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Progress Report

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Title: Structure and Regulation of an Archaeobacterial Promoter: An In Vivo Study.
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In our initial proposal we defined three specific questions for study:

1. What constitutes a halobacterial tRNA promoter in vivo?
2. Are halophilic tRNA promoters regulated by growth rate and nutrient levels?
3. Will non-halobacterial promoters function in the halobacteria?

During this time period we have made significant progress in the addressing questions 1 and 3, and are developing systems for examining gene regulation strategies. In addition to these questions, we have made a new discovery concerning transcription termination and are pursuing this problem. A summary of our accomplishments is provided below.

Halobacterial Promoter Analysis:

In the period prior to this grant we had established that a 120 bp DNA fragment from the *Haloferax volcanii* tRNALys gene directed transcription in vivo (see Appendix III; 15). This element then served as the starting point for a detailed analysis of the sequence requirements for in vivo transcription. Among several gene tentatively identified as reporter elements, we found that a eukaryotic intron-containing tRNAPro gene (and a splice site mutant form of this gene, tRNAProM) was ideal for our analysis. This gene, when placed under the transcriptional control of the *H. volcanii* tRNALys promoter fragment, produces a single small transcript (see Figure 1). Transcript analysis (S1 mapping and primer extension) showed that this RNA initiated at the expected tRNALys BoxB sequence and terminated in the tRNAPro RNA Pol III termination element present on the DNA fragment. The yeast tRNAProM RNA does not enter into the 5', intron, or 3' processing pathways and remains as a single stable primary transcript. No degradation or processing products could be detected by Northern analysis. In initial studies we determined that the 3' proximal 40 bp region of this tRNALys promoter element was sufficient for transcription initiation in vivo (data not shown). Removal of the distal 80 bp slightly decreased the expression (down by 30-40%), and this region was absent from all further constructs. The transcriptionally active 40 bp region contains only the BoxA and BoxB elements and short purine rich sequences 5' to both BoxA and BoxB (see Figure 2A). Using the tRNAPro gene as the reporter, and this minimal promoter, we performed a comprehensive analysis of the BoxA region. Each position of the BoxA region was converted to all four possible nucleotides and the transcription of 36 mutants was examined (see Figure 2B). Transcripts were quantitated by Northern analysis using a 5' end labeled tRNAPro oligonucleotide probe and a BetaGen scanner to measure radioactivity in the bands. The amount of tRNAPro hybridization was then normalized to an internal control, *H. volcanii* tRNALys RNA. The results of this analysis are summarized in Figure 2C. Among the sites analyzed, only the first five positions showed high levels of

discrimination; the preferred BoxA element was 5'-(T/C)(A/T)TA(A/G)NNNN-3'. Mutational analysis demonstrated that a transition from T-rich to A-rich sequences in the BoxA element is essential and that there is some flexibility in the location of the "TA" sequence. In addition to its effect on promoter strength, the "TA" sequence appears to determine the location of the transcription start site. The BoxA element defined in this study is similar to those observed *in vitro* for *Sulfolobus* (16) and the methanogen promoters (17), and supports the hypothesis that a similar "core" promoter element is used by all archaeal RNA polymerases (Figure 3). We have tested this hypothesis *in vivo* by introducing a methanogen gene, with its presumed promoter element, into *H. volcanii* and found that this DNA directs transcription (see below).

Figure 3: Comparison of *H. volcanii* tRNALys promoter mutants with consensus BoxA sequences and BoxA sequence preferences determined *in vitro*.

Expression of non-halobacterial genes in *H. volcanii*:

We reasoned that the similarity of the halobacterial BoxA element to other archaeal promoters indicated a common promoter recognition by the archaeal RNA polymerases. If true, the halobacterial system could then provide a model system to decipher the requirement for expression and regulation. Moreover, the halobacterial system could act a surrogate host system for the expression and analysis of genes originating from organisms that do not have tractable systems for *in vivo* analysis. To test this possibility we choose to introduce the *Methanopyrus kandleri* tRNAGln gene (18) into *H. volcanii*. This organism is distantly related to the halophiles (*M. kandleri* is the deepest branching methanogen known) and represents a class of organisms for which there is no *in vivo* analysis system. A 900 bp fragment carrying the tRNAGln gene was introduced into *H. volcanii* on the plasmid pWL102. Sequence analysis of this fragment indicates that the tRNA gene is preceded by a sequence element that closely resembles a methanogen BoxA sequence, 5'-TTATATAGG-3' (see Figure 4). Northern analysis of RNAs derived from the plasmid bearing strain indicate that specific RNAs, in the expected size range, are present; this strongly suggests use of the methanogen promoter (see Figure 4). Transcript mapping is currently underway to determine the precise transcription start site.

Transcription Termination:

As mentioned above, we observed that the tRNA^{Pro} RNA Pol III termination element appeared to direct specific and strong termination in *H. volcanii*. A study of transcription termination was not one of our original goals however, since virtually nothing is known about transcription termination in the archaea, we chose to examine this phenomena. To test whether this sequence element functions as a termination signal, and not a RNA processing site, we moved the Pol III termination element from the tRNA^{Pro} gene and placed it between the tRNA^{Lys} promoter and another reporter gene, tRNA^{Trp} O16M-ΔGGAG. The tRNA^{Trp} O16M-ΔGGAG gene encodes a mutant form of the tRNA^{Trp} RNA that does not undergo intron processing (19); instead the transcripts from this gene undergo processing at their 5' and 3' ends, producing three large stable RNAs. These RNAs are detected by Northern analysis using a probe directed against the modified acceptor stem of this mutant (19). If the element was a terminator, no transcription of the reporter would be observed. In contrast, if it was a processing signal a shorter stable RNA containing the tRNA^{Trp} reporter would be generated. When the element was introduced in both orientations, we observed that the forward orientation resulted in strong termination, while the reverse orientation allowed transcriptional read-through (see Figure 5). A comparison of the forward sequence with the sequences of archaeal RNA mapped 3' termini indicates that there is a correlation between pyrimidine-rich sequences and termination sites (Figure 6). It was interesting to note that the reverse orientation containing the T-rich sequence 5'TTAATTT-3', which has been implicated to function as a terminator in vitro with the methanogen RNA polymerase (20), was not sufficient to cause strong termination in *H. volcanii*. It appears that a defined sequence or sequences plus structure are required for efficient termination in vivo. The halophilic gene expression systems we have developed now provide a means to probe the requirements for transcription termination in vivo.

Gene Regulation Strategies:

In our original study we proposed to examine the regulation of the tRNA^{Lys} promoter in response to growth rate and nutrient levels. We reasoned that the "stringent-like" response, typical of the bacteria, could serve as a model for studying the molecular mechanism used by the archaea to control gene expression at the transcriptional level. The central question is whether the archaeal RNA polymerase, with its apparent close relationship to eukaryotic RNA polymerases and their transcription signals, utilizes bacterial (sigma factors, inducers-repressors) or eukaryotic (transcription factor) mechanisms for controlling transcription. While analysis of the tRNA promoter was possible with the available experimental systems, two recent findings led us to pursue the heat shock response as a model for transcriptional control in the archaea. The first was the description of archaeal DNA sequences that were similar to the eukaryotic transcription factors TFIIS (21) and TFIIB (22), suggesting further similarity between the eukaryotic and archaeal transcription complexes. The second was a description of heat shock responsive DNA elements in *H. volcanii* (14). In this study several DNA elements were identified that gave strong hybridization signals with cDNAs derived only from heat shock RNAs. This supported an earlier observation that this group of organisms has a typical heat shock response (24) and indicates that this induction takes place at the transcriptional level. Unlike the regulation of stable RNA synthesis, detailed information is available on the molecular mechanisms of transcriptional control of heat shock genes in both bacteria and

eukaryotes, and in both cases the control mechanisms are paradigms for the regulatory strategies utilized by these two types of cells (a more detailed description of these systems is present in the main proposal). The ability to carryout in vivo regulatory experiments on a system that has well defined models in both groups of organisms (each using a mechanism representative of the group), warranted this shift.

In collaboration with Dr. Rob Charlebois, we identified a DNA fragment on cosmid A199 (the entire *H. volcanii* genome has been mapped and is available on cosmids) that potentially encoded a heat shock gene. Two Mlu1 fragments within this cosmid were found to give strong transcriptional induction in response to heat shock (Figure 7). Both fragments (2 and 4kb) hybridized to a single 2800 nt RNA, and this RNA was strongly induced during heat shock and followed the reported time course for protein induction (23). The information content of this RNA is sufficient to encode 113 kDa of protein, enough for an operon of smaller heat shock proteins (eg. groES-groEL) or a single large protein (eg. HSP90). With the tools we have developed for monitoring in vivo transcription, and the knowledge we will gain in the "core" promoter experiments, we have all the needed tools to analyze the molecular mechanisms of heat shock transcriptional regulation in the halophiles.

Summary:

In the initial grant period we have devised an in vivo assay system for the analysis of gene expression in the halophilic archaea. This system has been used to analyze the *H. volcanii* tRNA^{Ala} promoter where it was found that the a 40 bp fragment carrying BoxA and BoxB sequences is sufficient for in vivo expression. Detailed analysis of the BoxA element indicates that the BoxA TA sequence is essential for efficient expression. Support for the hypothesis that all archaea share common transcriptional signals was obtained when a methanogen tRNA^{Gln} gene, with its associated BoxA sequence, was found to direct its own transcription in *H. volcanii*. In related experiments a eukaryotic RNA polymerase III terminator was found to act as a strong termination signal in *H. volcanii*. Sequence comparisons between this element and mapped RNA 3' ends indicates that T-rich sequences may be important role in directing termination in vivo. Finally, in an attempt to establish a model system to study regulated gene expression, we have isolated a DNA fragment that encodes a heat shock inducible transcript. This gene will now serve as a model for detailed studies of the mechanisms of gene expression in the archaea.

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Presentations and Publications:

This work has been presented at three meeting:

1. In vivo studies on the expression and processing of tRNA gene transcripts in the halophilic archaea. Gordon Conference on Methanogenesis; July 1993.
2. Transfer RNA gene expression in the halophiles. *Molecular Aspects of Archaea*; Max-Planck-Institut fur Biochemie, May 1993.
3. Evolution of tRNA expression and processing signals. Canadian Institute for Advanced Research; September 1992

Three manuscripts are in preparation:

1. Expression of a eukaryotic intron-containing tRNA gene in an archaea. D.T. Nieuwlandt, J. R. Palmer, Y-P. Kuo, and C.J. Daniels. For submission to *Mol. Microbiol.*
2. In vivo analysis of an archaea promoter. J. R. Palmer and C.J. Daniels. For submission to *J. Bacteriol.*
3. Transcription termination in the archaea by a eukaryotic RNA polymerase III terminator. Y-P. Kuo and C.J. Daniels. For submission to *J. Bacteriol.*

A **B**

Figure 1: Expression of Yeast tRNA^{Pro}M gene in *H. volcanii* produces a single stable transcript. Panel A: The yeast tRNA^{Pro}M gene, with its associated RNA polymerase III terminator, was placed downstream of the *H. volcanii* tRNA^{Lys} promoter in the plasmid pWL221. Also shown are the locations of the S1 probe (124 nt expected product) and the primer extension primer (PROEX1). Panel B: Transcript analysis. Left panel is the stained gel for the separation of total RNAs on a 6% denaturing polyacrylamide gel; center panel is the result of primer extension analysis using pWL221 total RNA and PROEX1 as the primer; right panel is the result of S1 mapping with pWL221 total RNA and 5' end labeled Xba1 fragment as the probe.

C

	A	G	C	T
Position				
-28	3	2	62	100
-27	212	11	8	100
-26	52	4	5	100
-25	258	108	17	100
-24	100	57	8	29
-23	166	93	100	72
-22	78	91	100	95
-21	79	77	100	93
-20	100	10	37	115

Figure 2: In vivo analysis of the *H. volcanii* tRNALys BoxA promoter element. Panel A: Sequence of the 40 bp test promoter element fused to the yeast tRNAProM reporter gene. Also shown are the locations of probes used for Northern analysis (TRNAPRO) and primer extension analysis (PROEXI). Panel B: Representative Northern analysis showing the results of changing position -25 to all four possible nucleotides. The amount of tRNAPro, and the internal standard tRNALeu, were quantitated using the BetaGen scanner. Panel C: Summary of the BoxA element analysis. Expression is presented as the percent activity, compared to the wild type nucleotide at that position (boxed nucleotide). All data are normalized to the control tRNALeu.

Methanopyrus kandleri tRNAGln (UUG) Gene

'BoxA'
┌───────────┐
 CGGCACACTACCGCTGCGACGTAATTGTTATATAGGATGC

'BoxB'
┌───┐
 ATAGAGGTTGGGCTGATGGGAGCAGGGCCGTGGGGTAGCG

MKTGLN
└──────────┘
GTCTATCCTGCGGGGCTTTGGACCCCGCGACCCCGGTTCA
ATAACGCGGCGGCCCTACCACTCCGAATCGTTTTGAGAACC
 ATAACAATAAGGGGACGGCTCTTTGGACGCGCTGCTGAAG

Figure 4: The expression of a Methanopyrus kandleri tRNAGln gene in H. volcanii. A 900 bp DNA fragment containing the M. kandleri tRNAGln (UUG) gene was introduced into H. volcanii on the pWL202 vector. Possible BoxA and BoxB regions are indicated; the tRNAGln region is underlined. Lower panel: Northern analysis of cells carrying an unrelated M. kandleri DNA fragment (control), and cells carrying the tRNAGln gene. Expression was monitored by using a probe directed against the M. kandleri tRNA sequences (MKTGLN). Some hybridization is observed in the control lane; this is most likely due to hybridization with the host tRNAGln RNA, which is 68% similar to the M. kandleri tRNA.

A **B**

Figure 5: Transcriptional termination in *H. volcanii* directed by the yeast tRNA^{Pro} RNA polymerase III terminator. Panel A: the RNA polymerase III terminator was introduced into the *H. volcanii* expression vector between the tRNA^{Lys} promoter and the reporter gene, tRNA^{Trp} O16M-ΔGGAG (19). Terminator was introduced in both forward and reverse orientations. Sizes of the expected transcripts from these genes are shown. The solid bar indicates the site of termination observed in vivo. Panel B: Northern analysis of RNAs carrying terminator constructs probed with an exon1 specific probe for tRNA^{Trp} O16-ΔGGAG. Also shown is a primer extension analysis of these RNAs using the tRNA^{Trp} O16M-ΔGGAG exon1 probe as primer.

		Ref
Hcut sod	CGCCCCGCCCCGCGGGGACACCCTGAAACGCCACG CTTTTTTCG CCGTGTAGCGTTCCGATAGCTTCTATACACAGCCCAG	1
Hcut slg	CCGAACACGCTCCCGTGT TTTTTTTC CGCGTCATGGCGGCTCATCAGTGGCTGGCGTGAGTGCGGCGACCGTGTGCTCG	2
Hhal BO	TCGCACACGCAGGACAGCCCCCACAACCGGCGCGGCTGTG TTCAAC GACACACGATGA	3
Hmar a operon	ACCGATGACCGCCCGCTCCCCGAGCGCGACGACCGAAAGGGG TTTTT ACGGGGCACGCAAAACGAAC	4
Hmar S9	<u>CGGTGCCGAGCCGACGTACGGACTCGACGACTTCGAGTCCGACATCTAACGTCCGTGAACCGTT</u> CGTTTTCTCGCTGT CGAAGTGC	5
SB12SSV1 T1	GGTGTGAGGGATGGAAATCAGTTTAAAGCCAATCATTTTTTTGGT CGTTTTTTATCATC GTAGGGATAGCACTATTTCG	6
SB12SSV1 T2	GCCCTTTATAAAGTCATATTCTTTTTCTTTCC CTGATGAGTGC GTTAGGGGATGTAATCTACA	6
SB12SSV1 T3	GCAAAATCTTTTTTACCT CTTTTT TAAATCTGTCTTATATGAAAAAAGTG	6
SB12SSV1 Tind1	CAATAAATGATTTGTTCTAAACTATTTTTTCT CTCT ATCTCTATATCTATATATATACATAACTAAA	6
SB12SSV1 Tind2	ACCATTATACAAACTCAGAAAACTATTTTTTTGTTATA CTCTTACCC CATATATATATAGATATATA	7
Hvan tRNAVal	GTTCGAATCCGGCTGGGTCC CTTAATTT TGAGCATATGTATC	8
Dmol 23S T1	GCAGCGCTCGTAACGGTTTTATTCTCTCC TGGCTTT CATCAACTAGTAGTGGTGGGTATG	8
Dmol 23S T2	AGATTATTTTTCAATGATGAGGATACC TAACTCCCTT ATGAGTGGTGTGGAGCAG	9
Tten 23S	GGCGTGGGGTACTCGTAATACTCC TTTTCT CCCCGGGCTCCGTTTGGCTACAGTGTG	10
Mtherm rRNA	TTAACGACTGATGCCCCAAGGTCCTTCTATG ACATGGGCTG ATGCCCGGGATGAT	11
Hvol rRNA	ATTCATACTTACAGACCGACTGGACGCGGT GTCCAGT GGGCTTTGTTTCATTTTATGCAGAGCGGCACGCACGG	12
Xlae tRNALys	CACACGTCCGTCAAGTTTAACCTTT TAACTC TTTTTACCTGCCTAACTGCC	12
Hum H3.3	ATTTTTAAAGAGGGACGT TTTTT TCCCTTTTTTGGAGAGGCGGAAACTTGATG	13

Figure 6: Comparison of archaeal DNA sequences in the vicinity of mapped RNA 3' termination sites. Experimentally determined sites are indicated by bold nucleotides. Sequences capable of forming hairpin structures are overlined. The potential *H. volcanii* rRNA operon terminator and representative eukaryotic RNA polymerase III (Xenopus tRNALys) and RNA polymerase II (Human histone H3.3) are shown for comparison.

***H. volcanii* Cosmid A199**
(34kb Mlu1 partial; 12 Mlu1 fragments total)

Figure 7: Identification of *H. volcanii* DNA fragments encoding heat shock (HS) responsive gene(s). Upper panel; coarse restriction map of the HS gene-containing cosmid A199 (14). Lower panel; Northern analysis of *H. volcanii* total RNAs probed with a 4 kb Mlu1 fragment derived from the cosmid A199. RNAs were isolated from cells at various times after a shift to 60°C.

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