Single stranded DNA SP6 promoter plasmids for engineering mutant RNAs and proteins: synthesis of a 'stretched' preproparathyroid hormone

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ABSTRACT

The intergenic region of bacteriophage f1 has been subcloned into the bacteriophage SP6 promoter plasmids, pSP64 and pSP65, in both orientations. Coinfection of *E. coli* with these SP6 promoter/phage f1 chimeric plasmids and the interference resistance phage, IR1, results in the replication and secretion of the pSP6-f1 plasmids as single stranded DNA. Bovine preProPTH cDNAs in both the native form and a form containing an insertion of 117 base pairs in the protein coding region have been inserted in these plasmids. The RNA transcribed from the SP6-f1/preProPTH cDNA constructs was efficiently translated in the wheat germ or reticulocyte cell free systems without addition of a 7-methylguanosine cap to the RNA. In the presence of dog pancreatic or chicken oviduct microsomal membranes, conversion of the resultant pre-proteins to pro-proteins was observed. Confirmation of the "mutated" preProPTH cDNA was determined by dideoxyribonucleotide DNA sequencing of single stranded plasmid DNA. These vectors are suitable for the efficient biosynthesis of large amounts of single or double stranded DNA, and translationally active RNA. The combined properties of single stranded DNA replication and the SP6 promoter simplify the engineering of mutant RNAs and their corresponding proteins. In addition, single stranded DNA or RNA corresponding to either complementary strand may be synthesized as nucleic acid hybridization probes.

INTRODUCTION

Derivatives of the filamentous phages (f1, fd, M13) have been particularly useful cloning vectors due to the nonlytic secretion of copious amounts of single stranded DNA (ssDNA) as viral particles (1). In addition there is little constraint on the size of inserted foreign DNA because the length of the virion is directly proportional to the length of the DNA packaged. The region of the phage genome required for ssDNA replication and morphogenesis of filamentous phages has been identified by analysis of phage-plasmid (phagemid) replicons (2-4). This region is located in the non-coding or intergenic region and contains signals for initiation and termination of viral strand synthesis (2) and a signal for morphogenesis (3). In the presence of helper phage, phagemids containing the functional replication origin and a morphogenetic signal are packaged and secreted as single stranded viral

particles capable of transducing plasmid markers (4).

The potential value of phagemid vectors for ssDNA sequencing and mutagenesis techniques (1) led to the development of the pEMBL cloning vectors which combine the pUC 8/9 plasmids with 1300 basepairs (bp) of phage f1 DNA (5). These vectors retain from the plasmid a selectable marker for ampicillin resistance, the ColEI origin of replication, the multiple cloning site, and the β -galactosidase assay for recombinants. In the presence of helper phage, plasmid ssDNA suitable for dideoxy sequencing can be obtained from secreted viral particles. These phagemids have the advantages of a ssDNA phage vector, but because of the plasmid mode of replication, they provide increased stability for larger DNA inserts and simplify the isolation of large amounts of double stranded DNA. These properties, combined with the smaller vector size, facilitates the analysis of recombinant plasmids (5).

A major obstacle in studying biochemical processes involving RNA transcripts has been the difficulty in obtaining sufficient quantities of purified RNA species. Two recently developed promoter plasmids, pSP64 and pSP65, are capable of directing in vitro transcription of large amounts of unique RNA (6). The value of these plasmids lies in the specificity of purified bacteriophage SP6 RNA polymerase for the SP6 promoter DNA and in a multiple cloning site, oriented in both directions, immediately following the promoter. The usefulness of these plasmids would be extended if they could be replicated as ssDNA. The powerful site-specific mutagenesis and enzymatic DNA sequencing techniques applicable to ssDNA combined with the specificity of the SP6 promoter should greatly facilitate engineering of RNA variants for studies on structure-activity relationships of the RNA or its protein product.

We now report the construction of a set of four ssDNA plasmids that contain the SP6 promoter. These vectors were used to construct an insertional mutation of bovine preProPTH. RNA transcribed from the preProPTH cDNA inserts was efficiently translated in wheat germ and reticulocyte cell free systems. The resulting prepro-proteins were converted to their respective pro-proteins by microsomal membranes.

MATERIALS AND METHODS

Enzymes and Chemicals

Restriction endonucleases were obtained from Bethesda Research Laboratories, New England Biolabs, or Promega Biotec. T4 DNA ligase, RNasin, and SP6 RNA polymerase were the products of Promega Biotec. E. coli DNA polymerase (Klenow fragment) was from Boehringer Mannheim. T4 DNA polymerase

and ribo-, deoxyribo-, and dideoxyribonucleotides were from PL Biochemicals. The Nco I linker, CCCATGGG, was purchased from New England Biolabs.

Bacteria and bacteriophage

Plasmids were propagated in the *E. coli* strain NM522 (7), an hsd $\Delta 5$ derivative of the lacZ strain 71-18 (8). The interference resistance phage IR1 (9) served as the helper phage and was obtained from R. Cortese (5).

Plasmids, ssDNA, and Dideoxy Sequencing

The plasmid pEMBL 8+ (5) was a gift of R. Cortese, pSP64 and pSP65 were obtained from Promega Biotec, pSP62-PL1 (10) was a gift of M. Green, pAT153 (11) was a gift of J. Scott, and pPTH4 and pPTH7 have been described previously (12). Template DNA for dideoxy sequencing was prepared as described (5). The method of infection, however, was to inoculate 5 ml of medium with 0.1 ml of an overnight culture. After shaking for 30 min at 37° C, cells were infected with IR1 at a multiplicity of infection of 20. DNA sequencing by the chain termination method was according to the procedure of Sanger *et al.* (13) using the "M13 reverse sequencing primer" (New England Biolabs) or an "SP6 promoter primer" (Promega Biotec).

Plasmid Constructions

For the construction of the plasmids, restriction fragments were fractionated by electrophoresis on polyacrylamide gels (14) or 1% agarose gels (15,16). Fragments were ligated overnight with T4 polynucleotide ligase and NM522 cells were transformed by the calcium shock technique (17). Recombinant plasmids were screened by restriction digests of DNA isolated by a rapid boiling procedure (18) or by sequencing the DNA (13). Large amounts of plasmid DNA were isolated according to a rapid boiling procedure (18) with the modification that after the isopropanol precipitation, RNA was removed by precipitation with 2.5 M ammonium acetate (19). Plasmid DNA was further purified by banding in CsCl-ethidium bromide density gradients. Cells containing plasmids derived from the pUC vectors were grown overnight in the absence of antibiotic and without chloramphenicol amplification.

In Vitro Transcription

Conditions for preparative transcription reactions were essentially as described (10). Reactions in 100 μ l containing 750 μ M NTPs, 200 μ g DNA/ml, 3000 units RNasin/ml, and 4 units SP6 RNA polymerase/ μ g DNA, were incubated at 41° C for 3 to 4 hr and stopped by phenol extraction. RNA was purified by oligo (dT) cellulose chromatography for subsequent *in vitro* translation. Analytical transcription reactions (10 μ l) were electrophoresed on 6% polyacrylamide gels containing 8 M urea. These reactions were stopped with

one-tenth volume of 10% diethyl pyrocarbonate and heated for 10 min at 60° C in the presence of 80% formamide prior to electrophoresis.

Cell-free protein synthesis

Conditions for PTH mRNA translation in the wheat germ system and reticulocyte lysate were as described previously (20) except that the final potassium concentration was maintained at 70 mM for uncapped RNA synthesized in vitro. The radioactive translational products were analysed on 15% acrylamide gels containing sodium dodecyl sulfate (20).

Dog pancreatic membranes were isolated according to the method of Meyer and Dobberstein (21). Preparation of chicken oviduct microsomal membranes was based on previously described methods (22,23). The magnum portion of chicken oviduct was rinsed in buffer A (50 mM triethanolamine acetate, pH 7.4, 50 mM KCl, 5 mM MgCl₂, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonylfluoride), cut into small pieces and suspended in 2 ml of buffer A containing 0.25 M sucrose per gm of tissue. The suspension was homogenized with 6-8 strokes of a motor-driven Potter-Elvehjem homogenizer followed by 4-6 strokes with a Dounce homogenizer. The homogenate was centrifuged for 10 min at 12,000 g. The pellet was resuspended in 1/2 volume of buffer A containing 0.25 M sucrose, homogenized with a Potter-Elvehjem homogenizer and centrifuged for 5 min at 3,000 g. Fifteen ml of the supernatant were layered on 5 ml of 1.5 M sucrose over 5 ml of 2.0 M sucrose in buffer A and centrifuged for 16 hr at 100,000 g in a Beckman Ti70 rotor. The microsomal membranes, banded between the 1.5 M and 2.0 M sucrose layers, were collected, diluted with buffer A and pelleted by centrifugation for 1 hr at 100,000 g. The membrane pellet was resuspended in buffer B (buffer A without MgCl₂ and phenylmethylsulfonyl-fluoride) to a concentration of 50-70 A₂₈₀ units/ml and treated with 15 mM EDTA as described (24). EDTA-treated membranes were pelleted by centrifugation for 60 min at 120,000 g. The pellet was resuspended in 20 mM HEPES, pH 7.5, 0.25 M sucrose, 2 mM dithiothreitol and small aliquots were stored frozen in liquid N₂.

RESULTS

Construction of an ϕ Intergenic Region Cassette Plasmid

The construction of plasmids containing the ϕ bacteriophage intergenic region is shown in Fig. 1. Although 1300 bp of phage ϕ DNA is present in pEMBL 8+, the functional replication origin and morphogenetic signal is present on a smaller 514 bp Rsa I fragment (3). The 514 bp Rsa I fragment corresponds to nucleotides 5487-6001 of the ϕ genome (25). This fragment was

Figure 1. Construction of ssDNA plasmids. A 514 bp Rsa I restriction fragment containing the replication origin and morphogenetic signal of phage fl was isolated from pEMBL 8+ and ligated into the Pvu II site of either pSP64 or pSP65, and into the Acc I site of pAT153. pEMBL 8+ is a derivative of pUC 8 containing 1300 bp of DNA from the ssDNA phage fl. The multiple cloning site is oriented in opposite directions in pSP64-fl or pSP65-fl, while the intergenic region is oriented in opposite directions in pSP6-fl- or pSP6-fl+. pDMI is a plasmid construction simplifying the isolation of the fl intergenic region which contains the ssDNA origin of replication and morphogenetic sequence. The Sal I, Acc I, and Hinc II sites bounding the fl intergenic region in pDMI are regenerated. The positions of the multiple cloning site (MCS), *E. coli* replication origin (Ori), the β -lactamase gene (Amp), and the tetracycline resistance marker (Tet) are shown, as is the location of the promoter-operator for the lac Z' gene (POZ') and the intergenic region of phage fl (fl IG). For simplicity the MCS of pEMBL 8+ is not shown. The ssDNA plasmids contain 3563 bp and 4172 bp for pSP64/65-fl and pDMI, respectively.

isolated and inserted at the Acc I site of pAT153. The Sal I, Acc I, and Hinc II sites are regenerated at the ends of the fragment. Therefore, the resulting plasmid, pDMI, contains an easily isolated fl intergenic region cassette for the conversion of essentially any plasmid into a ssDNA vector.

Construction of ssDNA SP6 Promoter Plasmids

The 514 bp Rsa I fragment was inserted in both orientations into the Pvu II sites of pSP64 and pSP65 (Fig. 1). The resulting four plasmids are identical in their DNA content, but differ in the orientation of the multiple

Figure 2. Orientation of the multiple cloning sites and the fl intergenic region of the pSP6·fl plasmids and sequences of the secreted ssDNA around the multiple cloning site region. (A) A schematic diagram of the region of the pSP6·fl plasmids which contains the SP6 promoter, the multiple cloning site, the fl intergenic region, and the ColE1 origin of replication (ORI). In addition to the restriction sites in the multiple cloning site, the Sph I and Nae I sites that were used to establish the orientations of the multiple cloning site and fl intergenic region are shown. Hd, Hind III; Ps, Pst I; Sl, Sal I; Xb, Xba I; Ba, Bam HI; Sm, Sma I; Sc, Sac I; Ec, Eco RI. (B) Analysis of restriction fragments of pSP6·fl plasmids. Samples were fractionated by electrophoresis on a 0.75% agarose gel. Molecular weight standards in base pairs are shown on the left. The sizes of the fragments observed are shown on the right. The Nae I digest is diagnostic of the orientation of the intergenic region while the Sph I plus Eco RI digest is diagnostic of the multiple cloning site orientation. (C) DNA sequence of the multiple cloning sites of pSP6 fl plasmids. ssDNA from pSP6·fl- was sequenced using the "SP6 promoter primer" while pSP6·fl+ was sequenced using the M13 "reverse sequencing primer". SP6 RNA polymerase initiates transcription at the nucleotide numbered 1. The pSP6·fl plasmids contain the promoter half of the lac Z' marker which is in the opposite orientation compared to the SP6 promoter and 3' of the multiple cloning site. The M13 "reverse sequencing primer" thus serves as a "universal primer" for sequencing ssDNA from pSP64/65·fl+. The individual DNA strands are labelled to show which of the strands is secreted in the ssDNA form for each of the four pSP6·fl plasmids.

cloning site and the fl intergenic region (Fig. 2A and B). Recombinant clones were selected by the ability of secreted single stranded vector DNA to transduce ampicillin resistance in subsequently infected cells. The correct construction of the plasmids was confirmed by restriction analysis and sequencing. The orientation of the fl fragment, which contains an asymmetrical Nae I site (Fig. 2A), was determined by Nae I cleavage. The fl+ plasmids produced a 590 bp fragment and the fl- plasmids produced a 845 bp fragment (Fig. 2B). The orientation of the fl fragment determines which of the two DNA strands of the resulting plasmids, pSP64/65•fl+ and pSP64/65•fl-, will be packaged as the viral particle. We have defined the plasmids for which the sequence of the secreted DNA strand is equivalent to that of the RNA transcript directed by the SP6 promoter as the fl+ plasmids. For the fl- plasmids, the DNA complementary to the RNA transcript is produced. The sequence of the secreted DNA around the multiple cloning site is shown in Fig. 2C. A commercially available oligonucleotide, the reverse sequencing primer, may be used as a direct primer for sequencing inserts in pSP6•fl+ ssDNA. A second oligonucleotide complementary to sequence in the SP6 promoter region may be used as a sequencing primer for pSP6•fl- ssDNA. The presence of helper phage DNA has not interfered with the sequencing procedure.

Construction of SP6 Promoter Plasmids Containing Bovine PTH cDNA Inserts

To obtain a full length bovine PTH cDNA insert, the 5' portion of the cDNA insert of pPTHi7 which contains a complete 5' region was combined with the 3' portion of the cDNA insert of pPTHi4 which contains the entire 3' untranslated region including 52 polyadenylate residues (12) (Fig. 3). In addition, the Sac I site in the 3' untranslated region was destroyed, leaving a single Sac I site in the coding region. This PTH construction, pPTHi74-Sx, contains an Acc I site at an in vivo transcription initiation site of the PTH gene (26). Therefore, the region from the Acc I site to the Pst I site at the 3' end of the cloned cDNA insert corresponds to the full length PTH mRNA and this fragment was inserted into the Hind III site in pSP62-PL1 to produce pSPPTH. To incorporate the PTH cDNA insert into a ssDNA vector a fragment of the cDNA from the Pvu II site (45 nucleotides 5' of the initiation codon) to a Sma I site on the 3' side of the insert was ligated into the Sma I site of pSP64•fl- to produce pUP47. In this plasmid, transcripts directed by the SP6 promoter contain 41 nucleotides from the multiple cloning site and 47 nucleotides of PTH cDNA prior to the initiation codon for preProPTH.

Our ultimate goal is to analyze the translocation and processing of preProPTH "mutants" by microsomal membranes in vitro. Processing of preProPTH

Figure 3. Construction of pSP6-f1 plasmids containing preProPTH cDNA. A plasmid containing full-length preProPTH cDNA (pPTHi74-Sx) and ssDNA SP6 promoter plasmids containing preProPTH cDNA (pUP47) or "stretched" preProPTH cDNA (pPNTH) were constructed as described in Methods. The maps are not drawn to scale; the arrows represent either the direction of transcription from the SP6 promoter or the 5' to 3' orientation of preProPTH cDNA. The open box represents the preProPTH coding region, while the closed boxes represent the 5' or 3' non-coding regions. The hatched box represents the SP6 promoter, while the raised box in pPNTH represents the direct repeat. The clusters of small lines represent the multiple cloning sites (MCS) of the SP6 plasmids. Selected restriction sites in the plasmids are indicated as S, Sac I; N, Nco I; H, Hind III; B, Bam HI; A, Acc I; P, Pst I; Pv, Pvu II; Sm, Sma I; Hc, Hinc II; T, Taq I; E, Eco RI. Incubations with bacterial alkaline phosphatase (BAP) and the Klenow fragment of *E. coli* DNA polymerase I (Klenow) are indicated. The sizes in bp are indicated for isolated fragments used in the constructions.

Figure 4. Nucleotide sequence analysis of "stretched" preProPTH cDNA and *in vitro* transcription of preProPTH cDNAs. (A) A schematic diagram of the cDNA insert of pUP47 is shown indicating the location and direction of transcription. The filled boxes represent the 5' and 3' noncoding regions, and the open boxes represent regions coding for preProPTH. The cDNA insert contains 52 deoxyadenylic acid residues (A52) and 30 deoxycytidylic acid residues (C30) at the 3' terminus. To construct pPNTH, a duplication of the Nco I to Taq I fragment was produced by inserting this fragment into the Nco I site of pUP47 (see Figure 3). This insertion results in the duplication of amino acids 19 to 52. In the lower portion of (A) the nucleotide and amino acid sequences of the fusion junction at the ends of the insert are shown with autoradiograms of the sequencing gels. Deoxyinosine was used instead of deoxyguanosine in these reactions. Sequence in pPNTH coding for amino acids A-E was generated by addition of Nco I linkers and filling in the staggered ends during the construction. (B) The cDNA inserts of both pUP47 and pPNTH were transcribed with SP6 polymerase. The products of the transcription reactions were analyzed by electrophoresis on 6% acrylamide gels containing 8M urea. For transcription, the plasmids were linearized by Eco RI cleavage at the site indicated in (A), and were incubated with SP6 polymerase as described in Methods. These templates produce RNA transcripts of 896 and 779 nucleotides for pPNTH and pUP47, respectively. The sizes of the RNA markers are indicated at the right (Riboprobe control, Promega Biotec) and the sizes of the major RNA transcripts are shown on the left. The 150 nucleotide product in lanes 1 and 2 presumably represents a prematurely terminated transcript.

in the reticulocyte system is more efficient than in the wheat germ system (20). However, the large amount of globin in the reticulocyte system comigrates with preProPTH and ProPTH, resulting in diffuse unresolved bands when analyzed by electrophoresis on SDS-polyacrylamide gels. To circumvent this problem we have designed an extended PTH cDNA that codes for a protein larger than preProPTH. A construction inserting 117 bp of DNA into the coding region of PTH is shown in Fig. 3. A direct repeat of the 103 bp Nco I to Taq I fragment of pUP47, which corresponds to amino acids 19 through 52 of the

Figure 5. Effect of potassium ion concentration on the translational efficiency of capped and uncapped mRNAs in wheat germ extracts. Translation reactions contained 100 ng of mRNA in a volume of 12.5 μ l and the 35 S-labelled proteins were analyzed by polyacrylamide gel electrophoresis and fluorography as described in Methods. Lanes a, b: 120 mM K^+ ; Lanes c, d: 70 mM K^+ ; Lanes a, c: uncapped PTH SP6/mRNA; Lanes b, d: capped PTH mRNA.

mature hormone, was inserted in the PTH cDNA to produce the plasmid, pPNT_H. An additional four amino acids not present in PTH resulted from the incorporation of the Nco I linker and the filling in of the Taq I site and the original Nco I site (Fig. 4A). The resulting "stretched" PTH cDNA codes for a protein 39 amino acids longer than preProPTH with the additional advantage of containing three more methionine residues in the mature hormone, which increases the amount of 35 S-methionine incorporated. The exact construction of pPNT_H was confirmed by DNA sequencing (Fig. 4A).

In vitro Transcription of preProPTH cDNA

After cleavage of the plasmids with EcoRI the expected truncated transcripts for PTH cDNA of 779 and 896 nucleotides were observed with pUP47 and pPNT_H, respectively (Fig. 4B). About 2 μ g of total RNA per 1 μ g of added plasmid DNA could be produced. Surprisingly, an additional strong band was observed that corresponded to an RNA transcript containing 150 nucleotides. This smaller fragment probably represents premature termination at a sequence which resembles an SP6 polymerase terminator. Cleavage of the template at restriction sites less than 150 nucleotides from the SP6 promoter eliminated the synthesis of this RNA (data not shown). Since the full length transcripts contain a polyadenylate region (Fig. 4A), isolation of poly(A) containing RNA by affinity chromatography on oligo(dT)-cellulose largely eliminated the prematurely terminated product. Poly(A) containing RNA ranged from 15% to 25% of total RNA synthesized.

Translation of mRNA Synthesized In Vitro

The PTH mRNA transcribed in vitro by SP6 RNA polymerase (SP6/mRNA) was efficiently translated in either the wheat germ or reticulocyte cell free systems (Figures 5 and 6). The translational efficiency of capped in vivo synthesized mRNA compared to uncapped synthetic mRNA is dependent on the potassium ion concentration. At the optimal K^+ concentration of 120 mM for translation of the natural mRNA, the uncapped SP6/mRNA is only 20% as

Figure 6. Analysis by gel electrophoresis of the ^{35}S -methionine labelled cell-free translation products. Either natural preProPTH mRNA or RNA transcribed *in vitro* with SP6 polymerase was translated in wheat germ (panel A) or reticulocyte (panel B) cell-free systems as described in Methods. The natural mRNA and uncapped RNAs made *in vitro* were translated at potassium ion concentrations of 120 mM and 70 mM, respectively. Microsomal membranes from either dog pancreas or chicken oviduct were added to some of the reactions. The products were analyzed by electrophoresis on 15% acrylamide gels containing sodium dodecyl sulfate and radioactivity was detected by autoradiography. The positions of preProPTH (pre), ProPTH (Pro), "stretched" preProPTH (Pre'), and "stretched" ProPTH (Pro') are indicated. Reactions contained the following mRNAs and amounts of microsomal membranes: Panel A, a) minus mRNA b-c) preProPTH mRNA; d-e) pUP47 SP6/mRNA; f-h) pPNTN SP6/mRNA; b,d,f) no membranes; c,e,g) 3.6 A_{260} units/ml of dog membranes; h) 0.32 A_{260} units/ml of chicken membranes. Panel B, a,b) pUP47 SP6/mRNA; c-h) pPNTN SP6/mRNA; b,h) no membranes; a,g) 3.6 A_{260} units/ml of dog membranes; c) 0.16 A_{260} units/ml of chicken membranes; d) 0.64 A_{260} units/ml of chicken membranes; e) 0.32 A_{260} units/ml of chicken membranes; f) 7.2 A_{260} units/ml of dog membranes.

efficient (Fig. 5, lanes a and b). However, at 70 mM K^+ the translational efficiency of the uncapped mRNA is similar to that of the natural mRNA (Fig. 5, lanes c and d) and is approximately 60% of the activity of capped mRNA at 120 mM K^+ . S-adenosylhomocysteine, an inhibitor of cap methylation, had no effect on the translational activity of SP6/mRNA (data not shown) indicating that synthetic mRNA is not capped in the cell free systems prior to translation.

A protein migrating with preProPTH was the major translation product of PTH SP6/mRNA in the wheat germ system (Fig. 6A, lane b,d). In the reticulocyte system a smeared radioactive band was observed that comigrated with the large amount of globin for translations of PTH SP6/mRNA (Fig. 6B, lanes a, b). For the "stretched" PTH SP6/mRNA, a single major larger protein was observed in both the wheat germ and reticulocyte systems (Fig. 6A, lane f; 6B, lane h). When either dog pancreatic or chicken oviduct microsomal

membranes were added to the cell free systems a new radioactive band was observed that corresponded to ProPTH (Fig. 6A, lanes c, e) or "stretched" ProPTH (Fig. 6A, lanes g, h; 6B, lanes c-g). Conversion to "stretched" ProPTH was more efficient in the reticulocyte cell free system than in the wheat germ system. In the wheat germ system the "stretched" preProPTH was more efficiently processed to the Pro form than the smaller preProPTH. The processing was dependent on the concentration of membranes added and up to 89% of preProPTH was converted to ProPTH (Figure 6B, lane d). The dog and chicken membranes were about equally effective in processing, although the background of peptides synthesized by endogenous mRNAs in the dog preparations was greater than that in the chicken system.

DISCUSSION

E. coli plasmids containing a promoter for SP6 polymerase have proven to be convenient and efficient templates for the in vitro synthesis of specific RNA transcripts from cloned DNA. RNA transcripts have been synthesized from SP6 promoter plasmids which are biologically functional in processing (6,10) or translation (28). In addition, the RNA transcripts are superior hybridization probes when compared to nick-translated DNA or ssDNA probes (6,29). We have increased the versatility of the SP6 transcriptional plasmids by incorporating the intergenic region of phage fl. These plasmids are secreted as ssDNA viral particles in cells coinfecting with IR1. The pSP6-fl vectors should prove particularly useful for the production of modified RNA molecules since mutations may be introduced by techniques that utilize either double stranded or ssDNA. Mutational changes can be confirmed by either the enzymatic or chemical DNA sequencing methods, and the mutant RNA can be synthesized using the same plasmid without shuttling fragments between vectors. Furthermore, the set of four vectors, with the polylinker and the fl intergenic fragment in opposite orientations, provides great versatility in cloning and mutagenesis strategies, and in selecting the strand of DNA to be secreted. These characteristics will be particularly important for rapidly engineering protein products and in heteroduplex studies requiring hybrid DNA-DNA or DNA-RNA products.

We have developed these vectors to facilitate our analysis of the structural determinants of the preProPTH signal sequence that specify its binding to the endoplasmic reticulum membrane, translocation, and processing. These processes can be studied in cell free translation systems that contain microsomal membranes (20). The single stranded SP6 transcriptional plasmids

will allow us to rapidly and specifically introduce mutations into the signal sequence. The construction of a variant preProPTH cDNA that codes for a "stretched" version of PTH exemplifies the capabilities of the pSP6-f1 vector system. The "stretched" preProPTH allows us to take advantage of the more efficient processing of preProPTH in the reticulocyte cell free system (20). An internal portion of PTH was used to construct the "stretched" protein since this sequence is part of a secreted protein and should not have properties that would prevent its translocation across the membrane.

Processing of preProPTH in the wheat germ cell-free system or its stretched version in the wheat germ and reticulocyte cell-free systems was observed with either dog pancreatic or chicken oviduct microsomal membranes. The stretched version of preProPTH was more efficiently processed than native preProPTH in the wheat germ system. The reason for this is not clear but may be related to the increased length of "stretched" preProPTH. In order to be successfully transported across the endoplasmic reticulum membrane, secreted proteins probably need to be at least 60 to 80 amino acids long (30). The growing nascent polypeptide must interact cotranslationally with signal recognition particle prior to interaction with the endoplasmic reticulum membrane (31). Once the nascent chain is completed and released from the ribosome, binding to the signal recognition particle will not occur. PreProPTH, with 115 amino acids, is relatively small and many nascent chains may be released without interacting with signal recognition particle in the cell-free systems. In support of this idea, even in intact parathyroid tissue incubated in vitro, a small amount of preProPTH can be detected which is apparently aberrantly released into the cytoplasm and degraded without being converted to ProPTH (32). The larger size of "stretched" preProPTH may allow more time for interaction with signal recognition particle before translation is completed, thus increasing the efficiency of its conversion.

The requirement of a 5' 7-methylguanosine cap for maximum activity of mRNA has clearly been demonstrated for natural mRNAs (33) and for SP6/mRNAs (28, see Fig. 5), at the optimal potassium ion concentration for the natural mRNA (120 mM). However, at the optimum potassium ion concentration for uncapped SP6/mRNAs (70 mM), the translational efficiency is 60% of the efficiency of the natural mRNA. This observation is consistent with experiments showing that the cap requirement is diminished at low potassium ion concentrations (27). Therefore, enzymatic capping is not required to obtain mRNA active enough for studies requiring protein synthesis in vitro.

The relaxed requirement for a 5' cap structure in in vitro systems may

also extend to other features of the mRNA, such as the sequence of the 5' untranslated region. We have observed similar translational activities for RNA transcripts from pSPTH and pUP47 (data not shown). pSPTH has the entire 5' non-coding region of preProPTH mRNA plus an additional 42 nucleotides of SP6 viral DNA while pUP47 lacks the first 52 nucleotides of the non-coding region but contains 41 bases from the polylinker. This result rules out at least the first half of the 5' untranslated region of preProPTH mRNA as being necessary for translation. A preProPTH cDNA construction deleting the entire 5' untranslated region was also efficiently translated in the wheat germ system (E. Skorupa and B. Kemper, unpublished observations). From a practical standpoint, these results suggest that any RNA synthesized in vitro, which has an appropriate methionine codon, can be actively translated.

We have observed a very strong terminator of SP6 RNA polymerase located in the coding region of PTH that gives rise to a truncated transcript of approximately 150 bases. While the position of the terminator-like site has not been mapped precisely, it is located near the end of the signal sequence in a T rich region which contains a stretch of 7 Ts out of 9 nucleotides. Although the propensity of SP6 RNA polymerase to prematurely terminate in eukaryotic sequences has been noted (34), several eukaryotic sequences have been transcribed by SP6 polymerase without evidence of any strong terminators (6). The sequence requirement for natural termination of SP6 polymerase, which is not known, may be similar to that of E. coli in requiring a string of Ts, although in this case there is no evidence for a hairpin loop structure prior to the Ts as is the case in E. coli (35). In theory, this strong terminator-like site could be placed near the end of inserted sequences so that the more efficient supercoiled template would not have to be linearized by restriction digestion before transcription. In practice, the termination at this site is much less efficient in supercoiled DNA (D. Mead and B. Kemper, unpublished observations).

We have exploited the ssDNA SP6 transcriptional plasmids to confirm by enzymatic sequencing the construction of an insertional mutation of PTH. By constructing a direct repeat of an internal portion of PTH cDNA we have "stretched" PTH in order to take advantage of the more efficient processing of preproteins in the reticulocyte cell free system. Utilizing this in vitro protein engineering system we intend to dissect the structure-activity relationship of the PTH signal sequence.

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