The binding of sigma factors to core RNA polymerase is essential for the specific initiation of transcription in eubacteria and is thus favorable 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 σ70 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 σ70 (region 2.2) that are largely responsible for sigma-core interactions (10, 12). These results have now been confirmed by the structures 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 σ70 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 σ70-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...
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 ICS (picoseconds up to a microsecond), so that LRET offers mainly technical advantages over FRET.

Heyduk and coworkers used LRET to measure DNA binding to σ70 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 ICS-labeled β’ fragment (residues 100 through 309 N-terminally fused to a heart muscle kinase [HMK] recognition site and a His\(_6\) tag). For the resulting homogeneous assay, we labeled σ70 with a europium–diethylenetriaminepentaacetic acid (DTPA)–7-amino-4-methylcoumarin-3-acetic acid (AMCA) complex as a donor and the HMK-His\(_6\)-β’(100-309) fragment with the Cy5 analogue ICS-maleimide (Dojindo) (purification and labeling procedures described in Bergendahl et al. [5]). It is possible to monitor complex formation between σ70 and β’ simply by looking at the long-lived emission of the acceptor (ICS) 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 multicolor 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 μl 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-His\(_6\)-β’(100-309) and σ70(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 were purchased from Sigma unless otherwise indicated in the text. A multiplate reader (VICTOR\(^2\) 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 μM 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 midaizole). Core RNAp was prepared according to the method described by Thompson et al. (20).

**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 σ70. Thus, the unlabeled β’ fragment itself

LRET assay to test for the inhibition of protein-protein interaction of labeled σ70 and β’. The LRET assay was performed in NTG buffer (200-μl total volume) plus 2.5% DMSO (when library samples were used) with 40 nM σ70* (labeled protein) and 30 nM β’ fragment (labeled protein). All assays were carried out in the standard reaction mixture. In the refolded 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 refolded σ70* in its refolded form to the reaction one hour after the addition of the donor and acceptor.

A stock solution (200 nM) of σ70* was prepared prior to the assay by a 1:20 dilution of labeled protein (40 μM) with NTG stored at –20°C in storage buffer. A stock solution (1.25 μM) of the β’ fragment was prepared by the dilution of labeled denatured protein (75 μM in TNTw buffer) to 1 M GuHCl with NTG and NTG plus 6 M GuHCl. First, 10 μl of that σ70* stock solution was mixed with NTG buffer (amount adjusted to give a final volume of 200 μl), the potential inhibitor (dissolved in 1 ml of methanol, diluted 1:50 in DMSO, and 5 μl of which was applied to the reaction mixture), salt, or solvent was then added, and finally, the 5 μl 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 (VICTOR\(^2\) 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 μs, and data were acquired for 50 μs at 615 and 665 nm). In fluorometric 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 multichannel reader. The ICS 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 multichannel 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 MgCl\(_2\), 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 σ70, 25 μM concentrations each of ATP and CTP, 10 μM GTP, and 20 μCi of [γ-\(^{32}\)P]GTP/100 μl 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 haldex complex at position +16 and the transcribed RNA oligonucleotide AUG GAG AGG GAC 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 haldex complex at position +16 and the transcribed RNA oligonucleotide AUG GAG AGG GAC AC-3’ and 5’-CAG TTC CCT ACT CTC TCG CAT G-3’.
represents a positive control for an agent able to interfere with
the binding of the labeled proteins $\beta'$ (residues 100 through 309) and $\sigma 70$. The fluorescence of the IC5-labeled $\beta'$ fragment has decayed during the delay of
the data acquisition 50 $\mu$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 graphs 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 acquisitions 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 $\beta'$ fragment.

Validation of the LRET assay to test for the inhibition of protein-protein interaction of labeled $\sigma 70$ and $\beta'$ with unla-
beled 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 $\beta'$ fragment as well as its inhibition. All results of the EMS
assay were reproducible by the LRET assay, and as an example, we were able to show competition of labeled \( \sigma70 \) binding to the \( \sigma70 \) fragment by increasing the amounts of unlabeled \( \sigma70 \) (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 \( \sigma70 \). A maximum of 250 nM labeled \( \sigma70 \) 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 \( \sigma70 \) fragment interaction with \( \sigma70 \) 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 \( \mu \)M (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 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. 4). The variance of the obtained values for the majority of samples spread over a wide range of up to 40% loss of signal.

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 \( \mu \)M 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 electrophoreograms 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 \( \mu \)M, assuming it had the mass and content of the sample.
mentioned above. There was no significant difference between the IC\textsubscript{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 \(\beta'\) 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 \(\beta'\) 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 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 \(\sigma70(442C)\) 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 σ70. With this data, we concluded that the assay can be used to monitor σ70 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 of natural and combinatorial libraries was demonstrated by resulting also in a very low cost per screen. The LRET assay turned out to be very sensitive and reliable. 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 of natural and combinatorial libraries was demonstrated by resulting also in a very low cost per screen.

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REFERENCES

9. Landick, R., D. Wang, and C. L. Chan. 1996. Quantitative analysis of tran-


