Transcription termination in the archaebacterium *Sulfolobus*: signal structures and linkage to transcription initiation

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Received January 11, 1988; Accepted February 22, 1988

ABSTRACT

The precise map positions were determined for the 3'-termini of five transcripts of the *Sulfolobus* virus-like particle SSV1. In all cases analyzed, these 3'-termini mapped immediately downstream of a sequence TTTTTYT which was part of a pyrimidine-rich region of 16-19 nucleotides length. No correlation was evident between the position of the 3'-termini and possible secondary structures within the RNA. In two cases, the 3'-termini of SSV1 transcripts mapped in the immediate vicinity of transcriptional initiation sites suggesting that transcription termination can be linked to the re-initiation of RNA synthesis.

INTRODUCTION

The investigation of the molecular biology of archaebacteria has revealed that some features of gene expression are shared by archaebacteria and eubacteria whereas other features are common to archaebacteria and eukaryotes (reviewed in ref. 1). Like in eubacteria, archaebacterial genes are frequently organized in transcriptional units (for review see ref. 2) and the mRNAs are non-capped and devoid of long poly(A)-tracks (3,4). The archaebacterial RNA polymerase, however, is of a eukaryotic type both in its subunit structure (5) and in its amino acid sequence (H. Leffers and G. Pühler, unpublished data). Furthermore, the signal structures used for transcription initiation are similar for the archaebacterial RNA polymerase and the eukaryotic RNA polymerase II (6,7). As far as transcription termination in archaebacteria is concerned, the available data have not yet allowed the derivation of general principles, which is partly due to the fact that so far there are only two reports on the map positions of 3'-termini of archaebacterial mRNAs (8,9). All

other mapping data on the 3'-termini of transcripts have been obtained for stable RNA genes (6,10,11,12) and in some of these cases the observed transcriptional termination sites may have been influenced by the extensive secondary structure within the transcripts.

To obtain more data on the termination sites of mRNAs in archaebacteria, we investigated the transcription from the *Sulfolobus* virus-like particle SSV1 (13). The genome of this UV-inducible genetic element is a 15.5 kb double-stranded circular DNA of known nucleotide sequence (P. Palm and B. Grampp, unpublished data). A transcription map of SSV1 and the precise location of six transcriptional initiation sites have been reported previously (7,14). We have used the S1 endonuclease technique for the precise mapping of the 3'-termini of SSV1 transcripts. These mapping data revealed the presence of a highly conserved sequence TTTTTYT immediately upstream of the transcriptional termination sites. In two instances a close linkage was observed between transcription termination and re-initiation of RNA synthesis.

MATERIALS AND METHODS

Materials.

S1 endonuclease and the Klenow fragment of *Escherichia coli* DNA polymerase I were from Pharmacia and M-MLV reverse transcriptase was from BRL. Restriction enzymes were obtained from Boehringer Mannheim or from Pharmacia. [α -³²P]dATP at 3000 Ci/mMol, [γ -³²P]ATP at 5000 Ci/mMol and [³⁵S]dATP α S at 800 Ci/mMol were from Amersham.

Purification of *Sulfolobus* RNA.

Total RNA from UV-irradiated *Sulfolobus* sp. strain B12 was purified as described previously (15). Cells were harvested 3 hours after UV-irradiation to obtain RNA for the mapping of the UV-inducible transcript T_{ind} and they were harvested 8 hours after UV-irradiation for all other mapping experiments.

S1 endonuclease mapping of transcripts.

For the precise determination of transcript lengths and for the mapping of 3'-termini, total *Sulfolobus* RNA was hybridized to uniformly labelled single-stranded DNA probes and

Table 1: Oligonucleotides, M13 clones and restriction enzymes used for the synthesis of S1 probes

Probe	Oligonucleotide	Label	Enzyme	Length (bases)	M13 clone
P1b	N1; d(ACTCCTATTTCTGAAGTA)	uniform	AluI	420	M23
P1c	N1; d(ACTCCTATTTCTGAAGTA)	uniform	Sau3AI	412	M23
P1d	N1; d(ACTCCTATTTCTGAAGTA)	5'	AluI	420	M23
P3a	N3; d(GCCTTATCGAGATAACT)	uniform	EcoRI	898	M23
P3b	N3; d(GCCTTATCGAGATAACT)	uniform	HindII	547	M23
P7	N7; d(AAATCCCCAAACCAGAG)	uniform	HaeIII	278	M6
P9b	M13 sequencing primer	uniform	SalI	759	M26

the resulting hybrids were digested with S1 endonuclease. For the synthesis of labelled DNA probes, oligonucleotide primers mapping downstream of the 3'-termini of transcripts were chosen from the known nucleotide sequence of the SSV1 genome and annealed to M13(+)-DNA containing the RNA-like strand of the transcribed region. In the case of probe P9b a universal M13 sequencing primer was used for the annealing reaction (Table 1). One microgram of M13(+)-DNA and 0.2 picomoles oligonucleotide primer were annealed in a volume of 10 μ l using the conditions and the buffer described previously (14). After cooling to room temperature, 4 μ l [α -³²P]dATP (40 μ Ci, 3000 Ci/mMol) and 1 μ l of a solution containing 1.2 mM dCTP, dGTP and dTTP were added and the primer was extended by incubating this mixture for 10 min with 1 U of Klenow enzyme. 1 μ l of 1.2 mM dATP were added and the incubation was continued for another 15 min. The enzyme was inactivated by heating to 70°C for 10 min and the partially double-stranded DNA was cleaved for 45 min with 10 U of a restriction enzyme to create a defined 3'-terminus. Electrophoresis on preparative polyacrylamide sequencing gels and electroelution of the single-stranded labelled DNA were carried out as described previously (14). The sequences of the oligonucleotide primers used for the synthesis of DNA probes, the restriction enzyme cleavage sites corresponding to the 3'-termini of these probes

Figure 1. Strategy used for the mapping of SSV1 transcripts.

The map positions of all identified SSV1 transcripts are indicated above the linearized EcoRI map of the SSV1 genome and the map positions of the oligonucleotide primers and the single-stranded DNA probes are indicated below this map. In the case of the M13 clones used for the synthesis of S1 probes (M6, M23 and M26) only the flanking restriction sites and the restriction sites corresponding to the 3'-termini of probes are shown.

and the lengths of all probes are listed in Table 1. The map positions on the SSV1 genome of all oligonucleotide primers and of the M13 clones used for the synthesis of S1 probes are shown in Fig. 1.

For the mapping of the 5'-terminus of transcript T9, the 5'-labelled DNA probe P9d was used. This probe was obtained by the general procedure described previously (14).

Hybridization of total *Sulfolobus* B12 RNA to labelled DNA probes, digestion of the resulting hybrids with S1 endonuclease and analysis of the digestion products on polyacrylamide sequencing gels were carried out as described previously (14).

Primer extension analysis.

The precise determination of the transcriptional initiation site of transcript T9 by oligonucleotide-primed

reverse transcription of RNA was performed as described previously (7). The sequence of the oligonucleotide N2 used for this analysis was d(AGGCGACTGTTCCTATT); its map position on the SSV1 genome is shown in Fig. 1.

DNA sequence analysis.

DNA sequences were determined by the enzymatic method (16) using the M13 cloning and sequencing technique (17,18). The general strategy for the sequence determination of the SSV1 genome has been described previously (15).

RESULTS

Mapping of the 3'-termini of the UV-inducible transcript T_{ind} .

T_{ind} is the only SSV1 transcript identified so far, which is not constitutively transcribed, but is induced within 2-3 hours after UV-irradiation of *Sulfolobus* B12. Two species of transcript T_{ind} (T_{ind}^1 and T_{ind}^2) with lengths of about 0.2 kb and 0.3 kb respectively have been identified by Northern analysis. These hybridization data indicated that both species of T_{ind} share the 5'-terminus but differ in their 3'-terminal regions (ref. 14, see Fig. 1).

Because of the small sizes of T_{ind}^1 and T_{ind}^2 , the map positions of their 3'-termini were deduced from the precise lengths of these RNAs together with the map position of their common 5'-terminus, which has been determined previously (7). The lengths of both T_{ind} species were measured by hybridization of total *Sulfolobus* B12 RNA to the uniformly labelled DNA probe P9b (Fig. 1 and Table 1), digestion of the resulting hybrids with S1 endonuclease and analysis of the lengths of protected fragments on polyacrylamide sequencing gels. The predominant fragments obtained by this analysis were in the size range between 275 and 282 nucleotides (Fig. 2B). In addition, a short ladder of fragments centered around a size of 171 nucleotides was observed (Fig. 2B). These sizes were in reasonable agreement with the lengths of the two T_{ind} species determined by Northern analysis (about 0.3 kb and 0.2 kb respectively). All of the S1-generated fragments described above were present in very low amounts when RNA from non-induced cells was used for the analysis. A semi-quantitative evaluation of the extent

Figure 2. Length determinations and mapping of 3'-termini by S1 endonuclease analysis.

Uniformly labelled DNA probes were hybridized to *Sulfolobus* B12 RNA and digested with S1 endonuclease (lanes S, S_b, S_c). Lanes marked "F" show undigested DNA probes and lanes marked "C" show controls lacking *Sulfolobus* RNA. S1-generated fragments were resolved on a 4% polyacrylamide sequencing gel in (A) and on 6% gels in (B)-(E). The sizes of fragments (in nucleotides) were determined from DNA size markers run in

parallel. These size markers were restriction fragments in (A) and sequencing reactions in (B)-(E).

(A) Length determination of T1 using 100000 cpm of probe P3a.

(B) Length determination of T_{ind}^1 and T_{ind}^2 using 80000 cpm of probe P9b.

(C) Mapping of the 3'-terminus of T3 using 90000 cpm of probe P7.

(D) Mapping of the 3'-terminus of T1 using 26000 cpm of probe P3a.

(E) Mapping of the 3'-terminus of T2 using 26000 cpm of probe P1b (lane S_b) or P1c (lane S_c).

of probe protection using different amounts of RNA indicated, however, that the protected fragments were about 100 times more abundant when RNA from cells 3 hours after UV-irradiation was used, confirming that the fragments obtained after S1 endonuclease digestion were derived from the UV-inducible transcript T_{ind} .

Mapping of the 3'-termini of transcripts T1 and T2.

The SSV1 transcripts T1 and T2 encode structural proteins of the virus-like particles. The map position of their common 5'-terminus has been determined previously (7,14). To map the 3'-terminus of transcript T1, probe P3b (Fig. 1 and Table 1) was used. After hybridization of this probe to total *Sulfolobus* B12 RNA and S1 endonuclease digestion of the resulting hybrids, three short ladders of fragments were observed which were centered around sizes of about 190, 182 and 170 nucleotides (Fig. 2D). In addition, the whole length of probe P3b was protected as expected from a hybridization with transcript T2 (Fig. 2D). Calculating the size of transcript T1 from the map position of its 5'-terminus and of its multiple 3'-termini, three subspecies with lengths of about 499, 491 and 478 nucleotides were expected. This was verified by measuring the size of T1 by S1 endonuclease analysis using probe P3a, which covers the whole length of this transcript (Fig. 1 and Table 1). In addition to a fragment of 860 nucleotides which was expected from the hybridization of this probe with transcript T2, a triplet of fragments with lengths of about 505, 495 and 485 nucleotides was observed (Fig. 2A), which was in reasonable agreement with the length of T1 predicted from the distance between the transcript termini. The multiple

Figure 3. DNA sequence comparison around SSV1 transcriptional termination sites.

The map positions of 3'-termini are indicated by arrows above the DNA sequences. Translational stop codons belonging to characterized protein-encoding genes (VP1 and VP2) or to a long open reading frame (ORF T3) are boxed. The ATG initiation codon of an 82 amino acid open reading frame contained within transcript T9 is marked by a bar above the DNA sequence. The conserved heptanucleotide sequence TTTTYYT preceding the transcriptional termination sites is underlined.

3'-ends of T1 were located 34-58 nucleotides downstream of the stop codon for the SSV1 major coat protein VP1 (Fig. 3) confirming that transcript T1 constitutes a monocistronic mRNA for this abundant structural protein (14).

To map the termination site of transcript T2, probe Plb (Fig. 1 and Table 1) was used. In addition to the fully protected probe, a fragment of 305 nucleotides and a ladder of short fragments in the size range between 116 and 136 nucleotides were observed (Fig. 2E). To achieve a clear assignment of these fragments, an additional probe (Plc) was used, which was shorter by eight nucleotides at the 3'-terminus than probe Plb (Table 1). After hybridization of probe Plc to total *Sulfolobus* B12 RNA and digestion with S1 endonuclease, the ladder of short fragments was shifted by about seven nucleotides to smaller sizes, whereas the large protected fragment (305 nucleotides) did not change in size compared to the analysis using probe Plb (Fig. 2E). This result indicated that the ladder of short fragments corresponded to multiple 3'-termini whereas the 305 nucleotide fragment was due to an

Transcript T9

Figure 4. S1 endonuclease and primer extension mapping of the SSV1 transcript T9.

For the S1 endonuclease analysis, 32000 cpm of probe P1d were hybridized to the amounts of *Sulfolobus* B12 RNA indicated above the lanes and the resulting hybrids were digested with S1 endonuclease. The lane marked "-S1" shows an undigested DNA probe and the lane marked "+S1 -RNA" shows a control lacking *Sulfolobus* RNA.

In the case of primer extension analysis the whole reaction mixture obtained by oligonucleotide-primed reverse transcription was applied to the gel in the lane marked "cDNA". The sequencing reactions (lanes G,A,T,C) were obtained using oligonucleotides N1 (left) or N2 (right) as primers.

RNA species mapping completely or only with its 5'-terminal region within both probes. The distance between the 5'-terminus and the multiple 3'-termini of transcript T2 was 1 kb, identical to the length of this transcript determined by Northern analysis (14). The most distal 3'-terminus of T2 mapped 53 nucleotides downstream of the stop codon of the gene encoding the SSV1 DNA-binding protein VP2 (Fig. 3). This gene is the last one within an operon comprising the coding regions for three SSV1 structural proteins (14,15).

The 305 nucleotide fragment obtained by the S1 analysis using probes Plb and Plc was also observed when the 5'-labelled probe Pld was used (Fig. 4), indicating that this fragment corresponded to the 5'-terminal part of a transcript. This transcript (T9) was not detectable on Northern blots, which may be due to a combination of low abundance and imprecise termination. Its 5'-terminus was also mapped by primer extension analysis with a result identical to that obtained by S1 endonuclease mapping (Fig. 4). According to these data, transcript T9 initiates within the termination region of transcript T2 (Fig. 3). An 82 amino acid open reading frame starts one nucleotide downstream of the 5'-terminus of T9 (Fig. 3). This open reading frame is followed by another five closely linked open reading frames with lengths between 92 and 277 amino acids. Since the length of T9 is not known, it is not clear whether all these reading frames are co-transcribed.

Mapping of the 3'-terminus of transcript T3.

Transcript T3 is an abundant constitutive SSV1-RNA which coincides in its map position with an unassigned open reading frame of 291 amino acids. The precise transcriptional initiation site for this transcript has been reported previously (7).

To determine the map position of the 3'-terminus of T3, probe P7 (Fig. 1 and Table 1) was used. Hybridization of this probe to total *Sulfolobus* B12 RNA and digestion of the resulting hybrids with S1 endonuclease resulted in a single fragment of 218 nucleotides length and a short ladder of fragments in the size range between 208 and 212 nucleotides (Fig. 2C). This corresponded to map positions between 20 and 30

nucleotides downstream of the stop codon of the long open reading frame contained within T3 (Fig. 3), a result which is in agreement with the proposed role of T3 as a monocistronic mRNA for a 291 amino acid hypothetical protein (7,14). The overall length of transcript T3 determined from the distance between the separately mapped termini was between 897 and 907 nucleotides which is in agreement with the size of this transcript determined by Northern analysis (0.9 kb; see ref. 14).

DNA sequence comparison around the 3'-termini of SSV1 transcripts.

In all five cases where 3'-termini of SSV1 transcripts have been mapped by S1 endonuclease analysis, these 3'-termini mapped within or immediately downstream of a pyrimidine-rich region (>80% C+T) about 16-19 nucleotides long (Fig. 3). A heptanucleotide sequence TTTTTYT (Y=C,T) was present in the 5'-terminal part of these pyrimidine-rich regions (Fig. 3). A computer search of the complete nucleotide sequence of the SSV1 DNA indicated that this sequence is not particularly frequent on the SSV1 genome. In addition to the five occurrences immediately upstream of transcriptional termination sites, only four other copies of this sequence were found and in these four cases the corresponding strand was non-transcribed. A search for possible inverted repeat sequences in a conserved position relative to the mapped 3'-termini yielded a negative result, indicating that the potential for the formation of secondary structures within the RNA is not required for transcription termination in *Sulfolobus*.

DISCUSSION

The comparative DNA sequence analysis around the 3'-termini of SSV1 transcripts indicated that in all cases analyzed multiple 3'-termini mapped several nucleotides downstream of a sequence TTTTTYT which was part of a pyrimidine-rich region of 16-19 nucleotides length. This high degree of sequence conservation suggests that all mapped 3'-termini were generated by the same molecular mechanism which could be transcription termination or RNA processing. The S1

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T9:   TTAAGCGGATGCACCGCAAGTAGGCCCTTTATAAAGTCATA.TTCCTTTCTTTCCCTGATGAGTGCCTTAGGGGATGAATCTACA
      |  ||  |  |  ||||| ||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
T3:   AGCATAATATCTGCGTTAGTGTAGGCTCTTTTAAAGTCTACCTTCTTTTTC.GCTTACATGAGGAAGTCCCTTCTAGCCCTACTAA
      |  ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||

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Figure 5. Comparison of the DNA sequences around the initiation sites of transcripts T3 and T9.

Transcriptional start sites are indicated by arrows and the ATG initiation codons belonging to open reading frames are boxed. Putative promoter elements are underlined and the oligo-pyrimidine sequences within the promoter regions are indicated by bars above the DNA sequence.

analysis of 3'-termini of *in vivo* transcripts does not allow a strict distinction between these possibilities if processing occurs by rapid exonucleolytic degradation or by endonucleolytic cleavage followed by the rapid degradation of one of the cleavage products. Although this represents a limitation in interpreting the experimental data, all available evidence speaks against a processing mechanism in the case of the SSV1 transcripts. If the mapped 3'-termini were generated by endonucleolytic cleavage of a larger precursor molecule, transcripts with the corresponding 5'-termini should be present. Such transcripts were not detectable by Northern analysis. In the case of transcript T2, the more sensitive S1 and primer extension assays were used for the detection of possible processing-derived 5'-termini leading to the result that the "termination region" of this transcript contains the single defined 5'-terminus of transcript T9. It appears highly unlikely, however, that this 5'-terminus is generated by RNA processing: Firstly, many of the multiple 3'-termini of T2 map in a position 3' to the 5'-terminus of T9 and secondly, the region around this 5'-terminus conforms to the highly conserved promoter consensus sequence for SSV1 promoters (7) indicating that the 5'-terminus of T9 is due to transcription initiation (Fig. 5). The pyrimidine-rich region correlating with transcription termination of T2 is located between the promoter elements "box A" and "box B" which are highly conserved between transcriptional initiation sites on the SSV1 genome (7) suggesting a spatial relationship between a promoter region and a termination signal.

A linkage between transcription termination and transcription initiation has also been found for the SSV1 transcripts T6 and T3. In this case, it has been established by Northern analysis in conjunction with S1 endonuclease mapping of transcript starts that the termination site for transcript T6 maps in the immediate vicinity of the initiation site for transcript T3 (7,14; see Fig. 1). Interestingly, an eleven-nucleotide sequence solely consisting of pyrimidine nucleotides and highly homologous to the putative termination signal for SSV1 transcription is located between the two promoter elements for transcript T3 (Fig. 5). This finding suggests that the signal structure for the termination of transcript T6 is located within the promoter region of transcript T3, a situation which is very similar to that found for the transcripts T2 and T9.

A re-initiation of RNA synthesis at transcriptional termination sites also occurs in the case of the rRNA operon of *Desulfurococcus mobilis* (6), an archaebacterium belonging to the same phylogenetic branch as *Sulfolobus* (19). This suggests that a linkage between transcription termination and transcription initiation may be a general feature of extremely thermophilic archaebacteria. A close spatial relationship between transcriptional termination sites and promoter elements has also been described for eukaryotic pol I transcription (reviewed in ref. 20).

As far as signal structures for transcription termination in archaebacteria other than *Sulfolobus* are concerned, pyrimidine-rich sequences unconnected with secondary structures have been implicated in transcription termination for the rRNA operons of the extremely thermophilic archaebacteria *Thermoproteus tenax* (11) and *Desulfurococcus mobilis* (6). In the case of methanogenic archaebacteria, oligo-pyrimidine tracks followed by an inverted repeat sequence have been described as putative terminators for stable RNA genes (10,12). Transcription termination of a highly expressed protein-encoding gene in *Methanococcus voltae* occurs in the 3'-terminal part of a twelve-nucleotide sequence solely containing pyrimidine nucleotides (9). Though eight of these bases are

involved in a possible secondary structure preceding the termination site, this observation suggests that long pyrimidine-rich tracks may constitute a termination signal conserved throughout the archaeobacteria. It must be borne in mind, however, that the available collection of archaeobacterial termination sites is strongly biased in favour of extreme thermophiles and that more data on mesophilic archaeobacteria are required to draw safe conclusions about common principles involved in transcription termination in archaeobacteria.

A comparison of the available sequence data with corresponding sequences in eubacteria and in eukaryotes indicates that with one possible exception (9) archaeobacterial terminator sequences are not of the rho-independent type recognized by eubacterial RNA polymerase. There is some similarity, however, between archaeobacterial terminator sequences and the pyrimidine-rich sequences which have been implicated in transcription termination by eukaryotic RNA polymerase I (21). Despite this similarity, the precise molecular mechanism of transcription termination in archaeobacteria remains elusive and the establishment of *in vitro* systems is clearly required for more detailed investigations.

ACKNOWLEDGEMENTS

We thank B. Grampp for excellent technical assistance and F. Pfeifer for critical reading of the manuscript.

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