Production of Biologically Active N^{α}-Desacetylthymosin α_1 in *Escherichia coli* through Expression of a Chemically Synthesized Gene†

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ABSTRACT: Thymosin α_1, an immune restorative polypeptide hormone, was synthesized in *Escherichia coli* by using recombinant DNA cloning techniques. Based on the known amino acid sequence, a gene coding for the thymosin α_1 polypeptide chain was designed and enzymatically assembled from chemically synthesized oligodeoxyribonucleotide fragments. The gene was ligated into plasmid pBR322 and placed under *lac* operon control, and N^{α}-desacetylthymosin α_1 was expressed as part of a β-galactosidase chimeric protein. Cyanogen bromide cleavage of this protein gave a mixture of polypeptides, among which thymosin α_1 activity was detected by radioimmunoassay (RIA). The *E. coli* product is identical with native thymosin α_1 isolated from calf thymus in the amino acid sequence but lacks the N-terminal acetyl group. Results of a guinea pig migration inhibition factor (MIF) assay, a terminal deoxyribonucleotidyl transferase (TdT) assay, and radioimmunoassay indicate that the N^{α}-desacetylthymosin α_1 produced by deoxyribonucleic acid (DNA) cloning techniques has biological activity equivalent to that of the native hormone.

Thymosin fraction 5 is a partially purified bovine thymic preparation containing over 30 peptides with molecular weights ranging from 1000 to 15000 (Hooper et al., 1975). Fraction 5 has been demonstrated to be an effective immunopotentiating agent and can act in lieu of the thymus gland to reconstitute the immune function in immunosuppressed animals and in humans with a number of primary and secondary immunodeficiency diseases (Wara et al., 1975; Cohen et al., 1979; Low & Goldstein, 1978). Thymosin α_1, a purified polypeptide component of this preparation, possesses a subset of fraction 5 activities associated with helper and amplifier functions and is a potent inducer of several of the later stages in T-cell[1] differentiation (Low & Goldstein, 1978; Goldstein et al., 1977a; Low et al., 1979). These results suggest that thymosin α_1 and fraction 5 may be useful restorative therapeutic agents in the treatment of some immunodeficiency diseases (Wara & Amman, 1978; Goldstein et al., 1977b) and immunosuppressed conditions (Goldstein et al., 1977b; Schafer et al., 1977) associated with thymic malfunction.

Thymosin α_1 is an acidic polypeptide (p*I* 4.2, M_r 3108) of 28 amino acid residues blocked at the N terminus by an acetyl group (Low & Goldstein, 1979). The amino acid sequence of human thymosin α_1 (Low & Goldstein, 1978) is probably identical with the bovine sequence (Goldstein et al., 1977a; Low & Goldstein, 1979) (Figure 1). With the elucidation of the sequence, it became possible to synthesize thymosin α_1 by chemical means (Wang et al., 1979; Birr & Stollenwerk, 1979).

An alternative to large-scale extraction from thymus glands or chemical synthesis is the production of thymosin α_1 in specially constructed strains of *Escherichia coli*. We have already reported the syntheses in *E. coli* of human somatostatin (Itakura et al., 1977) and human insulin A and B chains (Goeddel et al., 1979a), expressed as chimeric proteins, and of human growth hormone (Goeddel et al., 1979b), expressed directly. In this paper we extend the use of these techniques to the production of N^{α}-desacetylthymosin α_1.

Experimental Procedures

Materials

Analytical reagents were used for the chemical synthesis of deoxyribonucleic acid (DNA). Pyridine was dried over KOH, distilled, and stored over molecular sieves (type 4A). (2,4,6-Triisopropylbenzenesulfonyl)tetrazole (TPSTe) was synthesized as described (Stawinski et al., 1977). Fully protected trideoxyribonucleotides were synthesized as described (Hirose et al., 1978). Sources of bacterial strains and enzymes were described previously (Goeddel et al., 1979a). Phage λ p*lac* 5 L8UV5 was a gift of Dr. Joe Hedgpeth. High-performance liquid chromatography was performed on Spectra-Physics SP-8000 liquid chromatographs. Water for peptide high-performance LC was distilled from glass and passed through a column of Merck LiChrosorb RP-8 resin (25–40 μm). Solvents and reagents for buffers were high-performance LC grade. Synthetic thymosin α_1 and a tyrosine analogue were obtained from Drs. S.-S. Wang and J. Meienhofer.

Methods

Design of the Thymosin α_1 Gene. The synthetic gene for thymosin α_1 (Figure 1) was based on the known amino acid sequence (Low & Goldstein, 1979). The primary nucleotide sequence was derived from the restrictive criteria applied previously (Itakura et al., 1977) (i.e., use of preferred prokaryotic codons, elimination of sequences with multiple complementarities, and minimization of AT- or GC-rich regions). Additional features of the gene include (a) the terminal restriction endonuclease site sticky ends, to facilitate incorporation into plasmids, (b) a methionine (ATG) codon placed

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[1] Abbreviations used: T cell, thymus-dependent lymphocyte; TPSTe, (2,4,6-triisopropylbenzenesulfonyl)tetrazole; BSA, benzenesulfonic acid; TLC, thin-layer chromatography; high-performance LC, high-performance liquid chromatography; MIF, macrophage migration inhibitory factor; TdT, terminal deoxyribonucleotidyl transferase; RIA, radioimmunoassay; PBL, peripheral blood lymphocytes; PEC, peritoneal exudate cells; 3-SPITC, 3-sulfophenyl isothiocyanate; Me_2SO, dimethyl sulfoxide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CNBr, cyanogen bromide; HI, hydriodic acid.

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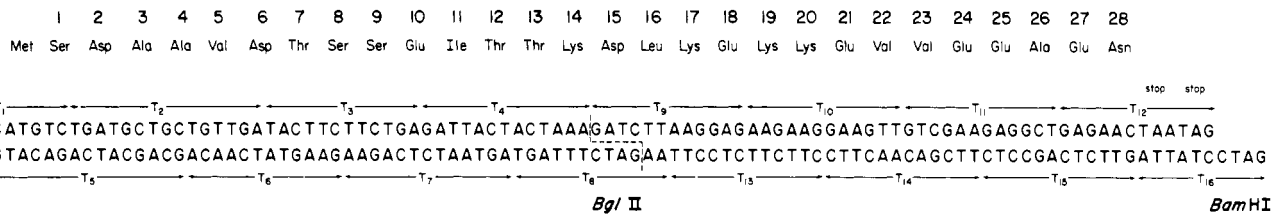
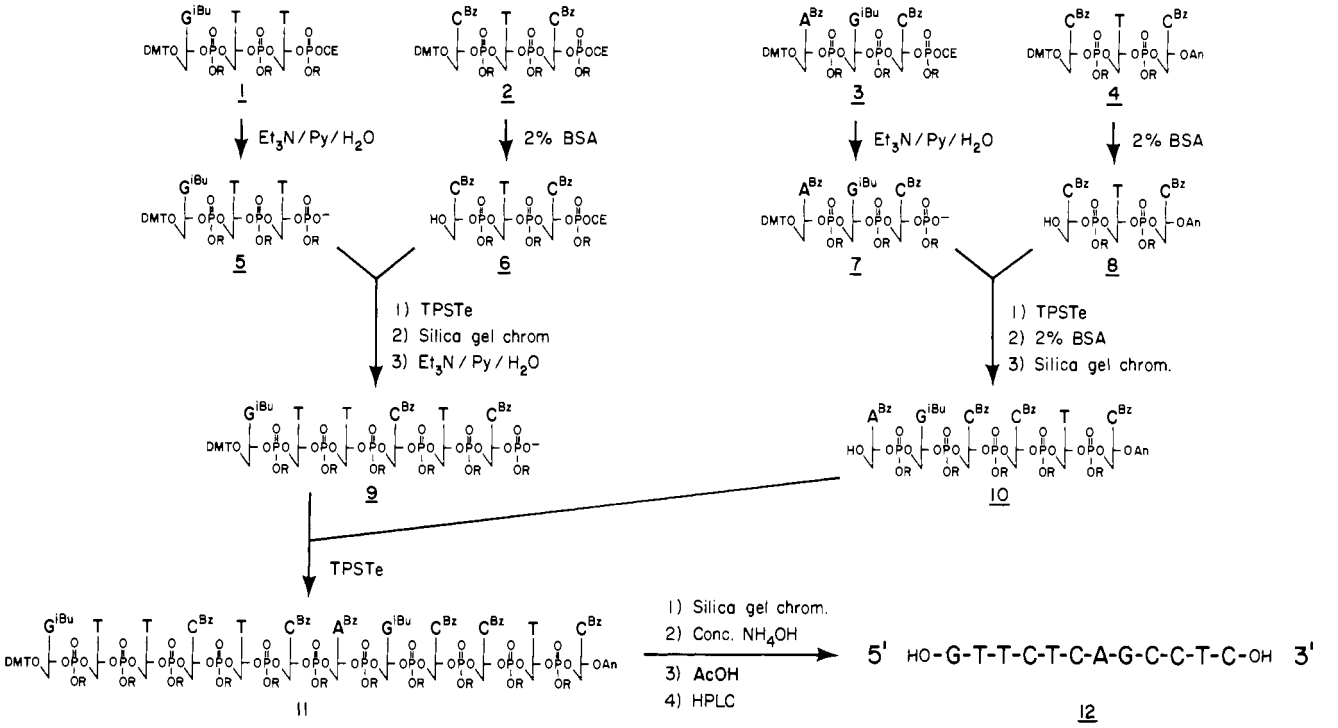


FIGURE 1: Design of a gene for thymosin α_1. The synthesis of the double-stranded DNA involved the 16 oligonucleotides (T_1–T_{16}) indicated by doubled-headed arrows. The 5′ ends have single-stranded cohesive termini to facilitate joining to plasmids cleaved with *Eco*RI and *Bam*HI. A *Bgl*II site in the center of the gene assists in the analysis of recombinant plasmids. The Met codon ATG is inserted at the N terminus to allow CNBr cleavage of N^{α}-desacetylthymosin α_1 from the expressed chimeric protein.

Scheme I: Synthetic Route to Fragment T_{15} Starting from Fully Protected Trideoxyribonucleotides (See Methods)[a]



[a] Abbreviations: DMT, 4,4′-dimethoxytrityl; BSA, benzenesulfonic acid; CE, 2-cyanoethyl; R, *p*-chlorophenyl; Bz, benzoyl; An, anisoyl; *i*-Bu, isobutyl; TPSTe, (2,4,6-triisopropylbenzenesulfonyl)tetrazole; Py, pyridine; AcOH, acetic acid; Et_3N, triethylamine.

just before the N-terminal codon to allow N^{α}-desacetylthymosin α_1 to be released from the chimeric protein by cyanogen bromide (CNBr) cleavage, (c) a central restriction site (*Bgl*II) for analysis of plasmids containing the gene, and (d) tandem stop codons.

Three considerations guided the division of each strand into the eight oligonucleotide fragments (10–15 bases) indicated in Figure 1: (a) Nucleotide overlaps of at least six base pairs with each of the opposing fragments of the complimentary strand were chosen for proper alignment during enzymatic ligation, (b) each oligomer was synthesized by using the minimum number of chemical condensations, and (c) the overall synthesis required a minimum variety of trideoxyribonucleotide building blocks.

Synthesis of Fragment T_{15}, *d*(G-T-T-C-T-C-A-G-C-C-T-C) (**12**). Oligodeoxyribonucleotides T_1–T_{16} were synthesized by a modified phosphotriester method using fully protected trideoxyribonucleotide building blocks (Itakura et al., 1977; Crea et al., 1978). The synthesis is typified by the following procedure for fragment T_{15}, as summarized in Scheme I.

The fully protected trideoxyribonucleotides **4** (85 mg, 0.05 mmol) and **2** (180 mg, 0.1 mmol) were deblocked at the 5′-hydroxyls by treatment with 2% benzenesulfonic acid (BSA) in 7:3 (v/v) chloroform/methanol (10 and 20 mL, respectively) for 10 min at 0 °C. Reactions were stopped by addition of saturated aqueous ammonium bicarbonate (2 mL), extracted with chloroform (25 mL), and washed with water (2 × 10 mL). The organic layers were dried (magnesium sulfate), concentrated to small volumes (~5 mL), and precipitated by addition of petroleum ether (bp 35–60 °C fraction). The colorless precipitates were collected by centrifugation and dried in a desiccator in vacuo to give **6** and **8**, respectively, each homogeneous by silica gel thin-layer chromatography (TLC) (Merck 60 F254; chloroform/methanol, 9:1).

Trimers **1** and **3** (270 mg, 0.15 mmol, and 145 mg, 0.075 mol, respectively) were converted into their phosphodiesters **5** and **7** by treatment with triethylamine/pyridine/water (1:3:1 v/v, 10 mL) for 25 min at room temperature. Reagents were removed by rotary evaporation and the residues dried by repeated evaporations with anhydrous pyridine (3 × 10 mL). Trimer **8** (0.05 mmol) and trimer **7** were combined with TPSTe (50 mg, 0.15 mmol) in anhydrous pyridine (3 mL), and the reaction mixture was left in vacuo at room temperature for 2 h. TLC analysis showed that 95% of the trimer **8** had been converted into hexamer product [visualized by detection of the 4,4′-dimethoxytrityl (DMT) group by spraying with 10% aqueous sulfuric acid and heating at 60 °C]. The reaction was quenched by addition of water (1.0 mL) and the solvent evaporated under reduced pressure. After removal of pyridine by coevaporations with toluene, the hexamer was deblocked

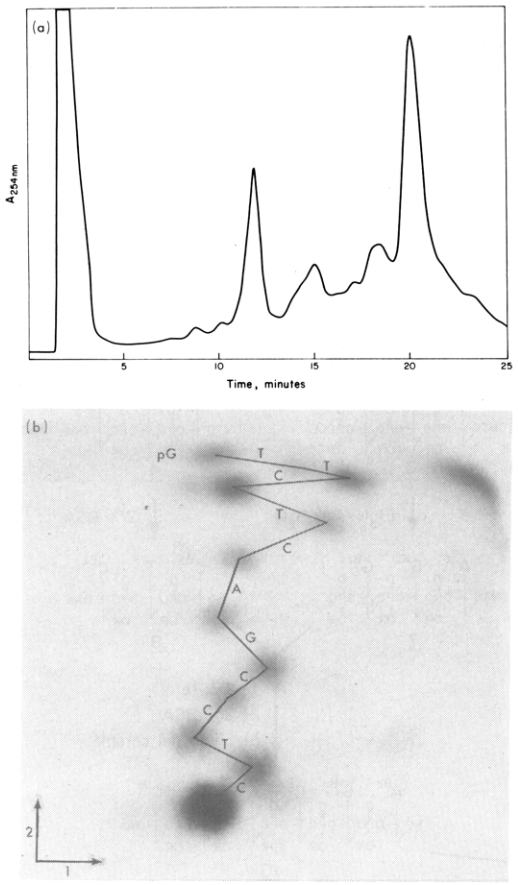


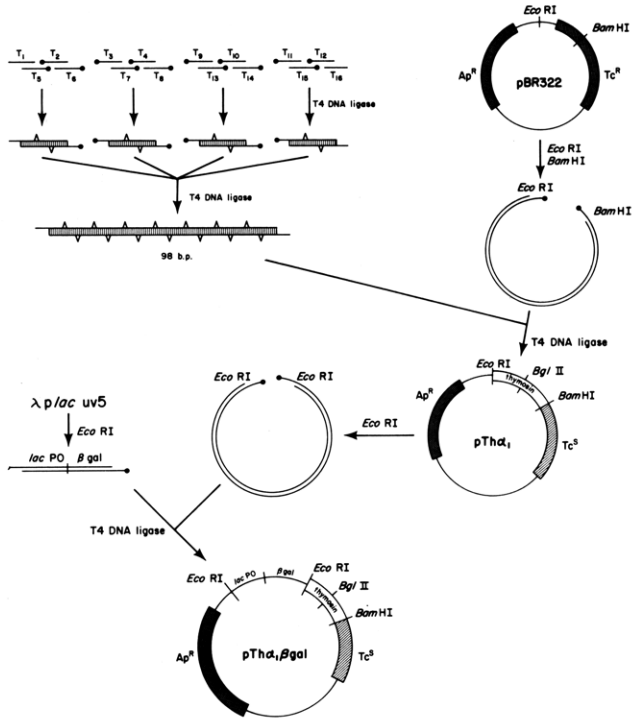
FIGURE 2: (a) High-performance LC analysis and purification of deblocked oligomer T_{15} on a Permaphase AAX (Du Pont) column (0.4 × 50 cm). Product elution was accomplished by a linear gradient of 0–1.0 M KCl in 5 mM potassium phosphate, pH 4.5, buffer at a rate of 3%/min and 3 mL/min at 60 °C. The desired product eluted at 20.2 min. (b) Two-dimensional sequence analysis (18) of high-performance LC purified T_{15}. The 5′-^{32}P-labeled fragment was partially digested with snake venom phosphodiesterase and the resulting mixture of labeled products submitted to 2D separation and autoradiography: first dimension, electrophoresis on a cellulose acetate strip at pH 3.5; second dimension, DEAE-cellulose TLC eluted with RNA homomix.

at the 5′ position with 2% BSA (8 mL) as described above for trimers **4** and **2**. The product **10** was purified on a silica gel column (Merck 60 H, 3.5 × 5 cm) by step-gradient elution with chloroform/methanol (98:2 to 95:5 v/v). Fractions containing product **10** were evaporated to dryness.

Similarly, trimer **5** was coupled to **6**, and the fully protected product was directly purified on silica gel. This latter compound was deblocked at the 3′ end by triethylamine/pyridine/water as described above to give fragment **9**.

Finally, hexamers **9** and **10** were coupled in anhydrous pyridine (2 mL) with TPSTe (75 mg, 0.225 mmol) as the condensing agent. Upon completion (4 h, room temperature) the mixture was rotary evaporated and the residue chromatographed on silica gel. Product **11** (160 mg) was obtained by precipitation with petroleum ether and appeared homogeneous on TLC. A portion of compound **11** (20 mg) in pyridine (0.5 mL) was completely deblocked by treatment with concentrated ammonium hydroxide (7 mL, 8 h, 60 °C and subsequent treatment in 80% acetic acid (15 min, room temperature). After evaporation of acetic acid, the solid residue was dissolved in 4% aqueous ammonium hydroxide (v/v, 4 mL) and extracted with ethyl ether (3 × 2 mL). The aqueous phase was concentrated to 1–2 mL and applied to high-performance LC (Figure 2a) for purification of **12**. The fractions

Scheme II: Summary of Ligation Reactions for Gene and Plasmid Constructions Described under Results[a]



[a] Heavy dots indicate 5′-phosphate groups. Gene fragments are not drawn to scale.

corresponding to the major peak were pooled (~2.0 OD_{254} units) and concentrated to ~5 mL. The final product **12** was desalted on Bio-Gel P-2 (1.5 × 100 cm) by elution with 20% aqueous ethanol, reduced to dryness, and resuspended in water (200 μL) to give a solution of A_{254} = 10. The sequence of **12** was confirmed by two-dimensional sequence analysis (Figure 2b).

Construction of the Thymosin α_1 Gene and Plasmids.[2] The thymosin α_1 gene was assembled from the 16 synthetic oligonucleotides by methods previously described in detail for somatostatin (Itakura et al., 1977), insulin (Goeddel et al., 1979a), and growth hormone (Goeddel et al., 1979b). Ten-microgram quantities of oligonucleotides T_2–T_{15} were quantitatively phosphorylated with [γ-^{32}P]ATP (New England Nuclear) in the presence of T_4 polynucleotide kinase (Goeddel et al., 1979a) to give specific activities of approximately 1 Ci/mmol. Radiolabeled fragments were purified by 20% polyacrylamide/7 M urea gel electrophoresis, and sequences of the eluted fragments were verified by two-dimensional electrophoresis/homochromatography (Jay et al., 1974) of partial snake venom digests (see Figure 2b). Fragments T_1 and T_{16} were left unphosphorylated to minimize undesired polymerization during subsequent ligation reactions. These oligonucleotides (2 μg each) were assembled in four groups of four fragments by T_4 DNA ligase by using published procedures (Goeddel et al., 1979a) (see Scheme II). The reaction products were purified by gel electrophoresis on a 15% polyacrylamide gel containing 7 M urea (Maxam & Gilbert, 1977). The four isolated products were ligated together and the reaction mixture was resolved by 10% polyacrylamide gel electrophoresis. DNA in the size range for the thymosin α_1

[2] All work involving recombinant DNA in *E. coli* was performed at Genentech in accordance with current NIH guidelines in certified host vector systems. Cloning and growth of cultures up to 10 L were with derivatives of plasmid pBR322 in *E. coli* K12 (P3/HV1 containment).

gene (90–105 base pairs) was electroeluted.

Plasmid pBR322 (Bolivar et al., 1977) (0.5 μg) was treated with *Bam*HI and *Eco*RI restriction endonucleases, and the fragments were separated by 10% polyacrylamide gel electrophoresis. The large fragment was recovered from the gel by electroelution and subsequently ligated to the assembled synthetic DNA (Goeddel et al., 1979b). This mixture was used to transform *E. coli* K12 strain 294 (Backman et al., 1976). Plasmid DNA was prepared from ampicillin-resistant, tetracycline-sensitive transformants (Goeddel et al., 1979a). These recombinant plasmids were screened for the presence of a unique *Bgl*II restriction endonuclease site, the *Eco*RI/*Bam*HI inserts were analyzed for the expected size, and the inserted DNA was sequenced by the Maxam–Gilbert technique (Maxam & Gilbert, 1977). An *Eco*RI fragment promotor and β-galactosidase gene isolated from λ p*lac* 5 L8UV5 DNA (Arditti et al., 1968; Polisky et al., 1976) containing the *lac* UV5 was ligated to the thymosin α_1 gene at the *Eco*RI site of plasmid pTh α_1 in the corrected orientation to give pTh α_1 βgal (Goeddel et al., 1979a).

Growth of Culture. E. coli strain 294/pTh α_1 βgal was grown to late log phase (A_{600} = 2.0) in 10 L of LB medium with 20 mg/L ampicillin in a 10-L fermentor (New Brunswick Scientific) at 37 °C. Cells were killed by incubation in the fermentor with 0.25% each of toluene and phenol for 30 min, then collected by centrifugation, and frozen. The yield was 30 g of wet cells.

Purification of N^{α}-Desacetylthymosin α_1. Purification steps were monitored by radioimmunoassay (see below) and high-performance LC (Figure 3c). Twenty-four grams of thawed cells were lysed by sonication at 4 °C in 135 mL of lysis buffer (10% sucrose, 0.2 M sodium chloride, 50 mM EDTA, 0.1 M Tris-HCl, pH 7.9, and 0.1 mM phenylmethanesulfonyl fluoride). The lysate was centrifuged (7000 rpm, 20 min) and the pellet suspended in 100 mL of 7 M guanidine hydrochloride by stirring overnight at 4 °C. This slurry was centrifuged (7000 rpm, 30 min) and the supernatant added to 10 volumes of cold water. The precipitate (1.8 g dry weight) was collected by centrifugation (4000 rpm, 30 min) and shown to contain the chimeric protein by $NaDodSO_4$ gel electrophoresis. This material was supended in 200 mL of 88% formic acid, 1.6 g of CNBr (Sigma) (40-fold molar excess over total methionine) was added, and the stoppered mixture was stirred overnight at room temperature in a high-draft hood. The resulting solution was rotary evaporated (oil pump with dry ice and NaOH traps in a high-draft hood) at 30 °C, and the residue was suspended in 7.5 M urea (10 mL), adjusted to pH 5 with ethanolamine and stirred 2 h at room temperature. This solution was diluted with cold water (200 mL) and stored at 4 °C overnight, resulting in the precipitation of a large percentage of contaminating proteins. After centrifugation (9000 rpm, 35 min) the supernatant containing N^{α}-desacetylthymosin α_1 was loaded onto a DEAE-cellulose column (Whatman DE-52, 2.5 × 60 cm) equilibrated at room temperature with 20 mM ammonium bicarbonate, pH 7.9, and eluted with a linear gradient of 0.0–0.5 M sodium chloride in the same buffer to give several radioimmunoassay (RIA) positive peaks (Figure 3a). The major peak fractions were pooled, lyophilized, and size fractionated on an Ultrogel AcA 202 column (1 × 100 cm) by elution with 10 mM ammonium bicarbonate, pH 7.9 (Figure 3b). The N^{α}-desacetylthymosin α_1 containing fractions were further purified by preparative high-performance LC (Figure 3c).

Chemical Characterization

Amino Acid Analysis. Samples were hydrolyzed with twice-distilled 6 N HCl at 110 °C for 24 h in sealed evacuated Pyrex glass tubes. Analyses were performed with a Beckman-Spinco amino acid analyzer, Model 119 CL, employing single-column methodology on Beckman W-3 resin.

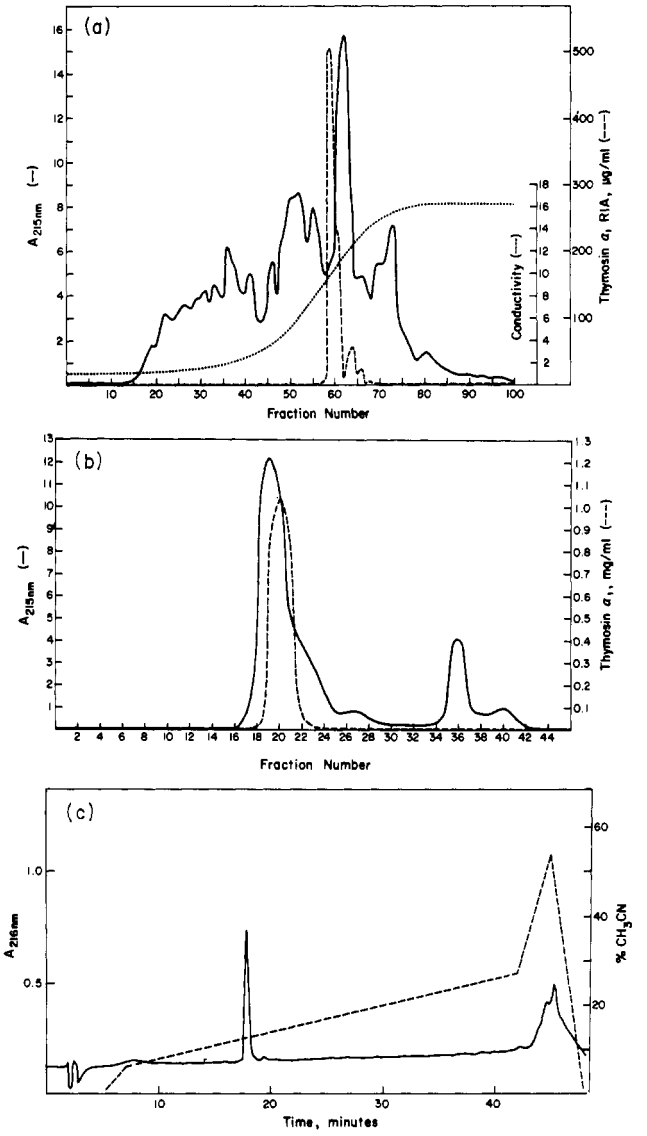


FIGURE 3: Purification of N^{α}-desacetylthymosin α_1. (a) DEAE-cellulose eluted with a linear gradient of NaCl (···) as described under Methods, showing N^{α}-desacetylthymosin α_1 (estimated by RIA) (---) and A_{215} (—). (b) Ultrogel AcA 202 gel filtration of the main DEAE peak, with high-performance LC estimated (see below): N^{α}-desacetylthymosin α_1 (---) and A_{215} (—). (c) High-performance LC analysis and purification of the Ultrogel pool on a 0.4 × 30 cm LiChrosorb C-18, 10-μm (Merck) column at 35 °C with a resolving gradient from 6 to 21% acetonitrile (Fisher HPLC grade) in 50 mM ammonium acetate, pH 7.1, at 0.6%/min and a flow rate of 1.5 mL/min. About 0.2 mg of N^{α}-desacetylthymosin α_1 (elution time 18.3 min) was purified in this run. By comparison, synthetic thymosin α_1 elutes at 18.7 min.

Enzymatic Digestion. Thermolysin digestion was performed in 1 M ammonium bicarbonate at pH 8.3 at 37 °C. Thermolysin was added to the protein solution to a final ratio of enzyme to substrate of 1:50 (wt/wt). The enzymatic digest was lyophilized immediately after 2.5 h.

Peptide Mapping. Thermolysin digests were separated by paper chromatography and electrophoresis as described previously (Low & Goldstein, 1979). Peptides were detected by staining with cadmium–ninhydrin reagent. For analysis of the amino acid composition of the peptides, the unstained chromatogram was stained with fluorescamine. The fluorescent

spots were cut out, eluted with water, hydrolyzed, and analyzed.

Isolation and Purification of Peptides. Peptides used for sequence studies were purified by paper chromatography and/or electrophoresis as described (Low & Goldstein, 1979).

Manual Sequence Determination. The sequences of small peptides were determined by manual Edman degradation using dansyl-monitored Edman (Gray, 1967) or subtractive Edman procedures (Konigsberg, 1967).

Automated Sequence Analysis. Automated amino acid sequence analysis was performed with a Beckman sequencer (Model 890C) using the DMAA program (Beckman peptide program 102974). N^{α}-Desacetylthymosin α_1 was precoupled with 3-sulfophenyl isothiocyanate (3-SPITC) (Dwulet & Gurd, 1976) before the sequencer program was initiated. The products were identified by high-performance LC and by amino acid analysis after back-hydrolysis with hydriodic acid (HI) (Smithies et al., 1971).

Terminal Deoxyribonucleotidyl Transferase (TdT) Assay. The in vitro TdT suppression assay was performed as described (Hu et al., 1980; Hu, T.L.K. Low and A.L. Goldstein, unpublished results).

(A) Cell Preparation. Six- to eight-week-old Balb/c mice were sacrificed by cervical dislocation and the thymus glands removed. Cells were dispersed over a fine metal screen, aspirated through a 25-gauge needle, and spun at 250*g* for 10 min. Cells were then resuspended in Hepes-buffered RPMI-1640 (Gibco, Grand Island, NY) at 5×10^6 cells/mL.

(B) Incubation with Thymosin. Approximately 10^8 cells were cultured with thymosin fractions or saline for 20 h at 37 °C. The cells were prepared in a sterile petri dish containing 20 mL of RPMI-1640 supplemented with penicillin, streptomysin, L-glutamine, 25 mM Hepes, and 10% fetal calf serum.

(C) Enzyme Extraction. The cells were harvested and ruptured by a sonicator (Heat System-Ultrasonics, Inc., Model W-225R) in potassium cacodylate buffer (pH 7.5). The cell homogenate was then ultracentrifuged at 40 000 rpm for 1 h.

(D) Enzyme Assay. The supernatant (25 μL) was incubated with 1 mL of assay reagents (0.2 M potassium cacodylate, 8 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.02 mM $p(dA)_{100}$ (Bethesda Research Lab, Bethesda, MD), and 1 mM [8-^{3}H]dGTP) for 30 min at 37 °C. The incubation mixture (100 μL) was removed and sprayed onto a GF/c filter. The filters were washed successively in 5% trichloroacetic acid containing 1% sodium pyrophosphate, 1 N HCl, and 95% ethanol, dried (110 °C, 10 min), and counted in a scintillation counter.

One unit of enzyme activity is defined as the amount catalyzing the incorporation of 1 nmol of dGTP into acid-insoluble material in 1 h at 37 °C. Specific activity was calculated from the enzyme activity per 10^8 viable cells.

Macrophage Migration Inhibitory Factor (MIF) Assay. (A) Preparation of Cells. The MIF assay is a modification of a method reported previously (Thurman et al., 1977). Hartley guinea pigs (400–600 g) were injected intradermally with 0.1 mL of Freund's complete adjuvant (3113-60, Difco, Detroit, MI) containing 10 μg of preservative-free tuberculin-purified protein derivative (PPD-CT68, Connaught Labs, Ltd., Willowdale, Toronto, Canada). Ten days later, the animals were thymectomized. Animals were bled by cardiac puncture 48 or 72 h postsurgery, and peripheral blood mononuclear cells (PBL) were obtained by centifuging the heparinized blood through a Ficoll metrizoate gradient (Lymphoprep, Nyegaard and Co.) at 400*g* for 40 min at 20 °C. The cells recovered from the interface were washed twice in HRPMI medium (RPMI-1640 with 25 mM Hepes) containing 100 μg/mL streptomycin and 100 units/mL penicillin and adjusted to 5×10^6 cells/mL. Rate-frozen peripheral blood lymphocytes (PBL) (−1 °C/min, 9% Me_2SO), maintained for 4 weeks at −70 °C, were thawed, washed, and utilized as outlined below. Peritoneal exudate cells (PEC) were obtained from nonsensitized guinea pigs injected intraperitoneally 3–5 days previously with 20–30 mL sterile light mineral oil by using a 23-gauge needle. These cells were washed 3 times to remove residual oil and adjusted to 45×10^6 cells/mL. The PBL were mixed with the PEC at a 1:9 ratio by adding 1 volume of PBL to 1 volume of PEC, and a migration inhibitory factor (MIF) assay was performed by using the microdroplet technique described below.

(B) MIF Microdroplet Thymosin Assay. To a stock solution of 0.45% Sea Plaque agarose (Microbiological Associates, Bethesda, MD) that had been melted in boiling water was added an equal volume of 2 × HRPMI, yielding a 0.225% agarose in HRPMI solution. This was maintained at 37 °C in a water bath. The PBL–PEC cell mixture was centrifuged at 200*g* for 10 min. All supernatant fluid was removed and the cell button adjusted to 37 °C in a water bath. One microliter of 0.225% agrose per 10^6 cells (e.g., 100 μL per 10^8 cells) was added to the cell button, and cells were resuspended by using a 50-μL Hamilton syringe in a Hamilton repeating dispenser (Hamilton Co., Reno, NV). Cell agarose droplets, 1 μL in size, were placed in the center of wells of an unprocessed Costar microtiter plate (Microbiological Associates, Bethesda, MD). The droplets were allowed to solidify at 20 °C for 10–20 min in a humidified atmosphere. HRPMI was then added (containing either no additives, PPD, thymosin, or thymosin and PPD) to the microtiter plates to bring the total volume to 100 μL/well. PPD was added at 5 μg/mL, and thymosin preparations were added at various concentrations. Following a 24-h incubation at 37 °C in a humid atmosphere (5% CO_2 in air), the areas of migration were measured by using a B and L Tri-Simplix microprojector. The major and minor axes were measured and the areas of macrophage migration were computed as ellipsoids. The areas of the agarose droplets were subtracted from the total areas to give the areas of cellular migration. The means and standard errors of the areas of migration of replicate cultures were computed. The percent specific inhibition was calculated as

$$\mathrm{PSI} = 100 - \left[\frac{\text{area (thymosin + PPD)}}{\text{area (thymosin)}} \times \frac{\text{area (HRPMI)}}{\text{area (PPD)}} \times 100 \right]$$

Radioimmunoassay for Thymosin α_1. A detailed description of the radioimmunoassay for thymosin α_1 is in preparation (J. E. McClure, N. Lameris, D. Wara, and A. L. Goldstein, unpublished results). Antiserum to synthetic thymosin α_1, produced in rabbits by immunization with the synthetic peptide coupled to hemocyanin by means of glutaraldehyde, was used in the RIA without purification and at final dilutions between 1:10 000 and 1:20 000. Labeled thymosin α_1 of high specific activity (150–200 Ci/μg) was purified by gel filtrations on Sephadex G-10 and Sephadex G-25 and found to retain immunoreactive integrity as assessed by incubation with excess antibody. The assay conditions included incubation of the standard or unknown with antiserum in phosphate-buffered saline for 1 h, addition of radioactive thymosin α_1, and successive incubations at 37 °C (1 h) and 4 °C (48 h). A second antibody (goat anti-rabbit γ-globulins) was used to precipitate the immune complexes formed during competitive binding. By use of the highly purified synthetic thymosin α_1 standard, a

Table I: Trimer Library for Thymosin α_1 Synthetic Gene[a]

		R_f					R_f		
		a	b	present in			a	b	present in
1	AAT	0.24	0.52	T_1, T_7	20	GAA	0.15	0.39	T_6, T_{10}, T_{11}
2	AAC	0.28	0.55	T_{12}, T_{14}	21	GAT	0.22	0.49	T_4, T_9, T_{16}
3	AAG	0.15	0.40	T_8, T_9, 2 × T_{10}, T_{11}	22	GAC	0.23	0.52	T_5, T_{14}
4	ATT	0.26	0.56	T_{16}	23	GAG	0.09	0.36	T_{11}, T_{12}
5	ATC	0.28	0.58	T_8	24	GTT	0.18	0.45	T_{15}
6	AGA	0.24	0.50	T_7	25	GTA	0.25	0.42	T_6
7	AGT	0.14	0.40	T_7	26	GTC	0.22	0.51	T_{11}
8	AGC	0.19	0.48	T_{15}	27	GCA	0.14	0.39	2 × T_5
9	TAA	0.17	0.44	T_4, T_{12}	28	ATG	0.11	0.32	T_5
10	TAC	0.22	0.55	T_3, 2 × T_4	29	ACA	0.12	0.40	T_6
11	TTT	0.24	0.53	T_8	30	AGT	0.14	0.42	T_8
12	TTC	0.26	0.52	2 × T_3, T_{14}	31	TAG	0.10	0.32	T_{12}
13	TCA	0.28	0.53	T_1, T_5, T_6	32	TTC	0.19	0.50	T_{14}
14	TGA	0.12	0.37	T_2	33	TGA	0.13	0.37	T_2, T_3
15	TGT	0.13	0.41	T_1, T_2	34	CTT	0.19	0.49	T_{13}
16	TGC	0.18	0.47	2 × T_2	35	CTC	0.20	0.56	T_{15}
17	CTT	0.25	0.55	T_9, 2 × T_{13}	36	GAG	0.04	0.22	T_9
18	CTC	0.26	0.59	T_7, T_{13}, T_{15}	37	GTT	0.07	0.29	T_{10}
19	CCT	0.30	0.55	T_{16}	38	GCT	0.10	0.35	T_{11}

[a] Trimers 1-27: 5′-*O*-(dimethoxytrityl)-3′-*O*-(2-cyanoethyl) *p*-chlorophenyl phosphate derivatives. Trimers 28-38: 5′-*O*-hydroxy-3′-*O*-anisoyl derivatives. Thin-layer chromatography (silica gel) was in methanol/chloroform: (a) system a, 0.5:9.5 v/v; (b) system b, 1.0:9.0 v/v.

calibration curve was generated which had a minimal detectable dose of 30–50 pg and an ED_{50} (50% response) of 150–400 pg. Measurements of the radioactivity bound in the immunoprecipitate were reduced by a four-parameter logistics program to a graph of (counts bound at defined dose)/(counts bound at zero dose) ($B_2/B_0 \times 100$) vs. log dose of peptide.

Results

Synthesis of Oligodeoxyribonucleotides. Oligomers T_1–T_{16} were synthesized as described for fragment T_{15} under Methods by using the fully protected trimer building blocks listed in Table I. The fully protected oligomeric products were deblocked and purified by high-performance LC (see Figure 2a). Each purified oligomer was 5′-phosphorylated and its size and sequence were confirmed as described under Methods (Figure 2b). Table II is a list of the oligomers synthesized in this way.

Gene and Plasmid Construction. As shown in Scheme II, ^{32}P-labeled oligomers were built into the thymosin α_1 gene by a series of T_4 DNA ligase catalyzed reactions using the complementarity of overlapping fragments to ensure proper ordering. Since fragments T_1 and T_{16} contain restriction site sequences, they are self-complementary and could polymerize during the ligation reaction; thus, they were used in their unphosphorylated forms. The final ligation product was partially purified by electrophoresis on 10% polyacrylamide slab gel and elution of the region between 90 and 105 base pairs. This fraction was ligated into the large *Eco*RI/*Bam*HI fragment of pBR322 and transformed into *E. coli* K12/294. Five percent of the transformation mixture was plated on LB plates containing 20 μg/mL ampicillin. The four ampicillin-resistant colonies obtained were sensitive to tetracycline, suggesting insertion into the tetracycline resistance gene. Analysis of the plasmids from these four colonies showed that in each case the plasmid contained (a) a *Bgl*II site not found in pBR322 itself, indicating the presence of the thymosin α_1 gene (Figure 1) and (b) a fragment of approximately 105 base pairs generated by *Bam*HI/*Eco*RI cleavage. The thymosin α_1 DNA sequence of one of these plasmids (pTh α_1) was confirmed by Maxam–Gilbert sequencing techniques (Maxam & Gilbert, 1977). The *Eco*RI fragment of λ p*lac* 5 L8UV5 containing the UV 5 *lac* promotor mutation and most of the β-galactosidase gene (Arditti et al., 1968; Polisky et al., 1976)

Table II: Synthetic Oligonucleotides for Thymosin α_1 Gene

compd	sequence	length	RT[a] (min)
T_1	A-A-T-T-C-A-T-G-T-C	10	17.4
T_2	T-G-A-T-G-C-T-G-C-T-G-T-T-G-A	15	24.3
T_3	T-A-C-T-T-C-T-T-C-T-G-A	12	20.3
T_4	G-A-T-T-A-C-T-A-C-T-A-A-A	13	22.0
T_5	G-C-A-G-C-A-T-C-A-G-A-C-A-T-G	15	24.8
T_6	G-A-A-G-T-A-T-C-A-A-C-A	12	20.1
T_7	A-G-T-A-A-T-C-T-C-A-G-A-A	13	22.6
T_8	A-A-G-A-T-C-T-T-T-A-G-T	12	20.2
T_9	G-A-T-C-T-T-A-A-G-G-A-G	12	20.4
T_{10}	A-A-G-A-A-G-G-A-A-G-T-T	12	21.1
T_{11}	G-T-C-G-A-A-G-A-G-G-C-T	12	20.5
T_{12}	G-A-G-A-A-C-T-A-A-T-A-G	12	20.4
T_{13}	C-T-T-C-T-T-C-T-C-C-T-T	12	19.9
T_{14}	T-T-C-G-A-C-A-A-C-T-T-C	12	20.5
T_{15}	G-T-T-C-T-C-A-G-C-C-T-C	12	20.2
T_{16}	G-A-T-C-C-T-A-T-T-A	10	17.2

[a] Retention time in high-performance LC analysis.

was inserted into the *Eco*RI site of pTh α_1. Transformants of *E. coli* K12/294 were screened for blue colonies on X-gal indicator plates containing ampicillin (Goeddel et al., 1979a). Restriction analysis confirmed the presence and proper orientation of the *lac* fragment in several of the selected colonies. Plasmid isolated from one of these colonies was designated pTh α_1 βgal.

Expression of N^{α}-Desacetylthymosin α_1. *E. coli* K12/294 containing pTh α_1 βgal was grown to an OD_{600} of ~1.0 in Luria broth containing ampicillin, and the pelleted cells were treated overnight with cyanogen bromide (CNBr)/formic acid. RIA analysis of the residues gave values in the vicinity of 1 μg of N^{α}-desacetylthymosin α_1 per mL fermentation broth (about 100000 molecules/cell). A control of *E. coli* K12/294 containing pBR322 gave RIA interference levels of about 0.01 μg/mL.

Purification of N^{α}-Desacetylthymosin α_1. Twenty-four grams (wet weight) of *E. coli* 294/pTh α_1 βgal cells was lysed and the chimeric protein partially purified as described under Methods to give 1.8 g of dry weight protein. This preparation was cleaved with CNBr to give a mixture of fragments including N^{α}-desacetylthymosin α_1. Many of the contaminating fragments were eliminated by a precipitation step. The

Table III: Amino Acid Composition[a] of *E. coli* N^{α}-Desacetylthymosin α_1

	determined	expected		determined	expected
Lys	3.50	4	Gly	0	0
His	0	0	Ala	3.13	3
Arg	0	0	Val	2.37	3
Asp	4.10	4	Met	0	0
Thr	2.84	3	Ile	0.89	1
Ser	2.86	3	Leu	0.97	1
Glu	6.24	6	Tyr	0	0
Pro	0	0	Phe	0	0

[a] The data are presented as assumed number of residues per molecule. The molecular weight is assumed as 3100. All values were from a 24-h hydrolysate in 6 N HCl at 110 °C. No correction was made for threonine, serine, valine, isoleucine, or leucine.

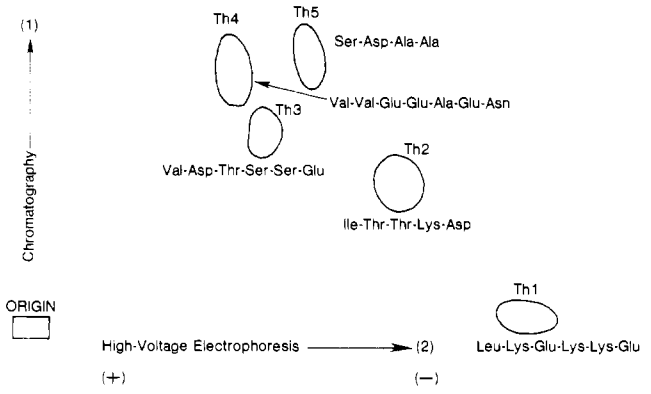


FIGURE 4: Thermolysin peptide map of N^{α}-desacetylthymosin α_1 from *E. coli* (see Methods). Fragments were eluted and subjected to amino acid analysis and Edman degradation (Table IV).

N^{α}-desacetylthymosin α_1 containing supernatant was further resolved by DEAE-cellulose chromatography (Figure 3a), Ultrogel AcA 202 chromatography (Figure 3b), and preparative high-performance LC (Figure 3c). The 4.5 mg of N^{α}-desacetylthymosin α_1 obtained in this way was used for chemical and biological characterization.

The RIA profile of the DEAE-cellulose column (Figure 3a) indicates the presence in small amounts of other RIA-positive peptides. Amino acid analysis of the largest of these minor peaks shows it to be a peptide containing β-galactosidase as well as thymosin α_1 amino acids (data not shown), consistent with incomplete CNBr cleavage.

Chemical Characterization. N^{α}-Desacetylthymosin α_1 synthesized in *E. coli* has an elution time (18.3 min) almost identical with that (18.7 min) of synthetic thymosin α_1 in the high-performance LC gradient described in Figure 3c, the difference being due to the absence of the *N*-acetyl group in the former. The high-performance LC purified material gave an amino acid analysis consistent with the proposed structure (Table III). Analysis of the intact molecule gave a sequence identical with that of natural thymosin α_1 at 26 identifiable positions (Table IV). The peptide map (Figure 4) of a thermolysin digest contained all the peptides found in native thymosin α_1 plus the N-terminal peptide which could not previously be visualized by amine-dependent stains. Amino acid analysis and N-terminal end group analysis (Table V) on each of the eluted thermolysin peptides were consistent with the thymosin α_1 sequence.

In radioimmunoassay, *E. coli* N^{α}-desacetylthymosin α_1 is indistinguishable from both bovine and synthetic *N*-acetylthymosin α_1. The slopes of the curves for binding to rabbit antisynthetic thymosin α_1 antibody (Figure 5) indicate that the three preparations share complete immunochemical identity.

Table IV: Automated Sequence Analysis of *E. coli* N^{α}-Desacetylthymosin α_1[a]

cycle	amino acid	recoveries (nmol) HI hydrolysis	LC	cycle	amino acid	recoveries (nmol) HI hydrolysis	LC
1	Ser	–[b]	–	15	Asp	13.9	ND
2	Asp	32.8	29.0	16	Leu	11.7	ND
3	Ala	37.4	31.5	17	Lys	8.5	ND
4	Ala	32.4	32.2	18	Glu	11.0	ND
5	Val	25.1	–	19	Lys	9.2	ND
6	Asp	14.3	20.5	20	Lys	7.1	ND
7	Thr	15.7[c]	2.5	21	Glu	8.8	ND
8	Ser	3.9[d]	–	22	Val	5.1	ND
9	Ser	2.4	–	23	Val	6.0	ND
10	Glu	14.3	17.6	24	Glu	6.6	ND
11	Ile	17.8[e]	ND[f]	25	Glu	6.9	ND
12	Thr	16.1	ND	26	Ala	4.3	ND
13	Thr	15.8	ND	27	Glu	1.9	ND
14	Lys	9.3	ND	28	Asn	–	–

[a] Partial structural analysis of N^{α}-desacetylthymosin α_1 was obtained by automated sequence analysis using the DMAA program on a Beckman 890C sequencer. A total of 120 nmol was applied. Values are for amino acids recovered after HI hydrolysis and analyzed on an amino acid analyzer. No correction was made for destruction of PTH derivatives. [b] A minus indicates not identifiable. [c] Values for threonine by amino acid analysis were calculated as 2-aminobutyric acid. [d] Serine residues were calculated as alanine after HI hydrolysis. [e] Isoleucine values were calculated as the sum of isoleucine and alloisoleucine. [f] ND = not determined.

Table V: Amino Acid Composition[a] and N-Terminal Residue of Thermolysin Peptides of N^{α}-Desacetylthymosin α_1 from *E. coli*

	Th1	Th2	Th3	Th4	Th5
Lys	2.40 (3)	1.02 (1)			
His					
Arg					
Asp		0.91 (1)	0.98 (1)	0.91 (1)	0.98 (1)
Thr		1.82 (2)	0.85 (1)		
Ser			1.46 (2)		0.71 (1)
Glu	2.05 (2)		1.12 (1)	2.93 (3)	
Pro					
Gly					
Ala				1.63 (1)	2.00 (2)
Val			1.21 (1)	1.54 (2)	
Met					
Ile		1.20 (1)			
Leu	0.91 (1)				
Tyr					
Phe					
total	6	5	6	7	4
N-terminal[b]	Leu	Ile	Val	Val	Ser

[a] Results from 6 N HCl hydrolysates at 110 °C for 24 h. Numbers in parentheses refer to the amino acid composition of expected thermolysin fragments. [b] Determined by dansylation and/or subtractive Edman procedure.

Biological Activity. (A) TdT Assay. Although the biological function of TdT is not clear, its restricted localization in thymus and its low levels in bone marrow cells but not in spleen or peripheral lymphocytes (Chang, 1971) make it a specific marker for T-cell differentiation and maturation. Thymosin fraction 5 and thymosin α_1 (both natural and chemical synthesized) are able to induce a marked decrease of TdT activity in murine thymocytes (Hu et al., 1980). The results (Table VI) indicate that *E. coli* N^{α}-desacetylthymosin α_1 suppresses TdT activity in murine thymocytes in a manner similar to the chemically synthesized thymosin α_1.

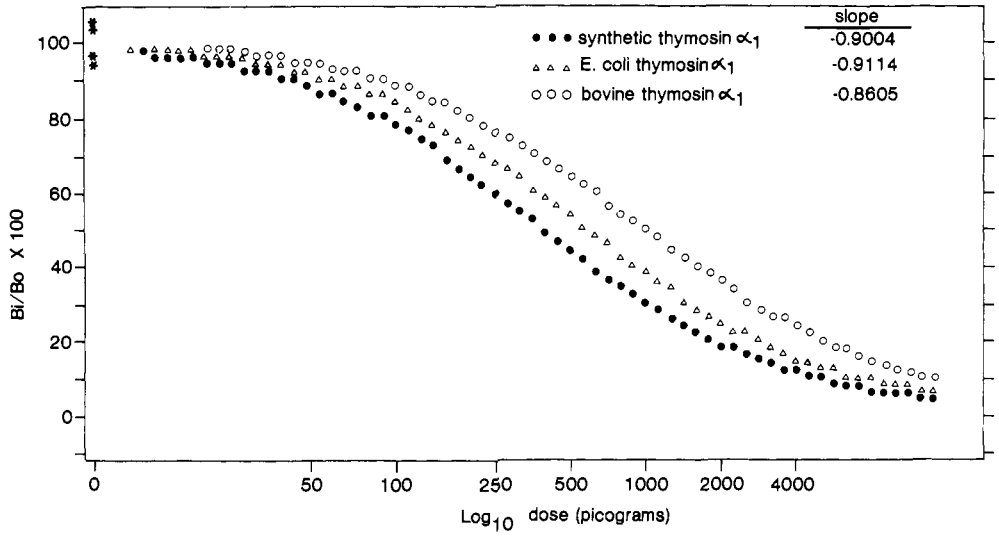


FIGURE 5: Dose–response curves in radioimmunoassay (see Methods) for chemically synthesized thymosin α_1 (●), N^{α}-desacetylthymosin α_1 from *E. coli* extract (△), and *N*-acetylthymosin α_1 isolated from bovine thymosin fraction 5 (○). Parallel responses indicate that complete immunochemical identity is shared by the three preparations. The lines represent graphs generated by a computer utilizing a program for a four-parameter logistics method.

Table VI: Suppression of TdT Activity by Chemically Synthesized and *E. coli* Thymosin α_1 in Murine Thymocytes[a]

preparation	concn (μg/mL)	% decrease[b]
thymosin α_1, chemically synthesized	0.02	20
	0.2	62
	0.4	23
N^{α}-desacetylthymosin α_1 from transformed *E. coli*	0.02	8
	0.2	55
	0.4	34

[a] Cells are incubated with thymosin fractions for 20 h at 37 °C in HRPMI and 10% fetal calf serum. See Experimental Procedures for details. [b] Decrease in TdT activity relative to cell culture without thymosin fraction.

Table VII: Reconstitution of MIF[a] Production in Peripheral Blood Lymphocytes (PBL) of Thymectomized Guinea Pigs by Addition of Thymosin α_1

		specific inhibition (%)			
preparation	concn (μg/mL)	expt 1	expt 2[b]	expt 3	expt 4[b]
thymosin fraction 5 (lot C100496)	200	24.7	29.1	28.9	38.9
	20	14.1	19.5	15.9	12.5
	2	9.5	12.1	15.5	1.7
thymosin α_1, chemically synthesized	1	52.3	46.8	48.4	59.1
	0.1	34.6	37.3	39.7	51.4
	0.01	11.0	27.2	25.5	33.4
	0.001	NT[c]	NT	10.6	7.8
thymosin α_1, from transformed *E. coli*	1	50.8	48.2	53.2	39.7
	0.1	29.4	33.3	34.4	32.2
	0.01	27.5	27.6	16.0	23.5
	0.001	NT	NT	8.6	14.4
no thymosin, PPD only		4.7	−3.4	5.0	5.0

[a] MIF assay microdroplet technique. [b] Rate-frozen PBL (−1 °C/min, 9% Me_2SO), maintained for 6 weeks at −70 °C. Viability = 75%. [c] NT = not tested.

(B) In Vitro Induction of MIF by Thymosin α_1. Table VII shows that N^{α}-desacetylthymosin α_1 synthesized in *E. coli* is also identical in activity with synthetic thymosin α_1 in the macrophage migration inhibition factor (MIF) assay. As can be seen in Table VII, the *E. coli* produced thymosin α_1 is about 10000 times more active than thymosin fraction 5 and is active at concentrations as low as 10 ng/mL. This assay demonstrates the ability of an added factor, like thymosin α_1, to induce development of immature lymphocytes, as measured by their ability to respond to an antigenic challenge by releasing the lymphokine MIF. As expected, crude fraction 5 is much less active and antigen alone gave no MIF release.

Discussion

Our approach to the bacterial expression of a cloned thymosin α_1 gene was dictated by the following considerations. Since the polypeptide product is relatively small, a total chemical synthesis of the gene (Itakura et al., 1977; Goeddel et al., 1979a; Crea et al., 1978) was preferable to its more laborious isolation from natural genetic material (Goeddel et al., 1979b). Experience has shown that the expression in *E. coli* of heterologous polypeptides ranging in size from 14 to 30 amino acids does not lead to stable products; on the other hand, insulin A and B chains and somatostatin are stable in *E. coli* when expressed as β-galactosidase chimeric proteins (Itakura et al., 1977; Goeddel et al., 1979a). The absence of methionine in the thymosin α_1 sequence allowed us to design a chimeric protein in which methionine separates the β-galactosidase leader from the C-terminal thymosin α_1. The protein can then be cleaved with CNBr to yield as series of fragments which include N^{α}-desacetylthymosin α_1.

Gene synthesis and cloning based on this approach gave a strain of transformed *E. coli* which produced about 400 mg of isolated N^{α}-desacetylthymosin α_1 per kg cells (roughly 100000 molecules/cell), a value consistent with the molar yields of other polypeptides produced in the same way. After chemical cleavage from chimeric protein and purification, N^{α}-desacetylthymosin α_1 was obtained in ~50% yield with a purity greater than 95%.

The progress of bone marrow stem cells to prothymocytes and thymocytes is accompanied by an increase in activity of terminal deoxyribonucleotidyl transferase (TdT), while in the further development of thymocytes to mature peripheral lymphocytes, this activity decreases (Low & Goldstein, 1978). The drop in measurable TdT activity in thymocytes incubated with thymosin α_1 but not with other peptides from the thymus or spleen (Hu et al., 1979, 1980) suggests an important and specific role for this polypeptide in the process of T-cell ma-

turation. The inability of peripheral lymphocytes from sensitized, thymectomized animals to produce and release lymphokines such as macrophage migration inhibition factor (MIF) upon challenge with the sensitizing antigen reflects the positive role of the thymus in T-cell maturation. The ability of thymosin α_1 to stimulate such a maturational sequence in vitro also points to the role of this molecule in T-cell development. In both tests, N^{α}-desacetylthymosin α_1 made in *E. coli* was found to have activity identical with both natural and chemically synthesized materials.

Thymosin α_1 made by the approach outlined in this paper lacks the N-terminal acetyl group of the molecule isolated from calf thymus. So far this difference has not affected its RIA response or in vitro biological activities. Preliminary results show that the free amino terminus of N^{α}-desacetylthymosin α_1 made in *E. coli* can be chemically acetylated to produce material identical with the isolated form of the natural polypeptide (R. Wetzel, unpublished results). Expression in *E. coli* of the gene for thymosin α_1 provides a promising alternative to large-scale isolation from thymus tissue or chemical synthesis.

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