CD-1 (Charles River), and MF1 (Harlan Sprague-Dawley) females were used as the source of blastocysts. (C57Bl/6 × CBA) F_1, (C57Bl/6 × DBA) F_1 (Charles River), or CD-1 females were used for foster mothers. Cells for injection were thawed and maintained in culture for no more than six additional passages. Approximately 12 to 15 cells were injected into blastocysts collected 3.5 days post coitus, as previously described (7) [A. Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, Ed. (IRL Press, Oxford, 1987), pp. 113–151]. Microinjected blastocysts were introduced into the uterine horns of pseudopregnant mice 2.5 days post coitus. Chimerism was scored by coat and eye pigmentation in the CD-1 and MF1 albino background and by the presence of agouti coat color in the C57Bl/6 background.
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Thymotaxin, a Chemotactic Protein, Is Identical to β_2-Microglobulin

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Thymotaxin, an 11-kilodalton protein chemotactic for rat bone marrow hematopoietic precursors, was purified from media conditioned by a rat thymic epithelial cell line. The NH_2-terminal sequence of thymotaxin was identical to that of rat β_2-microglobulin (β2m). Antibodies to β2m removed thymotaxin activity from the fraction containing the 11-kilodalton protein. Chemotactic activity was observed with rat plasma β2m, human β2m, and mouse recombinant β2m, further supporting the identity of thymotaxin with β2m. The directional migration, as opposed to random movement, of the cells was also confirmed. The only rat bone marrow cells that migrated toward β2m were Thy1$^+$ immature lymphoid cells devoid of T cell, B cell, and myeloid cell differentiation markers.

CHEMOTAXIS IS THE DIRECTIONAL migration of cells along soluble gradients of chemical substances. In mammals, several cell types are able to migrate in a chemotactic manner, especially cells from the hematopoietic system, for example, polymorphonuclear cells (*1*), monocytes, and mature T lymphocytes (*2*). It has been suggested (*3*) that chemotaxis is involved in directing the migration of hematopoietic precursors from their site of emergence, the bone marrow, to the thymus. Indeed, we showed that avian thymic peptides attract T cell precursors from quail bone marrow in vitro (*4*). In vivo, migration of hematopoietic precursors into the thymus is a prerequisite for T cell differentiation, which requires the influence of the thymic epithelium and thymic accessory cells (*5*). We also showed that secretion products of rat thymic epithelial cells induce the migration of the hematopoietic precursors from rat (*6*) and mouse (*7*) bone marrow. This chemotactic activity was due to an 11-kD protein called thymotaxin (*6*). In Boyden migration chambers, thymotaxin selectively attracted immature lymphoid cells, devoid of mature T and B cell differentiation markers. These nonreplicating cells failed to grow in methylcellulose when stimulated with growth factors. The selected population did acquire T cell differentiation markers and synthesized T cell receptor α and β chain transcripts on coculture with embryonic thymic tissue (*8*); thus, this population contained T cell precursors. We now report that thymotaxin is biochemically and functionally identical to rat β_2-microglobulin (β2m).

Thymotaxin, secreted in a serum-free medium conditioned by IT-45 R1 rat thymic epithelial cell line (*9*), was purified by gel filtration or reversed-phase high-performance liquid chromatography (HPLC). Column fractions were tested for their ability to induce the migration of rat bone marrow cells in a Boyden chamber assay (*10*). Bone marrow cells were partially depleted of erythroid and myeloid cells and enriched in low-density hematopoietic precursors by centrifugation on a 28% bovine serum albumin (BSA) gradient (*6*). In both gel filtration and reversed-phase HPLC, a cell migration activity was associated with the same 11-kD peptide when analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1, A and B). The active reversed-phase fraction was electroblotted onto a polyvinyl membrane and sequenced directly from membrane strips in a gas phase microsequencer (*11, 12*). The 11 NH_2-terminal amino acids were IQKTPQIQVYS, identical to those of rat β2m (*13*). Since the amino acid sequence of bovine β2m can easily be distinguished from that of rat β2m in this region, we confirmed that thymotaxin was a product of thymic epithelial cells and not a contaminant from the fetal calf serum used in the culture medium.

The biological activity of thymotaxin produced by IT-45 R1 cells was then further investigated. The thymotaxin fraction from reversed-phase HPLC (Fig. 1B, lane c) showed a peak of maximal activity at $10^{-11}M$ and another slightly lower peak at $3 \times 10^{-9}M$ (Fig. 2A). The activity found at $10^{-11}M$ was completely removed by passage over an affinity column prepared with rabbit polyclonal antibodies to mouse β2m (anti-β2m), which cross-react with rat β2m (Fig. 2A). The activity found at $3 \times 10^{-9}M$, which was not retained on the anti-β2m affinity column, seemed to be due to a second peptide of 8 kD, which comigrated with thymotaxin on reversed-phase HPLC (Fig. 1B, lane c) and which could be removed by running the reversed-phase fraction on SDS-PAGE and electroeluting the

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thymotaxin. Attempts to characterize the 8-kD molecule have been hampered because the NH_2-terminus is blocked, and all further studies have been made with β2m free of this molecule. A checkerboard analysis (*14*) with thymotaxin (free of the 8-kD molecule) revealed that the migration of bone marrow hematopoietic precursors results from a gradient of thymotaxin concentration. Thus, thymotaxin acts by a chemotactic mechanism (oriented migration toward a concentration gradient) (Fig. 2B), whereas cell migration induced by the 8-kD purified protein is chemokinetic (random migration) (*15*). Cells that migrated toward electroeluted thymotaxin were lymphoid cells with an immature $Thy1^+$ $CD4^-$ $CD8^-$ IgM^- phenotype (*8*).

To establish that β2m secreted by IT-45 R1 cell line and plasma β2m show the same activities, we prepared β2m from rat plasma by ultrafiltration and electroelution from an SDS gel. In Boyden chambers, it attracted hematopoietic cells from bone marrow at an optimal concentration of $10^{-11}M$ (Fig. 3A). The effect of β2m from other species on rat bone marrow cells was also tested. Activity was found with commercial human β2m purified from urine (Serotec, Oxford, England): cell migration was maximal between $3 \times 10^{-11}M$ and $3 \times 10^{-12}M$ (Fig. 3A) and was oriented as demonstrated by checkerboard analysis (Fig. 3B). Moreover, as reported for thymotaxin-responding cells, the low-density cells that had migrated toward human β2m displayed an immature $Thy1^+T^-B^-M^-$ phenotype (devoid of T cell, B cell, and myeloid cell differentiation markers) and did not give rise to granulocyte and macrophage colonies in semisolid cultures under stimulation with interleukin-1, interleukin-3, and colony-stimulating factors (*16*). Finally, recombinant mouse β2m was produced in a baculovirus system and further purified. This β2m (95% pure) attracted low-density rat bone marrow cells when added at $10^{-11}M$ (Fig. 3A), suggesting that no additional posttranslational maturation of β2m is necessary for chemotactic activity. Whatever the source of β2m, the cell migration indices were close to 1.5, and about 1.3% of the total low-density cells migrated specifically. Because we are dealing with a heterogenous cell population, the observed indices must underestimate the ability of the subset of responding cells to migrate toward β2m. In any event, these migration indices are compatible with those reported for lymphoid cells (*2*).

All of the above experiments were made with low-density bone marrow cells, which account for 10% of the total bone marrow. We also tested the migration ability of the high-density cell fraction. This high-density fraction is depleted in hematopoietic precursor cells (95% fewer colonies than from the low-density fraction in semisolid assays) and contains a large number of committed myeloid cells, especially cells from the granulocyte lineage, which migrate toward many soluble factors. Nevertheless, in our migration assay, essentially no lymphoid cells or myeloid cells migrated specifically toward β2m (Table 1), indicating that the responding cells are enriched in the low-density fraction. Thus, in the rat bone marrow, the cells able to migrate toward β2m belong to a small subset of immature $Thy1^+T^-B^-M^-$ precursor cells.

β_2-Microglobulin is the common small subunit of the major histocompatibility complex (MHC) class I molecule (*17*). Association with β2m is generally required for the transport of class I heavy chain from the endoplasmic reticulum to the cell surface (*18*), and β2m contributes to stabilizing the heavy chain conformation recognized by alloantisera (*19*). In addition, soluble β2m is produced, in vitro, by rabbit synovial fibroblasts stimulated with phorbol myristate acetate and can induce, in these cells, collagenase synthesis in an autocrine manner (*20*). The mechanism of this autocrine induction is not yet understood but could be involved,

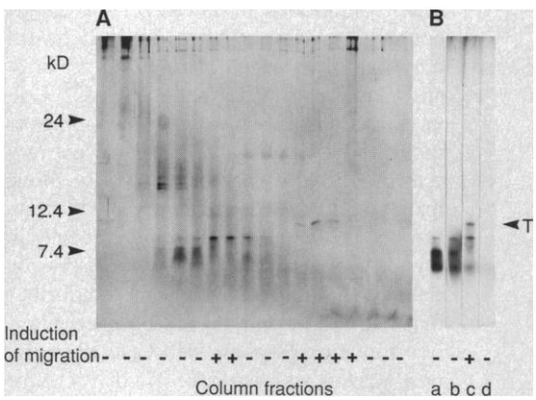
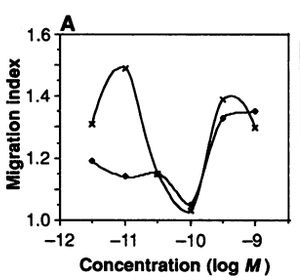
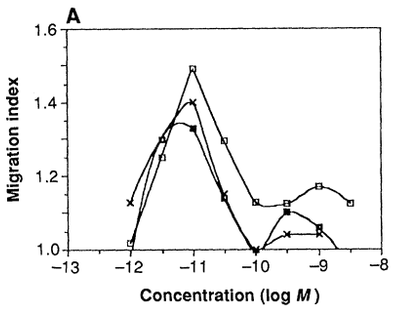


Fig. 1. Purification of thymotaxin by chromatography. (**A**) Serum-free medium from the IT-45 R1 thymic epithelial cell line was prepared and purified on Sep-Pack cartridges as described (*6*). This material was further purified by HPLC gel filtration on a Superose 12 column (1 ml/min) (Pharmacia), and fractions were analyzed on 15% SDS-PAGE (reducing conditions) stained by silver nitrate (*26*). Each fraction was tested at different concentrations for its ability to induce the migration of bone marrow–derived hematopoietic precursors in Boyden chambers (*10*); (+) represents active fractions and (−) represents fractions without activity. Active peptides had apparent molecular masses of 11 and 8 kD. (**B**) In a parallel experiment, Sep-Pack–purified thymotaxin was applied to a reversed-phase HPLC column (TSK ODS-120T; 5 μm; 1 ml/min; LKB). The column was loaded in an aqueous phase and eluted by a gradient of 35% to 39% acetonitrile, 0.1% trifluoroacetic acid during 20 min. Fractions (1 ml) were immediately lyophilized and reconstituted; representative portions were analyzed on 15% SDS-PAGE (reducing conditions), stained by the silver nitrate method (*26*), and tested at different concentrations for their ability to induce cell migration (*10*); (+) and (−) are as in (A). Fraction (a) corresponds to 35% acetonitrile, (b) to 36%, (c) to 37%, and (d) to 38%. The active peptide (thymotaxin) elutes at 37% acetonitrile. T, thymotaxin.



Upper / Lower	DMEM	$10^{-11}M$
DMEM	1.0	1.21
$10^{-11}M$	2.59	1.30

Fig. 2. Chemotactic activity of purified thymotaxin. (**A**) Thymotaxin from reversed-phase HPLC (Fig. 1B, lane c) was tested for its ability to induce migration of bone marrow hematopoietic precursors in a Boyden chamber assay (*10*). Thymotaxin from reversed-phase HPLC is indicated by (x). Flow-through of reversed-phase thymotaxin fraction passed over an affinity column prepared with rabbit polyclonal antibodies to mouse β2m bound to protein A–Sepharose (Pharmacia) is indicated by (◆). (Without antibodies on the column, the flow-through had the same activity as thymotaxin.) Results are given as mean of four separate experiments (six wells per experiment). The difference between the number of cells that migrated toward reversed-phase thymotaxin and the number of cells that migrated toward the flow-through of reversed-phase thymotaxin passed over the affinity column was statistically significant at $0.10 < P < 0.15$, according to a Student *t* test applied to paired samples. (**B**) Discrimination between chemotactic and chemokinetic effects of electroeluted thymotaxin. Thymotaxin was purified on a reversed-phase HPLC column, as described in Fig. 1B, and run on 15% SDS-PAGE (reducing conditions); the band with a molecular mass of 11 kD was electroeluted for 3 hours at 200 mA in a Schleicher & Schuell apparatus with 192 m*M* glycine, 25 m*M* tris, *p*H 6.8, as elution buffer. The purity of electroeluted thymotaxin was checked on 20% SDS-PAGE stained by the silver nitrate method. Concentration of thymotaxin was estimated according to a dilution series of cytochrome c (Sigma) on the same gel. Chemotactic activity of thymotaxin was determined by a checkerboard experiment (*14*) with $10^{-11}M$ thymotaxin and control Dulbecco's minimum essential medium (DMEM) in Boyden chambers as attractants for bone marrow hematopoietic precursors. Chemotactic activity was achieved when $10^{-11}M$ thymotaxin was placed in the lower chamber. These typical results are from a single experiment.



B

Upper / Lower	DMEM	3×10^{-12} M	10^{-9} M
DMEM	1	0.99	1.07
3×10^{-12} M	1.52	1.13	1.08
10^{-9}M	1.17	1.17	1.05

Fig. 3. Characteristics of β2m from other origins. (**A**) Chemotactic activity of rat plasma β_2m (x), human β2m (□), and mouse recombinant β2m (■). β_2-Microglobulin from rat plasma was prepared by ultrafiltration on a Diaflo UM20 membrane; the flow-through was applied to a Lichroprep reversed-phase column, electroeluted with 50% acetonitrile, and lyophilized. This material was loaded on 15% SDS-PAGE, and β2m was electroeluted. Human β2m was from Serotec, and recombinant mouse β2m was produced in a baculovirus system. The results are given as mean of 5, 11, and 6 experiments (six wells per experiment), respectively. The degree of purity of these proteins was tested on SDS-PAGE. The difference between the number of cells that migrated toward each β2m at $10^{-11}M$ and the number of cells that migrated toward DMEM was statistically significant at $0.025 < P < 0.05$, according to a Student *t* test applied to paired samples. (**B**) Commercially available purified human β2m (Serotec) was tested for its chemotactic activity on rat bone marrow hematopoietic precursors in Boyden chambers. Checkerboard analysis (*14*) revealed chemotactic activity of this molecule at $3 \times 10^{-12}M$. This molecule was completely devoid of chemokinetic effect. Results are given as mean of three separate experiments (six wells per experiment).

in vivo, in the modulation of connective tissue breakdown. We have shown that β2m can exert a chemotactic activity on immature lymphoid rat bone marrow cells in vitro. Since β2m is present on most cells and in body fluids at high concentration in adult mammals (*21*), the molecular mechanism by which such a molecule could be responsible for a specific chemotactic process is unclear.

If we consider that only free β2m and not the bound protein exerts a chemotactic activity, this biological effect could be regulated in vivo by the plasma concentration of free β2m. Indeed, different β2m-binding proteins have been described in the adult rat plasma (*22*), and the concentration of free circulating β2m is unknown. The plasma concentration of β2m during embryogenesis and its sites of production have not yet been studied, although MHC class I and β2m antigens are known to be expressed at lower levels on cells (*23*).

The specificity of the migration response may be due to a cell surface receptor that is restricted to a subset of bone marrow cells. Although MHC class I heavy chain could be such a receptor, a number of proteins from the immunoglobulin superfamily, including CD1 (*24*), have been reported to bind β2m. Notably, β2m associates with a class I–like molecule that acts as an Fc receptor on intestinal epithelial cells of the neonatal rat and allows the transcytosis of immunoglobulin G from mother's milk (*25*). Thus, it seems possible that another protein anchoring to the immunoglobulin superfamily could be involved in the chemotactic migration toward β2m. Finally, it is possible that β2m binds to responding cells by a nonspecific receptor (for example, the heavy chain of MHC class I) but exerts its chemotactic effect by an intracellular response specific to these cells. There may also be other pathways for this newly described activity of β2m.

Table 1. Cytological phenotype of bone marrow cells that migrated specifically toward β2m. Bone marrow–derived cells were taken after BSA gradient. High-density cells (pellet) were treated with a hypotonic medium in order to lyse red cells. Low- and high-density cells before migration were then centrifuged and stained in May-Grünwald and Giemsa solution. The percentage of cells of each cytological phenotype was determined by scoring at least 250 cells per experiment. The percentage of specifically migrated cells is obtained from the difference between cells that migrated toward human β2m and cells that migrated toward DMEM for each type of cell. Abbreviations: L, lymphoid cells; M, myeloid cells; and E, erythroid cells.

Cell density	Cytological phenotype (%)			Specifically migrated cells (%)	
	L	M	E	L	M
Low	43	20	37	2.21	<0.1
High	15	42	43	<0.1	<0.1

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10. Migration of bone marrow–derived hematopoietic precursors was measured by the Boyden chamber assay. Cells were counted with a Coulter Counter ZM equipped with a 256 channelizer; the counting window was 5 to 7 μm. Approximately 2.5×10^5 bone marrow cells (5 to 11 μm), depleted of erythroid and myeloid cells and enriched in hematopoietic precursors by centrifugation on a 28% BSA gradient (*6*), were loaded in the upper compartment of Boyden chambers. Proteins to be assayed were placed into the lower blind well chamber, which was separated from the cell-containing compartment by Nuclepore filters (pore size, 5 μm; Nuclepore, Pleasanton, CA). After 3 hours of incubation at 37°C, the cells of the upper compartment were removed by washing four times with phosphate buffer saline (PBS). The chamber was then centrifuged at 100*g* to detach weakly adherent cells from the lower surface of the filter, the Nuclepore filter was discarded, and the cells that had migrated were collected from the lower compartment and suspended in Isoton II (Coultronics, Margency, France). The migration index was defined as the ratio between the number of cells that had migrated toward testing protein and the number of cells that had migrated toward control medium (DMEM). If the counting window for migrating cells was 5 to 11 μm, results were not changed because the percentage of specifically migrating large cells is very low.
11. In a preparative batch, 15 liters of IT-45 R1–conditioned medium was passed on a column of 50-ml Lichroprep RP-18 (Merck); the column was washed with 5% acetonitrile, and the peptides were eluted at 80% acetonitrile. The eluate was lyophilized, and thymotaxin was purified on reversed-phase HPLC columns (TSK ODS-120T; 5 μm; LKB). Fractions containing thymotaxin were pooled and run on preparative 15% SDS-PAGE in 45 m*M* tris, 40 m*M* borate, 2 m*M* EDTA, and 1% SDS. The gel was electrotransferred for 45 min at 200 mA on a polyvinyl membrane in the same buffer without SDS. Blotted proteins were stained by Coomassie blue G-250. The band with a molecular mass of 11 kD (thymotaxin) was cut and analyzed into a gas phase sequencer (Applied Biosystem 470A, Foster City, CA). The amino-acid sequence of the NH_2-terminus of thymotaxin is IQKTPQI-QVYS (Abbreviations: I, Ile; Q, Gln; K, Lys; T, Thr; P, Pro; V, Val; Y, Tyr; and S, Ser). According to the chromatograms, this sequence, identical with the NH_2-terminus of rat β2m (*13*), was not contaminated by another protein.
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Unusual Pattern of Accumulation of mRNA Encoding EGF-Related Protein in Sea Urchin Embryos

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A sea urchin (*Strongylocentrotus purpuratus*) messenger RNA encoding a protein (SpEGF2) related to epidermal growth factor (EGF) was identified. The full-length complementary DNA sequence predicts a protein with an unusually simple structure, including four tandem EGF-like repeats and a hydrophobic leader, but lacking a potential transmembrane domain. Sequence similarities suggest that the peptides are homologous to two peptides from a different sea urchin species, which cause a classic developmental defect, exogastrulation, when added to the seawater outside of embryos. The SpEGF2 messenger RNA begins to accumulate at blastula stage, and in pluteus larvae it is distributed in discrete regions of ectoderm that are not congruent with known histological borders. One region corresponds to that expressing the homeodomain-containing protein, SpHbox1. The structure of the SpEGF2 protein and the pattern of accumulation of its messenger RNA suggest that it may have important functions as a secreted factor during development of sea urchin embryos.

THE FAMILY OF PROTEINS THAT CONTAIN domains similar to epidermal growth factor (EGF) includes many that are membrane-bound or diffusible mediators of cell-cell interactions. The proteins encoded by the *notch* (*1*) and *delta* (*2*) genes of *Drosophila* and by the *lin-12* (*3*) and *glp-1* (*4*) genes of *Caenorhabditis* are expressed during development of these invertebrate embryos in which they function in decisions of cell fate. The structure of the mRNA encoding a new member of this family, SpEGF2, which is expressed in embryos of the sea urchin *Strongylocentrotus purpuratus*, is shown in Fig. 1A. This message contains an open reading frame of 975 nucleotides beginning with an ATG codon. The deduced protein contains 325 amino acids and has a predicted molecular mass of 36.9 kD. Most (at least 263 of 325 amino acids) of the predicted protein consists of four tandem EGF-like repeats that can be aligned by the six cysteine residues found in each repeat spaced as $CX_6CX_5CX_{8-13}CXCX_{11-12}C$ (Fig. 1B). These are within the range of spacings ($CX_{4-14}CX_{3-8}CX_{8-14}CXCX_{8-14}C$) for 63 EGF-like repeats from functionally diverse proteins (*5*). The SpEGF2 repeats have several highly conserved residues proposed to be important for protein conformation (*6*), including Gly^{18}, Phe/Tyr^{29}, Gly^{36}-Phe/Tyr^{37}, and Gly^{39}. They lack two of six residues, Arg^{41} and Leu^{47}, conserved in ligands that bind to vertebrate EGF receptors (*7*). They also lack the site of β-hydroxylation at Asp/Asn^{22} and several other residues thought to be important for high-affinity Ca^{2+} binding characteristic of some EGF-like repeats of plasma proteins (*5*). Computer-assisted analysis of secondary structure (*8*) predicts that repeats 1 to 3 have a central β-sheet structure similar to that demonstrated for EGF (*9*).

The sequence around the ATG at the beginning of this open reading frame strongly suggests that it is the true initiation codon. First, there are stop codons in all three reading frames upstream of this ATG. Second, the 17 amino acids following it constitute a putative hydrophobic signal peptide, a feature common to all EGF-related proteins. Third, primer extension identified the A residue 70 base pairs upstream of this ATG codon as the unique transcription initiation site. Furthermore, sequence upstream from this initiation site includes a TATA box at base −32 to −26 and (G)CCAATT motifs at bases −52 and −171. Several other close matches to known regulatory motifs, whose functional significance in the SpEGF2 gene remains to be established, are described in the legend to Fig. 1A. The 3′ untranslated region is approximately 500 bases long and contains a known variant signal for transcript cleavage and polyadenylation, ATTAAA (*10*), which begins 19 bases upstream from a stretch of A residues that presumably represent the beginning of the polyadenylate tail. These features predict an mRNA length which matches that determined by blotting analysis.

The EGF-like domains ultimately have extracellular destinations, either as secreted peptides or in extracellular regions of membrane-bound proteins. In addition to their initially identified roles as growth factors [for example, EGF and transforming growth factor–α (TGF-α)], EGF-like domains are found in mosaic proteins that function in diverse biological processes and usually contain various other functional domains. However, several lines of evidence suggest that the SpEGF2 mRNA encodes a protein of relatively simple structure that is secreted into the blastocoel and whose function is mediated largely, if not entirely, by the EGF-like domains. (i) Suyemitsu *et al.* (*11*) recently reported the sequences of two of four peptides found in the blastocoel of embryos of a different sea urchin, *Anthocidaris crassispina*, which are similar to the SpEGF2 repeats (Fig. 1B). The similarity is greatest for peptide A, which has about 60% identity to repeats 2 and 3 of SpEGF2 and 64% to 68% similarity when conservative amino acid substitutions are included. This is a strong similarity in view of the time since divergence of the lines leading to these two sea urchin species [30 to 45 million years ago (*12*)]. EGF and TGF-α are only 55% identical in amino acid sequence, but bind to the same receptor with similar affinity (*7*). Furthermore, the peptides from *A. crassispina* have the same developmental effects on embryos of both genera. In contrast to their similarity with peptides of *A. crassispina*, the SpEGF2 repeats differ from the other EGF-related protein identified in *S. purpuratus*, uEGF1, which contains at least 20 relatively precise repeats of the EGF-like domain as well as other functional domains (*13*). Thus, it seems likely that *A. crassispina* peptides and SpEGF2 protein encode functionally equivalent products of homologous genes. (ii) The SpEGF2 protein includes a probable leader peptide but lacks a sequence suitable to serve as a transmembrane domain. (iii) At late blastula stage, SpEGF2 mRNA is concentrated at the basal sides of cells (*14*), a phenomenon we have observed for only one other mRNA, one that encodes an extracellular arylsulfatase (*15*). (iv) Other than the four EGF-like repeats and hydrophobic leader, the protein contains two stretches of 30 or fewer amino acids surrounding the EGF-like repeats, which do not correspond to any sequence in the National Biomedical Research Foundation protein data base. (v) Several other features

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