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Nucleolar dominance: uniparental gene silencing on a multi-megabase scale in genetic hybrids

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Abstract

Nucleolar dominance is a phenomenon in hybrids or allopolyploids in which nucleoli form on chromosomes inherited from only one of the two parents. The molecular basis for nucleolar dominance is the transcription by RNA polymerase I of only one parental set of ribosomal RNA genes (rRNA genes). These rRNA genes are clustered by the hundreds, or thousands, of copies, often spanning tens of millions of basepairs of chromosomal DNA at loci known as nucleolus organizer regions (NORs). Enforcement of nucleolar dominance appears to be accomplished by selectively silencing one set of rRNA genes via chemical modifications of chromatin. However, the mechanisms responsible for initially discriminating among the parental sets of rRNA genes and establishing nucleolar dominance remain unclear. Possibilities include mechanisms that act on each rRNA gene or mechanisms that affect whole NORs or even larger chromosomal domains. This review provides a historical perspective of nucleolar dominance research, explores the most popular hypotheses and their shortcomings, and offers some speculations concerning alternative hypotheses to be considered.

Abbreviations: NOR, nucleolus organizer region; rRNA, ribosomal RNA

Overview

Plants and other eukaryotes have high copy numbers of ribosomal RNA genes whose expression by a dedicated transcription system (RNA polymerase I) is tightly regulated to accommodate the cellular demand for ribosomes and protein synthesis. In many interspecies hybrids, the hundreds (sometimes thousands) of ribosomal RNA (rRNA) genes inherited from one parent are transcribed but the rRNA genes derived from the other progenitor are silent. As a result, nucleoli, the sites of ribosome assembly, form at the chromosomal loci where active rRNA genes are clustered, but not at the inactive loci. Originally termed 'differential amphiplasty' by Navashin (Navashin, 1928, 1934), this epigenetic phenomenon is now best known as nucleolar dominance (Honjo and Reeder, 1973; for reviews, see Reeder, 1985; Flavell, 1986; Neves *et al.*, 1997a, b; Pikaard and Chen, 1998; Pikaard, 1999).

Nucleolar dominance was first discovered in plants, and has been described in interspecific hybrids within numerous plant genera, including *Salix* (Wilkinson, 1944), *Ribes* (Keep, 1960, 1962), *Solanum* (Yeh and Peloquin, 1965), *Hordeum* (Kasha and Sadasivaiah, 1971; Lange and Jochemsen, 1976; Nicoloff, 1979; Schubert and Kunzel, 1990), *Triticum* (Crosby, 1957; Flavell and O'Dell, 1979; Martini *et al.*, 1982; Flavell *et al.*, 1988; Thompson and Flavell, 1988; Sardana *et al.*, 1993), *Agropyron* (Heneen, 1962), *Brassica* (Chen and Pikaard, 1997a, b) and *Arabidopsis* (Chen *et al.*, 1998) and in hybrids of inbred maize (Jupe and Zimmer, 1993; McMurphy and Rayburn, 1994). At least one intergeneric hybrid, *Triticale* (wheat × rye), also displays nucleolar dominance (Lacadena *et al.*, 1984; Appels *et al.*, 1986;

Amado *et al.*, 1997; Neves *et al.*, 1997a, b; Silva *et al.*, 1995; Viera *et al.*, 1990a, b). In the animal kingdom, important studies of nucleolar dominance have been conducted with *Drosophila* and *Xenopus* hybrids (Blackler and Gecking, 1972; Cassidy and Blackler, 1974; Durica and Krider, 1977, 1978) and mammalian somatic cell hybrids (Elicieri and Green, 1969; Bramwell and Handmaker, 1971; Miller *et al.*, 1976; Croce *et al.*, 1977; Soprano *et al.*, 1979; Soprano and Baserga, 1980; Onishi *et al.*, 1984).

Clever experiments in the mid-1980s, primarily with the frog genus *Xenopus*, suggested that dominant rRNA genes are selectively activated due to their superior ability to recruit one or more limiting transcription factors for RNA polymerase I (Reeder and Roan, 1984). More recent evidence in the plant genus *Brassica* has suggested that inactive genes are selectively silenced through covalent chromatin modifications (Chen and Pikaard, 1997 a, b). The inactive 'under-dominant' (the term 'recessive' is inadequate) rRNA gene arrays often span tens of millions of base-pairs of chromosomal DNA, thus gene silencing in nucleolar dominance occurs on a vast scale. In fact, X-chromosome inactivation in the somatic cells of female mammals may be the only example of silencing on a larger scale (Kay *et al.*, 1994; Willard, 1996; Heard *et al.*, 1997).

Cytosine methylation and histone deacetylation are implicated as partners in the enforcement of rRNA gene silencing in nucleolar dominance (Chen and Pikaard, 1997a, b). However, the critical targets of these chromatin modifications are unclear. Possibilities include the individual rRNA genes, the large chromosomal domains that encompass rRNA gene clusters (nucleolus organizer regions; NORs), or other, possibly unlinked regulatory loci such as genes encoding *trans*-acting factors. Even less understood are the mechanisms by which dominant and under-dominant rRNA genes are discriminated in newly formed hybrids, leading to the initial establishment of nucleolar dominance.

About 70 years of research has contributed to our current understanding of nucleolar dominance. I have attempted to distill the accumulated knowledge to provide a background on rRNA genes, their regulation, the discovery of nucleolar dominance, and key cytogenetic and molecular genetic experiments that provide clues concerning the mechanisms responsible.

The first 40 years of nucleolar dominance research

Nucleolar dominance was first described by the cytogeneticist Navashin who was examining metaphase chromosomes in dividing root-tip cells of various *Crepis* species in an effort to understand how karyotype (chromosome number and morphology) relates to genotype and phenotype (Navashin, 1934). A technical problem was that chromosome size and shape varied considerably between experiments, making between-species comparisons problematic. Navashin's solution was to make hybrids so that chromosomes of the two parent species could be compared side-by-side in the same cell. To his surprise, he found that hybridization consistently induced changes in chromosome morphology. Changes that affected all chromosomes, such as thickening or shortening, he termed 'amphiplasty' (Navashin, 1928). What he termed 'differential amphiplasty' was a striking change that affected only the 'D' chromosomes (Navashin, 1934). In the parental species, each D chromosome displayed a small, distal segment of the chromosome (satellite) that was attached to the remainder of the chromosome by a thin strand of chromosome known as the secondary constriction (the primary constriction is the centromere). In 13 of the 21 different hybrid combinations tested, the D chromosomes derived from one progenitor species failed to display the satellite and secondary constriction (Figure 1). This was true in every root-tip cell of every individual examined. Reciprocal crosses showed that it was always the D chromosome of the same species that was suppressed regardless of whether the chromosome was contributed by a maternal or paternal gamete. Evidence that the D chromosome itself might be responsible came from examination of aneuploid hybrids that had inherited a dominant D chromosome as part of an incomplete chromosome set. Importantly, suppression of satellite formation was not due to permanent damage or loss of the region, because Navashin stated (as data not shown) that satellites and secondary constrictions could again form in the next generation, presumably as a result of backcrossing the hybrid with the under-dominant species. These observations suggested that failure to form the secondary constriction and satellite was a reversible phenomenon caused by interactions between the parental genomes (Navashin, 1934).

Heitz showed that secondary constriction and satellite formation at metaphase was related to nucleolus formation during interphase and that nucleoli

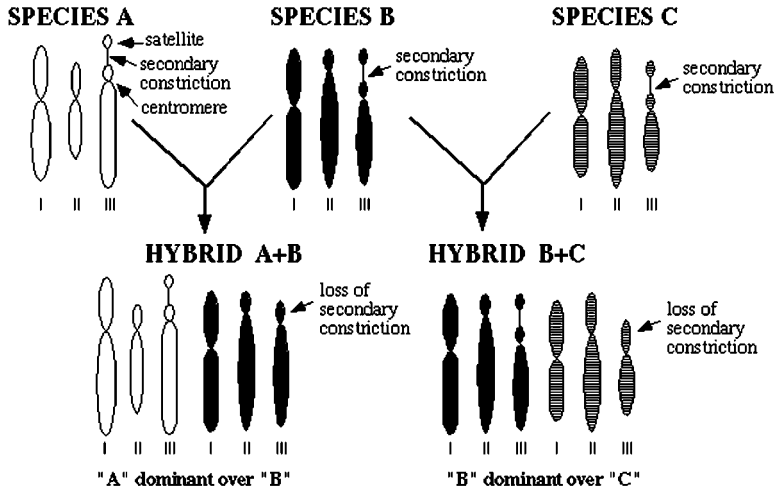

Figure 1. A cytogenetic manifestation of nucleolar dominance is the loss of a secondary constriction at the NOR at metaphase. In this cartoon, haploid chromosome sets of three related species are depicted. In each species, chromosome III has an NOR that organizes a nucleolus throughout interphase and displays a secondary constriction at metaphase. The different species can be crossed to form hybrids in which only chromosome III from one progenitor displays its characteristic secondary constriction. In this example, modeled after Navashin's analyses of *Crepis* hybrids, the NOR of chromosome III of species B is suppressed in a hybrid of species A and B, but is dominant in a hybrid of species B and C.

form at or very near the constrictions (Heitz, 1931). Convincing evidence was subsequently provided by McClintock, who used the term 'nucleolar organizer' to describe the loci (McClintock, 1934). In maize, a single NOR is located at the top of chromosome 6 (Figure 2). The region, as she described it, includes a dark-staining knob of heterochromatin (a chromomere) on the centromere-proximal side, the secondary constriction, and a distal satellite. McClintock identified a maize line in which a break occurred in this region, and another break occurred in chromosome 9, leading to a reciprocal translocation (Figure 2A). Two nucleoli now formed in this mutant line, one on each translocated chromosome (Figure 2B). Chromosome 9^6 had the centromere-containing portion of chromosome 9 fused to the distal end of chromosome 6. The reciprocal chromosome, designated 6^9, included the centromere-containing portion of chromosome 6. Both translocated chromosomes included a portion of the chromomere that had abutted the nucleolus of wild-type chromosome 6. McClintock concluded that the chromosome breakage event must have occurred within the chromomere, dividing it unequally among the two translocated chromosomes. This suggested to her that the chromomere must be the 'nucleolar organizer body' and that the genetic information within it must be redundant. Interestingly, she did not think the secondary constrictions were directly involved in nucleolus formation, even though they traversed the nucleolus and their length was better correlated with nucleolus size than were the masses of the adjacent chromomeres (McClintock, 1934). Nearly forty years later, after it had been demonstrated that NORs are the sites where rRNA genes are clustered in hundreds, sometimes thousands, of copies (Wallace and Birnstiel, 1966; Phillips *et al.*, 1971), McClintock's observations were reinterpreted to suggest that the chromomere is heterochromatin composed of excess, inactive rRNA genes whereas the secondary constrictions represent the transcribed genes (Wallace and Langridge, 1971) (Figure 3). In fact, the explanation for the secondary constrictions is thought to be that rRNA transcription late into the cell cycle and/or physical association of rRNA genes with structural components of nucleolus impedes chromosome condensation (Wallace and Langridge, 1971). According to this revisionist view, the 9^6 chromosome organizes a larger nucleolus because it includes the distal portion of chromosome 6 that is normally most active (and forms the secondary constriction) in wild-type maize. Importantly, the formation of a second nucleolus on chromosome 6^9 implied that rRNA genes that had been inactive and packaged in the chromomere of the chromosome 6 NOR had been activated (or derepressed) as a result of the translocation event (Wallace and Langridge, 1971).

McClintock saw a connection between Navashin's work and her own based on changes in the nucleolus-

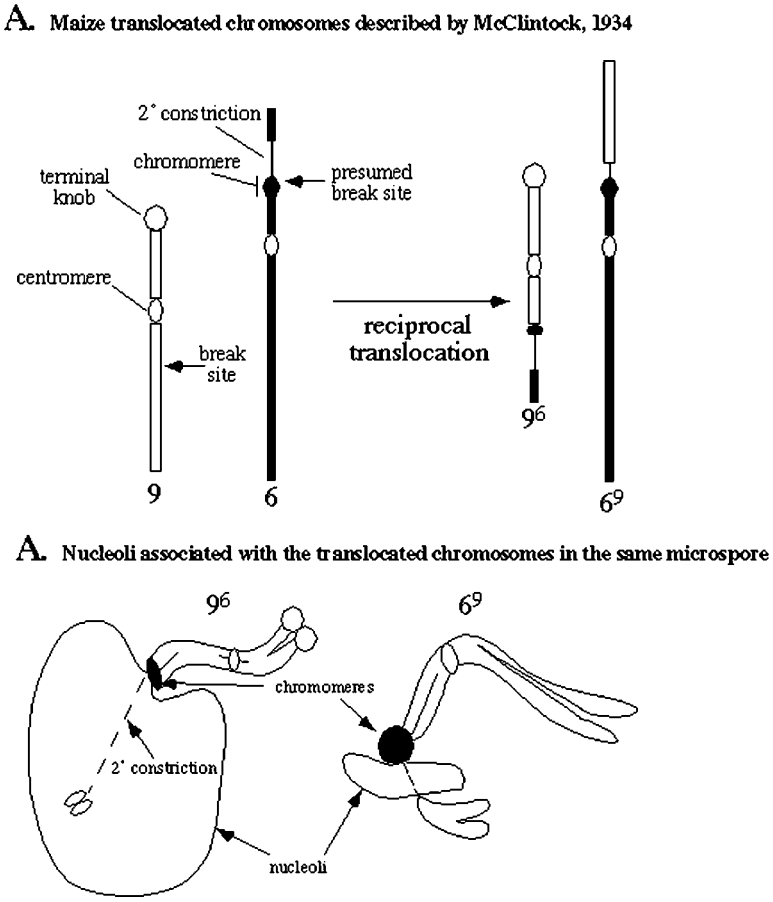

Figure 2. Diagrammatic representations of McClintock's observations which defined a specific locus on chromosome 6 as the nucleolus organizer region in maize. A. Chromosomes 6 and 9 and their reciprocal translocation products. In wild-type maize, a single nucleolus is associated with chromosome 6 on 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).

forming ability of wild-type and translocated maize chromosomes as they segregated in various combinations in pollen (McClintock, 1934). When chromosome 6^9 was the only nucleolus-forming chromosome in a spore, it formed a normal, large nucleolus. However, if combined with a wild-type chromosome 6 or with chromosome 9^6, nucleolus formation on $6^9$was reduced (see Figure 2B). These results suggested to McClintock that the NORs were competing for a substance that was present in limiting amounts. Navashin's observations in *Crepis* might then be explained by differing competitive strengths of NORs from different species. She reasoned that if the NOR of species A was dominant over species B and if B was dominant over species C, then A should be dominant over C. If so, species could be arranged in a simple dominance hierarchy. Navashin had not commented on this possibility, but the *Crepis* hybrid combinations displaying differential amphiplasty in his study (Navashin, 1934) fit neatly into such a scheme. Decades later, test crosses confirmed the hypothesis and showed that *Crepis* species could be arranged in a hierarchy with four tiers (Wallace and Langridge, 1971). Species in top tiers are dominant over all species below and species within a tier are co-dominant. An analogous dominance hierarchy has been demonstrated within the genus *Brassica* (Chen and Pikaard, 1997a, b), as shown in Figure 4.

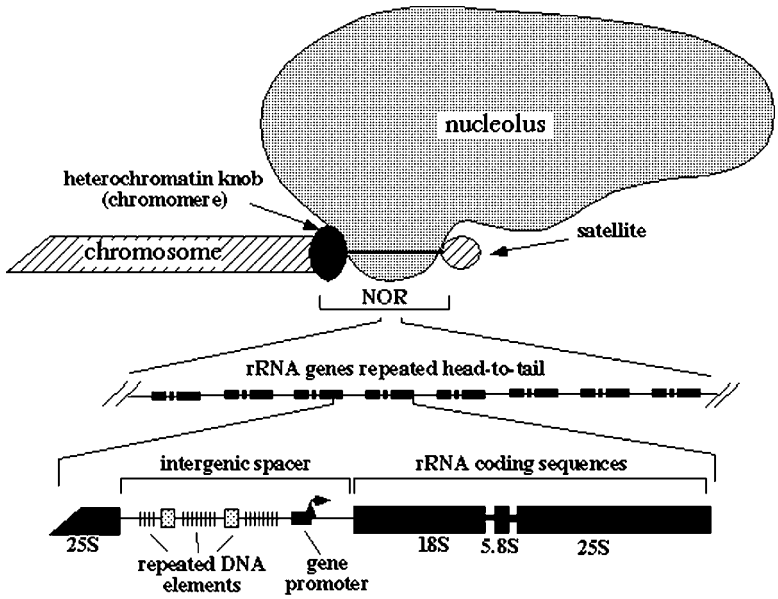

Figure 3. Organization of a generic nucleolus organizer region. NORs consist of long head-to-tail repeats of the genes encoding the precursor of the three largest ribosomal RNAs (18S, 5.8S and 25S). The NOR includes both transcriptionally active rRNA genes, which give rise to the secondary constriction 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.

The level at which nucleolar dominance operates was first made clear by studies using the frog *Xenopus*. Cytological examinations had shown that hybrids of *Xenopus laevis* and *X. borealis* did not express nucleoli at the NORs inherited from both parents (Blackler and Gecking, 1972; Cassidy and Blackler, 1974). Shortly thereafter, the new tools of molecular biology were employed to show that only *X. laevis* rRNA was synthesized during early development in *Xenopus* hybrids, showing that nucleolar dominance was a phenomenon controlled at the level of gene expression (Honjo and Reeder, 1973).

rRNA gene structure and function

Eukaryotes have three nuclear RNA polymerases, one of which (RNA polymerase I) is dedicated to the transcription of the ribosomal RNA genes in the nucleolus (for reviews see Gerbi, 1985; Flavell, 1986; Sollner-Webb and Tower, 1986; Reeder, 1992; Paule, 1994; Jacob, 1995; Moss and Stefanovsky, 1995; Hannan *et al.*, 1998). Current concepts of nucleolar dominance are shaped significantly by what is known about rRNA gene expression, thus a brief discussion of the RNA polymerase I transcription system is needed to consider several of the hypotheses for nucleolar dominance. In plants, as in other eukaryotes, NORs are made up of hundreds to thousands of rRNA genes in tandem arrays spanning several megabase-pairs (Reeder, 1974; Ingle *et al.*, 1975; Flavell, 1986; Rogers and Bendich, 1987) (see Figure 3). NORs can be among the most active genomic loci, with rRNA synthesis accounting for 40–80% of all nuclear transcription in actively growing cells (Sollner-Webb and Tower, 1986; Jacob, 1995; Nomura, 1999). Each transcription unit is separated from the next gene by an intergenic spacer. Unlike coding regions, which are highly conserved, intergenic spacers have little sequence similarity between species. Nonetheless, the intergenic spacers include the DNA elements that control ribosomal RNA gene transcription, including the gene promoter, transcription terminators, and repetitive enhancer elements.

Each rRNA gene in the NOR has the potential to be transcribed by polymerase I to produce a primary transcript that is subsequently cleaved to produce the 18S, 5.8S and 25S rRNAs. These three rRNAs, together with 5S RNA produced by RNA polymerase III, form the catalytic core of the ribosome. An additional ca. 85 proteins, whose mRNAs are transcribed

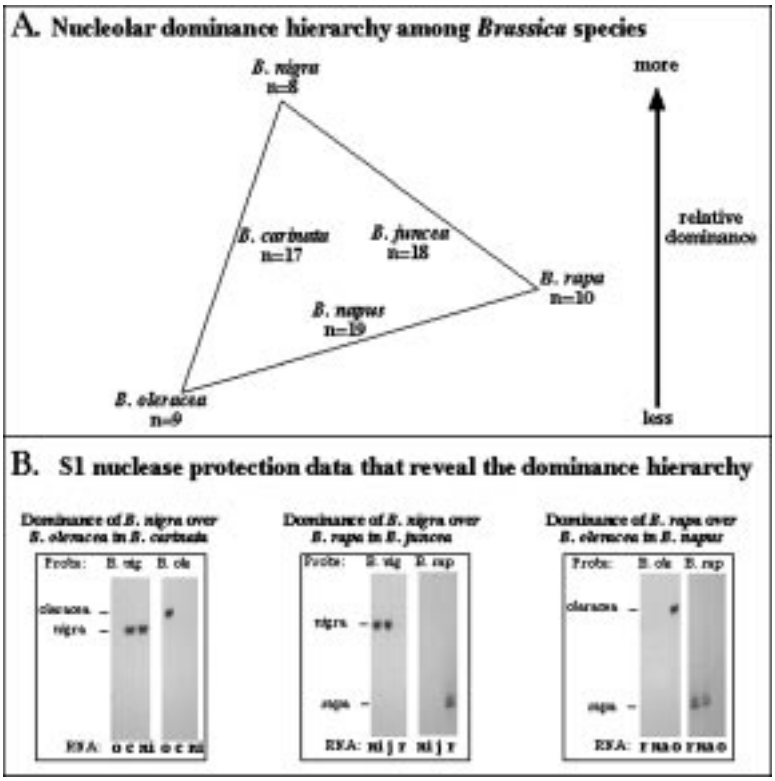

Figure 4. A hierarchy of nucleolar dominance can exist among species that hybridize. A. The triangle illustrates the genomic and nucleolar dominance relationships among six crop species in the genus *Brassica* (U, 1935). At the corners of the triangle are the diploid progenitors, *B. nigra*, *B. rapa* and *B. oleracea*. Their chromosome numbers (n) are shown. Three allotetraploid (amphidiploid) hybrids result from combining the genomes of these diploids, namely *B. carinata*, *B. juncea* and *B. napus*. *B. nigra* is at the top of the triangle and *B. rapa* is higher than *B. oleracea* to summarize the dominance hierarchy *B. nigra* > *B. rapa* > *B. oleracea*. B. The S1 nuclease protection data that reveal this hierarchy are shown for each leg of the triangle. RNA from leaves of *B. oleracea* (o); *B. carinata* (c); *B. nigra* (ni); *B. juncea* (j), *B. rapa* (r); or *B. napus* (na) was hybridized to 5′ end-labeled probes specific for rRNA transcripts from the appropriate diploid progenitor. After S1 digestion, protected probe fragments were resolved on a sequencing gel and exposed to X-ray film to produce the images shown. *B. nigra* transcripts are detected in *B. nigra*, and in both allotetraploids for which *B. nigra* is a progenitor. *B. rapa* transcripts are detected in *B. rapa* and *B. napus*, but not in *B. juncea*; *B. oleracea* transcripts are detected in *B. oleracea* but not in either allotetraploid for which *B. oleracea* is a progenitor.

by RNA polymerase II, are translated in the cytoplasm and imported into the nucleolus where they assemble with the four rRNAs to form the two ribosome subunits. Estimates suggest that as many as one million ribosomes are synthesized in each cell cycle and most, if not all, of this assembly takes place in the nucleolus (Warner, 1989, 1990) .

The driving force for the formation of the nucleolus and all of its associated activities appears to be the transcription of the rRNA genes by RNA polymerase I. A clear demonstration was the finding that rRNA transgenes integrated at ectopic locations away from the normal NORs in *Drosophila* are fully functional and organize mini-nucleoli in polytene tissues (Karpen *et al.*, 1988).

Presumably because growing cells need more ribosomes than do resting cells, rRNA transcription is coupled with the growth status of the cell. Theoretically, increases in rRNA transcription could result from increasing the amount of transcription per gene or from increases in the number of active genes. Electron microscopic studies in several organisms have shown that transcribed rRNA genes appear fully loaded with RNA polymerase and nascent RNA transcripts (McKnight and Miller, 1976; Trendelenburg and Gurdon, 1978; Morgan *et al.*, 1983). Adjacent genes can be completely inactive. These observations suggest that changes in rRNA transcription result mostly from changes in the number of active rRNA genes rather than by modulating the amount of transcription per gene. The on or off states of rRNA genes in nucleolar dominance may be an extreme reflection of this mode of regulation.

The search for molecular mechanisms to explain nucleolar dominance

Nucleolar dominance is a gene expression phenomenon (Honjo and Reeder, 1973) known to be controlled at the level of transcription, rather than RNA turnover (as shown by nuclear run-on assays; Chen and Pikaard, 1997a, b). Thus it has been logical to look to the pol I transcription system for an explanation. Two hypotheses stem directly from the biochemical characterization of pol I transcription: the species-specific transcription factor hypothesis and the enhancer-imbalance hypothesis.

The species-specific transcription factor hypothesis integrates the rapid evolution of rRNA genes (Reeder, 1974; Saghai-Maroof *et al.*, 1984; Dover and Flavell, 1984; Gerbi, 1985) with findings derived from the use of cell-free transcription systems. As mentioned previously, the intergenic spacers that contain the controlling elements for rRNA gene transcription evolve rapidly such that there is often little sequence similarity across species boundaries. Several groups showed that a murine or human rRNA gene promoter would not function in a cell-free transcription extract from the other species (Grummt *et al.*, 1982; Mishima *et al.*, 1982; Miesfeld and Arnheim, 1984; Miesfeld *et al.*, 1984; Learned *et al.*, 1985; Bell *et al.*, 1990; Schnapp *et al.*, 1991) suggesting that transcription factors had co-evolved with the changing DNA sequences. Biochemical dissection of the pol I transcription machinery led to the discovery that a mouse extract can be reprogrammed to transcribe a human rRNA gene promoter provided that a specific human transcription factor (SL1) is added to the reaction. Likewise, a mouse promoter can be recognized in a human extract if the equivalent mouse transcription factor is added (Mishima *et al.*, 1982; Miesfeld and Arnheim, 1984; Learned *et al.*, 1985; Bell *et al.*, 1990). The other transcription factors, and pol I itself, are apparently interchangeable between mouse and man.

A form of nucleolar dominance occurs in mouse-human hybrid somatic cells grown in culture. In some lines, mouse rRNA genes are expressed but human rRNA genes are not; in others, only human rRNA genes are active (Weiss and Green, 1967; Elicieri and Green, 1969; Miller *et al.*, 1976; Perry *et al.*, 1976; Croce *et al.*, 1977; Miesfeld and Arnheim, 1984; Miesfeld *et al.*, 1984). A reasonable hypothesis is that loss or inactivation of genes encoding components of the human or mouse species-specific transcription factor could silence the matching set of rRNA genes. However, the hypothesis does not appear to explain nucleolar dominance in species related closely enough to interbreed. For instance, rRNA gene promoters of *Brassica* or *Arabidopsis* species that exhibit nucleolar dominance when hybridized appear to be fully functional when transfected into protoplasts of the other species (Chen *et al.*, 1998; Frieman *et al.*, 1999). An *Arabidopsis* rRNA gene promoter is also functional across genus boundaries, being active in a cell-free transcription system from *Brassica oleracea* (broccoli) (Saez-Vasquez and Pikaard, 1997). These results suggest that dominant and under-dominant rRNA genes would use the transcription machinery of the other species even if their own transcription factors were unavailable, effectively ruling out the species-specific transcription factor hypothesis (Frieman *et al.*, 1999). Furthermore, McClintock's initial observations of NOR behavior among 6^9 and 9^6 chromosomes (McClintock, 1934), coupled with descriptions of nucleolar dominance among recombinant maize inbreds (Jupe and Zimmer, 1993; McMurphy and Rayburn, 1994), and hierarchical NOR expression in hexaploid bread wheat (Flavell, 1986) are all examples where differences in NOR expression occur within a species. The species-specific transcription factor hypothesis does not adequately explain differences in NOR activity within a species.

The enhancer imbalance hypothesis owes its origin to *Xenopus* oocyte injection experiments that revealed the DNA sequences required for rRNA gene transcription. Repetitive DNA elements just upstream of the gene promoter within the intergenic spacer act as orientation- and position-independent enhancers in oocytes and early embryos (Busby and Reeder, 1983; Moss, 1983a, b; Reeder *et al.*, 1983; Reeder, 1984; Labhart and Reeder, 1984, 1985; DeWinter and Moss, 1986, 1987; Pikaard and Reeder, 1988; Pape *et al.*, 1989). When cloned adjacent to an rRNA gene promoter, the enhancers dramatically stimulate transcription. However, when cloned within a separate plasmid coinjected with a promoter-bearing plasmid, enhancers compete, severely inhibiting transcription from the promoter (Labhart and Reeder, 1984). These data suggest that enhancers bind one or more transcription factors that also interact with the promoter, a prediction which was subsequently confirmed by the purification and analysis of the transcription factor UBF (Dunaway, 1989; Pikaard *et al.*, 1989, 1990).

Noting that *X. laevis* and *X. borealis* rRNA genes had different repetitive elements in their intergenic

spacers (Boseley *et al.*, 1979; Bach *et al.*, 1981), it seemed plausible that the more abundant *X. laevis* elements might titrate a critical transcription factor and sequester it, thus explaining the lack of *X. borealis* rRNA gene expression in hybrid frogs (Reeder *et al.*, 1983; Reeder, 1984). Co-injection into oocytes of *X. laevis* and *X. borealis* rRNA minigenes with complete spacers attached revealed that *X. laevis* minigenes were preferentially transcribed, in agreement with the *in vivo* situation in hybrids (Reeder and Roan, 1984). Using recombinant constructs in which promoter and intergenic spacer sequences were swapped, constructs that had *X. laevis* spacers were transcribed preferentially, supporting the hypothesis that the intergenic spacers, and not the gene promoters, were responsible for the differential expression (Reeder and Roan, 1984; Reeder, 1985) .

In plants, a correlation between intergenic spacer length and nucleolar dominance was noted in wheat. Hexaploid bread wheat has multiple NORs (Mukai *et al*., 1991), the most active of which are located on chromosomes 1B and 6B. Interestingly, the 1B NOR, with 1300 genes, organizes a larger nucleolus than the 6B NOR, with 2700 genes, showing that gene number is not the primary determinant of NOR activity (Flavell and O'Dell, 1979; Flavell, 1986). A fraction (ca. 10%) of the rRNA genes in the 1B NOR have long intergenic spacers compared to their counterparts at the 6B NOR (Flavell and O'Dell, 1979), suggesting that structural differences among rRNA genes may determine their relative activity. Furthermore, wheat NORs are suppressed in lines carrying NOR-bearing chromosomes of *Aegilops umbellulata*, a wild relative (Martini *et al.*, 1982). The *Aegilops* NORs, which organize large nucleoli in these lines, have rRNA genes whose intergenic spacers are longer than even the longest wheat spacers. Because spacer length variation is often due to variation in the number of repetitive elements (Reeder, 1974; Gerbi, 1985), which in *Xenopus* had been shown to be enhancers, these observations suggested that nucleolar dominance in wheat and *Xenopus* might be explained by the same mechanism (Reeder, 1985; Flavell, 1986).

The enhancer imbalance hypothesis is appealing because it suggests a simple biochemical basis for discriminating among rRNA genes based on transcription factor binding affinities. Such affinities, described by physical binding constants, would presumably be invariant and could explain the lack of maternal or paternal effect in nucleolar dominance. Furthermore, a NOR with relatively few genes could still be dominant over a NOR with more genes if the former had a higher binding affinity for transcription factors that were present in limiting amounts.

Despite its allure, several observations are inconsistent with the premises or predictions of the enhancer-imbalance hypothesis. The premise that rRNA genes with longer spacers (or more putative enhancer repeats) will be dominant is not the case in *Brassica* and *Arabidopsis* (Chen and Pikaard, 1997a, b; Chen *et al.*, 1998). Direct tests have also failed to reveal a superior ability of dominant genes to recruit transcription factors. In protoplasts, transfected dominant and under-dominant genes, with either minimal promoters or complete intergenic spacers, are transcribed at equivalent levels (Chen *et al.*, 1998; Frieman *et al.*, 1999). Even when co-transfected, no competition is observed. One could argue that, unlike *Xenopus* oocyte injection experiments, one cannot deliver into plant protoplasts sufficient amounts of template to observe competition. Using a cell-free transcription system, one can control the ratio of template to protein and thus address this concern. At high template concentrations, transcription factors should become limiting and dominant genes should recruit these factors preferentially. However, using a *Brassica in vitro* transcription system (Saez-Vasquez and Pikaard, 1997), dominant and under-dominant rRNA genes were found to be transcribed at equal levels over a broad range of template concentrations, including concentrations so high that they were inhibitory (Frieman *et al.*, 1999).

A possibility is that transcription factor competition only occurs at a specific time in early development when nucleolar dominance is first established (Neves *et al.*, 1995). If so, the lack of evidence that factors are limiting in vegetative cells used to make protoplasts or cell-free transcription extracts may be misleading. However, there is at least one genetic experiment using whole plants that have experienced the relevant developmental transitions that also argues against the hypothesis. In *Arabidopsis suecica*, an allotetraploid hybrid of *A. thaliana* and *A. arenosa* (also known as *Cardaminopsis arenosa*), the *A. thaliana* rRNA genes are normally repressed (Chen *et al.*, 1998). Backcrossing newly created *A. suecica* to tetraploid *A. thaliana* results in allotetraploids with a 3:1 *thaliana*:*arenosa* genome dosage (as opposed to the 2:2 ratio in *A. suecica*). As a result of this change in ploidy and/or rRNA gene dosage, *A. thaliana* rRNA genes become dominant and *arenosa* rRNA genes are repressed. This dominance reversal is inconsistent with the hypothesis

that *A. arenosa* rRNA genes are normally dominant because they have a substantially higher affinity for transcription factors than do *A. thaliana* genes. If they did, they would continue to titrate the factor until their binding sites were saturated, only then allowing any excess factors to be available for binding to under-dominant genes. Co-dominance might result, but dominance reversal is not predicted. The fact that dominance reversal does occur indicates that if transcription factors are involved in establishing dominance, cooperativity or some other property of rRNA gene clusters (or complete NORs) must be more important than primary rRNA gene sequence in dictating transcription factor recruitment.

Chromosome rearrangements affect nucleolar dominance

Studies in both plants and *Drosophila* have revealed chromosomal influences on nucleolar dominance that do not appear to involve changes in rRNA gene number or sequence. Initial evidence came from cytogenetic studies of nucleolar dominance in hybrids of *Drosophila melanogaster* and *D. simulans*. Both species have a nucleolus organizer on the X chromosome, and in hybrid XX females the *melanogaster* NOR is dominant. *D. melanogaster* has a second NOR on the Y chromosome. In a hybrid XY male, the *melanogaster* Y-associated NOR suppresses the NOR on the *simulans* X chromosome (Durica and Krider, 1977). Interestingly, rearrangements in the heterochromatin flanking either *melanogaster* NOR do not affect expression of the adjacent NOR but they prevent suppression of the *simulans* NOR *in trans*. Because *melanogaster* NORs appear to be fully functional, one infers that they make use of transcription factors as usual. This is clearly not sufficient to cause nucleolar dominance, as might be predicted if there were only enough transcription factor(s) for one parental set of rRNA genes (Durica and Krider, 1978).

Barley provides another example of a case where the chromosomal context of an NOR influences nucleolar dominance. Barley has two NORs, one on chromosome 6 and the other on chromosome 7. In wild-type barley, the two NORs are co-dominant. When both NORs are located on the same chromosome, as a result of a chromosome translocation, the chromosome 6 NOR becomes dominant (Nicoloff, 1979; Schubert and Kunzel, 1990). In a different translocation line that contains two copies of the chromosome 6 NOR on the same chromosome, both NORs are co-dominant (Schubert and Kunzel, 1990).

Chromosome rearrangements also affect nucleolar dominance in Triticale, the hybrid of wheat and rye. In Triticale, wheat NORs are expressed and the rye NOR, located on the short arm of chromosome 1R, is suppressed (Thomas and Kaltsikes, 1983; Lacadena *et al.*, 1984; Viera *et al.*, 1990a, b; Silva *et al.*, 1995; Amado *et al.*, 1997; Neves *et al.*, 1997a, b). However, if the short arm of 1R is translocated onto the long arm of wheat chromosome 1, the rye NOR is no longer suppressed, but becomes co-dominant with the wheat NORs (Viera *et al.*, 1990a, b). Interestingly, deletions or rearrangements in the long arm of rye chromosome 1R also lead to the derepression of the rye NOR, suggesting that it is the loss of the long arm rather than the translocation onto a wheat chromosome that is responsible for the derepression of the rye NOR (Viera *et al.*, 1990a, b). Substitution lines in which rye chromosome 2R is replaced by wheat chromosome 2D also results in the derepression of the NOR on rye chromosome 1R (Neves *et al.*, 1997a, b). These results suggest that sequences on the long arm of rye chromosome 1R and sequences on chromosome 2R are both needed to suppress the rye NOR in Triticale.

No obvious mechanism stems from these cytogenetic observations, but it seems clear that chromosomal context and loci unlinked to the NORs can be more important than rRNA gene sequences in determining nucleolar dominance relationships. Perhaps deletions adjacent to *Drosophila* NORs and translocations of NORs among barley chromosomes disrupt dominance by affecting processes such as chromosome pairing, replication timing or chromosome localization within the nucleus. Rye loci that are unlinked to the NORs but that affect nucleolar dominance in Triticale might regulate these processes without directly affecting the RNA polymerase I transcription system.

Evidence for selective rRNA gene silencing

Implicit in the enhancer imbalance and species-specific factor hypotheses is the idea that nucleolar dominance results from selectively activating only one set of rRNA genes. The alternative view, supported by the cytogenetic evidence, is that one set of rRNA genes is selectively repressed. In vertebrates and plants, cytosine methylation is often correlated with gene silencing (for reviews, see Chomet, 1991; Bird, 1992; Eden

and Cedar, 1994; Martienssen and Richards, 1995; Richards, 1997). Epigenetic phenomena for which this is the case include X chromosome inactivation, gametic imprinting, some cases of paramutation, some cases of homology-dependent gene silencing, and transposable element activation/inactivation (Gartler and Riggs, 1983; Grant and V.M., 1988; Matzke *et al.*, 1989; Li *et al.*, 1993; Lyon, 1993; Flavell, 1994; Gartler and Goldman, 1994; Rainier and Feinberg, 1994; Razin and Cedar, 1994; Bender and Fink, 1995; Federoff *et al.*, 1995; Martienssen and Richards, 1995; Matzke and Matzke, 1995; Federoff, 1996; Martienssen, 1996; Walker, 1998; Jeddeloh *et al.*, 1998; Walker, 1998; Vielle-Calzada *et al.*, 1999). In the case of nucleolar dominance, rRNA genes at active wheat and maize NORs are slightly less methylated and are more DNase-accessible than are rRNA genes at repressed NORs (Flavell *et al.*, 1988; Thompson and Flavell, 1988; Jupe and Zimmer, 1993).

Direct evidence that cytosine methylation plays a role in nucleolar dominance is that 5-aza-2′-deoxycytosine (aza-dC), an inhibitor of cytosine methyltransferase (Gabbara and Bhagwat, 1995), induces the dramatic reactivation of under-dominant rRNA genes (Chen *et al.*, 1998; Chen and Pikaard, 1997a, b) and suppresses NORs (Viera *et al.*, 1990a, b; Neves *et al.*, 1995, 1997a, b; Amado *et al.*, 1997). Chemicals that cause histone hyperacetylation by inhibiting histone deacetylase activity (Kruh, 1982) also induce rRNA gene derepression (Chen and Pikaard, 1997a, b), as is also the case in other epigenetic phenomena that involve protein-coding genes (Grunstein, 1997; Wade and Wolffe, 1997; Kadonaga, 1998). Interestingly, treatment with both aza-dC and trichostatin A is no more effective than either compound alone in derepressing rRNA genes subjected to nucleolar dominance, suggesting that cytosine methylation and histone deacetylation are partners that act in the same repression pathway (Chen and Pikaard, 1997a, b). This partnership between cytosine methylation and histone deacetylation in nucleolar dominance was the first clear demonstration that these processes can be linked to control a biological phenomenon.

Despite the effects of aza-dC, there is reason to question the regulatory potential of cytosine methylation in nucleolar dominance given that rRNA genes are so heavily methylated to begin with. For instance, in *Brassica napus*, both dominant and under-dominant rRNA genes appear to be methylated at every one of an estimated 50–60 *Hpa*II restriction endonuclease sites, making them insensitive to *Hpa*II cleavage (Chen and Pikaard, 1997a, b). Aza-dC treatment that causes only a modest decrease in methylation of these *Hpa*II sites nonetheless appears to fully derepress the under-dominant genes. Perhaps the extent of *Hpa*II site methylation cannot be extrapolated to other, more significant sites which might bind important regulatory proteins. However, *Brassica* rRNA minigenes methylated at all CG sites using *Sss*I methylase remain fully active for transcription *in vitro*, suggesting that cytosine methylation does not directly block the binding of transcription factors (Frieman *et al.*, 1999).

Instead of a direct effect, methylation might play a role in establishing a repressive chromatin state that prevents the transcription machinery from gaining access to the promoter. Supporting evidence is that in *Xenopus*, methylation can inhibit transcription of an rRNA minigene, but this inhibition is apparently dependent on the action of unidentified methylcytosine-binding proteins. If methylated competitor DNA is added to titrate these proteins, rRNA gene transcription is actually stimulated by methylation (Labhart, 1994).

Histone deacetylation is one process that can be brought about based on the methylation status of the template. Recent biochemical experiments have shown that proteins which bind specifically to methylated DNA are subunits of multi-protein complexes that include one or more histone deacetylases (Eden *et al.*, 1998; Jones *et al.*, 1998; Nan *et al.*, 1998). This suggests a model whereby cytosine methylation brings about the recruitment of one or more histone deacetylases which modify the local nucleosomal histones. Histone hypoacetylation, in turn, induces compaction of the chromatin in a way that inhibits transcription factors from being able to access their binding sites on critical elements such as enhancers and promoters (Grunstein, 1997; Kadonaga, 1998).

Thinking of nucleolar dominance from the perspective of genes organized in chromatin, as opposed to naked DNA, highlights the fact that either the DNA or the proteins that interact with the DNA are potential targets of regulatory modifications. An involvement of protein targets, such as histones, may help explain some of the uncertainty concerning the role of cytosine methylation in rRNA gene silencing. Furthermore, nucleolar dominance occurs in *Drosophila*, a species that does not methylate its DNA. Therefore, if nucleolar dominance involves the same mechanisms in plants and flies, something other than methylation must be involved. Histone acetylation or other chromatin modifications that occur in all eukaryotes are

likely candidates for such a (hypothetical) common mechanism.

Why should a hybrid cell care whose rRNA genes it expresses?

Ribosomal RNA-coding sequences are essentially identical in species that are related closely enough to interbreed. Therefore, ribosomes assembled by rRNA made by either of the two genomes should be identical. It is doubtful that silencing one set of rRNA genes avoids cellular catastrophe wreaked by ribosomes run amok. So what is the reason for nucleolar dominance?

McClintock's concept of nucleolar dominance involved competition for a positively acting substance that was not abundant enough to interact with all of the redundant information (McClintock, 1934). As a result some NORs were presumably better than others at assimilating the material, explaining why only some were active. If one substitutes 'transcription factor' for 'substance', one has the essence of the enhancer imbalance or species-specific transcription factor hypotheses discussed previously.

An alternative view has been that rRNA genes are negatively regulated even in non-hybrids so as to control the number of active genes. Nucleolar dominance may simply be a manifestation of this system (Wallace and Langridge, 1971; Flavell, 1986). Some species, such as maize, may have more rRNA genes than are needed to meet the physiological demands of the cell. Maize inbred lines can vary almost ten-fold in rRNA gene content (2500–24 000 rRNA genes in a diploid) yet have similar morphological characteristics and growth rates (Rivin *et al.*, 1986). Most of the maize rRNA genes are, in fact, condensed into tightly packed heterochromatin adjacent to the active genes that give rise to the secondary constriction (Givens and Phillips, 1976; Phillips, 1978) (Figure 3). Studies measuring the susceptibility of rRNA genes to psoralen crosslinking in animals and yeast have also suggested that only a fraction of the rRNA genes are in an accessible (presumably active) chromatin configuration (Conconi *et al.*, 1989; Lucchini and Sogo, 1992; Dammann *et al.*, 1995). Thus, it could be that the molecular mechanisms that control the number of active rRNA genes in all species are the same mechanisms responsible for nucleolar dominance in hybrids. The fact that the dominant rRNA genes in hybrids are up-regulated several-fold in response to aza-dC or histone deacetylase inhibitors, coincident with the derepression of under-dominant rRNA genes, is consistent with this hypothesis (Chen and Pikaard, 1997a, b).

A negative regulatory strategy may be important for controlling the effective dosage of active ribosomal RNA genes, but dosage compensation could be achieved by co-expressing both sets of rRNA genes at a lower level. Thus dosage compensation may be a reasonable explanation for why nucleolar dominance occurs, but it fails to explain why one set of genes should be selectively silenced.

Future directions

Though there is evidence that nucleolar dominance is enforced through changes in chromatin modification and selective gene silencing, many questions remain. As mentioned previously, methylation of rRNA genes may not directly control their activity, suggesting that methylation of an unlinked regulatory locus could be more important or that methylation is necessary but not sufficient to bring about downstream events such as histone deacetylation. Whether changes in histone acetylation status are directly correlated with rRNA gene activity is currently unknown. One way to address this question is to use the chromatin immunoprecipitation technique using antibodies specific for acetylated histones or deacetylated histones (Braunstein *et al.*, 1993). One can then determine if dominant genes are found preferentially in the chromatin fraction containing hyperacetylated histones, and if under-dominant genes are enriched in the hypoacetylated chromatin fraction.

The hypothesis that methylation acts upstream of histone deacetylation should also be tested. If the model is correct, aza-dC treatment should cause a decrease in cytosine methylation and an increase in histone acetylation coincident with the derepression of under-dominant rRNA genes. But by acting downstream of methylation, histone deacetylase inhibitors might derepress the under-dominant genes without affecting methylation.

It is important to know if gene silencing associated with nucleolar dominance acts at the level of the individual rRNA genes or at the level of the NOR. If silencing mechanisms act on the NOR, a prediction is that an rRNA transgene integrated at an ectopic location would escape silencing in a hybrid. If individual rRNA genes are targeted, chromosomal location may not be important. A related question is whether silenc-

ing is restricted to the NORs or if it spreads beyond the NOR to affect neighboring genes. Knowing where silencing ends and active genes begin on an NOR-bearing chromosome may define regions in which to search for a possible locus control center.

Remaining open-minded about the popular hypotheses of the past also seems wise. For instance, one can envision scenarios in which competition for transcription factors can occur even if dominant and under-dominant genes have identical binding affinities for these factors. Differences in replication timing of the NORs might provide dominant NORs with the opportunity to bind and titrate transcription factors at a time in the cell cycle when under-dominant NORs are still inaccessible. Changes in cytosine methylation or histone acetylation might affect this timing, thus explaining the abilities of these chemicals to derepress silent loci. Chromosomal translocations that move NORs to new locations might also affect dominance through changes in replication timing or chromosome localization within the nucleus. It is likely that understanding the mechanisms of nucleolar dominance can contribute substantially to a broader understanding of gene expression, chromosome dynamics, and allelic discrimination.

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