

The epigenetics of nucleolar dominance

Epigenetic phenomena are heritable, alternative states of gene activity that are not explained by mutation, changes in gene sequence or normal developmental regulation. Among the earliest examples was nucleolar dominance, a common phenomenon in interspecific hybrids in which only ribosomal RNA (rRNA) genes inherited from one parent are transcribed. Only active rRNA genes initiate formation of a nucleolus, hence the name for the phenomenon. As in other epigenetic phenomena, chromatin modifications enforce selective gene silencing in nucleolar dominance. However, the mechanisms that discriminate between parental sets of rRNA genes are unclear. Possibilities include sequence differences that affect transcription factor affinities. Other evidence suggests that chromosomal context is more important than rRNA gene sequences, implying control on a larger scale.

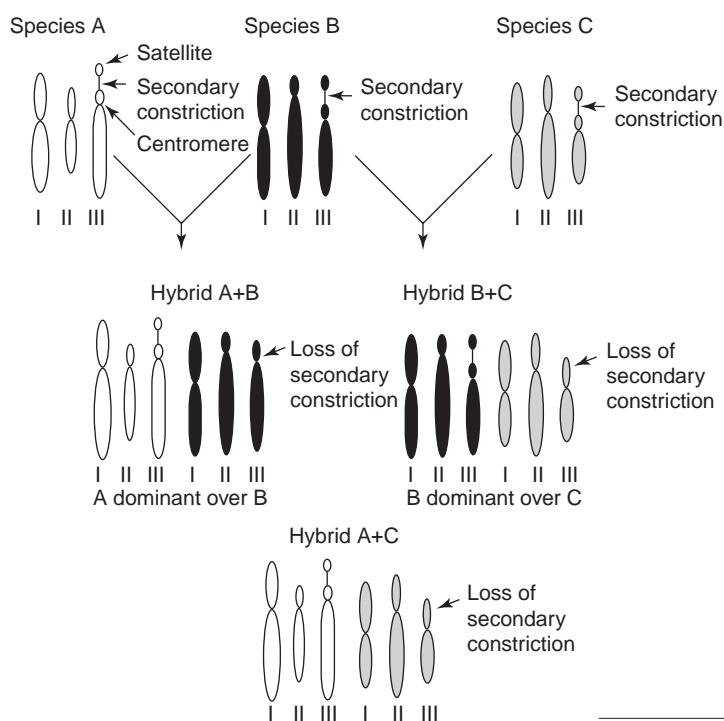
In the annals of nucleolar dominance research, 1934 was quite a year. Based on examinations of numerous *Crepis* species and their hybrids, Navashin noted¹:

It was a great surprise to find that the chromosomes of two or more different species brought together by hybridization in certain specific combinations suffer striking alterations of their individuality.

Navashin used the term ‘amphiplasty’ to describe the ability of metaphase chromosomes to adopt new forms². Changes that affected all chromosomes, such as thickening or shortening, he termed ‘neutral amphiplasty’. The most dramatic change, affecting only the ‘D’ chromosomes (*Crepis* chromosomes were designated with letters rather than numbers), he called ‘differential amphiplasty’. In ‘pure’ species (non-hybrids), D chromosomes at metaphase displayed a satellite attached to the rest of the chromosome by a thin strand known as the secondary constriction (the primary constriction being defined as the centromere). In only eight of 21 different hybrid combinations did the D chromosomes of both progenitor species display this characteristic morphology; in the other 13, the D chromosome of one progenitor had apparently ‘retracted its satellite’ (Fig. 1). This situation was observed in every root-tip cell regardless of which species was the maternal or paternal parent in the cross – the D chromosome of the same species was always the one suppressed (underdominant). Importantly, underdominant D chromosomes were not permanently altered because they were reactivated in F2 segregants that essentially recreated the genotype of the underdominant ‘pure species’. Thus, differential amphiplasty was interpreted to be a reversible phenomenon caused by interactions among the parental genomes¹. The reversibility of the phenomenon has since been demonstrated even within a generation, occurring as *Brassica* hybrids undergo the transition to flowering³ or as *Xenopus* hybrids develop into adults⁴.

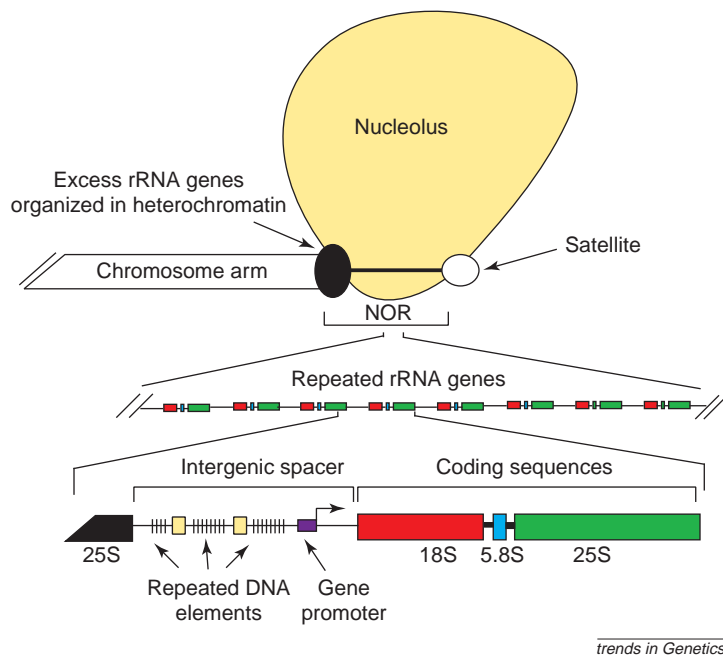
Also in 1934, McClintock made an important discovery showing that secondary constrictions correspond

FIGURE 1. The discovery of nucleolar dominance



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In this cartoon, inspired by Navashin's studies of *Crepis* hybrids², haploid chromosome sets of three ‘pure’ species and their hybrids are shown. Each pure species has one chromosome with a nucleolus organizer region (NOR) that forms a secondary constriction at metaphase. When crossed to form hybrids, only the NOR from one progenitor forms the characteristic secondary constriction. In the example shown, the NOR of species A is dominant over the NOR of species B in an A–B hybrid, the NOR of species B is dominant in a B–C hybrid, and the NOR of species A is dominant in an A–C hybrid. Such data suggested to McClintock that a series of species could be ordered in a simple nucleolar dominance hierarchy determined by the strength of their NORs⁵.

FIGURE 2. Organization of a typical nucleolus organizer region

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Nucleolus organizer regions (NORs) are head-to-tail arrays of genes encoding the precursor of the three largest ribosomal RNAs (18S, 5.8S and 25S in plants). NORs include active rRNA genes, which give rise to secondary constrictions of metaphase chromosomes, and silent rRNA genes, which are often highly compacted in dense heterochromatin. At metaphase, a proteinaceous remnant of the nucleolus often remains associated with the secondary constriction. Each rRNA gene at a NOR is nearly identical in sequence, although variation in size due to differences in the number of repeated DNA elements in the intergenic spacer region is common.

closely to sites of nucleolus formation⁵. In wild-type maize, chromosome 6 displays a secondary constriction at metaphase and is associated with a nucleolus throughout interphase. But in a line in which a reciprocal translocation divided the region near the secondary constriction among two chromosomes, nucleoli and secondary constrictions now occurred on both chromosomes. Thus the breakage site on chromosome 6 had defined a locus responsible for nucleolus formation, dubbed the 'nucleolar organizer'⁵. Clearly, the genetic information at this site was redundant, given that it could be divided. The latter conclusion was ultimately verified when it became clear that nucleolus organizer regions (NORs) correspond to loci where the middle repetitive rRNA genes are clustered by the hundreds (to thousands) in head-to-tail arrays (Fig. 2)^{6,7}. The appearance of secondary constrictions at NORs is thought to be due to rRNA transcription and/or structural features of the nucleolus impeding chromosome condensation⁸.

Studies in *Xenopus* elucidated the molecular basis for nucleolar dominance. Cytological examinations had shown that hybrids of *Xenopus laevis* and *Xenopus borealis* did not express the NORs of both parents^{9,10}. Molecular analyses then showed that only *X. laevis* rRNA transcripts could be detected in hybrid frogs during early development, indicating that differential rRNA gene expression was the basis for the phenomenon⁴. Nuclease protection assays have extended this conclusion to plants (Fig. 3)³. Nuclear run-on assays have confirmed that nucleolar dominance is controlled at the level of transcription rather than RNA turnover¹¹. As a result of the initial molecular studies, the term 'nucleolar dominance' was introduced into the

literature⁴ and has since gained wide acceptance (for additional minireviews, see Refs 12 and 13; for a more complete review of the literature see Ref. 14).

Early clues

The earliest studies suggested some regularity to nucleolar dominance in addition to its independence from maternal or paternal influences. McClintock saw a pattern in Navashin's data. She noted that when the NOR of species A was dominant over the NOR of species B, and B was stronger than C, then A was invariably dominant over C (see Fig. 1). This suggested that *Crepis* species could be ranked in a straightforward nucleolar dominance hierarchy⁵. Further test crosses ultimately confirmed this idea, showing that *Crepis* species could be arranged in a four-tiered hierarchy with species in top tiers dominant over all species below and species within a tier being codominant⁸. An analogous hierarchy describes nucleolar dominance in allotetraploid hybrids derived from three diploid *Brassica* species³. A hierarchy of wheat NOR activity has also been defined in wheat-rye hybrids by making use of a variety of genotypes and chromosome addition lines¹⁵.

One might imagine that differences in NOR activity are simply due to dominant NORs having more rRNA genes. However, this is not the case. For instance, in allohexaploid bread wheat (an ancient hybrid that combines the genomes of three progenitors) the most active NOR has only half as many rRNA genes as the second-most active NOR¹⁶. The total number of rRNA genes contributed by each progenitor is also not key because nucleolar dominance is often independent of ploidy. In *Crepis* hybrids, the NOR that was dominant when the parental genome doses were 1:1 (allodiploid) remained dominant even when outnumbered 2:1 or 3:1 in allotriploid or allotetraploid hybrids, respectively¹. The same is true for dominant NORs outnumbered 4:2 in *Brassica* allohexaploids³.

Hypotheses suggesting control at the level of individual rRNA genes

Because nucleolar dominance is controlled at the level of rRNA transcription, the RNA polymerase I system responsible for rRNA synthesis has been a logical place to look for explanations. Two hypotheses have resulted: the species-specific transcription factor hypothesis and the enhancer-imbalance hypothesis.

An early use of *in vitro* transcription systems for RNA polymerase I was the demonstration that a murine or human rRNA gene promoter would not function in a cell-free extract made from the other species (for reviews see Refs 17–19). The likely explanation was that one or more transcription factors had co-evolved with the changing DNA sequences so as to be species-specific. Subsequent studies showed that a mouse cell-free extract could be reprogrammed to transcribe a human rRNA gene promoter if a specific human transcription factor (SL1) was added to the reaction. Likewise, a mouse promoter could be transcribed in a human cell-free extract if the reaction were supplemented with mouse SL1.

A form of nucleolar dominance, achieved through somatic cell fusion, occurs in mouse-human hybrid cells. In some lines, mouse rRNA genes are expressed whereas in others only human rRNA genes are active^{20–24}. Loss or inactivation of genes for mouse or human SL1 could conceivably cause the failure to express the corresponding set of rRNA genes (although this has never been shown).

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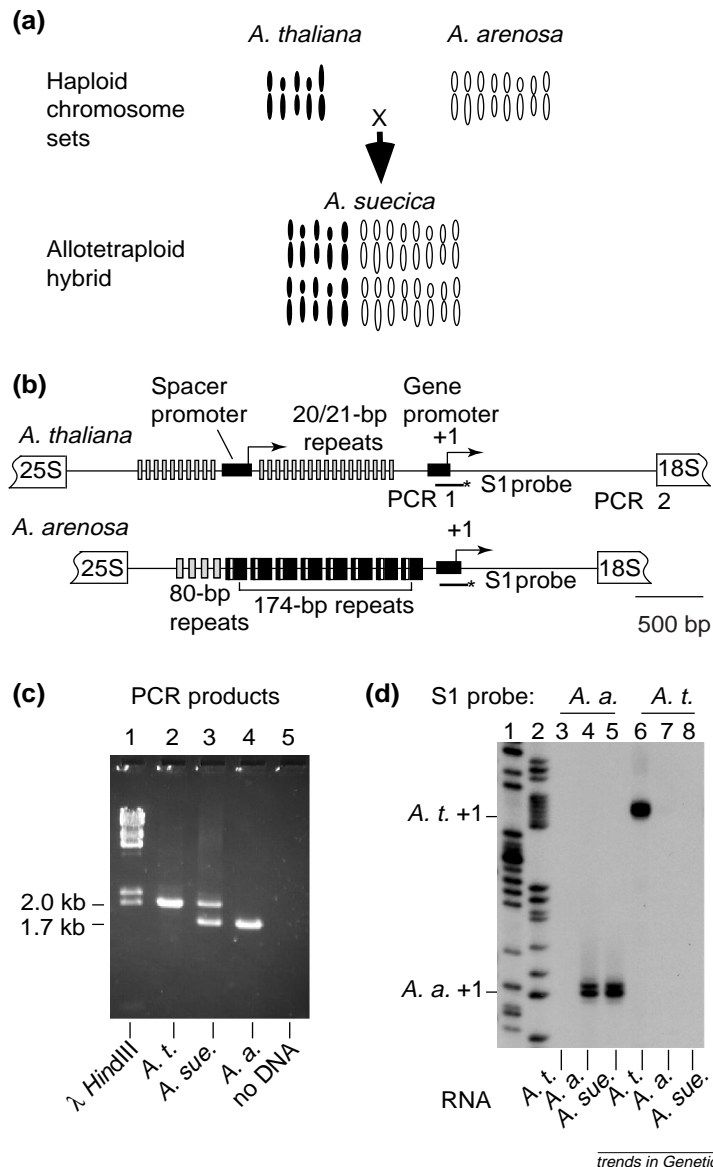
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However, this hypothesis is inadequate for nucleolar dominance in species that can interbreed. For instance, underdominant rRNA gene promoters of *Brassica* or *Arabidopsis* are fully functional following transfection into protoplasts of the dominant species^{25,26}. Thus, dominant rRNA genes could use the transcription machinery of the other species even if their own transcription factors were unavailable. Furthermore, nucleolar dominance can occur within a species, as in hybrids of recombinant maize inbred lines^{27,28}. For these reasons, the species-specific transcription factor hypothesis is not considered to be a likely explanation for nucleolar dominance in nature.

The enhancer imbalance hypothesis stems from studies conducted in *Xenopus*¹². In oocytes and early embryos, the repetitive DNA elements located just upstream of the rRNA gene promoter act as orientation- and position-independent enhancers of transcription (see Fig. 2). Importantly, transcription from a cloned promoter is severely inhibited when enhancers on a separate plasmid are coinjected, suggesting that enhancers and promoters compete for one or more transcription factors²⁹. *X. laevis* and *X. borealis* rRNA genes have different types and numbers of repetitive elements in their intergenic spacers (analogous to the situation for *Arabidopsis* rRNA genes; see Fig. 3b). This suggested a model whereby the more numerous enhancers of *X. laevis* might titrate a factor(s) and sequester it, thus denying the *X. borealis* rRNA genes access to the factor(s)^{12,30}. Experiments in which *X. laevis* and *X. borealis* rRNA minigenes were coinjected into oocytes showed that *X. laevis* minigenes were preferentially transcribed, in agreement with the situation in hybrids³⁰. Using recombinant constructs in which promoter and intergenic spacer sequences were swapped, it was demonstrated that the intergenic spacer of *X. laevis* was responsible for the differential expression³⁰.

The appeal of the enhancer imbalance hypothesis is that it suggests a mechanism for discriminating rRNA genes in a hybrid cell based on the affinities of DNA-binding transcription factors. This could explain independence from maternal or paternal effects. Furthermore, a NOR with relatively few genes could still be dominant over one or more NORs with greater numbers of genes if the genes of the smaller NOR had higher binding affinities for the limiting transcription factor(s). Yet, despite its allure, several results are inconsistent with the predictions of the enhancer-imbalance hypothesis. For instance, direct tests in plants have failed to reveal any differences in the competitive strength of dominant and underdominant genes for transcription factors, either *in vivo* or *in vitro*²⁶. Furthermore, an exception to the generalization of ploidy independence also raises doubts²⁵. In *Arabidopsis suecica*-like hybrids, dominance is reversed as the genome ratios are changed from 1:3 to 2:2 to 3:1 (*A. thaliana*:*A. arenosa*). If *A. arenosa* rRNA genes are normally dominant because they have a substantially higher affinity for transcription factors, they could be expected to bind the factors until their binding sites are saturated. Any factors in excess of the binding sites of *A. arenosa* genes would be available to underdominant genes, such that codominance might result, but dominance reversal is not predicted. Importantly, the observation that dominance reversal can occur does not disprove a role for transcription factor affinities in establishing dominance. However, some mechanism for cooperative recruitment of factors to NORs seems more likely than every rRNA gene competing independently for these factors.

FIGURE 3. Molecular analysis of nucleolar dominance in *Arabidopsis*



(a) *A. thaliana* has a haploid chromosome number of five. *A. arenosa* (until recently known as *Cardaminopsis arenosa*³¹) has a basic chromosome number of eight, as is true for most *Arabidopsis* species. Their hybrid, *A. suecica*, combines diploid genome complements of both progenitor species. (b) Diagram of the intergenic spacers of *A. thaliana* and *A. arenosa* highlighting the different numbers and types of repetitive elements upstream from the gene promoters. (c) PCR products using the primers shown in (b) (below the *A. thaliana* spacer drawing) with genomic DNA isolated from *A. thaliana* (lane 2), *A. suecica* (lane 3) or *A. arenosa* (lane 4). Size markers are in lane 1 and a control reaction, performed in the absence of template DNA, is in lane 5. (d) Only *A. arenosa* rRNA gene transcripts are detected in *A. suecica*. RNA isolated from *A. thaliana* (lanes 3 and 6), *A. arenosa* (lanes 4 and 7) or *A. suecica* (lanes 5 and 8) was subjected to the S1 nuclease protection assay with probes specific for *A. arenosa* (lanes 3–5) or *A. thaliana* (lanes 6–8) rRNA transcripts. Sequencing reactions served as size markers in lanes 1 and 2. These data are reprinted with permission and minor modifications from Ref. 25.

The chromosomal context of NORs and rRNA genes affects nucleolar dominance

The idea that nucleolar dominance results from differences in transcription factor binding affinities is hard to reconcile with chromosomal position effects that presumably do not alter rRNA gene sequences. Initial evidence came from cytogenetic analyses of hybrids of *Drosophila melanogaster* and *Drosophila simulans*^{31,32}. In both of these species a NOR is

located on the X chromosome. In hybrid XX females, the *D. melanogaster* NOR is dominant. *D. melanogaster* has a second active NOR, located on the Y chromosome and, in hybrid XY males, the *D. melanogaster* Y-associated NOR is dominant over the *D. simulans* X-associated NOR. Availability of *D. melanogaster* stocks bearing deletions or rearrangements in the heterochromatin flanking the X- and Y-associated NORs facilitated their use in hybrid crosses. Interestingly, chromosome alterations that had no effect on the *D. melanogaster* NORs allowed the expression of the *D. simulans* NOR. Because the *D. melanogaster* NORs appeared to be fully active, it was concluded that they continued to bind transcription factors as usual. Apparently, this is not enough to prevent expression of an underdominant NOR³².

Other examples of chromosomal context affecting NOR activity are found in cereals, such as barley and triticale. Barley has a NOR on chromosome 6 and another on chromosome 7. The two NORs are usually codominant but, in lines in which chromosome translocations place both NORs on the same chromosome, the chromosome-6-associated NOR is dominant^{33,34}. In a different translocation line that has two copies of the chromosome-6-associated NOR on the same chromosome, the duplicated NORs are codominant³³. These results suggest that different NORs respond differently, and in unpredictable ways, depending on their chromosomal environs.

In triticale, the hybrid of wheat and rye, wheat NORs are dominant and the rye NOR, located on the short arm of chromosome 1R, is inactive³⁵. Interestingly, following translocation of the short arm of rye 1R onto the long arm of wheat chromosome 1, the rye NOR becomes codominant with wheat NORs³⁶. Deletions or rearrangements in the long arm of rye chromosome 1R also lead to codominance of the rye NOR³⁶. Finally, the rye NOR is also expressed in lines in which rye chromosome 2R is substituted by wheat chromosome 2D³⁷. Apparently, sequences on the long arm of rye chromosome 1R and on chromosome 2R conspire to suppress the rye NOR in a rye-wheat hybrid.

Other evidence that chromosomal context matters comes from transient gene expression experiments. In *Brassica*, underdominant rRNA minigenes are fully expressed when transfected into the cells of a hybrid, even though the chromosomal underdominant genes are completely inactive in these same cells²⁶. This simple experiment shows that all the transcription factors necessary for expression of the underdominant genes are present in hybrid cells. One deduces that the chromosomal copies are denied access to these factors. Considered together with the evidence from *Drosophila* and cereals, these data suggest that underdominant rRNA genes are selectively repressed.

Chromatin modifications are implicated in uniparental rRNA gene silencing

In vertebrates and plants, cytosine methylation is often correlated with gene silencing³⁸. Evidence in wheat and maize had suggested that rRNA genes at dominant NORs were slightly less methylated than rRNA genes at repressed NORs^{39,40}, although this was not true in *Xenopus*⁴¹, making the role of methylation in nucleolar dominance controversial. Strong evidence for a role of cytosine methylation has come fairly recently by showing that 5-aza-2'-deoxycytosine (aza-dC), an inhibitor of cytosine methyltransferase, will derepress underdominant rRNA genes^{11,25} and suppressed NORs³⁵.

Chemicals that inhibit histone deacetylase activity (trichostatin A [TSA], sodium butyrate) also derepress underdominant rRNA genes¹¹, apparently without affecting rRNA gene methylation (Z.J. Chen and C.S. Pikaard, unpublished). Importantly, treatment with both aza-dC and trichostatin A is no more effective than treatment with either compound alone, suggesting that cytosine methylation and histone deacetylation are partners in the same repression pathway¹¹. Indeed, methylcytosine binding proteins and histone deacetylases are physically associated in mammals, providing a biochemical explanation for a linkage between DNA methylation and histone deacetylation^{42,43}. This linkage may help resolve some current controversies. For instance, the regulatory potential of cytosine methylation has been questioned in *Brassica* and *Arabidopsis* because the rRNA genes in these species are always heavily methylated. In *Brassica napus*, both dominant and underdominant rRNA genes are methylated at all 50–60 *Hpa* II restriction endonuclease sites, such that they are insensitive to *Hpa* II cleavage¹¹. A modest decrease in methylation of these *Hpa* II sites (caused by aza-dC treatment) induces a disproportionate, apparently complete, derepression of underdominant rRNA genes. Furthermore, *Brassica* rRNA minigenes methylated at all CG sites remain fully active for transcription *in vitro*, as is also true in *Xenopus*. Most probably, cytosine methylation does not block the binding of transcription factors directly²⁶. It seems more likely that methylation acts through histone deacetylation to establish a repressive chromatin state that blocks access of the transcription machinery to the promoter. Invoking histone deacetylation (or some other chromatin modification that occurs in all eukaryotes) as the proximal cause of rRNA gene repression might help account for nucleolar dominance in *Drosophila*, a species that does not methylate its DNA.

Nucleolar dominance might reflect a dosage compensation system

rRNA gene coding sequences are essentially identical in species that can interbreed. Therefore, ribosomes made with rRNAs of either species are probably indistinguishable. So what is the point of nucleolar dominance? The best answer at present is that nucleolar dominance may be a consequence of a regulatory process that controls the effective dosage of rRNA genes even in pure (non-hybrid) species. NORs can be very active loci, accounting for 40–80% of nuclear transcription in growing cells⁴⁴. In non-growing cells, rRNA transcription is downregulated to almost undetectable levels. Theoretically, changes in rRNA transcription could be achieved by changing the number of transcripts per gene or by changing the number of genes transcribed. Based on electron microscopic evidence, transcribed rRNA genes appear to be fully loaded with transcribing RNA polymerases^{45,46}. Adjacent genes can be completely inactive, but intermediate polymerase densities are not observed. Thus one can deduce that changes in rRNA transcription result mostly from changes in the number of rRNA genes that are active at any one time. Strains of some species, such as maize, probably have more rRNA genes than they ever use for rRNA synthesis. Different maize inbred lines can have anywhere from 2500 to 24 000 rRNA genes without displaying any obvious differences in growth or appearance⁴⁷. Most maize rRNA genes are packaged in highly condensed heterochromatin knobs adjacent to the active genes (see

Fig. 2)⁴⁸. By measuring the susceptibility of rRNA genes to psoralen crosslinking, studies in animals and yeast have also suggested that only a fraction of the rRNA genes are in an accessible (presumably active) chromatin configuration^{49,50}. Based on these considerations, it seems plausible that the mechanisms that control the number of active rRNA genes in pure species are the same mechanisms responsible for nucleolar dominance in hybrids. Consistent with this hypothesis, dominant rRNA genes in hybrids are upregulated by aza-dC or TSA coincident with the derepression of underdominant rRNA genes, suggesting that both classes of gene are subject to the same negative regulatory mechanisms¹¹. A caveat is that dosage compensation in hybrids could also be achieved simply by coexpressing both sets of rRNA genes at a lower level. Thus, the biased expression of only one parental set of rRNA genes is still in need of explanation.

Current challenges and future prospects

There are still many questions pertaining to the mechanisms responsible for establishment and enforcement of nucleolar dominance. Genetic analyses would seem to have great potential for identifying components of the system. Unfortunately, diploid interspecific hybrids are typically sterile and allopolyploids, although fertile, are not ideal because gene redundancy makes identification of loss-of-function alleles unlikely. None the less, it may be possible to obtain dominant gain-of-function mutations in such polyploid hybrids.

Other questions can be addressed with currently available systems and techniques. For instance, it should be possible to determine whether changes in histone acetylation are better correlated with rRNA gene activity than are changes in cytosine methylation. By using antibodies specific for either acetylated or deacetylated histones for chromatin immunoprecipitation, it should be possible to determine whether dominant genes are preferentially associated with hyperacetylated histones. Furthermore, if current models placing methylation upstream of histone deacetylation are correct, demethylation should induce histone hyperacetylation.

It might also be possible to determine whether nucleolar dominance mechanisms act on individual rRNA genes or on the NOR. If the NOR is the target of regulation, then an rRNA transgene integrated at an ectopic location should escape silencing. Conversely, if individual rRNA genes are targeted, localization within an NOR may be unimportant. A related goal is to determine whether silencing is restricted to the NORs or if it extends to neighbouring genes. Knowing the region at the boundary between silent and active chromatin may help to define important regulatory sequences.

Processes or events related only tangentially to rRNA gene transcription may turn out to be important. For instance, dominant and underdominant rRNA genes may use essentially identical transcription factors that have indistinguishable affinities for the two classes of genes. It is possible that differences in replication timing simply provide dominant NORs with the opportunity to bind these factors first. Cytosine demethylation and/or histone deacetylation could conceivably disrupt such timing differences, explaining the effects of the chemical inhibitors. Chromosomal translocations that move NORs to new locations might also affect replication timing. Alternatively, translocations or chromatin modifications may affect the location of an NOR within the nucleus, possibly affecting NOR activity in profound ways.

These may be idle speculations, but keeping one's mind as free from assumptions as possible seems wise given the large number of questions that remain.

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Perspective

The inheritance of mitochondrial DNA heteroplasmy: random drift, selection or both?

The mammalian mitochondrial genome (mtDNA) is a small double-stranded DNA molecule that is exclusively transmitted down the maternal line. Pathogenic mtDNA mutations are usually heteroplasmic, with a mixture of mutant and wild-type mtDNA within the same organism. A woman harbouring one of these mutations transmits a variable amount of mutant mtDNA to each offspring. This can result in a healthy child or an infant with a devastating and fatal neurological disorder. Understanding the biological basis of this uncertainty is one of the principal challenges facing scientists and clinicians in the field of mitochondrial genetics.

Here we consider the mechanisms behind the transmission of mtDNA heteroplasmy and focus on the key question: is there a selection bias operating during the transmission of heteroplasmic pathogenic mtDNA mutations, or are they inherited like neutral sequence variants?

In addition to the nuclear genome, mammalian cells also have multiple copies of a small (15–17 kb) circle of double-stranded DNA compartmentalized within individual mitochondria¹. The mammalian mitochondrial genome (mtDNA) codes for 13 polypeptides, each of

which is an essential subunit of the mitochondrial respiratory chain. Interspaced among the polypeptide-coding genes are 22 tRNA and two rRNA genes whose products are essential for intramitochondrial protein synthesis². Mammalian mtDNA is compact, with only ~1 kb of noncoding sequence (the D-loop). Unlike nuclear DNA, mtDNA is not coated by protective histones, and it is tethered to the inner mitochondrial membrane, close to the respiratory chain, which is a potent source of oxygen free radicals³. These factors are believed to contribute to a