

RNA Polymerase I Holoenzyme-Promoter Interactions*

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Julio Saez-Vasquez‡ and Craig S. Pikaard§

From the Biology Department, Washington University, St. Louis, Missouri 63130

In plants and animals, RNA polymerase I (pol I) can be purified in a form that is self-sufficient for accurate rRNA gene promoter-dependent transcription and that has biochemical properties suggestive of a single complex, or holoenzyme. In this study, we examined the promoter binding properties of a highly purified *Brassica* pol I holoenzyme activity. DNase I footprinting revealed protection of the core promoter region from ~ -30 to $+20$, in good agreement with the boundaries of the minimal promoter defined by deletion analyses (-33 to $+6$). Using conventional polyacrylamide electrophoretic mobility shift assays (EMSA), protein-DNA complexes were mostly excluded from the gel. However, agarose EMSA revealed promoter-specific binding activity that co-purified with promoter-dependent transcription activity. Titration, time-course, and competition experiments revealed the formation or dissociation of a single protein-DNA complex. This protein-DNA complex could be labeled by incorporation of radioactive ribonucleotides into RNA in the presence of α -amanitin, suggesting that the polymerase I enzyme is part of the complex. Collectively, these results suggest that transcriptionally competent pol I holoenzymes can associate with rRNA gene promoters in a single DNA binding event.

Eukaryotes transcribe their nuclear genes using three RNA polymerase systems. RNA polymerase I (pol I)¹ transcribes the genes that encode the precursor of the three largest ribosomal RNAs (rRNAs) (1–4), RNA polymerase II transcribes protein-coding genes and most small nuclear RNAs (5–9), and RNA polymerase III transcribes tRNAs and other small RNAs including 5 S ribosomal RNA (4, 10). None of the three polymerases can recognize a gene promoter on its own. Instead, polymerase-specific transcription factors mediate promoter recognition (11).

Transcription factors for all three polymerase systems can be purified independent of other factors or the polymerase core enzyme. However, all three nuclear polymerases can also be isolated in a form in which they are pre-associated with most or all of their required transcription factors (12–18). The resulting protein complexes, known as polymerase holoenzymes, may be

the relevant entities that accomplish promoter recognition and transcription initiation in the cell given that they can accomplish promoter-dependent transcription *in vitro* (19).

Initial evidence that a pol I holoenzyme might exist came from analyses of a cell-free transcription system from broccoli (*Brassica oleracea*) (15). Subsequently, we purified an analogous holoenzyme activity from cultured *Xenopus laevis* cells (17). In both cases, a portion (less than 15%) of the pol I activity co-fractionates with the ability to carry out promoter-dependent transcription *in vitro* and can be purified to near homogeneity by sequential preparative anion exchange, cation exchange, gel filtration, analytical anion exchange, and DNA-affinity chromatography (15, 17). Single fractions from each column retain the ability to program transcription initiation from an rRNA gene promoter *in vitro*. The holoenzyme activities initiate transcription from the same start sites used *in vivo* (defined as $+1$), are insensitive to high concentrations of α -amanitin (a toxin that inhibits RNA polymerases II and III), and are sensitive to promoter mutations that disrupt rRNA gene transcription *in vivo* (15, 17). Thus the purified holoenzyme activities can account for the accuracy and promoter specificity observed in intact cells.

Pol I holoenzyme activities have also been isolated from mouse (16) and rat (18) by making use of cells expressing epitope-tagged pol I subunits to facilitate their capture. The rat pol I holoenzyme is self-sufficient for promoter-dependent transcription (18), like the *Brassica* and *Xenopus* holoenzymes, whereas the mouse holoenzyme is apparently depleted for one transcription factor, TIF-IC (16). Based on gel filtration chromatography and/or glycerol gradient sedimentation, the four pol I holoenzyme complexes described thus far are estimated to be ~ 2 MDa in mass (15–18). This corresponds to a mass approximately 3–4-fold greater than the mass of pol I core enzyme (the form of the enzyme that can synthesize RNA but cannot recognize a promoter), which consists of ~ 11 –14 subunits (20–22). Accordingly, when purified holoenzyme fractions are subjected to SDS-polyacrylamide gel electrophoresis and stained, at least 30 major polypeptides are revealed. Definitive identities for most of these proteins have not been established, but biochemical assays and/or Western blotting have revealed protein kinase, histone acetyltransferase, and topoisomerase activities (17, 18) as well as components of the DNA replication/repair machinery (18).

The identification of polymerase holoenzymes has significant implications for how transcription pre-initiation complexes can be assembled on gene promoters, predicting that transcriptionally competent, polymerase-containing complexes can associate with a promoter in a single DNA binding event. The alternative is that transcription factors interact with promoters sequentially before polymerase recruitment, thus building transcription pre-initiation complexes in a series of steps (23–27). These scenarios need not be mutually exclusive, and both could play roles in establishing transcription complexes and then accomplishing multiple rounds of transcription. Nonetheless, because step-by-step assembly path-

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§ To whom correspondence should be addressed: Biology Dept., Washington University, Campus Box 1137, One Brookings Dr., St. Louis, MO 63130. Tel.: 314-935-7569; Fax: 314-935-4432; E-mail: pikaard@biology.wustl.edu.

¹ The abbreviations used are: pol I, RNA polymerase I; EMSA, electrophoretic mobility shift assay.

ways are well supported by experimental data, a persistent worry has been that holoenzyme activity might actually be attributable to multiple activities that co-purify (through numerous columns) or co-immunoprecipitate by chance. In the study reported here, we examined the DNA binding properties of highly purified *Brassica* pol I holoenzyme fractions in an effort to determine if multiple promoter-transcription factor sub-complexes could be detected. We found that only a single protein-DNA complex could be detected when holoenzyme fractions were incubated with an rRNA gene promoter fragment that was fully functional as a template for accurate transcription initiation. Using the electrophoretic mobility shift assay (EMSA), the promoter-protein complexes were mostly excluded from polyacrylamide gels, presumably because they are too massive, but they could be resolved on agarose gels. The presence of the polymerase I enzyme in these complexes was indicated by the fact that complexes could be labeled indirectly by incorporation of ^{32}P -ribonucleotide triphosphates into nascent RNA transcripts. These data support the hypothesis that the highly purified pol I holoenzyme activity can be attributed to a single multi-protein complex, which can establish a functional transcription preinitiation complex following a single promoter binding event.

MATERIALS AND METHODS

Purification of RNA Polymerase I—Nuclear extracts were fractionated using DEAE-Sepharose CL-6B, Biorex 70, Sephacryl S300, and Mono Q chromatography as described previously (15) and as diagrammed in Fig. 1. Peak Mono Q fractions were dialyzed against RB buffer (50 mM HEPES-KOH, pH 7.9, 10 mM EGTA, 10 mM MgSO_4 , 20% glycerol, 0.5 mM dithiothreitol) containing 100 mM KCl (designated RB100) diluted with an equal volume of KCl-free buffer (RB0) and subjected to chromatography on double-stranded calf thymus DNA-cellulose (Sigma; 0.3-ml bed volume) equilibrated in RB50 (numbers after the designation RB indicate the millimolar concentration of KCl). The column was washed with 2 ml of RB50. Bound proteins were eluted with RB175, then RB400. Fractions (~0.5 ml) were dialyzed against RB100 and stored at -80°C .

Western Blotting—50 μl of Mono Q-purified holoenzyme peak fractions were subjected to 12.5% SDS-polyacrylamide gel electrophoresis, and proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Protran BA85) at 30 V overnight in Tris/glycine/methanol buffer (25 mM Tris, 190 mM glycine, 20% methanol, pH 7.8) using a Bio-Rad Protean II apparatus. Transfer was verified by staining membranes with 0.5% (w/v) Ponceau S in 1% glacial acetic acid. Membranes were incubated 60 min at room temperature in TBST (20 mM Tris-HCl, 140 mM NaCl, 0.05% (v/v) Tween, pH 7.6) containing 5% (w/v) nonfat dry milk to block nonspecific protein binding sites. Membranes were rinsed twice with TBST followed by two washes, 10 min each, in a large excess of TBST. Filters were incubated with primary antibodies, diluted 1:1000 in all cases, for 3–6 h at room temperature in TBST, 5% (w/v) nonfat dry milk. After incubation with primary antibodies, filters were washed as described above then were incubated with horseradish peroxidase-conjugated sheep anti-rabbit secondary antibody (Amersham Pharmacia Biotech) in TBST for 1 h using conditions recommended by the supplier. Antibody-antigen complexes were visualized using an Amersham Pharmacia Biotech ECL kit (RPN2106) for enhanced chemiluminescence.

All primary antibodies used were raised in rabbits. Anti-pol I-LE (LE is last exon) is an antiserum raised against the last exon (~30 kDa) of the *Arabidopsis thaliana* pol I core enzyme largest subunit.² Anti-24.3 is an antibody raised against a 24.3-kDa *A. thaliana* polymerase subunit that is related to yeast RPB5 (28); in yeast this subunit is shared by all three nuclear RNA polymerases. Anti-14 is an antibody raised against the *A. thaliana* 14-kDa polymerase subunit related to yeast AC19 (29), a subunit shared by RNA polymerases I and III. Anti-24.3 and anti-14 antibodies were the generous gift of Dr. Thomas Guilfoyle (University of Missouri, Columbia).

rDNA Templates—pBor2 contains *B. oleracea* rRNA gene promoter sequences from -517 to $+104$ (relative to the transcription start site, $+1$), cloned as an *EcoRI-XbaI* restriction fragment into the plasmid pBluescript II KS $-$ (Stratagene). The template pBSII-Bor contains *B. oleracea* rRNA gene promoter sequences from -99 to $+42$ cloned into the *SmaI* restriction site of pBluescript II KS $-$.

In Vitro Transcription—20 μl of purified holoenzyme was mixed with 200 fmol (0.5 μg) of supercoiled pBor2. After 5 min at 25°C , 20 μl of $2\times$ transcription mix (30 mM HEPES, pH 7.9, 80 mM potassium acetate, 12 mM magnesium acetate, 1 mM dithiothreitol, 200 $\mu\text{g}/\text{ml}$ α -amanitin (Sigma), 1 mM of each ribonucleotide triphosphate) was added. After 2 h at 25°C , 360 μl of stop solution (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 250 mM sodium acetate, pH 5.3, 3 mg/ml yeast tRNA, 6 mM EDTA, pH 8.0) was added. After extraction with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), RNA was precipitated with 2.5 volumes of ethanol. Transcripts were hybridized to the *SphI-XbaI* fragment of pBor2 (sequences -116 to $+116$), which were 5' end-labeled at $+116$. Sequences $+104$ to $+116$ of this fragment are derived from the pBluescript plasmid polylinker. RNA-probe hybrids were subjected to S1 nuclease digestion, urea-polyacrylamide gel electrophoresis, and exposure to x-ray film as described previously (15).

For run-off transcription from linear promoter fragments, 20 μl of holoenzyme was mixed with 200 fmol of pBor2 digested with *SphI* (-116) and *XbaI* ($+116$) and 20 μl of $2\times$ transcription mix (same as above, except GTP was provided as 20 μM unlabeled GTP and 8 μCi of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (3000 Ci/mmol). Reactions were incubated for the times indicated in the figure legend, then processed for electrophoresis and autoradiography (30).

EMSA—The *SphI* to *XbaI* fragment (sequences -116 to $+116$) of pBor2 was used for all EMSA studies. The fragment was 5' end-labeled at the *XbaI* site using T4 polynucleotide kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Five to ten fmol of gel-purified promoter fragment was mixed with 10 μl of purified holoenzyme (unless otherwise stated) in a 20- μl reaction containing 50 mM HEPES-KOH, pH 7.9, 100 mM KCl, 10 mM EGTA, 10 mM MgSO_4 , and 20% glycerol. Binding reactions were incubated at 25°C for 25–30 min (unless indicated otherwise), then loaded onto a 0.8% agarose gel in TBE buffer (50 mM Tris, 50 mM borate, 1 mM EDTA; final pH 8.3). Electrophoresis was at 120 V (3.4 V/cm) for 2–3 h. Gels were vacuum-dried onto filter paper and exposed to x-ray film.

To label mobility-shifted complexes with radioactive RNA transcripts, purified holoenzyme (10 μl) was added to 200 fmol of unlabeled promoter fragment. $2\times$ run-off transcription mix containing $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (see above) was added. Incubation times are given in the figure legends. In some cases, reactions were treated with 1 μl of RNase (10 mg/ml), proteinase K (10 mg/ml), or RQ1 DNase I (Promega) for 5 min at 25°C before agarose EMSA. After electrophoresis, gels were washed twice (10 min each wash) in 250 ml of 50 mM TBE buffer to reduce background. Gels were then dried onto filter paper and exposed to x-ray film.

DNase I Footprinting—Plasmid pBSII-Bor was linearized with *EcoRI* (which cuts in the polylinker upstream of the promoter sequences, which end at -99) or with *XbaI* (which cuts in the polylinker ~20 nucleotides downstream of the promoter sequences, which end at $+42$). After end-labeling using T4 polynucleotide kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, probe fragments were released by digestion with *XbaI* (if *Eco*-labeled) or *EcoRI* (if *Xba*-labeled). After electrophoresis on 5% non-denaturing polyacrylamide gels, probe fragments were excised and recovered after diffusion overnight into 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. Footprinting reactions included 25 μl of purified holoenzyme, 25 μl of water, and 5–10 fmol of probe DNA. After 30 min at 25°C , 50 μl of 10 mM MgCl_2 , and 5 mM CaCl_2 was added. One minute later, 2 μl of DNase I, diluted at least 1:100 (determined empirically) from a 1 mg/ml stock, was added. Digestion was terminated 1 min later with 90 μl of stop solution (20 mM EDTA, 0.2 M NaCl, 1.0% SDS, 0.1 mg/ml yeast tRNA). Reactions were extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and DNA was precipitated with ethanol and subjected to denaturing urea-polyacrylamide gel electrophoresis, followed by exposure to x-ray film.

RESULTS

Purification of the RNA Polymerase I Holoenzyme—Previously, we showed that RNA polymerase I holoenzyme activity (defined as the ability of single fractions to carry out accurate, promoter-dependent transcription *in vitro*) could be purified to near homogeneity from *Brassica* or *Xenopus* cell-free extracts by sequential chromatography on DEAE-Sepharose, Biorex 70, Sephacryl S300, and Mono Q (15, 17). Because purified holoenzyme activities can recognize rRNA gene promoters to initiate transcription from the correct start sites, they must possess one or more intrinsic or associated DNA binding activities. Thus we expected holoenzyme activity to be retained on a DNA-cellulose column. This is the case, as shown in Fig. 1A. Highly purified *Brassica* holoenzyme fractions resulting from sequential puri-

² A.-C. Albert and C. S. Pikaard, unpublished information.

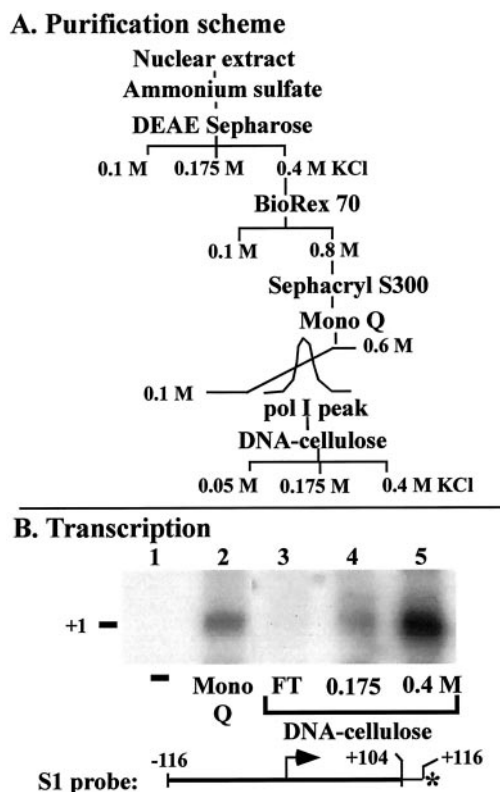


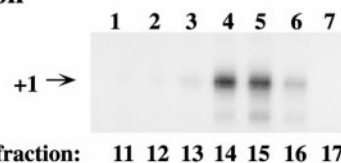
FIG. 1. Purification of RNA polymerase I holoenzyme activity from broccoli (*B. oleracea*). A, scheme for sequential chromatography using DEAE-Sepharose CL-6B, Biores 70, Sephacryl S-300, Mono Q, and double-stranded calf thymus DNA-cellulose. B, peak Mono Q and DNA-cellulose fractions program accurate transcription initiation from a cloned *B. oleracea* rRNA gene promoter (lanes 2–5). RNAs transcribed *in vitro* were detected using the S1 nuclease protection assay. The probe was 5' end-labeled at a plasmid polylinker site located at +116. Transcripts initiated at the correct start site are labeled "+1". Lane 1 is a control reaction with no protein added. FT, flow-through.

fication on four columns was subjected to DNA-cellulose chromatography. Flow-through fractions and fractions eluted with 175 and 400 mM KCl were then tested for their ability to program transcription from a cloned rRNA gene promoter (Fig. 1B). The fraction eluting at 400 mM KCl (DC400) was self-sufficient for transcription initiated accurately at +1 in the presence of α -amanitin (Fig. 1B, lane 5), as detected using the S1 nuclease protection assay with a probe labeled at +116 (see diagram at the bottom of Fig. 1B). A small amount of holoenzyme activity was also detected in the DC175 fraction (lane 4). Mixing the various DNA-cellulose fractions did not improve upon the activity in the DC400 fraction alone (data not shown).

The protein composition of the DC400 and Mono Q peak fractions were very similar following SDS-polyacrylamide gel electrophoresis and silver staining (data not shown), consistent with published evidence that Mono Q fractions are already purified to near homogeneity (15). At least 30 major polypeptide bands remain in peak Mono Q and DC400 fractions. Because of the similar compositions of Mono Q and DNA-cellulose fractions, most of the remaining experiments used Mono Q fractions for which the total holoenzyme activity was highest and for which we had multiple fractions defining peaks, allowing correlation to be made between transcription and DNA binding activities.

RNA Polymerase I Subunits Co-purify with Holoenzyme Activity—Mono Q peak fractions were tested for the presence of the 190, 24.3, and 14 kDa subunits of RNA pol I using Western blot analysis. Antibodies raised against the last exon of the largest RNA pol I subunit (190 kDa) of *A. thaliana* cross-

A. Transcription



B. Western blots

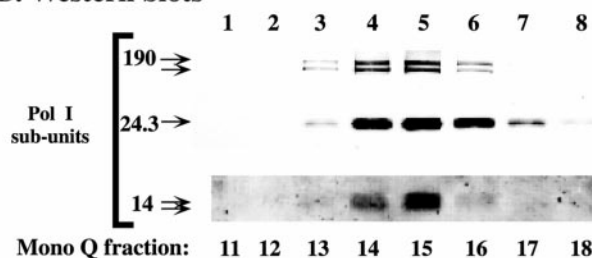


FIG. 2. RNA polymerase I subunits co-purify with holoenzyme activity. A, Mono Q fractions were tested for their ability to program accurate transcription initiation from a cloned *B. oleracea* rRNA gene promoter (pBor2) as in Fig. 1B. B, MonoQ fractions (50 μ l) were acetone-precipitated, subjected to electrophoresis on a 12.5% SDS-polyacrylamide gel, and blotted to nitrocellulose membranes. Western blots were probed with antiserum raised against the last exon of the largest *A. thaliana* RNA polymerase I subunit (190-kDa subunit) or against the 24.3- and 14-kDa RNA polymerase subunits.

reacted with a band of the appropriate size as well as a 170-kDa polypeptide in Mono Q fractions 13–16 (Fig. 2B, lanes 3–6). These same fractions correspond to the fractions capable of supporting promoter-dependent transcription (Fig. 2A, lanes 3–6). The identity of the ~170-kDa polypeptide is not known but may be a proteolytic product of the larger polypeptide migrating at 190 kDa (31). The relative abundance of the 190- and 170-kDa bands was not altered by treatment with calf intestine alkaline phosphatase, suggesting that they are not isoforms due to phosphorylation.

Antibodies against a 24.3-kDa polymerase subunit expected to be shared by all three nuclear polymerases (based on yeast) and against a 14-kDa polymerase subunit expected to be shared by polymerases I and III also cross-reacted with polypeptides of the appropriate sizes in Mono Q fractions 13–16 (Fig. 2B). Taken together with the Western blot data for the largest subunit and the ability of these same fractions to program transcription from the rRNA gene promoter, these data confirm that subunits of RNA polymerase I co-purify with holoenzyme activity. In agreement with our previous study using *Xenopus* (17), enzymatic assays showed that one or more protein kinases and histone acetyltransferase activities also co-purified with *Brassica* pol I holoenzyme activity (data not shown). The presence of a casein kinase 2 (CK2)-like protein in peak holoenzyme fractions was confirmed using an antiserum raised against the α subunit of human CK2 (data not shown).

Holoenzyme Proteins Protect the rRNA Gene Core Promoter Region from DNase Digestion—To assess the ability of proteins in peak holoenzyme fractions to bind to meaningful rRNA gene promoter sequences, protein-DNA contacts were examined using DNase I footprinting. Previous studies had shown that promoter sequences between -33 and +6 can program accurate transcription initiation *in vitro* using *Brassica* cell-free extracts (15). *In vivo*, sequences between -55 and +6 are needed for strong promoter function, although -33 to +6 defines a core promoter capable of minimal expression (32). In general agreement with these functional studies, the core promoter region from ~-30 to ~+20 was protected from DNase digestion by one or more proteins in the highly purified holoenzyme fractions (Fig. 3, compare lane 6 to the naked digestion

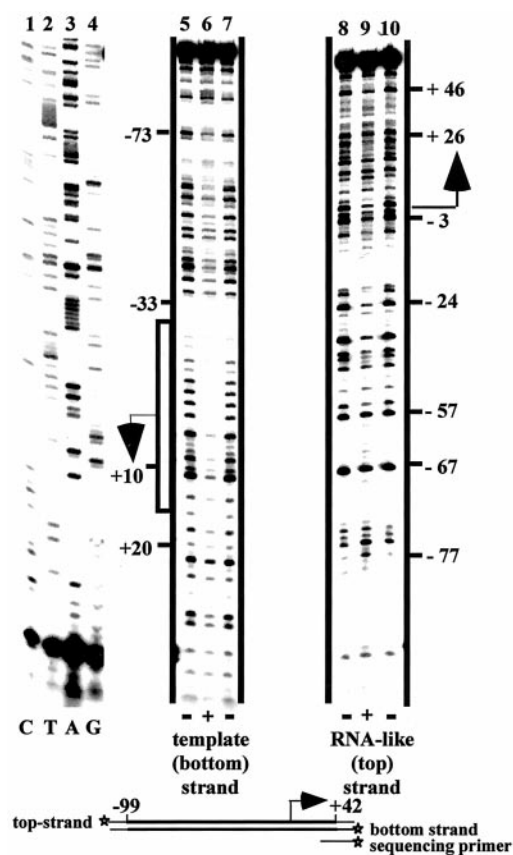


FIG. 3. Holoenzyme-promoter interactions mapped by DNase I footprinting. Purified holoenzyme activity was incubated with end-labeled promoter DNA (see the diagram at the bottom of the figure), then treated with DNase I (lanes 6 and 9). DNA digested in the absence of holoenzyme activity was run in lanes 5, 7, 8, and 10. Digestion products were subjected to denaturing polyacrylamide gel electrophoresis and were visualized by autoradiography. Lanes 5–7 show reactions using a double-stranded probe labeled on the template strand; for lanes 8–10, the RNA-like strand was labeled. Dideoxy sequencing reactions in lanes 1–4 were initiated from a primer end-labeled at the same nucleotide that was labeled for the template strand probe, allowing reactions in lanes 5–7 to be aligned with the sequence ladder. The arrows denote the transcription start site, +1. The bracket adjacent to lane 5 highlights the region protected most strongly by proteins present in the holoenzyme fraction. Note that protected sequences are also found both upstream and downstream of this bracketed region.

pattern in lanes 5 and 7). Interestingly, protection was observed most clearly on the template strand. On the RNA-like strand (lanes 8–10), the differences in DNase digestion were more subtle. Nonetheless, protections in the core promoter region are detected and a hypersensitive site at -77 is induced by proteins present in the peak holoenzyme fraction.

An Electrophoretic Mobility Shift Assay to Study Holoenzyme-Promoter Interactions—DNase I footprinting cannot determine how many promoter binding activities might be present within holoenzyme fractions. Therefore we turned to the EMSA. In a typical EMSA experiment, an end-labeled DNA fragment is incubated with protein fractions, and the resulting protein-DNA complexes are resolved from the free DNA due to the slower mobility of the complex on a native (non-denaturing) gel. Furthermore, multiple DNA binding activities can be resolved due to the different mobilities of these protein-DNA complexes (e.g. see Ref. 23).

Mono Q fractions that included the peak holoenzyme transcription activity were tested using EMSA with either a 4% polyacrylamide or a 0.8% agarose gel (Fig. 4). Peak promoter-dependent transcription activity was detected in Mono Q fractions 14–16 (Fig. 4A, lanes 4–6), with only trace amounts of

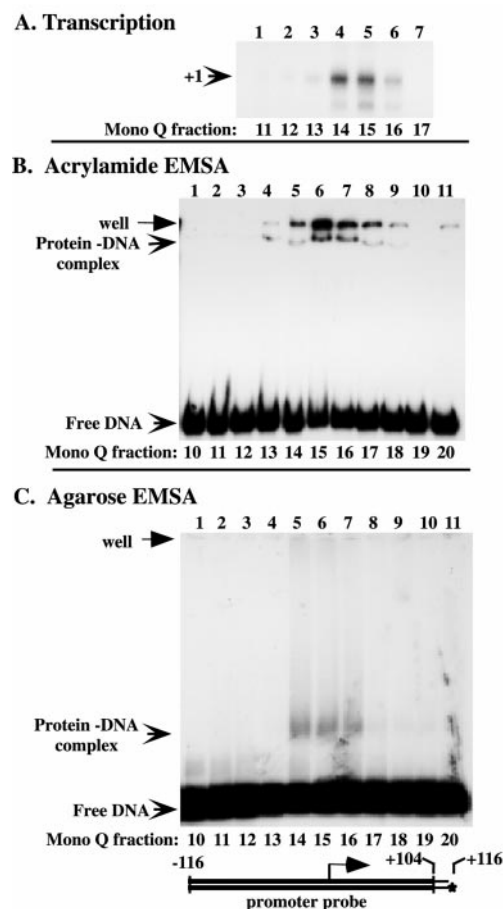


FIG. 4. Promoter binding activity co-purifies with holoenzyme activity. A, transcriptional activity of Mono Q fractions, defining the peak of holoenzyme activity. Fractions 11–17 were tested for their ability to program transcription using pBor2 template DNA. Transcripts were detected using the S1 nuclease protection assay. B and C, analysis of promoter binding activity using EMSA on 4% polyacrylamide or 0.6% agarose gels. Binding reactions incubated for 20 min were loaded into the wells of running gels. The EMSA probe diagrammed at the bottom includes promoter sequences -116 to $+104$ and is 5' end-labeled at a plasmid-encoded restriction site at $+116$. This same probe was for EMSA in all subsequent figures.

activity detected in flanking fractions. These same Mono Q fractions defined a peak of promoter-DNA binding activity using EMSA. Using polyacrylamide-EMSA, promoter binding activity was detected in Mono Q fractions 12–17 (Fig. 4B, lanes 4–9), with fraction 15 containing the highest binding activity. However, most of the protein-DNA complex ($\sim 60\%$) remained trapped in the well, a situation that persisted over a wide range of polyacrylamide gel concentrations and acrylamide:bisacrylamide cross-linking ratios (data not shown). The remaining $\sim 40\%$ formed a single band near the top of the gel.

Multiple mobility-shifted complexes were not detected in Fig. 4B, as would be expected if multiple activities bound independently and/or sequentially. However, a possibility was that sub-complexes were too large to enter the polyacrylamide gel. The use of agarose gels for EMSA circumvented the problem of having protein-DNA complexes trapped in the well (Fig. 4C). A single protein-DNA complex was observed using agarose EMSA, with peak activity in fractions 14–16 (Fig. 4C, lanes 5–7), again correlating well with the peak of transcription activity (Fig. 4A). A single protein-DNA complex was also formed using DNA-cellulose-purified holoenzyme activity, and its mobility was identical to the complex formed using Mono Q-purified holoenzyme activity (data not shown).

Specificity of Holoenzyme-Promoter Interactions—To test the

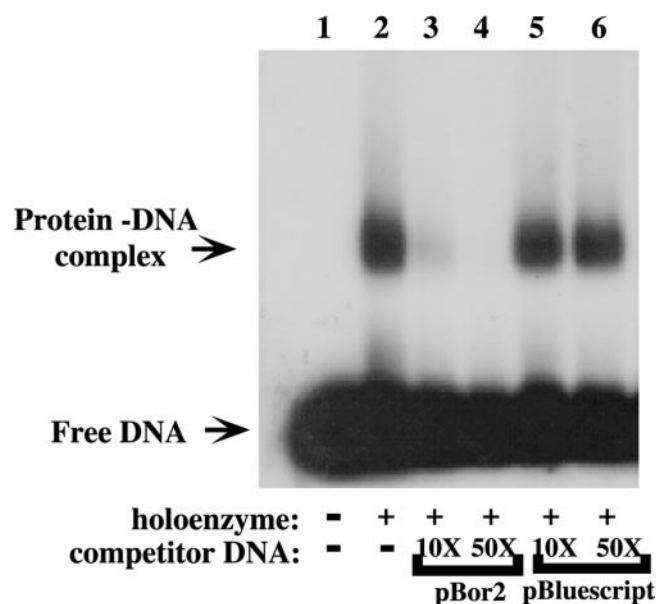


FIG. 5. Holoenzyme DNA binding activity is promoter-specific. Mono Q-purified holoenzyme (10 μ l) was preincubated 5 min without competitor DNA (lane 2) or with a 10- or 50-fold excess of unlabeled competitor DNA (lanes 3–6) before the addition of end-labeled promoter probe DNA. Reactions were incubated an additional 20 min, then loaded onto a 0.8% agarose gel. In lanes 3 and 4, the competitor DNA was plasmid pBor2, a pBluescript cloning vector containing the *B. oleracea* rRNA gene promoter region. The competitor in lanes 5 and 6 was pBluescript plasmid DNA lacking cloned sequences.

specificity of the DNA binding activity, end-labeled promoter probe was added to binding reactions preincubated with excess unlabeled plasmid pBor2 (a pBluescript KS plasmid containing a cloned *B. oleracea* promoter region) or with pBluescript plasmid lacking promoter sequences (Fig. 5). A 10-fold molar excess of pBor2 plasmid DNA reduced complex formation on the labeled probe approximately 10-fold (Fig. 5, compare lanes 2 and 3). Using a 50-fold molar excess of pBor2 competitor, only trace amounts of complex could be detected (lane 4). By contrast, neither a 10- nor 50-fold excess of pBluescript plasmid had a significant effect on complex formation on the labeled probe (lanes 5 and 6). Binding was also insensitive to synthetic competitor DNAs such as poly(dA-dT) (data not shown). These results suggest that the DNA binding activity in holoenzyme fractions is specific for the rRNA gene promoter, consistent with the DNase I footprinting results showing strong interaction with core sequences required for promoter function.

Titration and Time-Course Experiments Suggest the Binding of a Single Complex—The single protein-DNA complex observed in Figs. 4 and 5 could conceivably result from the binding of multiple activities if these activities bind cooperatively to the promoter. In an attempt to uncover such hypothetical intermediate complexes, we varied the amount of protein added to a fixed amount of labeled promoter DNA (Fig. 6). The addition of an increasing amount of holoenzyme fraction resulted in a linear increase in the amount of a single-shifted complex (Fig. 6, lanes 2–6), but no intermediate complexes were detected.

We next examined the formation of the mobility-shifted complex over time, again to see if intermediate complexes could be detected. Purified holoenzyme was mixed with end-labeled promoter DNA, and reactions were subjected to EMSA at timed intervals ranging from 0.25 to 20 min (Fig. 7A). Only one protein-DNA complex was observed in this experiment. The protein-DNA complex was detected even at the earliest time point (lane 1) and increased in abundance over time until reaching a maximum level after approximately 20 min.

To see how the kinetics of protein-DNA complex formation

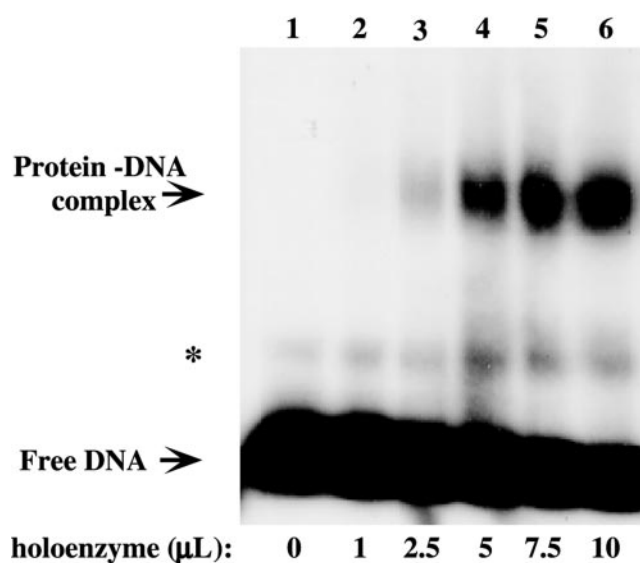


FIG. 6. Holoenzyme titration assayed by EMSA results in a single protein-DNA complex. Binding reactions containing 0–10 μ l of purified holoenzyme were incubated 40 min before agarose EMSA. The band denoted by an asterisk was endemic to the probe preparation.

visualized by EMSA compared with the kinetics of transcription using the same linearized promoter fragment used for EMSA, the experiment shown in Fig. 7B was performed. Preliminary experiments (not shown) demonstrated that the *Sph*I (–116) to *Xba*I (+116) promoter fragment yielded run-off transcripts of 116 nucleotides when incubated in transcription reaction buffer containing radioactive GTP. Likewise, an *Sph*I to *Sac*I (+136) promoter fragment yielded transcripts of 136 nucleotides. These results indicated that transcription occurred on linear fragments as short as 232 base pairs and was initiated accurately from +1 (note that the S1 nuclease protection data shown in previous figures utilized circular plasmid templates of ~3 kilobases). These results also showed that the probes used for EMSA were relevant as functional transcription templates and could bind whatever proteins in holoenzyme fractions were necessary for transcription initiation and elongation to at least +136. Building upon these controls, Fig. 7B shows a time-course of run-off transcription using the *Sph*I to *Xba*I promoter fragment used for all EMSA assays shown in this paper. Radioactive 116-nucleotide transcripts were detected within 5 min of adding purified holoenzyme to transcription reactions (Fig. 7B, lane 2) and increased linearly in the first 20 min of the reaction (lanes 3 and 4). However, if holoenzyme and template were preincubated for 45 min before the addition of nucleotide triphosphates, the amount of transcript detected after an additional 5, 10, or 20 min of incubation was similar (lanes 5–7). These results suggest that promoter binding is the limiting parameter affecting the amount of run-off transcripts produced under these conditions. The similar kinetics of promoter binding (detected by EMSA; Fig. 7A) and run-off transcription (Fig. 7B) over the first 20 min of the reactions is consistent with this interpretation.

Finally, to see if intermediate complexes would be revealed by dissociation of protein-DNA complexes, a 50-fold molar excess of unlabeled competitor DNA (plasmid pBor2) was added to binding reactions that had come to equilibrium after a 90-min incubation (Fig. 7C). Dissociated proteins are 50-fold more likely to bind to the competitor DNA than they are to reassociate on the labeled probe. As a result, complexes formed on the labeled probe are lost over time, a process that can be visualized using EMSA. In lane 2 of Fig. 7C, an aliquot of the binding reaction was loaded onto a running agarose gel after the 90-

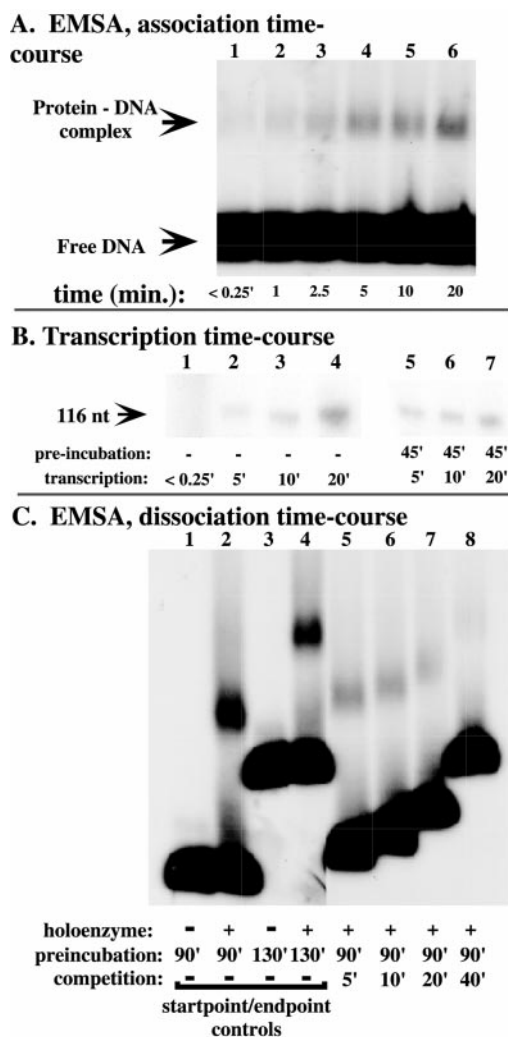


FIG. 7. Protein-promoter complex association and dissociation reveal only one protein-DNA complex. *A*, association time-course. Binding reactions containing 10 μ l of peak Mono Q fraction and 40 fmol of end-labeled promoter fragment were loaded onto a running gel within 15 s after mixing (*lane 1*) or after incubation for 1, 2.5, 5, 10, or 20 min (*lanes 2–6*, respectively). *B*, time-course of run-off transcription. An unlabeled *SphI-XbaI* (–116 to +116) promoter fragment was incubated with purified holoenzyme and NTPs (including [α - 32 P]GTP) for 0, 5, 10, or 20 min (*lanes 1–4*). In *lanes 5–7*, purified holoenzyme and template were first preincubated for 45 min before initiating transcription with NTPs and [α - 32 P]GTP. After an additional 5–20 min, reactions were stopped, and purified RNAs were subjected to denaturing polyacrylamide gel electrophoresis. Run-off transcripts were 116 nucleotides (nt) long, as expected. Accurate initiation was confirmed by S1 nuclease protection (not shown). *C*, dissociation time-course. A scaled-up reaction containing holoenzyme (10 μ l/gel lane) and promoter probe was preincubated for 90 min. A 50-fold excess of unlabeled pBor2 competitor plasmid was then added, and aliquots were removed and loaded onto a running gel after 5, 10, 20, or 40 min (*lanes 5–8*). Controls contained probe only (*lanes 1* and 3) or probe-holoenzyme binding reactions preincubated for 90 min, the start point for the experiment, or 130 min, the end point for the experiment (*lanes 2* and 4, respectively).

min preincubation period, thus representing the starting point control for the experiment. An equal aliquot was removed but was not loaded until 40 min later (*lane 4*), the end point of the experiment. Probe DNA not incubated with holoenzyme was loaded in *lanes 1* and 3. To the remaining reaction, competitor DNA was added. Aliquots were then removed after 5, 10, 20, or 40 min of competition and were loaded onto the running gel (*lanes 5–8*, respectively). The complex dissociated with a half-life of approximately 7 min. Importantly, as the complex dissociated, only free probe DNA was liberated; no intermediate

protein-DNA complexes were detected. Note that the protein-DNA complexes are stable during the time span of the experiment in the absence of competition (compare *lanes 2* and 4).

Mobility-shifted Complexes Can Be Labeled by Radioactive Nascent RNAs—Collectively, the data from Figs. 4–7 suggest that a DNA binding activity that interacts with the promoter correlates closely with pol I holoenzyme activity. The fact that this complex barely enters a polyacrylamide gel suggests that it might be large but does not address whether the mobility-shifted complex observed by EMSA might correspond to a promoter-holoenzyme complex capable of transcription. We reasoned that if RNA polymerase I were part of a mobility-shifted complex, it might be possible to indirectly label the complex with radioactive RNA transcripts retained in a promoter-polymerase-RNA ternary complex. To test this hypothesis, purified holoenzyme was incubated with an unlabeled promoter fragment in the presence of α -amanitin and nucleotide triphosphates, including [α - 32 P]GTP, then subjected to EMSA (Fig. 8). Indeed, RNA transcript-labeled complexes were produced and resolved in the EMSA assay (Fig. 8A, *lanes 3–7*). Maximal labeling was achieved within 20 min of incubation. The mobility of transcript-labeled complexes and complexes formed on end-labeled DNA were similar (compare *lane 2* with *lanes 4–7*). A consistent difference was that RNA-labeled complexes displayed a broader range of gel mobilities, thus producing more diffuse autoradiographic images than complexes formed on end-labeled DNA in the absence of nucleotide triphosphates.

The composition of mobility-shifted complexes was probed using RNase, DNase, and protease digestion (Fig. 8, *B* and *C*). As shown in Fig. 8B, complexes of similar mobility were formed using end-labeled DNA in the absence of nucleotide triphosphates (*lane 2*) or using unlabeled DNA under conditions in which radioactive transcripts were generated (*lane 4*). In the absence of added DNA template, no labeled species were generated in a mock transcription reaction (*lane 3*). RNase A treatment of a transcription reaction identical to the positive control in *lane 4* resulted in the loss of all labeled species (*lane 5*). DNase treatment eliminated the large protein-DNA complexes (*lane 6*). Protease K digestion generated a novel complex (*pt*) of higher mobility than the untreated complex, presumably due to the partial proteolysis of the ternary complex (*lane 7*). A labeled species (*r/d*) (*lanes 4* and 7) was sensitive to both RNase and DNase but not protease; the identity of this species is not known.

In Fig. 8C, protein-DNA complexes formed on a conventional end-labeled promoter DNA probe were also subjected to RNase, DNase, and protease digestion as a control for the experiment shown in *panel B*. As expected, these complexes were unaffected by RNase (compare *lanes 2* and 3) but were eliminated by DNase (*lane 4*) digestion. Protease digestion yielded a trimmed complex (*lane 5*) identical in its mobility to the protease-trimmed (*pt*) complex observed with transcript-labeled complexes (Fig. 8B, *lane 7*).

A trivial explanation for the results of Fig. 8 could be that strong but non-covalent [α - 32 P]GTP binding to one or more proteins is responsible for the indirect labeling of mobility-shifted complexes. Several results argue against this possibility. First is the RNase sensitivity of the GTP-labeled complexes (Fig. 8B, *lane 5*). Second, no labeling of complexes is observed if CTP, UTP, and [α - 32 P]GTP are included in the reactions but ATP is omitted (data not shown). The latter result is consistent with an inability to initiate transcription, which requires adenosines at positions +1 and +2 (the 5' end sequence of the pre-rRNA transcript, from +1 to +12, is AAGGGGTAGGCA).

DISCUSSION

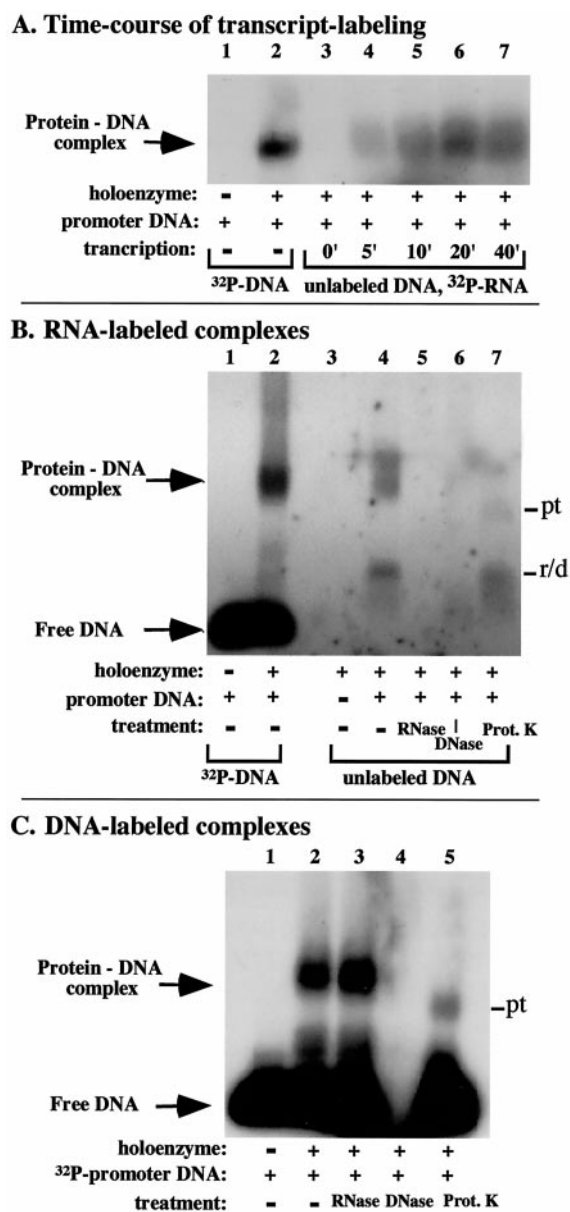


FIG. 8. Mobility-shifted complexes can be labeled indirectly by radioactive RNA transcripts. *A*, purified holoenzyme was incubated with an unlabeled *SphI-XbaI* promoter fragment (sequences -116 to +116) and nucleotide triphosphates (including [α -³²P]GTP) for 0, 5, 10, 20, or 40 min at room temperature (lanes 3–7) in the presence of α -amanitin. Reactions were then subjected to agarose EMSA. The controls in lanes 1 and 2 contain end-labeled promoter probe and probe incubated with purified holoenzyme, respectively. *B*, nuclease and protease sensitivity of RNA-labeled complexes. For lanes 3–7, purified holoenzyme was preincubated without template (lane 3) or with unlabeled promoter fragment (lanes 4–7), then NTPs including [α -³²P]GTP (lanes 3–7) were added. After an additional 5-min incubation, reactions were either left untreated (lane 4) or were treated with RNase I (lane 5), DNase I (lane 6), or proteinase K (lane 7) for 5 min. All reactions were then subjected to agarose EMSA. Lanes 1 and 2 are controls using end-labeled promoter DNA to show the mobility of the promoter and promoter-holoenzyme complexes, respectively. *r/d* denotes the position of a radioactive species sensitive to both RNase and DNase, and *pt* denotes the position of a “protease-trimmed” complex. *C*, reactions identical to those shown in panel *B*, lanes 3–7, were repeated except that end-labeled promoter DNA was used, and radioactive GTP was not included in the transcription reactions.

Collectively, these data support the interpretation that mobility-shifted complexes can be labeled by radioactive RNA transcripts synthesized in the presence of α -amanitin, suggesting that RNA polymerase I is integral to the complex.

To our knowledge, this study represents the first analysis of promoter binding by a highly purified RNA polymerase I holoenzyme activity. The study is made possible in large part by our ability to isolate biochemical quantities of holoenzyme activity from broccoli (*B. oleracea*), an inexpensive and abundant source of rapidly proliferating cells. Using *Xenopus* cells grown in culture (17), we were never able to obtain sufficient amounts of pol I holoenzyme activity that was concentrated sufficiently for assays such as DNase I footprinting, whereas this was possible using the *Brassica* system (Fig. 3). A current disadvantage of the plant pol I transcription system over the well established mammalian and *Xenopus* systems is that plant pol I transcription factors have not yet been defined. Furthermore, the rapid evolution of pol I transcription systems prevents the use of antibodies raised against vertebrate or yeast pol I transcription factors to determine if homologous factors are present in plant holoenzyme fractions. Thus, at present, we are limited to the use of antibodies against plant polymerase subunits. Nonetheless, we suspect that plant and vertebrate pol I holoenzyme activities are fundamentally similar because our previous work (15, 17) combined with the current study has shown that the biochemical characteristics of pol I holoenzyme activities isolated from *Brassica* and *Xenopus* are essentially identical. In both cases, the holoenzyme activities are purified using the same sequence of chromatography columns (DEAE, Biorex, Sephacryl, Mono Q, DNA-cellulose) with only minor differences in the salt concentrations used to elute the holoenzyme activities. In both cases, one or more protein kinases co-purifies with holoenzyme activity, as does histone acetyltransferase activity (Ref. 17 and data not shown).

DNase I footprinting revealed that one or more proteins present in *Brassica* holoenzyme fractions interact primarily with the rRNA gene core promoter region that was defined previously using promoter deletion mutants in transient expression and *in vitro* transcription assays (15, 32). Subtle changes in the DNase I digestion pattern were also detected both upstream and downstream of the core promoter. Whether these latter changes result from direct contact with proteins present within holoenzyme fractions or whether they result from conformational changes induced at a distance by proteins binding the core promoter cannot be determined at present. An interaction of plant holoenzyme proteins with the core promoter region is consistent with the finding that rat and *Xenopus* pol I holoenzyme fractions include subunits of the vertebrate pol I transcription factor SL1, which is required for core promoter function (17, 18). Upstream binding factor (UBF), a stimulatory but non-essential transcription factor that interacts primarily with an upstream domain of vertebrate rRNA gene promoters, is missing from the rat and *Xenopus* holoenzyme fractions (17, 18).

In our previous studies as well as the current study, we have used “holoenzyme activity” to refer to the ability of highly purified single fractions to carry out accurate, promoter-dependent transcription (15, 17). The fact that the activity can be purified so extensively suggests that all the needed transcription factors are stably associated with polymerase I to form a single multiprotein complex, or pol I holoenzyme. Other laboratories have arrived independently at the same conclusion (16, 18). Nonetheless, the existence of a single entity capable of promoter recognition and transcription has not been demonstrated, and one could argue that holoenzyme activity is actually due to multiple activities that co-purify during anion exchange, cation exchange, and size exclusion chromatography. If the latter were true, a prediction is that EMSA would reveal sub-complexes that are intermediates in the assembly of com-

plete transcription complexes, as was observed for pol II transcription complex assembly in the study by Buratowski *et al.* (23). However, this is not what we observed. Instead, only a single mobility-shifted complex was detected under a variety of experimental conditions that were designed to examine both the association and dissociation of promoter-protein complexes. This DNA binding activity co-purified precisely with transcriptional activity and with subunits of pol I core enzyme and showed specificity for rRNA gene promoter sequences. By using the same DNA fragment for footprinting, EMSA, and run-off transcription, we can correlate the results obtained in these assays to conclude that the DNA binding activity interacts primarily with the core promoter region and can position the polymerase to initiate transcription from the correct start site. Furthermore, time-course studies reveal a good correlation between the formation of mobility-shifted complexes and the production of run-off transcripts. Finally, the mobility-shifted complexes can be labeled by virtue of nascent RNA transcripts in the presence of α -amanitin, indicating that the complexes contain RNA polymerase I. Collectively, these observations suggest that the mobility-shifted complex observed by agarose EMSA is in fact a holoenzyme-promoter complex that is competent for transcription. The data further suggest that pol I transcription pre-initiation complexes can be assembled in a single DNA-binding event in which a holoenzyme complex interacts specifically with an rRNA gene promoter. The identification of the proteins within the holoenzyme, including the proteins that make direct contacts with the promoter DNA, is a priority for future studies.

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