Nucleic Acids Research, 1996, Vol. 24, No. 23 **4725–4732**

Species-specificity of rRNA gene transcription in plants manifested as a switch in RNA polymerase specificity

Jed H. Doelling[+] and Craig S. Pikaard*

Biology Department, Washington University, Box 1137, One Brookings Drive, St Louis, MO 63130, USA

Received August 14, 1996; Revised and Accepted October 16, 1996

ABSTRACT

Rapid evolution of ribosomal RNA (rRNA) gene promoters often prevents their recognition in a foreign species. Unlike animal systems, we show that foreign plant rRNA gene promoters are recognized in an alien species, but tend to program transcription by a different polymerase. In plants, RNA polymerase I transcripts initiate at a TATATA element (+1 is underlined) important for promoter strength and start-site selection. However, transcripts initiate from +32 following transfection of a tomato promoter into *Arabidopsis*. The rRNA gene promoter of a more closely related species, *Brassica oleracea*, programs both +1 and +29 transcription. A point mutation at +2 improving the identity between the *Brassica* and *Arabidopsis* promoters increases +1 transcription, indicating a role for the initiator element in species-specificity. *Brassica* +29 transcripts can be translated to express a luciferase reporter gene, implicating RNA polymerase II. TATA mutations that disrupt TATA-binding protein (TBP) interactions inhibit +29 transcription and luciferase expression. Co-expressed TBP proteins bearing compensatory mutations restore +29 transcription and luciferase activity, suggesting a direct TBP–TATA interaction. Importantly, +1 transcription is unaffected by the TATA mutations, suggesting that in the context of pol I recognition, the TATA-containing initiator element serves a function other than TBP binding.

INTRODUCTION

In eukaryotes, three nuclear RNA polymerases perform distinct functions. RNA polymerase I transcribes large ribosomal RNAs (rRNAs) (1–3), RNA polymerase II transcribes protein-encoding genes and most small nuclear RNAs (snRNAs) (4–6), and RNA polymerase III transcribes other small RNAs including tRNAs, 5S rRNA, U6 snRNAs and, in plants, U3 snRNA (6–8). The molecular mechanisms responsible for promoter recognition by the three polymerase systems is the subject of intensive investigation.

Ribosomal RNA genes transcribed by RNA polymerase I are located within the nucleolus and are arranged in tandem arrays of 18S, 5.8S and 25S rRNA coding sequences separated by an intergenic spacer that includes the gene promoter (9,10). In animals, rRNA gene promoters display little sequence similarity between species and are generally not functional across species boundaries (11–13). A subset of the transcription factors and promoter domains appear to be responsible for this species-specificity. Supporting evidence is that engineering 18 bp of mouse promoter sequences into a human promoter ~10 times larger is sufficient to convert the promoter into one efficiently recognized in a mouse extract (14). Likewise, half-helical turn spacing changes between the upstream and core promoter domains of the *Xenopus laevis* promoter convert it into a strong promoter in a mouse extract (15). Alternatively, addition of a specific transcription factor complex, SL1 (selectivity factor 1; also known as TIF-IB, factor D or Rib 1) can reprogram mammalian extracts to recognize an alien promoter. For instance, mouse SL1 added to a human extract allows efficient transcription of a mouse rRNA gene promoter and addition of human SL1 to a mouse extract facilitates transcription of a human promoter (12,13,16–18). Other essential activities, including upstream binding factor and polymerase I, can substitute between mouse and human. Collectively, these studies illustrate that precise protein–protein interactions, dictated in part by the spacing of promoter elements, are key to promoter recognition.

Polymerase specificity switching in snRNA genes has also provided important insights into promoter recognition processes. In animals and plants, the U2 snRNA gene is transcribed by RNA polymerase II whereas U6 snRNA is transcribed by RNA polymerase III. *Arabidopsis thaliana* U2 and U6 snRNA gene promoters are very similar in sequence, differing primarily in the spacing between a TATA element and an upstream sequence element. In both the U2 and U6 promoters, changing the spacing between the two elements by one helical turn can switch their polymerase specificity between pol II and pol III (8,19). In human cells, the U6 promoter is also transcribed by polymerase III but has a TATA element interchangeable with TATA boxes of genes transcribed by pol II. Ironically, the U2 promoter, which is recognized by RNA polymerase II, lacks a consensus TATA box and creating one by site-directed mutagenesis switches its specificity from polymerase II to polymerase III (6,20,21). Apparently promoter elements such as TATA boxes can be used by different polymerase systems and be interpreted in different ways based on their context relative to other promoter elements.

In the plant, *A.thaliana* we showed that sequences between –55/–33 and +6 are sufficient to program accurate pol I transcription initiation *in vivo* (22,23). At the start site is a sequence highly conserved in plants (24–33), approximating the consensus

*To whom correspondence should be addressed. Tel: +1 314 935 7569; Fax: +1 314 935 4432; Email: pikaard@biodec.urustl.edu

[+]Present address: Department of Horticulture, University of Wisconsin–Madison, 1575 Linden Drive, Madison, WI 53706, USA

TATATA(A/G)GGG (+1 is underlined) in dicots. Clustered point mutations within this conserved initiator element affected promoter strength and start site selection, showing that it is an important element of plant rRNA gene promoters (23). We speculated that the initiator element might be a binding site for TATA-binding protein (TBP) (23), first because TBP is known to be required by all three polymerases, from yeast to humans (4–7,34–37). In the pol I system, TBP is part of the species-specificity factor, SL1 (34–39). Second, pol II- and pol III-specific gene promoters can have TATA boxes that interact with TBP, suggesting that pol I promoters in plants might do so as well. Third, maize TBP had been shown to bind the maize rRNA gene promoter *in vitro* (40), though the functional significance of this observation was not known.

In this study, we investigated the species-specificity of plant rRNA gene transcription by transfecting promoters of a distantly related species (tomato) and a closely related species (*Brassica oleracea*) into *A.thaliana* protoplasts. Surprisingly, rather than displaying a simple on or off phenotype, the tomato and *Brassica* promoters preferentially programmed transcripts initiating ~30 bp downstream of the expected pol I start site (+1). In the case of *Brassica*, weak +1 transcripts were also detected. A point mutation that made the *Brassica* +1 region a better match to the corresponding *Arabidopsis* promoter improved the efficiency of +1 transcription, indicating a role for the initiator region in species specificity. We circumvented the lack of an *in vitro* transcription system using mutation-suppression analysis to show that *Brassica* +29 transcription is dependent on TBP–TATA interactions, but +1 transcription is not. These results suggest that the highly conserved initiator region of plant rRNA genes has a role other than direct TBP binding.

MATERIALS AND METHODS

Construction of rRNA gene promoter plasmids

Brassica oleracea rRNA gene promoter sequences from –518 to +106 were amplified from genomic DNA using the polymerase chain reaction and the primers 5′-CGGAATTCGGACCAAAA-TCACCCGGATAGTCCA-3′ and 5′-CGCGGATCCGGACCT-CAACCCAAGCATCATCG-3′. The amplification product was digested with *Eco*RI and *Bam*HI (these sites are underlined above) and ligated into pBluescript II KS– to generate pBor2. Two derivative plasmids of pBor2 were generated using site-directed mutagenesis. Changing A to G in the RNA strand at position +2 generated the 'A+2G' promoter. Substituting G for A at –4 and an A for T at –1 in the RNA strand generated the 'TGTAAA' mutant. The *Arabidopsis* clone pAt1 consists of sequences from –520 to +92 also cloned in pBluescript II KS– and has been described previously (22,23). The tomato promoter construct tested was a *Dra*I–*Sal*I fragment from clone pKU235 (26), including sequences from –321 to +156, cloned into pBluescript II KS–.

TBP expression plasmids pAt1wt, pAt1m, pAt2wt and pAt2m were generously provided by David Heard and Witold Filipowicz (41). Expression of wild-type (wt) and mutant (m) TBP coding regions was directed by the strong 35S promoter of cauliflower mosaic virus. The coding regions of wild-type and mutant TBPs differ at three amino acid positions in the DNA-binding domain. The mutant proteins can recognize the mutated TATA box sequence TGTAAA and suppress this mutation in TATA-dependent promoters (41).

To fuse the firefly luciferase coding region downstream of the rDNA promoter, the 138 bp *Hin*fI promoter fragments (–99 to +42) of pBor2 and the TGTAAA promoter mutant were blunt-ended with dNTPs and the Klenow fragment of DNA polymerase I and ligated into the *Sma*I site of pBluescript II KS–. The *Bgl*II fragment of pWB216 containing the luciferase coding region and the polyadenylation signal and 3′ flanking sequences of the tomato protease inhibitor gene (42) was introduced into the adjacent *Bam*HI site to complete the luciferase reporter constructs.

Plant growth, protoplast isolation and transient expression

Protoplast isolation from *A.thaliana* Columbia was by published methods (22,43). Protoplasts were transfected as described (22,23,43). For RNA studies, 3×10^7 protoplasts were transfected with 200 µg supercoiled plasmid DNA in the presence of 2 mg salmon sperm or calf thymus carrier DNA. For luciferase assays, the procedure was scaled down 20-fold. Transfected protoplasts were incubated 20–24 h in the dark at 24°C in liquid medium composed of 2× Gamborg salts and vitamins (Sigma) supplemented with 0.4 M mannitol, 20 g/l sucrose, 1 mg/l 2,4-dichlorophenoxy-acetic acid (Sigma) and 0.05 mg/l kinetin (Sigma), pH 5.7.

RNA isolation, S1 nuclease protection analysis, primer extension analysis and luciferase activity assays

Total RNA was isolated from protoplasts and further purified by precipitation with 2 M lithium chloride as described (22). Resulting RNA pellets were resuspended in water and quantified by UV absorbance. Fifty micrograms of RNA were hybridized to a 5′-end labeled DNA probe for analysis by S1 nuclease protection (44). S1 digestion in 300 µl reactions used 50 U S1 nuclease for 30 min at 22°C. Endogenous rRNA transcripts were isolated from non-transfected protoplasts. Transcripts from plasmid-borne promoters were detected using probes labeled within plasmid sequences adjacent to the cloned DNA. S1-digestion products were separated on 6% polyacrylamide sequencing gels adjacent to homologous sequence ladders generated from primers labeled at the same sites as the S1 probes.

Primer extension of *B.oleracea* rRNA involved 50 µg total RNA, a 5′-end labeled 62 bp *Ava*II–*Hin*fI fragment as the primer, and MMLV reverse transcriptase using standard methods (44). Extension products were subjected to electrophoresis beside a sequencing ladder generated using the same primer and the wild-type promoter as template.

Luciferase activity in protoplast lysates was assayed using an Analytical Luminescence Laboratory Monolight 2010 luminometer as described (43). Protein concentration of lysates was measured using the BioRad protein assay with BSA as a standard. Luciferase specific activity is reported as light units per µg protein; one light unit is defined as one-tenth of the total number of photons detected.

RESULTS

We examined the species-specificity of plant rRNA gene transcription by transfecting cloned tomato (*Lycopersicon esculentum* cv. Rutgers), *B.oleracea* and *A.thaliana* rRNA gene promoters into *Arabidopsis* protoplasts and detecting their transcripts by S1 nuclease protection (45). The three species tested are all dicots, but tomato is a member of the *Solanaceae* family whereas *Brassica* and *Arabidopsis* are related genera within

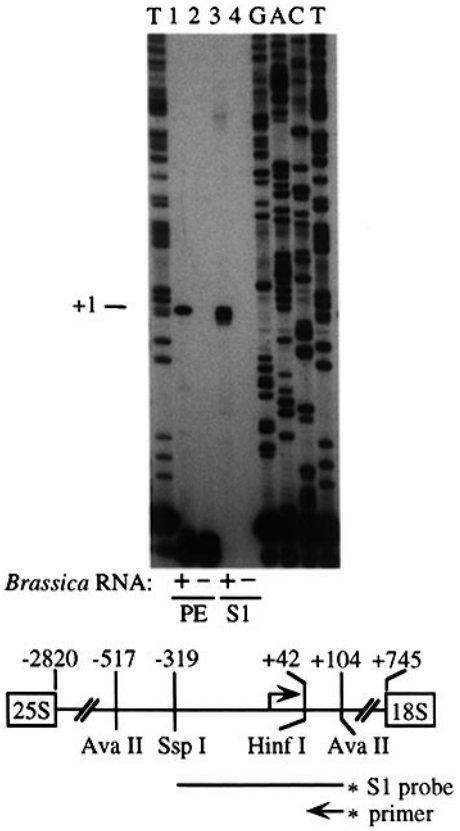

Figure 1. Determination of the transcription start site of chromosomal *B.oleracea* rRNA genes. Total RNA isolated from *B.oleracea* seedlings was subjected to primer extension (PE; lane 1) and S1 nuclease protection (lane 3). The S1 probe was the *Ssp*I (–319)–*Ava*II (+104) fragment; the primer was the *Hin*fI(+42)–*Ava*II (+104) fragment; both were 5′-end labeled at the *Ava*II site. Transcript 5′-ends mapped to the same site, TATAT**A**A (+1 is underlined). Dideoxynucleotide sequencing reactions (G, A, C, T) were generated using the *Hin*fI–*Ava*II primer. No primer extension or S1 nuclease protection products were detected in control reactions using yeast tRNA in place of *Brassica* RNA (lanes 2 and 4, respectively).

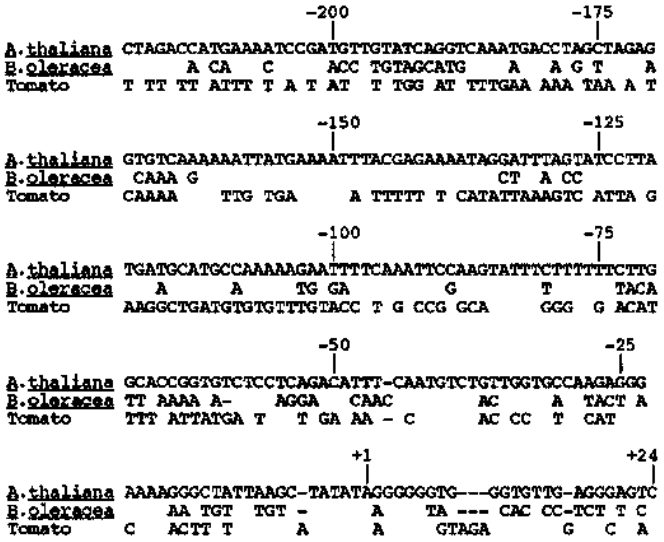

Figure 2. Comparison of *A.thaliana*, *B.oleracea* and tomato rRNA gene promoter regions. The *in vivo* transcription start site is defined as (+1). Only differences from the *Arabidopsis* sequence are shown. Note that Doelling and Pikaard (23) showed that the 5′ boundary of the *A.thaliana* promoter, defined by transient expression, lies between –55 and –33 and the 3′ boundary is near +6. In this minimal promoter region, the *Brassica* and tomato promoters share approximately the same similarity to the *Arabidopsis* promoter except that tomato has a 1 bp insertion at –6.

the *Cruciferae*. Transcription start sites of endogenous (chromosomal) tomato and *A.thaliana* rRNA genes have been mapped to the sequences TATAT**A**AGGG and TATAT**A**GGGG, respectively (+1 is underlined in both cases) (22,26). However, the transcription start site of *B.oleracea* rRNA genes had not been mapped prior to our study. Therefore, based on the published sequence of the complete intergenic spacer (46,47) and comparison to *Arabidopsis* sequences, we used primer extension (Fig. 1, lane 1) and S1 nuclease protection (Fig. 1, lane 3) to map the transcription start site in *B.oleracea* (Fig. 1). Both assays show that transcription initiates at TATAT**A**AGGG (+1 is underlined), as predicted. Importantly, we detected no transcripts other than those from the expected RNA polymerase I start site (+1). The same is true for tomato (26) and *A.thaliana* (22).

Comparison of *Arabidopsis*, *B.oleracea* and tomato promoter regions show that *Arabidopsis* and *B.oleracea* share blocks of sequence conservation from approximately –220 to +10, consistent with their phylogenetic relationship, whereas tomato and *Arabidopsis* are quite different except in the vicinity of the transcription start site (Fig. 2). Mutation analyses have shown that the conserved initiator region is important for promoter strength and transcription start site specificity in *A.thaliana* (23). If the initiator region is the dominant promoter element, we reasoned that species-specificity might not occur in plants such that tomato and *B.oleracea* promoters transfected into *Arabidopsis* protoplasts would program transcription from the normal start site (+1).

Cloned *A.thaliana* rRNA gene promoters transiently expressed in *Arabidopsis* protoplasts program transcription from +1, as expected (Fig. 3A, lane 1) (22,23). However, the tomato promoter gave rise to barely detectable transcription from +1 but strong transcription initiation from +32 (Fig. 3B, lane 1). The *B.oleracea* promoter gave rise to two prominent transcription start sites (Fig. 3C, lane 1); one mapping to +1 but a 5–10-fold stronger signal mapping to +29. Primer extension analysis also mapped RNA 5′-ends to +29, confirming the S1 data (data not shown). Note that S1 protected fragments were not detected in mock transfected (no plasmid DNA) protoplasts, showing that the probes specifically detect plasmid-derived transcripts (lane 2 of each panel) and not endogenous RNAs.

Tomato and *Brassica* transcripts initiated at +32 and +29 could be due to *Arabidopsis* RNA polymerase I transcribing the heterologous promoters from an unusual start site, in analogy to pol I initiation at –4 when a wild-type *X.laevis* promoter is expressed in a mouse extract (15,48). However, both +1 and +29 transcripts were observed with the *B.oleracea* promoter, suggesting that the *Arabidopsis* pol I machinery is capable of recognizing the promoter correctly. An alternative possibility was that +29 (or +32) transcripts were not synthesized by pol I but by another polymerase. This alternate hypothesis was suggested by the fact that polymerase II transcripts generally initiate 25–35 nucleotides downstream from a TATA box (49) and both the tomato and cauliflower rRNA initiation regions match the TATA box consensus TATAa/tAa/t (49,50). Unfortunately, α-amanitin cannot be used to selectively inactivate RNA polymerases II and III

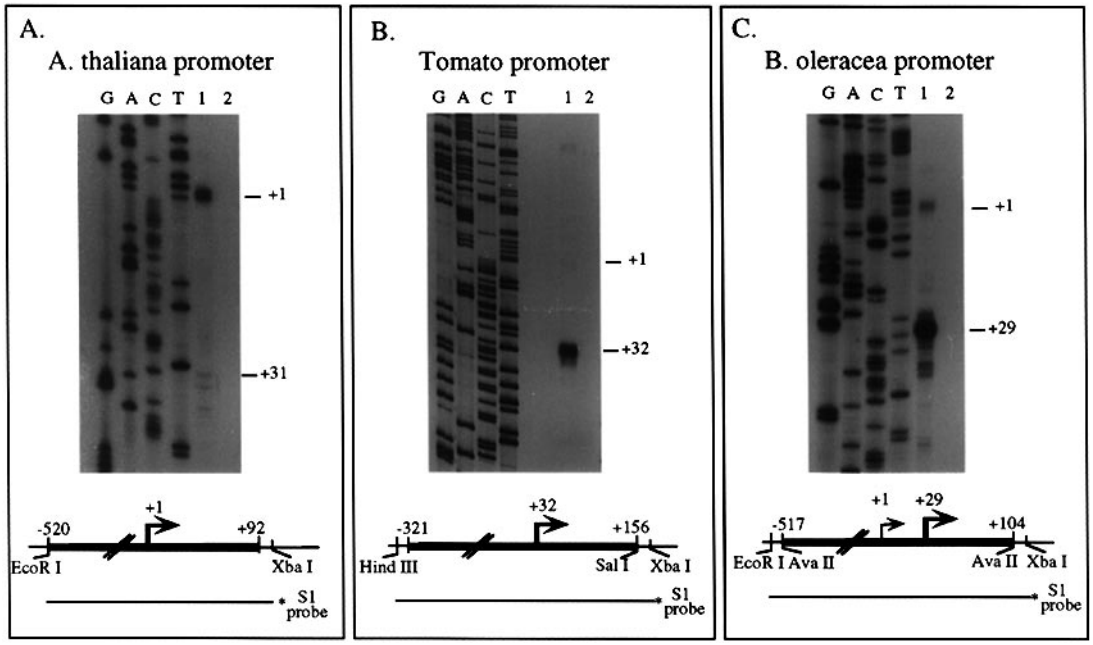

Figure 3. Transient expression of homologous and heterologous rRNA gene promoters in *A.thaliana* protoplasts reveals unexpected transcription start sites programmed by foreign promoters. In panels A, B and C, lane 1 shows the S1 protected products following transfection of a pBluescript plasmid bearing the cloned promoter regions, all in the same orientation within the plasmid (diagrammed at the bottom). Lane 2 shows that no S1 products are obtained with RNA from mock-transfected protoplasts. (**A**) The *A.thaliana* promoter programs transcription from the same start site used by endogenous, chromosomal rRNA genes, defined as +1. Note the faint bands at +31 to +34 discussed in the text. Similar results are obtained with a variety of clones with 5′ sequences ranging between –2590 and –55/–33 (22,23). (**B**) Transient expression of the rRNA gene promoter of tomato, a species distantly related to *Arabidopsis* programs strong transcription from +32 and only a trace signal at +1. (**C**) The rRNA gene promoter of *B.oleracea*, a species closely related to *Arabidopsis*, programs both accurate initiation from +1 and strong transcription from +29. Similar results were obtained with *B.oleracea* clones whose 5′ sequences extended to –2782, –68 or –39 (data not shown). For (A–C), sequence ladders were generated by the method of Sanger using an oligonucleotide primer whose 5′-end matched the labeled nucleotide of the S1 probe, allowing transcription initiation sites to be mapped precisely. Exposure times and probe specific activities were similar in each panel.

in intact plant cells, either due to poor uptake or inactivation of the drug (discussed in 22). Therefore, we needed to devise other means of determining if two polymerase systems were involved.

If +29 transcripts were due to TBP-dependent polymerase II or III transcription, we predicted TBP should bind directly to the TATA box at the +1 region. In the absence of a reliable plant *in vitro* transcription system, we tested this hypothesis using a molecular genetic approach based on mutagenesis. We focused on the *B.oleracea* promoter because +1 and +29 transcripts can be detected with a single probe and compared within a single lane of a gel, internally controlling each experiment. We changed TATATA to TGTAAA because this clustered set of point mutations disrupts TBP binding and RNA polymerase II transcription in yeast (51) and also disrupts TATA-dependent snRNA transcription by RNA polymerases II and III in *A.thaliana* (41). Transient expression of the TGTAAA mutant promoter in *Arabidopsis* protoplasts resulted in a dramatic reduction in +29 transcripts (Fig. 4, lane 1), compared to wild-type (lane 2), consistent with the hypothesis that +29 transcripts are dependent on TBP binding. Importantly, transcription from +1 was unaffected by the TGTAAA mutation (compare lanes 1 and 2). A second mutation was made that changed TATATAA to TATATAG to make the *B.oleracea* sequence match the corresponding *A.thaliana* initiator sequence (Fig. 2). Interestingly, the A to G change altered the ratio of +1 and +29 transcripts in favor of +1 (Fig. 4, lane 3), suggesting that making the initiator region more *Arabidopsis*-like improves recognition by pol I in *Arabidopsis* cells. However, +29 transcription was not eliminated, suggesting that other promoter domains must also be involved in programming accurate promoter recognition.

The results of Figure 4 suggested that +29 transcripts were dependent on the TATATAA sequence in the wild-type promoter, possibly due to the need for TBP to interact directly with this sequence. This assumption could be tested by suppressor analysis. In yeast, a three amino acid mutation in TBP allows recognition of the TGTAAA mutant TATA box (51). Analogous suppressor TBPs have been engineered within both *A.thaliana* TBP genes by altering the codons for the same three amino acids and were shown to compensate for TATA mutations in promoters recognized by both polymerases II and III in plants (41). Expression vectors for the two *Arabidopsis* suppressor TBP genes and the two wild-type TBP genes were generously provided by Heard and Filipowicz (41). Co-transfection of a suppressor TBP gene with the rRNA gene promoter bearing the TGTAAA mutation restored transcription from +29 (Fig. 5). Suppressor versions of both TBP genes were equally active (compare lanes 2 and 4). In contrast, co-transfection of the wild-type TBP genes failed to suppress the promoter mutation (lanes 1 and 3). Importantly, +1 transcription was unaffected by expression of the TBP proteins.

The data of Figure 5 suggested that +29 transcripts are the consequence of TBP–TATA interactions within the conserved +1 region. Though RNA polymerase II was implicated, certain genes transcribed by RNA polymerase III have TATA boxes that interact with TBP (6). As mentioned previously, α-amanitin is not useful for inactivating RNA polymerases II and III in intact plant

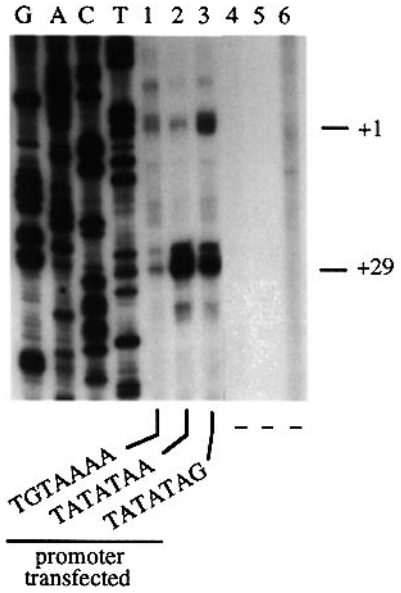

Figure 4. TATA box mutations known to disrupt TBP-dependent RNA polymerase II and III transcription reduce +29 transcripts. The wild-type *B.oleracea* rRNA gene promoter directs transcripts initiating weakly at +1 and strongly from +29 in transfected *Arabidopsis* protoplasts (lane 2). Changing the +1 region from TATATA to TGTAAA reduced +29 transcripts >10-fold (lane 1). Changing TATATAA to TATATAG (A+2G mutant) to resemble the start site of the *A.thaliana* promoter improved +1 transcription ~5-fold (lane 3). No transcripts were detected in mock-transfected cells using the construct-specific probes (lanes 4–6, respectively). The sequencing ladder was generated from the wild-type promoter and a primer 5′-end labeled at the *Xba*I site (as in Fig. 3C).

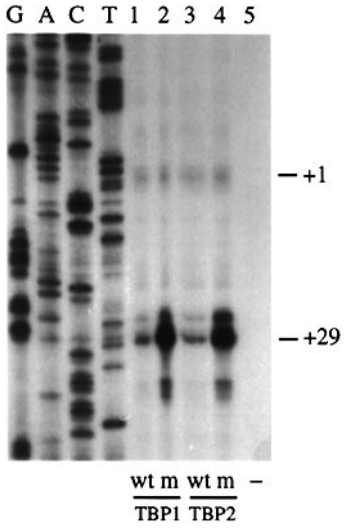

Figure 5. Suppressor TATA-binding proteins capable of binding TGTAAA restore +29 transcription. The TGTAAA promoter mutant was co-transfected into *Arabidopsis* protoplasts with a vector expressing *Arabidopsis* TATA-binding protein genes (TBP1 or TBP2) from the strong cauliflower mosaic virus 35S promoter (41). Both wild-type (wt) TBP and suppressor TBP (m) proteins were tested; these differ by three amino acids within the coding regions but are otherwise identical. Both suppressor TBPs rescued the TGTAAA mutation, restoring +29 transcripts (lanes 2 and 4). Co-expression of wild-type TBPs had no effect (lanes 1 and 3). No differences in the activity of TBP1 and TBP2 were detected (compare lanes 1 and 3 with lanes 2 and 4), suggesting that they are functionally equivalent (see also ref. 41). Note that transcription from +1 is unaffected by co-expression of wild-type or suppressor TBPs.

cells. Therefore, we exploited the fact that RNA polymerase II transcripts can be efficiently translated due to their 5′ 7-methyl-guanylate cap structures recognized by initiation factor complexes (52). In contrast, neither polymerase I nor polymerase III transcripts are translated at significant levels. A luciferase reporter gene was cloned downstream of a wild-type *B.oleracea* rRNA gene promoter or a promoter bearing the TGTAAA mutation and these constructs were tested by transient expression (Fig. 6A). The wild-type *B.oleracea* promoter directed high levels of luciferase expression (Fig. 6B; results of two independent trials are shown). Mutating TATATA to TGTAAA reduced luciferase activity 25–35-fold (Fig. 6B), consistent with the reduction in +29 transcripts (Fig. 4, compare lanes 1 and 2). Furthermore, co-transfection of suppressor TBP1 with the promoter mutant restored luciferase activity (Fig. 6C, compare to luciferase levels from the TGTAAA mutant promoter in Fig. 6B), whereas overexpression of wild-type TBP1 was 6–8-fold less effective. The restoration of luciferase activity with the suppressor TBP agreed with the restoration of +29 transcripts (Fig. 5, lanes 2 and 4).

It is noteworthy that the amount of luciferase expressed from the wild-type *Brassica* rRNA gene promoter was only 10-fold lower than what we measured in the same experiments using a control construct (42) which has luciferase expressed from the cauliflower mosaic virus (CaMV) 35S promoter with an extra copy of its enhancer, among the strongest known promoters for dicotyledenous plants (data not shown). For comparison, the 35S promoter is 50 times stronger than the nopaline synthase promoter and 10 times stronger than the CaMV 19S promoter, two pol II promoters well-characterized in plants (53). Therefore, the *B.oleracea* rRNA gene sequences constitute a remarkably strong promoter for reporter gene expression, similar in strength to the CaMV 19S promoter, and most consistent with expression by pol II. Also consistent with pol II transcription are the results of the mutation-suppressor analyses suggesting that polymerase II transcription is programmed by direct binding of TBP to the consensus TATA box at the RNA polymerase I initiation site. The latter results suggest that the TATA sequence of plant rRNA gene promoters is capable of interacting with TBP, but the differential response of the polymerase I and II systems to the A+2G and TGTAAA mutations (Fig. 4) suggests that TBP does not bind the start site directly during RNA polymerase I transcription complex assembly.

DISCUSSION

Our study shows for the first time *in vivo* that an rRNA gene promoter of one plant species is not correctly recognized in another species unless the two species are closely related. In general, this is the expected result by analogy to animal systems. What is different from reported animal systems is that an alien plant promoter is not simply inert, but tends to program transcription initiation from an altered site ~30 nucleotides downstream of the expected pol I transcription start site. We investigated the aberrant transcription to see if an alternative start site for RNA polymerase I was involved because this might tell us more about the sequences required for pol I transcription. Instead, we found that the alternative transcription start sites were due to initiation by another polymerase, most likely RNA polymerase II. A brief report of a crude cell extract from tobacco that supports pol I

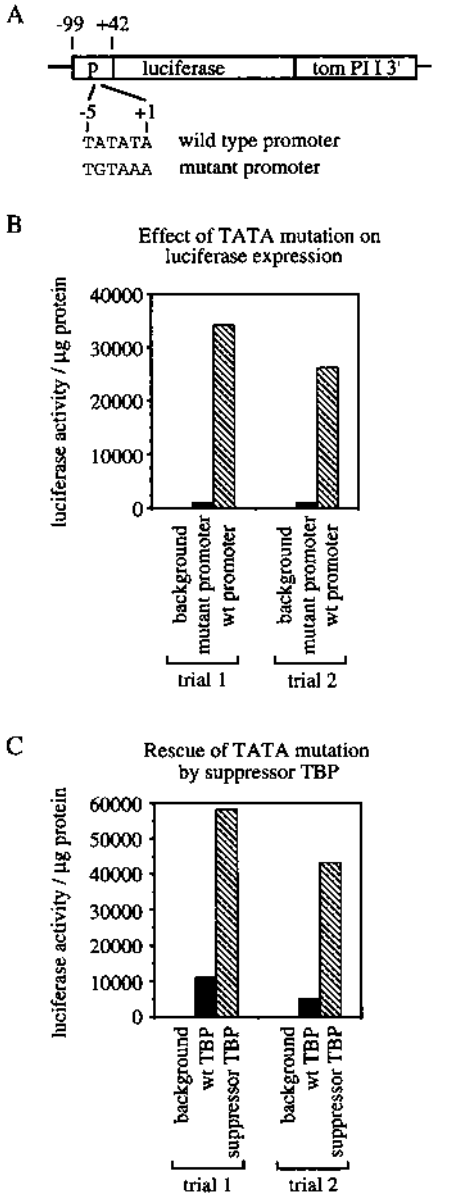

Figure 6. Transcripts initiating at +29 can be translated, suggesting transcription by RNA polymerase II. Wild-type (TATATAA) or mutant (TGTAAA) *B.oleracea* rRNA gene promoters were fused to a firefly luciferase reporter gene construct (**A**) and transfected into *Arabidopsis* protoplasts. Luciferase activity in two batches of protoplasts (trials 1 and 2) was determined 21 h following transfection (B) and (C). (**B**) The wild-type promoter (TATATAA) expressed high levels of luciferase whereas the mutant promoter (TGTAAA) did not. (**C**) Luciferase expression from the mutant promoter was rescued by co-transfecting suppressor TBP1, and partially rescued by co-expression of wild-type TBP1. The data of (B) and (C) are derived from the same two trials, the data are separated for the sake of clarity.

transcription *in vitro* showed no transcription signal with a bean promoter, also suggesting species specificity of plant rRNA gene transcription (54). These authors did not report aberrant start sites, but the capacity of the extract to program pol II transcription was not discussed.

One can argue that there is no *in vivo* significance to species-specificity or polymerase-specificity switching because these phenomena are due to introducing genes into species that do not interbreed or are the consequences of drastic promoter mutations, as in studies of snRNA genes. However, the insights these studies provide into promoter architecture and function are often important. Our results confirm and clarify the prediction that the TATA sequence at the pol I start site of plant rRNA genes can be a binding site for TBP (23,40). However, direct TBP–TATA interactions program pol II transcription, not pol I which appears unaffected by mutations that disrupt TBP binding and TATA-dependent luciferase expression. In hindsight, the experiments of Haass *et al*. are consistent with our results. Their study showed that maize TBP could bind the maize rRNA gene promoter, but only in the presence of yeast TFIIA (40), a transcription factor involved in recruiting the TBP-containing protein complex (TFIID) in the assembly of pol II pre-initiation complexes (55). Our results are also consistent with studies in other systems. *Acanthamoeba* has a TATA-like element at the rRNA transcription start site and requires a TBP-containing factor for pol I transcription (39). *Acanthamoeba* pol II and pol III transcription *in vitro* are sensitive to inhibition by a TATA-box containing oligonucleotide, but pol I transcription is resistant, suggesting that TBP does not directly interact with the TATA element of the rRNA initiator region (39). Interestingly, *Acanthamoeba* TBP can be cross-linked to promoter sequences near –40 (56), suggesting that lack of interaction with a TATA sequence does not preclude the possibility of interactions elsewhere. In mammals it appears that proteins tightly bound to TBP within the TIF-IB (SL1) complex, but not TBP itself, are in contact with the DNA and can be cross-linked to the promoter (38).

Our study also provides additional evidence for a functional role of the TATA-containing initiator element in plant rRNA genes. As shown in Figure 4 (lane 3) a single point mutation that makes the *Brassica* initiator a better match to that of *Arabidopsis* improves promoter recognition in favor of the authentic +1 start site. This demonstrates an involvement of the initiator region in promoter recognition. A parallel is that the 'core promoter' proximal to the transcription start site is also involved in species-specific pol I transcription in animals (14). However, the function of the initiator region in plant rRNA genes remains unknown. If not a binding site for TBP, what might it do? One possibility is that the TATA sequence is conserved because it is easily melted or has some other important structural characteristic. It may not be a direct binding site for a transcription factor.

Several control experiments done in the course of this study are worth mentioning, though the data have not been shown. We considered the possibility that aberrant promoter recognition in transient assays could result from excessive concentrations of template DNA transfected into plant cells, overwhelming the pol I transcription machinery. Several observations argue against this possibility. First, a transfected *Arabidopsis* promoter is correctly recognized in *Arabidopsis* cells, suggesting that the system has not been overwhelmed. Second, the number of transfected plasmids per cell approximates the number of endogenous rRNA genes using our standard conditions (23). We do not know how many transfected templates reach the nucleus, but their relatively weak expression signals compared to endogenous genes suggests that a small fraction are transcribed.

Another possibility is that differences in the promoter constructs might contribute to the apparent switch in polymerase specificity, possibly due to cryptic enhancers in the plasmid sequences. However, *Arabidopsis* promoter constructs containing essentially

complete spacers or minimal promoter sequences (e.g. –55 to +6) program transcription from the authentic +1 start site (23). Therefore, moving a hypothetical pol II enhancer in the plasmid closer to the promoter did not stimulate pol II transcription, nor did deletion of spacer sequences preclude pol I transcription. We also did the reciprocal experiment of Figure 4, lane 3; changing the G at +2 of the *Arabidopsis* initiator region to an A to resemble the initiators of tomato and *Brassica* in case this would allow a cryptic enhancer to act synergistically with a different TATA element. This change weakened the +1 signal slightly and led to a slightly increased intensity of the minor S1 protection products in the vicinity of +31 (Fig. 3A), suggesting that even an *Arabidopsis* promoter can be used (inefficiently) by pol II in *Arabidopsis* cells. However, the *Arabidopsis* G+2A mutation did not result in strong +30 transcription similar to tomato or *Brassica*. Furthermore, our *Brassica* templates were designed to resemble our best characterized *A.thaliana* templates, including their 5′ and 3′ boundaries and orientation within the pBluescript vector (see diagrams in Fig. 3A and C). Therefore, the differences in transcription from *Brassica* and *Arabidopsis* promoters is unlikely to be due to plasmid effects.

Another possible explanation for alien promoters being recognized by an alternative polymerase system might be that upon transient expression, foreign DNA becomes localized to a different nuclear compartment than does homologous DNA. A common misconception is that rRNA genes can only be transcribed within the nucleolus. A prediction is that only a transfected minigene localized to the nucleolus could be transcribed by pol I but a minigene elsewhere in the nucleus might only be accessible to pol II or pol III. Evidence against this notion is that in *Drosphila* (57), yeast (58) and plants (Doelling and Pikaard, unpublished) an rRNA transgene integrated at ectopic locations can still be transcribed by pol I. In fact, in *Drosophila*, ectopic rRNA genes assemble a mini-nucleolus, showing that the nucleolus is a consequence of rRNA gene expression and not a prerequisite. Second, reporter genes can be transcribed by pol II within the nucleolus of yeast when integrated among the rRNA genes (58–60). Therefore polymerase systems do not appear to be partitioned from one another within the nucleus. The fact that the nucleolus is not separated by a membrane or known physical barrier is consistent with this view (61).

A final set of controls were to test *B.oleracea* promoters with different amounts of spacer sequences attached to see if this suppressed transcription from +29, or contributed to pol I specificity and initiation at +1. Constructs with sequences extending upstream of the promoter to –2782 (essentially complete intergenic spacers), –518 (the construct used in Figs 3–5), –68 or –39 showed no significant differences in their expression from +1 and +29. We have not attempted to further define the sequences required for +29 transcription, but the fact that the –39 deletion programs transcription by both pol I and pol II suggests substantial overlap between the two promoter activities.

A case of rRNA gene promoter polymerase specificity switching with possible *in vivo* relevance has recently been reported in yeast. Nomura's lab initially showed that functional rRNA can be made by RNA polymerase II. They showed that a yeast strain with a lethal defect in the polymerase I enzyme could survive if rRNA was transcribed from a galactose-inducible promoter on a multi-copy plasmid (62). This suggested that RNA polymerase I was not strictly required in order to make functional rRNA. Recently, Conrad-Webb and Butow (63) studied respiratory deficient yeast 'petite' mutants that have lost their mitochondrial genomes and maintain a substantial portion of their nuclear rRNA genes as autonomously replicating extra-chromosomal circles. These episomal circles arise via recombination within the rDNA array and are maintained by virtue of a replication origin in the intergenic spacer of every rRNA gene. In the episomal state, they showed that the rRNA gene promoter was recognized by RNA polymerase II, initiating transcription from the same site as RNA polymerase I. Furthermore, petite mutants that were also defective for RNA polymerase I (same pol I mutant used by Nomura's lab) were viable under certain conditions, presumably surviving the pol I defect using pol II to transcribe episomal rRNA gene circles. Polymerase-switching has not been documented among yeast rRNA genes located at their normal chromosomal locations. Nonetheless, Conrad-Webb and Butow suggest that an RNA polymerase II promoter that overlaps the polymerase I promoter could provide cells with additional regulatory possibilities in the production of rRNA. If so, it is intriguing to speculate that plant rRNA genes may share this regulatory plasticity.

ACKNOWLEDGEMENTS

We thank David Heard and Witold Filipowicz (Friedrich Miescher Institute, Basel) for providing the TBP expression vectors, Wayne Barnes (Washington University) for the gift of the luciferase construct pWB 216, and Peter Palukaitis (Cornell University) for providing the tomato promoter clone pKU235. We thank Heather Conrad-Webb (Texas Women's University) for a pre-print of her paper. We are grateful to Robert Landick (University of Wisconsin) and David Ho (Washington University) for suggesting improvements to the manuscript. This work was supported by grants to C.S.P. from the National Science Foundation (DMB 9018428) and the USDA (NRICGP-Genetic Mechanisms Program 94-37301-0658). J.H.D. was supported, in part, by a Monsanto Pre-Doctoral Fellowship in Plant Biology.

REFERENCES

1 Reeder,R.H. (1992) In McKnight,S.L. and Yamamoto,K.R. (eds), *Transcriptional Regulation.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Vol. I, pp. 315–347.
2 Paule,M.R. (1994) In Conaway,R.C. and Conaway,J.W. (eds), *Transcription: Mechanisms and Regulation.* Raven Press, NY, Vol 3, pp. 83–106.
3 Moss,T. and Stefanovsky,V.Y. (1995) *Prog. Nucl. Acids Res. Mol. Biol.*, **50**, 25–66.
4 Serizawa,H., Conaway,J.W. and Conaway,R.C. (1994) In Conaway,R.C. and Conaway,J.W. (eds), *Transcription: Mechanisms and Regulation.* Raven Press, New York, Vol. 3, pp. 27–44.
5 Zawel,L. and Reinberg,D. (1993) *Prog. Nucleic Acids Res. & Mol. Biol.*, **44**, 67–108.
6 Lobo,S.M. and Hernandez,N.T. (1994) In Conaway,R.C. and Conaway,J.W. (eds), *Transcription: Mechanisms and Regulation.* Raven Press, New York, Vol. **3**, pp. 127–160.
7 Kassavetis,G.A., Bardeleben,C., Bartholomew,B., Braun,B.R., Joazeiro,C.A.P., Pisano,M. and Geiduschek,E.P. (1994) In Conaway,R.C. and Conaway,J.W. (eds), *Transcription: Mechanisms and Regulation.* Raven Press, New York, Vol. **3**, pp. 107–126.
8 Filipowicz,W., Kiss,T., Marshallsay,C. and Waibel,F. (1990) *Mol. Biol. Rep.*, **14**, 125–129.
9 Reeder,R.H. (1984) *Cell*, **38**, 349–351.
10 Reeder,R.H. (1989) *Curr. Opin. Cell Biol.*, **1**, 466–474.
11 Grummt,I., Roth,E. and Paule,M.R. (1982) *Nature*, **296**, 173–174.
12 Miesfeld,R. and Arnheim,N. (1984) *Mol. Cell. Biol.*, **4**, 221–227.
13 Mishima,Y., Financsek,I., Kominami,R. and Muramatsu,M. (1982) *Nucleic Acids Res.*, **10**, 6659–6670.

14 Safrany,G., Tanaka,N., Kishimoto,T., Ishikawa,Y., Kato,H., Kominami,R. and Muramatsu,M. (1989) *Mol. Cell. Biol.*, **9**, 349–353.
15 Pape,L.K., Windle,J.J. and Sollner-Webb,B. (1990) *Genes Dev.*, **4**, 52–62.
16 Learned,R.M., Cordes,S. and Tjian,R. (1985) *Mol. Cell. Biol.*, **5**, 1358–1369.
17 Bell,S.P., Jantzen,H.M. and Tjian,R. (1990) *Genes Dev.*, **4**, 943–954.
18 Schnapp,A., Rosenbauer,H. and Grummt,I. (1991) *Mol. Cell. Biochem.*, **104**, 137–147.
19 Waibel,F. and Filipowicz,W. (1990) *Mol. Biol. Rep.*, **14**, 149.
20 Lobo,S.M. and Hernandez,N. (1989) *Cell*, **58**, 55–67.
21 Lobo,S.M., Lister,J., Sullivan,M.L. and Hernandez,N. (1991) *Genes Dev.*, **5**, 1477–1489.
22 Doelling,J.H., Gaudino,R.J. and Pikaard,C.S. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 7528–7532.
23 Doelling,J.H. and Pikaard,C.S. (1995) *Plant J.*, **8**, 683–692.
24 Barker,R.F., Harberd,N.P., Jarvis,M.G. and Flavell,R.B. (1988) *J. Mol. Biol.*, **201**, 1–17.
25 Delcasso,T.D., Grellet,F., Panabieres,F., Ananiev,E.D. and Delseny,M. (1988) *Eur. J. Biochem.*, **172**, 767–776.
26 Perry,K.L. and Palukaitis,P. (1990) *Mol. Gen. Genet.*, **221**, 102–112.
27 Piller,K.J., Baerson,S.R., Polans,N.O. and Kaufman,L.S. (1990) *Nucleic Acids Res.*, **18**, 3135–3145.
28 Toloczyki,C. and Feix,G. (1986) *Nucleic Acids Res.*, **14**, 4969–4986.
29 Torres,R.A., Zentgraf,U. and Hemleben,V. (1989) Z. *Naturforsch.*, **44**, 1029–1034.
30 Zentgraf,U., Ganal,M. and Hemleben,V. (1990) *Plant Mol. Biol.*, **15**, 465–474.
31 Vincentz,M. and Flavell,R.B. (1989) *Plant Cell*, **1**, 579–589.
32 Gerstner,J., Schiebel,K., VonWaldburg,G. and Hemleben,V. (1988) *Genome*, **30**, 723–733.
33 McMullen,M.D., Hunter,B., Phillips,R.L. and Rubenstein,J. (1986) *Nucleic Acids Res.*, **14**, 4953–4968.
34 Comai,L., Naolo,T. and Tjian,R. (1992) *Cell*, **68**, 965–979.
35 Cormack,B.P. and Struhl,K. (1992) *Cell*, **69**, 685–696.
36 Schultz,M.C., Reeder,R.H. and Hahn,S. (1992) *Cell*, **69**, 697–702.
37 Rudloff,U., Eberhard,D. and Grummt,I. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 8229–8233.
38 Rudloff,U., Eberhard,D., Tora,L., Stunnenberg,H. and Grummt,I. (1994) *EMBO J.*, **13**, 2611–2616.
39 Radebaugh,C.A., Matthews,J.L., Geiss,G.K., Liu,F., Wong,J., Bateman,E., Camier,S., Sentenac,A. and Paule,M.R. (1994) *Mol. Cell. Biol.*, **14**, 597–605.
40 Haass,M., Griess,E., Goddemeier,M., Egly,J.-M. and Feix,G. (1994) *Plant Sci.*, **100**, 187–194.
41 Heard,D.J., Kiss,T. and Filipowicz,W. (1993) *EMBO J.*, **9**, 3519–3528.
42 Barnes,W.M. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9183–9187.
43 Doelling,J.H. and Pikaard,C.S. (1993) *Plant Cell Rep.*, **12**, 241–244.
44 Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
45 Berk,A.J. and Sharp,P.A. (1977) *Cell*, **12**, 721–732.
46 Bennett,R.I. and Smith,A.G. (1991) *Plant Mol. Biol.*, **16**, 1095–1098.
47 Tremousaygue,D., Laudie,M., Grellet,F. and Delseny,M. (1992) *Plant Mol. Biol.*, **18**, 1013–1018.
48 Wilkinson,J. and Sollner-Webb,B. (1982) *J. Biol. Chem.*, **257**, 14375–14383.
49 Breathnach,R. and Chambon,P. (1981) *Annu. Rev. Biochem.*, **50**, 349–383.
50 Wobbe,C.R. and Struhl,K. (1990) *Mol. Cell. Biol.*, **10**, 3859–3867.
51 Strubin,M. and Struhl,K. (1992) *Cell*, **68**, 721–730.
52 Rhoads,R.E. (1988) *Trends Biochem. Sci.*, **13**, 52–56.
53 Rogers,S.G., Klee,H.J., Horsch,R.B. and Fraley,R.T. (1987) *Methods Enzymol.*, **153**, 253–277.
54 Fan,H., Yakura,K., Miyanishi,M., Sugita,M. and Sugiura,M. (1995) *Plant J.*, **8**, 295–298.
55 Buratowski,S., Hahn,S., Guarente,L. and Sharp,P. (1989) *Cell*, **56**, 549–561.
56 Gong,X., Radebaugh,C.A., Geiss,G.K., Simon,M.N. and Paule,M.R. (1995) *Mol. Cell. Biol.*, **15**, 4956–4963.
57 Karpen,G.H., Schaefer,J.E. and Laird,C.D. (1988) *Genes Dev.*, **2**, 1745–1763.
58 Johnson,S.P. and Warner,J.R. (1989) *Mol. Cell. Biol.*, **9**, 4986–4993.
59 Szostak,J.W. and Wu,R. (1980) *Nature*, **284**, 426–430.
60 Petes,T.D. (1980) *Cell*, **19**, 765–774.
61 Scheer,U. and Weisenberger,D. (1994) *Curr. Opin. Cell Biol.*, **6**, 354–359.
62 Nogi,Y., Yano,R. and Nomura,M. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 3962–3966.
63 Conrad-Webb,H. and Butow,R.A. (1995) *Mol. Cell. Biol.*, **15**, 2420–2428.