Nucleotide sequence and analysis of the coliphage T3 S-adenosylmethionine hydrolase gene and its surrounding ribonuclease III processing sites

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ABSTRACT

To understand better the characteristics of the coliphage T3 S-adenosyl-L-methionine (AdoMet) hydrolase (AdoMetase, E.C. 3.3.1.2) and its expression in phage-infected *Escherichia coli*, we determined the DNA sequence of the cloned gene and its surrounding ribonuclease (RNase) III mRNA transcript processing sites. The AdoMetase gene contains two in-frame protein translation initiation sites specifying peptides 17105 and 13978 daltons in size. Both proteins terminate at the same ochre codon making the shorter peptide identical to the carboxy terminal 82% of the 17 kd protein. Our data explain the existence of two AdoMetase-related peptides in preparations of the purified enzyme as well as identify sequences that might serve to regulate the enzyme's expression. Comparisons between this T3 sequence and the homologous 0.3 gene region of the closely related coliphage T7 show both the nucleotide and amino acid sequences to be unrelated. The RNase III mRNA processing sites that bracket these genes in T3 and T7 are highly conserved in both their primary and secondary structures.

INTRODUCTION

The coliphage T3 S-adenosylmethionine (AdoMet) hydrolase (AdoMetase, E.C. 3.3.1.2) is responsible for inhibiting host-mediated restriction of the viral DNA (1). It performs this task through at least two independent mechanisms. Like the 0.3 gene anti-restriction protein produced by the related phage T7, AdoMetase binds to type I restriction endonucleases of various *E. coli* strains and forms a physical barrier to DNA cleavage (2). Unlike the T7 0.3 protein, T3 AdoMetase also hydrolyses AdoMet to homoserine and 5'-methylthioadenosine (3). This activity serves to remove AdoMet from the cell and eliminates a necessary cofactor in the restriction reaction (4). Either of these two mechanisms is sufficient to protect the viral DNA (1). See Kruger and Schroeder (5) for a review of these activities.

Our interest in AdoMetase concerns its AdoMet hydrolytic activity. In order to develop a research tool that would enable us to study the effects of depleting AdoMet pools in growing bacterial cells, we cloned the AdoMetase

gene into the M13mp8 expression vector (6). In this initial report, we described the cloning experiment, verified the presence of T3 AdoMetase in cells containing the cloned gene, and presented preliminary data indicating that *E. coli* tolerates high levels of AdoMetase expression.

To help clarify the nature of the expression of the AdoMetase gene and characteristics of the enzyme, we have determined the DNA sequence of the gene and its surrounding ribonuclease (RNase) III processing sites. This data explains the existence of two co-purifiable AdoMetase peptides and suggests reasons for the observed ratios of their expression as described by Spoerel and Herrlich (7), as well as its dependence on RNase III-mediated mRNA transcript processing for its efficient expression (8). In addition, the nucleotide sequence enables an examination of the relationship between AdoMetase and the coliphage T7 0.3 anti-restriction protein and gene sequences (9) and raises questions about how these two similar phage maintained the genetic location and activity of anti-restriction with two such different proteins.

MATERIALS AND METHODS

DNA Sequence Determination.

The DNA sequence of the AdoMetase gene and the surrounding RNase III sites were determined by the method of Sanger et al. (10) except that ^{35}S - α -dATP was used (11). Single-stranded DNA purified from recombinant derivatives of M13mp8 and M13mp9 (12) containing T3 restriction fragments (Fig. 1) were used as the sequencing templates. Nucleotides, sequencing primers, and enzymes for cloning and sequencing were purchased from New England BioLabs, Bethesda Research Labs, and Pharmacia Fine Chemicals while the radio-label was obtained from New England Nuclear/DuPont. DNA was manipulated, evaluated, and stored essentially according to Maniatis et al. (13).

Computer Analysis.

Computer programs used to identify open reading frames and analyze protein characteristics, determine the location and nature of inverted nucleotide sequence symmetries (such as for the RNase III sites), compare T3 and T7 DNA sequences, and locate restriction endonuclease cleavage sites were written in GWBasic/IBM Basic and compiled with the IBM Personal Computer Basic Compiler by J.A.H.

Fig. 1. Strategy for sequencing the T3 AdoMetase gene. DNA restriction fragments were cloned into M13mp8 and 9 and sequences determined by the dideoxy chain termination method. The numbers refer to distance (in bases) between each restriction site. The arrowhead indicates both the direction of sequencing and the approximate location of the most distally sequenced base for each clone. The names of the clones used (as described in ref. 6) are listed above each line.

RESULTS

Sequencing and Sequence Verification.

We recently described the restriction mapping of the 5' proximal T3 coliphage MboI "G" fragment and the cloning of specific AluI and HaeIII restriction fragments into M13mp8 and 9 (6). The clones diagrammed in Fig. 1 were used to sequence the AdoMetase gene and its surrounding DNA in both orientations. Sequence regions outside the AdoMetase gene which could not be determined from both directions were confirmed with multiple evaluations of independently produced sequencing templates. The resulting nucleotide sequence of this region of T3 is presented in Fig. 2.

Several restriction endonucleases were used to help verify the accuracy of this work. The 536-base pair insert containing the AdoMetase gene was liberated from the recombinant M13hb1 clone (see Fig. 1) by digesting the phage RFI DNA with EcoRI and HindIII, purifying the fragment from a low melting point agarose gel, digesting it with a series of restriction enzymes, and separating the reaction products on an acrylamide gel. All of the predicted restriction patterns were obtained (data not shown).

Other evidence supports the open reading frame described below as the one used to produce AdoMetase throughout its length. As described in our previous report, the M13mp8 clone containing the AluI fragment used to sequence the 5' end of this gene encodes a peptide of approximately 29

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1           20           40           60           80
AGCTCTTGAGTGTACCATATAAGCGAATAACTCAAGGTCGCACTGAAAGCGTGGCCTTTATGATATTCACCTTAACGAAT

81           100          120          140          160
AACATGAGGTAACACCAATGATTTTCACTAAGAGCCTGCGCACGTCTTCTATGTACTGGTTCCGCTTCCGTTCTAA
MetIlePheThrLysGluProAlaHisValPheTyrValLeuValSerAlaPheArgSerAs

161          180          200          220          240
CCTCTGCGATGAGGTGAATATGAGCAGACACGCCACATGTAAGCACTTACGTCCGCACCGGCTCTTATGGCTCCG
nLeuCysAspGluValAsnMetSerArgHisArgHisMetValSerThrLeuArgAlaAlaProGlyLeuTyrGlySerV

241          260          280          300          320
TTGAGTCAACCGATTTCACCGGTGCTATCGTGAGGCAATCTCAAGCGCACCAACTGAGGAAAAAAGTGTTCGTATACGC
alGluSerThrAspLeuThrGlyCysTyrArgGluAlaIleSerSerAlaProThrGluGluLysThrValArgValArg

321          340          360          380          400
TGCAAGGACAAAGCGCAGGCACTCAATGTTCACGCCCTAGCTTGTAAATGAGTGGGAGCAAGATTGCGTACTGGTATACAA
CylLysAspLysAlaGlnAlaLeuAsnValAlaArgLeuAlaCysAsnGluTrpGluGlnAspCysValLeuValTyrLys

401          420          440          460          480
ATCACAGACTCACACGGCTGGTCTGGGTACGCTAAAGGTATCGACGGGTATAAGGCTGAACGCTCTCCCGGTAGTTTCC
sSerGlnThrHisThrAlaGlyLeuValTyrAlaLysGlyIleAspGlyTyrLysAlaGluArgLeuProGlySerPheG

481          500          520          540          560
AAGAGGTTCTTAAAGGCGCACCGCTGCAAGGCTGCTTCACTATTGATGAGTTCGGTCCCGCTGGCAAGTACAATAAGTG
lnGluValProLysGlyAlaProLeuGlnGlyCysPheThrIleAspGluPheGlyArgArgTrpGlnValGlnEND

561          580          600          620
TTAAACTCAAGGTCATGCACGATGCGTGGCCTTTATGATTAACTTCTCTATAGAGGTATTGAATG
Met(?)

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Fig. 2. Nucleotide and amino acid sequence of the T3 AdoMetase gene and surrounding DNA. The sequenced region runs from the AluI site at approximately base number 790 on the T3 genome to the probable initiation codon for the gene immediately 3' to the second RNase III site (approximately base number 1420 on the phage genome). Shine-Delgarno and ATG protein translation initiation sequences are underlined.

kilodaltons (kd) in phage-infected *E. coli* JM103 cells induced with IPTG. Because the DNA sequence on the 5' end of this fragment shows termination codons in the reading frame used by the M13mp8 beta-galactosidase, the amino terminus of this protein must originate on the T3-derived AluI fragment, presumably at the AdoMetase translation start site. That this peptide is associated with a β -galactosidase activity suggests the AdoMetase and β -galactosidase genes have been fused in the same reading frame, a conclusion

Table 1. Codon usage frequencies for the 17 kd and 14 kd AdoMetase peptides.

UUU (phe)	0/0	UCU (SER)	1/0	UAU (tyr)	4/3	UGU (CYS)	1/1
UUC (PHE)	6/3	UCC (SER)	2/1	UAC (TYR)	2/2	UGC (CYS)	5/4
UUA (leu)	1/1	UCA (SER)	3/3	UAA (end)	1/1	UGA (end)	0/0
UUG (leu)	1/1	UCG (SER)	0/0	UAG (end)	0/0	UGG (TRP)	2/2
CUU (leu)	1/1	CCU (pro)	2/1	CAU (HIS)	0/0	CGU (ARG)	5/4
CUC (leu)	2/1	CCC (pro)	0/0	CAC (HIS)	4/3	CGC (ARG)	5/5
CUA (leu)	1/1	CCA (PRO)	1/1	CAA (gln)	5/5	CGA (arg)	0/0
CUG (LEU)	5/4	CCG (PRO)	3/3	CAG (GLN)	2/2	CGG (arg)	0/0
AUU (ile)	2/1	ACU (THR)	6/5	AAU (asn)	3/2	AGU (SER)	1/1
AUC (ILE)	2/2	ACC (THR)	2/2	AAC (ASN)	1/0	AGC (SER)	3/3
AUA (ile)	0/0	ACA (thr)	0/0	AAA (LYS)	6/5	AGA (arg)	1/1
AUG (MET)	3/2	ACG (thr)	1/1	AAG (lys)	2/2	AGG (arg)	0/0
GUU (VAL)	5/4	GCU (ALA)	5/4	GAU (ASP)	4/3	GGU (GLY)	5/5
GUC (val)	1/0	GCC (ala)	1/1	GAC (ASP)	2/2	GCC (GLY)	3/3
GUA (VAL)	6/5	GCA (ALA)	6/6	GAA (GLU)	2/2	GGA (gly)	0/0
GUG (VAL)	2/1	GCG (ALA)	2/1	GAG (glu)	9/7	GGG (gly)	2/2

Numbers represent how often specific codons are used in the sequence; those on the left of the slash depict the tally for the 17 kd protein, and those on the right for the 14 kd peptide. Amino acids printed in lower case letters indicate codons that tend to be used with reduced frequency by proteins expressed at high levels in *E. coli* (15).

supported by the predicted 27 kd size for such a fusion protein. This could happen only if the frame we have indicated for the AdoMetase gene is used through base number 360. Any other would either include a termination codon or fuse to the M13mp8 sequence at the *Sma*I site out of frame with the β -galactosidase gene. An error in sequencing after this point resulting in a frame shift is not likely. A peptide encoded by the other reading frames would either terminate earlier than predicted by SDS-PAGE estimates of the size of the pure enzyme (7, and our data) or be rich in "rare" codons throughout its length, a finding incompatible with the rapid and abundant expression of AdoMetase in T3-infected cells.

Open Reading Frames and Probable Protein Products.

The nucleotide sequence shown in Fig. 2 specifies an open reading frame starting at nucleotide number 99 and extending through an ochre termination codon at number 555. The ATG initiation codon is preceded by a strong Shine-Delgarno sequence from bases 83 through 90 that agrees with 6 of the 7 nucleotides on the 3' end of the 16S ribosomal RNA, a sequence that is purported to pair with mRNA transcripts and determine the location for the beginning of protein translation (14). The protein specified by this open

reading frame has a molecular weight of 17105 daltons and a basic pI. The AdoMetase gene uses codons commonly associated with abundantly expressed proteins (Table 1) and the "rare" codons are scattered evenly throughout the gene.

Because two peptides differing by three kd in size were selected by the purification scheme of Spoerel and Herrlich (7), it seemed possible that two transcription initiation sites could be used by the phage to result in the production of two proteins from the same DNA sequence. This requires a second translation start site, a prediction that was apparently confirmed by the discovery of a second strong Shine-Delgarno sequence located between bases 169 and 175. This sequence is followed by an ATG initiation codon beginning at base number 180. This ATG defines a methionine codon in the 17 kd protein described above and thus would serve to initiate the translation of a 13978 dalton protein identical to the carboxy terminal 125 residues of the AdoMetase described above.

Transcription/Translation Control Elements.

Besides the ATG initiation codons, the ochre termination codon, and the Shine-Delgarno sequences, several other possible control elements in AdoMetase expression can be seen in the sequence (Fig. 2).

The T3 early genes are transcribed by the host RNA polymerase into a long polycistronic messenger RNA that is subsequently processed by RNase III into five mature transcripts (reviewed in 5). RNase III cuts within long regions of double stranded RNA of no particular consensus primary nucleotide sequence (16,17) and also cleaves specific single stranded RNA sequences surrounded by duplex regions (18). Its recognition site in T7 consists of two short regions of inversely symmetrical sequences that produce duplex "stems" of RNA in which are located a short stretch of unpaired bases (19, and see Fig. 6). We found regions fitting this description on the T3 sequence spanning bases 20-73 and 556-607 (Fig. 3). These RNase III sites thus tightly bracket the AdoMetase gene and make its two products the only peptides translated off the first processed early mRNA transcript.

Interestingly, a 20 base region 5' to the first RNase III site (starting at the first sequenced base) is complementary to a sequence beginning at the first codon in the longer AdoMetase open reading frame, creating a potentially more complex secondary structure (Fig. 4). The significance of this structure may depend on whether the T3 sequence 5' to the first base of our sequence pairs further along the sequence for the 17 kd protein, but

Fig. 3. Location and structure of the T3 RNase III sequences in relation to the AdoMetase gene. The first Shine-Delgarno sequence, the AUG initiation codon for the 17kd AdoMetase, and the ochre termination codon are bold-faced. The first digit of the numbers underneath the sequence are positioned below their corresponding base along the nucleotide sequence.

possible functions for this added duplex RNA stem are suggested in the Discussion.

Finally, the same sequence identified in Fig. 4 as a possible binding partner for the 5' terminal 16 bases of the initial mRNA transcript encodes a region that resembles a T3 RNA polymerase-specific class III promoter. As shown in Fig. 5, the core of the consensus T3 promoter is perfectly represented over 9 bases beginning at nucleotide number 106, and a partial homology to the consensus phage promoter stretches for a total of 20 bases. Because it does vary from the consensus class III T3 RNA polymerase promoter, such a sequence would likely serve as either only a weak promoter or perhaps as a binding site for the T3 RNA polymerase, if in deed it is recognized at all. Though an active T3 RNA polymerase specific promoter has never been mapped to this region, the presence of such a long region of similarity to the consensus class III promoter at this position invites speculation about its possible regulatory and/or structural significance. This is especially

Fig. 4. Suggested mRNA secondary structure leading to the regulation of AdoMetase expression by RNase III. The sequence begins at base number one and ends at number 118. The first Shine-Delgarno (S/D) and AUG initiation codon sequences are typed in boldface while those for the 14 kd peptide are underlined. Numbers refer to positions along the sequence.

		+1
Consensus T3 promoter:	TATTAACCOCTCACTAAAGGGAGA	.
	CGA T	CA AC
		T
AdoMetase T3 promoter(?):	<u>AAATGATTTTCACATAAGAGCCT</u>	
	97	119

Fig. 5. T3 RNA polymerase class III promoter-like sequence located within the AdoMetase gene. The consensus promoter was obtained from a figure presented in McGraw et al. (20). Variations to the T3 sequence found in eleven different active promoters are presented above and below it and the initial base in the mRNA transcript is located by the "." and "+1". Locations on the AdoMetase sequence that match the T3 consensus promoter or any of its variations are underlined and those agreeing with the consensus promoter are bold-faced as well.

(A)

T7 5': ACUAGCGGAUAACUCAAGGUAUCGCA.....AG. GUGCCUUUAUGAUUUCACUAAUAA
 T3 5': UAAAGCGAAUAACUCAAGGU..CGCACUGAAAGCGUGGCCUUUAUGAUUUCACUUA
 T7 3' site: GAGUGAUAGACUCAAGGUCGCUCCUAGCGAGUGGCCUUUAUGAUUUCACUU
 T3 3' site: AAGUGUAAAACUCAAGGUC AUGCACGAUGCGUGGCCUUUAUGAUUAAAUUC

(B)

Fig. 6. Comparison of T3 and T7 RNase III site sequences. In (A), the T3 and T7 RNase III sites are aligned to show their primary nucleotide sequence similarity. The highly conserved sequences that comprise and surround the unpaired "bubble" structure and are common to all four sites are underlined. Spacing adjustments necessary to align similar sequences are denoted with "."s. (B) shows the secondary structures and demonstrates the locations of the known T7 and presumed T3 RNase III cleavage sites with the arrows.

true after also noting its potential for forming the mRNA secondary structure.

Comparison with the Homologous DNA Sequence of Coliphage T7.

The similar function and location of the T3 AdoMetase and T7 0.3 genes on their respective phage genomes suggests that the two genes and/or proteins may exhibit some sequence homologies. We devised a series of computer programs to evaluate this possibility.

Figure 6 compares the primary nucleotide sequence and possible secondary structural configuration of the RNase III sites that bracket the T3 AdoMetase and T7 0.3/0.4 gene pair. As shown in Fig. 6A, these sites have very similar base sequences. In fact, not only are the RNase III sites 5' and 3' to these genes similar to their homologs, but all four sequences have regions in common. The sequences comprising both sides of the unpaired bubble structures are highly conserved between all four sites, possibly reflecting their importance as the location of RNase III cleavage on the T7 (19, and see Fig. 6) and presumably T3 early gene transcripts. Differences in bases located on the double stranded stems are compensated for by changes in their counterparts to conserve the stem-bubble-stem secondary structures illustrated in Fig. 6B.

The high degree of sequence similarity exhibited by the RNase III sites does not seem to be represented in the structural genes. Despite extensive computer-assisted comparisons between the T3 and T7 DNA sequences, we could not identify any regions of extended homology. In fact, the conserved sequences noted for the 5' RNase III sites in Fig. 6 diverge soon after the end of the RNase III site. The only positions of further significant similarity between the two phage are scattered regions no more than 30 bases in length and probably represent coincidental matches.

Comparisons of the amino acid sequences show no regions of similarity between the two anti-restriction proteins. This is not surprising as previously published data concerning AdoMetase and the 0.3 gene product suggest them to be quite dissimilar (6,21).

DISCUSSION

Our attempt to understand better the T3 AdoMetase by DNA sequencing reveals that the gene encodes a 17 kd basic peptide with a codon usage pattern typical of an abundantly expressed protein. This data agrees with the findings of Spoerel and Herrlich (7) on the purified enzyme and the implications of their purification protocol on the physical nature of the AdoMetase enzyme itself. Furthermore, the discovery of a second Shine-Delgarno/ATG initiation codon pair in the same open reading frame which would produce a second peptide may explain the presence of two related, co-purifying proteins in "homogeneous" AdoMetase preparations. The resulting sequence similarity of these two peptides would account for their similar behavior in the AdoMetase purification scheme. Furthermore, both peptides adhere to the S-adenosylhomocysteine (AdoHcy) agarose column used in this

procedure, suggesting that binding AdoMet (or at least the AdoHcy ligand) is an activity independent of the amino terminus of the larger enzyme.

The duplex mRNA stem structure shown in Fig. 4 and the potential T3 RNA polymerase specific "promoter" illustrated in Fig. 5 suggest a possible function for those regions and may provide plausible explanations for previously observed traits of T3 early gene expression.

Although both the 17 and 14 kd peptides encoded in this DNA fragment are preceded by strong Shine-Delgarno sequences followed closely by methionine codons, the 14 kd moiety is much more abundant in phage-infected cells (7, and our results). This observation may be explained by the possible action of the T3 RNA polymerase class III promoter-like sequence found immediately after the initial codon of the larger peptide. No transcript has ever been demonstrated to originate at this position, but the similarity of this sequence to the consensus T3 RNA polymerase promoter (Fig. 5) suggests a function for it. While this "promoter's" regions of divergence from the consensus sequence are such that it is not likely to be an efficient initiator of transcription, any mRNA transcript started here would encode only the 14 kd peptide, thus enhancing its expression over the full-length protein. On the other hand, if the "promoter" only provides a binding site for the T3 RNA polymerase on the phage DNA, it could serve a regulatory role by physically blocking transcription of the early genes from this point on. This could conceivably help explain the cessation of early gene expression after the host has been successfully invaded.

In vitro studies on the expression of early gene products using extracts from RNase III mutants and wild type E. coli strains suggest that the AdoMetase activity is preferentially affected by the absence of this ribonuclease (8). The secondary structure of the 5' RNase III recognition sequences does not suggest a reason for this, but the added "stem" demonstrated in Fig. 4 could serve to form a structure that might sequester the Shine-Delgarno and AUG sequences on the unprocessed transcript from the ribosome. Alternately, because of its broad specificity for duplex RNA as a digestion substrate, the added stem described above could conceivably be cleaved by RNase III. Were this to happen, it would cut the transcript immediately 3' to the first Shine-Delgarno/AUG pair and make the 14 kd protein its only possible translation product, thereby explaining the predominant expression of the shorter peptide.

The functional roles of the two T3 AdoMetase peptides is not clear. As noted previously, the ability of both proteins to bind AdoHcy (and presumably

AdoMet) suggests both have a role in sequestering AdoMet from the host's type I restriction enzyme, but whether they serve as active and/or equally effective AdoMetases has not been demonstrated. Both also share the ability to bind the unidentified host protein (7, and our observations) and probably the EcoB and EcoK type I restriction endonucleases and thus contain the "ocr" anti-restriction activity (2). Perhaps some form of regulation or differential expression of one or more of their activities necessitates the expression of both peptides, but the exact mechanism or consequence of the deletion of the amino terminal 3 kd of the larger protein is a matter for further investigation.

Our inability to identify regions of sequence homology between the T3 AdoMetase and T7 0.3/0.4 region (and the succeeding 0.5/0.6/0.65 T7 gene sequences as well, results not shown) suggests this region evolved independently or has diverged greatly since the two phage became genetically distinct. The lack of homology was not surprising as comparisons between T3 and T7 in DNA:DNA and DNA:RNA hybridization studies (22), restriction mapping analyses (23), and the respective purified anti-restriction proteins imply significantly different nucleotide and amino acid sequences between the two phage in this region. Attempts to identify localized regions of conserved amino acid sequences were unsuccessful, consequently we have not been able to identify regions within the proteins that would account for their similar ability to bind type I restriction endonucleases. This suggests that either each protein may bind the restriction enzymes differently or that some secondary structural feature has been conserved in the two proteins despite their differences in amino acid sequence. Finally, it is interesting to note that T3 and T7 share similar sequences over long regions of their genomes. For instance, the two RNA polymerases are over 80% homologous in nucleotide and amino acid sequences (20) and many genes are so similar between the two phage that a wide variety of hybrid T3:T7 phage are viable (24). Thus, the base pair differences between T7 and T3 in the 0.3 gene region could conceivably result from the substitution of a gene into T7 and/or T3 rather than the progressive divergence of the two genes through base changes from a common ancestral DNA sequence. Because AdoMetase is dispensable for phage viability as long as the ocr function is maintained (1), speculation about the deletion of this activity during T7 development is not unreasonable. On the other hand, how and why these proteins diverged so greatly between T3 and T7 while most other essential elements have been conserved poses an interesting question in the evolution of these two phage.

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