

Developmental Expression and CORT-Regulation of TGF- β and EGF Receptor mRNA During Mouse Palatal Morphogenesis: Correlation Between CORT-Induced Cleft Palate and TGF- β 2 mRNA Expression

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ABSTRACT Glucocorticoids (CORT) have been shown to induce cleft palate in mice. Although the pathogenetic pathway of CORT-induced cleft palate has been investigated for several decades, the molecular details remain to be elucidated. Since growth factors have been shown to regulate palate morphogenesis, and the expression of several growth factors or their receptors, e.g. TGF- β , EGF receptor (EGF-R), are known to be modulated by CORT, we postulate that CORT modulation of growth factor (or receptor) gene expression is a key mechanism involved in CORT-induced cleft palate. To test this hypothesis, we analyzed the steady-state levels (Northern and RNase protection) and developmental expression (in situ hybridization) of four CORT-responsive genes—TGF- β 1, TGF- β 2, TGF- β 3, and EGF receptor (EGF-R)—in developing mouse palates in the presence or absence of exogenous CORT. Pregnant B10.A dams were injected on day 12 of gestation with CORT or sham-injected and embryonic palates were collected at 1, 2, and 3 days postinjection (E13–E15). During mouse palate development, significant increases in TGF- β 1 and TGF- β 3 mRNA levels, as well as a significant decrease in TGF- β 2 mRNA levels, are detected; no significant difference in EGF-R transcript level is observed with progressive development. In CORT-exposed palates, we demonstrate no significant differences in the direction or magnitude of change with time in TGF- β 1, TGF- β 3, and EGF-R mRNA levels compared to controls. However, CORT delays by 1 day the down-regulation of palatal TGF- β 2 transcript normally seen on day 14 of gestation.

TGF- β 2 is known to inhibit cell proliferation. The level of TGF- β 2 mRNA, the only isoform primarily expressed in the palatal mesenchyme, significantly decreases with progressive palatal development; this down-regulation in TGF- β 2 expression is associated with increased mesenchymal cell proliferation and palatal shelf growth. CORT, at a critical

stage of palatogenesis, induces a delay in the normal down-regulation of TGF- β 2 gene expression. Given that CORT is known to inhibit mesenchymal cell proliferation and palatal shelf growth, we conclude that the CORT-induced delay in the normal down-regulation of TGF- β 2 gene expression is probably a key event in the pathogenesis of CORT-induced cleft palate. © 1996 Wiley-Liss, Inc.

Growth factors and their receptors, e.g., transforming growth factor- β (TGF- β) and epidermal growth factor (EGF), have been shown to be present in the developing palate (Pelton et al., '90; Shiota et al., '90; Linask et al., '91; Gehris et al., '91; Abbott et al., '92). In addition, numerous in vitro studies have demonstrated that growth factors modulate palatal cell proliferation, extracellular matrix production, and shelf fusion (Hassell, '75; Silver et al., '84; D'Angelo and Greene, '91; D'Angelo et al., '94; Brunet et al., '93, '95). Taken together, these observations suggest that growth factors play important morphoregulatory roles during in vivo palatal development.

Furthermore, it is well established that glucocorticoids (CORT) induce cleft palate in mice (Fraser et al., '54; Biddle and Fraser, '77; Melnick et al., '81a,b; Abbott et al., '92; Jaskoll et al., '95). CORT treatment has been shown to inhibit palatal mesenchymal cell proliferation, resulting in smaller palatal shelves and cleft palate (Mott et al., '69; Jelinek and Dostel, '75; Nanda and Romeo, '78; Salomen and Pratt, '79; Potchinsky et al., '96). Although the pathogenetic pathway of CORT-induced cleft palate has been investigated for several decades, the precise mechanism(s) involved have not

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been fully delineated. Since growth factors are known to regulate palate morphogenesis (Brunet et al., '93, '95; D'Angelo et al., '94; Dixon and Ferguson, '92) and the expression of several growth factors or their receptors, e.g. TGF- β , EGF receptor (EGF-R), have been shown to be regulated by CORT (Danielpour et al., '91; Oberg and Carpenter, '89, '91; Nichols and Finch, '91; Abbott et al., '92; Jaskoll et al., '94; Melnick et al., '96), it is reasonable to postulate that CORT modulation of growth factor (or receptor) gene expression is a key mechanism involved in CORT-induced cleft palate.

In the present study, we analyzed the steady-state levels and developmental expression of four CORT-responsive genes, TGF- β 1, TGF- β 2, TGF- β 3, and EGF-R, in developing mouse palates in the presence or absence of exogenous CORT. During normal palate morphogenesis, we report significant increases in TGF- β 1 and TGF- β 3 transcript levels, as well as a significant decrease in TGF- β 2 transcript levels; EGF-R mRNA levels do not differ significantly with gestational age. CORT treatment only modulates TGF- β 2 mRNA expression, the only isoform specifically localized in the mesenchyme. CORT markedly alters the timing of the down-regulation of TGF- β 2 expression. Since TGF- β 2 is known to inhibit cell proliferation, the observed CORT-induced delay in the normal down-regulation of TGF- β 2 gene expression at a critical stage of palatogenesis is probably a key event in the pathogenesis of CORT-induced cleft palate.

MATERIALS AND METHODS

Tissue collection

Female B10A/SnSg mice, obtained from Jackson Laboratories (Bar Harbor, ME), were maintained and mated as previously described (Melnick et al., '81a). The B10.A mouse strain has been shown to be highly susceptible to CORT-induced cleft palate (Melnick et al., '81a; Jaskoll et al., '95). The date of plug detection was designated day 0 of gestation. Pregnant mice was injected with 6 mg/kg body weight triamcinolone hexacetonide (Aristospan, Fujisawa Pharmaceutical Co., Deerfield, IL) or sham-injected at 9 AM on day 12 of gestation. Triamcinolone hexacetonide is an oil-based, long-acting CORT agonist previously shown to elicit a response in pregnant mice similar to that observed with daily injections of dexamethasone on days 12, 13, and 14 of gestation (Melnick et al., '81a). This steroid treatment induces a cleft palate frequency of 94% (Jaskoll et al., '95). Pregnant females were anesthetized 1–3 days postinjection (days 13–15 of gestation) and euthanized by cervical dislocation. Embryos were dissected in cold phosphate-buffered saline (PBS) and staged according to Theiler ('72) as previously described (Melnick et al., '81a). For mRNA quantitation, palatal shelves were dissected, pooled by litter, and stored at -70°C ; for in situ hybridization studies, embryonic heads were fixed as described below. A mini-

mum of three litters of B10.A embryos were collected for each specific developmental stage, per each experimental protocol, unless noted otherwise.

RNA preparation

RNA was isolated from palates collected at 1 (E13), 2 (E14), or 3 days (E15) after hormone or sham injection. Two to three independent samples of control or treated animals from each developmental age were prepared with 5–18 palates pooled from single litters and assayed one to two times. Total cellular RNA was extracted with acidic guanidine isothiocyanate (Chomczynski and Sacchi, '87) by homogenizing frozen tissue in 500 μl guanidine solution, using a motor-driven polypropylene pestle. Purified RNA was quantified by ultraviolet (UV) spectrophotometry and its integrity checked by agarose gel electrophoresis.

Northern analysis of TGF- β transcripts

Steady-state levels of specific TGF- β transcripts were determined by Northern analysis as previously described (Jaskoll et al., '94). Briefly, RNA samples (20 μg) were electrophoresed through a 1% agarose/6.6% formaldehyde gel, blotted onto nylon membranes, and affixed by UV crosslinking (Stratagene, La Jolla, CA). To facilitate determination of transcript sizes, RNA size markers (0.24–9.5 kb) were prestained with ethidium bromide in sample buffer and detected directly on blots by ultraviolet-activated fluorescence. Blots were incubated with heat-denatured ^{32}P -cDNA probes and washed and hybrids visualized by screen-aided autoradiography with Kodak XAR film and quantified by phosphor image analysis with ImageQuant software and background correction (Molecular Dynamics, Sunnyvale, CA). Blots were stripped and reprobated for different transcripts. All results were normalized to the amount of β -actin mRNA in each sample and ratios arcsin-transformed for statistical analysis of effects of hormone treatment (*t*-test) or changes with developmental age [analysis of variance (ANOVA)].

Mouse subclones recognizing transcripts specific for TGF- β 1, TGF- β 2, or TGF- β 3 were provided by Dr. Stephen Yen (University of Southern California). To minimize the possibility of cross-hybridization, transcript-specific probes, consisting mainly of precursor-encoding regions of the TGF- β genes were used (TGF- β 1, Derynck et al., '86; TGF- β 2, Miller et al., '89; and TGF- β 3, Denhez et al., '90). The TGF- β 1 probe covers 350 bases of 5' untranslated sequence plus sequence for amino acids 1–311, including the mature N-terminus at residue 279; the only signal detected on Northern blots is the 2.5-kb β 1 transcript. The TGF- β 2 (amino acids 1–247) and TGF- β 3 (residues 1–168) probes do not contain sequence for mature peptides. The probe for murine β -actin was obtained from Ambion (Austin, TX). DNA was labeled with ^{32}P by random priming to specific activities of $\geq 1.0 \times 10^9$ cpm/ μg and purified

(Prime-It II and NucTrap systems, Stratagene) before use.

Analysis of EGF-R transcripts by RNase protection assay

Steady-state levels of EGF-R transcripts were measured with an RNase protection assay. Total cellular RNA was isolated from two samples of palates dissected from embryos 1–3 days postinjection (E13–E15). Each sample was a pool of 5–11 palates from one litter assayed one to two times. ³²P-labeled antisense riboprobe for EGF-R transcripts was obtained by transcription of plasmid pMEGFr containing a murine cDNA insert provided by Dr. Ginette Serrero (W. Alton Jones Cell Science Center, Lake Placid, NY) (Serrero et al., '92). Production of 221-nt full-length probe by T3 RNA polymerase from pMEGFr cleaved with *Bsa*HI was maximized by using the limiting nucleotide (UTP) at 11 μM and including human placental ribonuclease inhibitor in the reaction (MAXIscript kit, Ambion, Austin, TX). EGF-R mRNA protects a 162-nt fragment of the probe from digestion by RNases. β-Actin transcripts provided an internal standard for each assay; they were detected with a 168-nt T3 RNA polymerase transcript of *Dde*I-cleaved plasmid pTRI-mouse β-actin (Ambion), which yields a 131-nt protected fragment.

³²P-riboprobes (250,000 cpm at 3.7×10^6 cpm/μg EGF-R probe and 5,000 cpm at 6.7×10^6 cpm/μg β-actin probe) were hybridized to 10 μg total cellular RNA in a 40-μl reaction containing 50% formamide, 40 mM piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.4, 400 mM NaCl, 1 mM EDTA, and 10 μg yeast tRNA. Overnight hybridizations were conducted at 50°C after denaturing the RNA at 85°C for 5 min. A control reaction without palate RNA contained 20 μg tRNA. Nuclease digestion was done by adding 300 μl buffer (10 mM Tris-HCl, pH 7.9, 300 mM NaCl, 1 mM EDTA) containing 10 μg RNase A and 200 U RNase T1 (Ambion) and incubating for 30 min at 20°C. The nucleases were inactivated with 60 μg proteinase K (Ambion) and 0.5% sodium dodecyl sulfate (SDS) at 50°C for 30 min. Transcript-protected ³²P-labeled RNA was isolated by phenol-chloroform extraction, recovered by ethanol precipitation, dissolved in 5 μl loading buffer (80% formamide, 2 mM EDTA, 0.1% tracking dyes), denatured at 85°C for 3 min, resolved by gel electrophoresis (5% polyacrylamide, 8 M urea), displayed on film by screen-aided autoradiography, and quantified by phosphor imaging. All results were normalized to the amount of β-actin mRNA in each sample and ratios arcsin-transformed for statistical analysis of effects of hormone treatment or changes with developmental age by *t*-test or ANOVA, respectively.

In situ hybridization

Single-stranded RNA probes were prepared by *in vitro* transcription using standard protocols. To minimize cross-hybridization among the three TGF-β

RNAs, transcript-specific probes were constructed by deleting the highly conserved regions of the cDNAs. Digoxigenin (DIG)-labeled riboprobes were synthesized from the TGF-β2 and TGF-β3 cDNA mouse probes cited above; the TGF-β1 riboprobe was synthesized from a 314-bp insert of the rat TGF-β1 coding region (Nichols and Finch, '91). Labeled antisense and sense riboprobes were synthesized by incorporation of DIG-UTP (Boehringer Mannheim, Indianapolis, IN) using the T3 or T7 transcription system. The reaction was terminated by the addition of 50 μg DNase (Promega, Madison, WI) for 15 min at 37°C and ethanol precipitated. Newly synthesized DIG-RNA were reduced by limited hydrolysis to an average size of 100–150 bp.

Mouse embryonic heads were fixed overnight in 4% paraformaldehyde in PBS at 4°C, dehydrated through graded ethanol concentrations and xylene, and embedded in paraplast. Sections of 8 μm were cut and mounted on slides coated with 3-trithoxysilylpropylamine. *In situ* hybridization was performed basically as described by Cox et al. ('84). The slides were baked at 42°C for 18 hr, deparaffinized at 58°C for 2 hr, and dehydrated through decreasing concentrations of ethanol. The sections were treated with 20 μg/ml proteinase K (Sigma, St. Louis, MO) in 50 mM Tris buffer, 5 mM EDTA, pH 7.4 at 37°C for 7.5 min, postfixed in 4% buffered paraformaldehyde at room temperature (RT) for 5 min, and dehydrated through increasing concentrations of ethanol. The sections were prehybridized with 200 μl hybridization buffer: 50% formamide, 1.2 M NaCl, 20 mM Tris, pH 7.6, 2 mM EDTA, 0.2 mg/ml bovine serum albumin (BSA), 0.5 mg/ml sodium pyrophosphate, 0.4 mg/ml Ficoll, 100 μg/ml total RNA, and 0.5 mg/ml salmon sperm DNA] in 5 × SSC humidified chamber for 4 hr at 60°C. An equal volume of hybridization buffer containing 500 μg/ml DIG-RNA was overlaid on the sections. Hybridization was carried out for 16 hr at 65°C. After hybridization, the sections were washed in 4 × SSC buffer at RT and incubated in buffer containing 10 μg/ml RNase A and 100 U/ml RNase T (Boehringer Mannheim) for 30 min at 37°C. The sections were then washed in 0.2 × SSC at 72°C and RT for 1 hr and 5 min, respectively. The slides were washed three times in 0.1 M Tris buffer (pH 7.5), blocked with 10% sheep serum (Sigma) in 0.1 M Tris buffer for 1 hr, and incubated with anti-DIG antibody (1:2,000 in Tris buffer; Boehringer Mannheim) overnight at 4°C. After washes in Tris buffer, the sections are developed with nitroblue tetrazolium (NBT), 0.33 mg/ml, and 0.16 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega), with alkaline inhibitor (0.24 mg/ml levamisole). As a negative control, sections were hybridized with sense riboprobes. As a positive control, sections were pretreated with RNase prior to hybridization with the antisense riboprobes. For each probe, 3–5 individual heads/day of gestation/treatment protocol were analyzed.

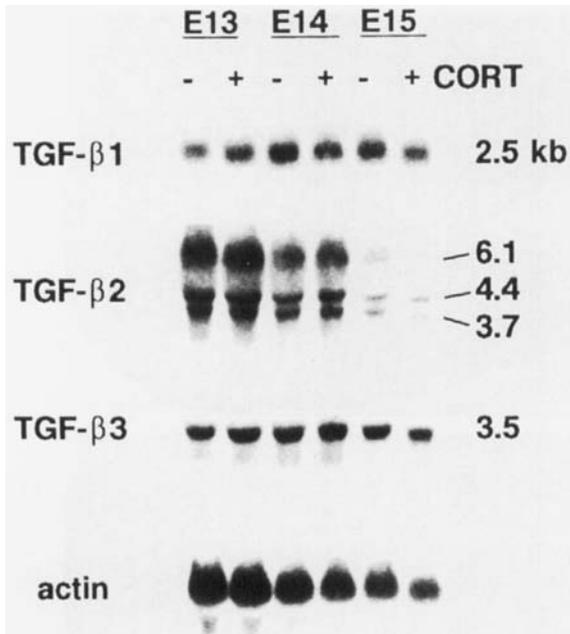


Fig. 1. Differential TGF- β gene expression in developing B10.A mouse palates. Steady-state levels of TGF- β transcripts were assessed by Northern analysis, as described in Materials and Methods. Blots of 20 μ g total cellular RNA from E13, E14, and E15 palates were probed for TGF- β 1, TGF- β 2, and TGF- β 3 transcripts (sizes in kilobases indicated), with β -actin as an internal reference. Representative autoradiograms are shown.

RESULTS

Quantitative analysis of TGF- β mRNA

To determine if CORT modulates embryonic palatal TGF- β expression, we analyzed the steady-state levels of TGF- β 1, TGF- β 2, and TGF- β 3 transcripts with progressive development in E13–E15 B10.A embryonic mouse palates in the presence or absence of maternal CORT treatment by Northern analysis. On all gestational days evaluated (E13–E15), the characteristic mRNA species for TGF- β 1 (2.5 kb), TGF- β 2 (6.1, 4.4, and 3.7 kb), and TGF- β 3 (3.5 kb) are observed (Fig. 1). Furthermore, the TGF- β mRNA steady-state levels significantly differ in the palate from E13 to E15 (Fig. 2). TGF- β 1 and TGF- β 3 transcript levels significantly increased from E13 to E15. Specifically, TGF- β 1 transcript levels significantly increased 96% from E13 to E15 ($t_3=6.45$; $P<0.01$) (Fig. 2A). There was a significant 25% increase in TGF- β 3 mRNA levels from E13 to E14/E15 ($t_2=9.65$; $P<0.02$); there was no change in transcript levels from E14 to E15 (Fig. 2C). By contrast, TGF- β 2 transcript levels significantly decreased 9% from E13 to E14 ($t_2=5.17$; $P<0.05$); there was a further drop from E14 to E15, for a significant overall reduction of about 20% over the E13–E15 period ($t_3=4.09$; $P<0.05$) (Fig. 2B).

CORT treatment has a marked effect on TGF- β 2 expression (Fig. 2B). Although a significant reduction in

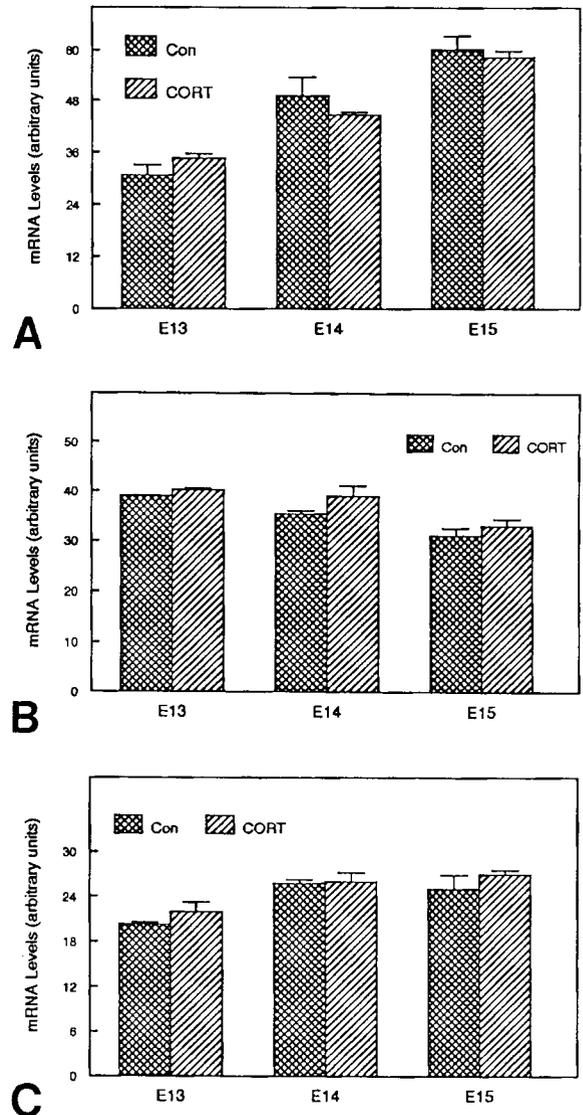


Fig. 2. Quantitative analysis of TGF- β transcript levels with progressive development in control (CON) and CORT-treated (CORT) palates. **A:** TGF- β 1. **B:** TGF- β 2. **C:** TGF- β 3. Northern blots were analyzed by phosphor imaging, as described in Materials and Methods. Ratios were arcsin transformed and mean differences between days of development were analyzed by ANOVA and mean differences between control and CORT-treated palates were compared by Student's *t*-test. TGF- β 1 and TGF- β 3 transcript levels significantly increase with development; CORT does not significantly affect the direction or magnitude of change of TGF- β 1 and TGF- β 3 transcript levels with time. By contrast, there is a significant drop in TGF- β 2 levels between E13 and E14 during normal palate development; there is a further drop from E14 to E15, for an overall significant 20% reduction in transcript levels. In the presence of exogenous CORT, there is virtually no drop in TGF- β 2 levels between E13 and E14. However a reduction in transcript levels is detected between E14 and E15, for a significant overall 18% decrease from E13 to E15. Vertical bar = 1 SEM.

TGF- β 2 transcript levels was detected in control palates between E13 to E14, there was virtually no drop from E13 to E14 (40.26 vs. 39.08) with CORT treat-

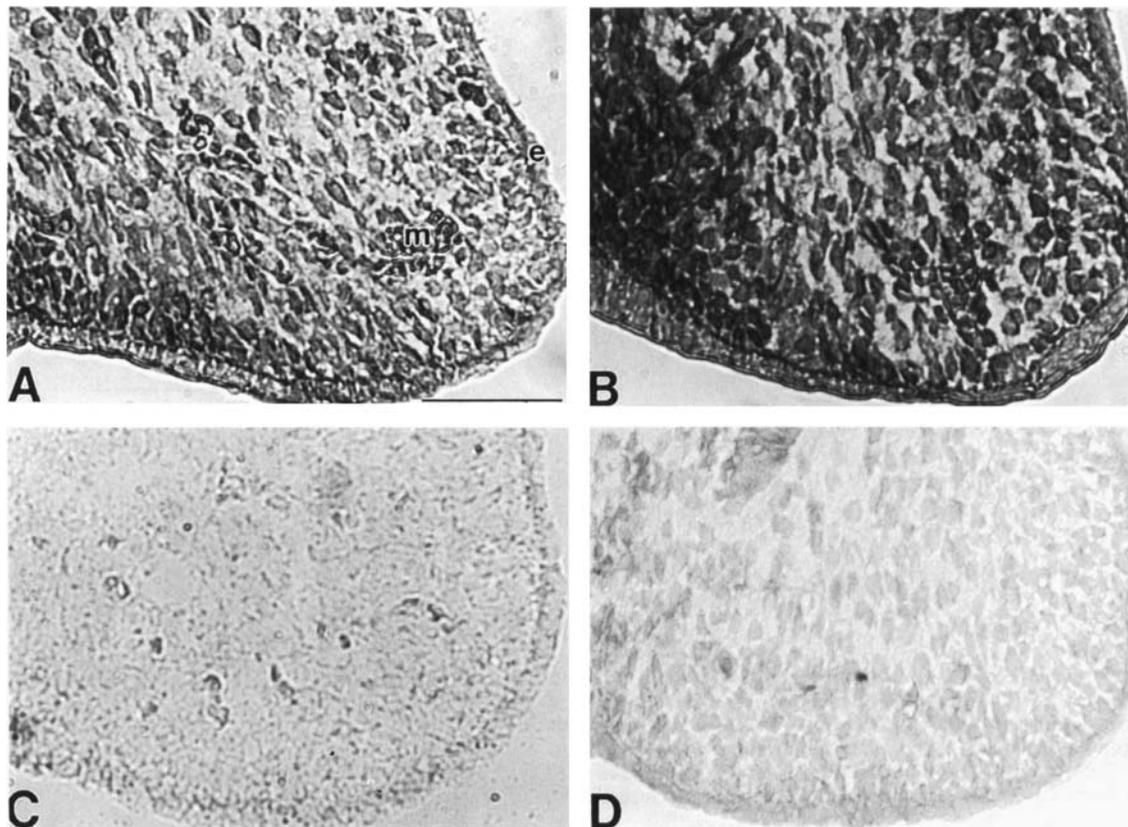


Fig. 3. In situ hybridization of TGF- β 2 mRNA in the E14 palate. **A:** Control palate hybridized with antisense probe. **B:** CORT-treated palate hybridized with antisense probe. **C:** Negative control consisting of a section hybridized with sense probe. **D:** Positive control consisting of a RNase pretreated section hybridized with antisense riboprobe. TGF- β 2 mRNA is primarily expressed in the mesenchyme (m). There is an increase in TGF- β 2 expression in the CORT-treated palatal mesenchyme compared to control. e: epithelium. Bar = 200 μ m.

ment; however, an 18% drop was detected by E15, this decrease being significant ($t_3 = 4.13$; $P < 0.05$). This CORT-induced decline in TGF- β 2 mRNA levels from E14 to E15 is similar in magnitude to the overall reduction in transcript levels seen in controls throughout the E13–E15 period (Fig. 2B). CORT does not significantly affect the direction or magnitude of the change with development of TGF- β 1 and TGF- β 3 transcript levels (Fig. 2A,C).

Tissue distribution of TGF- β mRNA

Since palatal development has been shown to be regulated by epithelial–mesenchymal interactions (see review, Ferguson, '88) and different cell types (i.e., epithelia or mesenchyme) may exhibit different responses to the CORT signal, we also investigated whether CORT modulates the spatial distribution of TGF- β 1, TGF- β 2, and TGF- β 3 mRNA in E13–E15 palates by in situ hybridization. The spatial distribution of TGF- β 1, TGF- β 2, and TGF- β 3 in control and CORT-treated palates is similar to that previously reported (Fitzpatrick et al., '90; Pelton et al., '90; Abbott et al., '92): TGF- β 2 was seen primarily in the mesenchyme (Fig. 3);

TGF- β 1 (Fig. 4) and TGF- β 3 mRNA (data not shown) was primarily expressed in the epithelia. In addition, the data derived from our in situ hybridization studies are consistent with our Northern data. There is a marked increase in TGF- β 2 mRNA in CORT-treated E14 palatal mesenchyme compared to controls (cf. Fig. 3B to 3A); no differences in the expression pattern of TGF- β 1 (cf. Fig. 4B to 4A) and TGF- β 3 mRNA (data not shown) were observed between control and CORT-treated palates.

Analysis of EGF-R expression

Given that the EGF/EGF-R signal transduction pathway is believed to play a morphogenetic role during *in vivo* palatogenesis (Hassell, '75; Ferguson, '88; Brunet et al., '93) and EGF-R mRNA (but not EGF) has been shown to be CORT responsive (Gross et al., '86; Oberg and Carpenter, '89, '91), we investigated the effect of CORT on the steady-state levels and developmental expression of EGF-R transcripts with progressive palatal development. Since the steady-state levels of EGF-R mRNA in embryonic mouse palates were dif-

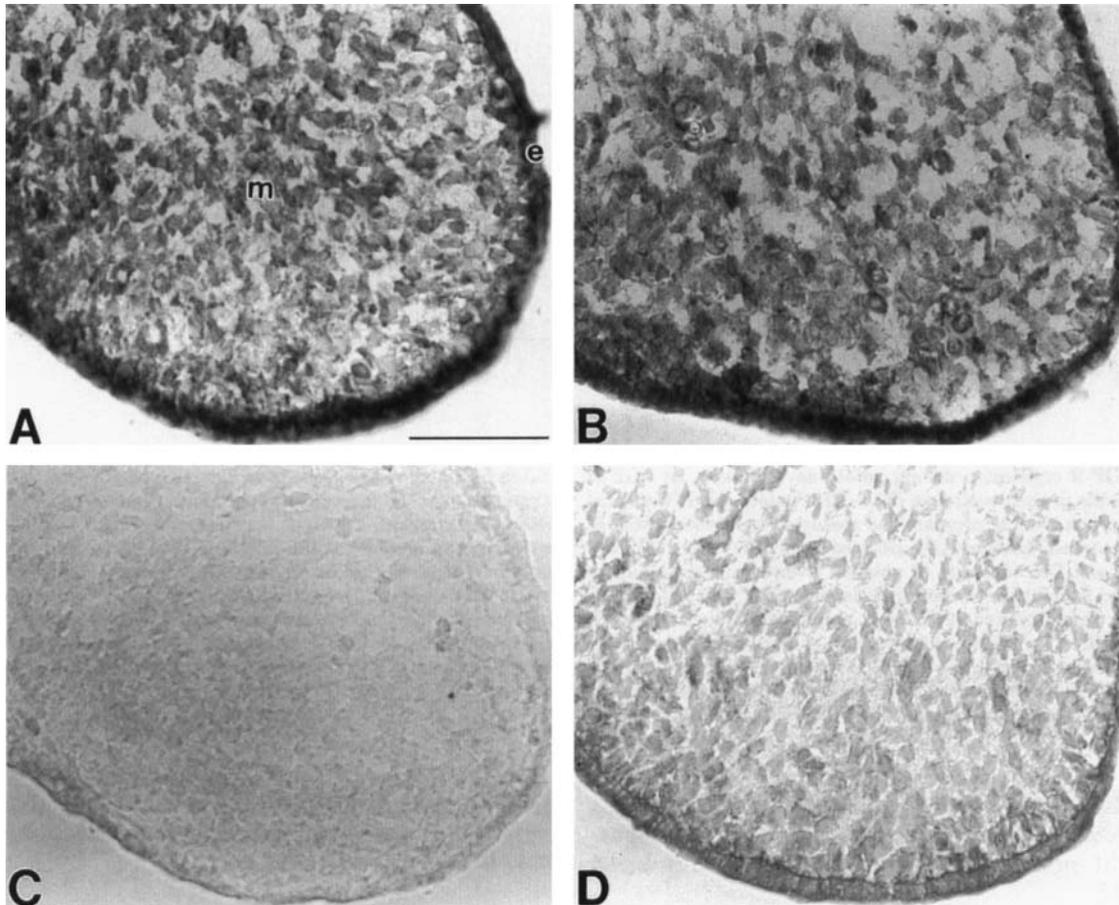


Fig. 4. In situ hybridization of TGF- β 1 mRNA in the E14 palate. **A:** Control palate hybridized with antisense probe. **B:** CORT-treated palate hybridized with antisense probe. **C:** Negative control consisting of a section hybridized with sense probe. **D:** Positive control consisting of a RNase-pretreated section hybridized with antisense ribo-

probe. TGF- β 1 is primarily localized in the palatal epithelia (e); this in situ expression pattern is similar to that seen for TGF- β 3 (data not shown). No marked difference in TGF- β 1 expression is seen between control and CORT-treated palates. m: mesenchyme. Bar = 200 μ m.

difficult to quantify by Northern analysis, we used RNase protection assays to analyze quantitative differences in palatal EGF-R transcript levels. As shown in Figure 5A, EGF-R transcripts are present in the palate on all gestational days evaluated. No significant difference in EGF-R transcript levels is observed from E13/E14 to E15 (Fig. 5B). In addition, CORT-treatment does not affect the direction or magnitude of change with progressive development compared to controls (Fig. 5B).

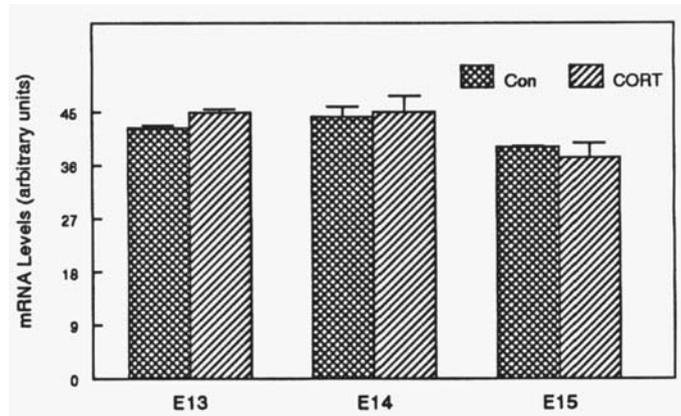
To determine whether CORT induces changes in the cell-specific expression of EGF-R, in situ hybridization studies were also conducted (Fig. 6). EGF-R mRNA was seen in palatal epithelia in both control and CORT-treated palates (Fig. 6A,B), this observation being identical with the previously reported localization of mouse EGF-R transcripts and protein (Abbott et al., '88, '92; Shiota et al., '90). CORT treatment does not change the in situ distribution or intensity of EGF-R mRNA (cf. Fig. 6B to 6A).

DISCUSSION

CORT has long been known to induce smaller palatal shelves and cleft palate by the inhibition of mesenchymal cell proliferation in the developing mouse palate (Mott et al., '69; Nanda and Romeo, '78; Salomen and Pratt, '79). However, the molecular details of the pathogenetic pathways(s) have not been fully characterized. This is particularly critical because numerous human and mouse teratogens known to induce cleft palate (e.g., phenytoin, smoking, diazepam, haloperidol, 2,4,5,T) also elevate maternal CORT levels in mammals (Barlow et al., '80; Hansen et al., '88; Sullivan-Jones et al., '92; Lieberman et al., '92; Hwang et al., '95). Thus, the underlying mechanism of seemingly disparate teratogens may be mediated through a common CORT-directed pathogenetic pathway (Fraser et al., '54; Barlow et al., '80; Hansen et al., '88; Sullivan-Jones et al., '92). Therefore, delineation of the molecu-



A



B

Fig. 5. EGF-R expression during palate development. **A:** EGF-R transcript levels in the developing (E13–E15) palate. An RNase protection assay was used to compare the steady-state levels of EGF-R mRNA in 10 μ g total cellular RNA isolated from E13 (lanes 1, 2), E14 (lanes 3, 4), and E15 (lanes 5, 6) palates. Even-numbered samples were prepared from litters of mothers treated on E12 with CORT; odd numbers are sham-injected controls. A no-palate RNA control contained yeast tRNA (-). The autoradiogram of a representative gel is shown. DNA size markers (*M*; pBR322-*Msp*I fragments) were used to locate 32 P-ribo probe fragments. The transcript-protected signals for EGF-R (*e*) and β -actin (*a*) are indicated. EGF-R mRNA protects a

162-nt fragment of the probe from digestion by RNases. β -Actin transcripts provided an internal standard for each assay; a β -actin mRNA protected fragment of 131 nt was detected. Small portions of undigested reactions show the full-length EGF-R (*E*; 0.4% of reaction) and β -actin (*A*; 20%) probes. **B:** Quantitative analysis of EGF-R transcript levels. Gels were analyzed by phosphor imaging, as described in Materials and Methods. In control and CORT-treated palates, no significant differences in EGF-R transcript levels were observed between days E13/E14 and E15 or between control and CORT-treated palates. Vertical bar = 1 SEM.

lar details of the relationship between CORT and growth factor expression *in vivo* could provide important insight into the pathogenesis of teratogen-induced cleft palate.

The TGF- β family of proteins is involved in regulating cell proliferation, differentiation, and extracellular matrix formation and degradation (Linask et al., '91; Derynck, '94). Several studies suggest that TGF- β isoforms (TGF- β 1, TGF- β 2, and TGF- β 3) play important morphogenetic roles during *in vivo* palate development:

1. The distinct cell-specific *in situ* distribution of each isoform in the developing palate; i.e., TGF- β 2 mRNA is expressed primarily in the mesenchyme, whereas TGF- β 1 and TGF- β 3 mRNA are expressed primarily in the epithelia (Pelton et al., '90; Fitzpatrick et al., '90; present study).
2. The presence in the palate of all three principal types of TGF- β receptors (Linask et al., '91)
3. The observations that exogenous TGF- β 1 or TGF- β 2 regulates extracellular matrix production, inhibits mesenchymal cell proliferation, and induces shelf fusion *in vitro* (Ferguson, '88; Sharpe and Ferguson, '88; Sharpe et al., '92; Linask et al., '91; D'Angelo and Greene, '91; D'Angelo et al., '94)
4. The observation that TGF- β 3 null mutant mice exhibit cleft palate due to the inability of palatal shelf medial edge epithelia to fuse (Kaartinen et al., '95)
5. The demonstration by Brunet et al. ('95) that the inhibition of TGF- β 3, but not TGF- β 1 or TGF- β 2, activity with antisense oligodeoxynucleotides (ODN) and

neutralizing antibodies *in vitro* prevents palatal shelf fusion (the addition of TGF- β 3 rescued antisense ODN- or antibody-exposed palates)

Together, these observations also argue that each TGF- β isoform plays a precise (and distinct) role during palatal morphogenesis.

We report a significant increase in TGF- β 1 and TGF- β 3 transcript levels and a significant decrease in TGF- β 2 transcript levels with progressive palatal development (Fig. 2). If TGF- β 3 activity is a necessary component of palatal fusion and a decrease in TGF- β 3-mediated signal transduction prevents palatal fusion (Brunet et al., '95; Kaartinen et al., '95), one would predict that an increase in TGF- β 3 mRNA is associated with palatal fusion. Our observation of increased TGF- β 3 transcript levels with increasing gestational age (E13–E15) is consistent with this prediction. Furthermore, if TGF- β 2, the only isoform primarily localized in palatal mesenchyme, inhibits palatal mesenchymal cell proliferation (Ferguson, '88) and palatal shelf growth is TGF- β -directed, TGF- β 2 transcript levels probably modulate mesenchymal cell proliferation and palatal shelf size. Thus, a reduction in TGF- β 2 transcript levels with progressive development would induce a decrease in TGF- β 2-mediated inhibition of mesenchymal cell proliferation, resulting in increased mesenchymal cell number and larger palatal shelves, followed by shelf apposition and fusion. Supporting evidence is provided by our demonstration of such a de-

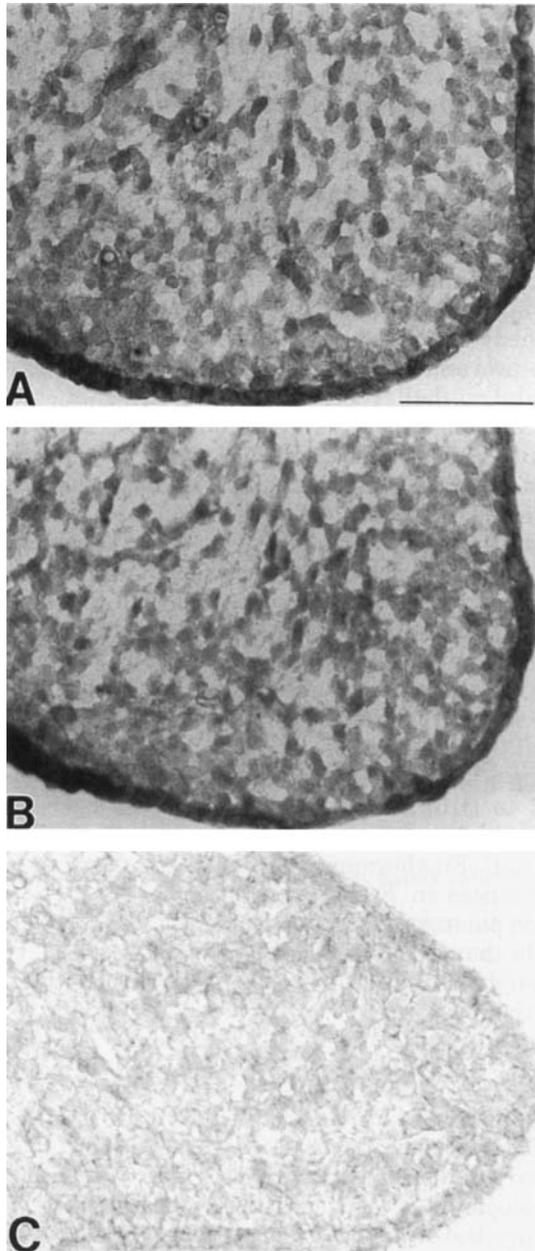


Fig. 6. In situ hybridization of EGF-R mRNA in E14 palate. **A:** Control palate. **B:** CORT-treated palate. **C:** Negative control consisting of a section hybridized with sense probe. EGF-R mRNA is primarily localized in the palatal epithelium. In situ expression of EGF-R mRNA is similar in control and CORT-treated palates. Bar = 200 μ m.

cline in TGF- β 2 transcript levels from E13 to E15 (Fig. 2B).

Given that (1) CORT-induced cleft palate is the result of decreased cell proliferation (Mott et al., '69; Jelinek and Dostel, '75; Nanda and Romeo, '78); (2) TGF- β gene expression has been shown to be regulated

by CORT in other embryonic and adult tissues and in cell lines (Danielpour et al., '91; Nichols and Finch, '91; Jaskoll et al., '94, '96; Melnick et al., '96); and (3) TGF- β regulates cell proliferation, including embryonic mesenchymal palatal cells in vitro (Markowitz et al., '95; Linask et al., '91; Ferguson, '88; Sharpe et al., '92; Polyak et al., '94a,b; Derynck, 1994), it was reasonable to postulate that CORT regulation of TGF- β gene expression is an important mechanism in the pathogenesis of CORT-induced cleft palate. To this end, we compared mRNA steady-state levels and developmental expression in E13–E15 CORT-exposed mouse palates to controls. To maximize the detection of possible CORT-induced changes in TGF- β gene expression, we employed an in vivo treatment strategy using a highly susceptible mouse strain (B10.A) and a CORT dose that results in a 94% cleft palate incidence in CORT-treated embryos (Jaskoll et al., '95).

CORT markedly alters the timing of the down-regulation of TGF- β 2 mRNA expression. The significant decrease in TGF- β 2 transcript levels detected in controls between E13 to E14 is absent in CORT-treated palates (Fig. 2B). However, a significant 18% drop in TGF- β 2 transcript levels is detected between E14 and E15. While the decline in TGF- β 2 transcript levels with CORT treatment is delayed from E14 until E15, the overall decrease in transcript levels is similar on E15 in control and CORT-treated palates. Our observation that the level of TGF- β 2 mRNA in E14 palates is greater with CORT treatment than in controls is confirmed by in situ hybridization studies (Fig. 3). Based on our Northern blot analysis and in situ hybridization studies, we conclude that CORT modulates TGF- β 2, but not TGF- β 1 and TGF- β 3, gene expression in vivo. Furthermore, TGF- β 2 is the isoform primarily expressed in palatal mesenchymal cells (Fig. 3) (Pelton et al., '90; Fitzpatrick et al., '90). Since extensive mesenchymal proliferation during critical stages of palatal morphogenesis is crucial for palatal shelf growth and fusion (Nanda and Romeo, '78; Jelinek and Dostel, '74), CORT inhibits palatal mesenchymal cell proliferation (Mott et al., '69; Jelinek and Dostel, '75; Nanda and Romeo, '78; Potchinsky et al., '96), and TGF- β inhibits palatal mesenchymal cell proliferation (Linask et al., '91; Ferguson, '88), a CORT-induced increase in TGF- β 2 transcript level on E14 would mediate an inhibition of mesenchymal cell proliferation, producing smaller palatal shelves and a cleft palate.

At present, it is difficult to assess the relative importance that small changes in the level of a specific growth factor might have on in vivo development. However, several in vitro studies provide evidence suggesting that small changes in growth factor expression may play an important role during morphogenesis: (1) low levels of TGF- β enhance embryonic epithelial proliferation and pulmonary branching, whereas high concentrations inhibit branching morphogenesis (Serra et

al., '94; Warburton et al., '94); (2) embryonic cells respond to morphogenetic concentration gradients to up- and down-regulate target gene expression, with the level of morphogens able to elicit these responses in the picomolar (pM) range (Gurdon et al., '94, '95); and (3) small changes in the expression of growth factor and hormone receptors can mediate large differences in target gene expression and subsequent phenotypes (Morrison et al., '94; Lillien, '95). Based on these reports it is reasonable to suggest that small (but significant) differences in the level of growth factors can elicit marked developmental differences. Thus, although the precise level of endogenous embryonic palatal TGF- β mRNA that produces a morphogenetic signal is, as yet, unknown, we propose that the reported 1-day delay in the down-regulation of TGF- β 2 mRNA in CORT-exposed palates results in detectable developmental differences (i.e., a cleft palate).

This pattern of CORT-induced modulation of TGF- β transcript levels in vivo appears to be tissue specific. A marked decrease in TGF- β 2 and/or TGF- β 3 mRNA levels in submandibular glands and lungs 3 days postinjection (E15) is observed (Jaskoll et al., '94, '96); this decrease is correlated with increased epithelial branching. Based on these embryonic in vivo studies, as well as numerous studies on cell lines showing that TGF- β regulates cell proliferation by arresting the G₁ phase of the cell cycle through the down-regulation of specific G₁ cyclins and cyclin-dependent kinases (cdk) (see review, Derynk, '94), we postulate that a critical mechanism of CORT-induced cleft palate involves CORT modulation of TGF- β 2-mediated inhibition of G₁ cyclins and cdk's. Further studies are needed to delineate the details of TGF- β 2 inhibition of mesenchymal cell proliferation.

Abbott et al. ('92) reported that exogenous CORT treatment induced a significant increase in TGF- β 1 expression in specific palatal regions using both immunohistochemical localization of the protein and quantitation of the immunostaining level by densitometry and in situ hybridization for the mRNA. In the present study, however, we detected no marked differences in TGF- β 1 mRNA steady-state levels (Fig. 2) or developmental expression (Fig. 4) with CORT exposure. The differences in these two studies are likely attributable to methodology differences. We evaluated CORT-induced changes in TGF- β expression in B10.A mice, a highly susceptible mouse strain, whereas Abbott and coworkers employed C57BL/6 mice, a relatively resistant strain of mice (Biddle and Fraser, '77; Melnick et al., '81b). In addition, our treatment protocol results in 94% of the CORT-exposed embryos exhibiting cleft palate compared to a 30% clefting rate obtained by Abbott and colleagues. Based on our Northern analysis and in situ hybridization studies, we conclude the CORT-induced modulation of TGF- β 1 gene expression is not involved in CORT-induced clefting in mice.

EGF-R and developing mouse palate

EGF, and its presumptive embryonic homologue, TGF- α , as well as its receptor, have been localized in the developing mouse and human palate in vivo (Abbott and Birnbaum, '90; Abbott et al., '88, '92; Shiota et al., '90; Dixon et al., '91; Citterio and Gaillard, '94). In addition, in vitro studies suggest that the EGF/EGF-R signal transduction pathway is involved in palate morphogenesis (Silver et al., '84; Ferguson, '88). For example, palatal mesenchymal cells are highly responsive to the stimulatory effect of exogenous EGF (and TGF- α) (Yoneda and Pratt, '81; Ferguson, '88). EGF (TGF- α) also prevents palatal shelf fusion and inhibits medial palatal epithelial cell degeneration (Brunet et al., '93). Since EGF-R, but not EGF, has been shown to be CORT-responsive and EGF-R transcript number has been shown to modulate cell proliferation and differentiation (Lillien, '95), we postulated that CORT-induced regulation of EGF-R mRNA expression is an important mechanism in the pathogenesis of CORT-induced cleft palate. In this study, we evaluated EGF-R gene expression by RNase protection assay and in situ hybridization in the presence or absence of CORT. Our results confirm the previous observation by Abbott et al. ('88, '92) that EGF-R transcripts are primarily expressed in palatal epithelia (Fig. 6). We report no significant difference in EGF-R transcript steady-state levels from E13 to E15; a similar result has also been seen for EGF-R protein in developing mouse palates (Shiota et al., '90). Furthermore, we demonstrate no significant differences in EGF-R transcript levels in CORT-exposed palates compared to controls (Figs. 5, 6) and conclude that CORT-induced cleft palate is not mediated through the regulation of the EGF/EGF-R signal transduction pathway.

CONCLUSION

A significant increase in TGF- β 1 and TGF- β 3 mRNA levels and a significant decrease in TGF- β 2 mRNA levels are demonstrated with progressive mouse palatal development. CORT treatment does not significantly change the direction or magnitude of change with time for TGF- β 1 and TGF- β 3 transcript levels. An important finding in our study is the demonstration that CORT markedly modulates TGF- β 2 gene expression. CORT delays the reduction of palatal TGF- β 2 transcript levels normally seen on day 14 of gestation until day 15, this delay probably associated with TGF- β 2-mediated inhibition of palatal mesenchymal cell proliferation and shelf growth at a critical stage of palatal morphogenesis. While it is known that cortisone and various analogs cause cleft palate in mice when administered at human-equivalent doses (Walker, '65; Pinsky and DiGeorge, '65), there is but scant evidence to support teratogenicity in humans (Fraser and Sajoo, '95). However, even if CORT-induced cleft palate is

rarely seen in humans, other drugs, known or "suspected" inducers of human cleft palate, also elevate maternal plasma CORT levels in mammals (Barlow et al., '80; Hansen et al., '88; Lieberman et al., '92; Hanson and Smith, '80). These drugs include phenytoin (Hanson and Smith, '75), smoking (Andrews and McGarry, '72; Hwang et al., '95), diazepam (Safra and Oakley, '75), and probably others yet unknown. In addition, stress-induced up-regulation of CORT may act alone or synergistically with other drugs to increase cleft palate incidence (Rosenzweig and Blaustein, '70; Rasco and Hood, '95). Thus, a common CORT-directed pathway may mediate the pathogenetic mechanism(s) of apparently disparate human teratogens. Finally, since CORT induces smaller palatal shelves in mice, and human cleft palate is due to insufficient palatal shelf size and rarely, if ever, associated with the inability per se of apposing palatal shelves to fuse, the delineation of the details of CORT-induced TGF- β 2-mediated palatal shelf growth inhibition and cleft palate pathogenetic pathway(s) in mice may provide insight into the pathogenesis of human cleft palate.

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