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Muriel Thomas‡, Stéphane Chédin, Christophe Carles, Michel Riva, Michael Famulok§¶, and André Sentenac‖

From the Service de Biochimie et de Génétique Moléculaire, CEA/Saclay, F-91191 Gif-sur-Yvette Cedex, France and §Institut für Biochemie, Genzentrum der Ludwig-Maximilians-Universität München, Feodor-Lynen-Strasse 25, 1377 München

To probe the complex nucleic acid binding domains of yeast RNA polymerase II (Pol II), we have isolated in the presence of heparin RNA molecules that selectively bind to yeast Pol II. A class of RNA molecules was found to bind and strongly interfere with enzyme-DNA interaction but not with RNA chain elongation. Remarkably, one selected RNA ligand was a specific inhibitor of *Saccharomyces cerevisiae* Pol II. *S. cerevisiae* Pol I and Pol III and Pol II from *Schizosaccharomyces pombe* or wheat germ cells were not affected. Photocross-linking experiments showed that the RNA ligand preferentially interacted with B220, the largest subunit of Pol II and, to a lesser extent, with B150, the second largest subunit. The selected RNA was expressed in yeast cells under the control of a Pol III promoter. Yeast cells that expressed the anti-Pol II aptamer grew normally. However, a cell growth defect was observed when expressing the RNA aptamer in cells having an artificially reduced level of Pol II.

During the transcription process, multisubunit RNA polymerases contact the DNA template and the RNA product at multiple sites to ensure the processivity of the polymerization reaction (Refs. 1 and 2 and references therein). All the subunits of bacterial RNA polymerase, including σ, participate in DNA binding (3, 4). Enzyme-DNA interaction is a dynamic process, and the extent of DNA protection does not remain invariant during RNA chain elongation (5, 6). Furthermore, the enzyme plays an active role in holding the RNA product. Current models assume the existence of at least two separate RNA binding sites to account for the high stability and processivity of the transcription complexes as well as other particular features of the elongating RNA polymerase complexes (5, 7, 8).

RNA polymerase-RNA interactions are functionally important at the various stages of the transcription reaction, yet our knowledge of the RNA binding sites is still very limited (2, 9). RNA polymerase binds free RNA tightly (10–12), preferentially single-stranded RNA, and RNA binding induces the release of the σ factor from the bacterial holoenzyme as during promoter clearance, when the growing RNA chain reaches 8–10 nucleotides long (11, 13). By filling the RNA product binding site, the nascent RNA causes a dramatic increase in the stability of the ternary complex (14, 15), probably in relation with the conformation difference exhibited by the core (elongating) and holo (promoter binding) forms of bacterial RNA polymerase (16). In addition, the product RNA plays an active role in pausing, termination, or anti-termination processes (5, 17, 18) by yet unclear mechanisms that probably involve interaction of the transcript with a site on elongating RNA polymerase.

The functional importance of RNA-enzyme interactions prompted us to screen for RNA aptamers that bind to and inhibit yeast RNA polymerases at different steps of the transcription reaction. In the present work, we isolated high affinity RNA ligands against yeast RNA polymerase II (Pol II)[1] using the Selex procedure (19, 20) starting from a randomized RNA library. This strategy can yield RNA ligands that have a higher affinity for their protein target than the natural nucleic acid ligands (21, 22) or even bind nonnucleic acid-binding proteins (23–27). One selected RNA ligand described here specifically bound to *Saccharomyces cerevisiae* Pol II with high affinity and was a strong inhibitor of transcription.

EXPERIMENTAL PROCEDURES

Purification of RNA polymerases—*S. cerevisiae* RNA polymerases I, II, and III were prepared as described previously (28–30). *Schizosaccharomyces pombe* Pol II was prepared analogously. Wheat germ Pol II was a generous gift from Dr. Dominique Job (Lyon).

Selex Assay—The initial random pool of RNAs was prepared as described (31). DNA molecules of the library contained 40 randomized nucleotides flanked by 5′ and 3′ constant sequences recognized by primers. 5′ Primer, 5′-TCTAATACGACTCACTATAGGGCGCTA-AGTCCTCGCTCA-3′; 3′ primer, 5′-GTCGGATCCGAGTCGCGCGT-3′.

For the first round of selection, RNAs were produced by *in vitro* transcription of a DNA library fraction (10 μg of DNA, complexity of 10^{15} molecules (31)) with T7 RNA polymerase (32). RNAs were separated by electrophoresis on a 7.5% polyacrylamide, 7 M urea gel, and RNA molecules of appropriate length (approximately 80 nucleotides) were eluted from the gel as described by Johnson and Chamberlin (17) and ethanol-precipitated.

RNAs (40 pmol) were incubated with *S. cerevisiae* Pol II (5 pmol) for 30 min at 30 °C in 25 μl of binding buffer (50 mM Tris-HCl, pH 8, 100 mM ammonium sulfate). The RNA·RNA polymerase complexes were filtered under a slight vacuum through a HA filter (2.5 cm; 0.45 μm, Millipore) pre-wetted in binding buffer. The filter was rinsed with 6 ml of binding buffer, and the retained RNAs were extracted by phenol/urea treatment as described by Kubik *et al.* (33). Selected RNAs were reverse-transcribed with the murine leukemia virus reverse transcriptase in the presence of the 3′ primer, and the cDNAs were amplified by polymerase chain reaction after the addition of the 5′ primer (Perkin-Elmer, Gene Amp^R). cDNAs were used as templates for a next round of transcription/selection/amplification. Before the incubation with Pol II, RNAs were filtered on a HA filter to eliminate molecules nonspecifically retained on the filter. Every three rounds, reverse transcription-poly-

[1] The abbreviations used are: Pol, polymerase; bp, base pair; CTD, carboxyl-terminal domain; RPR, RNase P RNA.
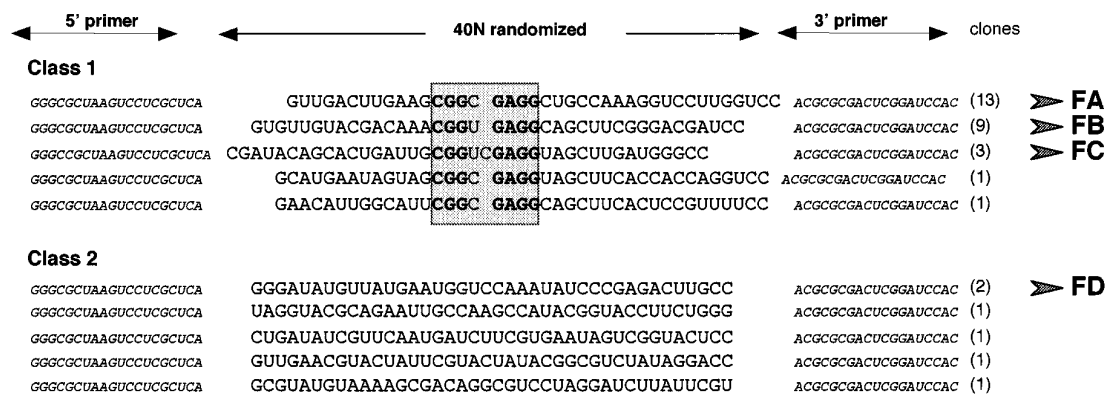
FIG. 1. **Sequences of selected RNAs.** Selected RNAs were obtained by a Selex procedure targeting *S. cerevisiae* Pol II (see "Experimental Procedures"). Each molecule was composed by constant 5′ and 3′ sequences surrounding a variable region of 40 randomized nucleotides. Class 1 and class 2 were, respectively, defined by the presence or the absence of a G-rich core region (*gray box*). The number of selected molecules having an identical sequence (or a very related sequence with minor base pair variation) is indicated in *parentheses*; ligands detected more than twice, and referred to as FA, FB, FC, and FD were further studied.

merase chain reaction products were purified by electrophoresis on a 7.5% polyacrylamide gel in denaturing conditions (see above). At the ninth round, cDNA (F9) were cloned and sequenced. Subsequently, six additional rounds of *in vitro* selection were performed under similar conditions, except that Pol II was preincubated with 250 ng of heparin (Sigma; H-2149 type) for 5 min at 30 °C in binding buffer before the addition of the RNAs. After the 15th round, cDNAs from the pool (F15) were cloned and sequenced.

RNA Polymerase Activity—Standard incubation mixture (10 µl) contained 70 mM Tris-HCl, pH 8, 5 mM dithiothreitol, 75 mM ammonium sulfate, 2.5 mM MnCl$_2$, 0.1 mM each ATP, CTP, GTP, UTP, 0.5 µCi of [α-^{32}P]UTP (400 Ci/mmol), 2 pmol of dC-tailed template (37-bp double strand oligonucleotide containing a (dC)$_8$ single strand extension at the 3′ extremity of the transcribed strand) or 1 µg of poly[d(A-T)]), 2.5 pmol of RNA polymerase, 1 unit of RNasin (Promega). For *S. cerevisiae* Pol I, MnCl$_2$ was replaced by MgCl$_2$ (5 mM), whereas for Pol III both divalent cations were present. After a 30 min incubation at 30 °C, the amount of transcribed RNAs was quantified by precipitation in 5% (w/v) cold trichloroacetic acid and scintillation counting of precipitated radioactivity. The radiolabeled transcripts from the dC-tailed template were also analyzed by electrophoresis on a 20% (w/v) polyacrylamide, 7 M urea gel and quantified with a PhosphorImager (Molecular Dynamics).

Filter Binding Assays—The evaluation of apparent K_d was performed by filter binding assays where 2 pmol of Pol II were incubated with varying amounts of FC- or FO-labeled RNAs (2×10^3 cpm/pmol). The binding reactions were filtered through a HA filter (2.5 cm, 0.45 µm; Millipore). After extensive washes with binding buffer, only bound RNAs were retained on filter. The radioactivity was then quantitated by scintillation counting. Apparent K_d values were calculated using the Eadie-Hofstee plot: [RNA·Pol II] = [pol II] − K_d [RNA·Pol II]/[RNA].

Gel Shift Assays—Pol II (0.8 pmol) was preincubated for 15 min at 30 °C with varying amounts of Pol II-specific aptamers in 15 µl of binding buffer. 5′-End-radiolabeled double-stranded dC-tailed template (see "RNA Polymerase Activity" above) (0.05 pmol, 10^5 cpm/pmol) was added, and incubation was continued for 15 min. After the addition of 2 µl of loading buffer (1 mM Tris-HCl, pH 8, 0.02% xylene cyanol, 0.02% bromphenol blue, 60% (w/v) sucrose), complexes were analyzed by electrophoresis on a 5% polyacrylamide gel and subjected to autoradiography.

Preparation of the Proteolyzed Form of Pol II Lacking CTD—Pol II lacking CTD was prepared by digesting purified Pol II with proteinase K (enzyme/substrate = 1/10,000 (w/w)) during 30 min at 37 °C. After evaluation of the specific proteolysis of the CTD by SDS-polyacrylamide gel electrophoresis, the proteolyzed Pol II was chromatographed on a Mono Q column (Smart system, Pharmacia Biotech Inc.) to remove proteinase K. Nonspecific transcriptional activity of this enzyme was determined on calf thymus DNA and was found not to be affected by proteolysis.

UV Cross-linking—For characterizing the Pol II-DNA interactions, the transcribed or the nontranscribed strand of dC-tailed DNA template (see "RNA Polymerase Activity") were independently 5′-end-labeled using [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Pharmacia). After hybridization of each labeled strand with its nonlabeled complementary strand, free [γ-^{32}P]ATP was eliminated by permeation chromatography on a Superdex 75 column in a 50 mM NaCl, 70 mM Tris-HCl, pH 8, buffer on a Smart system (Pharmacia). For cross-linking experiments, 30 pmol of DNA template labeled on the coding or on the noncoding strand (5×10^4 cpm/pmol) were incubated with 6 pmol of proteolyzed or nonproteolyzed Pol II in a transcription buffer (4 mM dithiothreitol, 2.5 mM MnCl$_2$, 30 mM Tris-HCl, pH 8, 130 mM ammonium sulfate, and 0.1 mM each of ATP, CTP, GTP, UTP) for 60 min at 30 °C. Irradiation was performed on ice for 8 min at a distance of 0.5 cm using a 254-nm transilluminator (model VL-6LC, Bioblock).

For characterizing the Pol II-FC interactions, FC RNA was body-labeled by *in vitro* transcription using T7 RNA polymerase and was purified by electrophoresis as described above. Pol II (2 pmol) was preincubated in the presence or the absence (see Fig. 6) of FO RNA (40 pmol) in binding buffer for 5 min at 30 °C in a final volume of 10 µl. Labeled FC RNA (4 pmol, 10^6 cpm) was added, and the incubation was pursued for 10 min. After irradiation, samples were supplemented with 20 µl of loading buffer and subjected to electrophoresis under denaturing conditions on a 7% polyacrylamide gel as described previously (34). Proteins bands were visualized by silver staining (35). The gel was dried and subjected to autoradiography on a Kodak Biomax film.

Expression Vectors and Strains—Expression vector pIIIex426 RPR was a gift from Dr. Engelke (University of Michigan). This high multicopy plasmid carried the *URA3* auxotrophy marker, allowing selection of yeast cells growing on plates lacking uracil (36). FO$_1$, FO$_2$, and FC cDNA were prepared by reverse transcription-polymerase chain reaction using 5′ and 3′ oligonucleotide primers that carried *Eco*RI recognition sites and cloned at the *Eco*RI site of pIIIex426 RPR, yielding recombinant plasmids pIIIexFO$_1$, pIIIexFO$_2$, and PIIIexFC. FO$_1$ and FO$_2$ cDNAs were derived from the unselected pool, whereas FC cDNA corresponded to the selected FC ligand after round 15. Nucleotidic sequences and insertion sense were determined by sequencing (Pharmacia).

Strain YF1971 (W303-1b with pLEU2-RPO21 LEU2) (37) was obtained by Dr. Friesen (University of Toronto). In this strain, the RPO21 gene that encodes the largest subunit of Pol II is placed under the control of a leucine-repressed promoter. Yeast transformation was done using the lithium acetate procedure. Yeast strains were grown at 30 °C on synthetic dextrose medium (38) supplemented or not with 0.2 mM leucine and isoleucine.

RESULTS

Selection of RNAs Ligands That Bind to Yeast Pol II—RNA aptamers that bind to *S. cerevisiae* Pol II were selected by the Selex strategy. The starting RNA library (FO) consisted of a randomized region of 40 nucleotides flanked by defined sequences at the 5′- and 3′-ends (Fig. 1). An excess of RNAs (40 pmol) was incubated with a highly purified preparation of Pol II (5 pmol). The RNA·protein complexes were retained on a nitrocellulose filter by filtration, whereas free RNAs passed through the membrane. After extensive washing of the membrane, the protein·RNA complexes were dissociated by phenol extraction, and the RNA was amplified. This selection/amplification procedure was repeated nine times and resulted in the RNA pool (F9). Gel shift assays of increasing amounts of Pol II
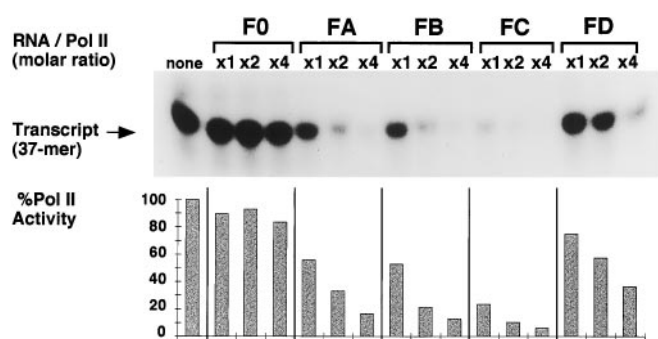
FIG. 2. **Inhibition of Pol II activity by selected RNAs.** Unselected FO or selected FA, FB, FC, and FD RNAs were preincubated with 2 pmol of Pol II at different molar ratios as indicated. The enzyme activity was then assayed in standard conditions using a 37-bp dC-tailed DNA template. Autoradiography of the 37-mer transcript analyzed by gel electrophoresis in denaturing conditions is shown. The diagram summarizes the percentage of residual Pol II activity quantified with the PhosphorImager.
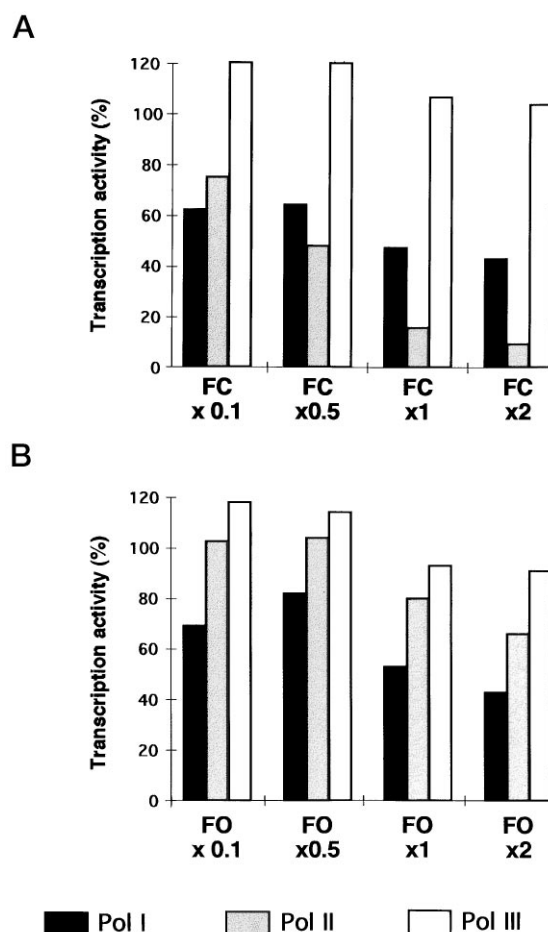

FIG. 3. **FC does not inhibit yeast Pol I and III.** *S. cerevisiae* Pol I, II, or III (2 pmol) were preincubated with FC RNA or FO RNAs at different molar ratio as indicated. The enzyme activity was then assayed in standard conditions using poly[d(A-T)] as the template. The enzyme activity is expressed in percent of activity of the enzyme without preincubation with RNAs.

incubated with an identical amount of radiolabeled FO or F9 RNAs demonstrated that F9 RNAs exhibited a higher affinity for the enzyme compared with FO RNAs (data not shown). A fraction of F9 RNA molecules was reverse-transcribed, cloned, and sequenced. The sequence analysis of F9 molecules showed a nonrandom sequence distribution, but selected molecules were still heterogenous (data not shown). Therefore, additional selection/amplification cycles were performed using a more stringent screen. At each cycle, Pol II was preincubated with heparin before addition of the RNAs ligands. In this way, a competition was imposed between heparin and RNA for interaction with the nucleic acid binding sites of Pol II. By saturating Pol II with heparin, one would expect to select RNA ligands with a high affinity for nucleic acid binding sites or that were directed to alternative sites of the enzyme.

After six rounds of high stringency selection, 34 clones of the final evolved population F15 were sequenced (Fig. 1). Based on sequence analysis, selected ligands were grouped into two classes: ligands of class 1 contained a conserved motif CG-GN$_x$GAGG ($_x$ = 1 or 2, where N is any nucleotide), whereas no consensus motif was found in ligands of class 2 (Fig. 1). Among 27 ligands of class 1, three of them named FA, FB, and FC were found 13, 9, and 3 times, respectively. Within class 2, all of the RNA sequences were unique, except for FD, which was found twice (Fig. 1). F15 population bound Pol II with higher affinity and showed a greater capacity to inhibit transcription compared with the F9 and FO populations (data not shown). Therefore, we decided to investigate the properties of the F15 population by studying the monoclonal ligands FA, FB, FC, and FD, which were not unique in the final F15-evolved population.

Selective Inhibition of Pol II Activity by Evolved RNA Ligands—Individual clones of RNA ligands FA, FB, FC, and FD were assayed for their ability to inhibit Pol II activity *in vitro* using a 37-bp dC-tailed DNA template that could be used for transcription and DNA binding studies as well as for cross-linking experiments. Transcription of this short double-stranded DNA can be easily monitored by gel electrophoresis of the 37-mer RNA transcript. Before the transcription reaction, Pol II was preincubated with varying amounts of the initial RNAs pool (FO) or of the monoclonal FA, FB, FC, or FD ligands. As shown in Fig. 2, the preincubation of Pol II with the selected FA, FB, FC, or FD ligands inhibited transcription in a concentration-dependent manner, whereas at the same concentrations FO RNA pool had only a weak effect (Fig. 2). Note that the FD RNA, which does not contain the consensus motif previously defined, had the lower inhibitory effect. During the selection, the apparent K_d of ligands for Pol II evolved from

approximately 180 nM for FO to 20 nM for FC (data not shown). Compared with the other selected ligands, the FC RNA displayed the highest inhibitory potential. For this reason, FC was chosen for further investigations.

Since the three forms of yeast RNA polymerase are highly homologous, in particular the two largest subunits, which represent two thirds of the mass of the enzymes, we analyzed the effect of the FC RNA on the *in vitro* transcriptional activity of Pol I, II, or III. Each enzyme was incubated with increasing amounts of FC RNA or FO pool and then assayed for their activity on a poly[d(A-T)] template. As previously observed with the dC-tailed template, Pol II activity was strongly inhibited by FC ligand at an equimolar ratio (Fig. 3A), whereas the FO pool had only a weak effect (10–20% inhibition; see Figs. 2 and 3B). Even when increasing the molar ratio FO/Pol II up to 6, FO RNA did not inhibit Pol II activity much (30% inhibition, data not shown). In the case of Pol I, a moderate inhibition of transcription was observed with a large amount of FC RNA (Fig. 3A). A similar result was obtained in the presence of nonselected FO pool RNAs (Fig. 3B), indicating that Pol I transcription shows a general, nonspecific sensitivity to RNA molecules. FC RNA or FO pool did not inhibit the Pol III activity but rather slightly activated the enzyme at sub-stoichiometric levels. These results indicated that the FC RNA was a specific inhibitor of the yeast Pol II. FC RNA was further examined for its ability to inhibit the activity of Pol II from various species. Before the transcription assay, each RNA po-
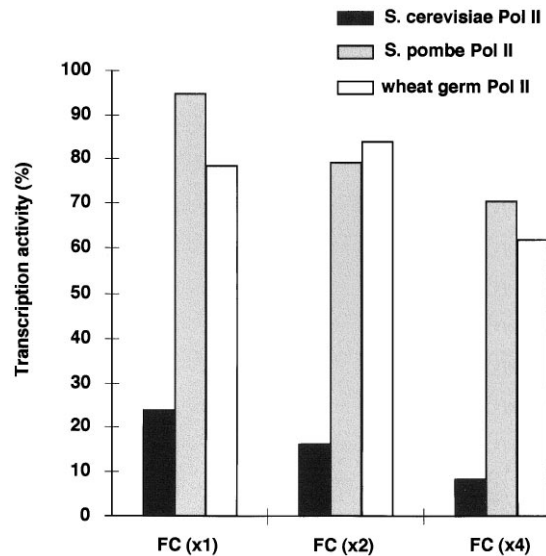
FIG. 4. **Species-specific FC inhibition.** Pol II (2 pmol) from *S. cerevisiae*, *S. pombe*, or wheat germ was preincubated with FC RNA at different molar ratio as indicated. The enzyme activity was then assayed in standard conditions using a 37-bp dC-tailed DNA template. Acid-insoluble RNAs were quantified by counting in a scintillation counter. The enzyme activity is expressed in percent of activity of the enzyme without preincubation with FC RNA.

FIG. 5. **Gel shift competition assay.** Pol II (1 pmol) was preincubated with FO pool (*lanes 3, 4,* and *5*) or FC ligand (*lanes 6, 7,* and *8*) before the addition of radiolabeled dC-tailed DNA template. After incubation, the complexes were separated from the free radiolabeled probe by electrophoresis in a nondenaturing polyacrylamide gel. The molar ratio between RNA ligands and Pol II is indicated. *Lane 1*, probe-incubated without Pol II.

lymerase was incubated with increasing amounts of FC RNA (Fig. 4). In contrast to Pol II from *S. cerevisiae*, Pol II from *S. pombe* and wheat germ were not inhibited by the FC ligand. Thus, the FC ligand appeared to be a species-specific inhibitor of *S. cerevisiae* Pol II.

We noted that the inhibition of Pol II transcription by FC RNA was independent of template concentration (from 0.5 to 3 µg per assay), suggesting that FC binding was noncompetitive with DNA (data not shown).

Binding of Selected RNA Ligands to Pol II—To test whether FC RNA interfered with the binding of Pol II to a DNA template, we performed gel shift competition assays using the labeled dC-tailed template. Binding of purified Pol II to this probe led to the formation of two protein·DNA complexes of different electrophoretic mobility (Fig. 5, *lane 2*). The low mobility band resulted from the interaction of nonproteolyzed Pol II (retaining the carboxy-terminal domain or CTD) with DNA, whereas the higher mobility band contained the proteolyzed form of Pol II missing the CTD. Indeed, only the upper band could be shifted by an anti-CTD monoclonal antibody (data not shown). Preincubation of Pol II with FC ligand (Fig. 5, *lanes 6–8*) dramatically affected the binding of the labeled DNA probe to the enzyme. In the presence of an equimolar amount of FC RNA and Pol II, DNA binding of the probe to both enzyme forms was completely abolished. FC ligand not only prevented the binding of DNA but also displaced prebound template (data not shown).

When exploring the effect of each selected RNA (FA, FB, FC, and FD) on the binding of Pol II on the DNA template using competition gel shift assays, we observed that in all cases the amount of DNA·Pol II complexes could be correlated to the inhibitory potency of the selected RNAs (data not shown). The differential effect of FC on Pol II activity as compared with Pol I and III (Fig. 3A) was also confirmed by gel shift experiments, since a 2-fold molar excess of FC RNA was unable to inhibit the formation of Pol I·DNA or Pol III·DNA complexes (data not shown). In contrast, the FO RNA pool only partially decreased enzyme-DNA binding when added in a 2-fold 2-fold molar excess relative to the Pol II (Fig. 5, *lane 5*). Surprisingly, FO RNA appears to diminish binding of the DNA template (Fig. 5,

compare *lanes 3, 4, and 5* with *lane 2*) at FO RNA/Pol II ratios that have almost no effect on Pol II transcriptional activity (see Fig. 2). The apparent discrepancy between binding assay and Pol II activity assay could be due to the presence of a nonactive subpopulation of Pol II in the enzyme preparation. Although experimental data supporting this hypothesis are lacking, FO RNA could possibly inhibit DNA binding by the transcriptionally incompetent enzyme population.

Using competition binding assays, we observed that the affinity of FC for Pol II did not depend on temperature (from 0 to 30 °C) or ammonium sulfate concentration (from 0 to 100 mM) (data not shown). To test whether the single-stranded DNA version of FC (FC DNA) displayed inhibitory properties as FC RNA, gel shift experiments as well as transcription assays were performed in the presence of FC DNA produced by asymetric polymerase chain reaction. In contrast to FC RNA, a 4-fold molar excess of FC DNA relative to the Pol II only partially impaired the dC-tailed DNA·pol II complex formation and did not inhibit the pol II-dependent transcription (data not shown).

FC RNA Preferentially Contacts the Largest Subunit of Pol II—To identify the polymerase subunit(s) contacted by FC RNA ligand, Pol II was cross-linked to ^{32}P-labeled FC RNA by UV irradiation, and the labeled subunits were identified after SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 6A). In a first experiment, under electrophoretic conditions at which all Pol II subunits could be visualized, we found that only the largest subunits were detectably labeled (data not shown). Fig. 6A, *lane 1* shows the two largest subunits (B220 and B150) of Pol II resolved on a 7.5% SDS-polyacrylamide gel electrophoresis. We determined by immunoblot analysis that the major intermediary B185 component derived from B220 by prote-
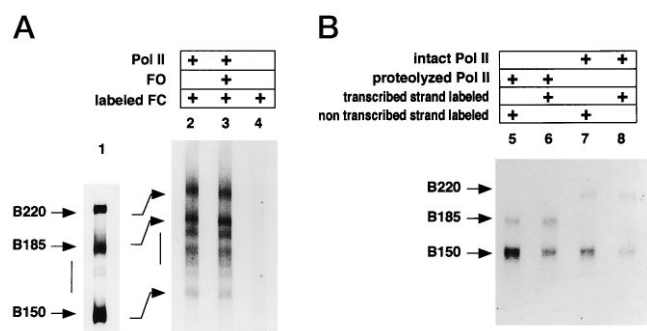
FIG. 6. **FC RNA and DNA contact the largest subunits of Pol II.** *A*, Pol II was preincubated with body-labeled FC RNA in the absence (*lane 2*) or in the presence (*lane 3*) of an excess of FO ligands. The complexes were UV-irradiated, and cross-linking products were detected by autoradiography after electrophoresis on a 7% polyacrylamide gel in the presence of SDS. Protein components were revealed by silver staining (*lane 1*). The silver-stained subunits (B220, B185, and B150) and their corresponding labeled products are indicated by *arrows*. Proteins indicated by a *vertical line* resulted from partial degradation of B220. *B*, Pol II or proteolyzed Pol II lacking the CTD were incubated with the 37-bp dC-tailed template ^{32}P labeled either on the transcribed strand or the nontranscribed strand and subjected to UV irradiation. The protein·DNA complexes were analyzed by electrophoresis on a 7% polyacrylamide gel in presence of SDS and autoradiographed.

olysis of the CTD and the minor products ranging from 160 to 185 kDa (indicated by a *vertical line*) corresponded to further proteolysis of B220 (data not shown). Most of the FC RNA covalently bound to Pol II reflected a preferential cross-linking to the B220 and B185 subunits and suggested that the CTD is not involved in this interaction (Fig. 6*A*, *lane 2*). The weakly labeled bands that migrated faster than B185 were derived from the cross-linkage of B220 derivatives (*vertical line*) or B150. In a control experiment, no radioactive band was detected upon UV-irradiation of the FC RNA in the absence of RNA polymerase (Fig. 6*A*, *lane 4*). Furthermore, the preincubation of Pol II with a large excess of nonradioactive FO RNA pool did not reduce the labeling of the subunits targeted by FC RNA (Fig. 6*A*, *lane 3*), hence confirming the specificity of the binding and cross-linking reactions.

We next compared the Pol II-binding sites of the FC RNA ligand with those of a DNA template, since the FC RNA competed for DNA binding. This was achieved by cross-linking of Pol II to a ^{32}P-labeled dC-tailed DNA template. Analysis of cross-linked subunits was performed as before (Fig. 6*B*). As previously mentioned, CTD is not involved in the interaction of FC RNA with Pol II. Nevertheless, Suzuki (39) demonstrated that a synthetic peptide corresponding to the CTD could bind *in vitro* to DNA. To check the involvement of the CTD in the binding of Pol II to DNA, two Pol II preparations were used for the characterization of the Pol II-DNA template interactions: one essentially containing the intact B220 subunit, the other containing the proteolyzed B185 form. Enzyme-DNA UV photocross-linking was performed using a DNA template selectively labeled either in the transcribed or the nontranscribed strand. We first determined that among all subunits of Pol II, only the two largest, B220 and B150, were cross-linked to the template (data not shown) as well as B185, the form of B220 lacking the CTD. Comparison of the relative amount of the B220·DNA and B185·DNA complexes (Fig. 6*B*, compare lanes *5* with *7* or *6* with *8*) revealed that the CTD was not involved in the interaction of B220 subunit with the DNA strands. Note that the largest subunit was cross-linked to the same extent to the transcribed and the nontranscribed strands, whereas the B150 subunit was predominantly cross-linked to the non-transcribed strand (Fig. 6*B*, compare *lanes 5* with *6* and *7* with *8*). In contrast, FC RNA was preferentially cross-linked to the
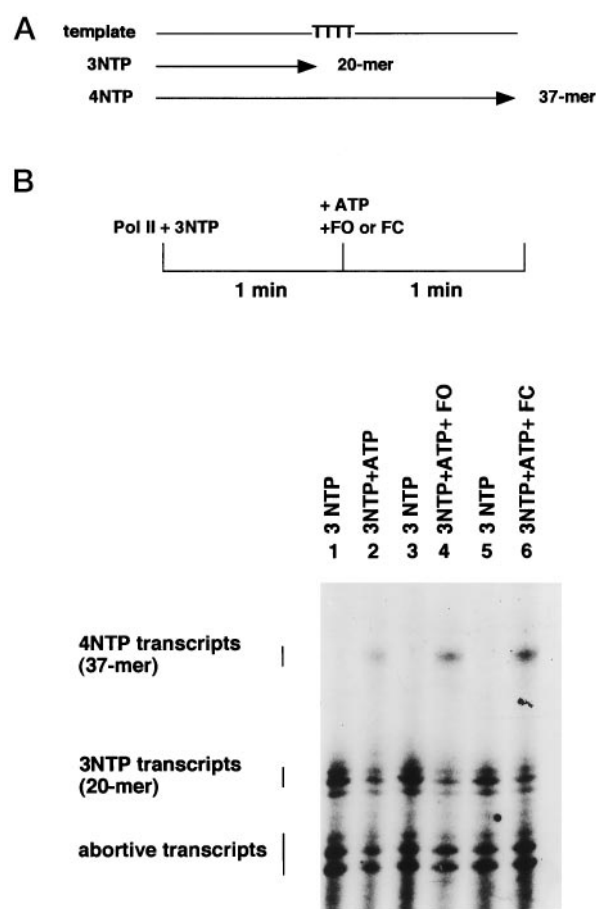

FIG. 7. **FC ligand did not inhibit Pol II during elongation.** *A*, schematic description of the 37-bp dC-tailed template containing a stretch of thymidines as an artificial stop in its middle and of the size of the transcripts expected in the presence of 4 NTPs or 3 NTPs (ATP omitted). *B*, Pol II was allowed to initiate and translocate to residue +20, where it will stall in the absence of ATP (*lanes 1, 3,* and *5*). Transcription complexes could resume elongation by the addition of ATP (*lane 2*) in the absence or in the presence of 10 pmol of FO (*lane 4*) or FC (*lane 6*) ligands. 2 pmol of DNA template and 2.5 pmol of Pol II were used in the reaction. Transcripts were analyzed by electrophoresis on a 20% polyacrylamide gel in the presence of 7 M urea and autoradiographed.

largest subunit. Using highly purified Pol II preparations with different specific enzymatic activities, we noted that the level of photocross-linking of B220 and B150 subunits to the DNA template was related to the transcriptional activity of the enzyme (data not shown).

FC RNA Does Not Inhibit Pol II during Elongation—We then investigated whether the RNA could block the enzyme during the elongation step. On the dC-tailed template, transcription complexes can be arrested at position 20 by omitting ATP in the transcription mixture (Fig. 7, *A* and *B*, *lanes 1, 3,* and *5*). The appearance of three transcripts at the 20-mer level is due to heterogenous initiation (see below). Three bands were also observed at higher gel resolution for the full-length transcripts (data not shown). When adding ATP, we observed that a small proportion of arrested complexes could resume elongation to form the 37-mer run off RNA (Fig. 7*B*, *lanes 1* and *2*). The addition of a large excess of FC RNA and ATP did not interfere with elongation (Fig. 7*B*, compare *lane 2* with *lane 6*) nor did the FO population (Fig. 7*B*, *lane 4*). The reason for the slight increase of 37-mer transcript observed in the presence of FO or FC RNAs (Fig. 7*B*, compare *lanes 4* and *6* with *lane 2*) is still unclear. These data indicated that FC ligand did not block Pol II molecules once engaged in the elongation step.
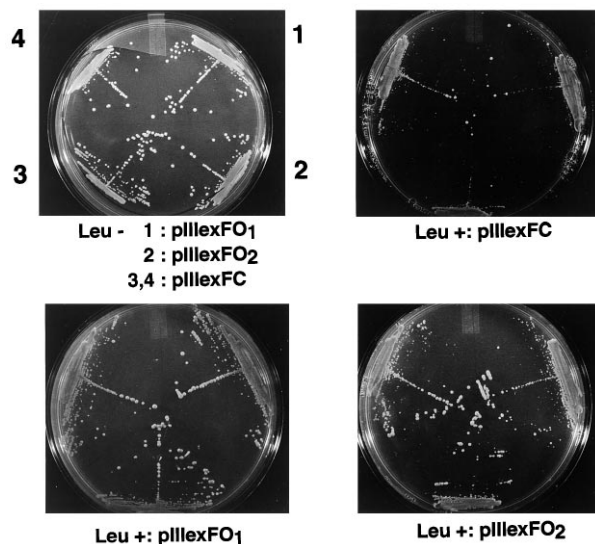
FIG. 8. **Growth of cells expressing FC RNA.** Strain YF1971 (W303-1b with pLEU2-RPO21 LEU2) was transformed either by pII-IexFC expression vector producing FC RNA or by pIIIexFO1 and pII-IexFO2 expression vectors producing nonselected, independent FO1 and FO2 RNAs, respectively. Yeast cells were grown on a leucine-lacking medium (leu⁻) and were then streaked on leucine-rich medium (leu⁺) on which the endogenous amount of Pol II is reduced.

Production of FC RNA in Yeast—Since monoclonal FC RNA inhibited Pol II *in vitro*, we wondered if *in vivo* expression of FC affected Pol II activity and cellular growth. To produce FC RNA in yeast, we used an expression vector containing a Pol III-dependent promoter (36). This promoter, derived from the RNase P RNA gene (*RPR1*), contains two intragenic control elements, the A and B blocks. This intragenic promoter was previously fused to 5 S RNA to produce an artificial 5 S RNA precursor allowing cell growth (40, 41). This RPR1–5S RNA is matured *in vivo* by cleavage of a 84-nucleotide 5′ leader sequence of RPR1. Three expression vectors were constructed: pIIIexFC, pIIIexFO₁, and pIIIexFO₂, expressing FC RNA and two independent nonselected RNAs, FO₁ and FO₂, respectively. The FO₁ and FO₂ sequences showed a randomized nucleotidic distribution over the 40-nucleotide variable region (Fig. 1) and were unrelated to FC (data not shown). In contrast to the RPR1 precursor RNA or the chimeric RPR1–5S RNA, Northern blot analysis indicated that the leader sequence was not cleaved from FC RNA (data not shown). In FC RNA expressing yeast, no detectable growth phenotype was observed (data not shown). This could be due to a very low FC RNA/Pol II ratio in cells. To verify this hypothesis, we expressed FC RNA in YF1971 mutant strain, where the endogenous amount of Pol II depends on the expression level of the large subunit gene that was placed under the control of the *LEU2* gene promoter (37). In the presence of leucine, the expression of the *RPO21* gene encoding the largest subunit of Pol II is reduced, and thereby the level of assembled Pol II is repressed. In the absence of leucine, the promoter of *RPO21* gene is activated, allowing the formation of a large quantity of Pol II. On a leucine-rich medium, the pIIIexFC-containing strain grew more slowly than the strains containing pIIIexFO₁ or pIIIexFO₂ (Fig. 8). Moreover, this growth defect was reversed upon transfer to a leucine-lacking medium (Fig. 8), suggesting that the FC RNA *in vivo* inhibitory effect depended on the amount of Pol II. Under the same conditions, the control FO1 and FO2 RNAs had no effect on the cell growth rate. Similarly, a yeast strain transformed with the expression vector without insert has no growth defect on leucine-rich medium (data not shown).

DISCUSSION

Multisubunit RNA polymerases are engaged in complex interactions with the two strands of the DNA template, the nascent RNA molecule and also a variety of polyanions like heparin. Here we show that RNA molecules designed as "aptamers" can be isolated, which *in vitro* bind to yeast Pol II with high affinity and inhibit transcription by competing with the DNA template in a highly specific manner. Indeed, the other forms of yeast RNA polymerases, Pol I and Pol III, were largely insensitive to the selected RNA ligands as was Pol II from different yeast species or other eukaryotic organisms. The possibility that such RNA transcripts could be toxic *in vivo* has been explored.

By selecting RNA molecules displaying a high affinity for Pol II in the presence of a large excess of heparin, one might expect to isolate RNA ligands directed to part of the nucleic acids binding site of the enzyme. Like heparin, the RNA dissociated enzyme·DNA binary complexes but did not affect elongating ternary complexes. Gel shift experiments showed that FC binding prevented the DNA·Pol II complex formation, whereas *vice versa* a large excess of DNA template did not interfere with the FC·Pol II complex formation (data not shown). This suggested that FC prevents DNA binding on Pol II by interacting with the enzyme at a site distinct from the DNA binding site. This hypothesis was supported by the fact that even in the presence of high concentrations of DNA template, FC still inhibited transcription to the same extent. Altogether, these data suggest that FC is not a competitive inhibitor of DNA binding.

Cross-linking experiments allowed comparison of the subunit binding pattern of the FC RNA with that of the transcribed or nontranscribed DNA strand. The nontranscribed strand was preferentially cross-linked to the β-like subunit and, to a lesser extent, also to the β′-like subunit. This differential binding probably reflects the relative extent of interaction of the 37-bp DNA strand with the two subunits. In contrast, the transcribed strand was cross-linked with the same efficiency to the two large subunits. Therefore, it was interesting to observe that the FC RNA was preferentially cross-linked to the largest, β′-like subunit. FC RNA also cross-linked to a lesser extent to the B150 subunit. On the contrary, a preferential cross-linking on the B150 subunit was observed when the DNA template was labeled on the nontranscribed strand. This suggests that the FC RNA might bind close to the binding site for the transcribed strand. At least the FC RNA binding site was clearly protected within the ternary transcription complex, since FC RNA did not inhibit Pol II paused during the elongation step.

Class 1 RNA ligands FA, FB, FC share a purine-rich sequence CGGN$_x$GAGG ($_x$ = 1 or 2) related to a GNRA motif (see review in Ref. 42). Due to the formation of an essential GA base pair, the GNRA tetraloop displays a stable structure involved in RNA stability. Such a motif has been recently shown to be essential for AMP-RNA aptamer complex formation (43). Among the four RNA molecules studied, those containing the conserved motif more strongly inhibited Pol II activity (compared FA, FB, and FC with FD). Mutational analysis should determine whether this conserved region is involved in the recognition of the selected RNA by Pol II. In a preliminary experiment, we generated a truncated version of FC RNA (delFC) lacking 19 nucleotides at the 3′ end that correspond to the 3′ primer sequence (see Fig. 1). When delFC was incubated with Pol II before the transcription reaction, the inhibition of activity was 2-fold lower than with the full-length FC RNA (data not shown). Thus, the length of the RNA appears important for FC inhibitor potency, suggesting that the core sequence is not sufficient for complete inhibition.

Finally, it was interesting to test whether the FC RNA was able to affect yeast cell growth. This possibility would open up a new experimental avenue for identifying vital functional domains of Pol II by genetic approaches. The FC RNA was expressed as a fusion with the transcribed promoter region of *RPR1* by the Pol III that was refractory to inhibition. In a first experiment, when FC RNA was produced in a wild-type strain, no effect on the cell growth was observed. This negative result can be due to the out-numbering of FC RNAs by Pol II in cells. Pol II is relatively abundant in the cell, since 10,000–14,000 molecules of Pol II are present in a wild-type cell according the growth medium (44). In addition, Archambault *et al.* (37) demonstrated that a decrease of approximately 90% of the Pol II level affected the cell growth rate but was not lethal. Therefore, this enzyme is present *in vivo* in excess. Moreover, we observed that FC RNA was poorly expressed *in vivo*. As judged from Northern analysis, the amount of FC represented approximately 0.3% of endogenous U6 RNA (data not shown). Since we could not improve the production of FC RNAs by testing different expression vectors, we performed the *in vivo* inhibition experiment in a yeast strain where the Pol II level was artificially lowered (strain YF1971: W303-1b with pLEU2-RPO21 LEU2 (37)). In such a particular genetic context, FC RNA was able to impair yeast cell growth (Fig. 8), suggesting that the *in vivo* FC effect depends on the Pol II amount. Importantly, this growth defect was specific to FC RNA, since it was not observed when expressing *in vivo* two independent nonselected RNAs from the FO pool in the same low level Pol II strain.

Since FC RNA was a strong inhibitor of Pol II, we searched the yeast genome for sequences similar to the 40-nucleotide central region of FC without success. One can imagine that such toxic or suicidal RNA molecules must have been counter-selected during evolution.

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NUCLEIC ACIDS, PROTEIN
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**Selective Targeting and Inhibition of Yeast
RNA Polymerase II by RNA Aptamers**

Muriel Thomas, Stéphane Chédin, Christophe
Carles, Michel Riva, Michael Famulok and
André Sentenac