Identification of Previously Unrecognized Common Elements in Eukaryotic Promoters

A RIBOSOMAL RNA GENE INITIATOR ELEMENT FOR RNA POLYMERASE I

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A new ribosomal RNA promoter element with a functional role similar to the RNA polymerase II initiator (Inr) was identified. This sequence, which we dub the ribosomal Inr (rInr) is unusually conserved, even in normally divergent RNA polymerase I promoters. It functions in the recruitment of the fundamental, TATA-binding protein (TBP)-containing transcription factor, TIF-IB. All upstream elements of the exceptionally strong Acanthamoeba castellanii ribosomal RNA core promoter, to within 6 base pairs of the transcription initiation site (tis), can be deleted without loss of specific transcription initiation. Thus, the A. castellanii promoter can function in a manner similar to RNA polymerase II TATA-less promoters. Sequence-specific photo-cross-linking localizes a 96-kDa subunit of TIF-IB and the second largest RNA polymerase I subunit (Δ123) to the rInr sequence. Also photo-cross-links when polymerase is stalled at +7.

Because promoters for some small nuclear RNA genes switch between polymerase II and III as a result of simple sequence/spacing alterations, promoters for eukaryotic RNA polymerases II and III are considered more similar to each other than to the RNA polymerase I promoter (reviewed in Ref. 1). Most polymerase II promoters contain a TATA box, or an initiator element (Inr),1 or both (2). The Inr surrounds the transcription initiation site (tis) and the TATA box is upstream about 30 base pairs. The TATA box is the specific binding site for TATA-binding protein (TBP), the subunit common to the fundamental transcription factors (3) for all polymerases (4). On polymerase II genes with TATA boxes, TBP alone can function in a manner similar to the RNA polymerase II initiator; tis, transcription initiation site; TIF-IB, transcription factor IB; TFIID, transcription factor IID; TBP, TATA-binding protein; TAF1, and TAF150, TBP-associated factors for RNA polymerase I and II, respectively.

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Ribosomal RNA Gene Initiator Element

The rRNA promoter of A. castellanii can be deleted to −12 or −6 without complete loss of specific transcriptional activity. A, 3.5, 75, or 150 ng of DNA fragments containing the wild type or a 5' deletion to −12 or −6 (relative to the tis) were used in a runoff transcription assay. Specific transcripts of the expected 207 nucleotide length are labeled. B, primer extension analysis of the RNA products from the runoff assay. A T3 RNA polymerase transcript (lane 1) containing rRNA gene sequence from −55 to +19 was used as a control for premature termination by reverse transcriptase. The transcripts from the reactions containing the wild type (W), −12, or −6 deletion templates with TIF-IB (lanes 2, 4, and 6) all gave products that correspond to the correct initiation site. The T3 control transcript (lane 1) and the reactions without TIF-IB (lanes 3, 5, and 7) did not contain any primer extension products in this region.

Promoter. Previous studies (15, 19) identified the region from −31 to +8 as the minimal sequence necessary to obtain specific transcription initiation. Deletion beyond −31 resulted in a loss of transcription runoff RNA detectable by autoradiography. However, phosphorimaging technology offers an approach which is capable of detecting isotopically labeled products at a significantly lower level. When the same deleted templates used previously were assayed using storage phosphor screens, transcription of genes deleted from their 5' end to −12 or even to −6 was detected (Fig. 1A, lanes 2, 3, 5, and 6). The wild type sequence in these templates ends at +19, with the pBR322 sequence beyond. The efficiency of these deleted templates is significantly lower than the wild type template; for comparison, the amount of wild type template was decreased to 5% that used in the deleted template reactions (lane 1). Such weak transcripts would not have been detected using autoradiography and the correct exposure for the template containing the full promoter.

These transcripts are correctly initiated. 1) They are dependent upon TIF-IB (lanes 4 and 7), which is required for RNA polymerase I to recognize the promoter (19, 20). 2) Restriction enzyme fragment templates produce correct runoff transcripts of predicted lengths (data not shown). 3) Both wild type and deletion mutant genes exhibit identical 5' ends in primer extension assays (Fig. 1B, lanes 2, 4, and 6). The primer used in these assays is complementary to the pBR322 vector sequence, so the assay is not detecting any contaminating rRNA which might be present in the protein preparations. The latter is extremely unlikely because these proteins have each been purified through multiple chromatographic columns. Multiple primer extension products have been observed before for in vitro rRNA transcripts in this and other systems and result either from artifacts of the method or heterogeneity of tis selection (20). They do not arise as artifacts because of secondary structure in a readthrough transcript originating upstream of tis. To test this notion, a T3 RNA polymerase transcript which originates upstream of the normal tis, thus having the same sequence as an RNA polymerase I readthrough product, was analyzed by primer extension. The T3 readthrough product does not produce any primer extension products in the region of the tis (lane 1), but does produce a strong full-length extension product (data not shown).

The minimal promoter detected in these assays is much smaller than in our previous study (15). Remarkably, both −12 and −6 are outside the region footprinted by the fundamental transcription initiation factor, TIF-IB, from A. castellanii (21, 22). However, this region does exhibit enhanced bands in some footprinting experiments, and point mutations in this region affect transcription efficiency (11).

To map the TIF-IB and RNA polymerase I subunits along the template, we subjected template-committed complexes to site-specific photo-cross-linking by incorporation of 5'-[N-(p-azidobenzoyl)-3-aminooallyl]-deoxyuridine monophosphate into the template (18). 5'-[N-(p-azidobenzoyl)-3-aminooallyl]-DUMP was incorporated into positions −1, −3, −5, and −7 in place of dTMP. In 9 separate experiments with several preparations of TIF-IB at various stages of purity, a specific 98-kDa protein photo-cross-linked weakly to the putative rInr sequence (Fig. 2A, lane 3). This protein was identified as TAF996 of TIF-IB based upon previously published criteria: TAF9,145 yields cross-linked bands of 153–154 kDa, TAF9,99 yields cross-linked bands of 109–110 kDa, and TAF9,66 yields 96.8–98-kDa, TAF9,91 yields 92.5–93-kDa, and TBP yields 39-kDa bands because of the short covalently linked DNA tag. (See also Ref. 17 for a complete discussion of assignments.) The experiment shown in Fig. 2A used TIF-IB in which no proteins other than TBP and the four TAF9s could be identified in silver-stained polyacrylamide gels. However, even in impure preparations of TIF-IB in which the TAF9s are not easily identified in the mass of contaminating proteins, no other proteins photo-cross-link to this position, indicating the specificity of this photo-cross-linking technique.

When RNA polymerase I is added to the committed complex, a protein of approximately 135 kDa photo-cross-links (Fig. 2A, lane 4). To verify that this is a component of RNA polymerase I, photo-cross-linking was performed across the peak of RNA polymerase I from a glycerol gradient using the same affinity probe (Fig. 2, B and C). The amount of photo-cross-linked 135-kDa product (Fig. 2C) correlates closely with RNA polymerase I activity determined in a specific runoff assay (Fig. 2B). The homogeneous RNA polymerase I used in this experiment only contains the two large subunits, 185 and 133 kDa, in this size range (14, 23). On this basis, the photo-cross-linked subunit was identified as the second largest subunit, A233, of RNA polymerase I.

When RNA polymerase I is bound to the promoter, the efficiency of photo-cross-linking of TAF9,96 is significantly increased (lane 4). Thus, the binding of polymerase results in closer proximity of TAF9,66 to the template. Based on this and other data, we propose that polymerase stabilizes TIF-IB binding and suggests an interaction between TAF9,96 and po-

rRNA which might be present in the protein preparations. The latter is extremely unlikely because these proteins have each been purified through multiple chromatographic columns. Multiple primer extension products have been observed before for in vitro rRNA transcripts in this and other systems and result either from artifacts of the method or heterogeneity of tis selection (20). They do not arise as artifacts because of secondary structure in a readthrough transcript originating upstream of tis. To test this notion, a T3 RNA polymerase transcript which originates upstream of the normal tis, thus having the same sequence as an RNA polymerase I readthrough product, was analyzed by primer extension. The T3 readthrough product does not produce any primer extension products in the region of the tis (lane 1), but does produce a strong full-length extension product (data not shown).

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3 C. Radebaugh, unpublished data.
polymerase I. Perhaps this interaction induces a conformational change in TIF-IB making it more accessible to the photo-cross-linking reagent. Following initiation and stalling of the polymerase at +7 by addition of ATP and GTP, but omission of CTP, both the second largest (A133) and the largest (A185) subunits of polymerase I photo-cross-linked (lane 5). Under single-round transcription conditions, once polymerase has cleared the promoter following addition of all four NTPs, the 8.0-Å probe is most efficient at cross-linking TAF196 and A133 far more strongly than any of the other probes. In contrast, A185 is cross-linked about equally by the 8.0- and 12.8-Å probes, but the 12.3-Å probe and probes longer than 12.8 Å are less efficient at photo-cross-linking this subunit. These probes also differ in the hydrophobicity of the linker arm, which may account for the apparent anomalous length dependence for the A185 subunit.

We propose ribosomal RNA promoters contain a sequence which is functionally similar to the Inr element found in RNA polymerase II promoters, the rInr, and defines yet another class of Inr (2). Importantly, a similar small minimal promoter was found when large amounts of truncated (to −9) X. laevis rRNA genes were injected into oocytes (9), also demonstrating functionality of an Inr-like element in a non-polymerase II promoter. In further support of this notion, we note that all eukaryotic rRNA promoters have an element near the tis whose mutation alters promoter activity. We previously described a remarkably conserved sequence which is present in a large number of rRNA promoters (8): a/gr/a/t(A/Gt/Gt/T/A)ATAGGGGA/ggAn, where the underlined A is the tis. The conservation of this sequence in rRNA genes is significantly stronger than the consensus sequence for the polymerase II Inr (25).

Point mutations of the A. castellanii rInr affect transcription (11). Similarly, point mutants of the homologous rInr sequence in A. thaliana affect transcriptional activity (10). However, even deletion of the A. castellanii rInr in the context of the full-length promoter does not affect subsequent RNA polymerase I binding (20). Thus, as is the case for Inr-less polymerase II promoters, TAF196 can be tethered to the tis by upstream promoter elements just as the Inr can tether TBP to TATA-less promoters (6). This reveals a functional similarity between promoters for RNA polymerases II and I which has previously gone unappreciated.

It has been argued that RNA polymerases II and III are more similar to each other because the sequences of their largest subunits are evolutionarily more similar to each other than to polymerase I (26), and several snRNA gene promoters can be switched between polymerase II and III by rather simple deletions or promoter element spacing changes (reviewed in Refs. 1

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4 J. Persinger and B. Bartholomew, manuscript in preparation.
and 27). However, this ignores the fact that polymerases I and III share more subunits in common than polymerases II and III (in yeast, the AC40 and AC19, in addition to the five subunits common to all three enzymes (28)). The demonstration here that the structure of the promoters for polymerases I and II might be more similar than previously recognized suggests this supposition should be reexamined.

REFERENCES
1. Lobo, S. M., and Hernandez, N. T. (1994) in Transcription: Mechanisms and Regulation (Conaway, R. C., and Conaway, J. W., eds) pp. 127–159, Raven Press, Ltd., New York
2. Weis, L., and Reinberg, D. (1992) FASEB J. 6, 3300–3309
3. Paule, M. R. (1990) Nature 344, 819–820
4. Hernandez, N. (1993) Genes Dev. 7, 1291–1308
5. Martinez, E., Chiang, C.-M., Ge, H., and Roeder, R. G. (1994) EMBO J. 13, 3115–3126
6. Verrijzer, C. P., Yokoorni, K., Chen, J., and Tjian, R. (1994) Science 264, 933–941
7. Kaufmann, J., Verrijzer, C. P., Shao, J., and Smale, S. T. (1996) Genes Dev. 10, 873–886
8. Perna, P. J., Harris, G. H., Iida, C. T., Kownin, P., Bugren, S., and Paule, M. R. (1992) Gene Expr. 2, 71–78
9. Windle, J. J., and Sollner-Webb, B. (1986) Mol. Cell. Biol. 6, 1228–1234
10. Doelling, J. H., and Pikaard, C. S. (1995) Plant J. 8, 683–692
11. Kownin, P., Bateman, E., and Paule, M. R. (1988) Mol. Cell. Biol. 8, 747–753
12. Iida, C. T., and Paule, M. R. (1992) Nucl. Acids Res. 20, 3211–3221
13. 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
14. Spindler, S., Duester, G. L., D'Alessio, J. M., and Paule, M. R. (1978) J. Biol. Chem. 253, 4669–4675
15. Kownin, P., Iida, C. T., Brown-Shimer, S., and Paule, M. R. (1985) Nucl. Acids Res. 13, 6237–6247
16. Paule, M. R., Iida, C. T., Perna, P. J., Harris, G. H., Shimer, S. L., and Kownin, P. (1984) Biochemistry 23, 4167–4172
17. Imboden, M. A., Matthews, J. L., Lofoquist, A. K., and Paule, M. R. (1992) J. Biol. Chem. 267, 24601–24610
18. Gong, X., Radebaugh, C. A., Geiss, G. K., Simon, M. N., and Paule, M. R. (1995) Mol. Cell. Biol. 15, 4956–4963
19. Iida, C. T., Kownin, P., and Paule, M. R. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1668–1672
20. Kownin, P., Bateman, E., and Paule, M. R. (1987) Cell 50, 693–699
21. Bateman, E., Iida, C. T., Kownin, P., and Paule, M. R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8004–8008
22. Bateman, E., and Paule, M. R. (1986) Cell 47, 445–450
23. D’Alessio, J. M., Perna, P. J., and Paule, M. R. (1979) J. Biol. Chem. 254, 11252–11257
24. Paule, M. R. (1994) in Transcription: Mechanism and Regulation (Conaway, R., and Conaway, J. eds) pp. 83–106, Raven Press, Ltd., New York
25. Zawel, L., and Reinberg, D. (1995) Annu. Rev. Biochem. 64, 533–561
26. Ménet, S., Saurin, W., and Sentenac, A. (1988) J. Biol. Chem. 263, 10048–10051
27. White, R. J. (1994) RNA Polymerase III Transcription, R. G. Landes Co., Austin
28. Sentenac, A., Riva, M., Thuriaux, P., Buhler, J.-M., Treich, L., Carles, C., Werner, M., Ruet, A., Huet, J., Mann, C., Chiannilkulchai, N., Stettler, S., and Mariotte, S. (1992) in Transcriptional regulation (McKnight, S. L., and Yamamoto, K. R. eds) pp. 27–54, Cold Spring Harbor Laboratory Press, New York