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CHARACTERIZATION OF A FULL-LENGTH cDNA WHICH CODES FOR THE HUMAN SPERMIDINE/SPERMINE N^1-ACETYLTRANSFERASE

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SUMMARY: Spermidine/spermine N^1-acetyltransferase is the rate-limiting enzyme in the catabolism of cellular polyamines. Using a combination of cDNA library screening and anchored PCR methodologies, a full length cDNA designated AP3/F7 corresponding to the human SSAT was cloned using RNA from the human large cell undifferentiated lung carcinoma line NCI H157. The resulting cDNA clone is 1,060 base pairs with a 513 base open reading frame coding for a 171 amino acid protein, with a predicted subunit molecular weight of 20,023. The 5' non-coding region of AP3/F7 is 165 bases and the 3' untranslated region is 382 bases with a polyadenylation site 20 bases 5' to the poly(A) tail. This full length cDNA should be an aid in the study of the regulation of spermidine/spermine N^1-acetyltransferase expression and the significance of the acetyltransferase in polyamine metabolism.

INTRODUCTION: Intracellular polyamine content is under the control of several enzymatic steps associated with their synthesis and breakdown (1,2). The highly inducible ornithine and S-adenosylmethionine decarboxylases are the major control points of the synthetic pathway (1). However, the activity of spermidine/spermine N^1-acetyltransferase (SSAT) is the rate limiting step of the catabolic pathway (2-4). We and others have previously shown SSAT to be highly inducible by a number of stimuli including cellular exposure to the natural polyamines and their analogues (5-8). Additionally, this enzyme has been associated with the cytotoxic response of specific human tumor phenotypes to treatment with a class of polyamine analogues (5,9-11).

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Abbreviations used: BESpm, N^1,N^{12}-bis(ethyl)spermine; PCR, polymerase chain reaction; RT, reverse transcriptase; SSAT, spermidine/spermine N^1-acetyltransferase.

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Recently, we reported a cDNA which contains the coding region for an active human SSAT (12). We now report the full length sequence of the polyamine induced message and discuss the possible mechanisms for the observed increase in steady-state mRNA and SSAT enzyme activity.

METHODS AND MATERIALS

Materials

N^1,N^{12}-bis(ethyl)spermine (BESpm) was synthesized and kindly provided by the laboratory of Dr. Raymond J. Bergeron, University of Florida, Gainesville (13-15). DNA modifying and restriction enzymes were purchased from Bethesda Research Laboratories. [1-^{14}C]Acetyl Coenzyme A (55mCi/mmol), [α^{32}-P]dCTP (3000 Ci/mmol), and [α-^{35}S]dATP (1000 Ci/mmol) were obtained from ICN Radiochemicals.

cDNA Library Construction and Screening.

The λZAPII cloning vector system was purchased from Stratagene, LaJolla, CA. Poly(A) RNA from BESpm induced NCI H157 large cell undifferentiated lung carcinoma was used to construct cDNA libraries in λZAPII as previously published (12). The cDNA libraries were screened with the coding region of the previously reported SSAT plasmid pSATH1 (12). Phage plaques which continued to hybridize through three levels of screening were converted to the Bluescript plasmid by the *in vivo* excision methods as outlined by the manufacturer.

Anchored PCR for obtaining 5' and 3' untranslated regions

A portion of the 5' untranslated region was cloned using a primer extension/PCR technique. A 30 base oligomer (PE2, Table 1, Figure 1) complementary to the 5' coding region of pSATH1 was used in a reverse transcriptase reaction. Five μg of Poly (A) RNA from BESpm treated NCI H157 cells was combined with 350 ng PE2, dried under vacuum, and resuspended in annealing buffer [0.5 M NaCl, 10 mM tris pH 8.0, 1 mM EDTA]. This suspension was then heated to 75°C for 2 minutes, vortexed, heated again at 75°C for 2 minutes, vortexed, and incubated at 55°C for 30 minutes. After 30 minutes, the sample was allowed to cool to 50°C and immediately ethanol precipitated. The annealed RNA and primer was resuspended in 13 μl H_2O, 5 μl 5X BRL RT buffer, 2.5 μl 5 mM dNTP's (final concentration 500 μM), 20 units RNAsin, 200 units of MMLV reverse transcription and incubated for 30 minutes at 37°C followed by a 5' incubation at 48°C. The cDNA product was then phenol/chloroform extracted, ethanol precipitated, resuspended in a 90% formamide, Tris/Borate/ EDTA (89 mM, 89 μM, 5 mM respectively) with 0.2 mg each bromphenol blue and xylenecyanol and purified by polyacrylamide/urea gel electrophoresis. The cDNA was visualized by ethidium bromide staining, extracted from the polyacrylamide gel slice, and dG-tailed with terminal deoxytransferase (BRL) using conditions recommended by the manufacturer. After phenol/chloroform extraction, this cDNA was PCR amplified using PCR3 and the nested PCR1 oligomers as primers (Table 1 and Figure 1). The PCR products were then purified and subcloned into the Bluescript plasmid for sequencing.

Similarly, a variation of the anchored PCR technique of Frohman et al. was used to facilitate obtaining the 3' untranslated region (16). Briefly, total cellular RNA was prepared from BESpm treated NCI H157 cells. Six μg of this RNA was used as a template for cDNA production using primer FL2 (see Table 1). The reverse transcriptase (RT) reaction was performed for 30 minutes at 37°C in a total volume of 30 μl, (0.5 mM dNTPs, 20 units RNAsin, 0.5 μM FL2 oligomer, 1 X BRL RT buffer). As a control, the above reaction

mixture was prepared without the addition of RT. Two μl from each reaction was used with the primers indicated below for PCR with a program of 95°C x 1 minute denaturation, 58°C x 2 minutes annealing and 70°C x 2 minutes extension. Since the entire coding region sequence of the human gene was known from our previous work (12), three 17 base oligomers (P1,P3,P5) within the coding region and one 17 base plus an Eco R1 linker from the 5' untranslated region (FL1) (see Figure 1 and Table 1) were used as 5' primers in PCR experiments in combination with FL2 as the 3' primer. The use of 5' primers which were of increasing distance from the 3' end of the SSAT mRNA served as internal controls since the distance between each of the 5' primer was precisely known. The amplified products of the PCR reactions were subcloned into Bluescript for characterization and sequencing.

RNA and DNA Analyses

Northern and Southern blot analysis were performed as previously published (12).

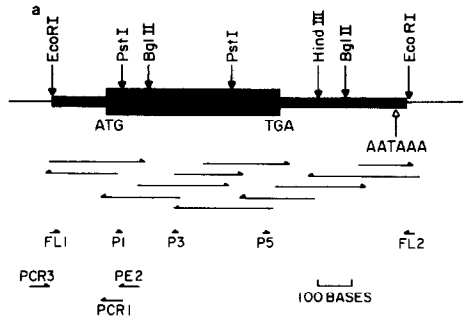
cDNA sequence determination

Double-stranded cDNA sequencing was performed from Bluescript plasmids containing inserts by the dideoxynucleotide method (17) using the Sequenase kit (United States Biochemical Corporation). The PCR products were sequenced by identical methods after subcloning into the Bluescript plasmid. Multiple PCR subclones were sequenced to obtain a consensus sequence to avoid the potential of PCR/subcloning introduced errors.

Translation of AP3/F7

T_3 RNA polymerase was used to generate sense transcripts from the linearized plasmid AP3/F7 by previously published methods (18,19). These transcripts were translated in a rabbit reticulocyte lysate system (BRL) (12). SSAT activity of the translated protein was measured by [^{14}C]N^1-acetylspermidine formation as published (5). SSAT antisera was also used to demonstrate the specificity of the acetyltransferase activity of the translated protein (12,20).

RESULTS: Two strategies were applied to obtain full length cDNA sequences: traditional library screening using the coding region of our previously reported cDNA clone (12) and PCR techniques. The standard library screening initially provided some additional information in the 5' region (data not shown). However, no clones containing homology 3' to the EcoRI site of the original pSATH1 clone (12) were found. Since the sequence 3' to the EcoRI site of pSATH1 could not be confirmed by protein sequence information (5) (it is entirely 3' to the coding region) and since no further 3' sequences were obtained by standard screening, PCR techniques (outlined in Figure 1) were used to clone the 3' region of the SSAT cDNA. This procedure produced four PCR amplified fragments with the expected differences in sizes based on the 5' primers used in each reaction (Figure 1 and Table 1). Each product was readily visible both after ethidium staining (not shown) and by standard southern analysis (Figure 2) using the coding region of pSATH1 as a probe. The resultant PCR products were subcloned into the Bluescript plasmid for sequencing and multiple clones were used to define a consensus sequence. Each



b

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   1  CGC GGG CCG ACT GGT GTT TAT CCG TCA CTC GCC GAG GTT CCT TGG GTC      48

  49  ATG GTG CCA GCC TGA CTG AGA AGA GGA CGC TCC CGG GAG ACG AAT GAG      96

  97  GAA CCA CCT CCT CCT ACT GTT CAA GTA CAG GGG CCT GGT CCG CAA AGG     144

 145  GAA GAA AAG CAA AAG ACG AAA ATG GCT AAA TTC GTG ATC CGC CCA GCC     192
   1                              M   A   K   F   V   I   R   P   A       9

 193  ACT GCC GCC GAC TGC AGT GAC ATA CTG CGG CTG ATC AAG GAG CTG GCT     240
  10   T   A   A   D   C   S   D   I   L   R   L   I   K   E   L   A      25

 241  AAA TAT GAA TAC ATG GAA GAA CAA GTA ATC TTA ACT GAA AAA GAT CTG     288
  26   K   Y   E   Y   M   E   E   Q   V   I   L   T   E   K   D   L      41

 289  CTA GAA GAT GGT TTT GGA GAG CAC CCC TTT TAC CAC TGC CTG GTT GCA     336
  42   L   E   D   G   F   G   E   H   P   F   Y   H   C   L   V   A      57

 337  GAA GTG CCG AAA GAG CAC TGG ACT CCG GAA GGA CAC AGC ATT GTT GGT     384
  58   E   V   P   K   E   H   W   T   P   E   G   H   S   I   V   G      73

 385  TTT GCC ATG TAC TAT TTT ACC TAT GAC CCG TGG ATT GGC AAG TTA TTG     432
  74   F   A   M   Y   Y   F   T   Y   D   P   W   I   G   K   L   L      89

 433  TAT CTT GAG GAC TTC TTC GTG ATG AGT GAT TAT AGA GGC TTT GGC ATA     480
  90   Y   L   E   D   F   F   V   M   S   D   Y   R   G   F   G   I     105

 481  GGA TCA GAA ATT CTG AAG AAT CTA AGC CAG GTT GCA ATG AGG TGT CGC     528
 106   G   S   E   I   L   K   N   L   S   Q   V   A   M   R   C   R     121

 529  TGC AGC AGC ATG CAC TTC TTG GTA GCA GAA TGG AAT GAA CCA TCC ATC     576
 122   C   S   S   M   H   F   L   V   A   E   W   N   E   P   S   I     137

 577  AAC TTC TAT AAA AGA AGA GGT GCT TCT GAT CTG TCC AGT GAA GAG GGT     624
 138   N   F   Y   K   R   R   G   A   S   D   L   S   S   E   E   G     153

 625  TGG AGA CTG TTC AAG ATC GAC AAG GAG TAC TTG CTA AAA ATG GCA ACA     672
 154   W   R   L   F   K   I   D   K   E   Y   L   L   K   M   A   T     169

 673  GAG GAG TGA GGA GTG CTG CTG TAG ATG ACA ACC TCC ATT CTA TTT TAG     720
 170   E   E                                                           171

 721  AAT AAA TTC CCA ACT TCT CTT GCT TTC TAT GCT GTT TGT AGT GAA ATA     768

 769  ATA GAA TGA GCA CCC ATT CCA AAG CTT TAT TAC CAG TGG CGT TGT TGC     816

 817  ATG TTT GAA ATG AGG TCT GTT TAA AGT GGC AAT CTC AGA TGC AGT TTG     864

 865  GAG AGT CAG ATC TTT CTC CTT GAA TAT CTT TCG ATA AAC AAC AAG GTG     912

 913  GTG TGA TCT TAA TAT ATT TGA AAA AAA CTT CAT TCT CGT GAG TCA TTT     960

 961  AAA TGT GTA CAA TGT ACA CAC TGG TAC TTA GAG TTT CTG TTT GAT TCT    1008

1009  TTT TTA ATA AAC TAC TCT TTG ATT TAA AAA AAA AAA AAA AAA AAA AAA    1056

1057  AAA A                                                           1060
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Figure 1.

a, Diagram of the human spermidine/spermine N^1-acetyltransferase cDNA clone AP3/F7. The heavy block represents the translated region with the translational start (ATG) and stop (TGA) sites indicated. The heavy line represents the untranslated region. The thin line represents the Bluescript cloning vector. The restriction sites of the AP3/F7 are as indicated. The sequencing strategy is as indicated by the long arrows. Labeled small arrows FL1, P1, P3, P5, and FL2 represent the oligomers used for PCR procedure to define the 3' end described in Methods and Materials. Labeled small arrows PE2, PCR1, and PCR3 represent the oligomers used for PCR procedure to define the 5' end as described. b, The sequence and predicted amino acid sequence of AP3/F7. The top number of the right and left columns represent the nucleotide number of the cDNA insert and the bottom number refers to the amino acid number with the initial methionine designated as 1.

Table 1. Oligomers used in PCR protocols

Oligomer[1]	Sequence
FL1 5′	ATA GGA ATT CCG TCA CTC GCC GAG GTT CCT 3′
P1 5′	GAT CTG CTA GAA GAT GG 3′
P3 5′	GCA TTG TTG GTT TTG CC 3′
P5 5′	CCA GTG AAG AGG GTT GG 3′
FL2 5′	ATA GGA ATT CCT TTT TTT TTT TTT TTT TTT TTT 3′
PE2 5′	GCC GCA GTA TGT CAC TGC AGT CGG CGG CAG 3′
PCR1 5′	CCG GAA TTC CGG CAG TGG CTG GGC GGA TCA 3′
PCR3 5′	GGG AAT TCC CCC CCC CCC CCC CCC CCC CCC 3′

[1]The oligomers FL1, P1, P3, P5, and FL2 were used to determine the 3′ end, including the poly(A) tail of the SSAT mRNA. The oligomers PE2, PCR1, and PCR3 were used to determine the 5′ translated region of the SSAT mRNA.

of the PCR generated clones defined precisely the same 3′ end of the SSAT gene product.

The 5′ untranslated region of the SSAT cDNA was defined by a similar technique. Using a PCR modification of standard primer extension techniques, an additional 146 bases 5′ to the translational start site were defined. Subsequently, using these PCR clones as probes for cDNA library screening, a clone designated AP3 containing 165 bases 5′ to the translational start site was identified. This clone extended 3′ past the translation stop site, spanning a single Hind III site at base 791 (Figure 1). Therefore, it was possible to construct a full length cDNA by ligating AP3 cut at this Hind III

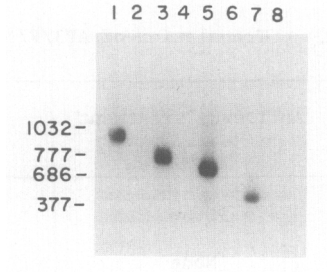

Figure 2.
Southern blot of anchored PCR products to define the 3′ end of the human SSAT gene. Five μl of each 50 μl RT/PCR reaction was separated by gel electrophoresis in a 1% agarose gel, transferred to a nylon membrane and probed with the coding region of the pSAThl clone as described in Methods and Materials. Lanes 1 and 2 primers, FL1 and FL2; Lanes 3 and 4, primers P1 and FL2; Lanes 5 and 6, primers P3 and FL2; Lanes 7 and 8, primers P5 and FL2. Lanes 2,4,6, and 8 represent negative control PCR reactions from RNA which had not first been reverse transcribed. The numbers at the left represent the size in bases, of the PCR amplified product.

site to a Hind III cut, PCR generated 3' sequence, which was identical to the consensus sequence described above (clone designated F7). The AP3/F7 clone is 1,060 bases with a coding region of 513 bases which is identical to the previously described pSATH1 (12). It contains 165 bases of 5' untranslated region with only one additional in-frame translation initiation codon at base 49 which is followed at base 61 by an in-frame stop codon. The translated region is followed by multiple, in frame stop codons, the first starting at base 679. The 3' untranslated region extends 359 bases from the translation stop site to the start of the poly(A) tail. A perfect polyadenylation signal sequence (AATAAA) starts 20 bases upstream from the poly(A) tail. This clone contains a tail of 27 adenosines.

In vitro translation was used to verify that AP3/F7 codes for an active human SSAT protein. The protein produced from the AP3/F7-dependent transcripts exhibit 5 high SSAT activity (>100-fold over background). Additionally, this activity is almost entirely precipitated by the specific antisera (Table 2).

The AP3/F7 clone was used to probe standard Northern and Southern blots. It is important to note that AP3/F7 recognizes precisely the same BESpm inducible message in the NCI H157 cells as the previously described pSATH1 clone (Figure 3). The size of the SSAT mRNA was approximated by RNA standards to be between 1100-1300 bases. For Southern analysis, DNA was isolated from the human lung cancer line NCI H157. Twenty μg of genomic DNA was digested with the restriction endonucleases as indicated in Figure 4, transferred, and probed with AP3/F7 as outlined in Material and Methods. The results of this analysis do not suggest that SSAT is part of a large gene family.

Table 2. Translation of AP3/F7 transcripts

mRNA added[1]	Antiserum treatment	SSAT activity (pmol/10 min)
None	None	0.6
AP3/F7	None	244
AP3/F7	Anti-SSAT	32

[1]RNA was transcribed from AP3/F7 and 0.5 μg aliquots were translated in a reticulocyte lysate for 1 h at 30°C. Aliquots were then removed and used for the assay of SSAT activity before or after precipitation with the specific antiserum (12). The activity reported represents the mean of the transcriptional/translational assays performed in duplicate. Each measurement was within 5% of the mean.

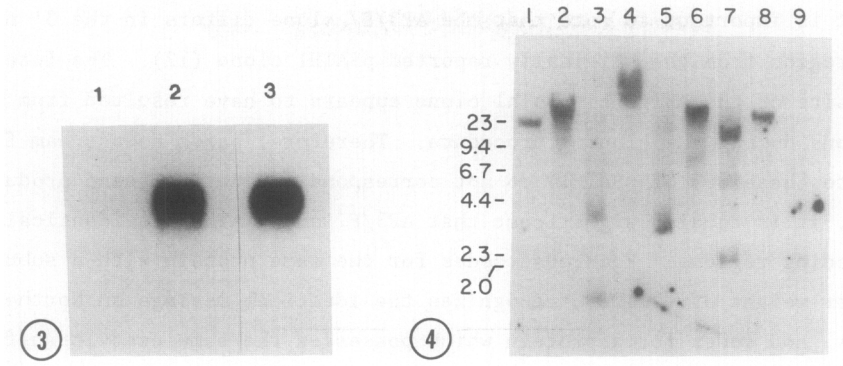

Figure 3.
Northern blot analysis of BESpm induced NCI H157. Large cell undifferentiated lung carcinoma probed with pSAThl or P3/F7. Each lane was loaded with 5 μg total cellular RNA and separated in a 1.5% denaturing agarose/formaldehyde gel, transferred to a nylon filter and probed with either the pSAThl or AP3/F7 cDNA. Lane 1, uninduced control RNA probed with pSAThl; Lane 2, 24 hr BESpm treated RNA probed with pSAThl; Lane 3, 24 hr BESpm treated RNA probed with AP3/F7. The size of the pSAThl/AP3/F7 recognized message is estimated from an RNA ladder to be between 1,100-1,300 bases.

Figure 4.
Southern anaylsis of NCI H157 DNA restricted with various restriction endonucleases and probed with the AP3/F7 probe. Twenty μg of DNA from NCI H157 cells was digested with the indicated restriction endonuclease, separated by gel electrophoresis in a 1% agarose gel, transferred to a nylon membrane and probed with the AP3/F7 cDNA. Lane 1, Bam HI; Lane 2, Hpa I; Lane 3, Pvu II; Lane 4, Sal I; Lane 5, Ava I; Lane 6, Hind III; Lane 7, Sph I; Lane 8, Eco RI; Lane 9, Pst I. The numbers at left represent the relative mobility of Hind III fragments of λ-phage DNA. Size of fragments is indicated in kilobases.

DISCUSSION: The inducibility of SSAT has been well documented (2-5,7,8). We have recently demonstrated that SSAT is highly induced in cells which respond cytotoxically to treatment with the polyamine analogue BESpm (9). Recently, Porter and colleagues have reported similar findings in human melanoma lines (11). We originally used the NCI H157 large cell lung carcinoma cell line to provide mRNA to clone a cDNA which coded for the active human protein (12).

We now report a cDNA clone which appears to represent the full length SSAT message. AP3/F7 contains a 165 base 5' untranslated region, a 513 base coding region and a 382 base 3' untranslated region. The total length of this cDNA, designated AP3/F7 is 1,060 bases, which includes 27 adenosines of the poly(A) tail. Assuming the SSAT mRNA contains a typical poly(A) tail (200-400 bp) the AP3/F7 clone probably represents most, if not all, of the entire human mRNA coding for SSAT (~1100-1300 bases). However, it will be necessary to clone and sequence the 5' region of the human gene corresponding to the SSAT message before the precise transcriptional start site and message size are known.

It is important to note that the AP3/F7 clone differs in the 3' non-coding region from the originally reported pSATH1 clone (12). The internal Eco R1 site of the original pSATh1 clone appears to have resulted from tandem insertions during the cloning procedure. Therefore, bases downstream from this site (base 665 of pSATH1) do not correspond to the SSAT gene product. However, it is equally significant that AP3/F7 and pSATH1 are identical in their coding regions. Each cDNA codes for the same protein with a subunit molecular weight of ~20,000, recognizes the identical message on Northern analysis, and codes for a protein which possesses the same acetyltransferase activity.

The current availability of a full length cDNA should be instrumental in determining the multiple levels of regulation of the human SSAT. We have previously shown that treatment of the human large cell lung cancer line NCI H157 responds to BESpm treatment with a time dependent increase in steady-state mRNA which is followed by a large increase in SSAT activity (10). The full length probe should facilitate the determination of mechanisms underlying this increase. The effects of polyamines on translational control for the ornithine decarboxylase and S-adenosylmethionine decarboxylase mRNA's has been demonstrated (21). With both the 5' and 3' untranslated regions of the SSAT cDNA characterized it will now be possible to examine whether the polyamines or their analogues effect translation of this gene product. Additionally, direct transcriptional studies in combination with SSAT mRNA half-life studies will answer whether the observed increase in steady-state message is a result of BESpm induced SSAT transcription, a stabilization of the SSAT mRNA, or a combination of the two. Finally, it should now be possible to examine whether the induced acetyltransferase plays a direct role in the cell specific cytotoxic response to the bis(ethyl)polyamines (9-11).

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