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A fluorescence-based assay for multisubunit DNA-dependent RNA polymerases

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Abstract

The properties of DNA-dependent RNA polymerases have been studied since the 1960s, but considerable interest in probing RNA polymerase structure/function relationships, the roles of different classes of RNA polymerases in cellular processes, and the feasibility of using RNA polymerases as drug targets still exists. Historically, RNA polymerase activity has been measured by the incorporation into RNA of radioisotopically labeled nucleotides. We report the development of an assay for RNA polymerase activity that uses the dye RiboGreen to detect transcripts by fluorescence and is thus free of the expense, short shelf life, and high handling costs of radioisotopes. The method is relatively quick and can be performed entirely in microplate format, allowing for the processing of dozens to hundreds of samples in parallel. It should thus be well-suited to use in drug screening and analysis of chromatographic fractions. As RiboGreen fluorescence is enhanced by binding to either RNA or DNA, template DNA must be removed by DNase digestion and ultrafiltration between the transcription and the detection phases of the assay procedure. Although RiboGreen fluorescence is sensitive to changes in solvent environment, solvent exchange in the ultrafiltration step allows comparison of transcription levels even under extremes of salt, pH, etc.

Keywords: RNA polymerase; Non-radioactive detection; Microplate assay; RiboGreen

DNA-directed RNA polymerases (RNAP;[2] EC 2.7.7.6) play a central role in cellular gene expression (reviewed in [1]), and, for this reason, they have been the subject of intense study for most of 4 decades. These enzymes fall into two main classes on the basis of structure: bacteriophage-type single-subunit RNAP of ~120 kDa and larger multisubunit polymerases that are typical of eubacteria, archaea, eukaryotic nuclei, and the RNAP encoded by the plastid genomes of green plants. The bacterial RNAP has a molecular mass of approximately 350,000 Da and consists of 4 core subunits ($\alpha_2\beta\beta'$), a subunit (ω) involved in assembly [2], and a specificity factor known as σ [3]. Archaeal and eukaryotic nuclear polymerases range in size up to nearly 800,000 Da and include 10 to 20 core subunits in addition to numerous specificity and regulatory factors [4]. The 4-subunit core of bacterial-type RNAP is sufficient for basic transcriptional activity in vitro (e.g. [5]) but requires the presence of the σ subunit for promoter-directed transcription initiation [6]. The most detailed biochemical information has been gathered on the RNAP of *Escherichia coli*, though recent years have seen an upswing of interest in other bacterial transcription systems, especially since the publication in 1999 of the first detailed crystal structure of a bacterial RNAP, that of *Thermus aquaticus* [3]. Isolation and characterization of RNA polymerases have been reported from bacterial groups including proteobacteria [7–9], cyanobacteria [10], gram-positive bacteria [11,12], and deinococcaceae [13].

Historically, RNAP activity has been monitored and measured by utilizing the incorporation of radioisotopically tagged ribonucleotides at the active site of the polymerase, and all activity assays of which we are aware follow this approach. However, the use of radioisotopic reagents is often associated with high

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[2] *Abbreviations used:* DIG, digoxigenin; ELISA, enzyme-linked immunosorbent assay; RNAP, DNA-directed RNA polymerase.

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reagent cost, high disposal cost of isotopically tagged materials, strict requirements for training of laboratory personnel and monitoring of lab areas where isotopes are in use, and (for high energy/short half-life isotopes such as ^{32}P) short reagent shelf life. For these reasons, we sought a nonradioisotopic technique for quantitative analysis of RNAP activity.

Our particular focus is on the plastid-encoded RNAP of flowering plants, a multisubunit RNAP that is structurally homologous [e.g., 3,14,15] and functionally analogous [14,16] to the eubacterial enzyme. To easily measure RNAP activity in large numbers of chromatographic fractions and to easily assess effects of structural alterations on RNAP activity, we have developed an alternative method for assaying the RNA-synthetic activity of bacterial-type multisubunit RNAP. This method appears to give results similar to those of previously reported assays utilizing radioisotopic labeling, is significantly less expensive, is well-suited to microplate, high-throughput use, and avoids many of the negative aspects of working with radioisotopic reagents.

Materials and methods

Enzymes and biochemicals

Ribonucleotides were purchased from Roche Applied Science, calf thymus and T7 phage DNAs from Sigma, T7 and *Escherichia coli* (both core and holoenzyme) RNA polymerases from Epicentre Technologies, and RNasin RNase inhibitor from Promega. Preparations of RNase-free DNase I were purchased from Promega, Ambion, and Roche. pHT-T7f1σ plasmid DNA [5] was the generous gift of Dr. Richard Ebright (Rutgers University). rRNA is included in the RiboGreen RNA Quantitation kit from Molecular Probes.

In vitro transcription reactions

Transcription was performed in 20-μL reaction volumes. The reaction mixture contained 40 mM Tris, pH 8.0, 10 mM $MgCl_2$, 100 mM KCl, 10% glycerol, 0.5 μg/μL bovine serum albumin, 2.5 mM dithiothreitol, 1 U/μL RNAsin (Promega), 200 μM each NTP, and varying amounts of RNA polymerase and template DNA. Our standard reaction uses 1 U of *E. coli* RNAP and 100 ng of plasmid or calf thymus DNA. Reactions were incubated at 37 °C for 30 min unless otherwise indicated; this time typically resulted in at least 90% maximal RNA synthesis (e.g., Fig. 4).

Detection of DIG- and biotin-labeled RNAs

In vitro transcription reactions were performed as described above except that the UTP concentration was reduced to 120 μM, and 70 μM DIG–UTP (Roche) and 10 μM biotin–UTP (Roche) were added to the reaction mixture, following the method of Eberle and Seibl [29] for measuring reverse transcriptase activity. After incubation at 37 °C for 60 min, reactions were transferred to a microtiter plate whose wells were precoated with avidin. Biotin- and DIG-labeled transcripts were detected by ELISA according to the instructions provided by the manufacturer (Roche).

In the experiment shown in Fig. 1, panels B and C, duplicate samples were analyzed by ELISA, run on a nondenaturing agarose gel (Invitrogen), and detected by ethidium bromide fluorescence (Sigma). DNA size standards (Hyperladder I, Bioline) are 200, 400, 600, 800, 1000, 1500, 2000, 2500, 3000, 4000, 5000, 6000, 7000, 8000, and 10,000 bp in length. Nucleic acids were blotted to a Nytran membrane (Schleicher and Schuell)

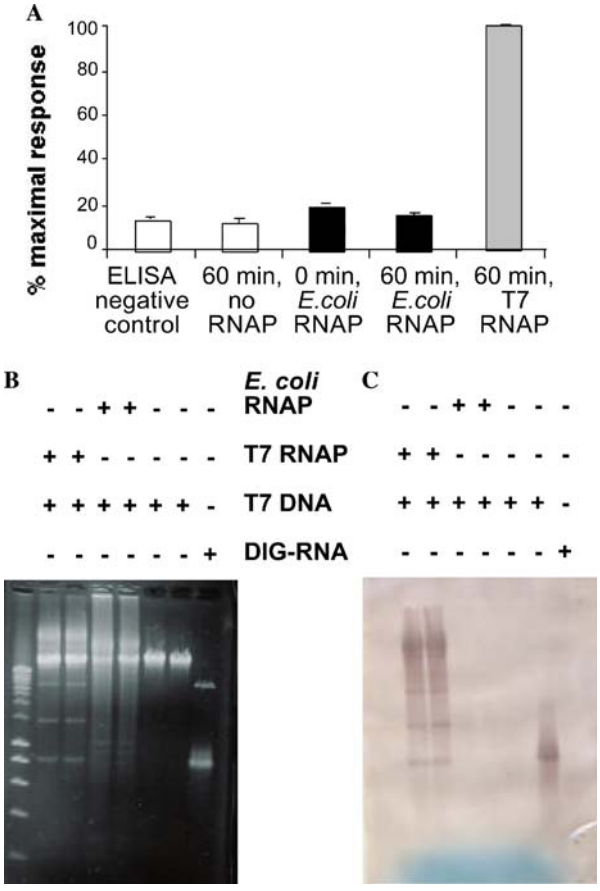

Fig. 1. *Escherichia coli* RNA polymerase does not incoporate DIG-conjugated nucleotides into transcripts in vitro. (A) T7 and *E. coli* RNAP were incubated with T7 DNA in the presence of biotin- and DIG-labeled UTP for the indicated amounts of time as described under Materials and methods. Samples were then transferred to avidin-coated microtiter plates and the amount of incorporated DIG was measured by ELISA. (B) Samples prepared as in A were run on an agarose gel and stained with ethidium bromide. The left most lane contains DNA size markers (see Materials and methods), and the right most lane contains a DIG-labeled RNA standard. (C) Anti-DIG immunoblot of the gel shown in B.

and tested immunochemically for presence of DIG using the DIG DNA Detection kit from Roche.

RNA polymerase assay with RiboGreen

After in vitro transcription (see above), samples were incubated with 4 U DNase I for 10–15 min at 37 °C to remove DNA template before RNA quantification. To enhance DNase efficiency [17], Ca^{2+} was introduced by adding 2 μL of vendor-supplied 10X buffer to each reaction (10X buffers contained 100 mM Tris, pH 8, 25 mM (Ambion) or 100 mM (Promega) $MgCl_2$, 5 mM (Ambion) or 10 mM (Promega) $CaCl_2$; both buffers gave equal activity in our hands). DNase I from Ambion seemed to give more complete digestion than DNase tested from Promega or Roche (data not shown; consistent with [18]). After digestion, sample volumes were increased to 200 μL by the addition of RNase-free TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), and DNA fragments were removed by ultrafiltration using either Microcon-100 centrifugal filtration units (Millipore) or Multiscreen-PCR 96-well vacuum filtration plates (Millipore). The most effective clearance of template DNA fragments was recorded with Microcon filters, but the filter plates more reproducibly yield a low DNA background.

Samples added to Microcon filters were spun at 510*g* for 12 min at room temperature, and then 20 μL of TE was added to facilitate sample recovery. Filters were then inverted and samples recovered by 3 more min of centrifugation at 1020*g*; 180 μL RiboGreen RNA Quantitation Reagent (Molecular Probes), diluted 1/900 in TE, was added to each sample and incubated at room temperature, protected from ambient light, for 10 min. This dilution of RiboGreen (1/1000 of the supplied stock solution) yields a final solution concentration of 150 nM. To reduce assay cost, Microcon filters can be used at least twice without apparent nucleic acid retention or loss (data not shown).

Samples in Multiscreen filter plates were filtered under vacuum following the manufacturer's instructions. Filters were washed once with 100 μL TE, based on experiments with Microcon filters as discussed under Results and discussion, and samples were recovered in 100 μL TE. Then 100 μL RiboGreen RNA Quantitation Reagent (Molecular Probes), diluted 1/500 in TE, was added to each sample and incubated at room temperature, protected from ambient light, for 10 min (final concentration is again 150 nM RiboGreen).

RiboGreen fluorescence was quantified by one of three methods. (1) Samples were transferred to low-volume cuvettes (National Scientific Products "microsert"s) and fluorescence was measured in a Turner Biosystems TD-360 fluorometer with blue-light source and microscale sample insert. (2) Samples in wells of a 96-well microtiter plate were placed on a blue-light box with orange filter (Claire Chemical), fluorescence was captured on a consumer-grade digital camera (Canon G1), and pixel density was quantified using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). (3) Fluorescence of samples in wells of a 96-well microtiter plate was determined in a fluorescence microplate reader (BioTek Instruments FLx800). The most inexpensive method—the blue-light box and digital camera—gave surprisingly good precision and linearity of response but suffered from a very small dynamic range; the Turner Biosystems fluorometer yielded a higher dynamic range but had the lowest linearity and highest noise of the three methods. The greatest throughput speed, precision, linearity, and dynamic range were found with the fluorescence microplate reader; its only drawback is cost, though this is still only roughly one-half that of a liquid scintillation counter. (The comparative data are not included in this report, but are available from the authors on request.)

Comparison of Figs. 4 and 5, performed several months apart with different ultrafiltration methods and different means of measuring fluorescence, illustrates that maximal signal may vary significantly between experiments over time, depending on reaction conditions, reagent age, and choice of equipment. Relative levels of transcription with regard to dependence on reaction duration and component concentrations are reproducible (data not shown), but comparison of absolute levels of transcription across assays requires additional internal controls.

Data analysis and presentation

All data are presented as means of duplicate samples, and error bars indicate ± one standard deviation. "Relative fluorescence" was calculated by first subtracting the mean fluorescence of the assay blank from readings for all other samples and second normalizing readings to the mean fluorescence intensity of 100 ng rRNA treated identically as experimental samples (defined as 100 units of relative fluorescence). For samples that were subject to ultrafiltration, rRNA control used for normalization was also filtered. All data are representative of at least two independent experiments.

Results and discussion

Basic transcription reaction conditions

The 4-decade history of study of RNA polymerases has seen a comparatively wide range of assay conditions used by various investigators. As our goal is to develop an assay for studying plastid RNA polymerase from flowering plants, incorporating commercially available bacterial RNAP as an assay control, we combined features of

several published in vitro transcription systems for our basic reaction mixture (see Materials and methods). This combination is based most closely on that of Stirdivant et al. [19], with minor modifications to broaden compatibility with other published studies and to optimize activity in our hands. Of greatest impact on transcription efficiency (data not shown) were the addition of magnesium chloride to 10 mM (following [20,21]) and bovine serum albumin to 0.5 mg/mL (following [22]). Investigators studying RNAP from plastids (e.g., maize [15], spinach [16,20], pea [23], mustard [21], and *Euglena* [24]), cyanobacteria (e.g., [10]), archaea (e.g., [25]), enteric bacteria (e.g., [9,26,27]), and gram-positive bacteria (e.g., [11]) have used similar reaction conditions, and so the system we describe should prove widely applicable.

The E. coli RNA polymerase active site appears to reject bulky modified nucleotides

All the published studies on quantitative analysis of RNAP of which we are aware detect RNA-synthetic activity via the incorporation of radiolabeled nucleotides into RNA. For reasons of logistics and cost, as discussed above, we sought a nonisotopic detection method. Our first idea was to incorporate modified nucleotides into the newly synthesized RNA and detect them immunochemically. This approach is broadly applied in molecular biological methods today, is commonly used with single-subunit phage-type RNAP (e.g., [28]), and forms the basis for several commercial RNA-labeling kits. To our surprise, although an ELISA-type method of detecting transcripts with modified nucleotides (see Materials and methods) effectively demonstrated RNA-synthetic activity of T7 RNAP, purified RNAP from *E. coli* did not generate any detectable production of dual-labeled transcripts (Fig. 1A). Further investigation confirmed that the *E. coli* enzyme was transcriptionally competent under these assay conditions, as shown by polymerase-dependent accumulation of nucleic acids visualized by ethidium bromide fluorescence in an agarose gel (Fig. 1B). When nucleic acids from the gel shown in (Fig. 1B) were blotted to nylon and probed for DIG-labeled transcripts, the apparent paradox was resolved: as shown in (Fig. 1C), in vitro transcription reactions from T7 RNAP but not from *E. coli* RNAP contain DIG-labeled RNAs. Probing blots for the presence of biotinylated bases by using alkaline phosphatase-coupled streptavidin gave similar results (data not shown). Thus it seems that under these assay conditions, the *E. coli* RNAP is transcriptionally competent but unable to incorporate biotin- and DIG-modified nucleotides. This conclusion is consistent with crystallographic studies of the structurally similar [30] *Thermus aquaticus* RNAP, which show a spatially constrained active-site channel and a narrow tunnel-like opening through which it is proposed that nucleotides diffuse into the catalytic site [3,30,31].

A three-step assay utilizing RiboGreen fluorescence

With the failure of the biotin/DIG dual-labeling strategy, we reviewed other labeling schemes that would not require the incorporation of bulky modified bases and elected to pursue an approach of in vitro transcription with unmodified nucleotides followed by noncovalent labeling of transcripts with the RNA-binding dye RiboGreen.

As described by Jones and co-workers [32], RiboGreen is a cyanine-based fluorophore that exhibits a fluorescence enhancement of greater than 1000-fold on binding to RNA in solution and has spectral properties that allow RNA/RiboGreen complexes to be detected using standard fluorescein filter sets. RiboGreen has been shown to be useful for detecting as little as 100 pg of RNA in a 100-μL volume and gives linear fluorescence response to increasing RNA concentrations over nearly two orders of magnitude ([32]; see also Fig. 2A). It thus seemed well-suited to this application. Unfortu-

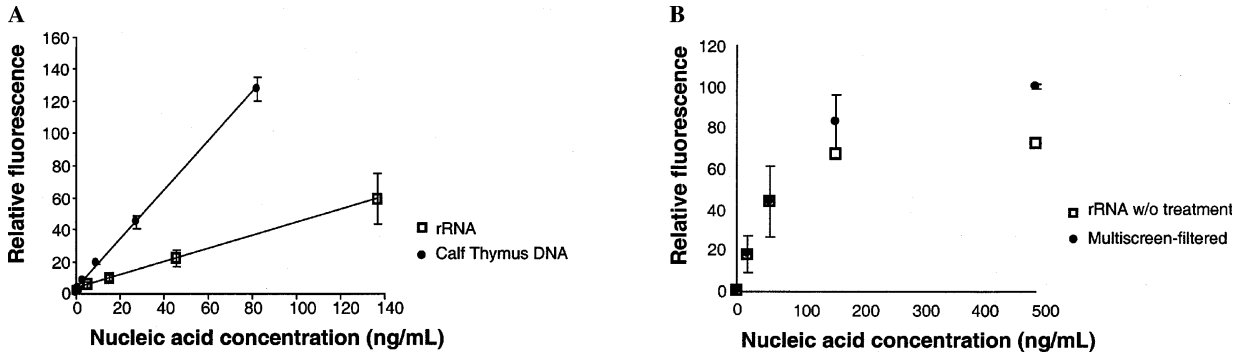

Fig. 2. RiboGreen fluorescence as a function of the amount of nucleic acid present in solution. (A) Varying concentrations of calf thymus DNA and rRNA were incubated with 150 nM RiboGreen in TE for roughly 10 min and fluorescence was quantified using a fluorescence microplate reader. Lines are linear least-squares fits to the data. (B) Replicate samples of rRNA either were transferred directly to a microtiter plate (open squares) or were first subject to ultrafiltration in a Multiscreen-PCR filter plate (closed circles). RiboGreen was added to all samples to a final concentration of 150 nM, and fluorescence was measured as in A.

nately, as shown in Fig. 2A, RiboGreen also binds to double-stranded DNA and indeed gives a ~5-fold higher fluorescence signal upon binding to DNA than to an equal mass of RNA. Given that transcription reactions must contain template DNA, this necessitates enzymatic digestion of the template DNA after transcription and before addition of RiboGreen.

Contrary to data reported by Jones et al. (Fig. 7 of [32]), we found simple DNase I digestion to be insufficient. In typical experiments with 100 ng to 1 μg of template DNA, DNase digestion alone resulted in a roughly 30% reduction in the fluorescence signal of RiboGreen binding to DNA (Table 1). The 70% signal remaining presumably reflects incomplete digestion of the DNA and the subsequent binding of RiboGreen to oligomers; staining of similar samples run on agarose gels revealed a smear of small DNA fragments up to about 200 bp (data not shown). These fragments can be effectively removed by the addition of an ultrafiltration step, which typically reduces the DNA background from ~200% to less than 10% of rRNA control fluorescence (2% in the experiment shown in Table 1). This background is further reduced if an additional wash step is added (Table 1).

The ultrafiltration does not significantly reduce the signal attributable to RNA and indeed typically increases it slightly (e.g., see Fig. 2B). This curious result is consistent with the inhibitory effect of some common buffer components on RiboGreen fluorescence as reported by Jones and co-workers [32]. Such inhibitors include many salts, organic solvents, and detergents. Table 1 shows that ultrafiltration before addition of RiboGreen largely ameliorates the inhibitory effect for two typical buffer components. Thus the ultrafiltration step provides an added benefit to this assay procedure, as it allows for measurement of enzyme activity under buffer conditions that would otherwise render transcript quantification unreliable.

We have used both Microcon-100 spin filters and Multiscreen PCR ultrafiltration plates (with a vacuum manifold), and both methods give satisfactory performance in this procedure. The use of filter plates cuts assay time significantly for large assays, making the processing of a 100 samples possible in a 4-h period by an investigator with minimal training. Thus this assay is suitable for the screening of libraries of enzyme inhibitor candidates or the monitoring of chromatographic fractions in enzyme purification, a key design goal of this investigation. Fig. 3 summarizes the three-step RiboGreen-based RNA polymerase assay procedure as compared to the conventional radioisotopic method.

The activity of Escherichia coli RNAP as measured by the RiboGreen method is consistent with published precedents with regard to reaction time course and assay sensitivity

To convince ourselves that this assay system yields the results expected on the basis of published studies using radioisotopic methods, we performed several preliminary tests on the activity of *E. coli* RNAP. As shown in Fig. 4, maximal synthesis of RNA under our assay conditions occurs in about 20 min, somewhat slower than data published by Mehrpouyan and Champney [26]. The nonzero level of transcription at the "zero time" point is a reproducible feature of these experiments and probably reflects transcription continuing during the first 5 min of template DNA digestion, as these experiments did not explicitly include any agent to denature or otherwise inhibit RNAP activity after the initial transcription period.

The ability of this method to detect RNA polymerase activity under conditions of limiting template or polymerase is also consistent with previous reports. As shown in Fig. 5A, transcription is easily detectable with as little as 5 ng of either plasmid or calf thymus DNA as template. Moreover, this preparation of sigma-saturated RNAP holoenzyme reaches a level of transcription that saturates the fluorescent detection at severalfold lower

Table 1
Effectiveness of ultrafiltration step in minimizing effect of agents that interfere with RiboGreen fluorescence in the presence of RNA

Interfering agent	Concentration expected to change fluorescence signal by ~10%[a]	Relative fluorescence[b]		
		Unfiltered	Filter[c]	Filter and wash[d]
None	–	100	100	100
DNA (0.5 μg/mL)	0.015 μg/mL	~400[e]	~400[e]	~400[e]
DNA (0.5 μg/mL, plus DNase)	–	~300[e]	102	100[f]
LiCl (190 mM)	20 mM	9	60	100[f]
$MgCl_2$ (2.4 mM)	0.5 mM	15	70	100[f]

[a] From [32].

[b] Fluorescence signal normalized to that of 0.5 μg/mL rRNA alone; see Materials and methods.

[c] "Filter" indicates treatment in the standard assay described under Materials and methods—sample prepared in 20 μL volume, 180 μL TE added, spun in Microcon-100 filter for 12 min at 510*g*, sample recovered.

[d] After standard filtration as described in footnote c, 200 μL of TE was added and sample was spun again for 12 min at 510*g* before recovery.

[e] Approximate value based on fluorescence of diluted samples; combined fluorescence of 0.5 μg/mL DNA + 0.5 μg/mL rRNA is beyond linear portion of fluorescence response curve (see Fig. 2).

[f] Statistically indistinguishable from RNA control.

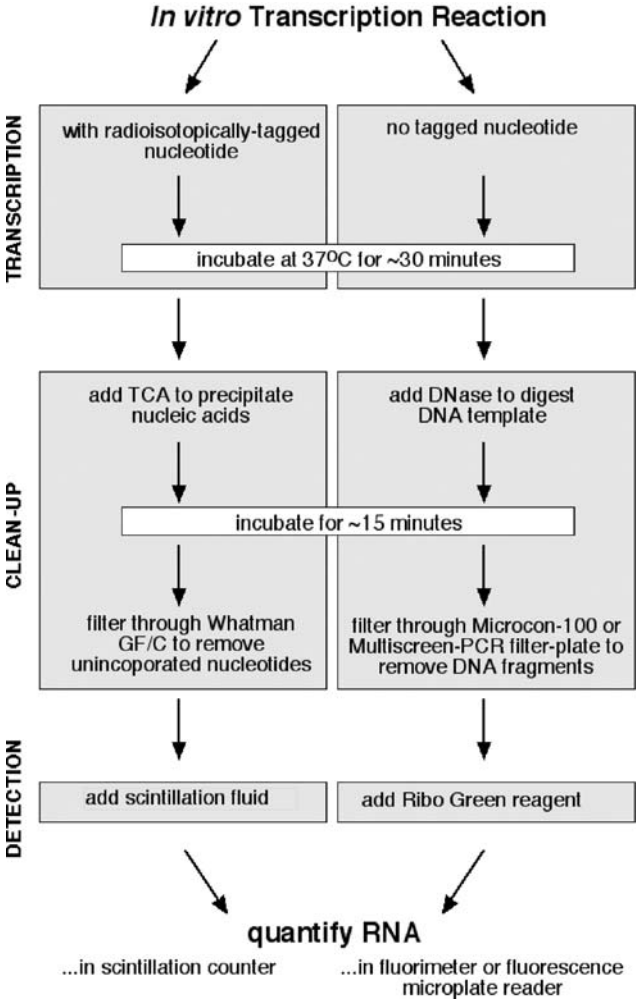

Fig. 3. Comparison of RNA polymerase activity assays using radioisotopic and RiboGreen detection. The right-hand side of this schematic summarizes the RiboGreen method for quantitative analysis of RNA polymerase activity. The left-hand side presents the standard method of RNA polymerase assay, which depends on the incorporation of radiolabeled ribonucleotides. Both methods are discussed in the text.

levels of plasmid template than bulk DNA template, as expected [9,33].

One unit of RNA polymerase activity is defined as the amount of active enzyme that yields 1 nmol of nucleotide incorporation in 10 min at 32 °C [27]. Investigators purifying a variety of multisubunit RNA polymerases have reported concentrations of approximately 10 Units/mL of crude lysate, with purified preparations ranging from 200 to 1000 U/mL (e.g., [9,27]). Fig. 5B shows that the present method allows measurement of the activity of as little as 0.1 Units of purified *E. coli* RNAP. Thus this method should be adequate to the detection of the RNAP in 10 μL of crude lysate or less than 1 μL of purified enzyme (as shown here). Indeed, increasing the total volume of the in vitro transcription reaction to 30 μL (to accommodate 10 μL of lysate) has minimal impact on RNA synthesis, and under these conditions we have measured RNAP activity in 10 μL of crude and fractionated chloroplast lysates (data not shown).

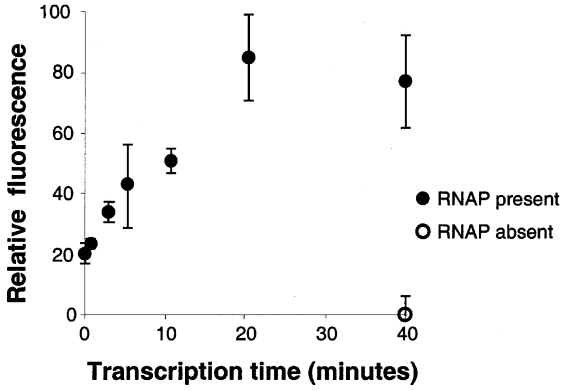

Fig. 4. Timecourse of *E. coli* RNA polymerase as measured by RiboGreen fluorescence. 1 Unit of RNAP holoenzyme (filled circles) or an equivalent volume of TE (open circle) was incubated with 1 μg pHT-T7f1σ plasmid DNA for the indicated amounts of time under standard assay conditions as described under Materials and methods. After DNase digestion, samples were filtered through Microcon-100 s. Fluorescence was measured on a fluorimeter.

Relative advantages of the RiboGreen method and radioisotopic precedents

When compared to the conventional radioisotopic procedures for quantifying activity of multisubunit RNAP, detection of transcripts with RiboGreen has three clear benefits. First, it avoids the short shelf life, high disposal cost, and high regulatory cost of working with radioisotopic tags. Second, although it still involves a reagent that is regarded as biohazardous, it is not added until the final step of the procedure, and so the risk of contamination and the need for special disposal steps are considerably reduced. (RiboGreen has not been tested for mutagenic or toxic effects, but because of its affinity for nucleic acids, the manufacturer recommends treating it as biohazardous and disposing of it in the same fashion as other nucleic acid-binding dyes.) Finally, despite the relatively high expense of ultrafiltration membranes, substitution of RiboGreen for ^{32}P- or ^{3}H-labeled ribonucleotides yields significant cost savings. Radioisotopic assay costs of ~$3 (1 μCi ^{3}H-UTP [22,34]) to ~$8 (10 μCi ^{32}P-UTP [20,21,24]) per sample (excluding cost of polymerase) are reduced to ~$1.75 with the use of RiboGreen and multiwell ultrafiltration plates. Moreover, the cost of equipment is considerably lower: a fluorescence microplate reader can typically be purchased for less than half the cost of a liquid scintillation counter. The reduction in cost should afford investigators greater flexibility in experimental design.

The ability to use 96-well microplates for all steps of the procedure, from transcription reaction through de-

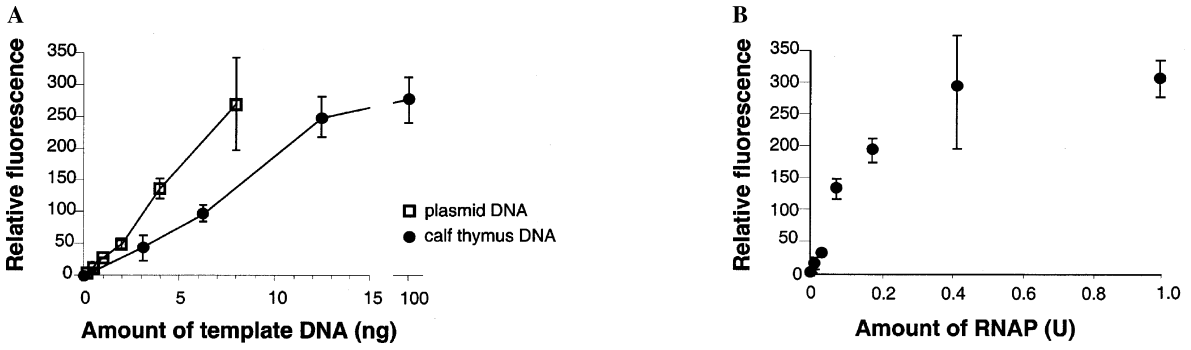

Fig. 5. Sensitivity of the RiboGreen-based RNA polymerase assay to low levels of transcription. (A) 1 Unit of *E. coli* RNAP holoenzyme was incubated with varying concentrations of calf thymus DNA (closed circles) or pHT-T7f1σ plasmid DNA (open squares) for 37 min under standard assay conditions. (B) Varying amounts of *E. coli* RNAP holoenzyme were incubated with 8 ng pHT-T7f1σ plasmid DNA for 37 min under standard assay conditions. For both panels, ultrafiltration utilized a Multiscreen-PCR filter plate, and fluorescence intensity was measured on a fluorescence microplate reader.

tection, also enhances the usefulness of this method. As noted previously, the desire to be able to assay large numbers of chromatographic fractions for RNAP activity has strongly motivated this study. Moreover, RNA polymerase, because of its central role in gene expression, is a tempting target for drug discovery. Several existing antibiotics (notably rifampicin [35] and α-amanitin [36,37]) and anti-cancer drugs (e.g., actinomycin D [37,38], ecteinascidin-743 [39]) act through effects on RNAP. Widespread resistance to rifampicin among clinical *Mycobacterium tuberculosis* isolates [40] is fueling a search for alternative molecular therapeutic agents. The RiboGreen-based assay should significantly increase the facility with which panels of all such compounds can be screened for their effects on polymerase activity. As test cases, we have verified the ability of this assay to detect the inhibitory action of actinomycin D and tagetitoxin, an inhibitor of eubacterial and plastid RNAP [41] (data not shown).

This procedure has been developed with the intent of simple quantification of transcription in vitro. Because labeling of RNA occurs posttranscriptionally and noncovalently, this method is unlikely to permit the specific identification of transcripts in high-resolution electrophoretic gels, as for mapping of transcription initiation or termination sites. For such applications, an alternative approach such as amino-allyl labeling [42,43] would probably be more appropriate even though it would necessitate additional filtration and labeling steps.

Conclusion

We have described a procedure for quantitative analysis of in vitro transcription by multisubunit RNA polymerases using the fluorescent RNA-binding dye RiboGreen. The method involves a transcription reaction with unlabeled nucleotides, followed by enzymatic digestion of template DNA and removal of DNA fragments by ultrafiltration before addition of RiboGreen and subsequent fluorometric quantification. This assay yields data consistent with published reports using radioisotopic detection with regard to transcription time course (Fig. 4) and sensitivity (Fig. 5) and has the benefits of greater convenience, reduced cost, and restriction of the most hazardous reagents to the final step of the procedure. All manipulations can be easily accomplished in multiwell plates, allowing high-throughput application in screening chromatographic fractions or libraries of inhibitors, promoters, etc. For most uses except those requiring covalent labeling of newly synthesized RNA, this should prove an attractive alternative to radioisotopic precedents.

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