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Biochemistry

Purification of thymus mRNA coding for a 16,000-dalton polypeptide containing the thymosin α_1 sequence

(thymic hormones/peptide hormones/cell-free polypeptide synthesis)

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ABSTRACT **A mRNA fraction purified by preparative polyacrylamide disc gel electrophoresis from calf thymus polysomes codes for a polypeptide(s) having a mass of 16,000–17,000 daltons. This polypeptide contains amino acid sequences corresponding to residues 11–18 and 19–25 of thymosin α_1. The yield of the octapeptide indicates that the 16,000-dalton peptide is the major product formed in the cell-free synthesis system containing the purified mRNA.**

The thymus gland has been shown to produce a variety of peptide hormones that appear to regulate the maturation and differentiation of T cells (for a review see ref. 1). One of these peptides, thymosin α_1, is of particular interest because its chemical structure is known (2, 3) and because it has been shown to be active in certain *in vitro* assays for thymic hormone function (1, 4). We have described evidence for a peptide, synthesized in a cell-free system containing mRNA isolated from the thymus gland, that may be the biosynthetic precursor of thymosin α_1 (5). In the present work we describe the purification of the specific mRNA and additional evidence that it encodes a 16,000-dalton peptide containing the thymosin α_1 sequence. Although the conversion of this peptide to thymosin α_1 remains to be demonstrated, it will, for convenience, be referred to here as the "thymosin α_1 precursor."

MATERIALS AND METHODS

Materials. [^{3}H]Leucine (56.5 Ci/mmol 1/Ci = 3.7×10^{10} becquerels) and [^{3}H]lysine (80.5 Ci/mmol) were purchased from New England Nuclear. Trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated) was from Millipore, and *Staphylococcus aureus* protease V8 was from Miles. Synthetic thymosin α_1 was provided by A. Felix of Hoffmann–La Roche. The octapeptide corresponding to residues 11–18 of the thymosin α_1 sequence was prepared by digestion of a sample of thymosin α_1 with *S. aureus* V8 protease and purified by high-performance liquid chromatography (HPLC). Its composition was verified by amino acid analysis. Other materials were from sources as described (5).

Methods. Polysomes were isolated from fresh calf thymus glands by the following procedure, which was carried out at 4°C under sterile conditions in containers that had been sterilized at 180°C for 12 hr or soaked in 0.1% diethyl pyrocarbonate for 30 min. The glands were freed of connective tissue, minced, and blended in 3 vol of 50 mM Tris·HCl buffer (pH 7.5) containing 25 mM NaCl, 5 mM $MgCl_2$, 2% (vol/vol) Triton X-100, 0.25 M sucrose, and 0.5 mg of heparin per ml, by using a Sorvall blender at half speed with five 5-sec bursts. The suspension was homogenized with five strokes at high speed in a glass/Teflon homogenizer. The homogenate was centrifuged at 20,000 × *g* for 10 min in a SS-34 Sorvall rotor. The polysomes were collected from the supernatant solution by aggregation with 100 mM $MgCl_2$ (6) followed by centrifugation at 27,000 × *g* for 20

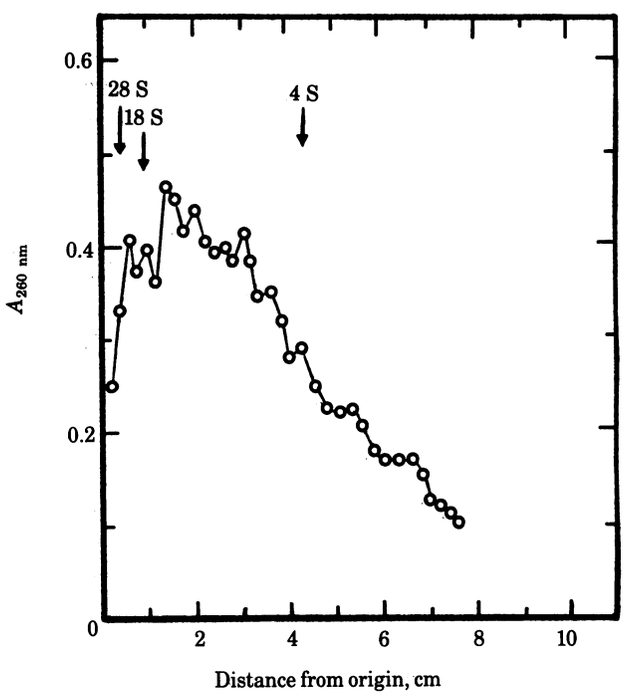

FIG. 1. Preparative polyacrylamide gel electrophoresis of thymus poly(A)-containing mRNA. Gels (10 × 80 mm) were prepared with 4% (wt/vol) acrylamide as described by Loening (8). The electrophoresis buffer was 40 mM Tris/20 mM NaOAc/2 mM EDTA adjusted to pH 7.7 with HOAc. Prior to loading the sample, the gels were electrophoresed at 5 mA per tube for 2 hr at 4°C, and the electrophoresis buffer was replaced with fresh buffer. The solution of poly(A)$^+$RNA was diluted to a final concentration of 8–10 A_{260} units in 70% (vol/vol) formamide (Merck) (deionized and vacuum distilled) containing 10% (vol/vol) of the electrophoresis buffer and 5% (wt/vol) of sucrose; this was heated to 65°C for 5 min, cooled, and applied to the top of the gel. Electrophoresis was carried out at 6 mA per tube for 3.5 hr at 4°C. The gels were sliced into 2-mm segments, and the RNA was eluted electrophoretically as follows. Each slice was placed on top of a small (1 cm) column of 3.6% (wt/vol) polyacrylamide gel (preelectrophoresed as before) in a 13 × 150 mm tube with a tapered tip and was covered with 1 ml of 80% formamide/5% sucrose/10% electrophoresis buffer. Elution with the same buffer was carried out at 7 mA per tube for 5 hr at 4°C. The RNA was collected into a dialysis bag tied to the bottom of the tube, and the *A* at 260 nm was determined. Reference tubes containing ribosomal RNA (28S, 18S) and wheat germ tRNA (4S) (Sigma) were run on parallel gels, stained with methylene blue [0.5% in 15% (vol/vol) HOAc] for 30 min, and destained with water.

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Abbreviation: HPLC, high-performance liquid chromatography.

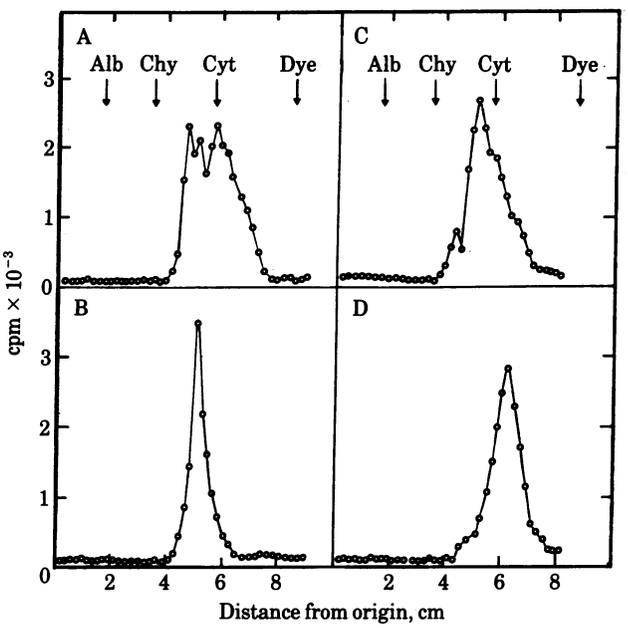

FIG. 2. Polyacrylamide gel electrophoresis of the labeled cell-free translation products of the purified mRNA fractions. Poly(A)$^+$mRNA fractions separated by polyacrylamide gel electrophoresis at 1.2–1.4 cm (*A*), 1.4–1.6 cm (*B*), 1.6–1.8 cm (*C*), and 1.8–2.0 cm (*D*) were evaluated in the wheat germ protein-synthesis system by using [^{3}H]leucine. Aliquots (15 μl) of the reaction mixtures were precipitated by the addition of trichloroacetic acid [final concentration, 10% (wt/vol)] and the precipitates were dissolved in 50 μl of 10 mM phosphate buffer, pH 7/1% $NaDodSO_4$/1% (vol/vol) 2-mercaptoethanol/10% (vol/vol) glycerol/0.002% Coomassie blue. After being heated at 90°C for 1 min, the solutions were applied to 12.5% polyacrylamide disc gels (0.6 × 9 cm). After electrophoresis, the gels were sectioned into 2-mm slices and extracted with 0.5 ml of 0.2 M $(NH_4)HCO_3$, (pH 7.8) for 2 hr at room temperature and then overnight at 4°C. Aliquots (200 μl) were taken for measurement of radioactivity. The arrows show the location of markers (Alb, albumin; Chy, chymotrypsin; Cyt, cytochrome *c*; Dye, Coomassie blue) run on parallel gels and stained with 0.1% Coomassie blue in methanol/glacial acetic acid/H_2O, 4.5:1:1.0 (vol/vol) for 8 hr.

min in a Sorvall SS-34 rotor through a cushion of 1 M sucrose in 50 mM Tris·HCl (pH 7.5) containing 25 mM NaCl, 100 mM $MgCl_2$, and 0.5 mg of sodium heparin (Sigma) per ml [polysome suspension/sucrose cushion, 17:26 (vol/vol)]. For extraction of RNA, the pellet was dissolved in 100 mM Tris·HCl buffer, pH 7.5/100 mM NaCl/1 mM EDTA/1% (wt/vol) $NaDodSO_4$ at a concentration of 30–40 A_{260} units/ml. The solution was extracted twice with phenol (buffer-saturated)/chloroform, 1:1 (vol/vol), and the RNA was precipitated with 2.5 vol of ethanol at −20°C. After 12 hr, the RNA was collected by centrifugation at 15,000 × *g* for 20 min, washed with ethanol and ether, dried in vacuo at 4 °C, and dissolved in 10 mM Tris·HCl, pH 7.5/0.5 M NaCl to a final concentration of 20–30 A_{260} units/ml.

Poly(A)-containing RNA was isolated from the polysomal RNA as described (5). The final concentration was 100–200 A_{260} units/ml.

For evaluation of the poly(A)$^+$RNA fractions in the cell-free protein synthesis system, the RNA fractions eluted from the disc gel segments (see Fig. 1 legend) were dialyzed for 1 hr against 50 mM Tris·HCl, pH 7.5/150 mM NaCl/0.2% $NaDodSO_4$, and the dialyzed solutions were extracted with chloroform/phenol, 1:1 (vol/vol). RNA was precipitated by the addition of 2.5 vol of ethanol and, after 48 hr at −20°C, was collected by centrifugation and dissolved in sterile water to yield a concentration of 20 A_{260} units/ml. The solutions were stored in liquid nitrogen.

Cell-free protein synthesis with the wheat germ system was carried out with 0.04 A_{260} units of purified mRNA in a total volume of 50 μl as described (5), and with 28 μg of phenylmethylsulfonyl fluoride (Serva) per ml included in the reaction mixtures. The labeled amino acid was either [^{3}H]leucine (10 μCi) or [^{3}H]lysine (10 μCi) as specified.

Disc gel electrophoresis of cell-free translation products was carried out as described in the legends to the figures and in ref. 5.

Peptides were analyzed by HPLC as described by Rubinstein (7).

RESULTS

Purification of mRNA Coding for the Thymosin α_1 Precursor. RNA was extracted from thymus polysomes, and the mRNA fraction was prepared by chromatography on oligo (dT)-cellulose. The mRNA was fractionated by preparative polyacrylamide gel electrophoresis (Fig. 1) and fractions were eluted from sections of the gel analyzed in the wheat germ system (Fig. 2). Fraction 8, located 1.4–1.6 cm from the origin, coded for a product that migrated as a single sharp peak in $NaDodSO_4$ disc gel electrophoresis, corresponding to a peptide with a mass of 16,000 daltons (Fig. 2*B*). This product also migrated as a single symmetrical peak on $NaDodSO_4$/urea/polyacrylamide gels, with an estimated mass of 17,000 daltons (Fig. 3). Elution of RNA from adjacent segments of the gel gave mixtures of peptides (Fig. 2*A* and 2*C*) or peptides of smaller size (Fig. 2*D*).

Characterization of the Thymosin Precursor. Our previous studies had identified a 16,000-dalton peptide, precipitated by antibodies against a thymic peptide fraction containing thymosin α_1, among the products formed with unfractionated mRNA from the thymus gland (5). To identify the 16,000-dalton product obtained with the purified mRNA, we examined peptides formed by digestion with trypsin or with *S. aureus* protease when it was labeled with [^{3}H]lysine. Analysis by reversed-phase

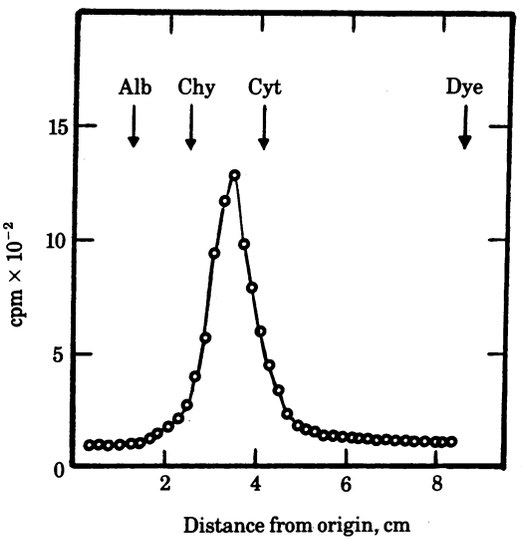

FIG. 3. Polyacrylamide gel electrophoresis in $NaDodSO_4$/urea of the leucine-labeled translation products formed with the purified mRNA fraction. An aliquot (15 μl) of the reaction mixture described in the legend to Fig. 2 for the 1.4- to 1.6-cm gel slice (Fig. 2*B*) was treated as indicated except that the solution buffer contained both 6 M urea and 1% $NaDodSO_4$ and the gels (15% polyacrylamide) also contained 6 M urea. Aliquots (100 μl) of the solutions obtained by extracting the 2-mm gel slices (5) were taken for measurement of radioactivity. The arrows indicate positions of marker proteins (identified in Fig. 2 legend) analyzed on parallel gels.

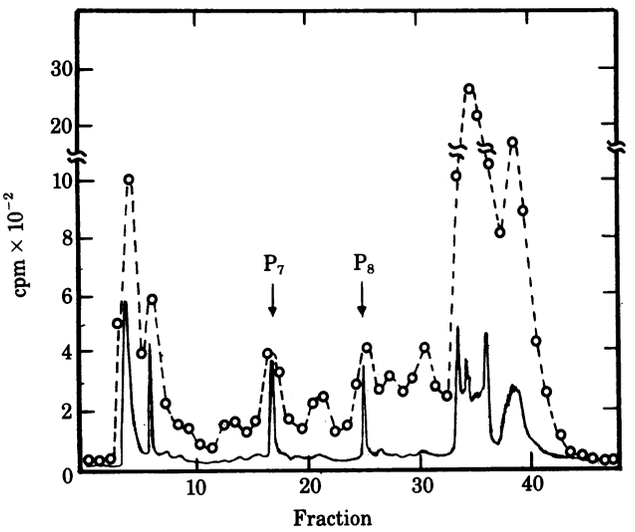

FIG. 4. High-performance liquid chromatography of the lysine-labeled peptides formed on digestion of the radioactive product with *S. aureus* protease. The translation of mRNA eluted from the 1.4- to 1.6-cm slice (see Fig. 1) was carried out with [^{3}H]lysine as described in the legend to Fig. 2. The reaction mixture (50 μl) was diluted to 0.8 ml with 0.1 M $(NH_4)HCO_3$, pH 7.8/5 mM unlabeled lysine and was centrifuged at 40,000 rpm for 1 hr in a SW 50.1 rotor on the Spinco ultracentrifuge. The supernatant solution was dialyzed overnight at 4°C against 0.1 M $(NH_4)HCO_3$, (pH 7.8) and was lyophilized. The residue (60,000 cpm of trichloroacetic acid-precipitable material) was dissolved in 50 μl of 0.2 M $(NH_4)HCO_3$, pH 7.8/2mM EDTA/3 M urea (9). *S. aureus* protease (50 μg) was added at time zero and after 8 hr and 16 hr. Incubation was at 25°C. Before the second addition of protease, 50 μg of carrier thymosin α_1 was added. After 24 hr the reaction was stopped by the addition of 25 μl of 70% (vol/vol) HCOOH. The solution was lyophilized, and the residue was dissolved in 200 μl of 0.2 M pyridine in 1 M HCOOH. Separation of peptides was carried out with a 10-μm, 300 × 4.6 mm Chromegabond C-18 column (ES Industries, Marlton, NJ) that had been equilibrated with the same buffer. Stepwise elution was carried out with the pyridine/formic acid buffer with additions as follows: (*i*) at 12 min, 8% acetonitrile; (*ii*) at 42 min, 15% acetonitrile; (*iii*) at 82 min, 23% acetonitrile; and (*iv*) at 100 min, 40% propanol. The flow rate was 0.35 ml/min, and fractions were collected every 2.5 min; at 20-sec intervals, 5-μl samples were diverted for analysis with fluorescamine (10). O---O, Aliquots (150 μl) taken for measurement of radioactivity; ——, tracing from the automatic peptide analyzer. P_7 and P_8 mark the positions of the heptapeptide Lys-Lys-Glu-Val-Val-Glu-Glu and the octapeptide Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu.

HPLC of the [^{3}H]lysine-labeled digestion products gave a number of radioactive peptides (Fig. 4), including the octapeptide Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu (P_8) and the heptapeptide Lys-Lys-Glu-Val-Val-Glu-Glu (P_7) that correspond to residues 11–18 and 19–25, respectively, of the thymosin α_1 sequence. Rechromatography of the radioactive fractions 16–17 and 24–25 gave sharp peaks coinciding with the carrier heptapeptide and octapeptide (Fig. 5). The structure of the labeled octapeptide was confirmed by digestion with trypsin, which formed peptides corresponding to residues 11–14 and 15–18 of thymosin α_1 (Fig. 6). Rechromatography of the peaks containing these peptides confirmed the coincidence of radioactivity with the peptides derived from the carrier thymosin α_1 (data not shown). The heptapeptide was further characterized by Edman degradation, which released most of the radioactivity in the first two cycles.

Recovery of Octapeptide. In order to assess the purity of the mRNA, we compared the radioactivity in the total cell-free translation products with that recovered in the octapeptide after purification of the latter by HPLC (Table 1). The recoveries in experiments with [^{3}H]lysine (Fig. 5) and with [^{3}H]leucine (data not shown) were in the range of 5–6.5% and 3.0–3.5%, respectively, compared with a predicted value of 5.9% calculated from the mass of the octapeptide (946 daltons) and from that of the precursor (*ca.* 16,000 daltons), with the assumption that the

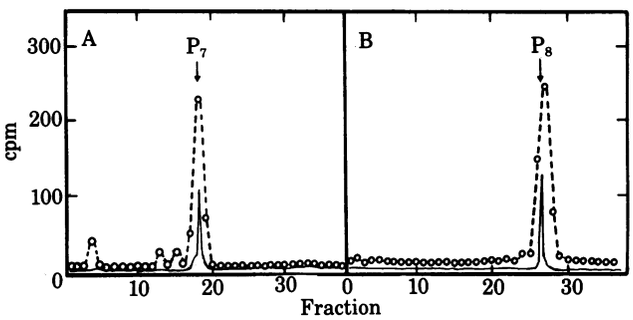

FIG. 5. Purification and chromatographic characterization of labeled heptapeptide and octapeptide formed by digestion of translation products with *S. aureus* protease. The fractions containing radioactivity corresponding to the positions of the authentic heptapeptide and octapeptide were pooled separately (8000 cpm in each pooled fraction), lyophilized, dissolved, and chromatographed as described in the legend to Fig. 4. (*A*) Fractions 16–17. (*B*) Fractions 24–25. The composition of the peptides was verified by amino acid analysis.

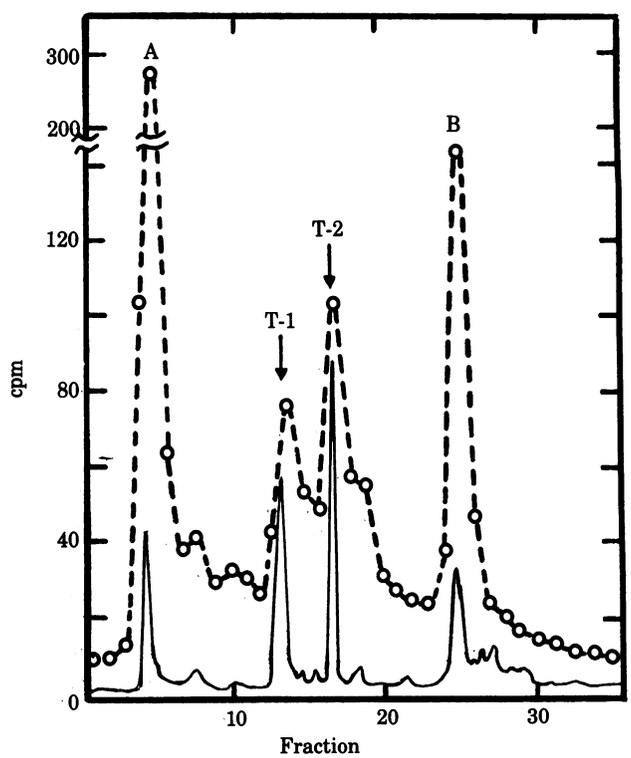

FIG. 6. Characterization of tryptic peptides derived from the octapeptide formed with the *S. aureus* protease. Fractions containing radioactivity corresponding to the purified octapeptide were pooled (6000 cpm) and after lyophilization were dissolved in 100 μl of 0.2 M NH_4HCO_3 buffer (pH 8.5). Digestion with trypsin (ratio 1:20, based on the yield of peptide expected from the addition of thymosin α_1 carrier before protease digestion) was for 14 hr at 37°C. The reaction was terminated with 50 μl of 70% HCOOH. The mixture was lyophilized, and the residue was dissolved and analyzed by HPLC as described in the legend to Fig. 4. The marker peptides Ile^{11}-Thr-Thr-Lys^{14} and Asp^{15}-Leu-Lys-Glu^{18} are indicated by T-1 and T-2, respectively. Peak A marks the position of free lysine; peak B marks the location of undigested octapeptide. The composition of each peptide was verified by amino acid analysis.

Table 1. Recovery of octapeptide following digestion of labeled 16,000-dalton translation product with *S. aureus* protease

	Radioactivity		
	Translation product, cpm	Octapeptide, cpm	Recovered as octapeptide, %
Labeled with [^{3}H]Lys	100,000	6,500	6.5
	75,000	3,800	5.0
Labeled with [^{3}H]leucine	80,000	2,900	3.6
	85,000	2,800	3.3

content of lysine and leucine in the octapeptide was similar to that in the precursor. Although this assumption introduces some uncertainty, the fact that the observed and predicted values are within a factor of two of each other suggests that the bulk of the translatable RNA in the purified mRNA codes for the thymosin α_1 precursor.

Estimate of the Size of the mRNA. Based on its migration in formamide gels (11), we estimate the purified mRNA to have a molecular weight of approximately 200,000, corresponding to 615 nucleotides (data not shown). Thus, the number of codons is nearly twice that needed for a peptide of 16,000 daltons.

DISCUSSION

In our earlier study of *in vitro* translation of thymus mRNA with the wheat germ protein-synthesizing system, we found that the peptides formed were distributed mainly in the size range of 10,000–20,000 daltons, as determined by NaDodSO$_4$/polyacrylamide gel electrophoresis (5). This suggested that the most abundant messages are those coding for peptides much smaller than the usual size for subunits of intracellular proteins. In the experiment shown in Fig. 1, 5% of the total mRNA was recovered in the fraction coding for the 16,000–dalton product, suggesting that the mRNA in this fraction falls into the class of abundant messages.

The mRNA purified by polyacrylamide gel electrophoresis appears to code for a single polypeptide chain having a mass of 16,000–17,000 daltons. The recovery of radioactivity in the octapeptide generated by the *S. aureus* protease suggests that the 16,000-dalton peptide is the major, if not the only, peptide translated by the purified mRNA fraction.

The previous characterization of the putative thymosin α_1 precursor was based on the identification of a tryptic pentapeptide, including residues 16–20 of the thymosin α_1 sequence. The present evidence for the sequence including residues 11–18 and 19–25 accounts for more than half of the total thymosin α_1 sequence and leaves little doubt that the entire sequence is present in the 16,000-dalton translation product. Availability of essentially pure preparations of radioactive peptide, synthesized in the cell-free synthesis system, should greatly facilitate its isolation and studies of the mechanism of its processing to form thymosin α_1 and other thymic peptides that may possess biological activity.

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