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Streptomyces Telomeres Contain a Promoter[▽]

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Bidirectional replication of the linear chromosomes and plasmids of *Streptomyces* spp. results in single-strand overhangs at their 3′ ends, which contain extensive complex palindromic sequences. The overhangs are believed to be patched by DNA synthesis primed by a terminal protein that remains covalently bound to the 5′ ends of the telomeres. We discovered that in vitro a conserved 167-bp telomere DNA binds strongly to RNA polymerase holoenzyme and exhibits promoter activities stronger than those of an rRNA operon. In vivo, the telomere DNA exhibited promoter activity in both orientations on a circular plasmid in *Streptomyces*. The telomere promoter is also active on a linear plasmid during exponential growth. Such promoter activity in a telomere has not hitherto been observed in eukaryotic or prokaryotic replicons. *Streptomyces* telomere promoters may be involved in priming the terminal Okazaki fragment (during replication) replicative transfer (during conjugation), or expression of downstream genes (including a conserved *ttrA* helicase-like gene involved in conjugal transfer). Interestingly, the *Streptomyces* telomeres also function as a promoter in *Escherichia coli* and as a transcription enhancer in yeast.

The linear chromosomes (33) and plasmids (38) of *Streptomyces* spp. are capped by terminal proteins (TPs) covalently bound to the 5′ ends of the DNA. Such TP-capped telomeres are also found in a variety of linear plasmid and viral DNAs of both eukaryotic and prokaryotic origins, including the well-studied adenoviruses and *Bacillus* ϕ29 phage, which are replicated from end to end using the TP as a primer for initiation (reviewed in reference 39). In contrast, the linear replicons of *Streptomyces* spp. are replicated bidirectionally from an internal origin. This mode of replication leaves single-stranded gaps of 250 to 300 nucleotides (nt) at the 3′ ends (16; C.-H. Huang, unpublished results), which are supposedly patched by TP-primed DNA synthesis (reviewed in reference 15). For essentially all linear chromosomes and most linear plasmids of *Streptomyces* spp., the telomere sequences are highly conserved for about 200 bp and contain extensive palindromic sequences capable of forming complex secondary structures (27). These archetypal telomeres are capped by TP encoded by *tpg* (4, 45). Several Tpg proteins have been identified (4, 45), and they are highly conserved in size (184 to 185 amino acids) and sequences.

Atypical telomere sequences have been found in a few linear plasmids (32, 47) and one chromosome (23) of *Streptomyces* spp. For example, the telomeres of linear plasmid SCP1 of *Streptomyces coelicolor* differ significantly from the archetypal telomeres in primary (32) and secondary (7) structures. They are capped by a 259-amino-acid TP (encoded by the *tpc* gene on SCP1), which shares no homology with Tpgs.

Little is known about the TP-primed end patching during replication of the *Streptomyces* linear replicons. An in vitro deoxynucleotidylation study using a crude extract of *Streptomyces* identified a Thr residue on Tpg (of *S. coelicolor* and "*Streptomyces lividans*") as the attachment site of the first nucleotide, dCMP (46). However, there is no clue as to which DNA polymerase in the extract is involved in the reaction. In a TP-primed replication of ϕ29 and adenoviruses, the DNA polymerase involved is of family B. However, no gene for a family B DNA polymerase is found in the genomic sequences of *S. coelicolor* (8) and *Streptomyces avermitilis* (29).

End patching requires the product of a gene named *tap*, which lies upstream of *tpg* in the same operon on *Streptomyces* chromosomes (4). Tap binds to the secondary structures formed by the 3′ overhangs at the archetypal telomeres formed during replication, and supposedly recruits Tpg to the telomeres to engage in end patching (2). Using Tap as a scaffold, Bao and Cohen (3) further identified DNA polymerase I (Pol I) and topoisomerase I as other components of the telomere complex. Intriguingly, these two proteins exhibit reverse transcriptase activity in addition to their predicted functions. The significance of this finding is not clear.

In this study, we attempted to isolate proteins that bind to the archetypal telomeres of *Streptomyces* spp. and unexpectedly found RNA polymerase among them. In an in vitro transcription system using purified *Streptomyces* RNA polymerase holoenzyme, the telomere DNA displayed strong promoter

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TABLE 1. Bacterial strains and plasmids used

Culture/plasmid	Genotype/description	Source/reference
Bacterial culture		
S. lividans ZX7	*pro-2 str-6 rec-46* Δ*dnd* SLP2$^-$ SLP3$^-$	48
S. coelicolor M145	SCP1$^-$ SCP2$^-$	26
S. coelicolor M600	SCP1$^-$ SCP2$^-$	44
S. coelicolor 3456	SCP1^{NF} SCP2$^-$ *pgl-1*	44
Plasmid		
pGEM-T Easy	*E. coli* TA cloning vector	Promega
pGEM-END167^{Sli}	pGEM-T Easy containing the 167-bp *S. lividans* telomere DNA	This study
pGEM-END167^{Sco}	pGEM-T Easy containing the 167-bp *S. coelicolor* telomere DNA	This study
pLacZi	*E. coli*-yeast shuttle vector for yeast one-hybrid assay	BD Biosciences Clontech
pIJ487	*Streptomyces* promoter-probe vector containing *tsr* and promoterless *neo*	43
pIJ487-END167(+)	pIJ487 containing END167^{Sco} inserted upstream of *neo* in the same orientation	This study; Fig. 5
pIJ487-END167(−)	pIJ487 containing END167^{Sco} inserted upstream of *neo* in opposite orientation	This study; Fig. 5
pQM5056	*E. coli* plasmid containing the *luxAB* operon	P. R. Herron
pLUS357L	Linear plasmid containing END167^{Sco} as one telomere	This study; Fig. 6
pLUS358L	pLUS357L containing promoterless *luxAB* operon downstream from END167^{Sco}	This study; Fig. 6
pLUS887	Derivative of pEGFP with the *lac* promoter of EGFP deleted	This study; Fig. 7
pLUS888	pLUS887 containing END167^{Sli} inserted upstream of EGFP in the same orientation	This study; Fig. 7
pLUS889	pLUS887 containing END167^{Sli} inserted upstream of EGFP in the opposite orientation	This study; Fig. 7
pLacZi-END167^{Sli}	pLacZi containing END167^{Sli} inserted upstream of the pCyc1 promoter	This study; Fig. 8
pBLCAT5	TK promoter-driven CAT reporter gene plasmid	10
pEND(F)tkCAT	Derivative of pBLCAT5 containing END167^{Sli} inserted upstream of the TK promoter in the same orientation	This study
pEND(R)tkCAT	Derivative of pBLCAT5 containing END167^{Sli} inserted upstream of the TK promoter in the opposite orientation	This study
pBLCAT6	Promoterless CAT reporter gene plasmid	10
pEND(F)CAT	Derivative of pBLCAT6 containing END167^{Sli} inserted upstream of the *cat* gene in the same orientation	This study
pEND(R)CAT	Derivative of pBLCAT6 containing END167^{Sli} inserted upstream of the *cat* gene in the opposite orientation	This study
pG5END(F)CAT	Derivative of pEND(F)CAT containing five copies of consensus GAL4	This study
pG5END(R)CAT	Derivative of pEND(R)CAT containing five copies of consensus GAL4	This study
pG5BLCAT6	Derivative of pBLCAT6 containing five copies of consensus GAL4	This study

activity. In vivo, the telomere DNA also exhibited promoter activity either on a circular DNA or at the end of a linear DNA. Interestingly, the telomere also functioned as a promoter in *E. coli* and as an enhancer of transcription in *Saccharomyces cerevisiae.* The possible mechanism and biological role of the novel promoter activity of the *Streptomyces* telomeres are discussed.

MATERIALS AND METHODS

General bacteriological and molecular biological procedures. Bacterial strains and plasmids used are listed in Table 1. Bacterial culture, DNA restriction, electrophoresis, hybridization, cloning, transformation, and other general biological and molecular procedures were according to Sambrook et al. (40) for *Escherichia coli* and Kieser et al. (31) for *Streptomyces*.

Synthesis of biotin-labeled telomere DNA by PCR. Biotin-labeled telomere DNA was prepared by PCR. The 167-bp terminal sequences of the *Streptomyces* chromosomes (END167^{Sli} and END167^{Sco}) (Fig. 1) were amplified by PCR using the following pairs of primers: for *S. lividans*, a forward primer (ACGCGTCGACCCCGCGGAGCGGGTACCC) containing a SalI site (underlined) and a reverse primer (CCGCTCGAGGCTCCGGGAGCGCAGCGG) containing a XhoI site (underlined); and for *S. coelicolor*, a forward primer (ACGCGTCGACCCCGCGGAGCGGGTACCA) containing a SalI site (underlined) and a reverse primer (CCGCTCGAGACTCCGGGAGCGCAGCGG) containing a XhoI site (underlined). Each 50-μl reaction mixture contained 5 μl 10× PCRx amplification buffer, 1.5 μl 50 mM $MgSO_4$, 2.5 U Platinum *Taq* DNA polymerase (Invitrogen), 1 μl 10 mM deoxynucleoside triphosphate mixture, 1 μl (25 pmol) of each primer, 20 ng template DNA (plasmid containing the cloned telomere DNA), and 10 μl 10× PCRx Enhancer System solution (Invitrogen). The PCR was performed in a PerkinElmer 9700 unit with an initial incubation at 95°C for 5 min followed by 30 cycles of denaturation (30 s at 95°C), annealing (30 s at 65°C), and extension (30 s at 72°C). The PCR products (designated DS167) were cloned in a TA cloning vector pGEM-T Easy (Promega) to give pGEM-END167^{Sli} and pGEM-END167^{Sco}, and the cloned sequences were confirmed by sequencing. For biotin-labeled DNA, the PCR primers were labeled with biotin at the 5′ end. Biotin-labeled forward primer and unlabeled reverse primer were used to generate DS167 DNA, which was denatured by boiling for 10 min and then quickly cooled on ice. Streptavidin-coated magnetic beads (Dynabeads M-280 streptavidin; Invitrogen) were used to isolate the biotin-labeled strand (designated SS167w) according to the manufacturer's specifications. For isolation of the complementary strand, biotin-labeled reverse primer and unlabeled forward primer were used. The resulting DNA was denatured, and the biotin-labeled strand (designated SS167c) was isolated by magnetic beads as described above.

Preparation of *Streptomyces* cell extracts and RNA polymerase. *S. lividans* ZX7 or *S. coelicolor* 3456 was cultured in Trypticase soy broth medium (31) at 30°C to an optical density at 600 nm (OD_{600}) of about 1.2 and harvested by centrifugation. The pellet was resuspended in low-salt buffer A (20 mM Tris-HCl [pH 7.4], 0.5 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 0.1 M NaCl, 5% glycerol, 0.5 mM dithiothreitol [DTT], and 5 mM $MgCl_2$) and disrupted by sonication on ice. Cell debris was removed by centrifugation. Protein concentrations were determined by the micro-Bradford method (Bio-Rad). The cell extract was divided into aliquots and stored at −20°C. RNA polymerase holoenzyme of *S. coelicolor* was purified as described previously (30).

Isolation of the telomere binding proteins (TBPs) and mass spectrometric analysis. Biotin-labeled terminal DNA (DS167, SS167c, or SS167w) was incubated with crude extract in ZTA buffer (0.1 mM DTT, 1 mM EDTA, 5% glycerol, 10 mM HEPES, 50 mM KCl, 0.1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100) at 4°C for 60 min. Streptavidin-coated magnetic beads were added, and the mixture was incubated at 4°C for 30 min, then washed three times with the same buffer and eluted with 0.5 M NaCl in ZTA buffer. Electrophoretic

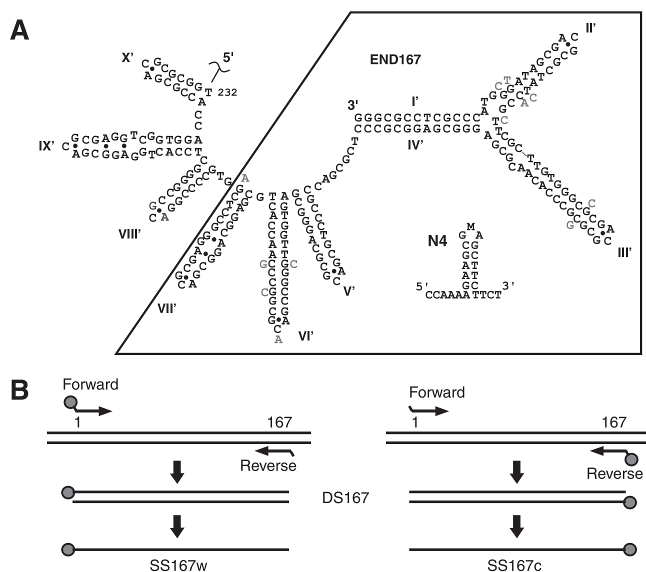

FIG. 1. Design and synthesis of telomere DNA probes. (A) The sequence and predicted secondary structure of the 3′ ends of the *S. lividans* chromosome (27). The secondary structures formed by the 10 palindromes (I′ to X′) with the lowest energy levels were constructed based on energy optimization using the DNA folding server (http://mfold.bioinfo.rpi.edu/) and G · A sheared pairings (solid dots) at the hairpin loops and at the GAA · GAA motifs (18, 27). The first 167 nt spanning the first seven palindromes is framed. The 12 substitutions (in lightface) in the corresponding terminal sequence of the *S. coelicolor* chromosome are shown next to the corresponding residues. Inset, secondary structure of the coliphage N4 promoter; M, A or G. (B) Synthesis of double-stranded and single-stranded terminal DNA. A pair of primers (Forward and Reverse), one of which was end labeled with biotin (filled circles), was used to produce the DS167 DNA by PCR. The two biotin-labeled strands (SS167w and SS167c) were separately purified by denaturation of DS167 DNA, followed by binding to streptavidin-coated magnetic beads.

separation in sodium dodecyl sulfate (SDS) gel, trypsin digestion, and mass spectrometric analysis of the proteins were as described previously (45).

In vitro transcription. In vitro runoff transcription assays were performed using the procedures of Fujita et al. (20) and Buttner et al. (14). The template DNA fragments were purified by electrophoresis in 5% polyacrylamide gel, eluted in 0.1% SDS overnight, and concentrated by precipitation with RNase-free ethanol. The template DNA (0.15 pmol) and RNA polymerase (1.5 pmol) were incubated at 30°C for 5 min in 15 μl transcription buffer (40 mM Tris-HCl [pH 7.9], 10 mM $MgCl_2$, 0.6 mM EDTA, 0.4 mM potassium phosphate, 1.5 mM DTT, 0.25 mg/ml bovine serum albumin, and 20% [vol/vol] glycerol). RNA synthesis was initiated by adding 3 μl of substrate mixture containing 2 μCi [α-^{32}P]CTP (400 Ci/mmol) and 0.4 mM each of UTP, ATP, and GTP. Heparin (3 μl, 0.1 mg/ml final concentration) was added after 2 min to prevent reinitiation, and the incubation was continued for 5 min before 2 μl cold CTP (1 mM final concentration) was added. After a 10-min incubation, the reaction was terminated by adding 50 μl of stop solution (375 mM sodium acetate [pH 5.2], 15 mM EDTA, 0.15% SDS, and 0.1 mg/ml calf thymus DNA). Transcripts were precipitated with ethanol, resuspended in formamide sample buffer (80% [vol/vol] formamide, 8% [vol/vol] glycerol, 0.1% SDS, 8 mM EDTA, 0.01% bromophenol blue, and 0.01% xylene cyanol), and electrophoresed on 5% polyacrylamide gel containing 7 M urea.

Gel electrophoresis mobility shift assay. DNA-protein binding reactions were carried out at room temperature for 30 min in 25 μl buffer containing 0.1 mM DTT, 0.1 mM EDTA, 3 mM $Mg(OAc)_2$, 50 mM NaCl, 50 mM Tris-HCl (pH 7.9), 25 μg/ml bovine serum albumin, 200 ng poly(dI/dC), 3 to 6 pmol RNA polymerase holoenzyme, and labeled DNA probe. For telomere DNA, the 205-bp EcoRI fragment containing the END167^{Sli} sequence on pGEM-END167^{Sli} was isolated and filled in with [α-^{32}P]dATP by the Klenow enzyme. For the *rrnD* promoter, a 245-bp AccI-EcoRI fragment from pIJ2820 (6) was isolated and filled in with [α-^{32}P]dATP by the Klenow enzyme. Electrophoresis was performed in 5% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer.

Promoter activity assays in *Streptomyces*. To test the promoter activity on a circular plasmid, a 206-bp EcoRI fragment containing END167^{Sco} from pGEM-END167^{Sco} was inserted into the EcoRI site upstream of the promoterless *neo* gene on pIJ487 (43) in two orientations. The resulting plasmids, pIJ487-END167(+) and pIJ487-END167(−), were introduced into *Streptomyces* isolates by transformation. Expression of the *neo* gene was tested by streaking 100 μl of spore suspensions of the transformants on an LB plate containing a kanamycin gradient, followed by incubation at 30°C for 7 days. To test the promoter activity of the telomere DNA on a linear plasmid, the linear plasmid pLUS357L (see Fig. 6A) was constructed based on a mini-linear plasmid, pLUS968 (28), on which a 365-bp *S. lividans* telomere was replaced by the END167^{Sco}DNA, and the *melC* operon was deleted and replaced by a synthetic linker. The *luxAB* operon (from pQM5065) was inserted into the linker to give rise to pLUS358L (see Fig. 6A). Spores of *S. coelicolor* M600 harboring these plasmids were inoculated in yeast extract-malt extract medium containing 5 μg/ml thiostrepton and shaken at 30°C. At a different time, aliquots of the culture were sampled for measurement of OD_{600} and luciferase activities. For the luciferase activity assay, 2 μl of *n*-decanal (Sigma) mixed with 200 μl of culture was incubated for 5 min at room temperature, and the luminance was determined in a luminometer (Wallac 1420 VICTOR^2; PerkinElmer). The luminance of each sample was normalized by the mass (OD), and the ratio of the normalized luminance for the pLUS358L-containing culture to that for the pLUS357L-containing culture was calculated and reported as relative luciferase activity.

Expression of the telomere promoter in *E. coli*. The *lac* promoter of the enhanced green fluorescent protein (EGFP) gene on pEGFP (BD Biosciences Clontech) was removed by digestion with PvuII and HincII and ligation to give pLUS887 (see Fig. 7). The END167^{Sli} DNA was inserted into the SmaI site upstream of the promoterless EGFP gene on pLUS887 in both orientations to give pLUS888 and pLUS889 (see Fig. 7). These plasmids were introduced into *E. coli* DH5α, and fluorescence produced by the transformants growing in LB medium was observed under a fluorescence microscope.

Yeast transformation and β-galactosidase assay. The yeast one-hybrid system (BD Biosciences Clontech) was used for constructing the reporter and effector plasmids. The END167^{Sli} DNA was removed from pGEM-END167^{Sli} by digestion with SalI and XhoI and inserted between the SalI and XhoI sites of the yeast one-hybrid reporter vector pLacZi (BD Biosciences Clontech). The resulting plasmid, pLacZi-END167^{Sli}, was linearized with NcoI (in the URA3 gene) and used to transform *S. cerevisiae* YM4271. Uracil prototrophic transformants were selected, and integration of pLacZi-END167^{Sli} into the chromosome was confirmed by PCR analysis. β-Galactosidase activities were detected by the colony-lift filter assay.

Testing the promoter activity of END167^{Sli} in human cell lines. Plasmids pEND(F)tkCAT and pEND(R)tkCAT are derivatives of pBLCAT5 (10) containing END167^{Sli} DNA inserted (in opposite orientations) upstream of the thymidine kinase (TK) promoter, which drives transcription of the *cat* gene. pEND(F)CAT and pEND(R)CAT are derivatives of pBLCAT6 (10) containing END167^{Sli} DNA inserted (in opposite orientations) in front of a promoterless *cat* gene. pG5END(F)CAT, pG5END(R)CAT, and pG5BLCAT6 are derivatives of pEND(F)tkCAT, pEND(R)tkCAT, and pG5BLCAT6 containing five copies of consensus GAL4 from pG5CAT (BD Biosciences Clontech). Plasmid DNA was introduced by transfection into HuH-7 and HeLa cell lines (grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum) by calcium phosphate coprecipitation, and 48 h after transfection, cells were harvested for CAT analysis (41). The concentration of plasmid DNA in the transfected cells was monitored by hybridization using a *cat* gene sequence as a probe after the removal of RNA by RNase treatment.

RESULTS

RNA polymerase binds *S. lividans* telomere DNA. In our initial attempt to isolate TBPs, we chose as the substrate the first 167 bp of the *S. lividans* chromosome spanning the first seven palindromes that are highly conserved among the archetypal telomeres of *Streptomyces* (Fig. 1A). This terminal sequence was designated END167^{Sli}. Biotin-labeled END167^{Sli} in the double-stranded form (DS167) and single-stranded forms (SS167w and SS167c) was prepared by PCR and purified using streptavidin-coated magnetic beads (Fig. 1B). These bi-

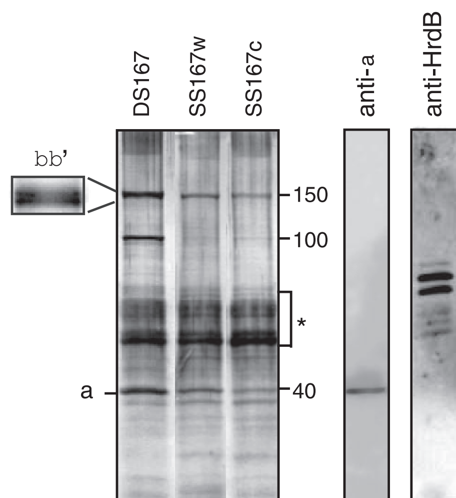

FIG. 2. Isolation of TBPs. Biotin-labeled double-stranded (DS167) and single-stranded (SS167w and SS167c) DNA fragments immobilized in a streptavidin column were used to purify binding proteins in crude extracts of *S. lividans*. The eluted proteins were separated by electrophoresis in SDS-polyacrylamide gel and silver stained (left). The sizes (in kDa) of the most abundant TBPs are indicated. The asterisk depicts nonspecific binding proteins that were also present in the flowthrough fractions. The region containing the β and β′ subunits (bb′) of RNA polymerase is enlarged in the insert on the left. The separated proteins were analyzed by immunoblots using anti-α (anti-a, middle) and anti-HrdB (right) antibody.

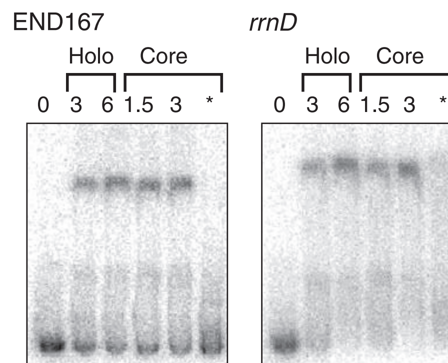

FIG. 3. Binding of RNA polymerase to telomere DNA. (Left) The gel mobility shift assay was performed between RNA polymerase holoenzyme (Holo) or core enzyme (Core) and ^{32}P-labeled telomere DNA (205-bp EcoRI fragment containing END167^{Sli}) in the presence of excess poly(dI/dC). (Right) For a comparison, the 245-bp ^{32}P-labeled AccI-EcoRI fragment containing the *rrnD* promoter of *S. coelicolor* was used in place of the telomere DNA. The amount of enzyme added is shown above (in nanomoles). The asterisk indicates an addition of 10-fold nonradioactive DNA probe.

otin-labeled DNA fragments were used for affinity purification of TBPs.

A crude extract from *S. lividans* ZX7 was incubated with the biotin-labeled DS167, SS167w, and SS167c DNA. The DNA-protein complexes were isolated using streptavidin-coated magnetic beads and separated by electrophoresis (Fig. 2, left). Four proteins of about 150 (two species), 100, and 40 kDa were eluted from the DS167 DNA. The 150- and 40-kDa proteins were also eluted from the SS167w and SS167c DNA. The addition of excess (50-fold by weight) salmon sperm DNA did not significantly alter the binding of these proteins, suggesting strong and specific interactions between them and the END167^{Sli} DNA.

The two 150-kDa proteins and the 40-kDa proteins agreed with the profile of the core RNA polymerase β, β′, and α_2. The reaction of the 40-kDa polypeptide to anti-α antibody (Fig. 2, middle) supported this notion. To test whether sigma factors also accompanied the RNA polymerase core enzyme, antibodies against sigma B, sigma R, and the major housekeeping sigma factor HrdB (12) were used to react with the isolated TBPs. Sigma R was not detected in either the crude extract or the isolated TBP, while sigma B was seen in the crude extract but not in the isolated TBPs. Anti-HrdB antibody detected several strong signals in the crude extract as well as in the isolated TBPs (Fig. 2, right). These reacting polypeptides consisted of two major species plus several minor species, in the range of 55 to 80 kDa. The molecular weight of HrdB is 56 kDa. Some of the proteins that reacted to the anti-HrdB antibody may represent nonessential homologs of HrdB in *S. coelicolor*, HrdA, HrdC, and HrdD (12, 13).

To test the archetypal telomere from another species, biotin-labeled END167 DNA was prepared from the corresponding 167-bp terminal sequence of the *S. coelicolor* chromosome (92% identity; designated END167^{Sco}). Binding using END167^{Sco} DNA produced a similar binding protein profile from a crude extract of *S. coelicolor*. The major subunits of RNA polymerase (β, β′, and α) were likewise detected. The availability of the genomic sequence of *S. coelicolor* allowed the identification of some of the TBPs by mass spectroscopy, such as Pol I (98.1 kDa, encoded by the gene SCO2003), BldD (18.1 kDa; SCO1489) and two hypothetical proteins, a 31-kDa AP endonuclease homolog encoded by SCO2111 and a 29-kDa exonuclease III homolog encoded by SCO6341. The possible biological roles of these proteins are being investigated.

The ca. 100-kDa protein bound to END167^{Sli} corresponds to the 98.1-kDa Pol I bound to END167^{Sco}. This protein, unlike the RNA polymerase, binds only to the double-stranded form and not the single-stranded forms (Fig. 2, left).

To confirm binding of RNA polymerase to the terminal DNA, gel electrophoresis mobility shift assays were performed with purified *S. coelicolor* RNA polymerase holoenzyme and END167^{Sli} DNA in the presence of excess poly(dI/dC). The results (Fig. 3, left) showed a shift of mobility of the DNA on binding of RNA polymerase in the form of holoenzyme, core enzyme, or reconstituted holoenzyme (core plus HrdB; not shown). Similar molecular shifts were observed when the promoter of an rRNA operon was used (Fig. 3, right). These results confirmed the strong affinity of the RNA polymerase of *Streptomyces* to the telomere DNA in vitro.

The telomere DNA exhibits strong promoter activity in vitro. The strong binding of RNA polymerase holoenzyme to END167^{Sli} suggested that END167^{Sli} might contain promoter activity. In an in vitro transcription assay using the DS167 DNA and purified RNA polymerase holoenzyme, two major transcripts (~100 and ~130 nt) were produced (Fig. 4, left). Interestingly, these in vitro promoter activities of the telomere DNA appeared to be significantly stronger than the three *Streptomyces* rRNA promoters (p2, p3, and

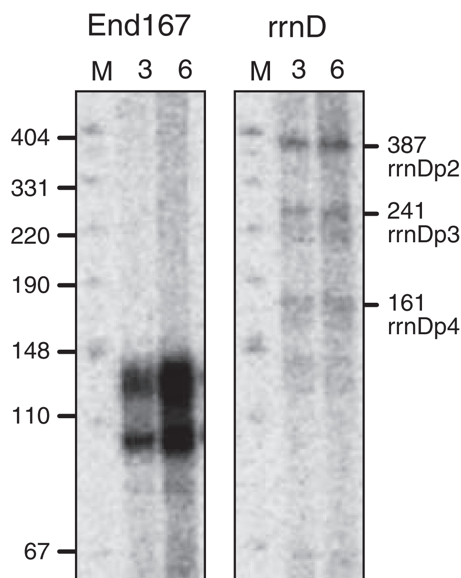

FIG. 4. Promoter activity of END167^{Sli} in vitro. Transcription assays were performed in vitro with 3 or 6 pmol (number of purified RNA polymerase holoenzyme from *S. coelicolor*). DNA fragments containing the END167^{Sli} and *rrnD* promoters were used as templates. The transcripts were analyzed by autoradiography after separation on a 7 M urea-5% polyacrylamide gel. Lane M, RNA size markers; lane 3, 3-pmol RNA polymerase; lane 6, 6-pmol RNA polymerase. The numbers on the side depict the size of the RNA (in nucleotides). Transcripts from the *rrnD* p2, p3, and p4 promoters are indicated.

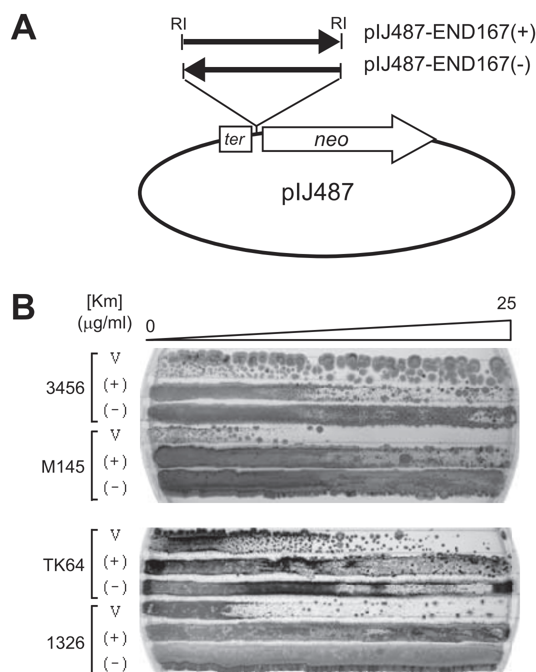

FIG. 5. Promoter activity of END167^{Sco} on a circular plasmid in *Streptomyces*. (A) The expression vector. pIJ487-END167(+) and pIJ487-END167(−) contain the END167^{Sco} DNA inserted at the EcoRI (RI) site of pIJ487, upstream of the promoterless *neo* gene in two orientations (+ and −). The arrows indicate the orientation of END167^{Sco} from nt 1 to 167. *ter*, phage fd transcription terminator. (B) Detection of promoter activity. Spores of *S. lividans* and *S. coelicolor* harboring pIJ487 (V), pIJ487-END167(+) (+) and pIJ487-END167(−) (−) were streaked and cultured on LB medium containing a kanamycin gradient.

p4) (Fig. 4, right). Multiple transcripts were detected from END167^{Sli}, probably resulting from different start sites and/or different directions of transcription.

The telomere DNA acts as a promoter in *Streptomyces*. *S. coelicolor* and *S. lividans* are very closely related. Their telomere sequences are more than 90% identical, and their capping TPs (Tpg^{Sco} and Tpg^{Sli}) are 100% identical (4, 45). Subsequent in vivo studies were performed primarily using the telomere DNA (END167^{Sco}) of the *S. coelicolor* chromosome, the complete sequence of which is available (8). To test the promoter activity in vivo, the END167^{Sco} DNA was inserted upstream of a *neo* (kanamycin resistance) reporter gene on pIJ487 (43) in two orientations (Fig. 5A). The resulting recombinant plasmids were introduced into *S. coelicolor* M145 and 3456 (44). Regardless of the orientation of the inserts, all the transformants exhibited elevated resistance to kanamycin compared to the control transformants containing pIJ487 (Fig. 5B). These results indicated that the telomere promoter was active in vivo on the circular plasmids. Identical results were obtained in *S. lividans* (wild-type 1326 containing linear plasmid SLP2 and a plasmidless derivative, TK64) (Fig. 5B).

To test possible promoter activity of the END167^{Sco} DNA as a telomere of a linear replicon, a recombinant linear plasmid, pLUS358L, was constructed, in which END167^{Sco} served as a telomere and a promoterless luciferase operon (*luxAB*) (1) was inserted 55 bp downstream from END167^{Sco} (Fig. 6A). *S. coelicolor* M600 harboring pLUS358L was cultured in liquid medium containing thiostrepton, and the expression of luciferase was monitored during growth (Fig. 6B). Luciferase activity was detected during the exponential phase and diminished toward the stationary phase. This indicated that the END167^{Sco} sequence acting as a telomere of a linear replicon could also function as a promoter in vivo during active growth.

The telomere DNA acts as a promoter in *E. coli*. END167^{Sli} was also tested for in vivo promoter activity in *E. coli*. END167^{Sli} was placed upstream in both orientations from the promoterless EGFP gene on pEGFP plasmid to give rise to pLUS888 and pLUS889, respectively (Fig. 7). Ampicillin-resistant *E. coli* transformants of the two plasmids were selected and inspected under a fluorescence microscope. Both groups of transformants emitted bright green fluorescence (Fig. 7), indicating that END167^{Sli} also acted as a promoter in *E. coli* in either orientation.

The telomere DNA enhances transcription in yeast. In a separate attempt to isolate TBP, we set up a yeast one-hybrid system to screen a *Streptomyces* genomic library. END167^{Sli} DNA was inserted upstream of the basal promoter pCyc1 of the *lacZ* reporter gene on pLacZi (Fig. 8). The resulting plasmid, pLacZi-END167, was introduced into *S. cerevisiae* YM4271 by transformation. Positive transformants were isolated, and integration of the plasmid into the chromosome was confirmed by PCR analysis (data not shown). Surprisingly, the transformants exhibited strong β-galactosidase activity by themselves (Fig. 8). In contrast, YM4271 containing pLacZi expressed only a low level of background β-galactosidase activity.

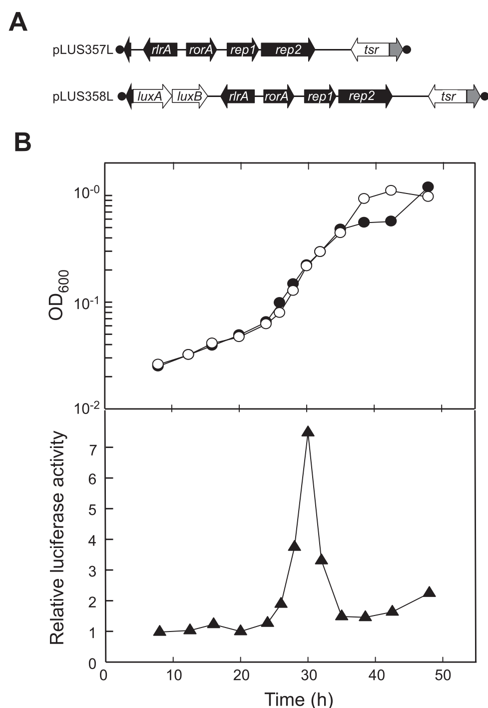

FIG. 6. Promoter activity of END167^{Sco} as a telomere of a linear plasmid in *Streptomyces*. (A) Reporter plasmids pLUS357L and pLUS358L. pLUS357L possesses END167^{Sco} as the left telomere (black arrow) and a 365-bp right telomere (gray arrow) sequence of *S. lividans*, the thiostrepton resistance gene (*tsr*; open arrow), and the ARS of pSLA2 (containing four genes depicted by the black arrows). pLUS358L contains the *luxAB* operon (open arrows) inserted 55 bp downstream of END167^{Sco} on pLUS357L. The TPs are represented by the filled circles. (B) Expression of *luxAB* from the telomere promoter. Spores of *S. coelicolor* M600 harboring pLUS357L (filled circles) and pLUS358L (open circles) were inoculated in yeast extract-malt extract medium containing 5 μg/ml thiostrepton and shaken at 30°C. At various times, aliquots were removed for measurement of OD_{600} (top) and relative luciferase activity (bottom).

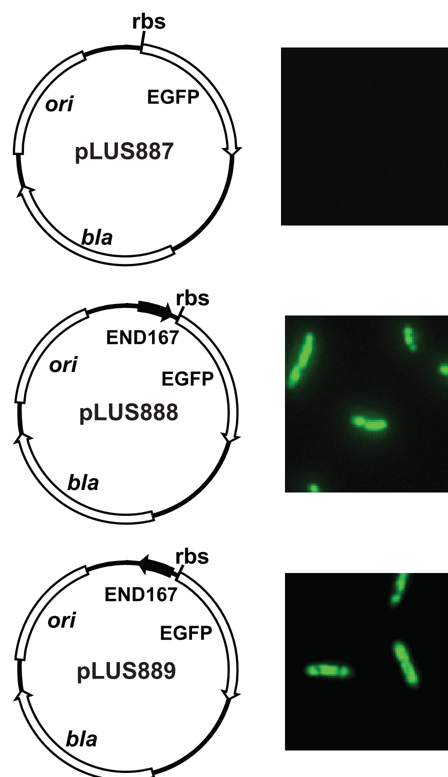

FIG. 7. Promoter activity of the telomere DNA in *E. coli*. (A) Expression vectors. pLUS887 contains a promoterless EGFP gene. pLUS888 and pLUS889 contain END167^{Sli} (filled arrows) inserted upstream of EGFP in opposite orientations. The orientation of the filled arrow points from nt 1 to 167. rbs, ribosome binding site; *ori*, replication origin of *E. coli* plasmid ColE1; *bla*, β-lactamase gene. (B) In vivo promoter activity of END167^{Sli}. *E. coli* transformants harboring the three plasmids were cultured in LB medium, and their fluorescence was photographed under a microscope. None was detected in the pLUS887 transformant.

On pLacZi-END167, END167^{Sli} could have acted as either a promoter or an enhancer. To distinguish these two possibilities, a 20-bp adapter sequence was used to replace the pCyc1 promoter. Transformants of this construct, pLacZi-END167ΔpCyc1, lost the ability to express β-galactosidase (Fig. 6). These results indicated that the pCyc1 promoter is required for transcription of the *lacZ* gene on pLacZi-END167 in yeast, and END167^{Sli} probably serves as a *cis*-acting element that activates transcription in the absence of activator proteins.

The telomere DNA does not enhance transcription in human cells. To explore whether END167^{Sli} could also function as a transcription promoter or enhancer in mammalian cells, END167^{Sli} was inserted in both orientations upstream of a promoterless *cat* (chloramphenicol acetyltransferase) reporter gene on pBLCAT6 (10) to produce pEND(F)CAT and pEND(R)CAT. These two plasmids were used to transfect HuH-7, a well-differentiated human hepatoma cell line established from a primary hepatocellular carcinoma (36). Transient expression of chloramphenicol acetyltransferase activity in the transfected cells was as low as that in cells transfected by pBLCAT6. To increase the sensitivity, an activator-binding DNA consisting of five copies of the GAL4 binding consensus sequence was placed upstream of END167^{Sli} on pEND(F)CAT and pEND(R)CAT to give pG5END(F)CAT and pG5END(R)CAT, respectively. Cotransfection of HuH-7 with these plasmids and pM3-VP16 (BD Biosciences Clontech) expressing a GAL_{BD}VP16 activator (a fusion protein of the GAL4 DNA binding domain and the VP16 activation domain) increased the expression level of CAT, but not to a level significantly higher than that after cotransfection with pBLCAT6 containing the activator-binding DNA, i.e., pG5BLCAT6 and pM3-VP16.

To explore whether END167^{Sli} might function as an enhancer, END167^{Sli} DNA was inserted in both orientations upstream of the TK promoter of pBLCAT5 (10), and the resulting plasmids, pEND(F)tkCAT and pEND(R)tkCAT, were introduced into HuH-7 and HeLa cell lines. No increase in CAT activity was observed in the transfected cells compared to those transfected by pBLCAT5. Thus, the *Streptomyces* telomere sequence could not function as a promoter or enhancer in human cells.

DISCUSSION

The discovery of RNA polymerase-binding and promoter activities of the *S. lividans* and *S. coelicolor* telomeres was

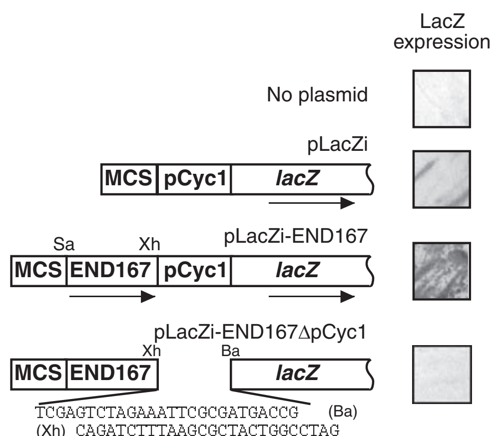

FIG. 8. Enhancer activity of END167^{Sli} in yeast. pLacZi, pLacZi-END167 (with END167^{Sli} inserted upstream of the pCyc1 promoter), and pLacZi-END167ΔpCyc1 (with pCyc1 replaced by a linker sequence) were introduced into *S. cerevisiae* YM4271, and the expression of *lacZ* in the transformants was assayed by colony lift assay (right). The N terminus of *lacZ* and its upstream elements on these plasmids are illustrated. The arrows indicate the orientations of END167^{Sli} (from nt 1 to 167) and *lacZ* (direction of transcription). MCS, multiple cloning site. Restriction sites are abbreviated as follows: Sa, SalI; Xh, XhoI; and Ba, BamHI.

unexpected. These two telomeres are more than 92% identical to each other in the END167 sequences that span the first seven palindromes. In fact, these seven palindromic sequences are also highly conserved among the telomeres of most *Streptomyces* chromosomes and many linear plasmids (27). Thus, it is likely that these archetypal telomere sequences possess a promoter function.

The END167 sequences contain high G+C contents (approximately 70%) and lack typical −35 and −10 RNA polymerase binding sequences or sequences similar to other known *Streptomyces* promoters. It is possible that, under negative superhelicity, the palindromic sequences in this stretch of DNA undergo a transition to form hairpin loops, which provide a ready access for RNA polymerase. While negative superhelicity is intrinsic to circular plasmids in vivo, it may also be found in TP-capped linear plasmids with interacting telomeres (H.-H. Tsai et al., unpublished data). The hairpins formed by the END167 sequences contain mainly GCA (3′ strands) and TGC (5′ strands) loops. The GCA loops are proposed to be closed by highly stable G · A sheared pairing, resulting in a single nucleotide (C) loop that is resistant to single-strand-specific nucleases (18, 27).

Promoter activity of hairpinned DNA has been previously documented. For example, Glucksmann et al. (21) and Glucksmann-Kuis et al. (22) described novel promoters of N4 phage that function efficiently in the single-stranded but not double-stranded form. These promoter sequences contain a palindrome, which extrudes from the double helix at physiological superhelical densities (19) and forms hairpins with a PyG GAPu or PyGAAPu loop (Fig. 1A, inset). The PyGGAPu loop has been found to be closed by G · A sheared pairing (25). The hairpin extrusion is necessary for promoter activity. Both the 3-base loop and a 5- to 7-bp stem are required for recognition by the phage RNA polymerase.

A different type of single-stranded DNA promoter (designated F*rpo*) was described by Masai and Arai (35) in the leading region of the F plasmid. This promoter is formed as a 329-bp secondary structure containing mispaired regions and a loop and is transcribed by the major RNA polymerase (containing σ^{70}) of *E. coli*. Similar promoters are also found in the leading region of plasmid ColIb-P9 (5). Interestingly, the predicted secondary structures of these promoters (5, 21) contain many G · A "mismatches" in the "bulges," which may potentially form sheared pairing (as in the *Streptomyces* telomeres) (Fig. 1B), thereby significantly reducing the numbers and sizes of single-stranded bulges and loops.

It is likely that the *Streptomyces* telomere promoters are active in the form of hairpin loops (Fig. 1A). The predominant hairpins with the PyGNAPu (N mostly C) loops closed by the G · A sheared pairing found in *S. lividans* and *S. coelicolor* are similar to those in the phage N4 genome. On the other hand, the complexity of the secondary structures formed by the *Streptomyces* promoters is more similar to that of F*rpo*. Both contain bulges formed by mispaired purines, which have been shown in nuclear magnetic resonance studies to form a stable duplex structure through purine-purine sheared pairings (18).

F*rpo* is transcribed by the major RNA polymerase in *E. coli*. Our in vitro results show that END167 is efficiently transcribed by the major RNA polymerase of *Streptomyces* containing the HrdB (σ^{70} ortholog) sigma factor (12). It is likely that it is also transcribed by the same holoenzyme in vivo.

F*pro* is located in the leading region of conjugal transfer on the F plasmid, and the RNA chain transcribed from this promoter serves for priming DNA replication during conjugation (35). In this regard, the telomeres of *Streptomyces* have been proposed to be the origins of conjugal transfer (17). It is tempting to speculate that the promoter activity at the telomeres is also involved in priming conjugal transfer. In this case, the plasmid would not be transferred in total, and the missing leading telomere sequence would have to be restored using the other (trailing) telomere of the plasmid (or the chromosome) as a template for recombination and synthesis. Such restoration of damaged telomeres on linear *Streptomyces* plasmids has been described by Qin and Cohen (37).

Alternatively, the telomere promoter may be involved in synthesis of the primer for the terminal Okazaki fragment during replication. The single-stranded gaps at the 3′ ends resulting from bidirectional replication from an internal origin were about 280 nt long for the plasmid pSLA2 (16) and from 250 to 300 nt long for three other linear replicons of *Streptomyces* spp. (C.-H. Huang, unpublished). Thus, the terminal Okazaki fragment is expected to be primed by an RNA transcribed within the telomere region. The promoter in END167 may be involved in synthesizing this primer.

A third possibility is that the telomere promoter may be involved in transcription of a downstream gene. A unique helicase gene, *ttrA*, involved in conjugal transfer of linear chromosomes and plasmids (28), is located in the terminal regions of the following four *Streptomyces* chromosomes: 1.6 kb from both ends in *S. lividans* (9), 1.7 kb from both ends in *Streptomyces scabies* (sequence data produced by the Sanger Institute and obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/ssc/), 1.3 kb from both ends in *S. coelicolor* (8), and 4.9 and 1.9 kb from the left and right ends, respectively, in *S. avermitilis* (29). These *ttrA* orthologs are

highly conserved in sequence, size, and direction of transcription (inward from the telomere), in sharp contrast to the surrounding nonconserved open reading frames in the terminal regions. It is possible that the telomere promoter controls the transcription of this strategically located *ttrA* gene. In this regard, the three F*rpo*-homologous promoters in the leading region of plasmid ColIb-P9 are supposed to be involved in the transcription of several genes downstream, including an antirestriction gene (*ardA*) during conjugal transfer (5). The transcription of *ardA* from a single-stranded promoter is of great strategic importance so as to inactivate the host restriction system before the incoming DNA strand is converted to a sensitive double-stranded form. In this regard, the TtrA sequences contain, in addition to helicase motifs, a short region of homology to some type I restriction enzymes and a long region of homology to some type III restriction enzymes.

In all, there are three plausible roles for the telomere promoter: (i) priming of the terminal Okazaki fragment, (ii) priming of DNA replication during conjugation, and (iii) transcription of the downstream gene(s). These functions may not be mutually exclusive, and it is possible that the promoter is activated when the telomere enters the single-stranded state during each round of replication in a cell cycle-dependent manner or during conjugal transfer. This may account for the observed sharp increase of the telomere promoter activity on a linear plasmid (Fig. 6) during the log phase of the liquid growth. Further investigation is required for clarification.

The observed in vivo promoter activity of $END167^{Sli}$ in *E. coli* may also involve the formation of hairpin structures (under the negative superhelicity of the circular plasmid) in view of the approximately equal activities in both orientations and the absence from END^{Sli} (and $END167^{Sco}$) of −35 or −10 motifs typical of *E. coli* promoters (24, 34). This raises the possibility that the telomere promoters of *Streptomyces* may also function in many other bacteria and consequently may be useful for construction of universal genetic markers on shuttle vehicles for these bacteria.

In contrast, the $END167^{Sli}$ sequence does not function as a promoter in yeast. It merely eliminates the need of an activator for initiation at the pCyc-1 promoter. It is possible that the palindromes in this sequence also form hairpin structures under a negative superhelical strain, thus facilitating the opening up of the downstream promoter. The inability of the same DNA sequence to provide an activating function in mammalian cells is probably because of the more complex regulation of gene transcription in higher eukaryotes. Transcription initiation is directed by various types of core promoters and their interaction with specific activators and enhancers (11, 42), and the $END167^{Sli}$ sequence, even if it contains a promoter or an activation or enhancer element, must be present in the appropriate genetic as well as epigenetic contexts to direct transcription of the reporter genes.

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