, GFP, luciferase) to indirectly measure rRNA gene promoter activity based on resulting enzymatic activity. Instead, one must measure rRNA transcript levels directly using techniques such as primer extension or S1 nuclease protection. Doelling devised a transient expression assay in *A. thaliana* protoplasts to show that sequences between –55 and -33 on the upstream side and +6 on the downstream side of the transcription start site (defined as +1) are sufficient to program accurate pol I transcription initiation *in vivo* (Doelling et al., 1993; Doelling and Pikaard, 1995). At the start site is a sequence that is highly conserved in plants (Barker et al., 1988; Delcasso et al., 1988; Gerstner et al., 1988; McMullen et al., 1986; Perry and Palukaitis, 1990; Piller et al., 1990; Toloczyki and Feix, 1986; Torres et al., 1989; Vincentz and Flavell, 1989; Zentgraf et al., 1990), approximating the consensus TATATA(A/G)GGG (+1 is underlined) in dicots. Mutations in this consensus region abolish or severely inhibit transcription, and sometimes alter the site of transcription initiation (Doelling and Pikaard, 1995). These observations suggest that the conserved core sequence plays a role in both start site selection and promoter strength. This led Doelling to propose (Doelling and Pikaard, 1995) that minimal rRNA gene promoters resemble promoters of those protein-coding genes that use an initiator element, or INR, at their transcription start sites {for reviews see (Gill, 1994; Roeder, 1991; Weis and Reinberg, 1992)}. Independent studies of a similar sequence element at the pol I transcription initiation site in the soil amoebae *Acanthamoeba castellanii* led to the

same conclusion (Radebaugh et al., 1997).

The function of the rRNA gene INR might be conserved in evolution given the similarity of the transcription start site in some (though not all) rRNA gene promoters. For instance, the -6 to +6 regions of *Arabidopsis thaliana* (CTATAT**A**GGGGG), *Brassica oleracea, B. rapa and B. nigra* (CTATAT**A**AGGGG), *Drosophila melanogaster* (TACTAT**A**GGTAG), *Acanthamoeba* (ATATAT**A**AAGGG), *Neurospora crassa* GTATAC**A**AGAAG), and *Homo sapiens* (TTATAT**G**CTGAC) are similar, especially upstream of the transcription start site (underlined and in bold in the sequences shown). Note that all have a TATA motif in the initiator region. Though TATA-binding protein (TBP) can interact with this sequence, it does not do so in the context of pol I transcription (Doelling and Pikaard, 1996). Therefore the identity of the pol I transcription factors that interact with the initiator region in plants and other eukaryotes remains an open question.

Unlike plants and *Acanthamoeba*, which have small rRNA gene promoters of similar size (~40-50 bp), the promoters in yeast, *Neurospora*, *Drosophila*, *Xenopus* and mammals have a bipartite structure consisting of a "core" domain and an upstream domain (for reviews see (Grummt, 1999; Hannan et al., 1998; Paule, 1994). The core promoter, from ~-40 to about +10, can direct accurate pol I transcription initiation, but only weakly. Full promoter activity requires the upstream domain, typically extending to ~ -150. No equivalent upstream domains have been revealed by transient expression or in vitro analyses of *Arabidopsis* or *Brassica* promoters (Doelling et al., 1993; Doelling and Pikaard, 1995; Doelling and Pikaard, 1996; Saez-Vasquez and Pikaard, 1997). This suggests that plants may have simple rRNA gene promoters equivalent to the core promoters in non-plant systems. However, it is interesting that the gene promoter and spacer promoters in *A. thaliana* share similarity that extends upstream to –92 even though transient expression assays have failed thus far to reveal functions for sequences upstream of -55. It is possible that a function for these additional sequences may only become apparent under different assay conditions, such as when a promoter is organized in chromatin.

Spacer promoters

In *X. laevis*, duplications of the gene promoter are located upstream of the gene promoter in the intergenic spacer (Boseley et al., 1979; Moss and Birnstiel, 1979). These spacer promoters are capable of programming pol I transcription initiation, but their transcripts are terminated by a "fail-safe terminator" element located just upstream of the gene promoter, at ~-215 (Moss, 1983). Because spacer promoter transcripts terminated in this way would never encode structural rRNAs, their function is unknown. However, there is some evidence that spacer promoters improve the performance of an adjacent gene promoter, especially in *Drosophila melanogaster* in which the intergenic spacer is composed largely of repeated spacer promoters (Grimaldi et al., 1988). *Arabidopsis thaliana* also has one or more spacer promoter located in the intergenic spacer. These spacer promoters program transcription initiation but do so only weakly, displaying ~10% of the activity of the gene promoter in the same assay (Doelling et al., 1993). *A. thaliana* spacer promoters share 90% similarity with the gene promoter from sequence positions –92 to +10. Presumably, the few nucleotide changes account for the different activities of the gene and spacer promoters, but this has not been investigated experimentally.

It is interesting that sequence analyses of intergenic spacers from other *Arabidopsis* species do not reveal any evidence for the existence of spacer promoters (Hayworth and Schaal, unpublished; Hayworth, Ph.D thesis, Washington University, St. Louis). This suggests that if spacer promoters were a feature of an ancestral rRNA gene prior to the divergence of the various species in the genus *Arabidopsis*, they have been lost in all but *A. thaliana*. A more likely explanation is that spacer promoters arose *de novo* in *A. thaliana* as a consequence of relatively recent (on an evolutionary time scale) promoter duplication events that became fixed and homogenized. A variety of patterns of complete promoter duplications, or promoter domain duplications are found in species as diverse as frogs, flies, mice and plants (see Figure 4). There is no obvious sequence similarity among the spacer elements of these eukaryotes, in contrast to the highly conserved coding sequences. Collectively, these observations suggest that promoter duplications occur *de novo* in different lineages, possibly as a consequence of the same unequal crossing-over mechanisms postulated to explain rRNA gene homogenization and concerted evolution.

Repetitive elements interposed between gene and spacer promoters in *Xenopus*, mouse and *Arabidopsis thaliana* have enhancer activity, but only in frog oocytes.

Xenopus laevis rRNA minigenes containing complete pro-

moter sequences are transcribed by pol I upon injection into *Xenopus* oocytes. In this assay, the 60/81bp intergenic spacer repeats act as strong orientation and position-independent enhancers of pol I transcription (Labhart and Reeder, 1984; Pikaard and Reeder, 1988) (Busby and Reeder, 1983; DeWinter and Moss, 1987; Pape et al., 1989). In *X. laevis*, each 60 or 81 bp repeat has a core sequence of ~42 bp that is ~80% identical to a sequence found in the upstream promoter domain from –114 to –72 (Boseley et al., 1979; Moss et al., 1980). Interestingly, *X. borealis* has several types of spacer repeats in the region between the gene promoter and spacer promoter, one of which is homologous to the 42 bp sequence found in *X. laevis* 60/81 bp spacer repeats and in the upstream promoter domain (Bach et al., 1981). Another repeated element in the *X. borealis* spacer is homologous to the sequence of the core promoter domain surrounding the transcription start site (Bach et al., 1981; Labhart and Reeder, 1987). When synthetic oligonucleotides corresponding to the upstream or core promoter domains of *X. laevis* are polymerized and cloned upstream of a gene promoter, they act as artificial enhancers (Pikaard, 1994). Collectively, these observations and experiments suggest that much of the spacer, particularly the spacer promoters and enhancer repeats in both *Xenopus laevis* and *X. borealis*, evolved via duplications of all or part of the gene promoter. Presumably, the enhancers bind one or more transcription factors and ultimately help recruit transcription complexes to the gene promoter. Indeed, it has been shown that spacer sequences will compete with the promoter and decrease transcription when both are co-injected into *Xenopus* oocytes on separate plasmids (Labhart and Reeder, 1984). Subsequent studies showed that the transcription factor UBF (Upstream Binding Factor), which is found only in vertebrates, binds to both enhancers and the promoter (Dunaway, 1989; Pikaard et al., 1989) and can account for enhancer activation *in vitro* (McStay et al., 1997).

In the *Arabidopsis thaliana* intergenic spacer, the repeated elements interposed between the gene promoter and duplicated spacer promoters are short, only 21 basepairs in size, and are characterized by the presence of a *Sal* I endonuclease recognition site (Gruendler et al., 1991; Gruendler et al., 1989). When cloned adjacent to an *Arabidopsis thaliana* ribosomal RNA gene promoter, and transfected into *Arabidopsis* protoplasts, the Sal repeats have little, if any, influence on promoter activity, stimulating transcription only two to threefold, at most (Doelling et al., 1993; Doelling and Pikaard, 1995). Sal repeats also have no obvious influence on the transcription of rRNA transgenes in *Arabidopsis* (Wanzenbock et al., 1997). Thus there is no compelling evidence that Sal repeats are enhancers of pol I transcription in *Arabidopsis thaliana*. Interestingly, when the *Arabidopsis* Sal repeats are cloned adjacent to a *Xenopus laevis* ribosomal RNA gene promoter that is then injected into frog oocytes, the Sal repeats display clear enhancer activity, stimulating transcription at least five to ten-fold (Doelling et al., 1993). Analogous observations have been made using the 140 base pair repeats interposed between the gene promoter and spacer promoter in mouse rRNA gene intergenic spacers. When these 140 bp repeats are attached to a mouse promoter transfected into mouse cells, they have little effect on transcription, stimulating only 2-3 fold. However, when the mouse 140 base pair repeats are cloned adjacent to the *Xenopus laevis* promoter and injected into frog oocytes, they display strong, orientation independent enhancer activity (Pikaard et al., 1990). Collectively, these experiments suggest that there is something special about *Xenopus* oocytes that makes them hyper-responsive to ribosomal RNA gene enhancers. Consistent with this hypothesis, *Xenopus laevis* 60/81 bp enhancers have little, if any, activity on ribosomal RNA gene constructs transfected into cultured *Xenopus* somatic cells (Pikaard and Reeder, unpublished), whereas they display clear activity in oocytes and early embryos.

It is intriguing that spacer repeats from *Xenopus*, mouse, and *Arabidopsis thaliana* should all have enhancer activity in the frog oocyte injection assay given that they lack obvious sequence similarity. The sequence-tolerant transcription factor UBF (Copenhaver et al., 1994) binds to both *Xenopus* and mouse intergenic spacer repeats (Pikaard et al., 1989; Pikaard et al., 1990), which might suggest a mechanism for cross-species enhancer function in vertebrates. However, no obvious UBF homolog has been found in the genomes of non-vertebrates, including *Arabidopsis thaliana*. If intergenic spacer repeats in plants and animals are not functionally related due to their shared ability to bind a highly conserved eukaryotic transcription factor, another possibility might be that these repeated elements adopt a conserved structural conformation. Intrinsic curvatures or sequence flexibilities can affect nucleosome positioning, and it is likely that a variety of primary sequences can have similar structural outcomes. If an ability to orient and phase nucleosomes is an important function for intergenic spacers in all eukaryotes, selective pressure might ensure that this property is conserved during the evolution of rRNA genes. The fact that the fundamental chromatin proteins, the histones, are so highly conserved in evolution makes this hypothesis worth considering. Hopefully, as our knowledge of plant chromatin components and RNA polymerases I transcription factors improves, it will be possible to test at least some of these speculations.

Species-specificity and promoter recognition

An intriguing aspect of ribosomal RNA gene transcription is that the rapid evolution of ribosomal RNA gene promoters is accompanied by the rapid evolution of RNA polymerases I transcription factors. As a consequence, pol I transcription is often species-specific (Grummt et al., 1982; Learned et al., 1985; Miesfeld and Arnheim, 1984; Mishima et al., 1982). For instance, a mouse promoter is not recognized in a human cell-free transcription system nor is a human promoter recognized in an analogous mouse extract. Subsequent fractionation of the essential pol I transcription factors in both mouse and human revealed that several of the essential transcription activities, such as pol I and UBF, are actually interchangeable between mouse and human (Bell et al., 1990; Schnapp et al., 1991). However, an essential transcription factor, SL1/TIFI-B, composed of TATA binding protein and three associated factors, is not interchangeable. As a result, mouse SL1 must be added to a human transcription extract for the mouse ribosomal RNA gene promoter to be recognized and transcribed in the human system. Likewise mouse SL1 must be added to human cell extract in order for the mouse ribosomal RNA gene promoter to be transcribed (Bell et al., 1990; Learned et al., 1985). In more distantly related species, such as human and *Xenopus*, additional transcription factors contribute to species-specificity. For instance, human and *Xenopus* UBF bind to DNA in virtually the identical way, producing DNase I footprints that are indistinguishable (Bell et al., 1989; Pikaard et al., 1989). Each is composed of multiple DNA binding domains known as HMG boxes due to their similarity to high mobility group (HMG) non-histone chromosomal proteins (Jantzen et al., 1990). However, *Xenopus* UBF has one fewer HMG box (McStay et al., 1991) than human (or mouse) UBF. This difference does not affect UBF's DNA binding characteristics on its own, but it does alter how UBF-SL1 protein complexes are positioned on the promoter, apparently disrupting their ability to recruit Pol I (Bell et al., 1989). Adding an extra HMG domain to *Xenopus UBF* is sufficient to make *Xenopus* UBF functional in the human pol I transcription system (Cairns and McStay, 1995).

The experiments discussed above suggest that an ability to bind DNA and establish protein-protein interactions with other transcription factors is not sufficient to bring about pol I transcription. An additional requirement is for the resulting protein complexes to be spaced just right on the promoter. An independent set of experiments that reached the same conclusion showed that a frog promoter can be recognized in the mouse pol I transcription system, though transcription is initiated at an incorrect site. Altering the distance between the upstream and core promoter domains by one half helical turn was sufficient to make the frog promoter a strong promoter in the mouse system and switched the transcription start site to the correct nucleotide used in *Xenopus* itself (Pape et al., 1990). The latter experiment reinforces the idea that in vertebrates, at least, evolution has tinkered with the spacing between binding sites for conserved transcription factors, requiring compensatory adjustments in the spacing of protein domains to allow transcription factor interactions.

Analysis of the species-specificity of pol I transcription in plants has provided some additional insights and interesting twists to this story. Ribosomal RNA gene promoters in unrelated plant species share little similarity beyond the initiator element discussed previously. Experiments that tested the ability of tomato and *Brassica oleracea* promoters to be recognized upon transfection into *Arabidopsis thaliana* protoplasts led to interesting results. Whereas *Brassica* and *Arabidopsis* are related species within the family *Brassicaceae*, tomato is a very distantly related dicot in the *Solanaceae*. When the tomato rRNA gene promoter is transfected into *Arabidopsis* protoplasts, it can program transcription initiation but transcription is initiated 32 base pairs downstream of the normal start site (Doelling and Pikaard, 1996). When the *Brassica* promoter is transfected into *Arabidopsis* protoplasts it programs transcription from two sites: the expected transcription start site, and an alternative start site located 29 base pairs downstream, a position similar to the site of transcription initiated from the tomato promoter (Doelling and Pikaard, 1996). An explanation for the unexpected transcription start sites programmed by the *Brassica* and tomato promoters in *Arabidopsis* protoplasts came from the realization that the pol I transcription start site overlaps a consensus TATA box. TATA boxes are a common motif found in promoters recognized by the RNA polymerase II transcription system and are typically located 25 -30 base pairs upstream of the transcription start site (Serizawa et al., 1994). This suggested a hypothesis whereby foreign ribosomal RNA gene promoters are mistaken in *Arabidopsis* as promoters of protein coding genes (due to the TATA box).

TATA boxes are bound by the TATA-Binding Protein (TBP) as a subunit of the transcription factor, TFIID (Berk, 1999; Serizawa et al., 1994). TBP is also a component of essential transcription factors for the RNA polymerase I and RNA polymerase III transcription systems, as well (SL1/TIFI-B, TFIIIB, respectively) (Cormack and Struhl, 1992; Schultz et al., 1992). An experiment to test whether or not TBP normally interacts with the TATA motif in the context of RNA polymerases I transcription was to use site-directed mutagenesis to alter the TATA sequence in a way known to prevent the interaction with TATA binding protein (Heard et al., 1993; Strubin and Struhl, 1992).

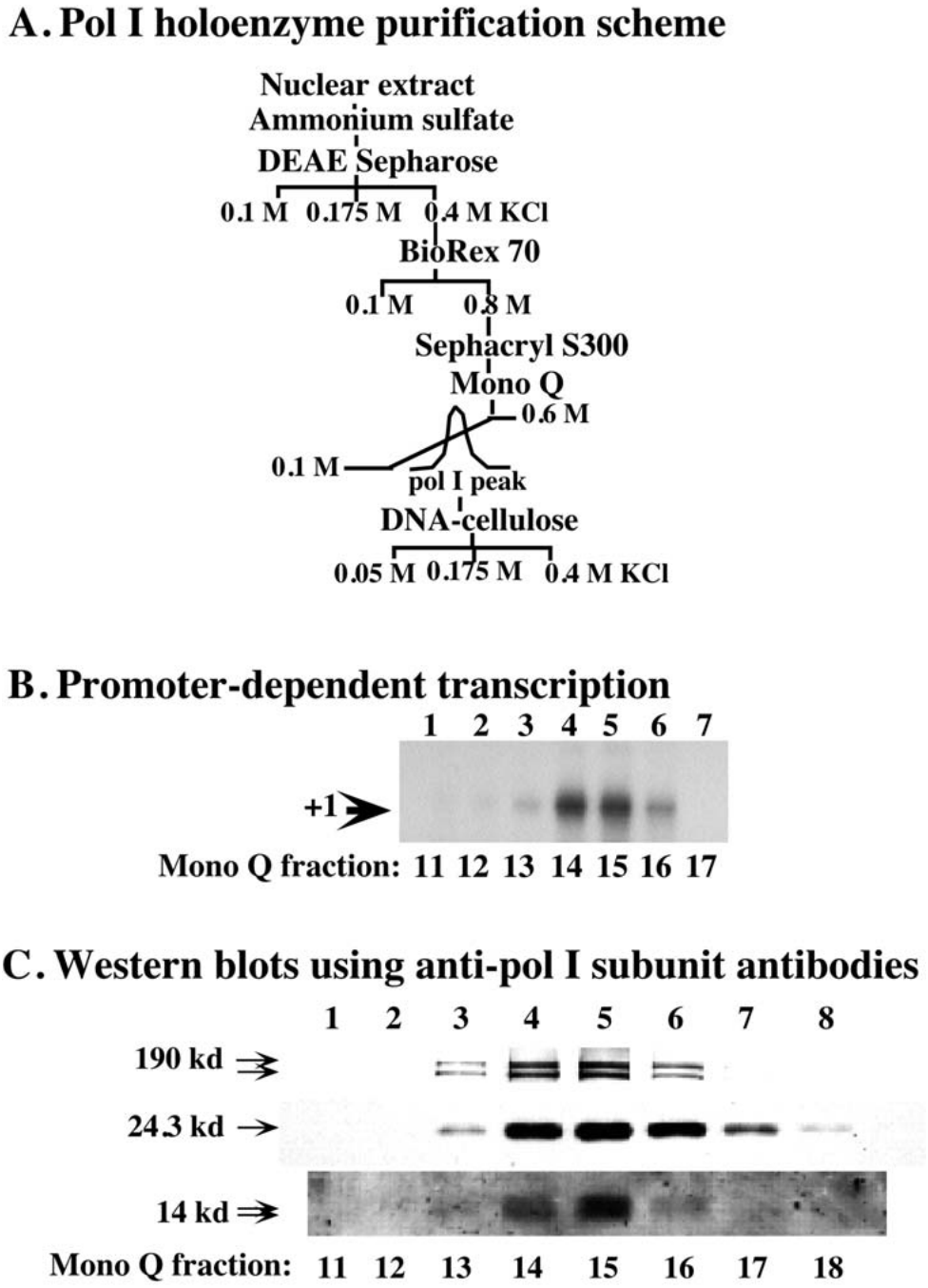

Figure 5. Purification of RNA polymerase I holoenzyme activity. **A.** Scheme for sequential chromatography of RNA polymerase I holoenzyme activity from broccoli (*Brassica oleracea*) using DEAE-Sepharose CL-6B, Biorex 70, Sephacryl S-300, Mono Q, and double-stranded calf thymus DNA-cellulose. **B.** Mono Q fractions were tested for their ability to program accurate transcription initiation from a cloned *B. oleracea* rRNA gene promoter. Accurately initiated transcripts were detected using an S1 nuclease protection assay. **C.** Mono Q fractions subjected to SDS-polyacrylamide gel electrophoresis and blotting to nitrocellulose were probed with antiserum raised against the last exon of the largest *A. thaliana* RNA polymerase I subunit (190 kd subunit), or against 24.3 kd and 14 kd RNA polymerase subunits. This figure is reprinted, with modifications, from (Saez-Vasquez and Pikaard, 2000) with permission from the publisher.

When this mutation was made in the *Brassica* promoter, which supports transcription initiated at +1 or + 29, the + 29 signal was reduced dramatically whereas the +1 signal, presumably resulting from accurate pol I transcription, was unaffected (Doelling and Pikaard, 1996). Furthermore, when a suppressor TBP, bearing a mutation which allows it to recognize the altered TATA box, was transfected into the cells along with the mutated promoter, transcription from + 29 was restored (Doelling and Pikaard, 1996). These experiments suggested that transcription initiation at +29 was dependent on direct TBP interactions with the TATA box whereas transcription from the normal pol I transcription start site (+ 1) was not, suggesting that two different polymerase systems might be at work (Doelling and Pikaard, 1996).

To test the hypothesis that + 29 transcription resulted from pol II transcription, the *Brassica* promoter was fused to a luciferase reporter gene, because only pol II is known to produce capped mRNAs that can be translated. Indeed, the wild type *Brassica* promoter drove expression of the luciferase reporter gene; the TATA box mutation which abolished + 29 initiation also abolished luciferase expression; and the suppressor TBP that restored +29 transcription restored luciferase expression (Doelling and Pikaard, 1996). Collectively, these experiments showed that species-specificity of ribosomal RNA gene transcription in plants can be manifested as a switch in polymerase specificity. The switch is presumably made possible by the inability of the pol I transcription machinery to engage the promoter and prevent the pol II transcription system from "hijacking" the promoter via TATA box recognition. An interesting parallel in yeast is that mutations in a transcription factor that interacts with the upstream domain of the ribosomal RNA gene promoter will also cause a switch from pol I to pol II transcription (Conrad-Webb and Butow, 1995; Oakes et al., 1999). Thus the ability of an rRNA gene promoter to be recognized by two distinct polymerase systems is not unique to plants.

Pol I holoenzymes and transcription

Identification of transcription factors is greatly facilitated by the ability to perform transcription *in vitro* using purified activities. A purified cell-free transcription system for pol I has been developed using broccoli (*Brassica oleracea*) inflorescence as the starting tissue (Saez-Vasquez and Pikaard, 1997). *Brassica* and *Arabidopsi*s are closely related and broccoli is a rich source of rapidly dividing cells available in kilogram quantities, making it ideal for biochemical studies. The *Brassica* pol I transcription system can be purified by successive chromatography on at least 5 columns (see Figure 5A): DEAE-Sepharose (anion exchange), Biorex 70 (cation exchange), Sephacryl S300 (gel filtration; size exclusion), Mono Q (analytical anion exchange) and DNA-cellulose (DNA-affinity). Single fractions that elute from each of the 5 columns can program accurate transcription initiation in the presence of high concentrations of alpha-amanitin, a fungal toxin which blocks pol II transcription at low concentration and which will also inhibit pol III transcription at high concentrations (Figure 5B). The fact that single fractions are competent for transcription suggests that transcription factors and the polymerase core enzyme co-purify because they are preassembled into a pol I "holoenzyme". Purified holoenzyme fractions program transcription initiation from the same start site and require the same core promoter sequences needed *in vivo*.

The pol I holoenzyme protein complex displays a molecular mass of ~2 Md based on gel filtration chromatography (Saez-Vasquez and Pikaard, 1997), and appears to consist of 30-40 polypeptides based on SDS-PAGE. Several of the proteins have been identified immunologically as subunits of the RNA polymerase I core enzyme (Figure 5C). Also co-purifying with pol I holoenzyme activity are protein kinase (Casein kinase 2) (Saez-Vasquez et al., 2001) and histone acetyltransferase activities (Albert et al., 1999), suggesting that pol I holoenzymes are equipped to respond to growth signals and to modify chromatin as necessary to activate transcription. The *Brassica* holoenzyme binds the promoter in a single step to form a complex that can be visualized using an agarose electrophoretic mobility-shift assay (Saez-Vasquez and Pikaard, 2000).

Identification of plant pol I holoenzyme subunits is a priority for future research. Are there specific transcription factors one can expect to find? Other than TBP, which is used by pol I, II and III, the answer is mostly no. As mentioned previously, vertebrate UBF has no known homolog in yeast , *Drosophila*, *Arabidopsis* or *C. elegans*. In yeast, there are two major activities called Upstream Activation Factor (UAF) and Core Factor (CF) (Keys et al., 1996; Lin et al., 1996). Yeast UAF is a multi-subunit complex of at least five proteins, none of which are homologs of known vertebrate transcription factors (Keys et al., 1996). Core Factor is probably analogous to vertebrate SL1 in that it is required for core promoter function and, like SL1, is composed of TBP and several TBP-associated factors (TAFs) (Comai et al., 1992; Lalo et al., 1996; Lin et al., 1996; Steffan et al., 1996). However, the yeast and mammalian TAFs share no obvious sequence similarity with one another. Based on these considerations, there is no reason to assume that plant pol I transcription factors will share obvious homology with UBF, yeast UAF, or known pol I TAFs.

Growth and hormonal regulation of pol I transcription.

Growth regulation of rRNA gene transcription is well documented in both prokaryotes and eukaryotes (Jacob, 1995; Waldron, 1977; Waldron and Lacroute, 1975). For instance, "stringent control" in bacteria and yeast involves the shut-down of rRNA production when cells are starved for carbon or nitrogen (Waldron, 1977; Waldron and Lacroute, 1975). Analogous down-regulation occurs in mammalian cells starved for serum or amino acids or in cells treated with cycloheximide, and transcription is up-regulated upon re-feeding or removing the inhibitor (for reviews see (Jacob, 1995; Tower et al., 1986)). rRNA gene transcription in mammals also responds to glucocorticoid hormones, and can be induced or repressed depending on the tissue (Cavanaugh and Thompson, 1985; Gokal et al., 1990; Mahajan et al., 1990; Mahajan and Thompson, 1990). In *Drosophila*, phorbol esters and serum induce rRNA transcriptional initiation via signaling pathways also used by pol II-transcribed genes (Chao and Pellegrini, 1993; Vallett et al., 1993).

In plants, Guilfoyle reported that auxin treatment increases the amount of extractable non-specific pol I activity (promoter-independent ribonucleotide incorporation using a nicked or sheared DNA template)(Guilfoyle, 1980). Gaudino subsequently showed that specific (promoter-dependent) rRNA transcription initiation is up-regulated by kinetin whereas auxin, surprisingly, had no effect (Gaudino and Pikaard, 1997). One possibility is that auxin up-regulates the amount of pol I in plant cells but cytokinin regulates a rate-limiting step in actual rRNA transcription initiation. An untested prediction is that auxin and kinetin might have synergistic stimulatory effects on pol I transcription.

What are the targets of regulation within the pol I system? Early studies suggested that the polymerase core enzyme itself might be a target because partially purified RNA polymerase I from actively growing mouse cells could restore activity to extracts made from stationary-phase or cycloheximide treated cells (Tower et al., 1986). Though some evidence has pointed to modification of RNA polymerase I itself (Bateman and Paule, 1986; Tower and Sollner-Webb, 1987) other studies suggested that one or more activities that co-purify with RNA polymerase I are probably responsible (Buttgereit et al., 1985; Schnapp et al., 1990).

In vertebrates there is good evidence that UBF phosphorylation regulates its activity. The degree of UBF phosphorylation, but not UBF abundance, varies with the growth status of cells (O'Mahony et al., 1992a; O'Mahony et al., 1992b; Voit et al., 1992). UBF dephosphorylated with alkaline phosphatase is a poor transactivator *in vitro* (O'Mahony et al., 1992a; Voit et al., 1992), though its DNA binding is unimpaired (Voit et al., 1992). Therefore, UBF phosphorylation may affect a step following assembly of the preinitiation complex, such as polymerase activation.

Multiple kinases appear to be responsible for UBF phosphorylation. One appears to be casein kinase 2 (CK2) (O'Mahony et al., 1992a; Voit et al., 1992). Evidence is that a UBF kinase in cell extracts is sensitive to inhibitors of CK2(such as heparin and 5,6,-dichloro-D-ribofuranosyl-benzimidazole) (Voit et al., 1992), the acidic tail of UBF contains numerous CK2 consensus sites (O'Mahony et al., 1992a; Voit et al., 1992), and CK2 can phosphorylate UBF *in vitro* at many of the sites phosphorylated *in vivo* (Voit et al., 1995). However, phosphorylation by CK2 is insufficient to restore transcriptional activity to recombinant UBF expressed in *E. coli*, suggesting that another kinase (or phosphatase), alone or in combination with CK2, may control UBF activity (Voit et al., 1995). Using biochemical and immunological techniques, we have shown that CK2 co-purifies with the *Xenopus* pol I holoenzyme (Albert et al., 1999). A kinase with all the characteristics of CK2 also co-purifies with the *Brassica* holoenzyme (Saez-Vasquez et al., 2001). Thus despite the lack of a UBF homolog in plants, CK2 may be playing a regulatory role that is conserved in plants and animals.

Nucleolar dominance

Epigenetic control of rRNA gene transcription

When two species are crossed to form an interspecific hybrid, frequently the NORs from one progenitor will form nucleoli but the NORs from the other parental species are inactivated. This still mysterious phenomenon, known as nucleolar dominance, is widespread in nature, occurring in plants, insects, amphibians and mammals (for reviews see (Pikaard, 2000a; Pikaard, 2000b; Reeder, 1985)). Nucleolar dominance results from the transcription of only one parental set of ribosomal RNA (rRNA) genes (see Figure 6C). This mitotically stable, but reversible change in gene expression state is not caused by alterations in rRNA gene sequences and is thus a classic example of an epigenetic phenomenon.

The biochemical and genetic mechanisms responsible for nucleolar dominance are not yet clear. Hypotheses include the idea that species-specific differences in rRNA gene sequences and/or pol I transcription factors lead to the preferential activation of only one set of rRNA genes. Alternative hypotheses suggest that one set of rRNA

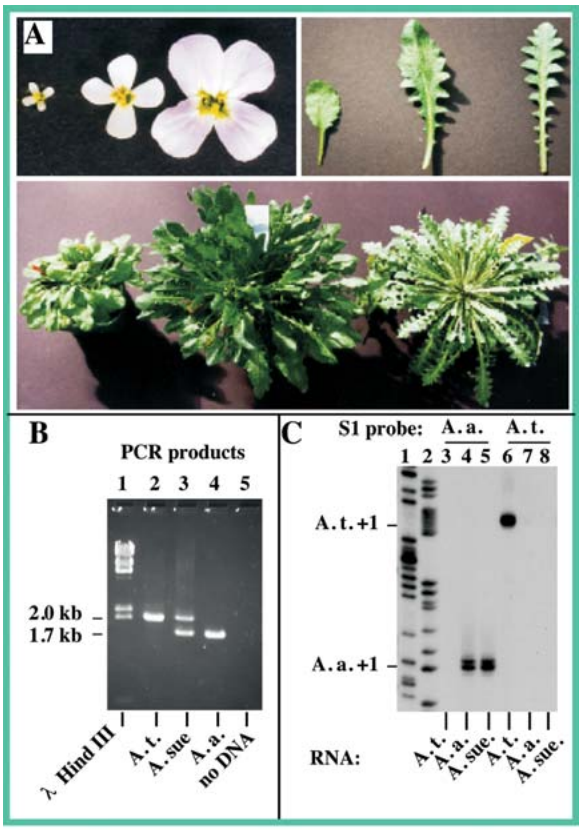

Figure 6. Molecular analysis of nucleolar dominance in Arabidopsis. **A.** Flower, leaf and whole-plant phenotypes of *Arabidopsis thaliana* (left), *Arabidopsis arenosa* (also known as *Cardaminopsis arenosa*; right) and their allotetraploid hybrid, *Arabidopsis suecica* (center). Note the intermediate phenotypes of flower and leaf morphologies in *A. suecica*. B: The ribosomal RNA genes from *A. thaliana* and *A. arenosa* are both present in similar abundance in *A. suecica.* Genomic DNA of *A. thaliana* (lane 2), *A. suecica* (lane 3) or *A. arenosa* (lane 4) was subjected to PCR using one primer corresponding to a region just upstream of the promoter and a second primer corresponding to the beginning of the 18S rRNA coding region. A control reaction in lane 5 lacked template DNA. Bacteriophage lambda DNA cleaved with *Hin*d III served as size markers in lane 1. C: Only *A. arenosa* ribosomal RNA genes are transcribed in *A. suecica*, as shown using the S1 nuclease protection assay (compare lanes 5 and 8). Equal aliquots of *A. thaliana*, *A. arenosa* or *A. suecica* RNA were analyzed with *A. arenosa* (lanes 3-5) or *A. thaliana* (lanes 6-8)-specific probes that detect rRNA gene transcripts initiated from the correct start sites (+1) of the respective gene promoters. Dideoxynucleotide sequencing reactions served as size markers in lanes 1 and 2. This figure is reprinted from (Pikaard, 1999) with permission from the publisher.

genes is singled out for repression, involving chromosomal influences not specified simply by rRNA gene sequences.

Transcription factor-based hypotheses

As discussed previously, RNA polymerase I transcription is frequently species-specific. Therefore, one can imagine that the silencing of a single species-specific transcription factor could cause the silencing of an entire set of ribosomal RNA genes. In fact, this may be the explanation for a type of nucleolar dominance that occurs in somatic cell hybrids resulting from the fusion of mouse and human cells. In these cell lines it is common for the human or mouse ribosomal RNA genes to be expressed, but not both (Elicieri and Green, 1969; Miller et al., 1976; Perry et al., 1976). Chromosome loss and rearrangement is common in such lines, thus the loss of one more genes encoding subunits of the mammalian species-specific transcription factor, SL1/TIF-IB is a possible cause for the expression of only mouse or human rRNA genes (Miesfeld et al., 1984). However, there are reports that the silent set of ribosomal RNA genes in somatic cell hybrids can be activated upon treatment with phorbol esters or upon viral infection (Soprano and Baserga, 1980; Soprano et al., 1979), suggesting an epigenetic basis for the phenomenon.

Obviously, mouse-human cell hybrids represent a wide cross not possible by natural mechanisms. What happens in species closely related enough to interbreed? In *Brassica* species that display nucleolar dominance when hybridized, it has been shown the ribosomal RNA genes from either species are functional when transfected into cells of the other species (Frieman et al., 1999). The same is true in *Arabidopsis suecica* (Chen et al., 1998). These experiments demonstrate that the RNA polymerases I transcription systems of closely related species are similar enough that the transcription factors are compatible. Thus differential expression of species-specific transcription factors is an unlikely explanation for nucleolar dominance in plant hybrids that occur in nature.

A more plausible explanation for nucleolar dominance, also based on transcription factor availability, is the so-called " enhancer imbalance hypothesis ". This model was first put forward as an explanation for nucleolar dominance in *Xenopus* hybrids (Reeder and Roan, 1984), but has also been suggested as an explanation for nucleolar dominance in wheat (Flavell, 1986; Martini et al., 1982). In hybrids of *X. laevis* and *X. borealis*, the *X. laevis* ribosomal RNA genes are dominant, at least early in development. Later, in adult tissues, the *X. borealis* rRNA genes are also expressed. Reeder and Roan showed that nucleolar dom-

inance could be mimicked using oocytes injected with *X. laevis* and *X. borealis* minigenes (Reeder and Roan, 1984). When two minigenes having complete intergenic spacers attached to the gene promoter were co-injected into oocytes, a minigene with an *X. laevis* spacer was preferentially expressed over a minigene with an *X. borealis* spacer. This competition effect was shown to be due to the intergenic spacer sequences upstream of the promoter. The promoters themselves had equivalent activity when no spacer sequences were attached. The dominance of the *X. laevis* ribosomal RNA genes in the oocyte injection assay is apparently due to the fact that *X. laevis* intergenic spacers have a greater number of enhancer elements than do *X. borealis* spacers (Reeder and Roan, 1984). In other experiments, it had been shown that these enhancer elements strongly stimulate an adjacent promoter but can compete against a promoter carried on a separate, co-injected plasmid (Labhart and Reeder, 1984). The latter experiments suggested that the enhancers and ribosomal gene promoter bind at least one transcription factor in common. Reeder and Roan speculated that if this transcription factor is only available in limiting quantities in oocytes, then the genes with the most enhancers might sequester the factor, making it unavailable to the genes from the other species.

The beauty of the enhancer imbalance hypothesis is that nucleolar dominance is proposed to be a consequence of protein-DNA interactions for which equilibrium constants are dictated by amino acid (in transcription factors) and DNA sequences. These sequences are not changed depending on whether a given species' chromosomes are contributed by the male or female parent, which might explain the lack of parent-of-origin effects in nucleolar dominance. The idea that dominant rRNA genes have a higher binding affinity for transcription factors might also explain why dominant genes can sometimes be vastly outnumbered by underdominant genes. For instance, in Navashin's studies of *Crepis* hybrids, he described instances in which hybrids displayed a variety of ploidys (Navashin, 1934). Remarkably, a single NOR-bearing chromosome from one species could remain dominant even when outnumbered 3:1 (in an allotetraploid) or 4:1 (in an allopentaploid) by underdominant NORs. This can be rationalized according to the enhancer imbalance hypothesis by assuming that transcription factors are present in limiting quantities such that there are only enough factors for one NOR to be expressed. Presumably the NOR with the highest binding affinity for a limiting transcription factor is the winner.

Despite the appeal of the enhancer imbalance hypothesis, there are a number of observations that are inconsistent with its predictions. For instance, there is at least one example in which nucleolar dominance is not independent of ploidy or gene dosage. In *Arabidopsis suecica*, which is the allotetraploid hybrid of *A. thaliana* and *A. arenosa*, the normal 2:2 dosage of progenitor genomes is correlated with the silencing of the *A. thaliana* rRNA genes (Chen et al., 1998) (see Figure 6). However, upon backcrossing *arenosa* to tetraploid *thaliana*, the resulting progeny, which have a 3:1 dosage of *thaliana: arenosa* genomes, show a reversal in the direction of nucleolar dominance such that *arenosa* rRNA genes become underdominant (Chen et al., 1998). This is not easily explained according to the enhancer imbalance hypothesis. If *arenosa* rRNA genes are normally dominant because they have the highest affinity for transcription factors, they should always be expressed, even when outnumbered. Reducing the relative number of *arenosa* genes might create a situation in which transcription factors are in excess over these genes and thus become available to the presumably weaker *A. thaliana* RNA genes. If so, one would expect both sets of genes to be co-expressed, but one would not predict a reversal in the direction of nucleolar dominance and silencing of *A. arenosa* genes. The fact that dominance reversal occurs indicates that it is not every rRNA gene for itself in the competition for transcription factors. Instead, it seems that there must be strong cooperatively among rRNA genes to cause an " all or none " wave of transcriptional activation at an NOR or there must be alternative mechanisms that dictate which NORs can be activated, perhaps independent of transcription factor availability.

Another strike against the enhancer imbalance hypothesis, at least in plants, is that transient expression experiments analogous to those performed in *Xenopus* oocytes do not yield similar results. Co-transfection of dominant and underdominant rRNA genes into *Brassica* hybrid protoplasts results in the equal expression of both types of genes, even though the chromosomal copies of the underdominant class are completely repressed in these same protoplasts (Frieman et al., 1999). The same is true in *Arabidopsis* hybrids (Chen et al., 1998). These simple experiments suggest that there is no intrinsic deficiency in the ability of underdominant rRNA genes to bind transcription factors in a hybrid cell. Instead, the results point to mechanisms that somehow prevent chromosomal copies of the underdominant genes from having access to the transcription machinery.

One can argue that protoplast transfection experiments do not deliver enough plasmid DNA into plant cells to truly mimic a *Xenopus* oocyte injection experiment in which 10 million copies or more of each competing minigene are delivered directly into the nucleus. However, using the *Brassica in vitro* transcription system, DNA can be added in excess of transcription factors such that genes with the highest binding affinities for transcription factors should be preferentially transcribed. Interestingly, the rRNA genes that are dominant in hybrids do not out-compete underdominant rRNA genes for transcription factors *in vitro*, fur-

ther suggesting that both classes of rRNA genes have similar affinities for general transcription factors (Frieman et al., 1999).

Chromosomal influences on nucleolar dominance

What are the DNA sequences that are necessary and sufficient to induce nucleolar dominance? This is a long-standing question for which there is still no clear answer. In the early studies of Navashin, aneuploid hybrids that sometimes inherited an incomplete chromosome set from the dominant parent were sometimes obtained. A NOR-bearing chromosome inherited as part of an incomplete set could nonetheless be the dominant NOR, suggesting that a NOR-bearing chromosome itself might be necessary and sufficient for nucleolar dominance (Navashin, 1934). Observations in *Drosophila* hybrids also support this idea. *Drosophila* species have NORs on the sex chromosomes. In XX female hybrids of *Drosophila melanogaster* and *D. simulans*, the *melanogaster* NOR is dominant. Likewise, in XY male interspecific hybrids, the NOR on the *melanogaster* Y is dominant over the NOR on the *simulans* X. In an X0 male, which lacks a sex chromosome from *D. melanogaster* but has a complete set of *D. melanogaster* autosomes, the NOR on the X chromosome of *D. simulans* is fully active (Durica and Krider, 1977). Collectively, these observations suggest that all sequences necessary for nucleolar dominance in *Drosophila* might be located on the sex chromosomes themselves. These findings could be interpreted as evidence supporting the idea that NORs compete for a limiting transcription factor and that the *melanogaster* rRNA genes are the stronger competitors when NORs from both species are present in the same nucleus. However, Durica and Krider noted that chromosome rearrangements in the heterochromatic regions flanking the X and Y-associated NORs in *D. melanogaster* prevented suppression of *D. simulans* NORs such that the NORs of both species became co-dominant in hybrids (Durica and Krider, 1978). The chromosome rearrangements did not affect nucleolus formation (or secondary constriction formation) at the adjacent *D. melanogaster* NORs, suggesting that these rRNA genes continued to recruit transcription factors as usual. Apparently, this is not enough to cause nucleolar dominance, thus Durica and Krider were the first to cast doubt on transcription factor competition models (Durica and Krider, 1978).

In barley and triticale, there is additional evidence that the chromosomal context of an NOR is important in determining whether or not nucleolar dominance will occur. Barley has two NORs on different chromosomes and these NORs are normally co-dominant. However, chromosome translocations that result in both NORs being on the same chromosome can induce the dominance of one NOR over the other (Nicoloff, 1979; Schubert and Kunzel, 1990). It is not simply that two NORs are not tolerated on a single chromosome because in other barley lines in which translocations have caused two copies of the same NOR to occur on a single chromosome, both NORs are co-dominant (Schubert and Kunzel, 1990). Apparently, NORs respond differently, and unpredictably, depending on their chromosomal context.

In triticale, the hybrid of wheat (genus *Triticum*) and rye (genus *Secale*), the wheat NORs are dominant and the rye NOR, located on the short arm of chromosome 1R, is suppressed (Lacadena et al., 1984; Silva et al., 1995; Thomas and Kaltsikes, 1983). However, if the chromosome arm bearing the rye NOR is translocated onto the long arm of wheat chromosome 1, the rye NOR becomes co-dominant with wheat NORs (Viera et al., 1990a; Viera et al., 1990b). Interestingly, deletion of the long arm of rye chromosome 1R, or substitution of rye chromosome 2R for wheat chromosome 2D can also cause the rye NOR to be expressed in triticale (Neves et al., 1997). Apparently, there are genes or sequences on the long arm of rye chromosome 1R and on chromosome 2R that play a role in suppressing the rye NOR in a rye-wheat hybrid.

One possibility could be that NOR silencing in nucleolar dominance is simply a by-product of a larger chromosome silencing event initiated elsewhere on a NOR-bearing chromosome. This does not appear to be the case based on results in *A. suecica*, in which NORs inherited from *A. thaliana* are repressed. Despite the essentially complete silencing of the two ~4 million basepair NORs, the three protein coding genes adjacent to NOR4, the nearest of which is only 3.1 kb away, remain active in hybrids (Lewis and Pikaard, 2001). These results suggest that the mechanisms responsible for nucleolar dominance are restricted to the NOR and do not act on larger segments of NOR-bearing chromosomes.

Chromatin modifications and nucleolar dominance

Though it is not yet clear how sequences adjacent to an NOR in *Drosophila* or at unlinked loci in rye can influence rRNA gene activity, these results suggest that underdominant rRNA genes are selectively repressed by mechanisms acting at a chromosomal level. Likewise, underdominant rRNA genes can be transiently expressed in a hybrid cell upon transfection even though their chromosomal counterparts are repressed, also pointing to mechanisms selec-

tively acting on the chromosomally encoded genes. Consistent with these observations, underdominant rRNA genes and NORs can be derepressed by a chemical inhibitor of DNA methylation, 5-aza-2'-deoxycytosine (aza-dC) (Amado et al., 1997; Chen et al., 1998; Chen and Pikaard, 1997; Neves et al., 1995; Viera et al., 1990a). Inhibitors of histone deacetylation, such as sodium butyrate and trichostatin A, can also derepress the underdominant class of rRNA genes in a hybrid (Chen and Pikaard, 1997). Collectively, these results suggest that underdominant rRNA genes are silenced as a consequence of covalent chromatin modifications.

Though preliminary evidence suggests that histones associated with dominant rRNA genes are more highly acetylated than are histones associated with underdominant genes (Chen, Lawrence and Pikaard, unpublished), it is not yet clear how methylation is involved. Thus far, no clear correlation has been observed between the extent of ribosomal RNA gene methylation (assessed using methylation sensitive restriction endonucleases) and nucleolar dominance in *Brassica* or *Arabidopsis* hybrids, though correlations have been reported in wheat (Flavell et al., 1988; Houchins et al., 1997). For instance, in *Brassica napus* both the dominant and the underdominant ribosomal RNA genes are methylated at essentially every methylation sensitive *Hpa* II restriction site (Chen and Pikaard, 1997), making one suspect that methylation could not carry much regulatory information. Another odd finding in *Brassica* is that aza-dC treatments that induce only about 10% demethylation of the ribosomal RNA genes cause a disproportionate, essentially complete, derepression of the silenced set of underdominant genes (Chen and Pikaard, 1997).

In different natural strains of *Arabidopsis suecica*, there is variability in the extent to which nucleolar dominance occurs, some strains showing almost complete silencing of the *A. thaliana* rRNA genes and other strains showing significant expression of *thaliana* genes (Lawrence, Chen and Pikaard; unpublished). The degree to which ribosomal RNA genes are methylated in the strains is also highly variable, but there is no obvious correlation between methylation levels and nucleolar dominance. In one of the best *A. suecica* strains for observing *A. thaliana* rRNA gene silencing, the rRNA genes are almost completely unmethylated. In other strains that also show *A. thaliana* rRNA gene silencing, methylation levels can be very high (Lawrence and Pikaard, unpublished). Although it is possible that the use of methylation sensitive restriction enzymes does not detect crucial methylation events at specific sequences within the ribosomal RNA genes, it is also possible that it is the methylation level of a locus other than the NOR that explains the de-repression of underdominant rRNA genes by aza-dC.

The logic of rRNA gene regulation in growth control and nucleolar dominance

NORs can be extremely active loci, typically accounting for 40 to 80% of all nuclear transcription in an actively growing cell. In non-growing cells, ribosomal RNA gene transcription is almost undetectable. Available evidence suggests that changes in ribosomal RNA gene transcription are achieved primarily by changing the number of genes that are transcribed, rather than changing the number of transcripts per gene. For instance, electron microscopic evidence in *Drosophila* and *Xenopus* suggests that active ribosomal RNA genes are fully loaded with RNA polymerase molecules whereas adjacent ribosomal genes can be completely inactive and free of polymerase molecules (McKnight and Miller, 1976; Trendelenburg and Gurdon, 1978). Interestingly, intermediate polymerase densities are not observed.

Strains of some species, especially in plants, probably have more ribosomal RNA genes than they normally use. For instance some maize inbred lines have only ~2500 ribosomal RNA genes whereas other lines have as many as 24,000 (Rivin et al., 1986). Most of these ribosomal RNA genes are packaged into condensed heterochromatin (e.g. see Figure 2) and are probably constitutively repressed (Givens and Phillips, 1976; Phillips, 1978; Phillips et al., 1971). In animals and yeast, which typically have ribosomal RNA gene numbers only in the hundreds, there is also evidence that only a fraction of the ribosomal RNA genes are expressed at any one time. This has been deduced by measuring the susceptibility of ribosomal RNA genes to psoralen chemical crosslinking (Conconi et al., 1989). Typically only one-third to one-half of the genes are susceptible to crosslinking (Conconi et al., 1989; Dammann et al., 1993; Dammann et al., 1995). This psoralen accessible class can be crosslinked to RNA transcripts, suggesting that this class represents the active group of genes whose more open chromatin structure allows psoralen accessibility. Based on these considerations, it seems reasonable that the mechanisms that control the number of active ribosomal RNA genes in a non-hybrid species could be the same mechanisms responsible for nucleolar dominance in hybrids. Thus nucleolar dominance might be a manifestation of a dosage compensation mechanism that is always at work to control the number of active ribosomal RNA

genes in a cell. The paradox is that dosage compensation in a hybrid could also be achieved by simply expressing both parental sets of rRNA genes at reduced levels. Thus the mechanisms leading to discrimination and preferential expression of only one parental set of ribosomal RNA genes in a hybrid is still a mystery in need of explanation.

Future directions

Many questions remain concerning the regulation of ribosomal RNA genes in eukaryotes, and *Arabidopsis* can continue to serve as an important model system. The mechanisms responsible for concerted evolution of ribosomal RNA gene sequences, for growth regulation of RNA polymerase I transcription, for chromosomal control of ribosomal RNA gene accessibility and for nucleolar dominance remain to be elucidated. Questions concerning the ways in which chromosomal domains are established and independently regulated can also be addressed using *Arabidopsis* NORs as a model system. The continued development of genetic resources in *Arabidopsis* combined with efforts to develop biochemical tools, such as *in vitro* transcription systems and *in vitro* RNA processing systems, is sure to have a large impact in unraveling the mysteries of rRNA gene biology.

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