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Developmental abnormalities in cultured mouse embryos deprived of retinoic acid by inhibition of yolk-sac retinol binding protein synthesis

(vitamin A deficiency/antisense oligonucleotide/morphogenesis)

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ABSTRACT **Presomitic and 3- to 12-somite pair cultured mouse embryos were deprived of retinoic acid (RA) by yolk-sac injections of antisense oligodeoxynucleotides for retinol binding protein (RBP). Inhibition of yolk-sac RBP synthesis was verified by immunohistochemistry, and the loss of activity of a lacZ-coupled RA-sensitive promoter demonstrated that embryos rapidly became RA-deficient. This deficiency resulted in malformations of the vitelline vessels, cranial neural tube, and eye, depending upon the stage of embryonic development at the time of antisense injection. Addition of RA to the culture medium at the time of antisense injection restored normal development implicating the role of RBP in embryonic RA synthesis. Furthermore, the induced RA deficiency resulted in early down-regulation of developmentally important genes including *TGF-β₁* and *Shh*.**

Total vitamin A (retinol) deficiency (VAD) is lethal to the embryo, while less severe VAD results in well-known developmental malformations (refs. 1–3 and the references therein). Compound mutant mice lacking retinoic acid (RA) receptors (RARs) and retinoid X receptors (RXRs) presented developmental abnormalities recapitulating those observed during VAD (refs. 1–7 and the references therein). However, additional abnormalities not reported in the fetal VAD syndrome were also found (3–7), indicating that total VAD would be required to reveal the full function of RA. It is likely that mouse embryos initially accumulate vitamin A in the visceral yolk-sac, the major site of embryonic retinol-binding protein (RBP) synthesis at this stage (8). We show here that yolk-sac injections of antisense oligodeoxynucleotides (9, 10) for RBP mRNA generate an RA deficiency in cultured mouse embryos that causes stage-specific defects accompanied by decreases in the expression of genes known to be involved in development—e.g., *TGF-β₁* and *Shh*.

MATERIALS AND METHODS

Oligodeoxynucleotide Design. A region of the mouse RBP gene (11) starting at the initiation codon for translation was chosen as a basis for the design of three oligodeoxynucleotides, 18 nt in length: "sense" is identical to the first 18 translated nt in the RBP gene; "antisense" (AS) represents the antisense configuration of "sense"; "rearranged" (RG) is identical to "antisense" in 14 out of 18 nt positions, but the positions of two pairs of nucleotides have been mutually exchanged leaving a mismatched oligodeoxynucleotide with preserved overall base composition. The position of the shifted nucleotides are underlined below: antisense (AS), 5′-CGCCCACACCCACTCCCAT-3′; rearranged (RG), 5′-CACCCTCACCCACACCGT-3′. No match was found between these sequences and gene sequences listed in the GenBank database. "Sense" (data not shown) and RG oligodeoxynucleotides gave indistinguishable results. As RG most closely resembles AS, RG was chosen as control.

Whole Embryo Culture and Microinjection Technique. CD1 strain or transgenic mouse embryos were cultured as described (12). Oligodeoxynucleotides were dissolved in sterile BGJb medium (Sigma) to a concentration of 100 μM and injected into the yolk-sac cavity immediately before culturing, using a pulled glass needle (20 μm diameter) connected to an air-injection system (Pico-injector, Medical Systems, New York). Care was taken to select embryos of similar developmental stages and to avoid damage to yolk-sac vessels. Injection volume was regulated to approximately one quarter the yolk-sac volume, giving a final oligodeoxynucleotide concentration of ≈25 μM. Embryos showing leakage of injected oligodeoxynucleotides were discarded.

Immunohistochemistry. Yolk-sacs were fixed at 4°C overnight in 3% paraformaldehyde. Endogenous peroxidases were inactivated with H_2O_2, and nonspecific binding of immunoglobulin was blocked by preincubation of the embryos with fetal calf serum. Yolk-sac expression of RBP was detected by overnight incubation with purified anti-RBP immunoglobulin at 40 μg/ml (13). The adsorption pattern was revealed by using a 1:4000 dilution of peroxidase-conjugated goat anti-rabbit IgG (Sigma).

Transgenic Animals. The transgenic mouse line used throughout this study expresses an RARβ₂ promoter–*lacZ* fusion (14). After culturing, embryos were dissected free from extraembryonic membranes prefixed, stained for β-galactosidase activity, and postfixed as described (14).

Histology, Electron Microscopy, and *in Situ* Hybridization. Embryos were fixed in Bouins' fixative overnight and then dehydrated through graded ethanols. Samples were cleared and embedded in paraffin wax following classical histological methods. Sections were cut 7 μm thick, mounted, and counterstained with hematoxylin/eosin. Embryos processed for scanning electron microscopy were fixed in 2.5% cacodylate-buffered glutaraldehyde (0.1 M, pH 7.2) overnight at 4°C and washed in cacodylate buffer for a further 30 min at 2°C before being postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (1 hr, 4°C). Embryos were dehydrated through graded alcohols (50, 70, 90, 100%) for each of 30 min and dried with critical point-drying apparatus. Embryos were mounted on

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Abbreviations: AS, antisense; p.c., post coitum; RA, retinoic acid; RAR and RXR, RA and retinoic X receptor, respectively; RBP, retinol binding protein; RG, rearranged; SP, somite pair; VAD, vitamin A deficiency.

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aluminum stubs and coated with palladium-gold using a cold sputter-coater, before photographing on a scanning electron microscope (Philips Electronics, XL20, Pays Bas). Whole mount *in situ* hybridization was performed on embryos fixed overnight in 4% paraformaldehyde as described (15), using RNA probes for *Shh* (16), *TGF-β_1* (17), or *Wnt-3a* (18).

RESULTS AND DISCUSSION

Yolk-sacs of mouse embryos were injected with RBP-antisense (RBP-AS) or control "rearranged" (RBP-RG) oligodeoxynucleotides at the 3- to 5-somite pair stage (3–5 SP) immediately before culturing and were immunohistochemically stained for RBP 12 hr later (Fig. 1). Staining of uninjected (Fig. 1*A*) and RBP-RG-injected yolk-sacs (Fig. 1*B*) verified the presence of strong RBP expression, whereas RBP-AS-injected yolk-sacs (Fig. 1*D*) were indistinguishable from "negative" controls (Fig. 1*C*). To investigate whether injected embryos could acquire retinoids from the yolk-sac, the activity of the RA-responsive RARβ_2 promoter was examined in transgenic embryos containing an RARβ_2 promoter–*lacZ* fusion gene (14), cultured from 3–5 SP (Fig. 1). RARβ_2 promoter activity was identical in RBP-AS (Fig. 1*H*) and RBP-RG-injected (Fig. 1*E*) embryos 6 hr from the time of injection, whereas it has ceased in RBP-AS-injected embryos after 12 hr (compare Fig. 1 *F* and *I*), with very little promoter activity in RBP-AS-injected (Fig. 1*J*) embryos after 24 hr. This result demonstrates that inhibition of yolk-sac RBP synthesis by RBP-AS creates an RA-deficient embryo within 6 to 12 hr of their injection.

RBP-AS-injected embryos displayed a range of defects depending on the stage of embryonic development at the time of injection. After injections of approximately day 7.5 post coitum (p.c.) presomitic embryos, 59% of RBP-AS-treated embryos lacked vitelline vessels after 48 hr of culture (compare Fig. 2 *A* and *B*), resulting in an accumulation of blood islands on the yolk-sac (bi in Fig. 2*B*). All RBP-RG-injected embryos developed vitelline vessels similar to those of untreated cul-

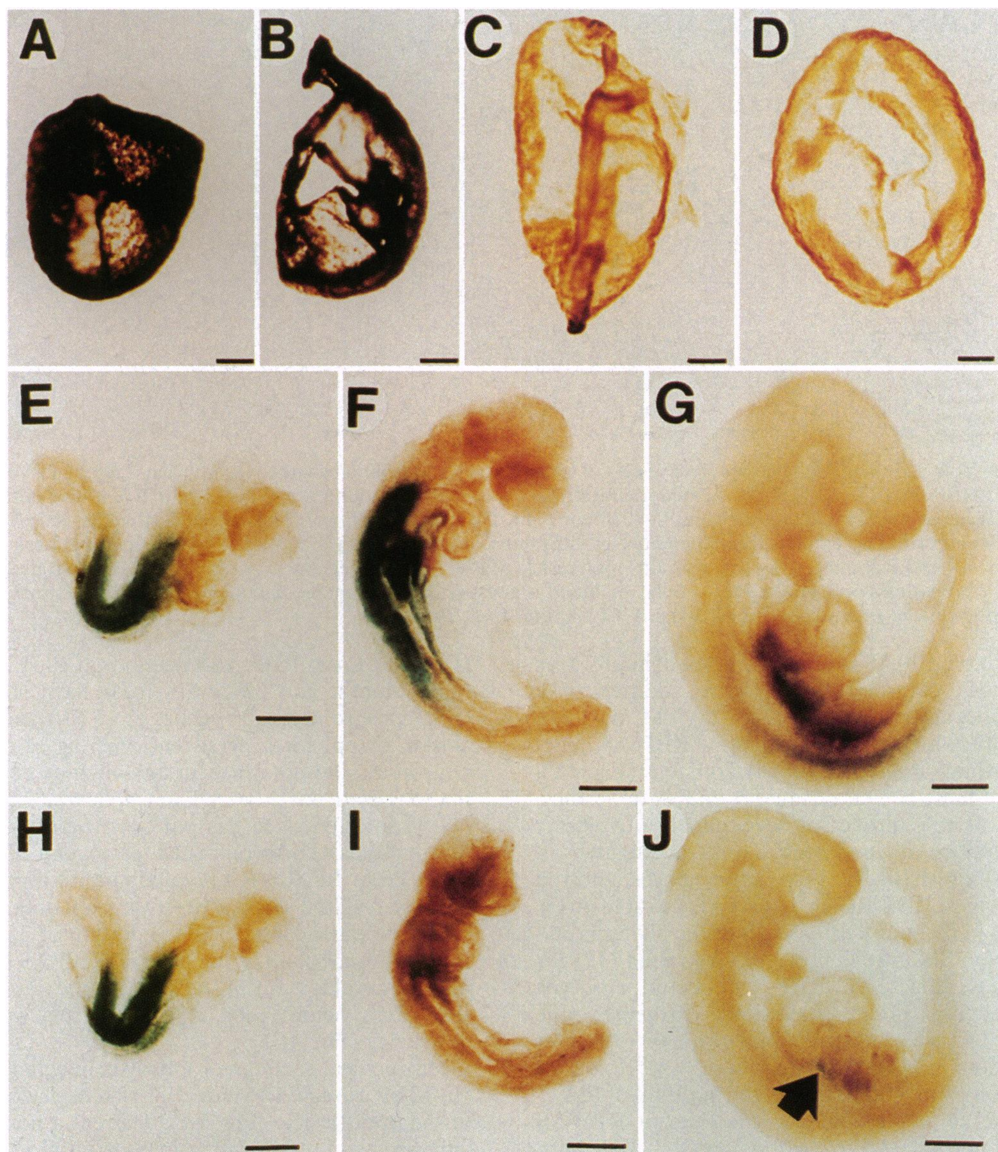

FIG. 1. (*A–D*) Immunohistochemical staining of RBP in yolk-sacs of embryos cultured from the 3- to 5-somite pair stage (3–5 SP) for 12 hr. (*A*) Normal embryonic yolk-sac. (*B*) Control RBP-RG-injected yolk-sac, showing similar RBP-staining as in *A*. (*C*) Negative control normal yolk-sac (normal embryonic yolk-sac treated identically to *A* but without exposure to primary antibody). (*D*) RBP-AS-injected yolk-sac. (*E–J*) Transgenic embryos stained for β-galactosidase activity after 6 hr (*E, H*), 12 hr (*F, I*) or 24 hr (*G, J*) of culturing from 3–5 SP. Yolk-sac cavities were injected with RBP-RG (*E, F,* and *G*) or RBP-AS (*H, I,* and *J*). Note the complete loss of staining in *I* and the weak staining in the area of the heart in *J* (arrow). (Bars = *A–D*, 2 mm; *E–J*, 0.5 mm.)

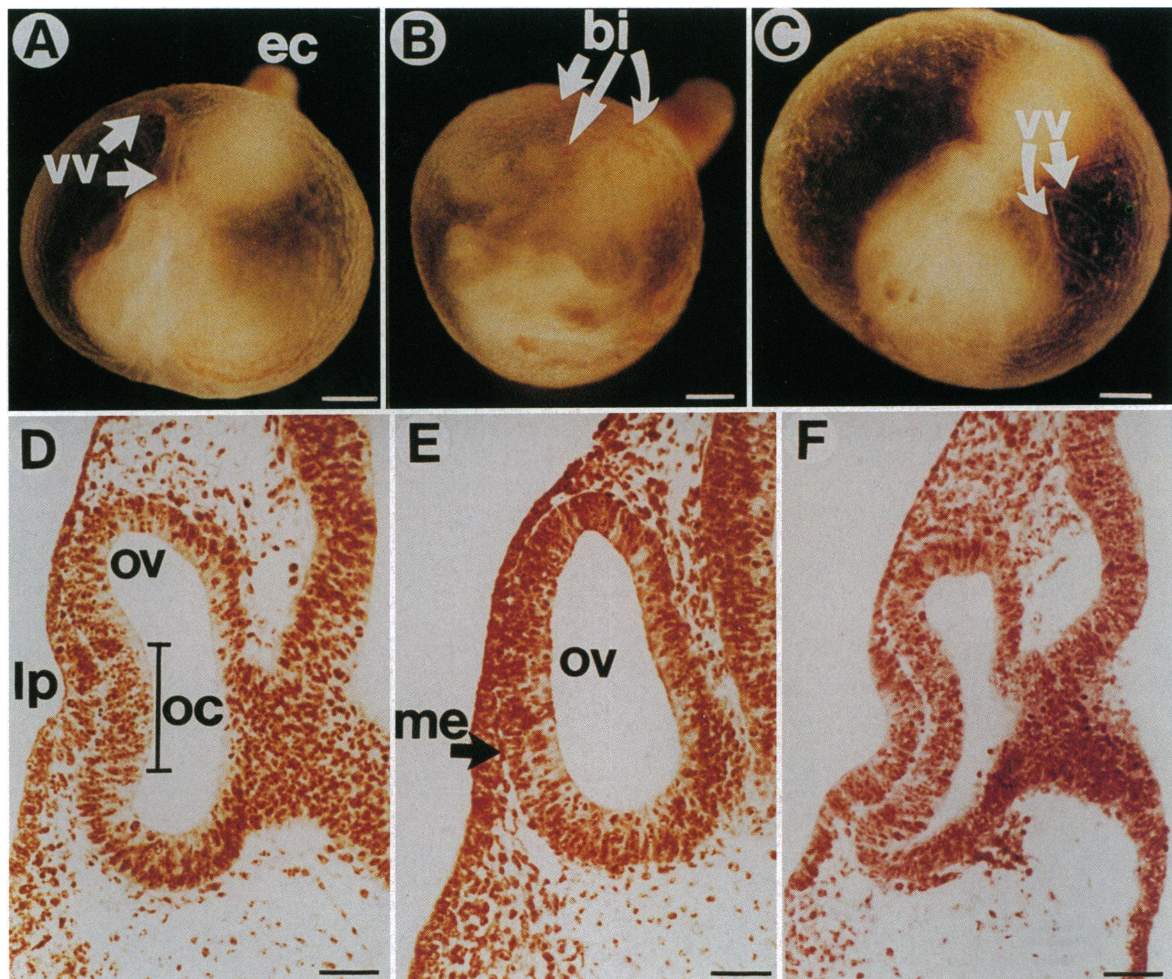

FIG. 2. (*A* and *B*) Yolk-sac circulation and vitelline vessels of embryos cultured from presomite stages (≈day 7.5 p.c.) for 48 hr, after injections of RBP-RG (*A*) and RBP-AS (*B*). (*C*) Embryo treated identically to *B* but with the addition of 3×10^{-8} M RA in the culture medium; (*D–F*) Histological frontal sections of embryos cultured from 10–12 SP for 24 hr. The yolk-sac cavity of *D* was injected with RBP-RG; *E* and *F* were injected with RBP-AS. (*F*) Section from an embryo treated identically to *E*, but with the addition of 3×10^{-8} M RA in the culture medium. Note that in contrast to control (*D*) and RA-treated (*F*) embryos, the optic vesicle of RBP-AS-injected embryos (*E*) was always separated from the ectoderm by mesenchymal cells, sometimes only one cell layer thick. bi, blood islands; ec, ectoplacental cone; lp, lens placode; me, mesenchymal cell layer; oc, optic cup; ov, optic vesicle; vv, vitelline vessel. Bars: *A–C*, 500 μm; *D–F*, 20 μm.

tured embryos, as did RBP-AS-injected embryos at later stages (3–5 and 10–12 SP, data not shown). The addition of a subteratogenic concentration of RA (3×10^{-8} M, data not shown) to the culture medium at the time of RBP-AS injections, restored normal vitelline vessel development (Fig. 2*C*), demonstrating that these defects resulted from RA-deficiency. *TGF-β_1* is known to be required for development of yolk-sac vessels (17); therefore its expression was investigated in RBP-AS (Fig. 3*B*) and RBP-RG (Fig. 3*A*) embryos cultured from presomite stages. RBP-RG-injected embryos selectively expressed *TGF-β_1* in the mesodermal cells of the allantois and extraembryonic blood islands as previously reported (19) for untreated embryos (Fig. 3*A*). RBP-AS-injected embryos showed only weak, if any *TGF-β_1* expression in extraembryonic tissues (Fig. 3*B*) 12 hr after injection, thus within 6 hr of onset of VAD (see above), indicating that early expression of *TGF-β_1* depends on RBP. Furthermore, the addition of RA (3×10^{-8} M) to the culture medium at the time of RBP-AS injection restored normal *TGF-β_1* expression (Fig. 3*C*), demonstrating the requirement of RA for *TGF-β_1* expression in the yolk-sac and allantois.

The rostral neural tube of embryos RBP-AS-injected at 3–5 SP failed to close in the region of the fore- and midbrain (38% open midbrain and forebrain; Fig. 4*B*). No malformations of the neural tube were observed in RBP-RG-injected embryos (Fig. 4*A*). Later RBP-AS-injections at 10–12 SP, a stage when the midbrain and hindbrain folds have started converging (final closure normally occurs at 17 SP) and the forebrain is closed, except for a persistent opening of the most anterior neuropore, did not result in any obvious neural tube malformations (data not shown). Exencephaly caused by failure of the neural folds to close has not been reported before in VAD fetuses but is a commonly seen teratogenic effect of excess RA (20, 21). In RARα/RARγ double-null mutant fetuses exencephaly resulted from a persistent opening of the rhombencephalic neural tube (5). The addition of 3×10^{-8} M RA to the culture medium at the time of RBP-AS injection restored normal development of the neural tube, demonstrating again that these neural tube closure defects were due to RA-deficiency (Fig. 4*C*).

Histological sections of embryos injected with RBP-AS at 10–12 SP, revealed no induction of lens placode or indentation of the optic vesicle (Fig. 2*E*), whereas a normal lens placode and optic cup was forming in control-injected embryos (Fig. 2*D*). Again, the addition of 3×10^{-8} M RA to the culture medium restored normal eye development (Fig. 2*F*). Agenesis of the lens and aphakia (failure of lens formation) have been reported in RAR double-null mutant embryos (5).

Sonic hedgehog (*Shh*) is known to act as an inductive signal in several regions of the developing embryo (16, 22, 23) and its

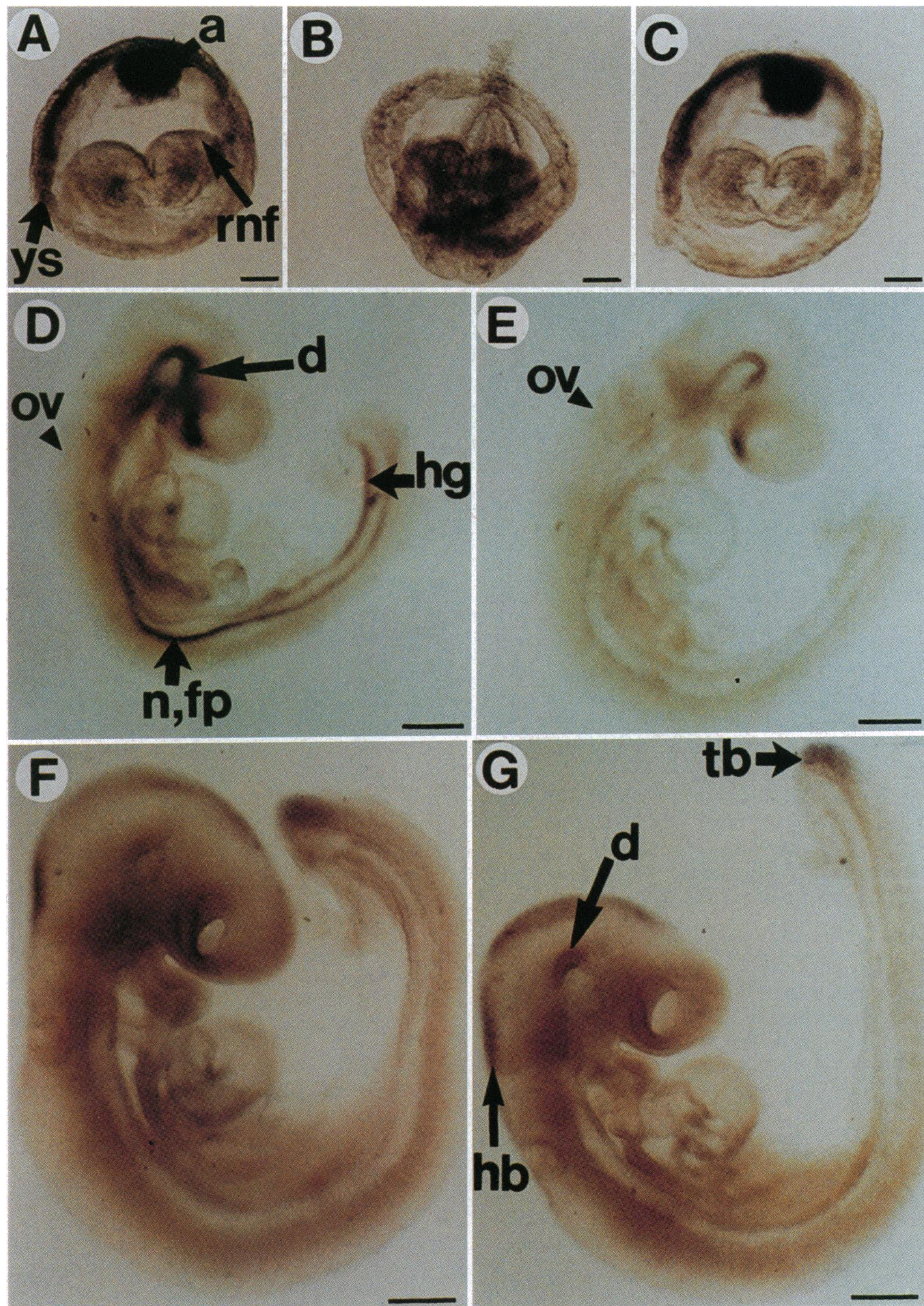

FIG. 3. Whole-mount *in situ* hybridizations using RNA probes for *TGF-β_1* (*A, B,* and *C*), *Shh* (*D* and *E*), and *Wnt-3a* (*F* and *G*). Embryos were examined 12 hr after injection, thus within 6 hr of onset of VAD. Embryos were cultured from the presomite stage (*A, B,* and *C*) and from 3–5 SP (*D, E, F,* and *G*). Yolk-sac cavities of embryos in *A, D,* and *F* were injected with RBP-RG, whereas those of embryos in *B, C, E,* and *G* were injected with RBP-AS. RA was added to the culture medium of *C* to a final concentration of 3×10^{-8} M. a, Allantois; d, diencephalon; fp, floor plate; hb, hindbrain; hg, hind gut; n, notochord; ov, otic vesicle; rnf, rostral neural fold; tb, tail bud; ys, yolk-sac. (Bars = 500 μm.)

expression can be induced by RA-excess treatment (16). The expression pattern of *Shh* in RBP-RG-injected 3- to 5-SP embryos (Fig. 3*D*) did not differ from normal patterns previously reported (16), whereas embryos injected with RBP-AS showed a reduced expression, being almost completely lost caudal to the otic vesicle (Fig. 3*E*). Surprisingly, *Shh* expression remained normal after RBP-AS injections at 10- to 12-SP, indicating that the dependency on retinoids for its expression is stage-specific (data not shown). To verify that decreased expression was specific for *Shh* and not general for any gene normally expressed in these areas, the expression pattern of *Wnt-3a* was investigated. The normal expression pattern of *Wnt-3a* (18) was unchanged in embryos injected at 3–5 SP with RBP-RG (Fig. 3*F*) or RBP-AS (Fig. 3*G*). As expected, *Wnt-3a* was expressed in the diencephalon, dorsal hindbrain, and tail bud.

This study shows that injection of RBP-antisense oligodeoxynucleotides can efficiently inhibit RBP synthesis in the yolk-sac of developing presomitic and 3- to 12-SP mouse embryos in culture. Consequentially, the loss of RARβ_2 promoter activity demonstrates that RA synthesis in the preplacental embryo requires yolk-sac-derived RBP. Furthermore, inhibition of RBP synthesis leads to stage-specific

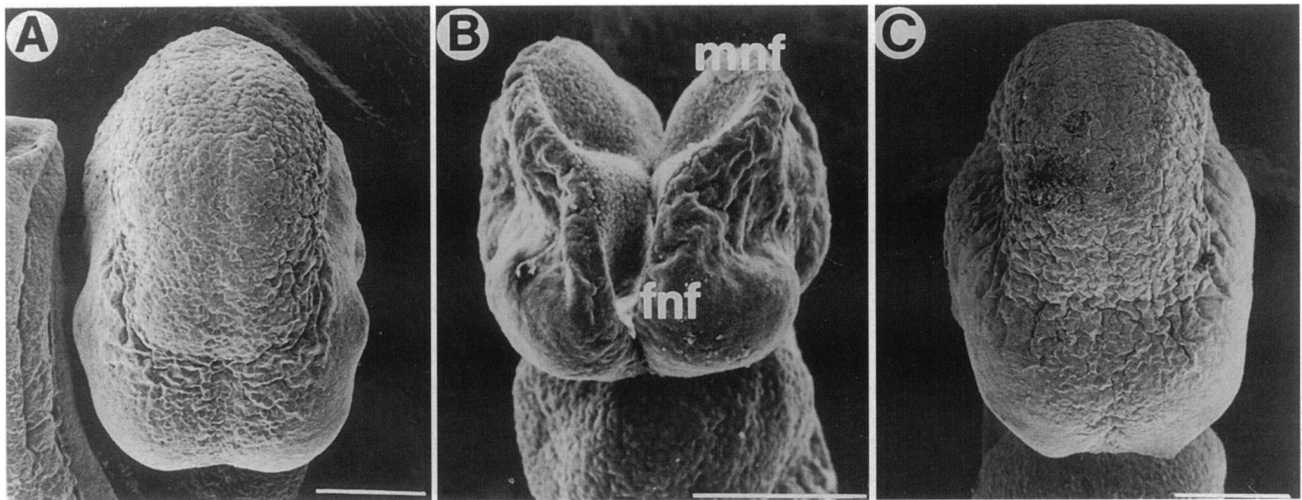

FIG. 4. *A–C* show scanning electron micrographs of embryos cultured for 24 hr from 3–5 SP treated with RBP-RG (*A*) and RBP-AS (*B*). (*C*) Embryo treated identically to *B*, but with addition of 3×10^{-8} M RA in the culture medium. fnf, forebrain neural fold; mnf, midbrain neural fold. (Bar = 200 μm.)

malformations of the vitelline vessels, cranial neural tube, and eye. That each of these malformations can be prevented by the addition of exogenous RA further implicates the role of RBP in embryonic RA synthesis. Finally, this study establishes that "normal" stage-specific expression of *TGF-β_1* and *Shh* during embryogenesis depends upon endogenous RA. Interestingly, injection of RBP-antisense oligodeoxynucleotide at later stages of development (≈ day 8.5–9.0 p.c.) also resulted in RA deficiency in the embryo (data not shown). Thus total RA deficiency, as achieved in this study, provides a valuable tool for future studies, enabling the investigation of RA function at different stages of embryonic development before the haemochorial placenta functionally supersedes the yolk-sac placenta (≈day 9.5 p.c.).

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