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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 (Navashin 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. Favored 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-IB 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-

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man rRNA genes, but not both, in mouse-human somatic cell hybrids (Elicieri and Green 1969; Miller *et al.* 1976; Perry *et al.* 1976; Soprano *et al.* 1979; Soprano and Baserga 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 coinjected on a separate plasmid, the enhancers compete against the promoter (Labhart 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 set of experiments yielded results consistent with this hypothesis, showing that preferential transcription of *X. laevis* rRNA minigenes in *borealis* oocyctes 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, *Aegilops 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 show that the differences in *B. rapa* and *B. oleracea* rRNA 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 *Ava*II-*Hin*fI fragment (sequences −517 to +42 relative to the transcription start site, +1) of pBor2 (Doelling and Pikaard 1996) into the *Sma*I site of the pBluescript plasmid (Stratagene, La Jolla, CA) pBSII 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 *Dra*I-*Bst*BI fragment of the *B. oleracea* genomic clone pBOB6 (Bennett and Smith 1991) into *Eco*RV-*Bst*BI-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 (Bhatia *et al.* 1996) as an *Acl*I-*Sna*BI fragment. This fragment was cloned into the *Eco*RI-*Bam*HI sites of pBSII KS- after blunting all ends. An *Eco*RV-*Sac*II fragment containing the inserted DNA was subsequently subcloned into pBSII SK-, resulting in the construct pBra-F. *B. oleracea* and *B. rapa* rRNA minigenes lacking extensive 5′ flanking sequences were engineered by removing intergenic spacer sequence (IGS) upstream of the conserved *Xmn*I 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 (5×10^6) 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 polyethylene 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 1917-bp *Pvu*I fragment of

pBluescript SK(−) was used as the probe to subject aliquots of total nucleic acid to agarose gel electrophoresis and Southern blotting. RNA was purified from total nucleic acid by lithium chloride precipitation (Doelling *et al.* 1993) and the S1 nuclease protection assay was used to detect minigene transcripts (Berk and Sharp 1977). S1 probes were 5′ end-labeled at the *Bss*HII restriction sites located in the plasmids downstream of the cloned Brassica sequences such that minigene transcripts can be discriminated from rRNA transcripts from endogenous chromosomal genes. For the *B. oleracea* minigene, the probe was the *Acc*I-*Bss*HII (−41 to +124) fragment of pBol-F labeled at +124. The probe used to detect *B. rapa* minigene transcripts was the *Bst*BI-*Bss*HII (−93 to +159) fragment of pBra-F labeled at +159. The *Acc*I(−39) to *Ava*II (+103) gene fragment labeled at +103 was used to detect chromosomally encoded *B. oleracea* rRNA gene transcripts (Figure 2B). The *Sph*I(−110) to *Ava*II (+76) gene fragment labeled at +76 was used to detect chromosomally encoded *B. rapa* transcripts. S1 digestion products were resolved on urea-PAGE sequencing gels, which were subsequently dried onto filter paper and visualized by exposure to X-ray film.

***In vitro* transcription:** Broccoli (*B. oleracea*) nuclear extract proteins purified by successive DEAE, Biorex, and Mono Q chromatography were used for *in vitro* transcription experiments, as described previously (Saez-Vasquez and Pikaard 1997). Single Mono Q fractions contain all activities necessary for accurate, promoter-dependent transcription. These activities appear to be physically associated to comprise an RNA pol I holoenzyme. Transcription reactions contained template DNA, 20 μl of dialyzed holoenzyme (in 50 mm HEPES pH 7.9, 20% glycerol, 10 mm EGTA, 10 mm $MgSO_4$, 1 mm DTT, 100 mm KCl), and 20 μl of 2× transcription reaction mix (30 mm HEPES pH 7.9, 80 mm potassium acetate, 12 mm magnesium acetate, 1 mm DTT, 200 μg/ml α-amanitin, 1 mm each nucleotide triphosphate). Transcription reactions were incubated for 2 hr at 25°. Stop solution (360 μl) was then added (150 mm NaCl, 50 mm Tris-HCl pH 8.0, 250 mm sodium acetate pH 5.3, 3 μg/ml yeast tRNA, 6 mm EDTA pH 8.0). Reactions were extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) followed by extraction with chloroform:isoamyl alcohol (24:1 v/v). The aqueous phase was ethanol precipitated with excess end-labeled probe, resuspended in hybridization buffer, and subjected to S1 nuclease protection as described previously (Doelling *et al.* 1993; Chen and Pikaard 1997b).

***In vitro* methylation:** Supercoiled plasmid DNA was methylated on cytosines in CpG motifs using *Sss*I methylase (New England Biolabs, Beverly, MA) in a reaction buffer supplemented with 0.2 mm *S*-adenosyl methionine (supplied by the manufacturer) for 2 hr at 37°. Reactions were stopped by heat treatment at 65° for 20 min, followed by phenol/chloroform extraction and ethanol precipitation. The extent of methylation was estimated by inhibition of digestion by *Hpa*II.

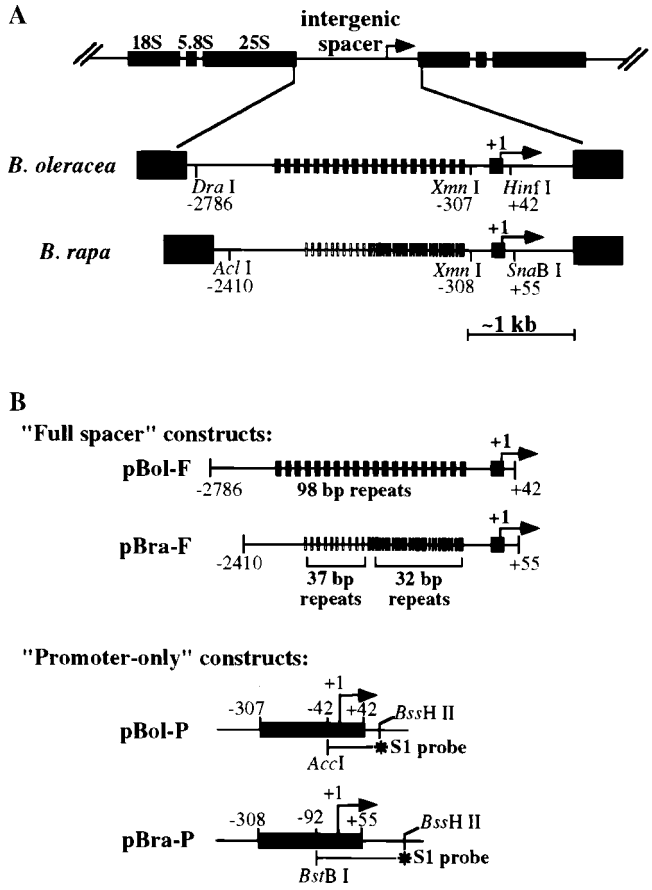

Figure 1.—*B. oleracea* and *B. rapa* rRNA gene organization and minigenes used in the study. (A) Ribosomal RNA genes encoding the precursor of the three largest rRNAs are tandemly arrayed at nucleolus organizer regions of eukaryotic chromosomes. Transcribed regions are separated by intergenic spacers that include the gene promoter and numerous repetitive elements represented by small solid or shaded boxes. *B. rapa* and *B. oleracea* rRNA gene intergenic spacers differ in length and in the types and numbers of repetitive elements. The locations of restriction endonuclease sites used to construct the minigenes for *in vivo* and *in vitro* experiments are shown. Arrows labeled +1 denote the transcription start sites mapped in previous studies (Doelling and Pikaard 1996; Chen and Pikaard 1997b). (B) Minigenes used in the study include the "full-spacer" constructs pBol-F and pBra-F and the "promoter-only" constructs pBol-P and pBra-P. For the pBol-P and pBra-P constructs, solid boxes denote rRNA gene sequences and thin lines denote plasmid sequences. The locations of restriction fragments end-labeled and used as S1 nuclease protection probes for pBol and pBra minigenes are shown below the promoter-only constructs.

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 the *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 transfection across species boundaries, the *B. oleracea* minigene was active in *B. rapa* protoplasts (Figure 2A, lane 2) as

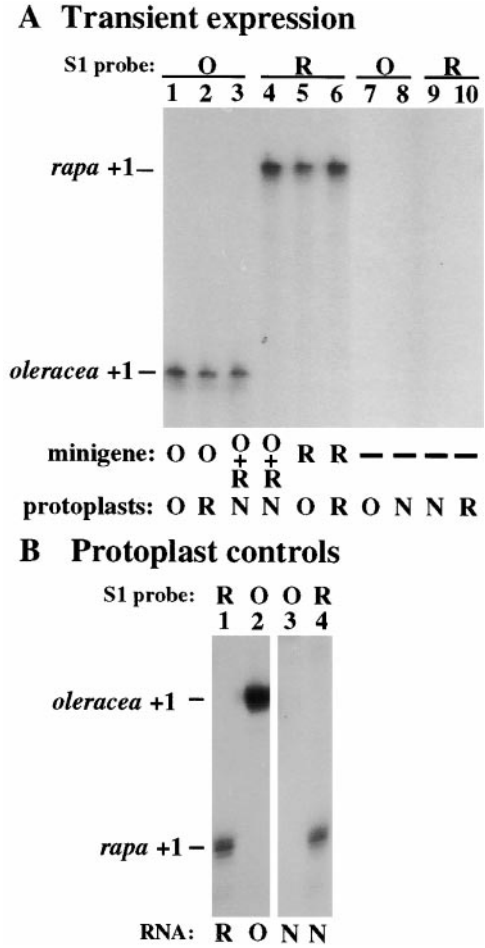

Figure 2.—rRNA gene transcription in the allotetraploid, *Brassica napus* and its progenitor species is not species-specific. (A) Equimolar amounts of the pBol-P and pBra-P minigene plasmids containing cloned rRNA gene promoter sequences of *B. oleracea* (O) or *B. rapa* (R), respectively, were transfected alone (lanes 1, 2, 5, and 6) or together (lanes 3 and 4) into protoplasts of *B. oleracea*, *B. rapa* or *B. napus* (N). Probes specific for the *B. oleracea* (lanes 1–3) or *B. rapa* (lanes 4–6) minigenes were used to detect transcripts by S1 nuclease protection. RNA from the same number of protoplasts was probed in all lanes. For the cotransfection experiment (lanes 3 and 4), the reaction was scaled up twofold and the purified RNA split into two equal aliquots for hybridization to the *B. oleracea* and *B. rapa* minigene-specific probes. Lanes 7–10 are controls to show that the probes do not protect RNAs in untransfected *B. oleracea*, *B. napus* or *B. rapa* protoplasts. (B) Protoplast generation does not derepress *B. oleracea* rRNA genes in *B. napus* cells. Probes specific for chromosomally encoded *B. rapa* or *B. oleracea* rRNA genes were used to subject RNA isolated from protoplasts of *B. rapa* (R), *B. oleracea* (O), or *B. napus* (N) to S1 nuclease protection.

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 a 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

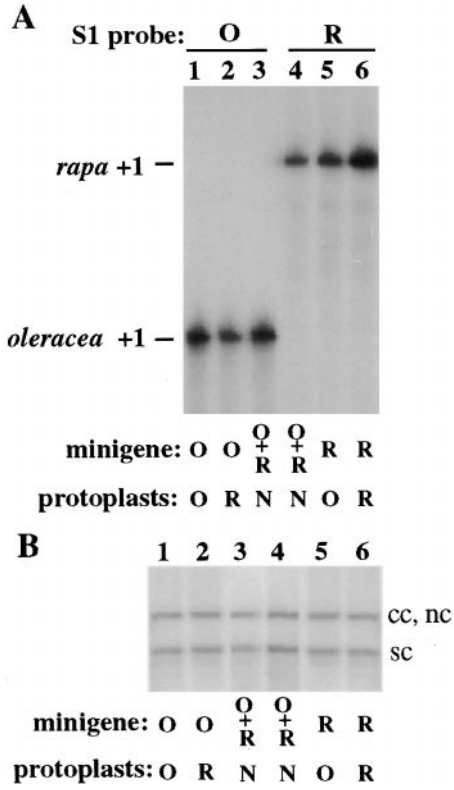

Figure 3.—Repetitive elements of the intergenic spacer (putative enhancers) do not influence the competitive strength of *B. oleracea* and *B. rapa* minigenes transfected into protoplasts. (A) Equimolar amounts of minigene plasmids pBol-F and pBra-F were transfected alone or together into protoplasts and their transcripts were detected as in Figure 2A. (B) The pBol-F and pBra-F constructs are transfected with similar efficiency into protoplasts of *B. oleracea*, *B. rapa*, and *B. napus*. A Southern blot is shown for which equal aliquots of total nucleic acid purified from transfected protoplasts were probed with a radiolabeled plasmid (pBluescript) fragment. sc, supercoiled; nc, nicked circular DNA; cc, closed circular DNA.

fully active in *B. napus* protoplasts cotransfected with an equimolar amount of pBra-F (lane 3). In the experiment shown, pBra-F appears to be less highly expressed in *B. napus* protoplasts than in *B. oleracea* or control *B. rapa* protoplasts (compare lanes 4 and 6). However, this was not consistently observed, which suggests that experimental variation is the likely explanation for the relatively low *B. rapa* signal in lane 4.

Another trivial explanation for our inability to observe nucleolar dominance in cotransfected *B. napus* protoplasts could be that more copies of the *B. oleracea* construct are taken up by protoplasts, thus masking a competitive advantage of the *B. rapa* minigene. To test this possibility, a probe that recognized the pBluescript portion of the minigene was used to subject equal aliquots of total nucleic acid isolated from the washed protoplasts (same total nucleic acid preparations from which RNA was further purified and probed in 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 ~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 Xenopus. 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 ~20- to 40-fold molar excess over the endogenous, amplified rRNA genes. In contrast, our transient expression procedure results in the uptake of only ~2000 copies of each minigene plasmid into *B. napus* cells estimated to have ~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

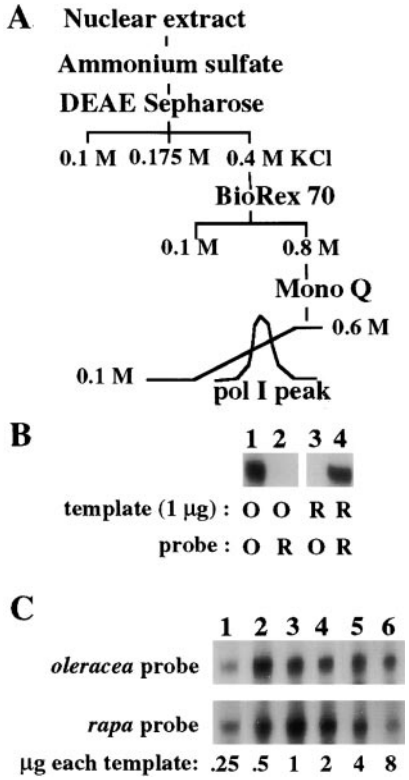

Figure 4.—*B. oleracea* and *B. rapa* rRNA genes compete equally for pol I transcription factors *in vitro*. (A) Purification scheme used to obtain broccoli (*B. oleracea*) RNA polymerase I holoenzyme fractions that support accurate, promoter-dependent rRNA gene transcription. (B) The full-spacer constructs 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. oleracea* 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: Differential 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 (Neves *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 (Gruenbaum *et al.* 1981; Jeddeloh and Richards 1996), and in Brassica, we have shown that ~80% of the cytosines in genomic *Taq*I 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 *Sss*I methylase and, after the reactions were stopped, the extent of methylation was estimated by examining the extent to which digestion by *Hpa*II 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 *Msp*I and its isoschizomer, *Hpa*II (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 MspI, which is insensitive to methylation of the central cytosine (lane 5). However, cleavage of the template by *Hpa*II, 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

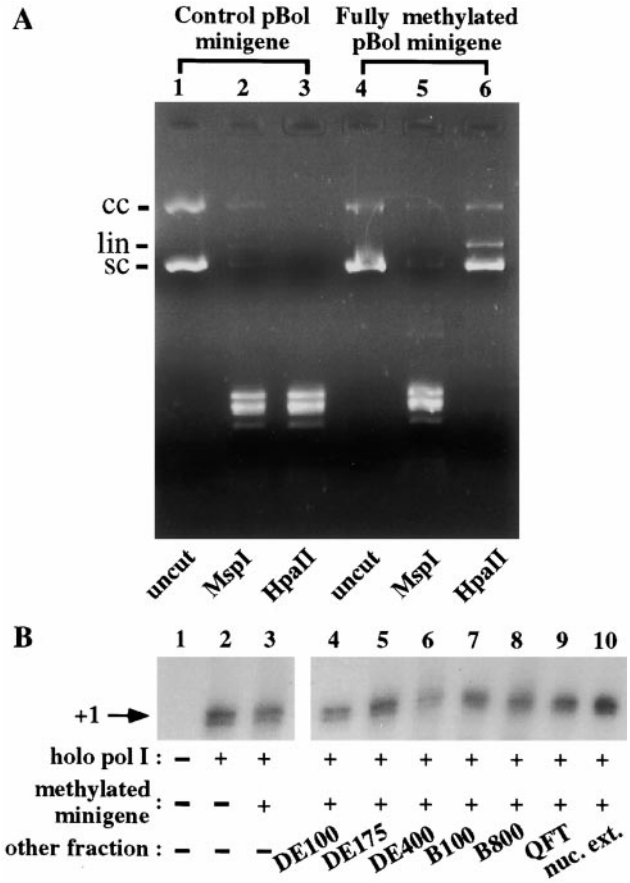

Figure 5.—CpG methylation does not inhibit transcription of *B. oleracea* minigenes *in vitro*. (A) A *B. oleracea* minigene, including sequences from −517 to +104, was subjected to digestion with the restriction endonuclease isoschizomers *Msp*I or *Hpa*II before (lanes 2 and 3) or after (lanes 5 and 6) *in vitro* methylation using *Sss*I methylase. The extent of methylation was estimated by the degree to which *Hpa*II digestion was inhibited. Supercoiled (sc), linear (lin), and closed circular (cc) forms of the plasmid are indicated to the left of the ethidium bromide-stained agarose gel. (B) *In vitro* transcription is not inhibited by CpG methylation. Aliquots of the 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 (Boyes 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 Xenopus 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 this out, genetic evidence in Arabidopsis argues against this possibility. In *A. suecica*, an allotetraploid 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 all 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 decreased accessibility to DNase I digestion and 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; Pennock 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 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'Dell 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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