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A Transcriptional Reporter for In Vivo Promoter Analysis in the Archaeon *Haloferax volcanii*

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We have used a modified intron-containing $tRNA^{Pro(UGG)}$ gene ($tRNA^{ProM}$), derived from the *Saccharomyces cerevisiae* $tRNA^{Pro(UGG)}$ gene, as a reporter to measure in vivo transcription from a halophilic archaeon promoter. Coupling of the yeast $tRNA^{ProM}$ gene to the *Haloferax volcanii* $tRNA^{Lys}$ promoter on the *H. volcanii* plasmid pWL201 led to the production of a single stable transcript that was readily quantitated by Northern (RNA) blot analysis. Comparison of $tRNA^{ProM}$ RNA production from constructs containing the wild-type $tRNA^{Lys}$ promoter and those containing mutant $tRNA^{Lys}$ promoters demonstrated that this assay system can be used to measure expression from strong and weak promoters.

There is growing evidence that the transcription systems of the *Archaea* and the *Eucarya* are closely related. Comparative sequence analysis of archaeal genes has revealed a consensus promoter element that resembles the eukaryal TATA-like element (4), and participation of these sequences in transcription initiation has been verified in in vitro studies with purified *Sulfolobus shibatae* and *Methanococcus vannielii* RNA polymerases (4, 5, 15). The relatedness of these two systems extends to the RNA polymerase complexes as well. Archaea contain a single multisubunit RNA polymerase that is similar in complexity to the eukaryal RNA polymerases (18), and the archaea appear to have proteins that are related in sequence and function to eukaryal transcription factors (3, 8, 12, 16).

While these studies support the hypothesis that the archaeal transcription complex is closely related to the eukaryal system, it has been difficult to investigate in vivo gene regulation because of the lack of available genetic exchange systems for most archaea. The recent development of an efficient transformation protocol (1, 2) and shuttle vectors (7) for the halophilic archaea has allowed the development of systems for in vivo analysis of promoter structure and gene regulation in these organisms. In this study, we exploited a modified yeast $tRNA^{Pro}$ gene, which produces a single stable RNA species in *Haloferax volcanii* (13), as a tool for measuring relative promoter strength in the halophilic archaea. The utility of this gene as a transcriptional reporter is demonstrated in an analysis of the *H. volcanii* $tRNA^{Lys}$ promoter and three promoter mutants, each containing a base substitution in the TATA-like box A element.

Construction of the reporter vector and preparation of *H. volcanii* $tRNA^{Lys}$ box A promoter mutants. We have previously established that a 112-bp *Hae*III restriction fragment containing the *H. volcanii* $tRNA^{Lys}$ promoter region is capable of initiating transcription in vivo when present on the *H. volcanii-Escherichia coli* shuttle vector pWL202 (10). To reduce the size of the promoter-containing fragment, a portion of the original *Hae*III restriction fragment which contains the box A (TATA-like) and box B (initiation site) regions was selectively amplified by PCR (14). Sequences between the regions of pWL202 complementary to the oligonucleotides PROMO5P (5′-CGTGCAAAGCTTGAAAGGAAAGTCATTTTACC-3′) and 0167INT (5′-CTCCGGTGTGCGCCAAGCCTC-3′) were amplified. PROMO5P encompasses the box A region of the $tRNA^{Lys}$ promoter fragment and has a *Hin*dIII site plus 6 extra nucleotides at its 5′ terminus, and 0167INT is complementary to the $tRNA^{Trp}$ intron sequence present in the original construction (10). The resulting 63-bp *Hin*dIII-*Xba*I fragment, containing the archaeal box A and box B elements, was removed from the amplified DNA by restriction digestion and ligated into the corresponding sites of pUC19 to produce plasmid pUC301. A modified form of the *Saccharomyces cerevisiae* $tRNA^{Pro(UGG)}$ gene, $tRNA^{ProM}$ (13), was subcloned as an *Xba*I-*Eco*RI fragment into the corresponding sites of pUC301 to produce plasmid pUC302. This strategy placed the $tRNA^{ProM}$ gene 21 nucleotides 3′ of the $tRNA^{Lys}$ promoter initiation site. Vector construction was verified by DNA sequence analysis. The *H. volcanii* shuttle-expression vector pWL222 was constructed by cloning the *Hin*dIII-*Eco*RI expression cartridge from pUC302 into the corresponding sites of pWL201, replacing the original sequences of this plasmid with the yeast tRNA gene expression module (Fig. 1A).

The box A promoter mutations, at position −27 of the *H. volcanii* $tRNA^{Lys}$ promoter, were synthesized by amplifying DNA between the regions of pUC302 complementary to the oligonucleotide MUTP1 (5′-CGTGCAAAGCTTGAAAGGAAAGTCATNTTACCCACCG-3′, where N is any nucleotide), which is specific for the box A sequence region and possesses fourfold degeneracy at position −27 of the *H. volcanii* $tRNA^{Lys}$ promoter, and the universal pUC primer M13/−40 (5′-GTTTTCCCAGTCACGAC-3′). The DNA fragment containing the $tRNA^{Lys}$ promoter and the $tRNA^{ProM}$ gene, including the associated yeast RNA polymerase III terminator element of the $tRNA^{Pro}$ gene, was removed from the amplified DNA as a *Hin*dIII-*Eco*RI fragment and cloned into the equivalent sites of pUC19. Plasmid DNA isolated from the resulting transformants was screened by DNA sequence analysis, and plasmids containing the wild-type promoter and each of the three possible mutant promoters were identified. The four individual *Hin*dIII-*Eco*RI expression cartridges were subcloned into the corresponding sites of *H. volcanii* shuttle vector pWL201. These plasmids were introduced into *E. coli* JM110, and plasmids isolated from these cells were used to transform *H. volcanii* (10). *H. volcanii* has a restriction system that degrades DNA methylated at adenosine residues, and passage

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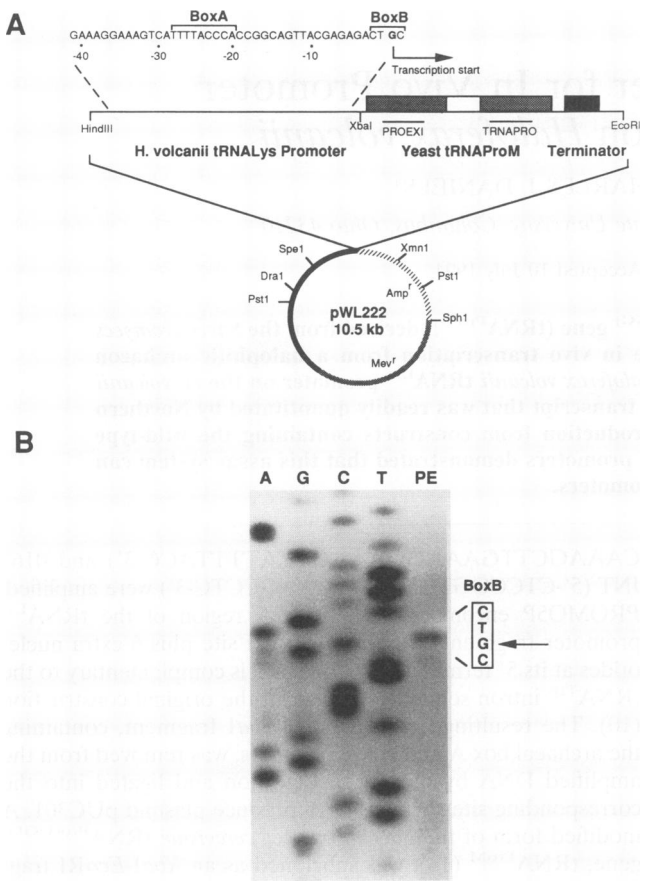

FIG. 1. Development of an *H. volcanii* transcription reporter gene. (A) Schematic representation of the expression module of plasmid pWL222, which contains the *H. volcanii* $tRNA^{Lys}$ promoter and the modified yeast $tRNA^{ProM}$ gene. The box A and B elements of the $tRNA^{Lys}$ promoter and the locations of the probes used in primer extension and Northern analysis are shown. (B) Results of primer extension analysis performed on RNAs isolated from cells carrying plasmid pWL222, with oligonucleotide PROEXI used as a primer. PE, primer extension.

of plasmids through *E. coli* strains carrying a *dam* mutation reduces restriction by *H. volcanii* during transformation (6).

Transcript analysis. Analysis of archaeal transcripts has shown that initiation usually occurs at a purine residue located approximately 25 bp 3′ of the box A element in the region referred to as the box B element (17, 19). To determine if the yeast $tRNA^{ProM}$ gene altered the site of transcription initiation directed by the *H. volcanii* $tRNA^{Lys}$ promoter, primer extension analysis was performed on RNA isolated from cells carrying the pWL222 plasmid. In these experiments, the $tRNA^{ProM}$ exon 1-specific oligonucleotide PROEXI (5′-CCC AAAGCGAGAATCATACCAC-3′) was used as a primer for DNA synthesis by avian myeloblastosis virus reverse transcriptase (11). The PROEXI oligonucleotide was also used to prime a DNA sequencing reaction with pUC302 as the template DNA. Both the primer extension and sequencing reaction products were separated by electrophoresis through a denaturing (8.3 M urea) 6% polyacrylamide gel, and the 5′ terminus of the $tRNA^{ProM}$ transcript was determined by comparison with the adjacent DNA sequence ladder. Figure 1B shows that transcripts of the $tRNA^{ProM}$ gene initiated at the expected G residue in the $tRNA^{Lys}$ promoter box B element (10). We have also determined that this RNA does not undergo 5′ or 3′ end maturation or intron removal (13). The inability of this RNA to undergo 5′ or 3′ end maturation is due to the presence of the non-Watson-Crick U6-U67 pair in the acceptor stem. The occurrence of this nucleotide pair specifically blocks RNase P cleavage of the 5′ leader sequence of $tRNA^{ProM}$ RNA, and this processing step is required for subsequent 3′ processing (13). This RNA also does not undergo intron processing, and the detected $tRNA^{ProM}$ RNA therefore represents the primary transcript from this gene. Northern analysis with probes directed against $tRNA^{ProM}$ sequences revealed a single hybridizing species, indicating that there is no detectable processing or degradation of the $tRNA^{ProM}$ RNA (13) (see Fig. 2). Thus, quantitation of the $tRNA^{ProM}$ RNA provides a direct measurement of transcription.

To test the sensitivity of the $tRNA^{ProM}$ gene as a transcriptional reporter, we introduced single-nucleotide mutations into the *H. volcanii* $tRNA^{Lys}$ box A sequence, changing T−27 to each of the other three possible nucleotides. These changes lie within the TATA-like sequence of the box A element. On the basis of in vitro transcription studies with RNA polymerase from *M. vannielii* (5) and *S. shibatae* (4), these changes were expected to alter the efficiency of this promoter. By Northern analysis with probes directed against the $tRNA^{ProM}$ RNA sequences and the chromosomally encoded $tRNA^{Leu}$ RNA as an internal control, the relative amount of transcript produced from each promoter was determined. The $tRNA^{ProM}$ exon 2-specific oligonucleotide TRNAPRO (5′-CGAGCTGGGAA TTGAACCCAGG-3′) was used to determine $tRNA^{ProM}$ transcript levels. Each $tRNA^{ProM}$ signal was normalized by comparison with its companion signal derived from the oligonucleotide LEU3E (5′-GGGGACGAGATTCGAACTCGCGAA CCCCTACG-3′), which is complementary to the *H. volcanii* chromosomal $tRNA^{Leu}$ sequence. Prior to hybridization, each oligonucleotide was end labeled with [γ-^{32}P]ATP by using T4 kinase (9). Approximately 2.0×10^6 cpm of each probe was added to the hybridization mixture, and the incubation was allowed to continue at 50°C for 24 h. The membranes were then washed three times at room temperature (10 min each wash) in 250 ml of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate. The amount of hybridizing RNA in counts per minute was determined with a Betagen Betascope 603 blot analyzer (Betagen, Waltham, Mass.). The relative amount of expression was determined by measuring the radioactivity associated with the $tRNA^{ProM}$ and $tRNA^{Leu}$ bands and expressing the results as the ratio of $tRNA^{ProM}$ to $tRNA^{Leu}$. This value was then compared with the relative expression of the wild-type $tRNA^{Lys}$-$tRNA^{ProM}$ construct, which was set at 100%.

Visual inspection of the Northern hybridization patterns revealed that each of these mutations altered the expression level of the $tRNA^{ProM}$ gene (Fig. 2). These data show that the A−27 substitution resulted in an "up-promoter" mutant with a concomitant twofold increase in transcriptional activity to 212% of the wild-type level. In contrast, the G−27 and C−27 substitutions resulted in "down-promoter" mutants with activity levels of 11 and 8% of the wild-type activity level, respectively. Reproducibility of the assay was tested with the wild-type $tRNA^{Lys}$-$tRNA^{ProM}$ construct. For 17 independent determinations, the level of $tRNA^{ProM}$ activity relative to that of the $tRNA^{Leu}$ internal control was found to be 33% ± 6.0%.

These data illustrate the ability of the yeast $tRNA^{ProM}$ gene to function as an in vivo transcriptional reporter in *H. volcanii* by directly reflecting the transcriptional strengths of both

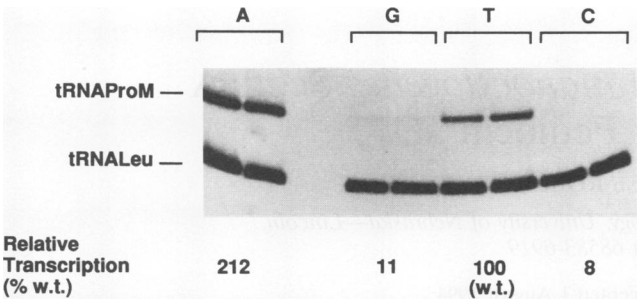

FIG. 2. Transcription from the *H. volcanii* $tRNA^{Lys}$ promoter and three promoter mutants each possessing a base substitution at position −27 (Fig. 1A). RNAs were hybridized in a Northern blot to probes directed against the plasmid-encoded $tRNA^{ProM}$ and chromosomally encoded $tRNA^{Leu}$ sequences. Levels of the promoter activities, normalized to the $tRNA^{Leu}$ internal standard, are shown below the lanes as percentages of the wild-type (w.t.) activity level.

strong and weak promoter elements. The absence of processing of the $tRNA^{ProM}$ transcript, in conjunction with its stability and relative ease of detection, makes this modified yeast $tRNA^{Pro}$ gene a good transcriptional reporter for studies of the regulation of *H. volcanii* promoters. Its use as an in vivo reporter may also be applicable to other halobacteria that possess characterized genetic exchange systems.

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