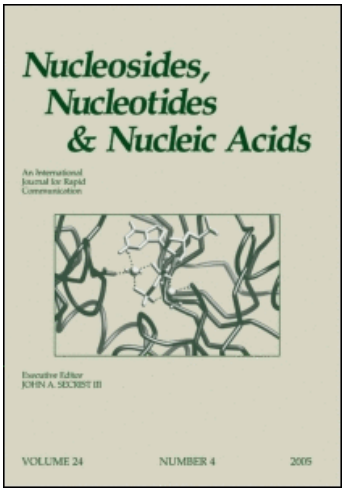

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SYNTHESIS OF A GENE CODING FOR HUMAN VASOACTIVE INTESTINAL PEPTIDE (VIP)

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Abstract

A gene coding for human Vasoactive Intestinal Peptide (VIP) was designed as a double-stranded 99 base pair DNA sequence. The sixteen fragments of the gene were chemically synthesized using a solid-phase phosphoramidite triester coupling approach and enzymatically assembled using T4 DNA ligase. The resulting gene was cloned into pBR322 and sequenced using the Maxam-Gilbert sequencing procedure.

Introduction

Vasoactive Intestinal Peptide (VIP), a highly basic single chain polypeptide of twenty-eight amino acid residues in length, was first isolated in 1970 from the hog small intestine[1,2]. Its amino acid sequence was determined by Mutt and Said in 1974[3] (Figure 1). VIP has subsequently been isolated from chicken[4,5], cow[6] and man[7]. Porcine, bovine and human VIP have identical amino acid sequences. Avian VIP differed from mammalian VIP by the replacement of Thr-11, Leu-13, Ile-26 and Asn-28 by Ser-11, Phe-13, Val-26 and Thr-28 respectively.

Originally isolated from gut, VIP has been found widely distributed throughout the body with significant concentration in the brain and gut[8,9]. VIP, now classified as a brain-gut peptide, plays a dual role as gastrointestinal hormone and as a putative neurotransmitter[10]. Some of its biological actions include vasodilation, lowering of arterial blood pressure, increase in cardiac output and relaxant of airway smooth muscle[8]. Our interest in VIP lies in its

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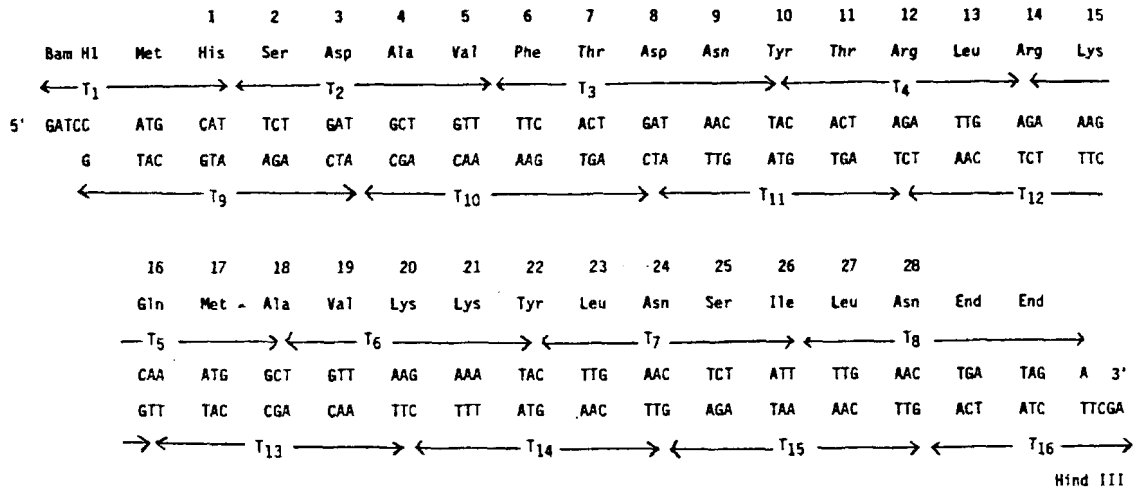


FIG. 1: Amino acid sequence of human VIP and a designed nucleotide sequence coding for VIP. Numbers above each amino acid refer to amino acid positions. Arrows with T_N represent the synthetic oligodeoxyribonucleotides.

potential usefulness in the treatment of bronchial asthma or other respiratory diseases in human subjects. The synthesis of a VIP gene will then allow us to produce VIP by recombinant DNA technology.

Because of recent advances in the solid-phase synthesis of oligonucleotides, the chemical synthesis of a gene is now a feasible task. There are many advantages to the synthesis of a gene: 1) the exact desired sequence can be obtained; 2) the amino acid composition of a protein can be altered by replacing a specific codon in the DNA sequence and 3) no isolation of a cDNA library is required. In the past few years, the human genes for insulin[11], interferon α-1[12] and somatostatin[13] have been synthesized using the phosphotriester procedure. Recently, the synthesis of human interferon-γ[14] using the phosphoramidite approach was reported.

In this study, we describe the overall strategy in the design of a gene coding for VIP, the synthesis of the sixteen fragments by the phosphoramidite method and their ligation, the cloning of the gene into pBR322 and its sequencing.

In 1981, Obata *et al.* found that human VIP was synthesized from a precursor, pro-VIP in human neuroblastoma cells[15]. A cDNA clone coding for human Preprovasoactive Intestinal Polypeptide was isolated[16] and its sequence determined. It differed slightly from the one we have designed in this paper.

Results and Discussion

(a) Design of the synthetic VIP gene

A VIP gene sequence, made up of sixteen oligodeoxynucleotides, was designed from the amino acid sequence of human/porcine VIP as shown in Figure1. Due to the degeneracy of the genetic code, 2.8×10^{13} different nucleotide sequences can correctly code for VIP. In arriving at one particular sequence, the following discriminators were employed[13]: 1) codon frequencies for expression in yeast[17]; 2) minimization of AT- and GC-rich regions; 3) elimination of regions of self-complementarities; 4) placement of restriction endonuclease sites for incorporation into the pBR322 vector; 5) addition of an ATG codon for translation initiation and 6) introduction of translation stop codons. The second and third operations were conveniently accomplished with a computer program[18]. The choice for expression of VIP in

yeast was based on (1) that Met-17 could complicate the isolation of a chimeric VIP expressed in *E. coli* by CNBr cleavage and (2) that expression, stability and secretion were less favorable in *E. coli*. The minimization of AT- and GC-rich regions was required to avoid premature termination of transcription. The restriction endonuclease sites chosen were BamH1 at the 5'-terminal and HindIII at the 3'-terminal.

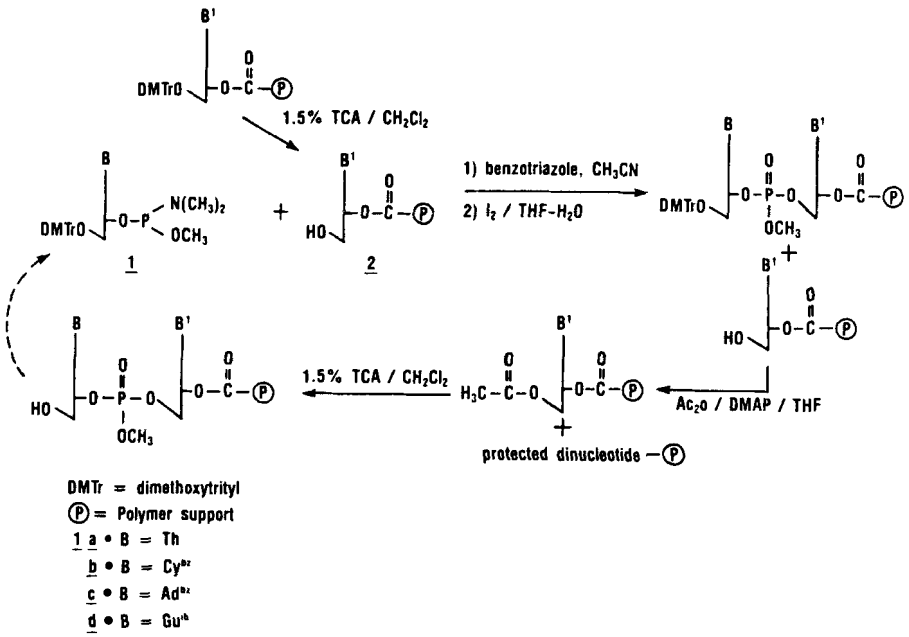
The following criteria were considered in the division of the ds DNA into oligodeoxynucleotide fragments for chemical synthesis. First, a minimum overlap of six base pairs were deemed best for efficient ligation using T4 DNA ligase. Second, the length of the oligonucleotide fragments was restricted to less than 20-mer for synthesis ease and purification. Third, regions of four base pairs or more which could associate in either the primary sequence or the complement were avoided. Therefore, for some amino acids, two different codons were chosen at different locations in the gene sequence (e.g. Lys-21 is AAA while Lys-15 and 20 are AAG. His-1 was changed from CAC to CAT).

(b) Chemical synthesis of gene fragments

The chemical synthesis of the deoxynucleoside phosphoramidites and the subsequent coupling reactions were adapted from the procedure developed by Beaucage and Caruthers[19] with slight modifications as shown in Scheme 1.

For the synthesis of the deoxynucleoside N,N-dimethylaminophosphoramidites 1 (a-d), either acid free $CHCl_3$ or dry CH_2Cl_2 were used as reaction solvent. The order of addition of reagents was very important in order to minimize the formation of 3', 3'-dimers. We also found that the use of deoxygenated solvents during work-up eliminated the formation of side products (presumably phosphoramidates) appearing in the -20 to 0 ppm region of the ^{31}PNMR spectrum. The purity of these mononucleotides was of prime importance for a high yield in the coupling reaction.

To test for the optimal conditions for the activation of the nucleoside phosphoramidites, we compared the efficiency of two activators, benzotriazole and tetrazole, and two solvent systems, THF and CH_3CN, during the first coupling step. As can be seen in Table 1, freshly distilled CH_3CN gives a more quantitative yield of product than THF. Although a much larger excess of benzotriazole was required, it is much less expensive than tetrazole



Scheme 1

TABLE 1 Efficiency of coupling using various conditions in the synthesis of DMTrd(T_{pOCH_3} T-Ⓟ)

Mononucleotide	Activator	Solvent	% yield[a]
40 eq	tetrazole, 142 eq	THF	79%
40 eq	tetrazole, 132 eq	CH_3CN	90%
42 eq	tetrazole, 400 eq	THF	79%
39 eq	benzotriazole, 119 eq	CH_3CN	73%
37 eq	benzotriazole, 385 eq	CH_3CN	98%
36 eq	benzotriazole, 973 eq	THF	26%

a. Reading of the absorbance at 504 nm after detritylation with 3% TCA/CH_2Cl_2

and can be used directly from the bottle without sublimation. The coupling reactions therefore were carried out in dry CH_3CN using a 40 eq excess of mononucleotide and a 400 eq excess of benzotriazole.

The synthesis cycle described in Scheme 1 involves the detritylation of the monomer on the polymer support using 1.5% TCA in CH_2Cl_2 to yield 2. It was previously observed by other researchers that the use of TCA (or DCA) minimized depurination which is known to be the major side product with N-protected adenosine[20]. After activation of the mononucleotides (1 a-d) with benzotriazole, coupling with the free 5'-hydroxyl group of 2 occurs followed by oxidation of the phosphite to a phosphate using iodine in tetrahydrofuran-water. Any unreacted nucleoside on the polymer support was capped with acetic anhydride/tetrahydrofuran/dimethylaminopyridine to minimize the amount of fragment with shorter sequences[21]. The overall yields based on the absorbance at 504 nm from the detritylation step ranged from 15% to 40% (see Table 2) which indicated >90% coupling at each step.

(c) Deprotection and purification of the gene fragments

The deprotection procedure described in the experimental section was used in order to minimize the possibility of internucleotidic bond breakage or rearrangement products that could arise from the cleavage of the methoxy groups using ammonium hydroxide. Such neighboring group participation of the free 5'-hydroxyl group in the base cleavage of protecting groups at the phosphate linkage has previously been observed by deRooij *et al.*[22]. The fully deprotected fragments were purified by polyacrylamide gel electrophoresis, desalted on a G-25 Sephadex column, verified for their size and analyzed for their purity by HPLC (see Experimental). All fragments were purified to homogeneity as determined by polyacrylamide gel electrophoresis and anion-exchange HPLC. A typical HPLC chromatogram is illustrated in Figure 2. Figure 2A shows the profile of the T_4 reaction mixture after deprotection and Figure 2B shows the purified fragment T_4. Anion-exchange HPLC is a fast and useful tool for determination of the purity of oligonucleotides and also can be used on a routine basis for purification of the fully deprotected oligonucleotides.

(d) Ligation of the gene fragments

The sixteen fragments were enzymatically assembled using T4 DNA ligase following three different strategies as outlined in Scheme 2. In the

TABLE 2 Sequences and overall yields of fragments of the gene

Fragment	Sequence (5'→3')	Length	% yield[a]
T_1	GATCCATGCAT	11-mer	28.7%
T_2	TCTGATGCTGTT	12-mer	22.1%
T_3	TTCACTGATAACT	13-mer	30.1%
T_4	ACACTAGATTGA	12-mer	20.1%
T_5	GAAAGCAAATGG	12-mer	18.2%, 40.3%
T_6	CTGTTAAGAAAT	12-mer	23%
T_7	ACTTGAACTCTATT	14-mer	30%
T_8	TTGAACTGATAGA	13-mer	28.7%
T_9	ATCAGAATGCATG	13-mer	21.4%
T_{10}	CAGTGAAAACAGC	13-mer	36.6%, 35.8%
T_{11}	TAGTGTAGTTAT	12-mer	42.8%
T_{12}	GCTTTCTCAATC	12-mer	22.7%
T_{13}	TAACAGCCATTT	12-mer	40.9%, 32.1%
T_{14}	TTCAAGTATTTCT	13-mer	purchased[b]
T_{15}	GTTCAAAATAGAG	13-mer	20.6%[c]
T_{16}	AGCTTCTATCA	11-mer	15.3%

a. From reading of the absorbance at 504 nm after detritylation with 1.5% TCA/CH_2Cl_2;
b. T_{14} was purchased from Applied Biosystems;
c. T_{15} was synthesized manually using the sintered glass funnel technique

ligation reactions where the sticky ends of the restriction enzyme sites were involved (in fragments T_1 and T_{16}), these were not phosphorylated because of their self-complementarity nature which would favor self-ligation. In the first strategy (I), the fragments were divided into three groups and ligated to yield the products A-36 B-26, C-24 D-36 and E-39 F-37. The extent of ligation was analyzed by 15% polyacrylamide gel electrophoresis, under non-denaturing conditions. The ligated products of expected size (slowest moving band on gel) (results not shown) were detected by autoradiography and isolated by electroelution followed by ethanol precipitation.

The three ligated products were then mixed in annealing buffer and incubated at 65°C followed by annealing and ligation. The VIP gene was

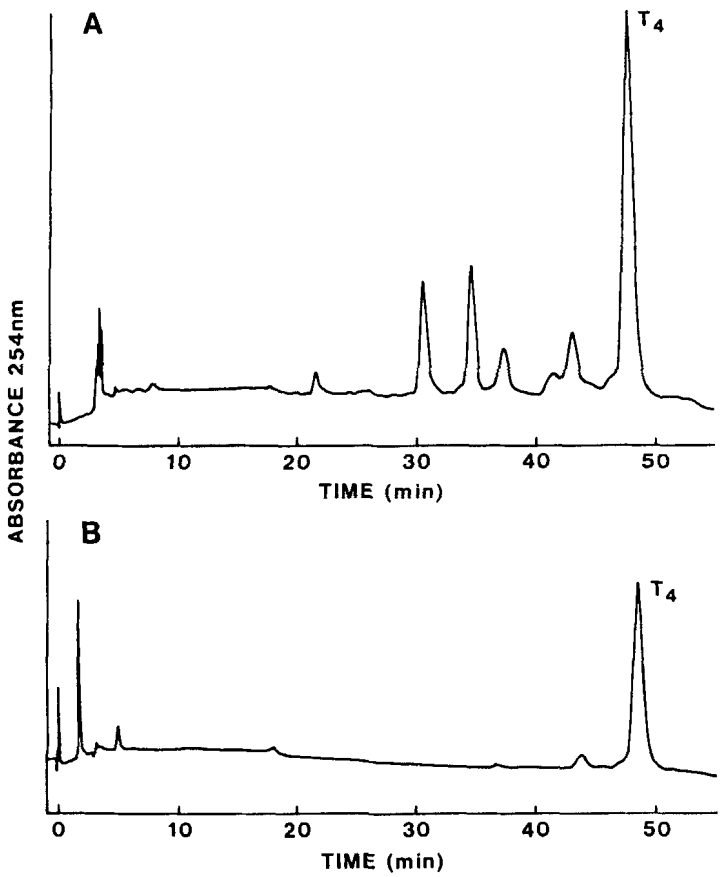
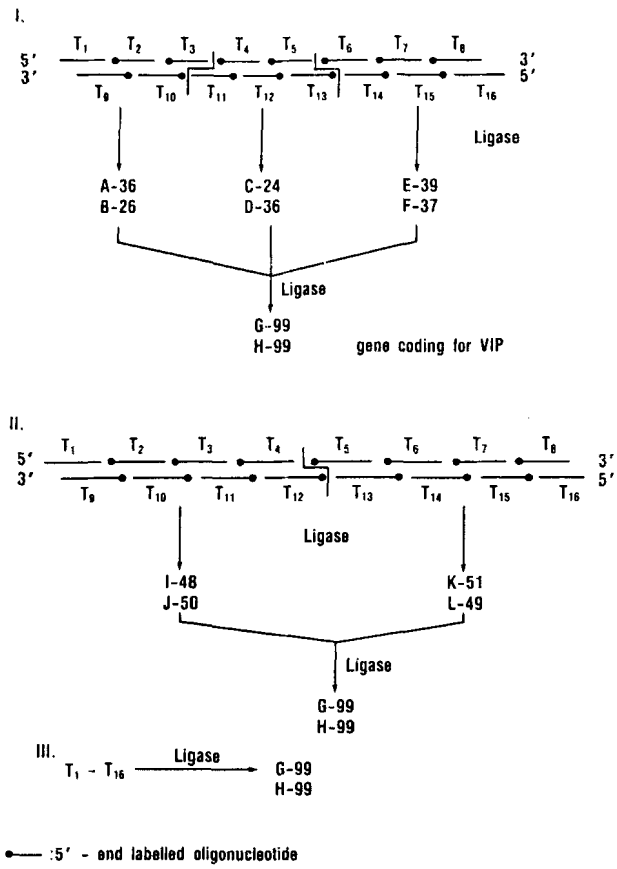


FIG. 2: HPLC chromatograms of T_4 reaction mixture (A) and gel purified T_4 (B).

isolated from 10% polyacrylamide gel electrophoresis under non-denaturing conditions as shown in Figure 3. The purified VIP gene was found to be homogeneous with the expected size as analyzed by polyacrylamide gel electrophoresis under denaturing and non-denaturing conditions. This VIP gene was then used in the cloning experiment described below.

Two other strategies were also explored for the enzymatic assembly of the sixteen fragments. Following the second strategy (II), eight fragments were involved in each ligation reaction. The ligated products of expected size (I-48 J-50 and K-51 L-49) were purified by 10% polyacrylamide gel electrophoresis under non-denaturing conditions, annealed and ligated. As expected, a 99 bp product corresponding to the size of the VIP gene was



Scheme 2

observed on a non-denaturing polyacrylamide gel (Figure 4). The faster moving band corresponds to fragment I-48 J-50 which is in excess in the reaction mixture.

In the third strategy where the sixteen fragments were all mixed together, annealed and ligated, a number of products were observed on a non-denaturing polyacrylamide gel (Figure 5). Although a relatively low yield of product was obtained, it did work. It is interesting to speculate that the efficiency could be improved by the judicious, sequential addition of fragment oligonucleotides to the one-pot strategy. From these results, we observed that more efficient ligation was achieved following the second strategy. The final ligation in strategy II did give a quantitative yield of

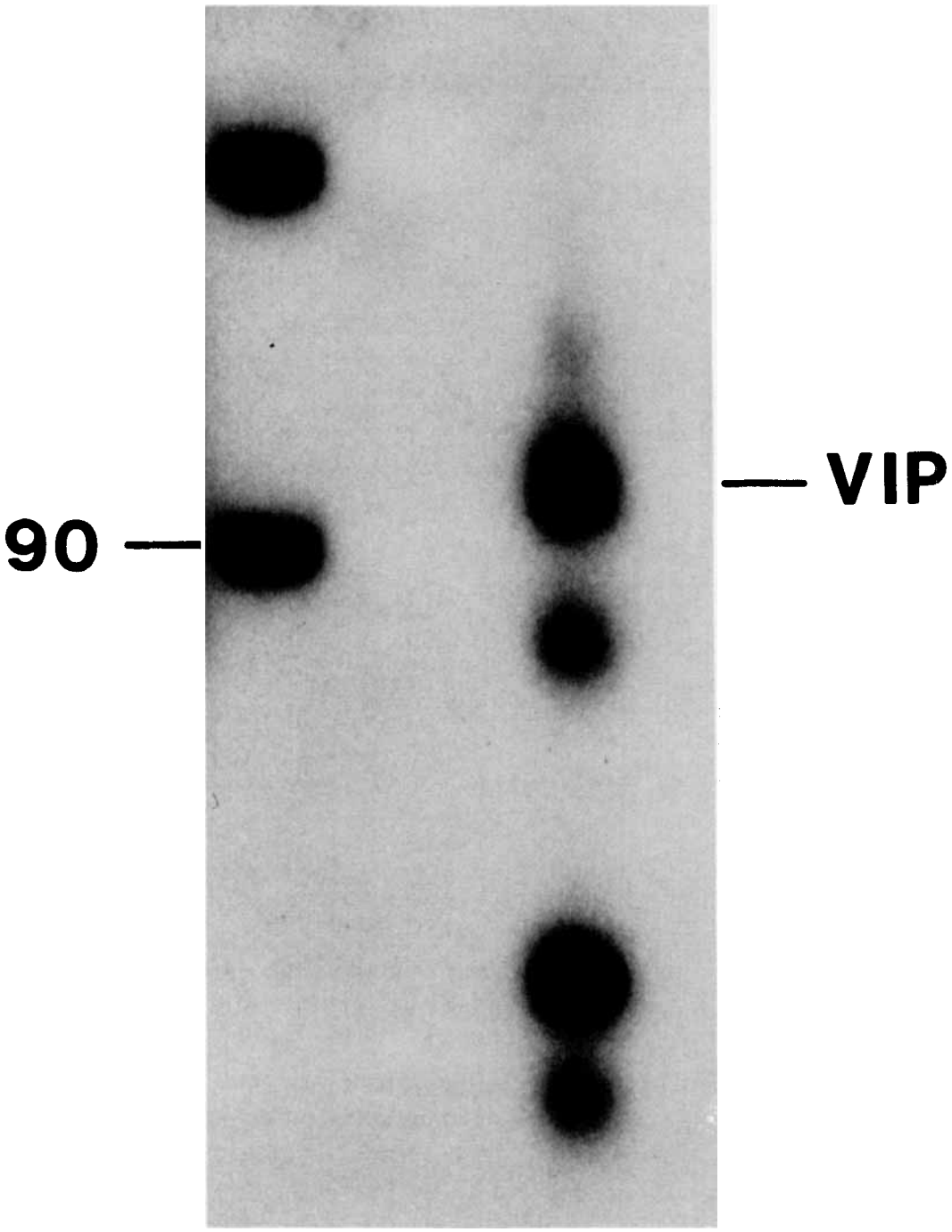


FIG. 3: Autoradiogram of the VIP gene reaction mixture resulting from the final ligation in strategy I of Scheme 2. Left: 90 bp marker (from pKC7 treated with BglII, 3'-end labeled and digested with PvuII); Right: VIP gene reaction mixture.

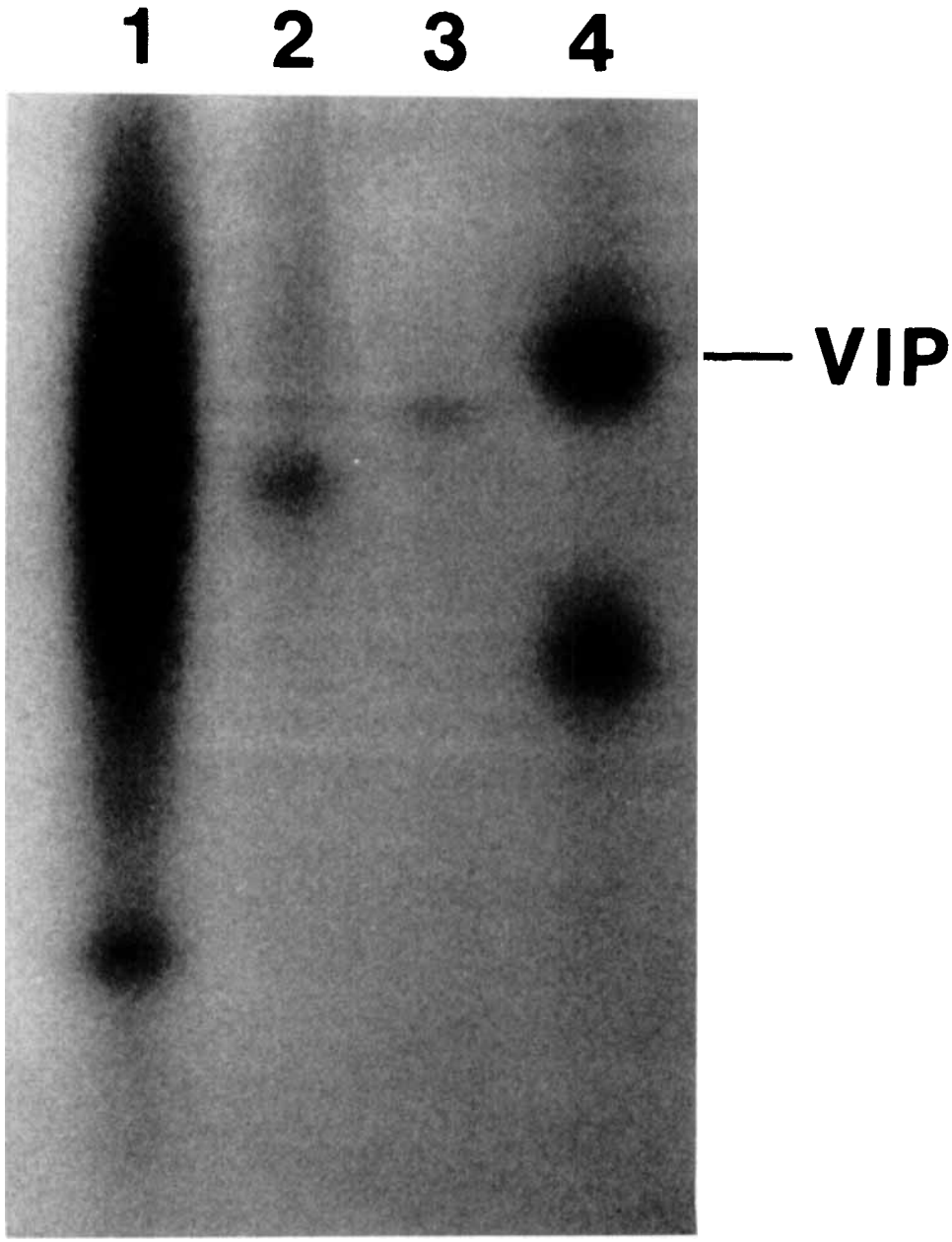


FIG. 4: Autoradiogram of the VIP gene reaction mixture (lane 4) resulting from the final ligation in strategy II of Scheme 2. Lanes 1,2,3 are markers of 30 bp (pBR322/HindIII/EcoR1), 75 bp (pSK102/Cla1/PvuII) and 90 bp (pKC7/BglII/PvuII) respectively.

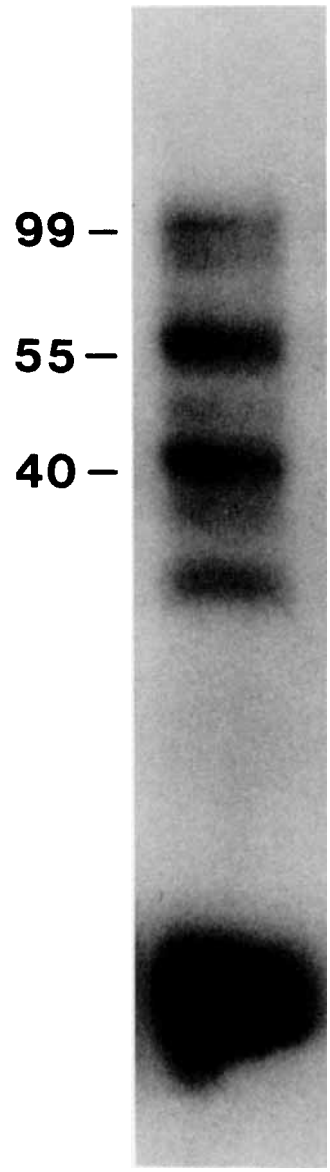


FIG. 5: Autoradiogram of the VIP gene reaction mixture from the ligation in strategy III of Scheme 2. The slowest moving band corresponds to the desired ligated fragment.

product unlike strategy I where a significant amount of unligated fragments as well as a side product were observed (see Figure 3).

(e) Cloning and sequencing of the VIP gene

The gene coding for VIP was cloned into pBR322, replacing the small HindIII-BamH1 fragment in the tetracycline-resistance determinant. The pBR322 was digested with BamH1 and HindIII and the large fragment was isolated by agarose gel electrophoresis. This fragment was ligated to the VIP gene (with its BamH1 and HindIII terminal restriction enzyme sites) to yield the plasmid pVIP1. In pVIP1, the VIP gene is oriented counterclockwise.

Both strands of the VIP gene in pVIP1 were sequenced as described below. First, pVIP1 was digested with HindIII, 3'-end labelled with [α-^{32}P]dCTP and digested with Ava1 to yield a 1.15 kb fragment. The fragment purified by agarose gel electrophoresis was subjected to Maxam-Gilbert sequencing analysis and the results are shown in Figure 6. Analysis with 20% polyacrylamide gel confirmed bases 2 to 50 while the 8% gel identified bases 20 to 102 of the primary strand of the VIP gene. The pVIP1 was also digested with BamH1, 3'-end labelled with [α-^{32}P]dCTP and digested with Pst1 to yield a 885 bp fragment which was purified and used for sequence analysis. The results (not shown) also confirmed the sequence of the complementary strand of the VIP gene.

Experimental

(a) Materials and enzymes

N-protected 5'-ODMTr 2'-O-deoxynucleosides were purchased from Cruachem, BioSearch or Vega Biochemicals. Vydak A and Fractosil 500 silica gel supports were obtained from The Separations Group and Merck respectively. Functionalized polymer supports were bought from BioSearch. Acrylamide, bis-acrylamide, bromophenol blue and xylene cyanol were purchased from BioRad, urea from Bethesda Research Laboratories and agarose from Sigma. T4 Polynucleotide kinase, T4 DNA ligase and the restriction enzymes were purchased from New England BioLabs. Klenow fragment of *E. coli* DNA polymerase I was obtained from Boehringer Mannheim and bacterial alkaline phosphatase was from Bethesda Research Laboratories. [γ-^{32}P]ATP and [α-^{32}P]dCTP were purchased from Amersham.

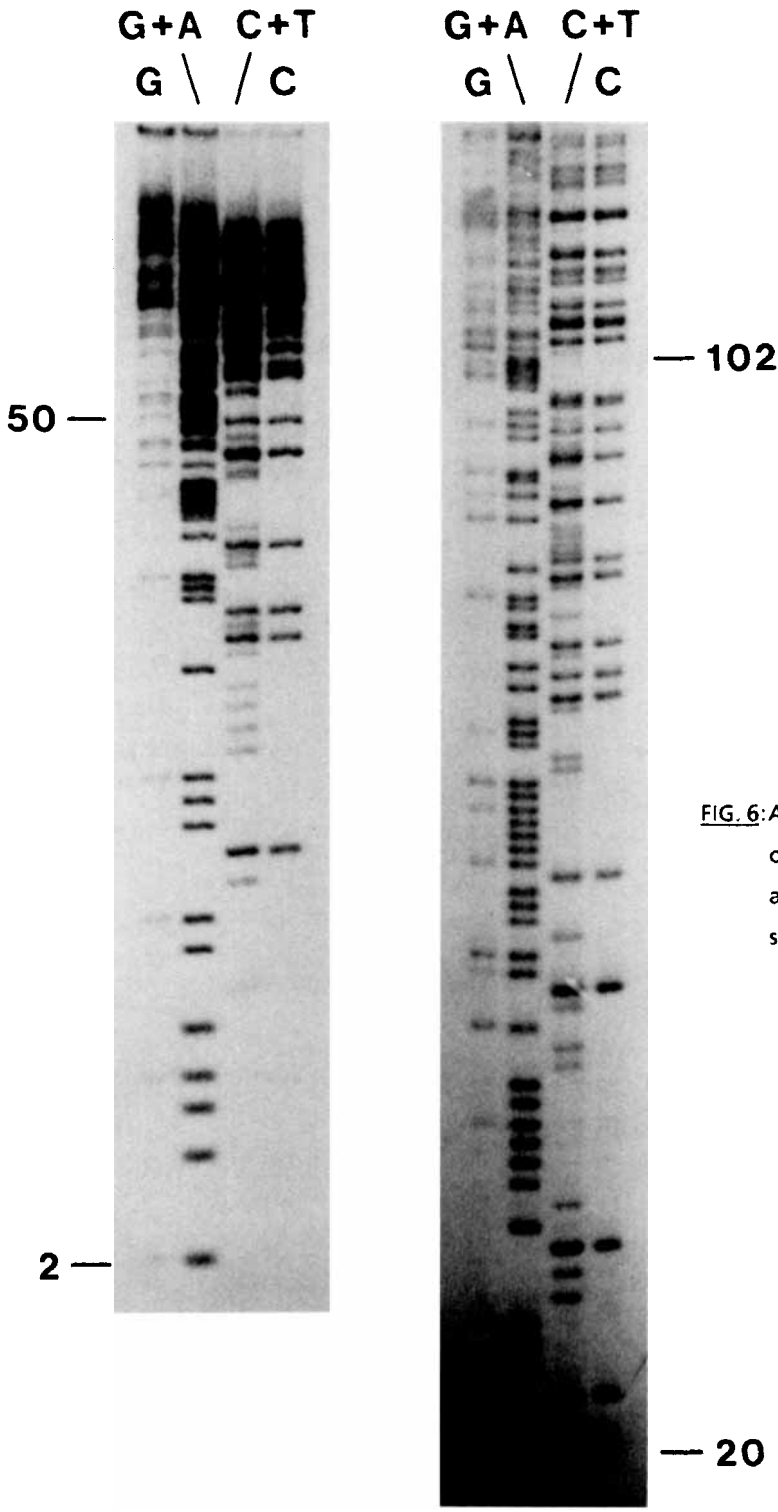


FIG. 6: Autoradiogram of the 20% (left) and 8% (right) sequencing gels.

Hydrazine was obtained from Eastman Kodak and dimethylsulfate, benzotriazole, tetrazole and N,N-dimethylaminopyridine (DMAP) were from Aldrich. DMAP was recrystallized from ether. Reagent grade acetonitrile was refluxed and distilled over calcium hydride immediately before use. Reagent grade THF was refluxed, under nitrogen, with sodium and benzophenone for a few hours and distilled prior to use. The capping solution (Ac_2O/DMAP/THF) was prepared fresh before use.

The ^{31}PNMR spectra were recorded on a Varian XL-200 spectrophotometer and the UV and visible spectra on a Perkin Elmer Lambda 3 UV/VIS spectrophotometer. HPLC analyses were done using a Varian Vista system fitted with a Rheodyne manual loop valve injector and a Varian UV-200 programmable wavelength UV-VIS detector. A polyethyleneimine (PEI) coated Hypersil 5 μm silica column (Supelco, 15 x 0.46 cm ID) functionalized in situ according to a published procedure by Pearson and Regnier[23] was used. BUDGE was used as the cross-linking agent. A linear gradient of 0→30% buffer B in 120 min at a flow rate of 0.6 ml/min (22°C) was employed. Buffer A was 0.05M potassium phosphate plus 30% MeOH adjusted to pH 5.9 and buffer B was 1M $(NH_4)_2SO_4$ in buffer A.

(b) Oligonucleotide synthesis

Unless stated otherwise, the oligonucleotides were synthesized on a BioSearch SAM ONE synthesizer using 50 mg of the appropriately functionalized polymer support as previously described[24]. The silica gel polymer support was functionalized using two different routes as published by Matteucci and Caruthers[25] and Chow *et al.*[21]. Monomer loadings of 20-40 μmole/gm of support and 60-80 μmole/gm were obtained respectively. The 3'-N,N-dimethylaminophosphoramidites of the appropriately protected nucleosides: 5'-O-dimethoxytrityl with N-benzoyl deoxyadenosine, N-benzoyl deoxycytidine, N-isobutyryl deoxyguanosine and thymidine were prepared in very good yields (75-90 %) as previously described by Beaucage and Caruthers[19].

(c) Oligonucleotide purification

After synthesis, the oligonucleotide on the polymer support was deprotected by treatment with 1 ml of φSH:TEA:dioxane (1:1:2v/v/v) at room

temperature for 2 hours. The polymer support was recovered by centrifugation and washed with methanol and ether. The oligonucleotide was released from the polymer support by treatment with 1 ml of NH_4OH at room temperature for 15 hours and at 50°C for 3-5 hours. After centrifugation and washing of the polymer support with H_2O, the eluates and washings were pooled and lyophilized. Depending on the loading on the polymer support, 60-150 O.D. units of reaction mixture were usually obtained.

All the fragments were purified by preparative 20% polyacrylamide gel electrophoresis (29:1 acrylamide:bis-acrylamide, 7M Urea, 3mm thick). Twenty O.D. units of each oligonucleotide reaction mixture were suspended in 40% formamide, 10% sucrose, 0.015% bromophenol blue, 0.015% xylene cyanol, applied to a 13 mm wide well and electrophoresed at 250V in 0.09M Tris base, 0.09M boric acid, 0.25mM EDTA for 2-3 hours.

The product band was visualized by UV after putting the gel over a silica gel plate with UV indicator. The oligonucleotides were recovered from the acrylamide by incubation at 37°C in 0.5M ammonium acetate, 1mm EDTA for 24-48 hours. Three to seven O.D. units were usually recovered from the gel. These oligonucleotides were desalted on a Sephadex G-25 (superfine) column (16 cm long x 0.7 cm diameter) using H_2O for elution.

To verify the size of the oligonucleotides, the fragments were labelled with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase and analyzed by 24% polyacrylamide gel electrophoresis using oligonucleotides of 8-, 10-, 12- and 14- bases long as standards.

(d) Annealing and ligation

Equimolar amounts (0.2 nmole) of fragments were mixed and phosphorylated with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP followed by a chase with cold ATP. Equimolar amount of the 5'-terminal fragment and annealing buffer (50mM Tris HCl pH 7.8, 10mM $MgCl_2$) were added to the reaction mixture. It was then incubated at 90°C for 10 min, transferred to a 65°C bath and slowly cooled down to room temperature over a 5-7 hour period. After incubation at 15°C for one hour and addition of ATP to 0.5mM, DTT to 20mM and T4 DNA ligase, the reaction mixture was kept at 15°C overnight.

(e) Construction of recombinant plasmids

Conditions for all DNA modifying enzymes used were according to that of the supplier. Restriction fragments were separated by agarose gel electrophoresis and isolated by adsorption onto glass beads[26]. Large scale and rapid plasmid preparations were according to procedures described in Maniatis *et al.*[27]. Transformations were performed by a low pH method[28].

(f) Sequencing analysis of cloned VIP gene

The procedure described by Maxam and Gilbert[29] was used except that formic acid was used for the A + G reaction. Fragments were labeled at 3'-end by the use of DNA polymerase I (Klenow fragment) with [α-^{32}P]dCTP in the presence of the other 3 dNTP's, followed by a chase with dCTP.

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