RNA Polymerase I Transcription in a Brassica Interspecific Hybrid and Its Progenitors: Tests of Transcription Factor Involvement in Nucleolar Dominance

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ABSTRACT

In interspecific hybrids or allopolyploids, often one parental set of ribosomal RNA genes is transcribed and the other is silent, an epigenetic phenomenon known as nucleolar dominance. Silencing is enforced by cytosine methylation and histone deacetylation, but the initial discrimination mechanism is unknown. One hypothesis is that a species-specific transcription factor is inactivated, thereby silencing one set of rRNA genes. Another is that dominant rRNA genes have higher binding affinities for limiting transcription factors. A third suggests that selective methylation of underdominant rRNA genes blocks transcription factor binding. We tested these hypotheses using Brassica napus (canola), an allotetraploid derived from B. rapa and B. oleracea in which only B. rapa rRNA genes are transcribed. B. oleracea and B. rapa rRNA genes were active when transfected into protoplasts of the other species, which argues against the species-specific transcription factor model. B. oleracea and B. rapa rRNA genes also competed equally for the pol I transcription machinery in vitro and in vivo. Cytosine methylation had no effect on rRNA gene transcription in vitro, which suggests that transcription factor binding was unimpaired. These data are inconsistent with the prevailing models and point to discrimination mechanisms that are likely to act at a chromosomal level.

Nucleolar dominance describes the phenomenon in which ribosomal RNA genes inherited from only one parent are expressed to form a nucleolus in an interspecific hybrid. First described in plants (Nava-shin 1928, 1934), nucleolar dominance also occurs in insects, amphibia, and mammals (Reeder 1985; Pikaard and Chen 1998). Honjo and Reeder were first to show that ribosomal RNA from only one parent could be detected in newly formed Xenopus hybrids, suggesting that nucleolar dominance was a transcriptional phenomenon (Honjo and Reeder 1973). More recently, nuclear run-on experiments confirmed that nucleolar dominance is controlled at the level of RNA polymerase I (pol I) transcription (Chen and Pikaard 1997a).

At least two sets of mechanisms are likely to be responsible for nucleolar dominance: those that discriminate the rRNA genes from each progenitor and first establish nucleolar dominance and those that subsequently enforce dominance through successive mitoses (Pikaard and Chen 1998). Cytosine hypermethylation and histone deacetylation appear to be partners in the enforcement mechanism because inactive rRNA genes can be derepressed by chemical inhibitors of cytosine methyltransferase or histone deacetylase (Chen and Pikaard 1997a). It is not yet clear whether these chromatin modifications act on the rRNA genes themselves or on other regulatory loci.

Mechanisms that discriminate between parental sets of rRNA genes and initially establish nucleolar dominance remain obscure. Favorable hypotheses share the premise that dominance is controlled at the level of RNA pol I transcription complex assembly. The simplest model stems from the rapid evolution of rRNA genes and the coevolution of pol I transcription factors, such that rRNA gene transcription is often species-specific (Grummt et al. 1982; Miesfeld and Arnheim 1984). For instance, a mouse promoter will not be transcribed in a human cell extract nor will a human promoter be transcribed in a mouse extract. However, a mouse extract can be reprogrammed to transcribe a human rRNA gene template if the human transcription factor SL1/TIF-1B is added to the reaction (Learned et al. 1985; Bell et al. 1990; Schnapp et al. 1991; Heix and Grummt 1995). Likewise, addition of mouse SL1 to a human extract facilitates transcription of a mouse promoter. The other required transcription factors are functionally equivalent in mouse and human. Therefore, loss or inactivation of genes encoding mouse or human SL1 subunits might explain the expression of mouse or hu-
man rRNA genes, but not both, in mouse-human somatic cell hybrids (Ellicieri and Green 1969; Miller et al. 1976; Perry et al. 1976; Soprano et al. 1979; Soprano and Baser 1980; Miesfeld et al. 1984). Obviously, mouse-human cell hybrids represent a wide cross not possible via normal reproductive mechanisms. However, pol I transcription has also been shown to be species-specific between Drosophila melanogaster and D. virilis (Kohorn and Rae 1982), which suggests that the species-specific transcription factor mechanism could be a plausible explanation for nucleolar dominance in other Drosophila hybrids (Durica and Krider 1977).

A second hypothetical discrimination mechanism is the “enhancer imbalance” model put forward to explain nucleolar dominance in Xenopus and wheat (Reeder 1985; Flavell 1986). In hybrids of Xenopus laevis and X. borealis, the laevis rRNA genes are dominant during early development (Honjo and Reeder 1973; Cassidy and Blackler 1974). Compared to X. borealis rRNA genes, X. laevis genes have more repetitive DNA elements in the intergenic spacers upstream of the gene promoter (Bach et al. 1981). When cloned in cis to a ribosomal gene promoter injected into oocytes or embryos, these repetitive elements stimulate transcription (Busby and Reeder 1983; Moss 1983; Labhart and Reeder 1984). However, when co-injected on a separate plasmid, the enhancers compete against the promoter (Lahbort and Reeder 1984). These results inspired the hypothesis that nucleolar dominance might result from sequestration of critical transcription factors by the more abundant (or stronger) enhancers of dominant genes. A subsequent series of experiments yielded results consistent with this hypothesis, showing that preferential transcription of X. laevis rRNA minigenes in borealis oocytes was due to some feature of the X. laevis rRNA gene intergenic spacer, presumably the enhancer repeats (Reeder and Roan 1984). Likewise, in allohexaploid bread wheat (Triticum aestivum) and in crosses of bread wheat with a wild relative, Agllops umbellulata, dominant nucleolus organizer regions were those where rRNA genes with the longest intergenic spacers were located (Martini et al. 1982). Because most spacer length variation results from differences in the number of repetitive elements, a reasonable deduction was that an enhancer imbalance might also explain nucleolar dominance in wheat (Martini et al. 1982; Flavell 1986).

A third hypothesis is that cytosine methylation may play a role in establishment, as well as enforcement, of nucleolar dominance by selective hypermethylation of underdominant rRNA genes, which thus blocks the binding of pol I transcription factors (Flavell et al. 1988; Sardana et al. 1993; Houchins et al. 1997). Decreased binding affinity of activator proteins to methylated DNA has been shown for some RNA polymerase II transcription factors (Eden and Cedar 1994).

In the study reported here, we used transient expression and in vitro transcription assays to design direct tests of the three prevailing hypotheses discussed above. We show that Brassica rapa and B. oleracea rRNA gene promoters are functional in protoplasts of either species or in protoplasts of B. napus, the allotetraploid in which chromosomal B. oleracea rRNA genes are silent but B. rapa genes are expressed. These results argue against the existence of species-specific transcription factors among these plants. We also showed that the differences in B. rapa and B. oleracea gene intergenic spacers do not cause any detectable differences in their abilities to recruit transcription factors in vivo or in vitro. Last, we show that B. oleracea rRNA gene transcription in vitro is insensitive to cytosine methylation at CpG sequences, the predominant sites of DNA methylation in plants. The latter result suggests that pol I transcription complex assembly, transcription initiation, and polymerase elongation are not directly affected by DNA methylation. Collectively, these results suggest that nucleolar dominance in plants is unlikely to be controlled through activator protein levels or their binding affinities but, instead, is a chromosomal phenomenon primarily involving negative control.

MATERIALS AND METHODS

Construction of rRNA minigenes: A B. oleracea minigene, pBor-, was constructed by ligating the Avai-HinfI fragment (sequences -517 to +42 relative to the transcription start site, +1) of pBor2 (Doelling and Pikaard 1996) into the SmaI site of the pBluescript plasmid (Stratagene, La Jolla, CA) pBSI KS- (J. H. Doelling and C. S. Pikaard, unpublished results). Addition of sequences flanking the promoter on the 5′ side was accomplished by ligating a DraI-BstBI fragment of the B. oleracea genomic clone pBOB6 (Bennett and Smit 1991) into EcoRV-BstBI-digested pBor-. The resulting minigene construct, pBol-F (where F designates the presence of a full intergenic spacer), includes rRNA gene sequences from -2786 to +42. An equivalent B. rapa minigene including sequences from -2410 to +55 was derived from the genomic clone pBCIGS (Batcha et al. 1996) as an Acl-SnaBI fragment. This fragment was cloned into the EcoRI-BamHI sites of pBS1I KS- after bluntling all ends. An EcoRV-Sacl fragment containing the inserted DNA was subsequently subcloned into pBS1 SK-, resulting in the construct pBraF. B. oleracea and B. rapa rRNA minigenes lacking extensive 5′ flanking sequences were engineered by removing intergenic spacer sequence (IGS) upstream of the conserved XmnI sites at -307 and -308 of pBol-F and pBra-F, respectively. Thus, the B. oleracea minigene, pBol-P (P designates “promoter-only”), includes sequences from -307 to +42. The analogous B. rapa minigene, pBra-P, contains sequences from -308 to +55 (see Figure 1).

Transfection and transient expression: Protoplasts (5 × 10⁶) of B. rapa, B. oleracea, or B. napus, isolated from 3- to 4 wk-old plants grown under sterile conditions, were transfected with 50 pmol of CsCl-purified supercoiled minigene plasmid DNA using the polycethylene glycol-calcium nitrate procedure, as previously described (Doelling and Pikaard 1996). After transfection, protoplasts were incubated for 18-20 hr to allow for transcription of the minigenes. After the protoplasts were washed and pelleted, total nucleic acid was isolated (Chen et al. 1998). To verify that equal amounts of plasmid DNA were taken up by the protoplasts, the 1997-bp PvuI fragment of
RESULTS

Pol I transcription factors and rRNA gene promoters are functional across species boundaries in Brassica: To determine if RNA polymerase I transcription might be species-specific in Brassica, we transfected B. rapa and B. oleracea “promoter-only” rRNA minigenes (see Figure 1B) into B. rapa, B. oleracea, or B. napus protoplasts and detected their transcripts using the S1 nuclease protection assay (Figure 2A). As expected, transcripts from B. oleracea construct pBol-P were readily detected in B. oleracea protoplasts (lane 1) as were transcripts from the B. rapa construct pBra-P in B. rapa protoplasts (lane 6). Endogenous rRNA gene transcripts present in B. oleracea, B. napus, and B. rapa protoplasts were not detected (lanes 7-10), which verified that the probes (diagrammed in Figure 1B) were specific for transcripts of the transfected minigenes. Upon transcription across species boundaries, the B. oleracea minigene was active in B. rapa protoplasts (Figure 2A, lane 2) as
was the B. rapa minigene in B. oleracea protoplasts (lane 5). Both minigenes appeared to be slightly less active in the protoplasts of the other species (compare lanes 1 and 2; 5 and 6). Nonetheless, these results show that pol I transcription systems of B. oleracea and B. rapa are sufficiently similar such that the promoters of either species can be recognized by the transcription factors of the other species.

To examine the possibility that preferential transcription of B. rapa rRNA minigenes might be apparent only under competitive conditions in allotetraploid B. napus cells, equimolar amounts (50 pmol each) of the B. oleracea and B. rapa minigenes were cotransfected into B. napus protoplasts (Figure 2A, lanes 3 and 4). Both minigenes were fully active, directing transcription at levels indistinguishable from those in the control transfections that used homologous protoplasts (compare lanes 1 and 3; 4 and 6). Because underdominant B. oleracea and dominant B. rapa rRNA minigenes appear to be equally active in transfected B. napus, this suggests that the pol I transcription machinery in the allotetraploid is available to the rRNA genes of both progenitors without apparent bias.

Our previous studies showed that in vegetative leaves of B. napus plants, B. rapa rRNA genes are active but B. oleracea rRNA genes are silenced (Chen and Pikaard 1997a,b). A trivial explanation for the transient expression of both B. oleracea and B. rapa rRNA genes in B. napus protoplasts in Figure 2A could be that nucleolar dominance occurs in whole plants but not in protoplasts. This possibility was ruled out by analysis of endogenous chromosomal rRNA gene expression in isolated protoplasts (Figure 2B). Using species-specific S1 nuclease probe, B. rapa rRNA gene transcripts were readily detected at similar levels in B. napus and B. rapa protoplasts (compare lanes 1 and 4). A B. oleracea-specific probe (of specific activity higher than that of the B. rapa probe) was used to detect B. oleracea transcripts in B. oleracea protoplasts (lane 2), but did not detect any in B. napus protoplasts (lane 3). These results match those obtained when intact plants are used (Chen and Pikaard 1997a,b). Together, the data of Figure 2, A and B, show that transfected B. oleracea rRNA gene promoters can be active in cells in which their chromosomal counterparts are repressed.

The results of Figure 2A suggest that B. oleracea and B. rapa promoters have similar abilities and opportunities to recruit pol I transcription factors in B. napus. However, the constructs tested in Figure 2 lacked the repetitive elements of the intergenic spacer postulated to be important in the establishment of nucleolar dominance via titration of a limiting transcription factor. Therefore, we repeated the transfection experiment of Figure 2 using B. rapa and B. oleracea minigenes that have nearly complete intergenic spacers upstream of their promoters (Figure 3). The B. oleracea minigene pBol-F included sequences from −2786 to +42; the B. rapa minigene pBra-F included sequences from −2410 to +55 (see Figure 1B). The same radiolabeled probes employed in Figure 2 were used to detect transcripts from these minigenes by S1 nuclease protection. The results obtained with the full-spacer constructs were essentially identical to those obtained with the promoter-only constructs. As shown in Figure 3A, the pBol-F construct was fully active in B. oleracea protoplasts (lane 1), slightly less active in B. rapa protoplasts (lane 2), but
transfected plasmid DNA were detected in each batch of probes are minigene specific). A series of reactions was

Figure 3A) to Southern blot analysis (Figure 3B). Equal amounts of transfected plasmid DNA were detected in each batch of protoplasts (lanes 1–6). Similar amounts of supercoiled (sc) and circular (cc, nc) topoisomers were detected (under these gel conditions, closed and nicked circles comigrate). Using DNA from untransfected protoplasts, no hybridization signals were detected in other controls (data not shown). We also compared Southern blot hybridization signals from transfected protoplasts with the signals obtained when serially diluted purified plasmid DNA was run on the same gel. On the basis of this quantitative comparison, we estimate that an average of \( \sim 2000 \) plasmid molecules were taken up by each B. oleracea, B. napus, or B. rapa protoplast (data not shown), in agreement with our previous estimates for DNA uptake in transfected Arabidopsis protoplasts (Doelling and Pikaard 1995).

Collectively, the results of Figure 3 suggest that the intergenic spacer of the naturally dominant B. rapa rRNA genes does not confer any obvious competitive advantage to the B. rapa minigene in the transient expression assay. The alternative hypothesis, that the intergenic spacers of B. oleracea might preferentially recruit one or more transcriptional repressors, is likewise not supported by the results.

**Dominant and underdominant Brassica rRNA genes compete equally for transcription factors in vitro**

Lack of competition between transiently expressed B. rapa and B. oleracea minigenes in transfected B. napus protoplasts contrasts with results in X. laevis. In the latter case, nucleolar dominance was mimicked when competing X. laevis and X. borealis minigenes with full intergenic spacers were coinjected into oocytes (Reeder and Roan 1984). One explanation might be that in oocyte injection experiments, plasmid DNA is injected directly into the nucleus at \( \sim 20 \) to 40-fold molar excess over the endogenous, amplified rRNA genes. In contrast, our transient expression procedure results in the uptake of only \( \sim 2000 \) copies of each minigene plasmid into B. napus cells estimated to have \( \sim 9000 \) endogenous rRNA genes (Bennett and Smith 1991). Thus, it is possible that we cannot deliver sufficient DNA to make pol I transcription factors limiting in transfected plant cells, whereas this was more likely to have been the case in injected oocytes. We recently developed a cell-free RNA pol I transcription system from broccoli (a cultivated variety of B. oleracea) that allows us to circumvent this caveat due to our ability to control the DNA-to-protein ratio in transcription reactions (Saez-Vasquez and Pikaard 1997). A fully functional RNA pol I holoenzyme can be purified by successive chromatography on multiple columns, yielding single fractions that support accurate, promoter-dependent transcription initiation (Saez-Vasquez and Pikaard 1997). Holoenzyme fractions purified by ammonium sulfate precipitation, DEAE-Sepharose, Biorex 70, and Mono Q chromatography (Figure 4A) programmed transcription from both B. oleracea and B. rapa minigenes (Figure 4B, lanes 1 and 4; the controls in lanes 2 and 3 show that the S1 probes are minigene specific). A series of reactions was
A

Nuclear extract

Ammonium sulfate

DEAE Sepharose

0.1 M 0.175 M 0.4 M KCl

BioRex 70

0.1 M 0.8 M

Mono Q

0.1 M 0.6 M

pol I peak

B

template (1 ng) : O O R R

probe : O R O R

C

oleracea probe

1 2 3 4 5 6

rapa probe

1 2 3 4 5 6

| µg each template | .25 .5 1 2 4 8 |

Figure 4.—B.oleracea and B. rapa rRNA genes compete equally for pol I transcription factors in vitro. (A) Purification scheme used to obtain broccoli (B.oleracea) RNA polymerase I holoenzyme fractions that support accurate, promoter-dependent rRNA gene transcription. (B) The full-spacer constructs pBol-F and pBra-F are similarly active as templates for in vitro transcription (lanes 1 and 4). Transcripts were detected by S1 nuclease protection. Lanes 2 and 3 are controls to test the minigene-specificity of the S1 probes. (C) The pBol-F and pBra-F minigenes are similarly active when competed against one another in vitro. B.oleracea and B. rapa minigenes were both added to six transcription reactions, representing a range of 0.25–8 µg of each plasmid. After incubation of the reactions, RNA was purified and split into two equal aliquots such that transcripts from each minigene could be detected by S1 nuclease protection.

then set up, which contained equal amounts of the B. rapa and B. olereacea full-spacer constructs pBol-F and pBra-F, spanning a range of 0.25–8.0 µg of each plasmid per reaction (Figure 4C). Our rationale was that if dominant B. rapa rRNA genes are better able to recruit one or more transcription factors, this advantage might only become apparent at high template concentrations that cause transcription factors to become limiting. As can be seen in Figure 4C, the optimal amount of template for each minigene was found to be between ~0.5 and 1 µg (lanes 2 and 3), and at the highest template concentrations transcription was inhibited severalfold (lane 6). Inhibition at high template concentrations is thought to be due to the disruption of the holoenzyme complex after transcription is initiated, allowing released factors to bind independently to the excess DNA, making reassociation of holoenzyme complexes inefficient (J. Saez-Vasquez and C. S. Pikaard, unpublished results). Importantly, no preferential transcription of the B. rapa construct was observed in Figure 4C at any template concentration tested. We conclude that dominant and underdominant rRNA genes compete equally for pol I transcription factors, both in vitro (Figure 4) and when transiently expressed in vivo (Figures 2 and 3).

Effects of CpG methylation on pol I transcription:
Different cytosine methylation of dominant and underdominant rRNA genes has been observed in wheat (Flavell et al. 1988; Sardana et al. 1993), and we and others have shown that underdominant genes can be derepressed by 5-aza-2′-deoxycytidine, an inhibitor of cytosine methyltransferase (Neyes et al. 1995; Chen and Pikaard 1997a). Changes in cytosine methylation have also been correlated with developmental and light-regulated expression of an rRNA gene variant class in pea (Watson et al. 1987). It has been proposed that methylation of rRNA genes might inhibit the binding of one or more pol I transcription factors to methylated target sites (Houchins et al. 1997). If so, selective hypermethylation of underdominant genes could lead to preferential association of transcription factors with dominant genes.

In plants, the majority of DNA methylation occurs on cytosines at symmetrical CpG or CpNpG motifs (Grundenbaum et al. 1981; Jeddeloh and Richards 1996), and in Brassica, we have shown that ~80% of the cytosines in genomic TaqI sites (TCGA) are methylated (Chen and Pikaard 1997a). Therefore, we examined the sensitivity of B. oleracea rRNA minigene transcription in vitro after CpG methylation (Figure 5). Plasmid pBor2 (sequences —517 to +104; Doelling and Pikaard 1996) was treated with Sssl methylase and, after the reactions were stopped, the extent of methylation was estimated by examining the extent to which digestion by H palI was inhibited (Figure 5A). As can be seen in the ethidium bromide-stained agarose gel of Figure 5A, unmethylated template DNA was digested efficiently by both M splI and its isoschizomer, H palI (compare lanes 2 and 3 to the uncut control in lane 1), both of which recognize the sequence CCGG. After in vitro methylation, template DNA was still cut to completion by M splI, which is insensitive to methylation of the central cytosine (lane 5). However, cleavage of the template by H palI, which is blocked by methylation of the internal cytosine, was inhibited (lane 6), suggesting that the template was nearly fully methylated.

The relative abilities of unmethylated and fully methylated B. oleracea minigenes to program transcription in vitro were compared in Figure 5B (lanes 2 and 3). Methylation had no effect, which suggests that the binding of the pol I transcription machinery is insensitive to cytosine methylation. Though a direct inhibition of transcription factor binding seems unlikely, cytosine methylation might inhibit rRNA gene transcription indirectly if CpG binding proteins and associated repressors
had no significant effect on transcription (Figure 5B, (presumably enhancers) of dominant rRNA genes X. laevis would inhibit holoenzyme transcription. Addition of X. laevis be missing in highly purified pol I holoenzyme fractions. tion mimicked nicely the dominance of et al. Jones Boyes vertebrates (DE100, DE175, and DE400 indicate the protein pools eluted of these Brassica species are very similar and that all the from a DEAE column at 100, 175, or 400 mKCl, respectively. transcription is not inhibited by CpG methylation. Aliquots of the the ethidium bromide-stained agarose gel. (B) methylation was estimated by the degree to which in vitro transcription results show that the pol I transcription systems in vitro the other species, which might indicate a suboptimal same unmethylated (lane 2) or fully methylated B. oleracea minigene DNA (lanes 3–10) that were tested in A were transcribed in vitro using purified pol I holoenzyme only (lanes 2 and 3) or holoenzyme supplemented with other dialyzed column fractions or crude nuclear extract (lanes 4–10). DE100, DE175, and DE400 indicate the protein pools eluted from a DEAE column at 100, 175, or 400 mm KCl, respectively. B100 and B800 are Biorex 70 fractions named according to the same scheme. The flow-through of the final Mono Q column is labeled QFT.

are recruited to hypermethylated DNA in plants, as in vertebrates (Boy es and Bird 1991; Lewis et al. 1992; Jones et al. 1998; Nan et al. 1998). Such activities might be missing in highly purified pol I holoenzyme fractions. Therefore, we tested whether addition of other fractions would inhibit holoenzyme transcription. Addition of DEAE, Biorex, or Mono Q fractions (see Figure 4A) had no significant effect on transcription (Figure 5B, lanes 4–9), nor did addition of crude nuclear extract (lane 10). Similar results were obtained when methylated pBol-P and pBol-F minigenes were used (data not shown). We conclude that binding of the pol I holoenzyme to the B. oleracea rRNA gene promoter is not blocked directly by cytosine methylation. At present, we also have no evidence for methylcytosine binding proteins that might play an indirect role in rRNA gene repression.

DISCUSSION

Previous studies showed that ribosomal RNA gene transcription in plants, as in animals, can be species-specific. For instance, a tomato (Lycopersicon esculentum) rRNA gene promoter was not recognized properly when transfected into Arabidopsis thaliana protoplasts (Doelling and Pikaard 1996) nor was a tobacco (Nicotiana tabacum) promoter recognized in a bean (Vicia faba) cell-free transcription extract (Fan et al. 1995). Furthermore, a B. oleracea rRNA gene promoter was inefficiently recognized by the pol I transcription machinery in protoplasts of the related crucifer, A. thaliana. Instead, the Brassica promoter was aberrantly, but efficiently, recognized by the RNA polymerase II transcription machinery, leading to transcription initiation ~30 bp downstream of a consensus TATA sequence present at the site where pol I normally initiates (Doelling and Pikaard 1996). On the basis of these initial studies of species specificity in plants, the possibility existed that B. oleracea and B. rapa rRNA genes might be recognized efficiently only by transcription factors that have coevolved with these genes in the same species. If so, inactivation of one or more B. oleracea-specific transcription factors might explain the silencing of B. oleracea rRNA genes in B. napus. Our in vivo and in vitro tests argue against this hypothesis. Though B. rapa and B. oleracea rRNA genes appear to be slightly less active when transfected into the other species, which might indicate a suboptimal interaction with one or more required transcription factors, both gene types were fully active when cotransfected into protoplasts of B. napus. These transient expression results show that the pol I transcription systems of these Brassica species are very similar and that all the transcription factors needed for B. oleracea rRNA gene expression are present in B. napus cells.

Experiments in X. laevis showed that the rRNA gene promoters of X. laevis and X. borealis were equally active when injected into X. borealis oocytes, but that minigenes with X. laevis intergenic spacers attached were transcriptionally dominant over minigenes bearing X. borealis spacer sequences (Reeder and Roan 1984). This situation mimicked nicely the dominance of X. laevis over X. borealis rRNA genes during the early development of X. laevis × X. borealis hybrids (Cassidy and Blackler 1974), leading to the hypothesis that spacer sequences (presumably enhancers) of dominant rRNA genes titrate a limiting transcription factor(s), thus making the
factor(s) unavailable to underdominant genes. Despite the appeal of this model, we have been unable to find any evidence that dominant and underdominant Brassica rRNA genes differ in their abilities to recruit the pol I transcription machinery. Both classes of genes compete equally for highly purified B. oleracea pol I holoenzyme in vitro. This result can be criticized in at least two ways, namely (1) that a hypothetical protein(s) distinct from the holoenzyme might be responsible for rRNA gene discrimination or (2) that the results might have been different if a B. rapa or B. napus in vitro system were available and tested. However, dominant and underdominant rRNA genes are also equally transcribed in vivo upon transfection into B. rapa or B. napus protoplasts. Any potentially important factors missing in our B. oleracea extracts should have been present in these living cells. The fact that chromosomal copies of the underdominant B. oleracea genes are repressed in B. napus protoplasts but transfected B. oleracea genes are expressed in these same cells suggests that the chromosomal copies are somehow denied access to the transcription factors.

Another argument one could make is that competition for transcription factors might be the basis for establishment of nucleolar dominance in early embryos but that other mechanisms, such as chromatin modifications, then enforce nucleolar dominance in vegetative cells, such as those we have used to isolate protoplasts or to make in vitro transcription extracts. Though we cannot rule out this possibility, genetic evidence in Arabidopsis argues against this possibility. In A. suecica, an allo-tetraploid hybrid of A. thaliana and Cardaminopsis arenosa, the thaliana rRNA genes are normally repressed (Chen et al. 1998). Upon backcrossing newly created (synthetic) A. suecica to tetraploid thaliana, we found that the progeny still had active thaliana rRNA genes but, in some cases, had silenced the arenosa rRNA genes, showing that the direction of dominance can be switched. If the normally dominant arenosa rRNA genes have a superior binding affinity for one or more limiting transcription factors, they should have competed best for these factors at the critical stage of development and escaped inactivation. The fact that this is not the case argues strongly against the hypothesis (Chen et al. 1998).

Collectively, the results of our genetic and biochemical studies in Brassica and Arabidopsis are hard to reconcile with any model that suggests that it is “every rRNA gene for itself” in the competition for transcription factors. Instead, it seems likely that rRNA genes of one parental type are coordinately silenced through changes in chromatin that sequester them from the transcription machinery. Early evidence that chromatin was involved was that in wheat, nucleolar dominance was correlated with increased methylation of inactive genes (Flavell et al. 1988; Sardana et al. 1993; Houchins et al. 1997). In Xenopus, similar changes in DNase I accessibility occurred, but without any detectable change in DNA methylation (Macleod and Bird 1982). In fact, methylated templates were found to be fully active for transcription in Xenopus (Macleod and Bird 1982; Pennonck and Reeder 1984), which suggests that methylation did not impair transcription factor binding. Macleod and Bird (1982) did note, however, that methylation might be necessary, but not sufficient, for silencing. Labhart et al. later showed that Xenopus rRNA gene transcription in vitro could be inhibited by repressor activities that bind preferentially to methylated DNA (Labhart 1994). Our finding that inhibitors of either cytosine methylation or histone deacetylation will derepress silenced B. oleracea rRNA genes in B. napus reinforces the idea that methylation and other chromatin modifications are partners in rRNA gene repression (Chen and Pikaard 1997a). The recent finding that methylcytosine-binding proteins are part of a complex that includes histone deacetylase activity further suggests that methylation may exert its influence on transcription through changes in histone acetylation status (Jones et al. 1998; Nan et al. 1998).

It is not clear whether the rRNA genes themselves, other regulatory loci, or both, are the primary targets of cytosine methylation and histone deacetylation events that result in the coordinate repression of whole parental sets of rRNA genes. Evidence for the involvement of loci unlinked to the NORs has been known for some time (Flavell and O’De l l 1979; Neves et al. 1997), and genes encoding species-specific transcription factors have been proposed as logical candidates for such loci (Neves et al. 1997). However, transient expression results effectively rule out the involvement of species-specific transcription factors in B. napus (this study) or A. suecica (Chen et al. 1998). Other evidence points to the involvement of chromosomal regions adjacent to the NORs on both the X and Y chromosomes of D. melanogaster. Rearrangement or deletion of these regions results in the failure of the D. melanogaster NORs to be dominant over the single NOR on the X chromosome of D. simulans in XX female or XY male hybrids (Durica and Krider 1978). Interestingly, these rearrangements do not appear to negatively affect the expression of the adjacent melanogaster NORs. The latter observation indicates that expression of the dominant set of rRNA genes is not sufficient to cause the repression of the underdominant set as predicted by transcription factor competition models (Durica and Krider 1978).

Evidence that rRNA genes are coordinately controlled, combined with the various lines of evidence that suggest a chromosomal basis for the phenomenon, lead us to speculate that NORs may be the units of regulation in nucleolar dominance, rather than individual rRNA genes. There is precedent for chromatin-based repression mechanisms operating on the multimegabase scale needed to suppress an NOR. The best example is
X-chromosome inactivation in somatic cells of female mammals, in which most of the genes on one X-chromosome are silenced (Rastan 1994; Penny et al. 1996; Willard 1996; Heard et al. 1997; Lee and Jaenisch 1997). A specific locus, the X-inactivation center, is required in cis for silencing to occur. Like nucleolar dominance, X-inactivation involves both cytosine hypermethylation and histone deacetylation. However, unlike nucleolar dominance, the choice of which X-chromosome to inactivate appears to be random in somatic cells, which suggests that a counting mechanism rather than an allele discrimination mechanism is responsible for X inactivation.

If NORs are controlled by an adjacent locus analogous to the X-inactivation center, a prediction is that an rRNA gene located outside of an NOR should not be subjected to nucleolar dominance. This prediction can be tested using rRNA transgenes integrated at ectopic locations. It would also be instructive to know whether silencing is restricted to the rRNA genes within the NORs or whether neighboring genes are also affected, as might be the case if silencing affects the entire chromosomal region where NORs are located. These experiments should be possible using the Brassica and Arabidopsis species we have chosen as our model systems.

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