

Luminescence Resonance Energy Transfer-Based High-Throughput Screening Assay for Inhibitors of Essential Protein-Protein Interactions in Bacterial RNA Polymerase

Veit Bergendahl,¹ Tomasz Heyduk,² and Richard R. Burgess^{1*}

McArdle Laboratory for Cancer Research, University of Wisconsin—Madison, Madison, Wisconsin 53706,¹ and Edward A. Doisy Department of Biochemistry and Molecular Biology, St. Louis University Medical School, St. Louis, Missouri 63104²

Received 23 September 2002/Accepted 18 December 2002

The binding of sigma factors to core RNA polymerase is essential for the specific initiation of transcription in eubacteria and is thus critical for cell growth. Since the responsible protein-binding regions are highly conserved among all eubacteria but differ significantly from eukaryotic RNA polymerases, sigma factor binding is a promising target for drug discovery. A homogeneous assay for sigma binding to RNA polymerase (*Escherichia coli*) based on luminescence resonance energy transfer (LRET) was developed by using a europium-labeled $\sigma 70$ and an IC5-labeled fragment of the β' subunit of RNA polymerase (amino acid residues 100 through 309). Inhibition of sigma binding was measured by the loss of LRET through a decrease in IC5 emission. The technical advances offered by LRET resulted in a very robust assay suitable for high-throughput screening, and LRET was successfully used to screen a crude natural-product library. We illustrate this method as a powerful tool to investigate any essential protein-protein interaction for basic research and drug discovery.

The need for new antimicrobial drugs has become an obvious scientific challenge since inappropriate use and clinical conditions have favored selection for strains resistant to an increasing number of antibiotics. In order to accelerate the pace at which we compete with nature, high hopes lie in the exploitation of recent advances in genomic and proteomic research. Due to the increasingly detailed structural and mechanistic information about proteins involved in the central processes of the life cycle of a cell, such as its replication, transcription, and translation, rationally designed assays can be developed to find inhibitors of very specific and vulnerable targets within these biochemical machines. With this paper, we illustrate the development of a luminescence resonance energy transfer (LRET)-based high-throughput screen for antimicrobial drugs based on the identification of a crucial protein-protein interaction in RNA polymerase (RNAP).

Like the ribosome and DNA polymerase, the bacterial transcription machinery appears to offer an attractive target for drug discovery and rational drug design. Any inhibitor of the core RNAP assembly with a major sigma factor to form the holoenzyme would inhibit the initiation of transcription and therefore prevent the growth and eventually the survival of a cell. Researchers have previously identified by biochemical methods the regions in *Escherichia coli* RNAP (a coiled-coil region between amino acid residues 260 and 309 in the β' subunit of *E. coli*) (3, 4) and its major sigma factor $\sigma 70$ (region 2.2) that are largely responsible for sigma-core interactions (10, 12). These results have now been confirmed by the struc-

tures of *Thermus aquaticus* holoenzyme (14, 22). Each protein has a remarkably conserved (>80%) amino acid sequence (11, 13) among all known eubacteria within these regions. These homologies suggest a highly conserved structure and function within the holoenzyme form of RNAP, which is crucial for the correct initiation of transcription. Any inhibitor of this interaction can thus be expected to be a broad-spectrum antibiotic. This is supported by the fact that the induction of expression of the β' fragment (residues 260 through 309) stops cell growth in *E. coli*. (2). No $\sigma 70$ homologue has been found in archaea and mammalian cells except for sigma factors in mitochondria (21) and chloroplasts (1). However, these do not show a significant homology to their bacterial counterparts. This fact implies that there is very little chance of a potential new antibiotic interfering with eukaryotic RNAP assembly, which could otherwise lead to serious side effects if it were used as a drug.

In order to screen for inhibitors of RNAP assembly with sigma, an assay simpler, faster, and more reliable than the ones currently available (6) was developed for the formation of the $\sigma 70$ - β' complex of *E. coli*. We decided to use LRET, a recent modification of fluorescence resonance energy transfer (FRET) that can create the desired signal upon protein binding (15, 17, 18). The more general term “luminescence” instead of “fluorescence” (as in FRET) indicates that lanthanide emission is technically not considered fluorescence (i.e., arising from a singlet-to-singlet transition). The details of LRET have been elegantly described in recent reviews by Selvin (8) and Heyduk (16) and will be covered only briefly here. A quantitative description of the effect is based on the Förster theory that describes the decrease of energy transfer as inversely proportional to the sixth power of the distance between the two dyes and is applicable to FRET and LRET. The advantages of LRET result from the prolonged fluorescent lifetimes of the

* Corresponding author. Mailing address: McArdle Laboratory for Cancer Research, University of Wisconsin—Madison, 1400 University Ave., Madison, WI 53706. Phone: (608) 263-2635. Fax: (608) 262-2824. E-mail: burgess@oncology.wisc.edu.

lanthanide-based donor fluorophores like Eu and Tb (more than microseconds up to several milliseconds) compared to the short lifetime of most organic-based fluorophores used in FRET, like Cy5 or IC5 (picoseconds up to a microsecond), so that LRET offers mainly technical advantages over FRET.

Heyduk and coworkers used LRET to measure DNA binding to $\sigma 70$ in holoenzyme by using an Eu chelate as a donor and Cy5 as an acceptor (7). They were able to show its applicability within this system. We adapted the principle by exchanging the Cy5-labeled polynucleotide with an IC5-labeled β' fragment (residues 100 through 309 N-terminally fused to a heart muscle kinase [HMK] recognition site and a His₆ tag). For the resulting homogenous assay, we labeled $\sigma 70$ with a europium–diethylenetriaminepentaacetic acid (DTPA)–7-amino-4-methylcoumarin-3-acetic acid (AMCA) complex as a donor and the HMK-His₆- β' (100-309) fragment with the Cy5 analogue IC5-maleimide (Dojindo) (purification and labeling procedures described in Bergendahl et al. [5]). It is possible to monitor complex formation between $\sigma 70$ and β' simply by looking at the long-lived emission of the acceptor (IC5) due to LRET as an optically measurable signal of complex formation. Measuring time-resolved fluorescence allows one to start signal acquisition after the background fluorescence (potentially from a natural product) and intrinsic acceptor fluorescence have decayed so that all short-lived background fluorescence can be excluded from the measurement. This leads to a highly favorable signal-to-noise ratio and a higher sensitivity and confidence. This is especially important when working with natural-product samples, which often produce a high background fluorescence. The principle of the assay and the structures of the dyes are described in Fig. 1.

The assay can be performed in a multiwell plate and measured by a multiplate reader to accomplish a high-throughput screening of a large number of samples from any chemical library in an automated way. Typical reaction volumes were 10 to 200 μ l where the components, including the test substances, were mixed directly in the multiwell plate before the plate was measured in the reading device. The very sensitive nature of such a fluorescence-based assay (typically in the low nanomolar range) provides good accuracy and signal-to-noise ratio, avoiding false-positive hits in the measurement. We have chosen to screen a marine sponge library (19) since it has been successfully screened for cytotoxic compounds. Due to its complexity, it demanded the high performance of the assay and was therefore well suited to evaluate its applicability. We show the robust nature of the assay and its suitability for use as a high-throughput screen.

MATERIALS AND METHODS

Materials and chemicals. Overproduction, labeling, and purification of HMK-His₆- β' (100-309) and $\sigma 70$ (442C) were described previously (5). The origin of the natural-product library comprises privately collected samples of marine sponges (19) extracted with acetone and ethylacetate. The samples (2 to 5 mg [dry weight]) were dissolved in 1 ml of methanol and diluted 1:50 in dimethyl sulfoxide (DMSO) for the assay. All chemicals used were purchased from Sigma unless otherwise indicated in the text. A multiplate reader (VictorV² 1420; Wallac) was used to perform the LRET assay. The following buffers were used: NTG buffer (50 mM NaCl, 50 mM Tris, 5% glycerol [pH 7.9]), storage buffer (50 mM Tris-HCl, 10 μ M EDTA, 0.5 M NaCl, 10% glycerol [pH 7.5]), and TNTw buffer (6 M GuHCl, 50 mM Tris-HCl [pH 7.9], 500 mM NaCl, 0.1% [vol/vol] Tween 20, 400 mM imidazole). Core RNAP was prepared according to the method described by Thompson et al. (20).

LRET assay to test for the inhibition of protein-protein interaction of labeled $\sigma 70$ and β' . The LRET assay was performed in NTG buffer (200- μ l total volume) plus 2.5% DMSO (when library samples were used) with 40 nM $\sigma 70^*$ (labeled protein) and 30 nM β'^* fragment (labeled protein). All assays were performed with refolded $\sigma 70^*$. In the standard assay, the β'^* fragment was added in denatured form directly into the reaction mixture, which, by 10-fold dilution of GuHCl to 0.15 M in the final assay, allowed instant refolding. This was done to prevent precipitation of the refolded labeled protein upon storage before the assay (5). In order to assure that refolding occur and to confirm the results from the experiments with denatured protein being added, some assays were carried out by adding the β'^* fragment in its refolded state.

A stock solution (200 nM) of $\sigma 70^*$ was prepared prior to the assay by a 1:200 dilution of labeled protein (40 μ M) with NTG stored at -20° C in storage buffer. A stock solution (1.25 μ M) of the β'^* fragment was prepared by the dilution of labeled denatured protein (75 μ M in TNTw buffer) to 1 M GuHCl with NTG and NTG plus 6 M GuHCl. First, 10 μ l of that $\sigma 70^*$ stock solution was mixed with NTG buffer (amount adjusted to give a final volume of 200 μ l), the potential inhibitor (dissolved in 1 ml of methanol, diluted 1:50 in DMSO, and 5 μ l of which was applied to the reaction mixture), salt, or solvent was then added, and finally, the 5 μ l of denatured, labeled β'^* stock solution was added. Salts or denaturants were dissolved in NTG buffer according to the desired final concentration to maintain standard buffer conditions. Mixing (pipetting up and down three times) after the addition of each component was very important for reproducible results. The mixture was incubated for 30 min at room temperature and measured in a 96-well plate (Costar 3650) with a multiplate reader (VictorV² 1420; Wallac). For this time-resolved fluorescence measurement, the manufacturer's protocol (LANCE high count 615/665) was used (excitation occurred with 1,000 flashes at 325 nm, measurement was delayed by 100 μ s, and data were acquired for 50 μ s at 615 and 665 nm).

In fluorimetric measurements, it is common to use a second emission wavelength as an internal standard. This allows for the correction of instrument noise but also normalizes the signal for the actual amount of donor, in this particular case. This was possible, since donor and acceptor emission wavelengths were well separated and could be acquired separately with the multiplate reader. The IC5 emission was corrected for the very small amount of signal from the Eu emission band (by cross-talk measurement of a standard) and then divided by the intensity of the Eu signal. The normalization could be included in the overall measurement protocol and is described by the manufacturer of the multiplate reader (Wallac). Fortunately, the nature of this method allowed us to differentiate the loss of signal due to the inhibition of protein-protein binding from simple absorption caused by the inner-filter effect of the substance. This helped us to identify false positives in the actual high-throughput screen.

In vitro transcription. The conditions and procedure in the in vitro transcription assay were according to those published by Landick et al. (9) except for omitting bovine serum albumin, since it could bind potential hydrophobic inhibitors. The assay conditions were as follows: 100 mM NaCl, 25 mM Tris HCl (pH 7.9), 10 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA, 5% glycerol, 0.15 mM ApU (dinucleotide), 20 nM template DNA, 20 nM core RNAP, and inhibitor in final concentration of 2% DMSO, 10 nM $\sigma 70$, 25 μ M concentrations each of ATP and CTP, 10 μ M GTP, and 20 μ Ci of [α -³²P]GTP/100 μ l, added in the order listed. For the template, we amplified a 694-bp fragment from the plasmid pCL185 by PCR with the primers 5'-GTT TTC CCA GTC ACG AC-3' and 5'-CAG TTC CCT ACT CTC TCG CAT G-3'. Omitting UTP in the reaction mixture and initiating with ApU dinucleotide resulted in a halted complex at position +16 and the transcribed RNA oligonucleotide AUG GAG AGG GAC ACG G. The assay tested for the ability of the RNAP holoenzyme (core plus sigma factor) to recognize a $\sigma 70$ -dependent promoter (T7A1; located after bp 280 of the 694-bp PCR fragment) and to initiate as well as elongate to yield the 16-mer oligonucleotide. Failure of sigma binding to the core resulted in a lack of product.

RESULTS

Labeled as well as unlabeled proteins were shown to be able to form a complex in electrophoretic mobility shift (EMS) assays by using native polyacrylamide electrophoresis gels (5). The different scanning techniques also confirmed the identities of the bands in the EMS assay. Furthermore, the EMS assay showed that the unlabeled β' fragment can compete for binding to the labeled $\sigma 70$. Thus, the unlabeled β' fragment itself

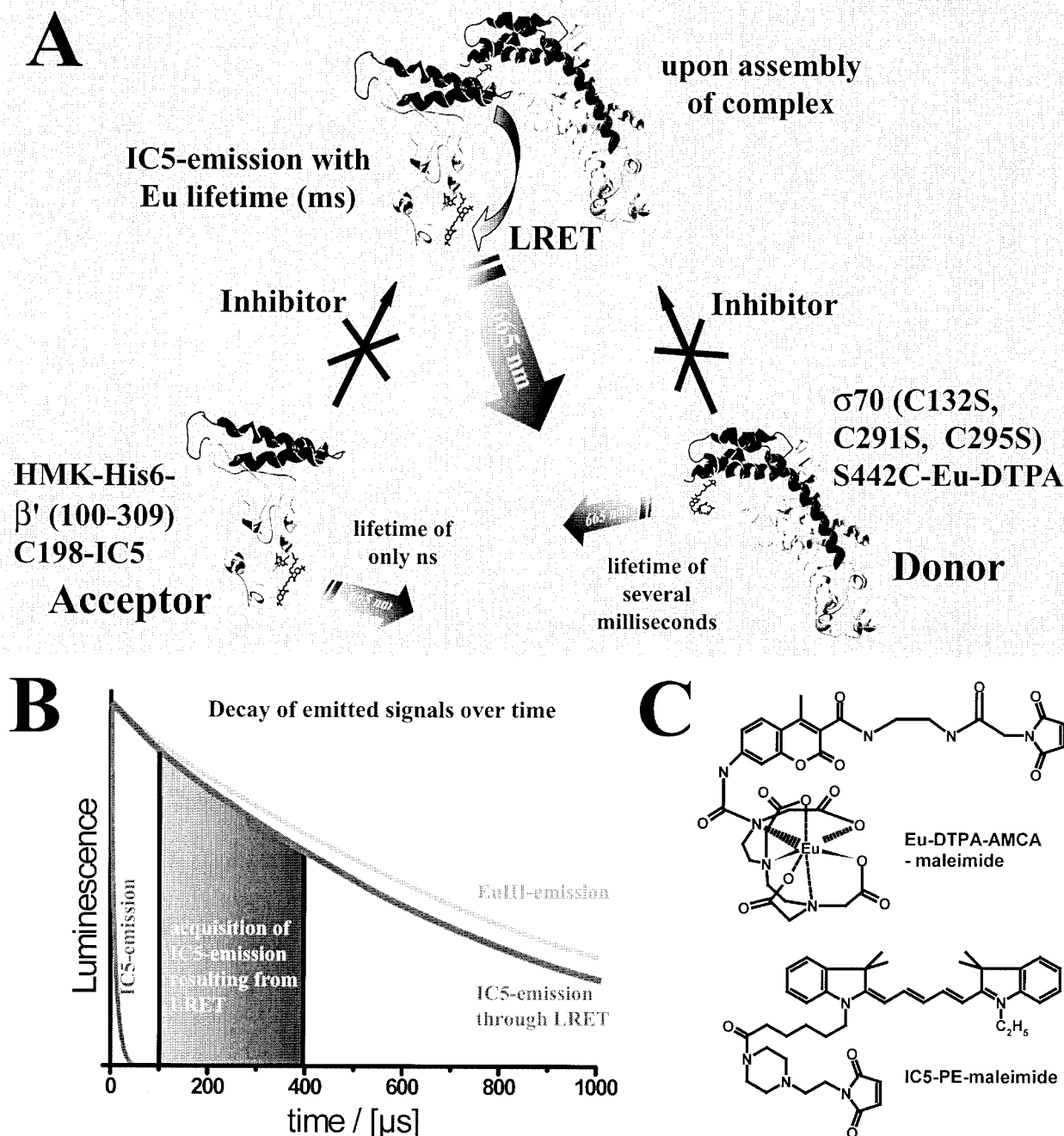


FIG. 1. Principle of the LRET assay. (A) The scheme above shows the principle of how the LRET signal in the assay is created upon binding of the labeled proteins β' (residues 100 through 309) and $\sigma 70$. The fluorescence of the IC5-labeled β' fragment has decayed during the delay of the data acquisition 50 μ s after excitation at 320 nm. Only the Eu emission of labeled $\sigma 70$ and the sensitized IC5 emission in the complex can be observed after the delay due to the characteristic long Eu luminescence lifetime of over 1 ms. (B) In the diagram, the luminescence is plotted versus the time. The graph shows the intensity of the luminescence emitted at 615 and 665 nm over time. Whereas the intrinsic IC5 emission has decayed long before the data acquisition starts, the emission from the IC5 sensitized by LRET goes on for over 1 ms. IC5 has a slightly faster decay than the Eu emission. That way, only the IC5 emission due to LRET is acquired in the data acquisition window, whereas the background emission and the intrinsic IC5 fluorescence is excluded. (C) The structures of the fluorophores that were used to derivatize the proteins are shown. The Eu chelate DTPA-AMCA-maleimide was used to label $\sigma 70$. IC5-PE-maleimide served as the label for the β' fragment.

represents a positive control for an agent able to interfere with the binding of the labeled β' fragment and $\sigma 70$ in the assay.

Validation of the LRET assay to test for the inhibition of protein-protein interaction of labeled $\sigma 70$ and β' with unlabeled proteins.

The LRET assay provides a fast and reproducible alternative to the EMS assay (5) to monitor the formation of a protein-protein interaction between the labeled $\sigma 70$ and β' fragment as well as its inhibition. All results of the EMS

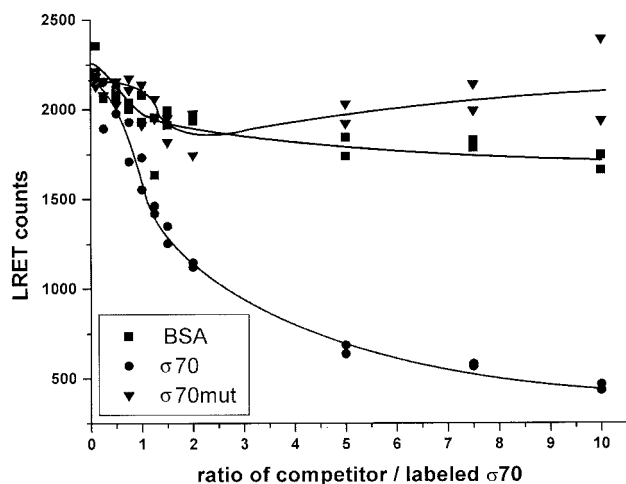


FIG. 2. Competition of unlabeled $\sigma 70$, a nonbinding $\sigma 70$ mutant (D403R) (3), and bovine serum albumin (BSA) with labeled $\sigma 70$ for binding to the β' fragment. Only the $\sigma 70$ (wild type) competes for binding to the β' fragment, while the other proteins show only non-specific binding resulting in a slightly lower LRET count.

assay were reproducible by the LRET assay, and as an example, we were able to show competition of labeled $\sigma 70$ binding to the β' fragment by increasing the amounts of unlabeled $\sigma 70$ (Fig. 2). As a very important feature of the assay, the signal-to-noise ratio was between 10 and 11. The limit of detection within the assay, using the described instrument, was 1 nM labeled $\sigma 70$. A maximum of 250 nM labeled $\sigma 70$ could be applied to the assay before diffusion-controlled LRET occurred.

Influence of salt and organic solvents on the LRET assay. In further experiments, the dependence of salt (NaCl and GuHCl) and solvents (methanol, ethanol, and DMSO) were characterized (Fig. 3). As can be seen, the salt concentration had a major effect on the assay, since the signal drops to 50% when the NaCl concentration was increased from 100 to 400 mM. It is known that this β' fragment interaction with $\sigma 70$ is weakened by increased NaCl concentration. On the other hand, DMSO as a common solvent for natural products to be tested had no significant effect on the assay. The signal in the LRET assay was not critically affected by the amount of DMSO present, up to 2.5%. In the same experiment, ethanol and methanol showed a more significant effect over the range of 1 to 5%.

Performance of the LRET assay in a preliminary screen of 100 sponge extracts. The concentration of inhibitor in the extracts of marine sponges was estimated to be less than 1 μM (assuming a 2-mg sample was 100% of a single component having an approximate molecular mass of 1,000 Da). Out of the 100 samples tested, sample D7 turned out to be the only extract to reduce the signal by roughly 90% in the assay. Samples E1, E12, G1, H4, and H6 showed a loss of signal of more than 50%, which served as an arbitrary threshold (between 55 and 75%). Most samples (like A7 and B12) were significantly less active in the inhibition of binding, judged by the loss of the LRET signal (Fig. 4). The variance of the obtained values for the majority of samples spread over a wide range of up to 40% loss of signal.

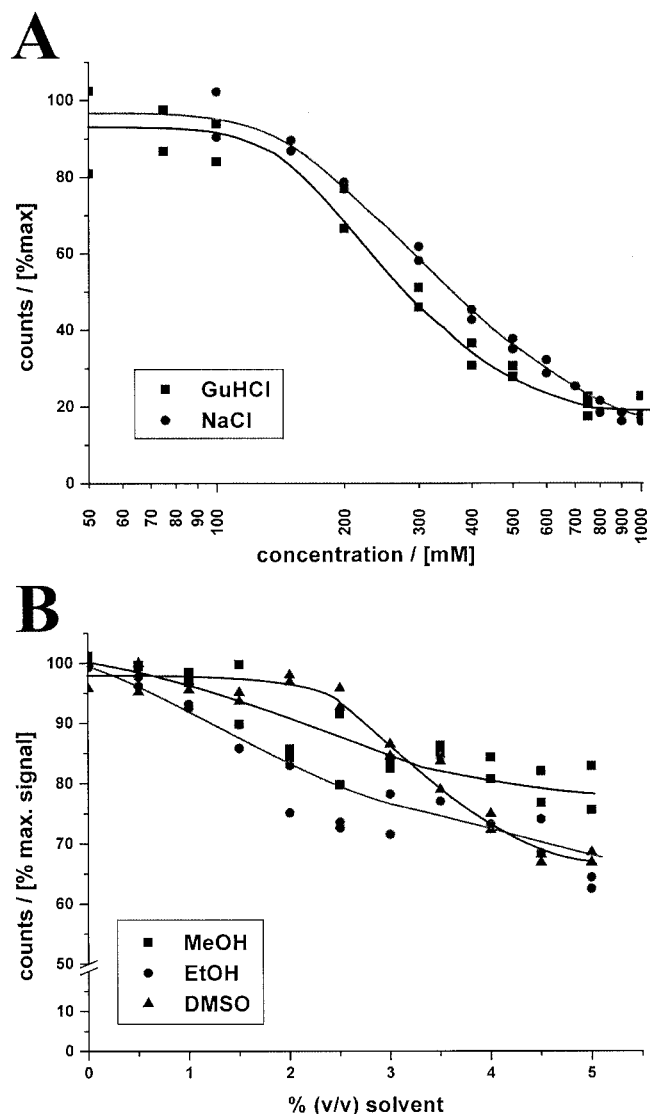


FIG. 3. Dependence of salt concentration and effect of solvent concentration on the LRET assay. (A) With increasing amounts of salt (NaCl, GuHCl), the LRET signal significantly decreases, which should be due to the decreased amount of the $\sigma 70$ - β' complex formed. (B) Increasing amounts of DMSO, methanol, and ethanol (0 to 5%) mixed with the assay buffer prior to the addition of proteins are shown.

Confirmation of hits by the LRET assay and by in vitro transcription. The concentration of inhibitor in the extracts of marine sponges was examined over the range of 30 nM to 2 μM in the LRET assay. Samples A5, B12, E1, E12, G1, H4, and H6 resulted in no or very minor loss of signal in the LRET assay. Sample D7 turned out to be the only extract to give a clear inhibition curve over the examined range (Fig. 5A). This was confirmed by in vitro transcription assays. Samples A5, B12, E1, E12, G1, H4, and H6 resulted in no loss of activity in the in vitro transcription assay, as judged by densitometry of the electrophoretograms of the gels (Fig. 5B). In this assay, only D7 showed a dose-dependent inhibition of transcriptional activity, with a 50% inhibitory concentration (IC_{50}) of around 1 μM , assuming it had the mass and content of the sample

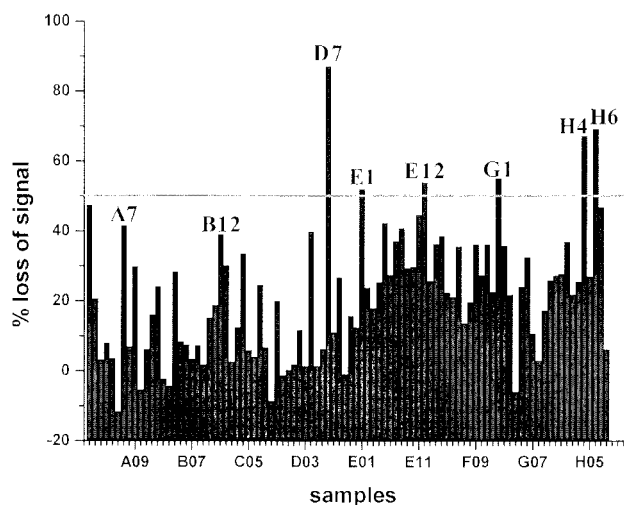


FIG. 4. Screen of 100 extracts from marine sponges by the LRET assay. Inhibition of $\sigma 70$ binding to the β' fragment was tested with 100 extracts from marine sponges (G. Marriott and J. Tanaka). Binding of $\sigma 70$ to the β' fragment could be significantly inhibited by fraction D7, while other fractions show lower inhibition under these conditions. Assuming a molecular mass of 1,000 Da and 100% active content in a 2-mg sample, concentrations used were in the low micromolar range.

mentioned above. There was no significant difference between the IC_{50} s determined by LRET and those derived from the *in vitro* transcription assays.

DISCUSSION

The use of LRET to investigate protein-protein interactions in a homogenous assay has been described before, along with its advantages and challenges. In spite of them, there are not many publications that describe the development of an assay based on this technique and actually document its final application. In this work, we describe a straightforward approach to designing a robust high-throughput assay that can be exploited for any other pair of proteins that appears to be a valid target for drug discovery.

There are several reasons to believe that the primary protein-protein interaction between bacterial core RNAP and sigma factors represents a prime target for drug discovery. The key to the potential of this target is the absolute requirement of sigma binding to core RNAP for the initiation of transcription; no bacterial cell can grow upon uptake of an inhibitor that effectively blocks this interaction. In addition to a very high bioactivity, a good specificity can also be expected since the binding region of both proteins is highly conserved among bacteria and is significantly different from any known eukaryotic analogue. This implies a very low probability for side effects to occur due to interference with human RNAP. The site itself offers another advantage over many potential and specific targets. Since the binding site on the β' subunit of RNAP is suspected to interact with many if not all of the sigma factors of a bacterium, the development of resistance via point mutations against an inhibitor that binds to the β' subunit of RNAP in the binding site is unlikely, since it may impair binding to any of the essential sigma factors. Due to the increasing incidence of antibiotic resistance and the growing

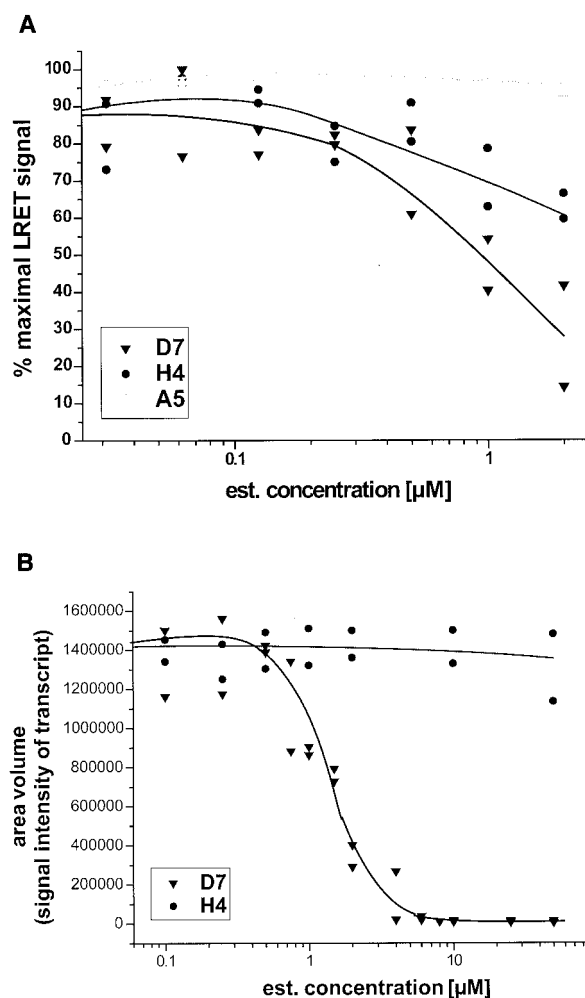


FIG. 5. Validation of the hits by LRET (A) and *in vitro* transcription (B) assays. (A) Some candidates (D7, H4, E1, and G1) resulting from the screen and a sample with no effect (A5) serving as control were tested in the LRET assay over a range of 33 nM to 2 μ M (estimated concentration). The y axis represents the fluorescence signal resulting from LRET due to binding of labeled $\sigma 70$ and the β' fragment. Data from samples E1 and G1 are represented by H4 since they behaved very similar in the LRET assay. (B) To determine the functional effect of an inhibitor of $\sigma 70$ - β' binding, we tested the candidates in an *in vitro* transcription assay. This assay requires the incorporation of $\sigma 70$ into holoenzyme RNAP and tests for its ability to initiate transcription and elongation. The autoradiogram of the sequencing gel was used to quantify the transcription product from a halted complex using a T-less cassette as a template and omitting UTP in the reaction mixture (see Materials and Methods). The resulting 16-mer oligonucleotide can be separated on the gel and quantified by using Molecular Dynamics software. The estimated concentrations in the assay were between 0.1 and 50 μ M (data from samples A5, E1, and G1 are represented by H4 since they behaved very similar in the assay; data from D7 are indicated by triangles).

need for new antibiotics, this has recently become a major issue in drug discovery.

Using LRET to measure sigma binding to core RNAP has been shown by Heyduk and coworkers to be an effective and very sensitive method. We used a well-characterized $\sigma 70(442C)$ mutant (7) that has all-natural cysteine residues mutated to serine residues and was shown to be fully active in

in vitro transcription assays (7). This mutant was derivatized with a DTPA-AMCA-maleimide Eu complex that serves as a donor in the LRET assay. A fragment (residues 100 through 309) of the β' subunit of RNAP with an N-terminal HMK recognition site and His₆ tag fusion was derivatized with IC5-maleimide as the LRET acceptor (5). We were able to show with EMS assays (5) and with spectrometric measurements by using time-resolved fluorescence that the labeled proteins can bind to each other in all combinations with or without the label. As controls, the unlabeled proteins were tested to determine if they could compete with their labeled counterparts. In both assays, EMS and LRET, the unlabeled β' fragment was able to compete with the labeled β' fragment for binding to labeled σ 70. With this data, we concluded that the assay can be used to monitor σ 70 to β' binding and can be used to screen for inhibitors of this protein-protein interaction. The assay represents a fast and sensitive probe for this particular complex formation. Substrates and material either are readily available or can be prepared in simple and efficient procedures. All the labeled protein components show excellent stability during storage, a great advantage when screening large libraries with 10,000 to 100,000 or more substances. Furthermore, the LRET assay has a very high sensitivity so that measurements can be performed at very low protein concentrations of 1 to 100 nM, resulting also in a very low cost per screen.

The suitability of the assay for the high-throughput screening of natural and combinatorial libraries was demonstrated by a screen of 100 extracts of marine sponges. The high demand on the performance of an assay when screening natural-product libraries such as this, containing very complex mixtures of possibly more than 30 compounds, becomes obvious by looking at the variance of the obtained values in the LRET assay. This variance occurs mainly due to fluorophores in the mixtures that can absorb at any of the crucial wavelengths of the assay and is generally referred to as the inner-filter effect. Such an effect could have led to the response of samples B12, E1, E12, G1, H4, and H6 in the LRET assay (Fig. 4 and, in part, 5A), which in the in vitro transcription assay (Fig. 5B) did not show an inhibitory effect on transcriptional activity. It is also conceivable that one of these compounds binds to a region of the β' subunit that is inaccessible in core RNAP and thus leads to an impaired binding by the distortion of protein structure. This underlines the technical and biochemical differences between the two described assays so that compounds that are active in the LRET assay might not have the same effect in the in vitro transcription assay. The LRET assay monitors particularly the binding of sigma to a fragment of the β' subunit from RNAP, whereas the in vitro transcription assay tests the ability of holoenzyme RNAP for transcriptional initiation and elongation. Due to its simplicity and suitability for use in a high-throughput mode, the LRET assay is used to select for potential inhibitors among a large number of candidates present in a library of compounds. This way, the number of samples that have to be investigated by a more informative but also significantly more time-consuming assay, like the in vitro transcription assay, can be minimized.

The LRET assay turned out to be very sensitive and reliable. A very good signal-to-noise ratio of above 10 and the internal standard method helped to distinguish between inhibition and fluorescence quenching by an inner-filter effect of the test

substance. This contributed enormously to avoiding false-positive readings. Also, the very good compatibility of the assay with the use of DMSO as a solvent to add test substances augments its applicability as a high-throughput screen. Since many natural products and most peptides or small molecules from combinatorial libraries have low solubility in water, it is necessary to use organic solvents. In this respect, DMSO represents the most versatile and potent solvent, and since it is often used in libraries, its compatibility is crucial for an assay. One sample (D7) could be identified by the LRET assay as an inhibitor containing extract, and this was confirmed by an in vitro functional assay. The assumptions made to estimate the concentration of the inhibitors should clearly overestimate the amount of active compound present so that it is fair to expect an IC of 10 to 100 nM or less. Initial studies on the ability of the D7 extract to inhibit the cell growth of *E. coli* cells on culture plates were inconclusive due to limited amounts of available material (data not shown). Considering the complexity of the samples, we plan to carry out an automated screen of over 1,500 more sponge extracts before deciding on which samples to characterize in detail.

In the case of natural-product libraries, a positive hit could represent a new class of antibiotic, since no substance with such a mode of action is known. On the other hand, the screen will help to identify known antibiotics for which the mode of activity has not yet been identified or that reveal more than one activity. Together with positive hits from combinatorial libraries, these substances can then serve as lead structures to design and tailor a new compound with desirable characteristics, such as high activity, specificity, stability, and ability to enter the cell and, on the other hand, low side effects, costs, chance, and likelihood of resistance development. In addition, this assay can serve as a powerful tool to investigate the relative binding of different sigma factors and sigma factor mutants to the core.

ACKNOWLEDGMENTS

We thank Katherine Foley, Katherine Coleman, and Nancy Thompson for their support in the lab. We also thank Junichi Tanaka and Gerard Marriott for providing the natural-product library.

This work was supported by NIH grant GM28575 to R.R.B.

REFERENCES

- Allison, L. A. 2000. The role of sigma factors in plastid transcription. *Biochimie* **82**:537-548.
- Arthur, T. M. 2000. Localization and characterization of a sigma subunit-binding site on the *Escherichia coli* RNA polymerase core enzyme. Ph.D. thesis. University of Wisconsin, Madison.
- Arthur, T. M., L. C. Anthony, and R. R. Burgess. 2000. Mutational analysis of $\beta'_{260-309}$, a σ^{70} binding site located on *Escherichia coli* core RNA polymerase. *J. Biol. Chem.* **275**:23113-23119.
- Arthur, T. M., and R. R. Burgess. 1998. Localization of a σ^{70} binding site on the N terminus of the *Escherichia coli* RNA polymerase β' subunit. *J. Biol. Chem.* **273**:31381-31387.
- Bergendahl, V., L. C. Anthony, T. Heyduk, and R. R. Burgess. 2002. On-column tris(2-carboxyethyl)phosphine reduction and IC5-maleimide labeling during purification of a RpoC fragment on a nickel-nitrilotriacetic acid column. *Anal. Biochem.* **307**:368-374.
- Burgess, R. R., T. M. Arthur, and B. C. Pietz. 2000. Mapping protein-protein interaction domains using ordered fragment ladder far-western analysis of hexahistidine-tagged fusion proteins. *Methods Enzymol.* **328**:141-157.
- Heyduk, E., and T. Heyduk. 1999. Architecture of a complex between the σ^{70} subunit of *Escherichia coli* RNA polymerase and the nontemplate strand oligonucleotide. Luminescence resonance energy transfer study. *J. Biol. Chem.* **274**:3315-3322.
- Heyduk, T. 2001. Luminescence resonance energy transfer analysis of RNA polymerase complexes. *Methods* **25**:44-53.
- Landick, R., D. Wang, and C. L. Chan. 1996. Quantitative analysis of tran-

- scriptional pausing by *Escherichia coli* RNA polymerase: his leader pause site as paradigm. *Methods Enzymol.* **274**:334–353.
10. **Lesley, S. A., and R. R. Burgess.** 1989. Characterization of the *Escherichia coli* transcription factor sigma 70: localization of a region involved in the interaction with core RNA polymerase. *Biochemistry* **28**:7728–7734.
 11. **Lonetto, M., M. Gribskov, and C. A. Gross.** 1992. The σ^{70} family: sequence conservation and evolutionary relationships. *J. Bacteriol.* **174**:3843–3849.
 12. **Lonetto, M. A., V. Rhodius, K. Lamberg, P. Kiley, S. Busby, and C. Gross.** 1998. Identification of a contact site for different transcription activators in region 4 of the *Escherichia coli* RNA polymerase σ^{70} subunit. *J. Mol. Biol.* **284**:1353–1365.
 13. **Mittenhuber, G.** 2002. An inventory of genes encoding RNA polymerase sigma factors in 31 completely sequenced eubacterial genomes. *J. Mol. Microbiol. Biotechnol.* **4**:77–91.
 14. **Murakami, K. S., S. Masuda, and S. A. Darst.** 2002. Structural basis of transcription initiation: RNA polymerase holoenzyme at 4 Å resolution. *Science* **296**:1280–1284.
 15. **Selvin, P. R.** 1995. Fluorescence resonance energy transfer. *Methods Enzymol.* **246**:300–334.
 16. **Selvin, P. R.** 1999. Luminescent lanthanide chelates for improved resonance energy transfer and application to biology, p. 457–487. *In* W. Rettig, B. Strehmel, and S. Schrader (ed.), *Applied fluorescence in chemistry, biology and medicine*, 1st ed. Springer-Verlag, Berlin, Germany.
 17. **Selvin, P. R.** 2000. The renaissance of fluorescence resonance energy transfer. *Nat. Struct. Biol.* **7**:730–734.
 18. **Stryer, L.** 1978. Fluorescence energy transfer as a spectroscopic ruler. *Annu. Rev. Biochem.* **47**:819–846.
 19. **Tanaka, J., G. Marriott, and T. Higa.** 2001. Cacosfurans A and B, new furanoditerpenes from a marine sponge. *J. Nat. Prod.* **64**:1468–1470.
 20. **Thompson, N. E., D. A. Hager, and R. R. Burgess.** 1992. Isolation and characterization of a polyol-responsive monoclonal antibody useful for gentle purification of *Escherichia coli* RNA polymerase. *Biochemistry* **31**:7003–7008.
 21. **Tracy, R. L., and D. B. Stern.** 1995. Mitochondrial transcription initiation: promoter structures and RNA polymerases. *Curr. Genet.* **28**:205–216.
 22. **Vassilyev, D. G., S. Sekine, O. Laptenko, J. Lee, M. N. Vassilyeva, S. Borukhov, and S. Yokoyama.** 2002. Crystal structure of a bacterial RNA polymerase holoenzyme at 2.6 Å resolution. *Nature* **417**:712–719.