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Detection of epidermal growth factor-urogastrone and its receptor during fetal mouse development*

(palate development/fetal growth factor/receptor programming)

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ABSTRACT **Using both a radioimmunoassay and a radioreceptor assay, we have estimated the content of mouse epidermal growth factor-urogastrone (EGF-URO) in fetal mice at 11½ to 17½ days of gestation. Concurrently, the amount of specific EGF-URO receptor binding was determined in crude membrane fractions from the embryos. EGF-URO receptor binding is readily detected in membranes from the youngest embryos (day 11½) and rises steadily up to parturition (18 days); the rise is more marked in embryo membranes derived from a potential target tissue, such as the maxilla and secondary palate. In the embryonic extracts, EGF-URO proved to be labile, requiring the presence of soybean trypsin inhibitor and sodium azide to stabilize the recovery of added EGF-URO in test samples. Even with added stabilizing agents, immunoreactive EGF-URO was barely detectable before day 14½ (less than 20 fmol per embryo), whereas a substantial increase was observed from day 15½ to 17½ (from 70 to 200 fmol per embryo). In contrast, the radioreceptor assay detected appreciable amounts of an EGF-URO-like substance at 11½ days (50 fmol per embryo); the values estimated by radioreceptor assay (about 10-fold higher than by radioimmunoassay) also increase markedly between days 15½ and 17½ (on average from 500 to 3000 fmol per embryo). We conclude that during fetal mouse development there is an increase both in the receptors for EGF-URO and in a substance (presumably a fetal growth factor) that can occupy the receptor. The differences between the radioreceptor and radioimmunoassay estimates for the EGF-URO content suggest that the fetal form of mouse EGF-URO differs from the adult molecule.**

Epidermal growth factor-urogastrone (EGF-URO) is a polypeptide of approximately 6000 daltons that is found in mice, in humans, and undoubtedly in a variety of other mammalian species (for reviews, see refs. 1–6). The recognized biological actions of EGF-URO are stimulation of cellular proliferation and inhibition of gastric acid secretion; in addition, the occurrence of receptors for EGF-URO in a large number of tissues, including the placenta (6, 7), and the effects of EGF-URO observed in organ cultures on the secondary palatal epithelium (8, 9) suggest that EGF-URO plays an important role in embryonic development. Although the time course of the postnatal accumulation of EGF-URO in male mice has been observed [the submaxillary gland content rises to a maximum at about 50 days (10)], no detailed studies have been made in prenatal animals. Because of our interest in the effects of EGF-URO on palatal development, we have determined the content of both EGF-URO and its receptor at various stages of development. We have used both a radioreceptor and a radioimmunoassay to measure increasing levels of EGF-URO in embryos at 11½ to 17½ days of gestation, and we have concurrently measured increases in EGF-URO binding in membranes obtained from embryos at 12 to 14 days of gestation.

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MATERIALS AND METHODS

Polypeptides and Antibodies. Mouse EGF-URO was either isolated from frozen male mouse submaxillary glands as described (11) or purchased from Collaborative Research (Waltham, MA). Radiolabeled peptide (about 250–450 cpm/pg; 85% counting efficiency) was prepared with carrier-free ^{125}I (Amersham/Searle) as described (12). Rabbit anti-EGF-URO antibodies were either purchased from Collaborative Research or raised in male albino rabbits by repeated subcutaneous injections with complete (first injection) or incomplete (all subsequent injections) Freund's adjuvant at 3-week intervals until a plateau of serum antibody was reached (50% of label bound at about 1:20,000 dilution of serum).

Radioimmunoassay. The assay, performed essentially by a routine method is, in brief, as follows: Embryo extract (100 μl) or standard EGF-URO solution (100 μl) was mixed with 100 μl of ^{125}I-labeled EGF-URO (^{125}I-EGF-URO) (about 100,000 cpm) in 0.1 M sodium phosphate buffer, pH 7.4, and 100 μl of diluted antiserum (produced by M. Hollenberg; 1:20,000 dilution vol/vol with 0.1 M sodium phosphate buffer, pH 7.4) was then added. After 1 hr at 23°C, 100 μl of diluted normal rabbit serum (1:60 dilution with 0.1 M phosphate buffer, pH 7.4) and 100 μl of diluted (1:5 vol/vol with phosphate buffer) goat anti-rabbit IgG (Miles) were added and the samples (total volume, 400 μl) were incubated overnight at 4°C. The immunoprecipitate was collected and washed on Millipore EGWP (pore size, 0.2 μm) filters for measurement of radioactivity. The concentrations of EGF-URO in the unknown solutions were estimated from standard curves determined for each assay.

Radioreceptor Assay. The assay, outlined elsewhere (13), is briefly as follows: Human placental membranes (about 10 mg of protein per ml in 25 mM Tris·HCl, pH 7.4) isolated by differential centrifugation after Polytron (Brinkmann) homogenization (14, 15) were diluted 1:20 vol/vol with a 0.1 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.1% wt/vol bovine serum albumin. An aliquot of the diluted membrane suspension (100 μl) was added to a mixture composed of 100 μl of ^{125}I-EGF-URO (about 100,000 cpm) in the phosphate buffer and either 100 μl of embryo extract or 100 μl of a standard solution of unlabeled EGF-URO in phosphate buffer. After 60 min at 24°C, the membranes were collected and washed on Millipore EGWP (0.2 μm pore size) filters for measurement of bound radioactivity. Standard curves, from which the concentrations of unknowns were estimated, were determined for each assay.

Abbreviation: EGF-URO, epidermal growth factor-urogastrone.

* Some of these data have been presented in preliminary form at the 1978 18th Annual Meeting of the American Society for Cell Biology (19).

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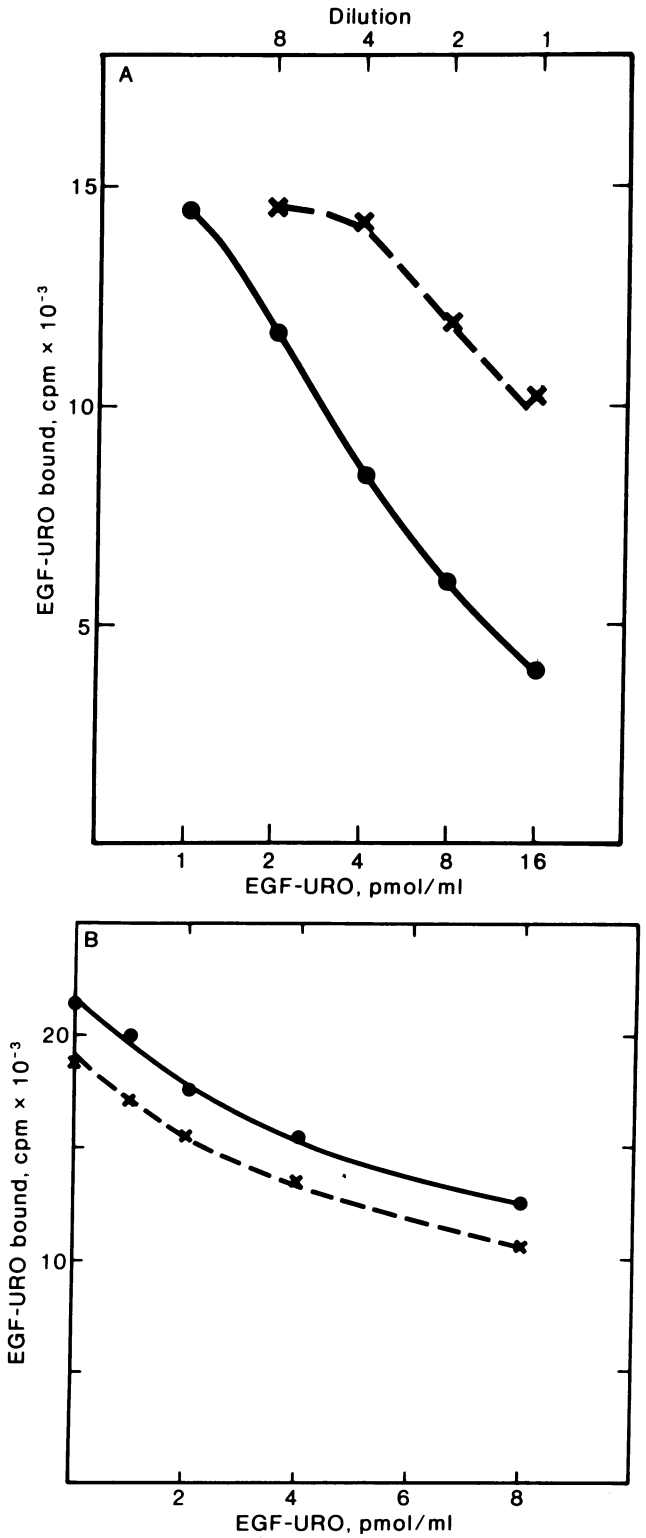

FIG. 1. Radioreceptor assay of EGF-URO. (*A*) Binding competition exhibited by an extract of day-13 embryos (1.56 embryos per ml) was measured at the indicated dilution (✕) and compared with the binding competition of pure mouse EGF-URO (●). (*B*) The binding competition curve for pure mouse EGF-URO was determined in the absence (●) and presence (✕) of an extract of day-13 embryos (0.78 embryo per ml).

Extraction and Recovery of EGF-URO. NIH Swiss Webster mouse embryos were dissected free from adventitial tissue, placed on ice, quickly frozen, and stored at −20°C until used. In the initial experiments, embryos were extracted at 0°C in 50 mM acetic acid (pH approximately 4) by the procedure used routinely for recovery of EGF-URO from mouse submaxillary glands (11). However, both immunoassay and radioreceptor assay of extracts to which unlabeled EGF-URO was added revealed a marked (up to 90%) loss of both immunoreactive and receptor-reactive material, both at 0°C and upon storing extracted samples at −20°C. In contrast, extraction of embryos with 0.1 M sodium phosphate buffer, pH 7.4, containing sodium azide at 1 μg/ml and soybean trypsin inhibitor (Sigma) at 10 μg/ml resulted in complete recovery of added EGF-URO, as determined by both immunoassay and radioreceptor assay. Embryo extracts (about 300 mg/ml, wt/vol) were prepared in this buffer with 10 strokes of a tight-fitting manual glass homogenizer. The extract was clarified by centrifugation (20,000 × *g* for 30 min at 4°C) prior to assay.

Preparation of Membranes. Crude membrane suspensions were prepared by homogenization of either whole embryos (three or four embryos per 2 ml) or dissected maxillas in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, with the use of both loose and tight-fitting glass homogenizers. Aliquots of the suspension were used to measure EGF-URO binding, as described (12); membranes were harvested on Millipore EGWP filters (0.2 μm pore size) for the measurement of specifically bound radioactivity.

RESULTS

Quantitation of EGF-URO in Embryos. Despite our success in improving the recovery of added EGF-URO from embryonic extracts, considerable variation was encountered upon analyzing the EGF-URO content of individual embryos. In a series of five day-13 (day 0 is the day when vaginal plugs were found) embryos assayed with the radioreceptor assay, values ranged from 0.1 to 2.4 pmol per embryo. According to stringent criteria (16), we were able to establish that receptor-destroying substances were not present in the embryo extracts in amounts sufficient to affect the assay. Further, in the receptor assay, the EGF-URO displacement curve for the embryo extract was parallel to the curve for pure EGF-URO (Fig. 1*A*) and the standard curve, assayed in the presence of embryo extract, was shifted to the left by a constant amount (Fig. 1*B*). We therefore conclude that the embryo extracts do contain an EGF-URO-like substance and that the variability may be due to uncontrolled destruction of the endogenous EGF-URO prior to the addition of proteolysis inhibitors.

Because of the variation in analyzing individual embryos, groups of embryos of increasing gestational age were collected and extracted simultaneously; the average values (Table 1),

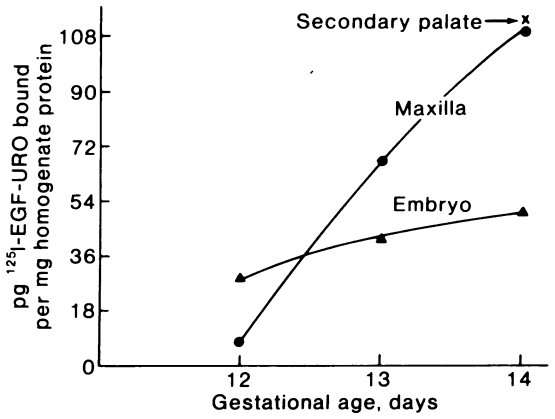

FIG. 2. Binding of ^{125}I-EGF-URO to membranes from homogenates of maxilla (●), secondary palate (✕), and whole embryo (▲).

Table 1. Embryonic levels of EGF-URO detected by radioreceptor and radioimmunoassay

Age, days	Embryo content, no./ml	Wet weight, g/ml	EGF-URO content					
			pmol/ml		pmol/embryo		pmol/g wet weight	
			RRA	RIA	RRA	RIA	RRA	RIA
11½	4.33	0.213	0.20	<0.02	0.05	ND	0.94	ND
12½	3.00	0.244	0.15	<0.02	0.05	ND	0.61	ND
13½	2.00	0.324	0.20	0.040	0.10	0.02	0.62	0.12
14½	0.83	0.262	0.20	0.045	0.24	0.05	0.76	0.17
15½	0.66	0.289	0.30	0.045	0.45	0.07	1.04	0.16
17½	0.33	0.325	1.00	0.065	3.03	0.20	3.08	0.20

ND, not detected; RIA, radioimmunoassay; RRA, radioreceptor assay.

while by no means precise, can be taken as indicative of the developmental trend. Until about day 13, we detected small amounts of EGF-URO in the extracts (10 or more embryos per extract). However, between days 13 and 17, there is a marked increase in EGF-URO, with the most dramatic rise occurring between about 15½ and 17½ days of gestation. A similar time course was observed in two independent series of embryos. Further, although the increase is shown in parallel qualitatively by both the radioreceptor and immunoassay, quantitatively the embryos appear to contain about 5- to 10-fold more receptor-reactive material than is detected by the immunoassay. Importantly, the identical standard solutions of mouse EGF-URO were used concurrently for both assays, done on the same embryo extract samples.

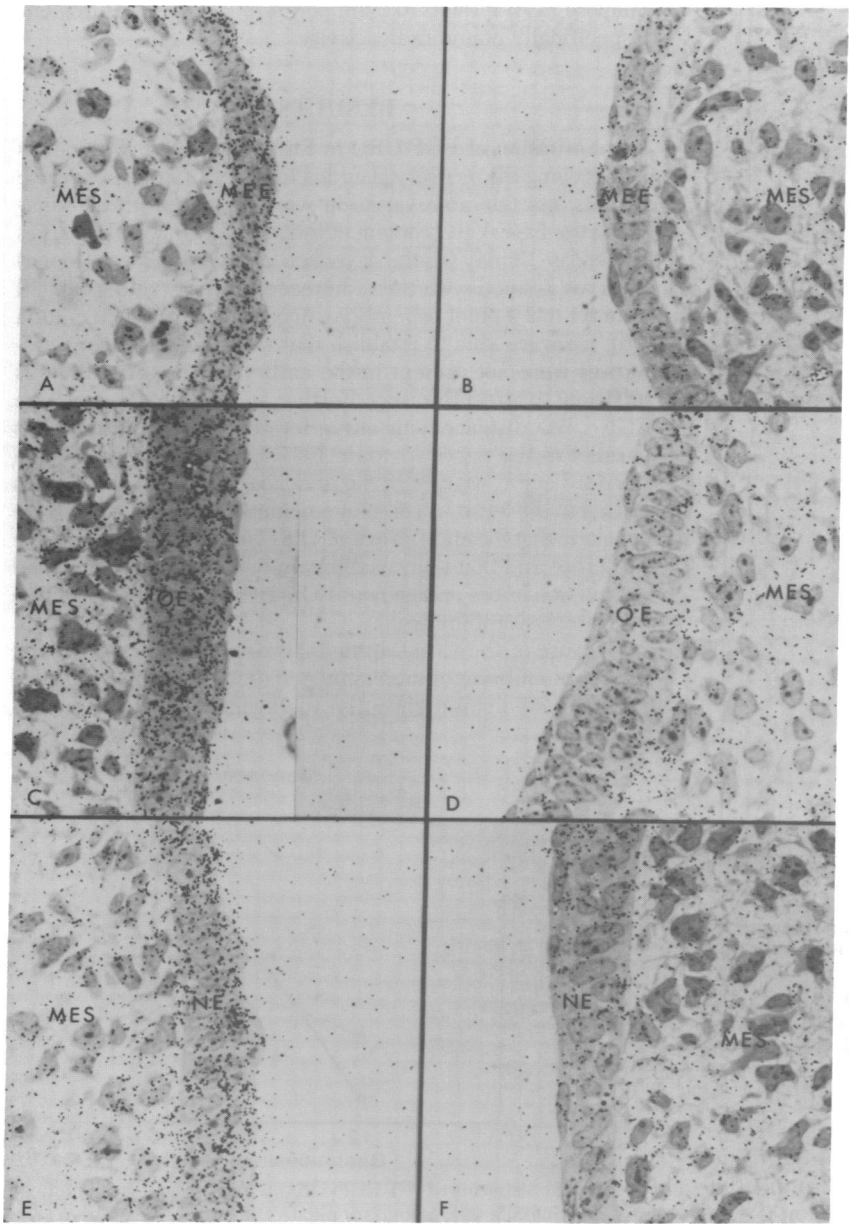

FIG. 3. Autoradiographic localization of ^{125}I-EGF-URO binding in the mouse secondary palate. Day-13 mouse palatal shelves were dissected and incubated in phosphate-buffered saline containing ^{125}I-EGF-URO (1 ng/ml) with or without unlabeled EGF-URO (1 μg/ml) for 45 min at 37°C. The palates were rinsed extensively in the buffered saline at 4°C, fixed in 2.5% (wt/vol) glutaraldehyde, pH 7.4, and embedded in plastic. Sections (1 μm) were cut and processed for autoradiography. (*A*, *C*, and *E*) ^{125}I-EGF-URO only; (*B*, *D*, and *F*) ^{125}I-EGF-URO plus unlabeled EGF-URO at 1 μg/ml. MEE, medial-edge epithelium; MES, mesenchyme; OE, oral epithelium; NE, nasal epithelium. (×300.)

Binding of EGF-URO by Embryo Membranes. Virtually all of the EGF-URO-binding activity in embryo extracts could be recovered in a crude membrane fraction (data not shown). The presence of EGF-URO receptor in the membrane was readily detected at a time when only minimal amounts of EGF-URO could be measured in the embryo extracts. Using the crude membrane preparations, we determined that neither binding to the membranes nor ligand destruction could account for the low levels of EGF-URO found in the extracts. When membranes from whole embryos were analyzed, the amount of EGF-URO binding present was observed to increase with gestational age; this increase appeared more dramatic when membranes from a potential target tissue (the maxilla) were analyzed (Fig. 2). The presence of specific binding sites for EGF-URO in the day-13 palatal epithelium was readily visualized by autoradiography of fixed replicate samples that had been exposed to ^{125}I-labeled mouse EGF-URO both in the presence and absence of unlabeled EGF-URO (Fig. 3).

DISCUSSION

The main finding of this study is that there is a time-dependent increase of both the EGF-URO receptor and an EGF-URO-like substance in mouse embryos during development. Because of the variability in the assays of EGF-URO in individual embryos, the exact correlation between embryonic EGF-URO levels and the content of receptor cannot be deduced. However, it is probable that there can be independent variations of EGF-URO serum levels and the tissue content of receptor. In particular, in a receptor-containing structure, such as the secondary palate, local variations in receptor content *per se* may play a regulatory role apart from any variations in peptide serum levels that may occur. Programmed receptor variations of this kind may provide a mode of cellular control apart from the homospecific hormone-mediated receptor down- and up-regulation that has been described in other systems (for a review, see ref. 17).

The source and exact composition of the EGF-URO extracted from the embryos are uncertain. Possibly, maternal EGF-URO could contribute to fetal levels via transplacental transport. However, maternal EGF-URO is equireactive in the radioreceptor assay and the immunoasssay. In contrast, the EGF-URO-like substance extracted from the embryo was much more reactive in the radioreceptor assay than in the immunoassay. This kind of reactivity is reminiscent of the sarcoma growth factor that can be isolated from mouse cells infected with murine or feline sarcoma virus (18); sarcoma growth factor is radioreceptor reactive in an EGF-URO assay but does not cross-react in an immunoassay. On the basis of our assays, we speculate that the fetal form of mouse EGF-URO may differ from the molecule present in the adult. The fetal form of EGF-URO, however, would be expected to be biologically active, because it can react with the membrane receptor.

In summary, we have described dynamic changes in both the EGF-URO receptor and the levels of an EGF-URO-like substance during an active phase of mouse embryonic development. In addition to our present findings, the occurrence of receptors for EGF-URO in a variety of organ systems as diverse as the liver, palate, and lens of the eye (summarized in ref. 6) may serve as evidence for the widespread influence that this polypeptide may have on fetal development. The exact function that EGF-URO may serve in the development of a number of individual organ systems remains an exciting subject for future work.

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