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Nucleolar dominance and silencing of transcription

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Nucleolar dominance is a phenomenon in plant and animal hybrids whereby one parental set of ribosomal RNA (rRNA) genes is transcribed, but the hundreds of rRNA genes inherited from the other parent are silent. The phenomenon gets it name because only transcriptionally active rRNA genes give rise to a nucleolus, the site of ribosome assembly. Nucleolar dominance provided the first clear example of DNA methylation and histone deacetylation acting in partnership in a gene-silencing pathway. However, the sites of chromatin modification and the ways in which one set of rRNA genes are targeted for repression remain unclear. Another unresolved question is whether the units of regulation are the individual rRNA genes or the multi-megabase chromosomal domains that encompass the rRNA gene clusters.

Nucleolar dominance was first discovered as a reversible change in chromosome morphology[1–3]. It was noted that the metaphase ‘D’ chromosomes in the root-tip cells of pure (non-hybrid) diploid species of the plant genus *Crepis* always had a ‘satellite’: a distal portion of the chromosome attached to the rest of the chromosome by a thin secondary constriction (the primary constriction being the centromere). However, in 13 out of 21 different F1 hybrid combinations a satellite and a secondary constriction formed on the D chromosome inherited from one species but not the other, a phenomenon called ‘differential amphiplasty’ (Fig. 1). Importantly, the same species’ satellite was suppressed (under-dominant) regardless of whether this species was the maternal or paternal parent in the cross. Suppressed D chromosomes could form satellites again in the next generation if the hybrids were backcrossed to the under-dominant parent, which suggested that the chromosome was not being damaged by passage through the hybrid. It was concluded that differential amphiplasty is a reversible phenomenon that is brought about by interactions between the parental genomes[1].

Coincident with the studies on *Crepis*, Barbara McClintock demonstrated that nucleolus formation and secondary constriction formation are causally related[4]. Convincing evidence was obtained from a maize line that had undergone a reciprocal chromosome translocation resulting from X-ray-induced chromosome breakage. One break occurred within the region on chromosome 6 where the nucleolus is associated (a locus McClintock named the nucleolar organizer); the other occurred within chromosome 9. Instead of the usual single nucleolus and secondary constriction observed in wild-type maize, two nucleoli and secondary constrictions were formed precisely at the sites where the pieces of chromosomes 6 and 9 were fused. McClintock concluded that to be divisible the chromosomal information at the nucleolus organizer region (NOR) must be redundant. She was correct, and decades later NORs were shown to be multi-megabase loci where rRNA genes are repeated[5,6], sometimes in thousands of copies (Fig. 2). Based on the relative nucleolus-forming ability of the translocated maize chromosomes (alone or in the presence of wild-type chromosomes) McClintock hypothesized that dominant and under-dominant NORs differed in their ability to organize nucleoli. Considering the *Crepis* data, McClintock suggested a simple dominance hierarchy based on the functional capacity of *Crepis* NORs (Ref. 4). Consistent with this prediction, it was

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shown nearly 40 years later that *Crepis* species could be arranged in a four-tiered hierarchy, with those at the top dominant over all the species below, and those species within a tier being co-dominant[7].

The knowledge that NORs are the sites where rRNA genes are clustered and that nucleoli contain rRNA transcripts[5,6], led to the proposal that is essentially the modern view of the NOR (Fig. 2). Based on McClintock's cytogenetic data, it was suggested that only a fraction of the rRNA genes are active at the NOR in wild-type maize[7]. These active genes were thought to form the secondary constriction because the nucleolus somehow interfered physically with chromosome condensation. It was further suggested that most maize rRNA genes are not active, but are condensed into a dark-staining, heterochromatic structure (chromomere) adjacent to the secondary constriction. Interestingly, McClintock had considered this chromomere to be the NOR, dismissing a role for the secondary constriction even though it traversed the nucleolus). Because McClintock's reciprocal translocation line resulted from a break within the chromomere, the formation of a second nucleolus implied a de-repression of 'excess' rRNA genes normally situated within the chromomere[7]. This interpretation suggested that the control of rRNA gene activity was a 'typical case of a repressible system'[7] analogous to a prokaryotic operon. Presumably, the same repression mechanisms that control the number of active genes within a pure species might be responsible for nucleolar dominance in hybrids[7]. The idea that nucleolar dominance reflects a dosage-compensation mechanism, which controls the number of active rRNA genes, remains in favor[2,3,8].

Hybrid frogs provided the first example of nucleolar dominance in the animal kingdom. During early development in hybrids of *Xenopus laevis* and *X. borealis*, only *X. laevis* rRNA was synthesized[9]. Subsequent S1 nuclease protection (Fig. 3) and nuclear run-on assays in plants have confirmed that nucleolar dominance is controlled at the level of transcription rather than RNA turnover[10].

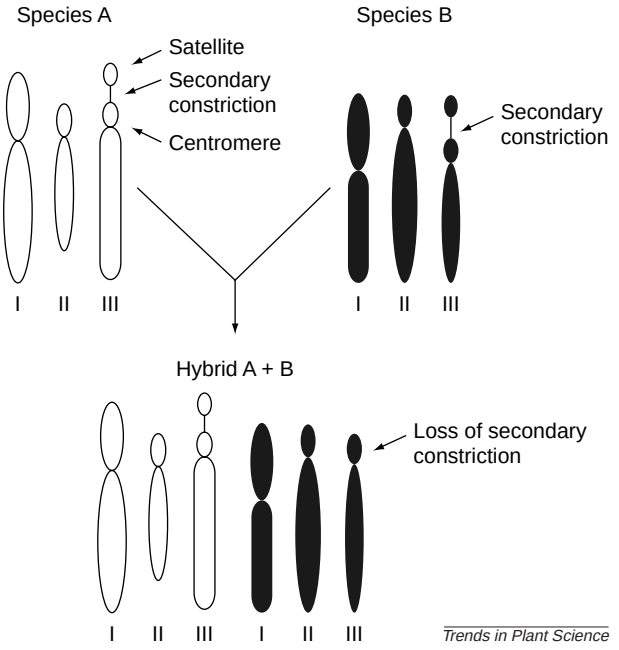

Fig. 1. Loss of the secondary constriction on metaphase chromosomes is the cytogenetic manifestation of nucleolar dominance, also known as 'differential amphiplasty'. Active rRNA genes that cause nucleolus formation during interphase[49] remain relatively uncondensed at metaphase, giving rise to a secondary constriction. In this diagram of a haploid chromosome complement from two related species at metaphase, each species has a nucleolus organizer region (NOR), which has a secondary constriction on chromosome III. However, in the hybrid progeny of these species, chromosome III of species A forms a normal secondary constriction but the constriction at the NOR of species B is suppressed.

A role for cytosine methylation in selective gene silencing

Although our understanding of nucleolar dominance is incomplete, there is substantial evidence that under-dominant rRNA genes are selectively repressed. Cytosine methylation appears to be a part of this silencing mechanism, at least in plants. Initial evidence suggested that the methylation of specific restriction endonuclease sites was correlated with rRNA gene inactivity at under-dominant NORs of wheat[11], Triticale[12–14] and maize[15] as well as at developmentally-regulated rRNA gene loci in pea[16]. More recently, treatment with 5-aza-2′ deoxycytosine (aza-dC) (an inhibitor of cytosine methyltransferase activity) has been shown to derepress under-dominant rRNA gene transcription[10,17] and to cause nucleoli to form at suppressed NORs (Refs 18–20).

Is the effect of cytosine methylation on rRNA gene activity direct or indirect? An example of a direct effect would be the inability of a transcription factor to recognize its binding site if one or more cytosines at the site were methylated. Arguing against this possibility is the observation that *Brassica* rRNA minigenes can be methylated at all CG sites using *Sss* I methylase, and remain fully active for transcription *in vitro*[21]. This suggests that nucleolar dominance in *Brassica* is unlikely to be due to the inability of transcription factors to recognize a methylated template. A more likely scenario is that cytosine methylation helps to bring about the assembly of a repressive chromatin state that excludes access by the transcription machinery, as has been shown for several genes[22]. The correlation between rRNA gene inactivity and decreased DNase accessibility (indicative of a more tightly packed chromatin structure) in wheat[11], Triticale[12–14], maize[15] and pea[16] is fully consistent with this alternative view. Likewise, a study in *Xenopus* has shown that methylation can inhibit transcription from an rRNA minigene, but only if the proteins that bind specifically to methylated DNA are present[23]. If these proteins are titrated with methylated competitor DNA, transcription from the rRNA gene promoter is stimulated by methylation. Collectively, these results favor indirect inhibition of rRNA gene transcription by cytosine methylation, mediated by changes in chromatin structure.

Changes in rRNA gene methylation might not even be needed to establish repressive chromatin structures. For instance, rRNA genes at dominant NORs in *Xenopus* hybrids are more accessible to DNase than genes at under-dominant NORs but no differences in methylation have been found[24]. The extent of rRNA gene methylation and rRNA gene activity is also poorly correlated in *Brassica*[10]. In the allotetraploid hybrid, *Brassica napus*, both active and inactive rRNA genes appear to be methylated at every *Hpa* II site (a methylation-sensitive restriction endonuclease) suggesting that rRNA genes are fully methylated even when active[10]. Aza-dC treatment, which causes only a modest decrease in the methylation of these *Hpa* II sites, causes full derepression of the under-dominant genes[10]. Perhaps methylation of *Hpa* II sites is not a good predictor of the methylation status of a crucial regulatory sequence within the rRNA genes. Or perhaps modest changes in methylation density bring about disproportionately strong effects by preventing cooperation among repressive chromatin proteins. A third possibility is that aza-dC causes the demethylation of a regulatory locus that is distinct from the rRNA genes. At present, these possibilities cannot be distinguished.

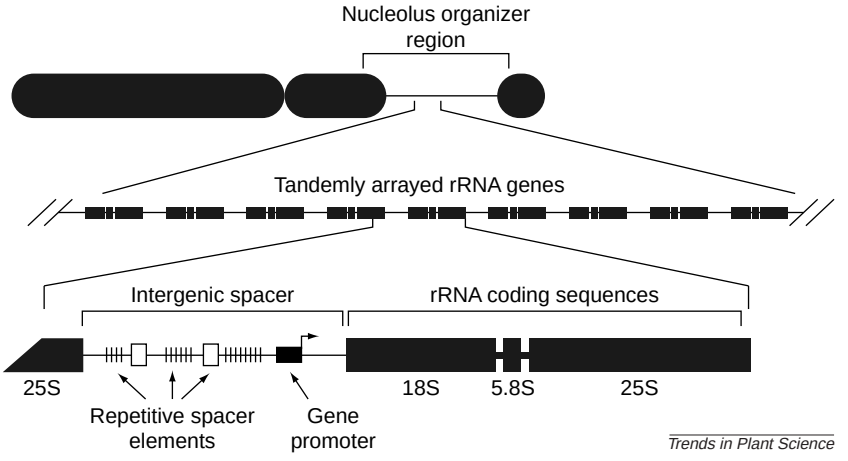

Fig. 2. Nucleolus organizer regions (NOR) consist of long head-to-tail repeats of the genes encoding the precursor of the three largest ribosomal RNAs (18S, 5.8S and 25S). An NOR can include both transcriptionally active rRNA genes, which give rise to the secondary constriction on a metaphase chromosome (top), and to silent rRNA genes. Thus an NOR is often larger than the secondary constriction. Within an NOR, each rRNA gene in the tandem array is almost identical in sequence, with some variation in length caused by differences in the number of repeated DNA elements in the intergenic spacers (bottom). These intergenic spacer regions evolve rapidly and can be different even between closely related species, whereas rRNA coding regions are highly conserved from bacteria to humans.

A role for histone modification

Although cytosine methylation is often a focus of gene-silencing studies[25,26], organisms including *Saccharomyces cerevisae*, *Drosophila* and *Caenorhabditis elegans* do not methylate their DNA (Refs 26,27). In these organisms (as well as in species that methylate), modification of the histones, around which DNA is wrapped, plays an important regulatory function[28,29]. Acetylation or deacetylation of lysines in the amino termini of several histones, especially histones H3 and H4, appears to control gene activity by altering the accessibility of transcription factor binding sites on the surface of the nucleosome[28,29]. In general, inactive genes are assembled in nucleosomes whose histones are mostly deacetylated whereas active genes tend to be associated with nucleosomes containing hyperacetylated histones. These acetylation levels reflect a steady state that results from the combined actions of histone acetyltransferases (which put acetyl groups onto histones) and histone deacetylases (which take them off).

Nucleolar dominance occurs in *Drosophila*[30], but methylation cannot explain it. This prompted an investigation to discover whether histone deacetylation might play a role in nucleolar dominance. *B. napus* allotetraploids showing nucleolar dominance were treated with sodium butyrate or trichostatin A (chemicals that block histone deacetylase activity and cause histones to accumulate in a hyperacetylated state). As with aza-dC treatment, silent rRNA genes are reactivated[10]. Interestingly, treatment with both aza-dC and trichostatin A is no more effective than either compound alone, suggesting that cytosine methylation and histone deacetylation are partners in the same repression pathway[10].

Recent biochemical studies in mammalian systems have suggested how the partnership between cytosine methylation and histone deacetylation probably works. Proteins that bind with high specificity to methylated DNA are part of a multi-protein complex that includes one or more histone deacetylases[31,32]. Thus transcriptional repression associated with cytosine methylation might come about by recruiting to methylated DNA a histone deacetylase complex that modifies the nucleosomal histones in the region. The resulting histone hypoacetylation is then thought to alter the local chromatin structure, such that transcription factors are denied access to the promoter. In this model, methylation acts upstream of histone deacetylation. This hypothesis predicts that aza-dC treatment should cause a decrease in cytosine methylation and an increase in histone acetylation in the course of derepressing the under-dominant rRNA genes. But by acting downstream of methylation, histone deacetylase inhibitors might derepress the under-dominant genes without affecting methylation. These predictions need to be tested. It should also be interesting to determine whether nucleolar dominance in *Drosophila* and *Xenopus* can be relieved by blocking histone deacetylase activity, as is predicted if nucleolar dominance operates by the same mechanisms in plants and animals. Likewise, the developmentally programmed derepression of under-dominant rRNA genes in all organs derived from the floral meristem in *Brassica*[33] can be investigated to determine if the change in gene activity is correlated with changes in cytosine methylation, histone acetylation, or both.

Histone deacetylase-targeting in *Drosophila*, yeast and *C. elegans* cannot be based on methylation; thus other molecular signals must be important for specifying where regulatory chromatin modifications occur. Sequence-specific DNA-binding proteins (e.g. Ume 6, Mad/Mxi1, SMRT, N-CoR) are known to play roles in histone deacetylase recruitment and in the repression of specific genes[34–36]. Thus, plants and other organisms that methylate their DNA might use both methylcytosine density and/or sequence-specific binding of repressor proteins to target chromatin modifications to specific genes. If this is so, a given methylation state might be necessary but not sufficient to explain gene silencing. This could be the explanation for situations in which cytosine methylation is not correlated with transcriptional silencing, as in some cases of paramutation in maize[37].

Silencing of transcription factor genes cannot explain nucleolar dominance

Under-dominant rRNA genes can be derepressed by inhibitors of cytosine methylation or by histone deacetylation, indicating that the silenced genes are not defective but are controlled at the level of chromatin. Whether the rRNA genes or another locus, such as a gene encoding a transcription factor, is regulated by chromatin modification remains unknown. For instance, the rapid evolution of rRNA gene regulatory sequences in the intergenic spacer (Fig. 2) and the co-evolution of the transcription factors that recognize these sequences often result in species-specificity of RNA polymerase I transcription[38]. In other words, an rRNA gene promoter from one species will not be recognized by the transcription machinery in the cell of another species. Thus, silencing the gene for a species-specific transcription factor encoded in one parental genome could, conceivably, result in the complete silencing of that parent's rRNA genes in the hybrid[2].

If nucleolar dominance were caused by the absence of a transcription factor, one would expect that an under-dominant gene introduced into a hybrid cell would not be expressed, but this is not the case. In both *Brassica* and *Arabidopsis*, under-dominant

and dominant rRNA minigenes (cloned in plasmid vectors) are expressed at the same level whether they are transfected alone or if they are in competition with each other in hybrid cell protoplasts[17,21]. Importantly, the chromosomal copies of the under-dominant genes remain silent in these same protoplasts. These results suggest that the transcription machinery necessary for the expression of under-dominant genes is present in hybrid cells, but that the chromosomal copies of these genes are denied access to this machinery. Presumably chromosomal genes are assembled in an inaccessible chromatin structure that is not assembled on transiently expressed genes carried within plasmids.

Transcription factor competition is unlikely in plants

Discrimination between dominant and under-dominant rRNA genes has been explained by a popular model in which the differences in sequence or number of regulatory elements in the intergenic spacers results in a greater binding affinity for transcription factors to the dominant genes[2,8,14,39]. Assuming that the number of rRNA genes in the nucleus is substantially in excess of these critical factors, the model predicts that dominant genes sequester the factors and are transcribed, whereas underdominant genes lose out in this competition and are inactive[2,8]. Subsequent changes in methylation and/or histone deacetylation might then lock-in or enforce the silencing of under-dominant genes[8,10].

Preferential transcription of *X. laevis* rRNA minigenes over *X. borealis* minigenes when both are co-injected into oocytes (which mimics the dominance relationship in hybrid frogs), favors the transcription factor competition model[40]. Differences in the intergenic spacers, presumably in the sequence or in the number of repetitive enhancer elements, appear to be responsible[40].

Although differences in gene structure have been correlated with nucleolar dominance in cereals[20,35], which indirectly suggests a transcription factor competition model similar to that proposed for *Xenopus*, no such correlation exists for *Arabidopsis* or *Brassica*[21,33]. Direct tests have also failed to detect any difference in the ability of dominant and under-dominant plant rRNA genes to compete for transcription factors, either *in vivo* or *in vitro*[17,21].

One might argue that transcription factor competition with plasmid-encoded and chromosomal rRNA genes might yield different results. However, dominant chromosomal genes can be made under-dominant by changing the parental chromosome dosage[17], a result that also argues against the hypothesis that dominant genes have higher affinities for transcription factors. If the hypothesis were correct, dominant genes should always recruit transcription factors best, even when outnumbered. Decreasing the number of dominant genes, such that they are no longer in excess over transcription factors, should allow under-dominant genes to recruit transcription factors, leading to apparent co-dominance. However, silencing of the normally dominant genes (dominance reversal) is not predicted – nonetheless, dominance reversal is what is observed[17].

Importantly, the results discussed here do not disprove the involvement of transcription factors in rRNA gene discrimination. The results simply argue against the notion of every gene for itself when competing for these factors. Instead, some undiscovered property of groups of rRNA genes, such as cooperativity, or some other feature of dominant NORs, perhaps unrelated to rRNA gene structure, is probably important.

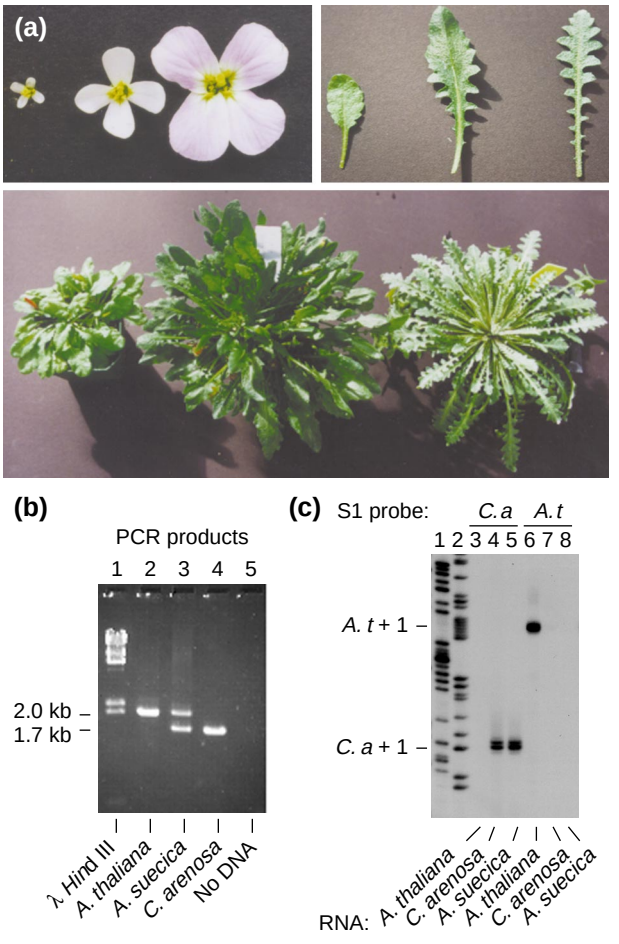

Fig. 3. Molecular analysis of nucleolar dominance in *Arabidopsis*. (a) Flower, leaf and whole-plant phenotypes of *Arabidopsis thaliana* (left), *Cardaminopsis arenosa* (also known as *Arabidopsis arenosa*; right) and their allotetraploid hybrid, *Arabidopsis suecica* (center). Note the intermediate phenotypes of flower and leaf morphologies in *A. suecica*, which is common in hybrids. (b) The ribosomal RNA genes from *A. thaliana* and *C. arenosa* are both present in similar abundance in *A. suecica.* Genomic DNA of *A. thaliana* (lane 2), *A. suecica* (lane 3) or *C. arenosa* (lane 4) was subjected to PCR using a 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 λ DNA cleaved with *Hin*d III served as size markers in lane 1. (c) Only *C. arenosa* ribosomal RNA genes are transcribed in *A. suecica*, as shown using the S1 nuclease protection assay (compare lane 5 with 8). Equal aliquots of *A. thaliana*, *C. arenosa* or *A. suecica* RNA were analyzed with *C. arenosa* (*C.a*; lanes 3–5) or *A. thaliana* (*A.t*; 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. (b) and (c) are reprinted

Chromosomal effects suggest regulatory signals external to NORs

Compelling evidence that rRNA genes themselves are not sufficient to dictate nucleolar dominance includes several cytogenetic studies that show that the chromosomal context of an NOR is important. For instance, barley has two co-dominant NORs: one located on chromosome 6 and the other on chromosome 7. When these two NORs are located on the same chromosome owing to a chromosome translocation (presumably involving breakage sites far from the NORs), the chromosome 6 NOR is dominant[41–43]. Triticale, the hybrid of wheat and rye, provides another example. Normally the rye NOR on the short arm of chromosome 1R is suppressed in Triticale and wheat NORs are active[44,45]. However, translocations that fuse the short arm of rye chromosome 1R onto

the long arms of any of several wheat chromosome 1 homeologs, in place of the short arms of the normal wheat chromosome 1, allow the expression of the rye NOR in addition to the wheat NORs (Ref. 46). Based on other observations, the loss of the rye 1R long arm appears to be responsible for the lack of rye NOR suppression. Interestingly, on an unrearranged chromosome 1R (where the long arm is present) in Triticale, the rye NOR is also expressed when the rye chromosome 2R is substituted by wheat chromosome 2D (Ref. 43). Together, these results suggest that one or more genes on the long arm of rye chromosome 1, and one or more genes on rye chromosome 2, interact to suppress the rye NOR in a wheat–rye hybrid. Complex chromosomal interactions also affect nucleolus expression in wheat[47].

Nucleolar dominance in *Drosophila* has also provided other evidence that chromosomal regions, in addition to the rRNA genes and NORs, can play a role in nucleolar dominance. *Drosophila melanogaster* and *D. simulans* each have an NOR near the centromere on the X chromosome. *D. melanogaster* has an additional NOR on the Y chromosome. Both *D. melanogaster* NORs are dominant over the *D. simulans* NOR (Ref. 30), as can be shown readily in hybrid XX females or in XY males (in which the Y is from *D. melanogaster*). Interestingly, rearrangement of the heterochromatin that flanks either of the *D. melanogaster* NORs allows normal nucleolus formation on the *D. melanogaster* sex chromosomes but causes a failure to suppress the *D. simulans* NOR (Ref. 48).

Questions, speculations and directions

The cytological data from *Drosophila* and cereals imply the existence of genes or sequences that suppress one parental set of rRNA genes as opposed to selectively activating the other. In flies, *D. melanogaster* sequences play a role in suppressing the *D. simulans* NOR. In Triticale, sequences on two rye chromosomes are implicated in suppressing their own (rye) NOR but not wheat NORs. One possibility might be that regulatory loci external to the NORs encode genes for freely diffusible repressor proteins, which bind to under-dominant rRNA genes in a sequence-specific manner, reminiscent of the operon-like regulation envisioned nearly 30 years ago[7]. However, this appears unlikely given the transient expression results in *Arabidopsis* and *Brassica*, which show that minigenes on plasmids are transcribed in the same cells in which their chromosomal counterparts are suppressed[21]. Perhaps the chromosomal loci external to the NORs encode chromatin-modifying activities, such as histone deacetylases or methyltransferases, which are specific for chromosomal rRNA genes. Other possibilities include genes of a hypothetical dosage-compensation mechanism[8,43] that control the number of transcribed rRNA genes by acting upstream of chromatin modification. How this system would function or choose one set of genes over another is unknown.

The cooperativity inherent in the silencing of complete sets of rRNA genes coupled with the cytogenetic studies discussed here suggest that the units of regulation in nucleolar dominance might be NORs, or even larger chromosomal regions that include NORs, rather than individual rRNA genes. Perhaps altering the genomic locations of NORs by chromosome rearrangement can disrupt NOR-silencing by separating the NORs from important chromosomal signals. For instance, NORs might be discriminated by the time at which they are replicated or by their location in the three-dimensional space of the nucleus. Genes that affect these chromosomal processes could affect nucleolar dominance without directly involving rRNA gene sequences or the RNA polymerase I transcription system.

Experiments are a logical antidote for ignorance. As a start, one can determine whether silencing in nucleolar dominance is restricted to the rRNA genes or if silencing extends beyond the NOR boundaries. Other experiments, such as determining whether rRNA transgenes located outside the NORs can be silenced should be informative. Together, these approaches might indicate whether nucleolar dominance mechanisms act primarily on each rRNA gene or if they act on the NOR as a single chromosomal domain. Identifying genes or sequences encoded by loci that affect nucleolar dominance would be illuminating.

Obviously, there is much hard work to be done, but the knowledge that nucleolar dominance holds important clues about the chromosomal control of gene expression is a wellspring of inspiration.

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