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Production of EGF-containing polypeptides in *Xenopus* oocytes microinjected with submaxillary gland mRNA

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The biosynthesis of epidermal growth factor (EGF), a 6045 dalton mitogen produced in the mouse submaxillary gland under androgen regulation, was studied using *Xenopus* oocytes. Microinjection of total, unfractionated gland mRNA together with [^{35}S]cysteine resulted in the production of a secretory polypeptide of ~9000 daltons, specifically immunoprecipitable with anti-EGF antibodies. A minor amount of a similarly immunoreactive 9000 dalton secretory polypeptide was produced from the sucrose gradient 9S fraction of gland mRNA. Other, more intensely labeled polypeptides, a cytoplasmic 125 000 dalton and a secretory 110 000 dalton protein were immunoprecipitated from oocytes injected with the >25S mRNA fraction. The biosynthesis of both can hardly be detected in oocytes injected with unfractionated mRNA. All three polypeptides are produced under androgen regulation and share common immunoreactive properties. Northern blot analysis using a 76 nucleotide synthetic EGF cDNA probe revealed hybridization with a single 28S mRNA species. This, and the apparent interrelation between the three polypeptides, suggest that a gland-specific processing protein, encoded by a 9S mRNA, is required to produce the 9000 dalton pro-EGF from the nascent translation product of EGF mRNA.

Key words: EGF/polypeptides/submaxillary gland/mRNA/*Xenopus* oocytes

Introduction

Epidermal growth factor [EGF, mol. wt. 6045 daltons] is a mitogen for various cell types *in vivo* and *in vitro* (for review, see Schlessinger *et al.*, 1983). EGF is produced in much higher quantities in the submaxillary glands of male compared with female mice (Byyny *et al.*, 1972). However, the amount of EGF produced in the submaxillary gland of female mice is increased by more than two orders of magnitude by treatment with androgens (Byyny *et al.*, 1974). EGF is stored and secreted from the gland in a high mol. wt. complex together with an arginine-esterase, the so-called EGF-binding protein (Burton *et al.*, 1978). This complex (mol. wt. 74 000 daltons) consists of two EGF molecules and two molecules of EGF-binding protein.

The biosynthesis of EGF has been studied by pulse-chase experiments, using cultured submaxillary glands (Frey *et al.*, 1979). A very low level of a biosynthetic precursor (pro-EGF) of mol. wt. 9000, having an extension at the C-terminal site, was found. Pro-EGF could be processed to EGF by addition of EGF-binding protein. These studies indicated that EGF mRNA is more scarce than may be expected from the high protein content (~1%) of EGF in the gland. As a lag-phase was observed in the labeling experiments before immunoreactive pro-EGF could be detected, the existence of a primary translation product of EGF, a pre-pro-EGF, which cannot be recognized by antibodies against native EGF, was postulated. The recent cloning of a male-specific cDNA corresponding to EGF mRNA (Gray *et al.*, 1983; Scott *et al.*, 1983) revealed a coding region for the synthesis of an EGF-containing 128 000 dalton protein, starting with a NH_2-terminal signal sequence, which indicates that it enters a secretory pathway in the cell. Both findings may explain why, up to now, there have been no reports on translation of EGF mRNA in any heterologous translation system.

We were not able to detect immunoreactive EGF in *in vitro* translation systems primed with submaxillary gland mRNA, either in the presence or absence of dog pancreas microsomes. We therefore assumed that both efficient translation and post-translational processing of the immediate translation product of EGF mRNA are required for the generation of a product which can be immunoprecipitated with anti-EGF antibodies. For these reasons, we selected *Xenopus* oocytes (Gurdon *et al.*, 1971; for recent review, see Lane, 1983) as a translation system. The oocytes efficiently translate microinjected heterologous mRNAs, perform correct post-translational processing, direct newly synthesized proteins into the correct subcellular compartment (Colman and Morser, 1979; reviewed in Lane, 1983), and export active secretory proteins into the incubation medium (Soreq *et al.*, 1982a; Miskin and Soreq, 1981). We now report that a single 28S mRNA species in the submaxillary gland can direct the androgen-regulated synthesis of three different proteins which are specifically immunoreactive with anti-EGF antibodies in microinjected *Xenopus* oocytes.

Results

Immunoprecipitation with anti-EGF antibodies from oocytes microinjected with unfractionated submaxillary gland mRNA

Since the 53 amino acid long EGF contains six cysteine residues (Gray *et al.*, 1983), we selected this amino acid for labeling. However, the oocytes contain a relatively large pool of endogenous cysteine (Bravo *et al.*, 1976) and hydrophobic cysteine residues tend to bind non-specifically to oocyte proteins. To circumvent these difficulties, *Xenopus laevis* oocytes were microinjected with poly(A)-containing mRNA from submaxillary glands, together with [^{35}S]cysteine (Koren *et al.*, 1983).

Extracts and incubation media of microinjected oocytes were immunoprecipitated with anti-EGF antibodies and protein-A Sepharose. About 0.2% of total labeled proteins from extracts and 2% from the incubation media were specifically immunoprecipitated. The accumulation of newly synthesized immunoreactive material in extracts of oocytes takes place mainly during the first 5 h of incubation; however, accumu-

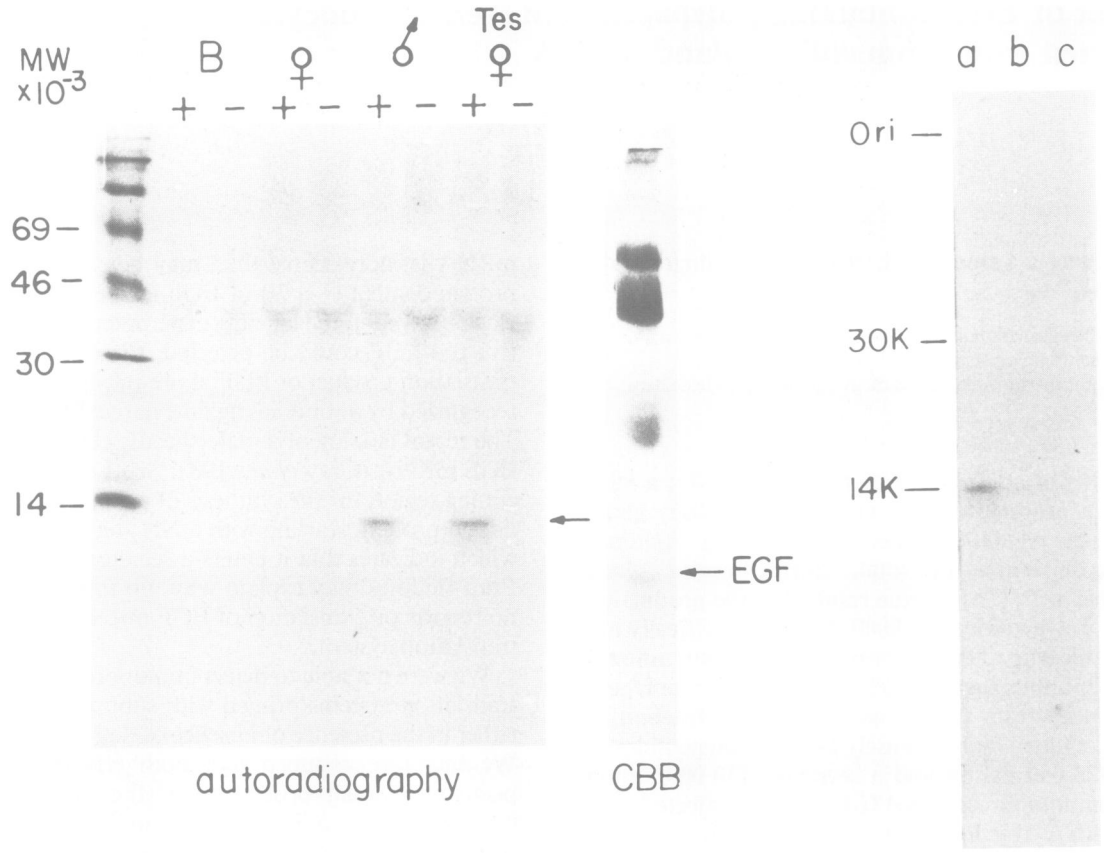

Fig. 1. Specific immunoprecipitation of pro-EGF from microinjected oocytes. Oocytes were injected with 50 nl Barth medium or total poly(A)$^+$ RNA (1 μg/ul) from the submaxillary glands of female (♀), male (♂) or testosterone-treated female (Tes ♀) mice, together with ~2 μCi [^{35}S]cysteine, and incubated for 10 h. Fluorograms of proteins immunoprecipiated with anti-EGF antibodies (+) or control antibodies (−) are presented (exposure 2 days). CBB: Coomassie brilliant blue stain of gel shown in the right lane. 5 μg EGF (marked by arrow) was added to this sample prior to separation. Arrow indicates positions of EGF (lower) and specifically immunoprecipiated pro-EGF (higher). The apparent mol. wts. differ by ~3000 daltons. **Right:** immunoprecipitation analyzed as above, from the incubation medium of oocytes injected with RNA of ~9S. Reaction was with anti-EGF antibodies **(a)**, anti-EGF antibodies and 2 μg cold EGF **(b)**, and control antibodies **(c)**.

lation continued in the medium between 5 and 10 h. We therefore routinely incubated the oocytes for ~10 h after microinjection. Analysis of precipitates by gel electrophoresis and fluorography showed specific precipitation of a low mol. wt. protein and ~90% of the precipitation was inhibited with excess cold EGF (Figure 1). Oocytes injected with Barth medium did not contain immunoprecipitable material, and no precipitation was observed from mRNA-injected oocytes with preimmune rabbit antibodies.

The precipitated material did not migrate as a sharp band, but tailed to lower mol. wts. Similar behavior was observed for EGF (Figure 1; Rose *et al.*, 1975). The apparent mol. wt. of the material immunoprecipitated from oocyte extracts and media was ~3000 daltons higher than that of native EGF, and similar to the mol. wt. reported for nascent pro-EGF (Frey *et al.*, 1979). Furthermore, partial proteolysis of the gel-eluted immunoprecipitated material indicated that this 9000 dalton polypeptide contains proteolytic sites similar to those present in EGF (Burmeister, 1983). These findings suggest that oocytes produce and secrete pro-EGF (mol. wt. 9000 daltons) and are not able to process it any further. When oocyte extracts were fractionated on a Sephadex G50 column, the ^{35}S-labeled immunoprecipitable pro-EGF appeared shortly before the position of [^{125}I]EGF, as expected for pro-EGF. No precipitable material was found in the void volume of this column, where a high mol. wt. complex is expected to migrate (Burmeister, 1983). This chromatographic separation, and the appearance of non-processed pro-EGF, thus indicate that active EGF-binding protein was not present in the extracts.

Characterization of the mRNA species translatable into immunoreactive material

The EGF-content of the submaxillary gland is androgen-dependent (Byyny *et al.*, 1974). We therefore compared the level of EGF mRNA of female, male and testosterone-treated female mice (see Figure 1). No EGF-like material could be precipitated from oocytes microinjected with mRNA from female mice, whereas male and testosterone-treated female mice contained translatable EGF mRNA. mRNA from testosterone-treated female mice contained 2- to 5-fold the amount of EGF mRNA found in male mice, as judged by scanning of fluorograms from precipitated proteins following controlled pre-activation of the film (not shown). These results suggest that the androgen regulation of EGF in submaxillary glands takes place at the mRNA level.

To estimate the size of the mRNA species producing the immunoprecipitable polypeptide, sucrose gradients of dimethylsulfoxide-denatured poly(A)-containing mRNA from the submaxillary glands were run. *In vitro* translation of sucrose gradient fractions revealed that cysteine-rich proteins with mol. wts. between 10 000 and 30 000 were mostly en-

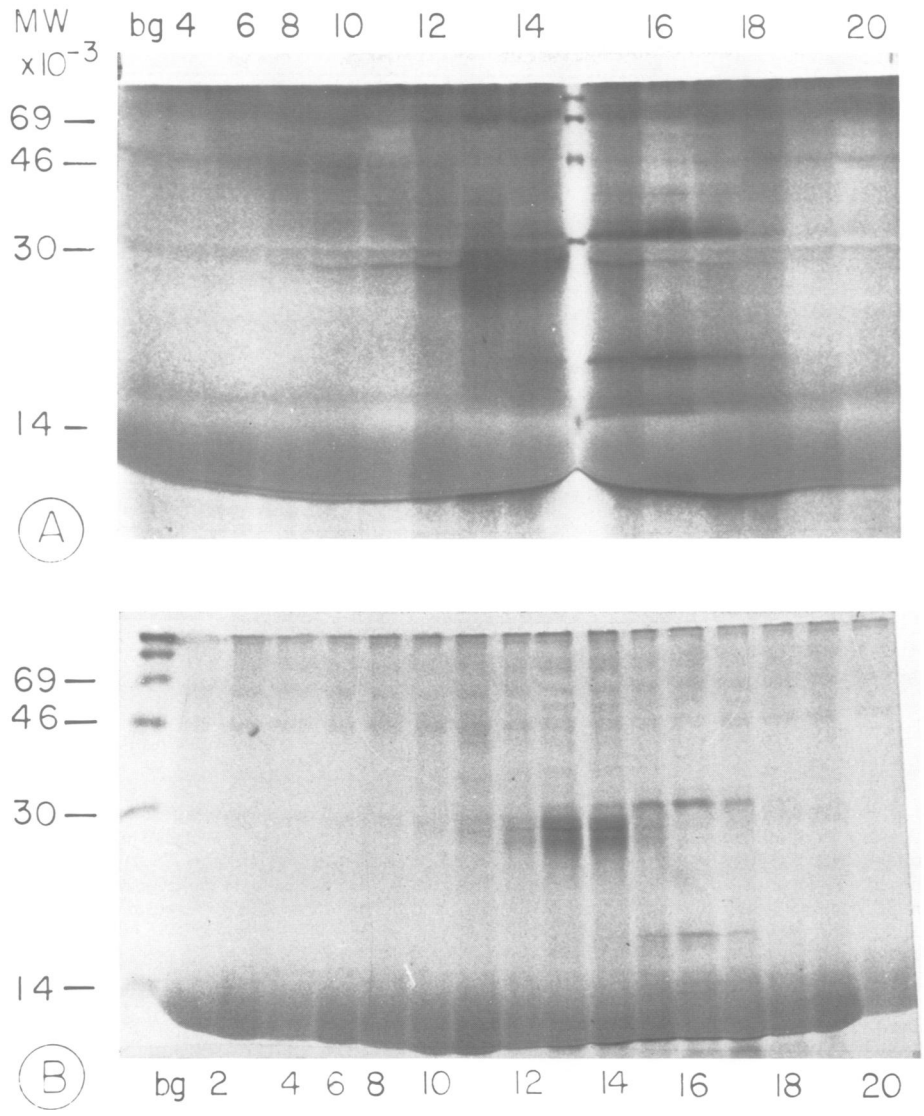

Fig. 2. *In vitro* translation products of sucrose gradient-fractionated mRNA. 2.5% of each fraction of a sucrose-gradient from poly(A)$^+$ RNA from male submaxillary glands was translated (Soreq *et al.*, 1982b) in reticulocyte lysate in the presence of 27 μCi of [^{35}S]cysteine and 27 μCi of ^{3}H-labeled amino acid mixture/12.5 μl assay. 3 μl were subjected to polyacrylamide gel electrophoresis. **(A)** Fluorography. **(B)** Autoradiography. bg = reticulocyte own background incorporation. Numbers refer to fractions of the sucrose gradient.

coded by mRNAs migrating as 7–12S (slots 12–14, Figure 2). However, high mol. wt. cysteine-containing polypeptides, of the range expected from the cloned EGF mRNA (Gray *et al.*, 1983), were preferentially produced by mRNAs migrating as 25–28S mRNA (slots 2–6, Figure 2). Each of the separated fractions was therefore injected into oocytes, and the level of newly synthesized immunoprecipitable material was monitored. Immunoprecipitable radioactivity was detected in two fractions of RNA, migrating as ~28S and 9S, respectively, from glands of both male mice and testosterone-treated female mice. In both sources, no immunoreactivity was detected in fractions containing mRNA of sizes in the range 11–20S.

High mol. wt. mRNA producing polypeptides immunoprecipitable with anti-EGF antibodies

When mRNA migrating higher than 25S was injected into oocytes, a strong double band of mol. wt. 125 000/110 000 was precipitated with anti-EGF antibodies from the extracts. Apparently, only the lower 110 000 dalton band was present in the incubation medium of oocytes. Small amounts of low mol. wt. (9000) immunoprecipitated material were also detected in these gels. This material co-migrated with the immunoprecipitable polypeptide as translated from total poly(A)$^+$ RNA. Both the 9000 and the 110 000 immunoreactive proteins were detected by immunoprecipitation in extracts as well as in the incubation medium of oocytes injected with high mol. wt. mRNA. Secretion was a specific and selective process, as indicated by the finding that the 125 000 dalton immunoreactive protein was apparently not secreted (see Figure 3). The precipitation of both the 125 000 and the 110 000 polypeptides was androgen-dependent (Figure 3) and was inhibited by an excess of cold EGF (Figure 4).

When total poly(A)-containing mRNA was injected, immunoprecipitation of the high mol. wt. double band was hardly detectable and the 9000 dalton polypeptide was the major protein to be precipitated (Figure 3), even after very short incubation periods (not shown). This observation might indicate that the 125 000/110 000 dalton proteins are efficiently translated but rapidly processed to yield the 9000

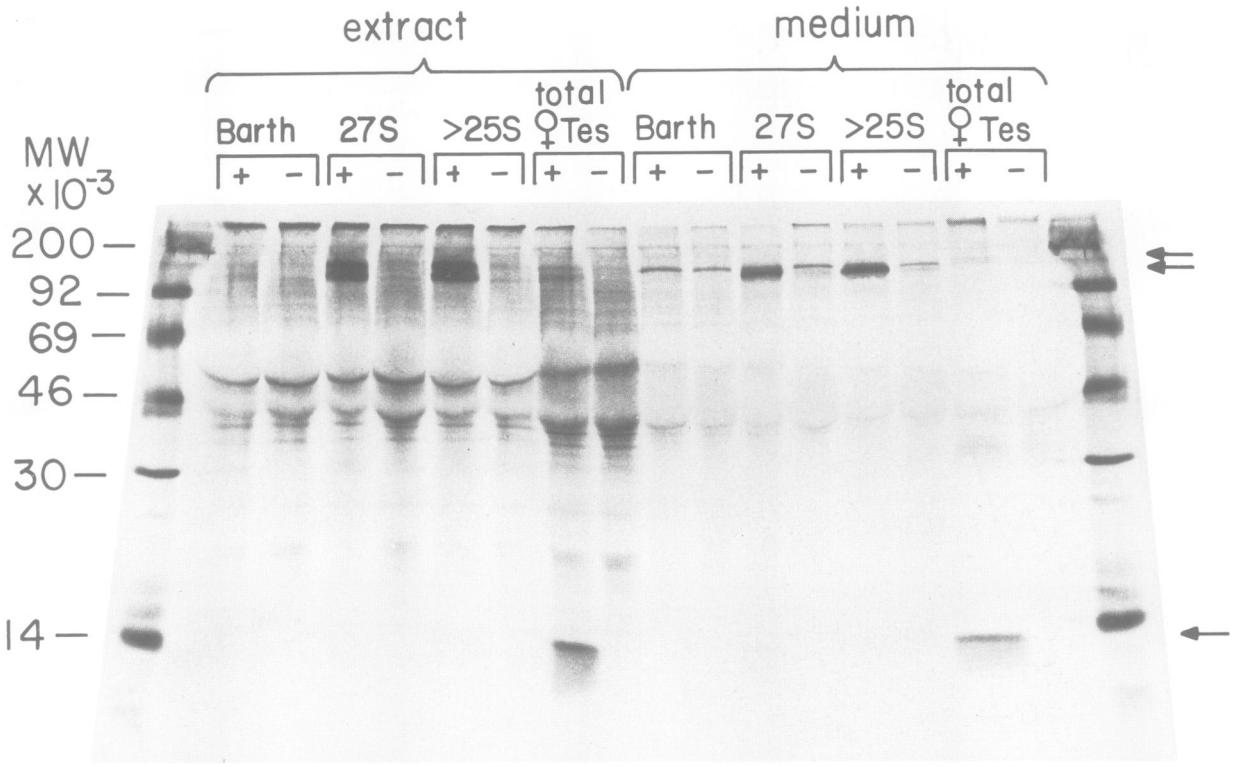

Fig. 3. Three distinguishable proteins immunoreactive with anti-EGF antibodies are produced in gland mRNA-injected oocytes. Oocytes were microinjected with [^{35}S]cysteine and mRNA as described in Figure 1. Poly(A)$^+$ mRNA injected was from the submaxillary glands of testosterone-injected female mice (Tes ♀); one sucrose gradient fraction containing RNA larger than 25S from male mice (>25S) or a fraction of ~27S from testosterone-injected female mice (27S). Barth medium was injected as control. After 10 h incubation, extracts and media were treated with anti-EGF antibodies (+) or control antibodies (−). Precipitates were analyzed on SDS-polyacrylamide gel electrophoresis (10−22%) and fluorographed. An overexposed (7 days) fluorogram is shown, in order to visualize faint bands.

dalton protein in oocytes injected with total poly(A)-containing mRNA. Alternatively, it is possible that the ability of the 28S mRNA to be translated is highly increased by fractionation.

Characterization of the immunoprecipitated polypeptide directed by 9S mRNA

Microinjection of submaxillary gland 7−12S mRNA resulted in the formation of fast-migrating material, immunoprecipitable by anti-EGF antibodies, with similar properties to those of the 9000 dalton protein precipitated from oocytes injected with unfractionated mRNA; it could only be precipitated with anti-EGF antibodies; its precipitation was inhibited with excess cold EGF and upon electrophoresis; it tailed to lower mol. wts. Furthermore, the 9000 dalton polypeptide directed by the low mol. wt. mRNA accumulated in the oocyte incubation medium up to 10 h post-injection, similar to the immunoprecipitable material obtained with unfractionated mRNA (Figure 5A).

Fractions of mRNA migrating at ~9S, from glands of both male mice and testosterone-treated female mice, had most of this activity (Figure 5B). There was practically no precipitation in oocytes injected with 11−20S mRNA fractions. However, it should be noted that more extensive electrophoresis and longer exposure of the autoradiograms revealed that minor amounts of the immunoprecipitable 110 000 dalton polypeptide were also present in these oocytes (not shown), indicating that this mRNA fraction also contained minor amounts of heavier mRNA species.

Northern blot analysis of EGF mRNA

The EGF cDNA sequence (Gray *et al.*, 1983; Scott *et al.*, 1983) is complementary to a 28S mRNA species, encoding the synthesis of a 128 000 secretory protein. To test whether this is the only mRNA species in the submaxillary gland mRNA which could specify EGF-containing polypeptides, we prepared a synthetic [76 nucleotide] EGF cDNA probe, using the published cDNA sequence (Gray *et al.*, 1983). This probe was radioactively labeled and hybridized to 'Northern'-blotted mRNA from the different sucrose gradient fractions (Figure 6). It hybridized, as expected, to a 4700 nucleotide long mRNA species present in unfractionated poly(A)$^+$ RNA and enriched in the heavier gradient fractions. A positive, although faint, hybridization with this 28S mRNA could also be seen in the faster sedimenting fractions, indicating an incomplete fractionation of the mRNA. There was no hybridization at all with poly(A)-free RNA, or myeloma mRNA, corroborating the specificity of the hybridization reaction (Figure 6). This indicates that the 28S mRNA species is the only one encoding the formation of EGF-containing polypeptides in the submaxillary gland, and must therefore be responsible for the production of all of the three polypeptides which we detect in microinjected oocytes.

Discussion

Injection of mRNA from mouse male, or testosterone-treated female, submaxillary glands into *Xenopus* oocytes elicits the production of three different proteins precipitable with anti-

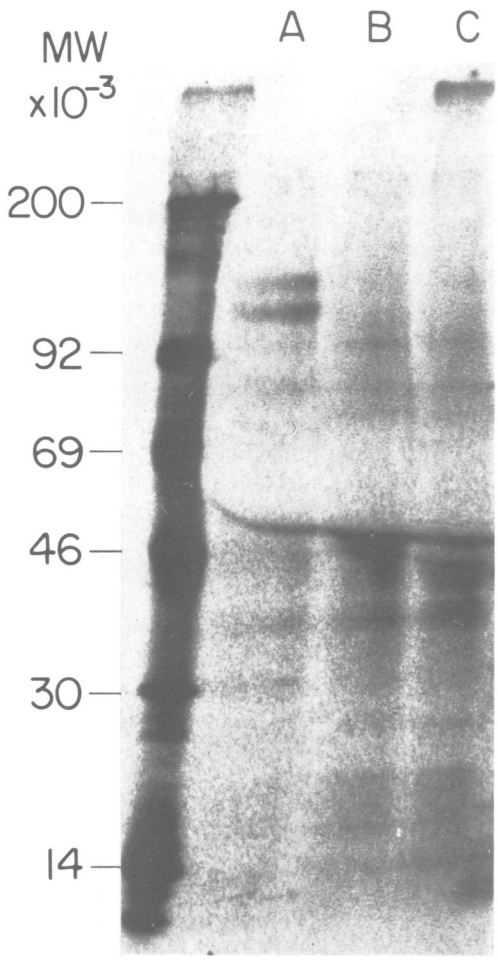

Fig. 4. Inhibition of immunoprecipitation of high mol. wt. EGF-containing proteins by excess cold EGF. Immunoprecipitation was performed from >25S mRNA injected oocytes as described in Figure 3. The precipitates were analyzed on a 5–15% polacrylamide gradient. The oocyte extracts had been treated with: anti-EGF antibodies **(A)**, anti-EGF antibodies together with 2 μg cold EGF **(B)**, control antibodies **(C)**.

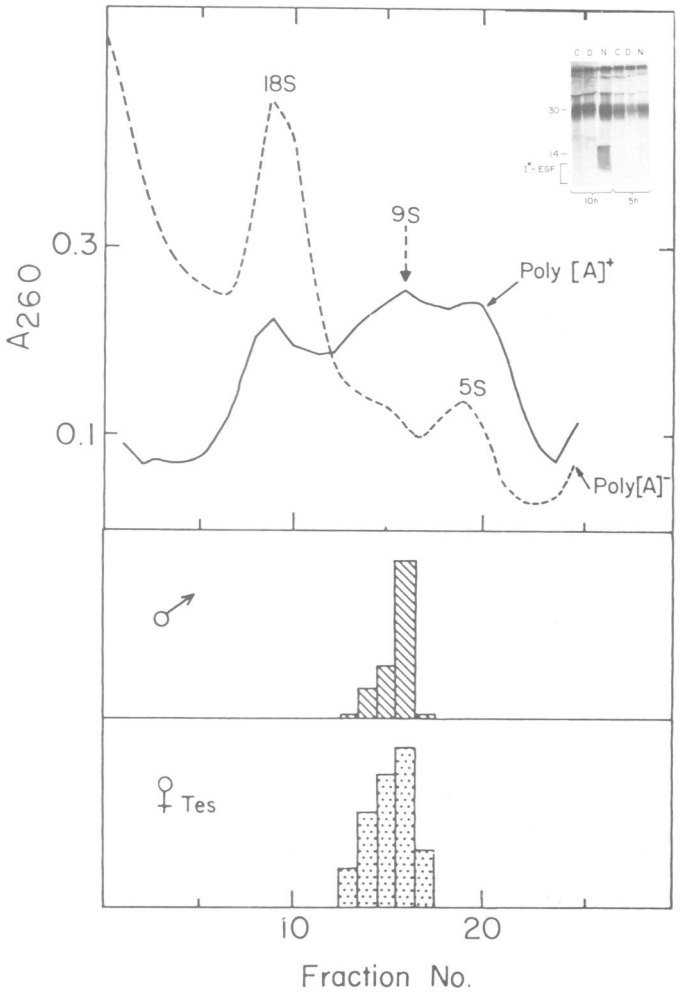

Fig. 5. Production of pro-EGF in oocytes injected with the 9S fraction of gland mRNA. Poly(A)-containing mRNA from the submaxillary glands of male mice was fractionated on a sucrose gradient. Optical density at 260 nm (line) was measured, and is compared with the OD of poly(A)$^-$ RNA run in parallel (dotted line). mRNA (0.8 μg/μl) of fractions 13–17 was injected into oocytes together with 50 μCi/μl [^{35}S]cysteine. Extracts and media were subjected to immunoprecipitation with anti-EGF antibodies. Immunoreactive material was quantitated by scanning of underexposed fluorograms and integration of peaks of pro-EGF by weighing. **Upper histogram:** immunoreactivity of fractions from the gradient prepared from male mice, whose profile is shown above; **lower histogram:** immunoreactivity of fractions from a similar gradient, prepared from testosterone-treated female mice. **Inset:** pooled RNA from fractions 13–17 was injected as above. Extracts of oocytes incubated for 5 and 10 h, respectively, were subjected to immunoprecipitation with anti-EGF antibodies (N), with irrelevant antibodies (D), or without antibodies (C). Electrophoresis was in 10–22% gel. Exposure was for 15 days.

EGF antibodies of 125 000, 110 000 and ~9000 daltons; the latter two species are secreted from the oocyte. All three proteins share common immunogenic properties and their synthesis is commonly regulated by androgens. Furthermore, there appears to be only one EGF mRNA species in the gland. All this suggests that the three immunoreactive proteins are interrelated.

The 9000 dalton protein was the main EGF-containing polypeptide produced in oocytes injected with unfractionated poly(A)$^+$ RNA. Based on SDS-gel analysis, column fractionation, specific immunoprecipitation and partial proteolysis, we believe it is pro-EGF. Since EGF cDNA hybridizes to a 28S mRNA species (Gray *et al.*, 1983, and Figure 6), we conclude that this pro-EGF is probably a processed product of a large precursor protein. The nucleotide sequence of EGF cDNA suggests that this nascent translation product should be a secretory protein of 1168 amino acids, such as the heavy immunoprecipitable protein(s) detected in oocytes that were injected with >25S gland mRNA. It is therefore most likely that there is a precursor-product relationship between the heavy and the light EGF-containing proteins produced in the oocytes.

The 125 000 dalton pre-pro-EGF is probably rapidly processed in the gland, which might explain why it is not recognized by anti-EGF antibodies in *in vivo* labeling experiments (Frey *et al.*, 1979). That this is also the case in microinjected oocytes is suggested by the observation that the heavy immunoprecipitable proteins are hardly detectable in oocytes injected with unfractionated gland mRNA, even after a very short incubation. Thus it appears that an additional androgen-regulated protein(s), encoded by other mRNA species, is necessary to direct the processing of the nascent translation product of EGF mRNA to yield the secretory pro-EGF sequences.

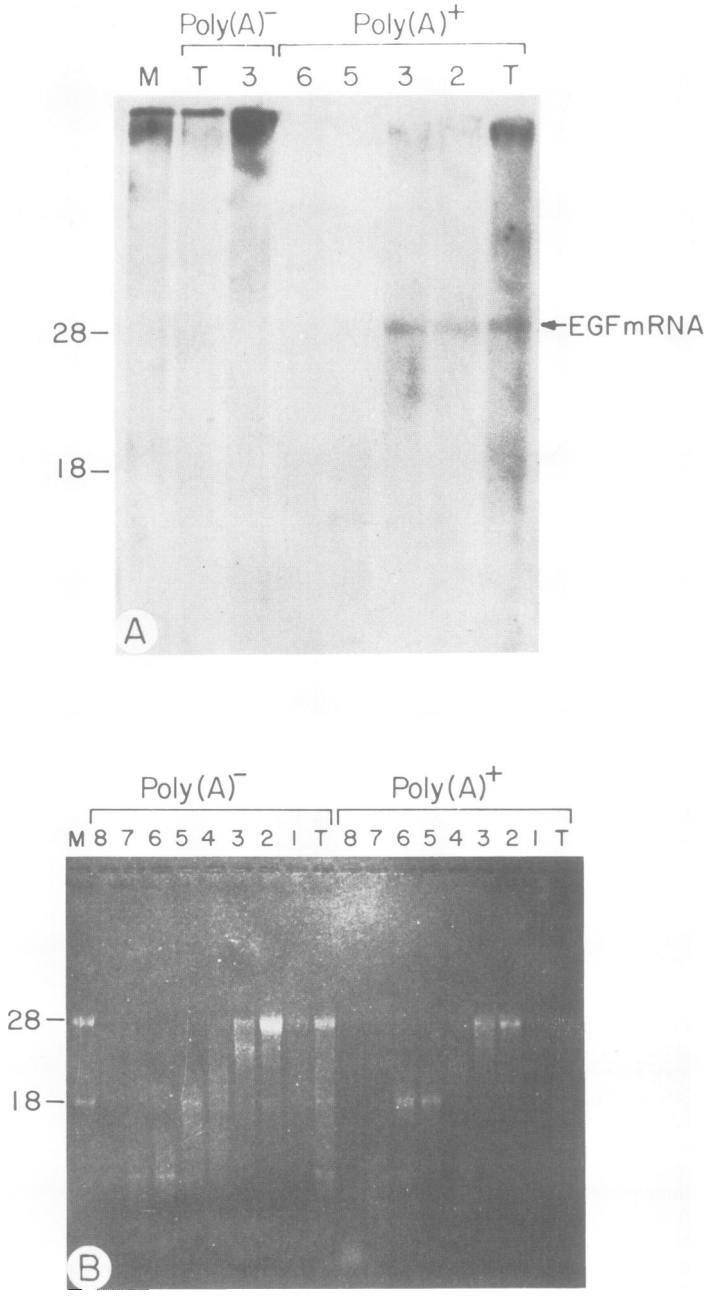

Fig. 6. Northern blot analysis of fractionated gland mRNA, using an EGF cDNA probe. Polyadenylated RNA from male submaxillary glands was fractionated by sucrose gradient centrifugation and ethanol precipitated. 10 μg RNA from each fraction were separated by agarose gel electrophoresis. RNA gel was blotted onto nitrocellulose filter and hybridized with an EGF cDNA probe. All procedures were as detailed in Materials and methods. **(A)** Autoradiograph of hybridized RNA. Poly(A)$^+$ RNA samples included: T, unfractionated poly(A)$^+$ RNA; and 2,3,5,6-RNA size-fractions from sucrose gradient, sedimenting as >28S, 26–28S, 18–21S and 8–12S, respectively. Poly (A)$^-$ RNA samples were: 3, fraction of poly(A)-free salivary gland mRNA, which sedimented as 26–28S on a parallel gradient; and T, unfractionated poly(A)-free salivary gland mRNA. An additional sample, designated M, included poly (A)$^+$ myeloma mRNA and served as a negative control. Note the presence of positively hybridized EGF mRNA migrating as 28S throughout the gradient of poly(A)$^+$ RNA and its absence in poly(A)$^-$ RNA and in myeloma RNA. **(B)** Ethidium bromide staining of RNA gel. The same gel presented in **A** was stained with ethidium bromide and photographed prior to blotting of the RNA. The photograph includes all of the slots presented in **A**, with similar designations, as well as additional slots which are not presented in **A**. Note the different migration of size-fractionated RNA samples.

What is the size of this putative mRNA species? This should be deduced from size fractionation of gland mRNA. Our Northern blot analysis reveals that each of the sucrose gradient fractions also contains a minor amount of the 28S EGF mRNA. The microinjected RNAs therefore represent mixed populations, composed of specifically enriched size groups together with some EGF mRNA. Enrichment of the mRNA-directed processing activity should thus result in the formation of processed pro-EGF. This is, indeed, the most likely explanation of our finding that some pro-EGF can be immunoprecipitated from oocytes injected with the 9S fraction of gland mRNA. In total, unfractionated mRNA samples there is a higher concentration of 28S mRNA and a relatively lower concentration of the mRNA species coding for the processing activity. This therefore becomes the rate-limiting element, and a minor amount of the 125 000 dalton polypeptide remains unprocessed in oocytes injected with total gland mRNA. On the other hand, there is almost no 9S mRNA in the heavy (>25S) fractions. The nascent chain therefore remains mostly unprocessed to pro-EGF in oocytes injected with heavy gland RNA.

The 110 000 dalton protein represents, probably, a partially processed secretory form of the 125 000 polypeptide, as indicated from its presence in the incubation medium. It thus appears that several post-translational processing events must be involved in the formation of secretory mature EGF from the nascent translation product of EGF mRNA. The first processing step, which results in the formation of the 110 000 dalton protein, appears to be necessary for translocation into the oocyte secretory vesicles. The next step, production of pro-EGF chains, should therefore take place within these vesicles. This implies that the 9S mRNA should also direct the production of a secretory protein, which would interact with the 110 000 dalton chains within the oocyte vesicles. It appears that the oocyte's ability to perform the first processing step (125 000 to 110 000) is rather limited, since it only takes place partially. Indeed, several reports refer to the limited capacity of oocytes to perform various post-translational processes (reviewed by Lane, 1983). The cleavage into pro-EGF, on the other hand, is indicated by our findings to be completely tissue specific, since it requires the co-injection of 9S gland mRNA. Further processing events, leading to the production of mature EGF in a high mol. wt. complex, cannot be performed in the oocyte at all, under our experimental conditions. This might imply that these require additional proteins, which are, perhaps, produced outside the gland.

Materials and methods

Animals

Mice (BALB/c) were obtained from the Weizmann Institute breeding center. Wherever stated, female mice were injected s.c. daily for 9 days with 1 mg testosterone-propionate in 0.1 ml sesame oil.

Antisera

Anti-EGF antibodies were prepared in rabbits and the γ-globulin fraction was purified by precipitation with ammonium sulfate. The solution contained 15 mg/ml protein. Similarly treated normal rabbit antibodies were used for control.

EGF

EGF was purified from the submaxillary glands of adult male mice by the method of Savage and Cohen (1972).

Preparation of poly(A)-containing RNA

5–10 g frozen salivary glands (from 50–150 animals depending on sex, hormone treatment and age) were used per preparation, which was performed as

reported (Soreq *et al.*, 1982b). Usually 1 – 3% of the total RNA was recovered as poly(A)$^+$, which was up to 1 mg/10 g tissue.

Sucrose gradient centrifugation of RNA

Sucrose gradient centrifugation was performed under partially denaturing conditions. 150 – 500 μg RNA were precipitated in 75% ethanol/0.3 M NaCl at – 20°C. The RNA was suspended in 60 μl dimethylsulfoxide and incubated for 20 min at 37°C. 10 μl sterile water was added to resolve RNA, which was then kept for another 20 min at 37°C. Water was added to a final volume of 250 μl. Gradient centrifugation was performed essentially according to Schechter (1975). A linear gradient of 15 – 30% sucrose in 5 mM Tris/HCl, 0.5 mM EDTA, 0.1% SDS was prepared in 15 ml nitrocellulose tubes. RNA was carefully loaded on the gradient, and centrifuged for 20 h at room temperature at 37 000 r.p.m. in a Beckmann SW 41 rotor. Poly(A)$^-$ RNA was run as size marker in parallel. Fractions of ~0.45 ml were collected and optical density at 260 nm was determined.

Injection into oocytes

Adult *X. laevis* females (South African Snake Farm, Windshoek, South Africa) were anesthetized by cooling in ice, ovarian lobes were removed, and individual ooctyes dissected. 50 μCi ^{35}S-labeled cysteine (~1000 Ci/mmol, Amersham Radiochemical Centre) per 1 μl of injection solution was lyophilized and dissolved in RNA solution or Barth medium (Gurdon *et al.*, 1971) shortly before injection. Stage 6 oocytes were microinjected with 50 nl/oocyte of RNA (1 μg/μl) or the same amount of Barth medium. Oocytes were incubated in groups of 10 at 19°C in 100 μl Barth medium containing 0.23 TIU/ml trasylol, 10 μg/ml soybean trypsin inhibitor, 2 x 10^{-3} M 6-aminocaproic acid, 5 x 10^{-4} M leupeptin and 10^{-4} M phenylmethylsulphonylfluoride (PMSF) to prevent proteolytic degradation by oocyte proteases (Soreq and Miskin, 1981). After incubation, oocytes and medium were frozen separately and stored at – 20°C.

Preparation of oocyte extracts

Ten oocytes were homogenized in 100 μl Barth medium containing protease inhibitors as above and layered into 200 μl nitrocellulose tubes over 50 μl of 1 M sucrose. On top of the homogenate, 50 μl H_2O were added. Centrifugation was performed for 15 min at 100 000 *g* in an air-driven centrifuge (Beckmann). The soluble phase was carefully taken out, avoiding the upper lipid phase. The lipid phase, when present during immunoprecipitation, severely increases non-specific precipitation.

Immunoprecipitation

We optimized the procedure by adding [^{125}I]EGF to *in vitro* labeled oocyte extracts. The procedure finally adopted (described in Burmeister, 1983) is a modification after Opdenakker *et al.* (1982). Equal samples (40 – 80 μl) of extracts or medium of microinjected oocytes were used for specific reaction with anti-EGF antibodies and for control samples with normal rabbit antibodies. After pre-absorption with normal rabbit antibodies to reduce the non-specific background, the samples were reacted with 45 μg anti-EGF antibodies or control antibodies. When inhibition by cold EGF was checked, 2 μg of EGF were added prior to immunoprecipitation. The immunocomplex was bound to 2.5 mg Protein A-Sepharose (Pharmacia) and washed thoroughly. The specifically bound proteins were then eluted at 100°C with sample buffer containing 3% SDS and 0.5% mercaptoethanol. Samples were taken for determination of radioactivity in a beta-scintillation counter. At least 70% of [^{125}I]EGF was recovered using this procedure and background, by non-specific sticking, was negligible (<0.1%). Although unlabeled L-cysteine was present in all buffers, some non-specific absorption of L-[^{35}S]cysteine to higher mol. wt. proteins was observed. It was not observed when media of oocytes were used in which no free labeled cysteine was present (Figure 1, right).

Gel analysis

Gel analysis was performed on slab gels in a discontinuous buffer system as introduced by Laemmli (1970). The stacking gel was 3% acrylamide (stock 30% acrylamide, 0.8% bisacrylamide), and the separating gel was a linear gradient of 10 – 22%. ^{14}C-Labeled protein mixture (Amersham) was used as mol. wt. markers. Gels were stained in 50% methanol, 7% acetic acid containing 0.1% Coomassie brilliant blue R and destained in 20% methanol, 7% acetic acid. Gels were fluorographed with 'Enlightening' (NEN), vacuum dried and exposed with pre-flashed Kodak X Omat films.

Quantitative analysis of gels

Underexposed pre-activated fluorograms were scanned in a Beckmann gel scanning device or in a Gilford spectrophotometer. Relevant peaks were integrated by weighing.

Northern blot analysis of gland mRNA using labeled EGF cDNA sequence as a hybridization probe

10 μg of RNA were loaded per slot. RNA was ethanol precipitated, dissolved in 20 μl of sample buffer and heated to 60°C for 10 min prior to loading. Electrophoresis (100 V, 4 h) was in horizontal 1% (0.5 cm thick) agarose gels.

Running buffer (MEN) was 20 mM MOPS (Sigma M 12S4), 5 mM Na acetate and 1 mM EDTA, in a final pH of 7.0. Sample buffer contained 50% deionized formamide (run twice through an amberlite column), 6% formaldehyde, 10% glycerol and some bromophenol blue in MEN buffer. Gel solution contained 1% agarose, 6% formaldehyde and 0.5 μg/ml ethidium bromide in MEN buffer. Following electrophoresis, gels were blotted overnight onto nitrocellulose, using 10 x SSC as the transfer solution. Filters were then soaked for 4 h at 42°C in pre-hybridization buffer (50% formamide, 20 mM potassium phosphate buffer, pH 6.8, 100 μg/ml denatured *E. coli* DNA, 5 x Denhardt solution and 6 x SCC). For hybridization, we used 50 – 100 ng of nick-translated, pre-boiled hybridization probe. This was a 76-bp double-stranded fragment, synthesized (D. Segev, A. Avivi, J. Schlessinger and D. Givol, unpublished results) according to the published EGF mRNA sequence (Gray *et al.*, 1983) and corresponding to the 22nd (arginine) up to the 45th (histidine) amino acids of the EGF protein. Hybridization was in 4 ml of pre-hybridization buffer for an 8-slot filter. Incubation was for 18 h at 42°C. Filters were then washed twice for 20 min each time, in 3 x SSC and 0.1% SDS at 65°C, dried and exposed.

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