Isolation and Sequenc ing of the rho Gene from Streptomyces lividans ZX7 and Characterization of the RNA-dependent NTPase Activity of the Overexpressed Protein*

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The gene for transcription termination factor Rho was isolated from Streptomyces lividans ZX7. It encoded a 77-kDa polypeptide (Rho 77) with considerable homology to known Rho factors. An atypical hydrophilic region of 228 residues was found within the N-terminal RNA-binding domain. Only Rho from Micrococcus luteus and Mycobacterium leprae (closely related GC-rich Gram-positive bacteria) had an analogous sequence. Rho 77 was overexpressed in Escherichia coli and purified using an N-terminal hexahistidine-tag. Rho 77 displayed a broad RNA-dependent ATPase activity, with poly(C) RNA being no more than 4-fold more effective than poly(A). This contrasts with the ATPase activity of Rho from E. coli which is stimulated primarily by poly(C) RNA. Rho 77 was a general RNA-dependent NTPase, apparent Km values for NTPs were: GTP 0.13 mM, ATP 0.17 mM, UTP 1.1 mM, and CTP >2 mM. Rho 77 poly(C)-dependent ATPase activity was inhibited by heparin, unlike the E. coli Rho. The antibiotic bicyclomycin inhibited the in vitro RNA-dependent ATPase activity of Rho 77, did not inhibit growth of streptomycetes but delayed the development of aerial mycelia. N-terminal deletion analysis to express a truncated form of Rho (Rho 72, 72 kDa) indicated that the first 42 residues of Rho 77 were not essential for RNA-dependent NTPase activity and were not the targets of inhibition by heparin or bicyclomycin.

The essential Escherichia coli transcription factor Rho mediates 3’ end point formation in RNA by termination at specific sites. Rho uses a primary RNA binding activity, located in the N-terminal RNA-binding domain, which associates with single-stranded, cytidine-rich RNA. Termination by Rho involves the hydrolysis of ATP (or other NTP) coupled with secondary RNA-binding interactions to translocate the protein along RNA, toward the RNA 3’ end and the paused RNA polymerase (reviewed in Refs. 1 and 2). Termination appears to be effected during this process by the RNA:DNA helicase activity of Rho (3). Rho-dependent terminators can be masked by the presence of ribosomes during translation and one major function of Rho may be to terminate untranslated mRNAs (4).

Recently, it has become apparent that the rho gene is widely distributed within the eubacteria (5, 6). Western blotting has detected the expression of Rho in a variety of Gram-negative bacteria (7). Only in the Mollicute Mycoplasma genitalium is a close Rho analogue apparently absent (8). However, there has been little characterization of Rho factor outside the enteric bacteria and a non-enteric Rho-dependent terminator has not been found. As the primary RNA binding activity of Rho shows a preference for C-rich RNA; it might be expected that Rho from an organism with an extreme GC content has an altered RNA binding specificity. We chose streptomycetes to address this question because of their extreme GC-rich bias, industrial importance as antibiotic producers, and because the role of Rho in Gram-positive bacteria is poorly defined. Here we report the isolation and sequencing of the rho gene from Streptomyces lividans. The protein has an extended RNA-binding domain which appears characteristic of Rho from GC-rich Gram-positive bacteria. The RNA-dependent NTPase activity of the over-expressed S. lividans Rho reveals significant differences to the enteric Rho which may relate to the GC-rich nature of target RNA.

MATERIALS AND METHODS

General DNA Manipulations—Transformation, purification, and manipulations of E. coli and streptomycete DNA were as described by Sambrook et al. (9) and Hopwood et al. (10), respectively. Purification of streptomycete genomic DNA was by the method of Hunter (11).

PCR Amplification of the Streptomyces Rho—Degenerate oligonucleotide primers were designed using conserved regions of ATPase domains of Rho (Fig. 1 and Ref. 6). These oligos were used to amplify a single band from 200 ng of mechanically sheared, heat-denatured genomic S. lividans ZX7 DNA (12) using 35 cycles of 94°C, 50–55°C, 72°C (1.5 min at each temperature) followed by one cycle of 72°C for 3 min. PCR used Promega Taq polymerase and buffers containing 1.5 mM MgCl2, 10% dimethyl sulfoxide, 1.25 mM of each dNTP, and 150 pmol of each primer.

Library Screening and DNA Analysis—A PCR-derived DNA fragment of the S. lividans rho gene was gel purified using a Quick Gel Purification kit (Qiagen) and radioactively labeled with [γ-32P]dCTP using a random-primer kit from Boehringer Mannheim. This probe was used to screen (by colony hybridization) a library of S. lividans ZX7 in cosmid pfD666 (13). The region of the recombinant cosmid DNA identified by the probe was subcloned into pUC18 and restriction endonuclease mapped. Various fragments were subcloned into M13 and sequenced by the dyeoxy method using a Sequenase 2.0 kit (Amersham) with deaza-GTP mixture and dITPs to resolve compressions. Sequencing was completed on both strands and across all restriction

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1 The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; NTP, nucleoside triphosphate; RBS, ribosome-binding site; poly(A), polyadenylate, poly(C), poly cytidylylate; poly(G), polyguanidylate; poly(U), polyuridylate RNA.
NTPase Activity of the Streptomyces Rho Protein

RESULTS

Detection and isolation of the rho Gene—A PCR product of approximately 530 bp was amplified from genomic DNA from Streptomyces ambfaciens, S. lividans ZX7 and Streptomyces coelicolor 1147 and J 1501 with primer annealing performed at 50 °C. At an annealing temperature of 55 °C, the 530-bp product was amplified from S. coelicolor 1147 and J 1501 but not S. lividans ZX7 DNA. Amplification required both primers and genomic DNA. Southern blotting at high stringency (washes at 65 °C, 0.1 × SSC) showed the PCR products from each species to be closely related, suggesting that a rho analogue is widely distributed in streptomycetes. The S. lividans PCR product was used to isolate four cosmids from a S. lividans ZX7 library. Restriction digests with BamHI suggested that all four cosmids were derived from an overlapping region of the genome. Southern blotting with the PCR product as a probe localized the PCR product to a 3-kilobase pair BamHI fragment both on a digest of genomic DNA and the cosmid clones (data not shown).

Sequence Analysis—The 3-kilobase pair BamHI fragment (EMBL accession no. X95444) was found to contain a single open reading frame with a strong similarity to the enteric rho gene. The open reading frame encoding Rho 77 (707 residues, 77 kDa, Fig. 2) is preceded by the sequence GGAGGACCCCATATGAGCATATGGTGCTGGCCGAG with translational fusion of the His- tag (Fig. 2), removing a GAGCATATGGTGCTGGCCGAG) with translational fusion of the His- Rho 72 but used the mutagenic oligo NDEM43 (5'-GTACCGGCCTC-676 I fragment (Fig. 1) into the N-terminal region after extension with the oligo NDEV1 of sequence 5'-GGAAGGACCCCATATGAGCATATGGTGCTGGCCGAG) with translational fusion of the His- tag (Fig. 1) in the His-tag expression vector pET15b (Novagen). The fidelity of PCR was checked by sequencing the N-terminal region after ligation with the rest of the rho gene (C-terminal PstI-BamHI fragment, Fig. 1) in the His-tag expression vector pET15b (Novagen). The fidelity of PCR was checked by sequencing the N-terminal region after ligation between oligo NDEV1 and the forward universal primer. This was accomplished by subcloning into PstI-BamHI fragment both on a digest of genomic DNA and the cosmid clones (data not shown).

Expression and His-tag purification of proteins and removal of the His-tag with thrombin protease were as described by Novagen (16). Expression of Rho and purity were monitored by PAGE (17) and storage of the protein from phosphate by chromatography in 0.35M KH2PO4, pH 3.4, with the ATP resolved from poly(A), Pharmacia, average length at least 180 nucleotides).

Sequence assembly and comparison were performed with the oligo NDEV1 of sequence 5'-GGAAGGACCCCATATGAGCATATGGTGCTGGCCGAG) with translational fusion of the His- Rho 77 and 72 open reading frames. Sequence analysis with the oligo NDEV1 of sequence 5'-GGAAGGACCCCATATGAGCATATGGTGCTGGCCGAG) with translational fusion of the His- tag (Fig. 1) in the His-tag expression vector pET15b (Novagen). The fidelity of PCR was checked by sequencing the N-terminal region after ligation with the rest of the rho gene (C-terminal PstI-BamHI fragment, Fig. 1) in the His-tag expression vector pET15b (Novagen). The fidelity of PCR was checked by sequencing the N-terminal region after ligation between oligo NDEV1 and the forward universal primer. This was accomplished by subcloning into PstI-BamHI fragment both on a digest of genomic DNA and the cosmid clones (data not shown).

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Within 50 bp downstream of the TGA stop codon was an...
overexpression of Rho 72 and 77 in E. coli—Optimal expression of Rho 72 and 77 occurred 1.5 to 2.5 h after isopropyl-1-thio-β-D-galactopyranoside induction with both attaining up to 10% of total cellular protein. Including the 2 kDa of the hexahistidine tag and associated residues, the sizes predicted were 74 and 79 kDa, respectively. Both polypeptides had PAGE mobility consistent with 85–90-kDa proteins, as estimated using Sigma VI h markers. Purification by nickel-agarose resulted in >90% pure protein as judged by Coomassie Blue-stained SDS-acrylamide gels.

RNA-dependent ATPase Activity—In the absence of RNA, the coupled assay did not detect ATPase activity in preparations of Rho 77. Homopolymeric C, U, and A RNAs were effective in stimulating ATP hydrolysis by Rho 77 while poly(G) was completely inert (Table II). Oligo(C) DNA did not stimulate ATPase activity (Table II) nor did single-stranded M13mp18 DNA (data not shown). The apparent $K_m$ value for poly(C)-stimulated ATPase activity (average length 284 nucleotides) was 0.5 μM using the malachite assay. Rho 72 activity was generally similar to Rho 77 (data not shown). Rho 77 was not only an RNA-dependent ATPase but could also hydrolyze GTP, CTP, and UTP, as demonstrated by the release of inorganic phosphate from these compounds. Apparent $K_m$ values of Rho 77 (Table III) showed that GTP and then ATP have the lowest $K_m$ values followed by CTP at millimolar CTP concentrations, phosphate contamination gave an unacceptable high background. Along with the particularly low rates of hydrolysis of CTP this made determination of an accurate $K_m$ for CTP (>2 μM) impossible. Rho 72 as an NTPase had apparent $K_m$ values that were indistinguishable from Rho 77 (data not shown).

RNA-dependent hydrolysis of ATP could also be demonstrated using a radioactive assay (Table IV). This was performed using higher concentrations of ATP (2 mM) than the coupled assay which partly accounts for the higher rates of hydrolysis. Rifampicin is an inhibitor of initiation of RNA polymerase. Any effect of rifampicin on Rho was assayed by the direct radioactive method because rifampicin was too intensely pigmented for the spectrophotometric assays. Rho 77 was not inhibited by rifampicin (Table IV) which makes it a suitable agent for blocking the reinitiation by RNA polymerase in Rho termination assays. Heparin is used in a similar fashion to rifampicin, but the ATPase activity of Rho 77 was completely inhibited by 100 μg/ml heparin in the direct assay (Table III). This was confirmed using the malachite ATPase assay: only 0.2 μg/ml heparin is required to reduce the ATPase activity of Rho 77 by 50%, when stimulated by either 2 or 20 μg/ml poly(C) RNA (Fig. 3A). Bicyclomycin also inhibited Rho 77 (Fig. 3B) with as little as 7 μM sufficient to reduce the RNA-dependent ATPase activity by 50%. The same sample of bicyclomycin, at a
soya-mannitol agar plates with 600...hydrophilic region was surprisingly variable. Apart from Gram-positive bacteria the sequence and extent of the addi-...demonstrated that poly(A) RNA was only 4-fold less effective than poly(U) RNA in stimulating the ATPase activity of Rho 77...was found in the RNA-binding domain. This feature is common to the RNA-binding domain, and contained both ATPase A and B motifs as well as regions characteristic of Rho-ATPases. The specificity of Rho factors as well as some other helicases. The specificity of Rho 77 for polymeric RNA was far less biased toward poly(C) RNA in the presence of heparin...and bicyclomycin. Such a region would be expected to form an unstructured random coil (24). An analogous hydrophobic region is found in the human U1A RNA-binding protein (data not shown) and is known to result in aberrant mobility by PAGE (28). The latter observation may explain why Rho 72 and 77 run as if they were larger proteins, something which is also true of the M. luteus Rho (22). An RNP-1 RNA-binding motif was found in the RNA-binding domain. This feature is common to all Rho factors although the RNP-1 motif from the GC-rich Gram-positive bacteria was somewhat distinctive. The ATPase domain of the S. lividans Rho was highly conserved, compared to the RNA-binding domain, and contained both ATPase A and B motifs as well as regions characteristic of Rho-ATPases. On the basis of alignment with the N-terminal regions of the M. luteus Rho we suggest Rho 77 is expressed in S. lividans. The truncated, in-frame Rho 72 was found to have a possible ribosomal-binding site upstream of the adjacent ATG and GTG start codons. As N terminally distinct isoforms are sometimes found in actinomycete transcriptional regulators, it cannot be excluded that Rho 72 is also expressed. The streptomycete transcriptional regulatory gene tipA (29) and the repressor locus of actinophage øC31 both express such truncations (30). Rho 77 had RNA-dependent ATPase activity, a property of Rho factors as well as some other helicases. The specificity of Rho 77 for polymeric RNA was far less biased toward poly(C) RNA than Rho from E. coli. Mori and co-workers (19) reported that poly(U) RNA was more than an order of magnitude less effective than poly(C) RNA in stimulating the ATPase activity of E. coli Rho, and did not detect activity with poly(A) RNA. They used a similar method to the results in Table II, which demonstrated that poly(A) RNA was only 4-fold less effective than poly(C). The RNA activation of ATPase activity of Rho 77 resembles mutants of the E. coli Rho more closely than the wild type, for example Rho-He235 (19), but no mutant enteric Rho has as broad a specificity as Rho 77. Effective poly(U/A) activation of the Rho 77 ATPase may indicate an adaptation in an organism which has an extreme GC-rich bias. The S. lividans Rho presumably must select termination sites from RNA in a manner methyltransferase. Such a region would be expected to form an unstructured random coil (24). An analogous hydrophilic region is found in the human U1A RNA-binding protein (data not shown) and is known to result in aberrant mobility by PAGE (28). The latter observation may explain why Rho 72 and 77 run as if they were larger proteins, something which is also true of the M. luteus Rho (22). An RNP-1 RNA-binding motif was found in the RNA-binding domain. This feature is common to all Rho factors although the RNP-1 motif from the GC-rich Gram-positive bacteria was somewhat distinctive. The ATPase domain of the S. lividans Rho was highly conserved, compared to the RNA-binding domain, and contained both ATPase A and B motifs as well as regions characteristic of Rho-ATPases. 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Similar results to S. lividans were obtained for S. coelicolor A3(2) 1980 on R2YE medium, and bicyclomycin also reduced the production of the blue-pigmented antibiotic actinorhodin.

**DISCUSSION**

The deduced gene product of Rho from S. lividans shows certain sequence characteristics in the RNA-binding domain that appear peculiar to the GC-rich Gram-positive bacteria. Most notable was a predominantly hydrophilic region of 228 residues, but a putative DDVXPVAGILD motif and a second minor insert were also characteristic. Within the GC-rich Gram-positive bacteria the sequence and extent of the additional hydrophilic region was surprisingly variable. Apart from an absence of hydrophobic residues, there appears to be few evolutionary constraints. The hydrophilic sequence of the S. lividans Rho resembles regions common to a number of RNA-binding proteins including the C terminus of the ErmE riboso-...spectrum of homology between Rho 77 and related proteins using the GCG program BESTFIT to calculate percentage identity (% I) and similarity (% S)

| Polypeptide  | Accession | % I  | % S  | Ref.            |
|-------------|-----------|------|------|----------------|
| Bacillus subtilis Rho | M97678 | 50.6 | 71.3 | 5              |
| Escherichia coli Rho | J01674 | 52.5 | 73.1 | 25             |
| Micrococcus luteus Rho | L27277 | 57.1 | 73.8 | 22             |
| Mycobacterium leprae Rho | U15186 | 59.4 | 75.2 | Genome Therapeutics Corp. |
| Yersinia pestis YscN | U15186 | 27.0 | 51.4 | 26             |
| Saccharopolyspora erythrae ErmE | X51891 | 24.6 | 45.1 | 24             |

**TABLE II**

Stimulation of Rho 77 ATPase activity (nmol of ATP hydrolyzed/µg Rho min, ± S.D.) with 20 µg/ml homopolymeric RNA or DNA measured using the indirect assay. Activity normalized to poly(C) stimulation is shown for poly(U) and poly(A) RNA.

| Nucleic acid | ATPase activity | Ratio |
|-------------|-----------------|-------|
| None        | <0.01           |       |
| rC          | 3.60 ± 0.54     | 1.00  |
| U           | 1.51 ± 0.11     | 0.42  |
| rA          | 1.02 ± 0.13     | 0.29  |
| rG          | <0.01           |       |
| dC          | <0.01           |       |

**TABLE III**

Apparent K_m values (mM) for NTPs for Rho 77 compared to literature values for the E. coli Rho (27).

| NTP       | Rho 77 | E. coli Rho |
|-----------|--------|-------------|
| ATP       | 0.17   | 0.009       |
| CTP       | >2.0   | 0.04        |
| GTP       | 0.13   | 0.06        |
| UTP       | 1.1    | 0.15        |

**TABLE IV**

Effect of inhibitors of the RNA polymerase on the RNA-dependent ATPase activity of Rho 77, measured using radioactive method with 2 nm ATP and 100 µg/ml poly(C) RNA.

| Activity | % activity |
|----------|------------|
| Control  | 7.6 ± 0.8  |
| Rifampcin (2 mg/ml) | 6.4 ± 0.7 |
| Heparin (100 µg/ml)  | <0.1       |

**FIG. 3.** Inhibition of ATPase activity of Rho 77, as measured by the malachite green assay with 0.5 nm ATP. A, with 2 µg/ml (○) poly(C) RNA in the presence of heparin. B, with 2 µg/ml poly(C) RNA and in the presence of bicyclomycin.
which cytosine-rich RNA tracts will be common. While interfering secondary structure may also be more frequent in GC-rich RNA, it is unlikely that this is sufficient to exclude a Rho factor of specificity similar to E. coli from inappropriate sites. Nowatzke and Richardson (22) have found a similarly broad RNA-dependent ATPase activity for Rho purified from M. luteus, so this appears to be a general adaptation of the GC-rich Gram-positive bacteria. The ATPase activity of Rho 77 was not stimulated by poly(G) RNA as a cofactor, also the case with the enteric Rho (27, 34). This is unsurprising, given the highly ordered secondary structure adopted by poly(G) RNA in solution which is likely to preclude Rho 77 from binding (34). Stimulation of ATPase activity with homopolymeric RNAs does not predict the sequence of a Rho-dependent terminator, but it does suggest that recognition of U- and A-rich RNA elements are more important to the GC-rich Gram-positive Rho factors than in E. coli. Defining the ability of an RNA-binding protein to select a particular target RNA is an important goal. Establishing the basis for the diversity of the interaction of Rho from different subacteria with RNA is likely to enhance our understanding of RNA recognition by proteins generally and Rho function specifically.

The NTPase activities of Rho 77 also differed from the enteric Rho and deletion of the N terminus to express Rho 72 made little difference. The S. lividans Rho was primarily a GTPase/ATPase, both on the basis of K_m (Table I) and rate of hydrolysis (data not shown). As little is known about NTP metabolism in streptomycetes, the significance of the higher NTP K_m values of Rho 77 compared to the E. coli Rho is unclear. The Rho family may prove a useful group of proteins to approach the question of recognition and hydrolysis of specific NTPs. Mutants altering the preference of the E. coli Rho for NTPs have not been found but this may be because the ATPase activity alone is most commonly assayed.

The poly(C)-dependent ATPase activity of Rho 77 was sensitive to heparin. This was a surprising result given the insensitivity of the E. coli Rho poly(C)-dependent ATPase activity to concentrations as high as 1 mg/ml (27), although stimulation of the enteric Rho by mRNA is heparin sensitive (31). If heparin is acting as a nucleic acid analogue in this experiment, then streptomycete Rho must interact with poly(C) RNA in a different way to the enteric Rho. Inhibition of Rho 77 by heparin did not display simple competitive kinetics with poly(C) RNA (Fig. 3A and data not shown) and the nature of the interaction is unclear.

Bicyclomycin, a known inhibitor of the RNA-dependent ATPase activity of the E. coli Rho, was also effective against the streptomycete Rho at a similar concentration to that required to inhibit the enteric Rho (32). Bicyclomycin retarded the sporulation of S. lividans and S. coelicolor A3(2), possibly a consequence of inhibiting Rho in vivo. This is interesting given that the Gram-positive bacteria, unlike enteric bacteria, are generally resistant to this antibiotic (33). Bicyclomycin may be inactivated or excluded in some fashion or the Gram-positive Rho may be inessential. Indeed, Quirk et al. (5) have shown that the B. subtilis rho gene can be disrupted.

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