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Retinoic acid modulation of the early development of the inner ear is associated with the control of *c-fos* expression

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Summary

The effects of retinoic acid (RA) on the early development of the inner ear were studied *in vitro* using isolated chick embryo vesicles. Low concentrations of RA (1–50 nM) inhibited vesicular growth in stage 18 otic vesicles that were made quiescent and then reactivated by either serum or bombesin. Growth inhibition was concentration-dependent and was paralleled by a reduction in the rate of DNA synthesis as measured by [^{3}H]thymidine incorporation. Half-inhibition occurred between 1 and 10 nM RA, and the full effect at 20 nM. Retinoic acid, in the presence of serum, induced the precocious differentiation of (1) secretory epithelium, the *tegmentum vasculosum* and endolymphatic sac and (2) early sensory and supporting epithelia. These structures were positioned in their corresponding normal presumptive areas. The overall direction of growth was reversed by RA and the ratio of the internal to the external vesicular surface area increased with RA concentration. The expression of the nuclear proto-oncogene *c-fos* in the developing otic vesicle was transient and stage-dependent. High levels of *c-fos* mRNA were positively correlated with cell proliferation. Incubation of growth-arrested otic vesicles with bombesin *plus* insulin at concentrations that induced cell proliferation produced a strong induction of *c-fos*. This mitogen-induced expression was suppressed by 25 nM RA. The results suggest (1) a role for retinoic acid in controlling the early development of the inner ear and (2) that this control is effected through the regulation of the proto-oncogene *c-fos*.

Key words: cell proliferation, oncogenes, retinoic acid, otic development, otic vesicle.

Introduction

Retinoic acid exerts a variety of biological effects. The teratogenic consequences of hypervitaminosis A and the administration of retinoids have been known for a long time and include alterations of growth and morphogenesis of the nervous system (Morriss, 1972; Geelen, 1979; Durston *et al.* 1989). A specific role for retinoic acid during normal development has been supported by experiments on the developing limb where it is thought to specify position across the limb anlage (Tickle *et al.* 1982; Thaller and Echielle, 1987). *In vitro* experiments, using cultured cells, have shown that retinoic acid inhibits cell proliferation and induces differentiation in transformed cells from embryonic origin (Jetten, 1986; Sidell *et al.* 1983). The mechanism by which retinoic acid exerts its actions is being extensively studied and the role of RA receptors as transcriptional factors is beginning to be understood (Petkovich *et al.* 1987; Evans, 1988). Modulation of gene expression by retinoic acid extends to a variety of genes including homeobox and proto-oncogenes (Adamson, 1987; Astigiano *et al.* 1989).

The development of the inner ear is an interesting example of organogenesis in the nervous system. At early developmental stages, it involves the formation of a transient structure, the otic vesicle, which undergoes a distinct period of cell proliferation that precedes the differentiation and histogenesis of various sensory, secretory and supporting elements (Van de Water, 1984; Giraldez *et al.* 1987; Swanson *et al.* 1990). This process can be reproduced *in vitro* and it can also be arrested, to then be reactivated by growth factors (Represa *et al.* 1988; Miner *et al.* 1988; Represa and Bernd, 1989). We exploit here this preparation to examine the effects of retinoic acid on the early development of the inner ear and its relation to the expression of the nuclear proto-oncogene *c-fos*. A possible association between RA and *c-fos* is relevant because the *c-fos* gene product acts also as a transcrip-

tional modulator whose expression is regulated by growth factors (see Curran, 1988). More generally, the possibility of regulatory roles for proto-oncogenes in normal growth during embryonic development is very attractive (Adamson, 1987).

The results show that physiological concentrations of RA (1–20 nM) produced a strong inhibition of cell proliferation along with a selective induction of cell tissue differentiation. These effects were associated with a rapid inhibition of mitogen-induced *c-fos* mRNA levels. They suggest a role for RA in controlling the early development of the inner ear and that this control is effected through the regulation of the proto-oncogene *c-fos*.

Materials and methods

Experiments were done on chick embryo otic vesicles that were isolated by microdissection at stage 18 and cultured according to procedures described previously (Represa *et al.* 1988; Represa and Bernd, 1989) but with the following modifications. The culture chamber contained 250 μl of antibiotic-free culture medium, vesicles were incubated at 37°C in an atmosphere of 5 % CO_2 and the standard culture medium consisted of serum-free M-199 medium with Earle's salts (Flow laboratories).

For morphometry, otic vesicles were processed as previously described (Represa *et al.* 1986; Giraldez *et al.* 1987). Briefly, isolated otic vesicles were fixed with Bouin's solution, dehydrated and embedded in paraffin. Serial frontal sections (8 μm) were then stained with haematoxylin-eosin. Morphometric measurements were made using graphical reconstructions in camera lucida. Some specimens (Fig. 5) were fixed in 2 % glutaraldehyde in 0.1 M cacodylate buffer, dehydrated in a graded series of acetones and propylene oxide and embedded in Epon resin. Frontal or sagittal sections (1 μm) were cut and stained with 1 % toluidine blue. Light microscopy radioautography was performed as described elsewhere (Bernd and Represa, 1989).

DNA synthesis was measured as acid-precipitable [^{3}H]thymidine incorporation. Vesicles were placed in standard incubation medium containing 0.4 μM (10 μCi ml^{-1}) [^{3}H]thymidine for periods of 24 h. Vesicles were then washed in cold Ringer, extracted with 10 % trichloroacetic acid and the radioactivity incorporated into acid-precipitable material was counted in Triton X-100–toluene using a scintillation counter.

For Northern Blot Analysis, total RNA was extracted by lysing isolated otic vesicles in 3 M guanidinium isothiocyanate and centrifugation over a CsCl gradient. RNA was quantitated by measuring OD_{260} and analysed by electrophoresis in formaldehyde 1.3 % agarose gels. After transfer to nylon membranes (Nytran, Schleicher and Schuell), the Northern Blots were hybridyzed with a random-priming labelled avian *c-fos* probe (2×10^9 cts min^{-1} μg^{-1}) in 5×SSPE, 50 % formamide, 0.1 % SDS for 20 h at 55°C, washed and autoradiographed. The *c-fos* probe (Mölders *et al.* 1987) was generously provided by Drs Rolf Muller and Martin Zenke.

Results

Retinoic acid inhibits cell proliferation

The early development of the vertebrate inner ear involves the thickening and invagination of the ectoderm and the formation of the otic vesicle. At developmental stage 18 (Hamburger and Hamilton, 1951), it consists of a fluid-filled cavity lined by a transporting epithelium (Represa *et al.* 1986; Giraldez *et al.* 1987). In about 48 h the otocyst goes through a period of intense cell proliferation and evolves to form a more complex structure with signs of growth and morphogenesis. Cell division in the otic vesicle can be arrested *in vitro* by incubation in serum-free media and then reactivated by growth factors and mitogens (Represa *et al.* 1988; Miner *et al.* 1988). Fig. 1 shows a typical experiment where the effects of retinoic acid on this growth-factor-reactivated growth were assayed. The photograph on the left shows an otic vesicle that was isolated at stage 18 and arrested for 24 h (0S). Two other vesicles reactivated to grow with 10 % serum (10S) or with 100 nM bombesin *plus* 5 μg ml^{-1} insulin (B+I) are shown. They displayed the characteristic signs of growth and were taken as control conditions. The presence of RA in the incubation medium produced a concentration-dependent inhibition of vesicular growth whether induced by serum or bombesin. The numbers in Fig. 1 indicate RA concentrations (nM) and it can be seen that the inhibitory effect was detectable at 1 nM RA and increased up to 50 nM. The incorporation of [^{3}H]thymidine into acid-precipitable material in cultures corresponding to the above experiments are illustrated in Fig. 2. DNA synthesis was reduced in parallel with the inhibition of growth. Inhibition of [^{3}H]thymidine incorporation was already detectable at 1 nM RA concentration and a maximal effect occurred between 10 and 25 nM RA in vesicles reactivated by bombesin and serum, respectively. Fig. 3 compares autoradiographic sections of otic vesicles incubated with serum (10 %) in the absence (Fig. 3A, C) or in the presence (Fig. 3B, D, E) of retinoic acid. An example of an arrested vesicle, incubated in the normal medium but in the absence of additives (Fig. 3F), is shown for comparison. The reduced uptake of labelled thymidine with retinoic acid was associated with the loss of labelling of nuclei in the epithelium. It should be noted that the regional pattern of cell proliferation in the otic vesicle, ventral and medial, was preserved in the presence of RA.

The effect of retinoic acid on cell proliferation was also estimated by measuring the volume of the *epithelial tissue* of the otic vesicle. This was obtained morphometrically from serial reconstructions of otic vesicles that were incubated in the presence of 10 % serum and with increasing concentrations of retinoic acid (Fig. 4). These experiments were done in parallel with those used for DNA incorporation measurements. Cell density values remained fairly constant at different RA concentrations. For example, cell density values ($\times10^{-8}$ cells cm^{-3}) were 95±26 for 10S in the absence of RA, 80±22 in 17 nM RA and 89±8 in 0S (42 fields per vesicle, 2 vesicles per condition). This indicates that the measurements of epithelial tissue volume should faithfully reflect the absolute number of epithelial cells in each condition. For the cell densities given above and the corresponding values of epithelial volume from

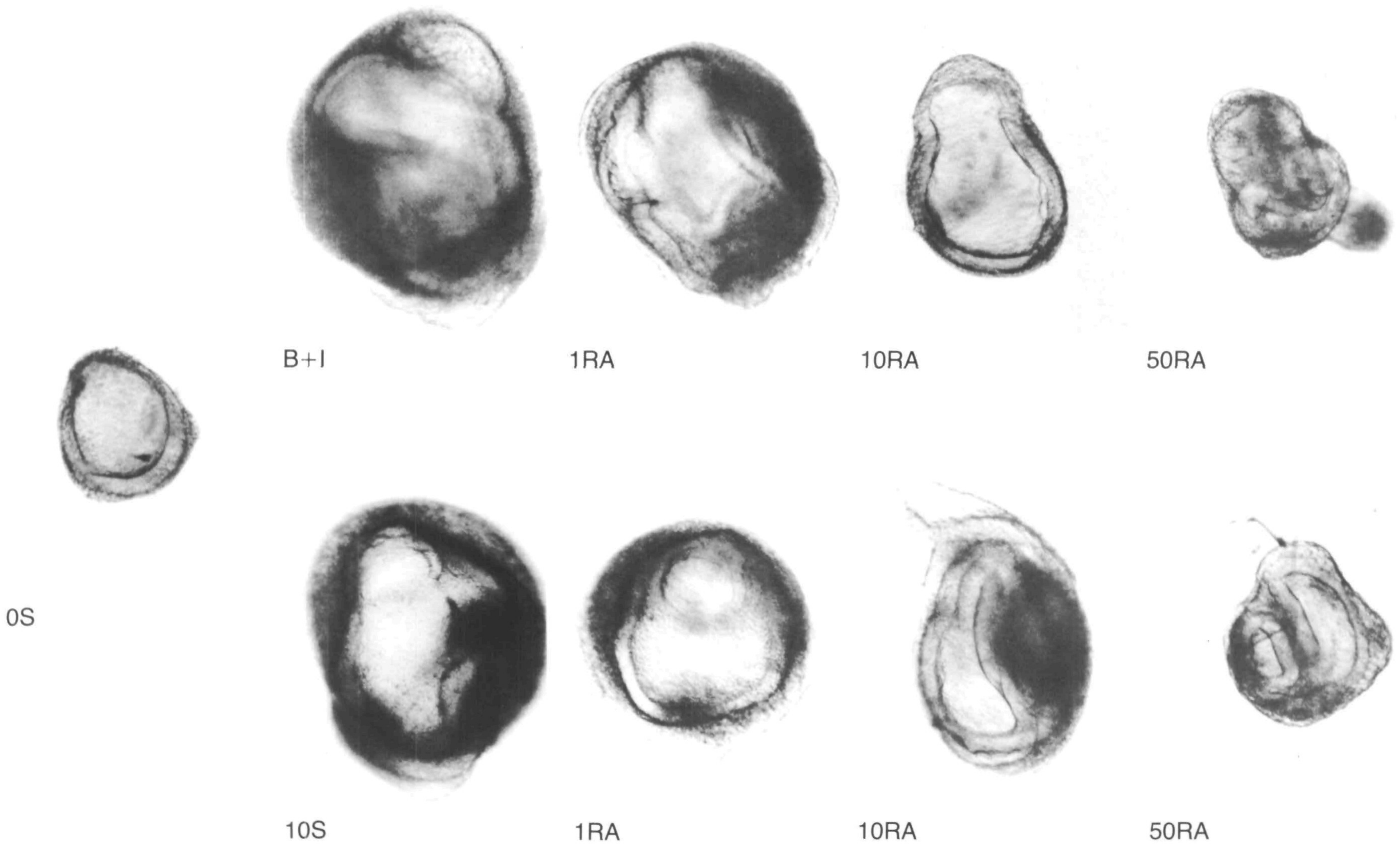

Fig. 1. Retinoic Acid (RA) inhibits growth of explanted otic vesicles. Otic vesicles were isolated at stage 18, made quiescent by incubation for 24 h in the absence of serum and then reactivated for another 24 h in the appropriate media. Reactivation was carried out either by the addition of 100 nM bombesin plus 5 $\mu g\,ml^{-1}$ insulin (B+I) (upper row) or 10 % foetal calf serum (10S) (lower row), in the presence of RA (nM) at the concentrations indicated by the numbers. Calibration bar=100 μm.

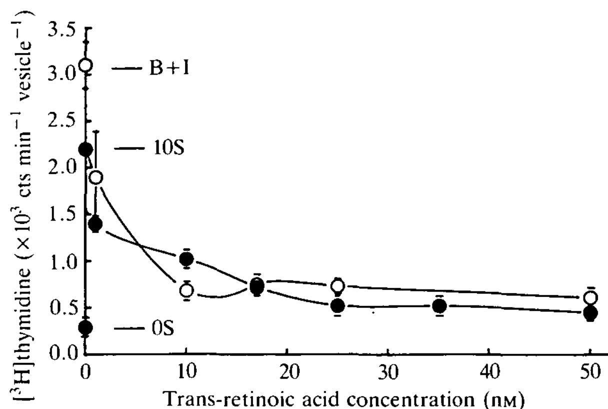

Fig. 2. Retinoic acid inhibits DNA synthesis induced by mitogens. Acid-precipitable [^{3}H]thymidine incorporation by reactivated otic vesicles as a function of RA concentration in the culture medium. Vesicles were reactivated with 10% FCS (filled circles) or 100 nM bombesin plus 5 μg ml^{-1} insulin (open circles). Values are mean±S.E. of 3 to 4 measurements.

Fig. 4, the number of cells ($\times 10^{-4}$) was 13.5 in 10S, 2.4 in 17 nM RA and 2.5 in 0S. The inhibition of RA on cell proliferation was, therefore, also demonstrated by direct estimates of the cell number.

Retinoic acid induces differentiation and reverses the direction of growth

Several morphological changes were also associated with the inhibition of cell proliferation by retinoic acid, and they were studied in 17 different vesicles cultured in serum *plus* different concentrations of retinoic acid 1 nM (4), 10 nM (9), and 25 nM (4). Another 8 vesicles incubated in serum with no added RA served as controls. First, low concentrations of RA (1–10 nM) in the presence of serum produced the induction of differentiated structures that are normally present in the otic vesicle at much more advanced developmental stages. Fig. 5A,B shows that incubation with serum and RA (10 nM) for 24 h induced the differentiation of dome-like epithelial structures, which strongly resembled the *tegmentum vasculosum* with characteristic large scale corrugations, and which normally do not appear until developmental stage 30–33 (5–6 days in culture). This is a secretory epithelium, which is the equivalent in birds of the mammalian *stria vascularis*. An example of a *tegmentum vasculosum* from a chick embryo otic vesicle after 5 days in culture is shown in Fig. 5C,D for comparison. No such structure was observed in 24–48 h cultures in the absence of RA in the present or previous work. Note that the RA-induced differentiation was located in the dorsal region of the otic vesicle which, during normal development, will also give rise to the physiological *tegmentum vasculosum*. The early induction of the *tegmentum vasculosum* was observed in 13 specimens (4, 5 and 4 in 1, 10 and 25 nM RA, respectively). Another sign of differentiation of the secretory system was the formation of the endolymphatic sac (see below).

Fig. 6 shows the thickening of defined areas of epithelium (Fig. 6A, area within arrows) which became stratified and contained both 'clear' and 'dark' cells with toluidine blue staining (Richardson blue). 'Dark cells' were flask-shaped and typically polarized towards the inner surface (Fig. 6B,C and inset). This epithelium was adjacent to the developing cochleo-vestibular ganglion (CVG in Fig. 6) and closely resembled intermediate stages of differentiation of the auditory and vestibular sensory epithelia, which again occurs much later during the course of normal development (days 5–6) (see Whitehead and Morest, 1985). This epithelium gives rise to hair cells by day 7 *in vivo* or the equivalent time in grafting experiments (Swanson *et al.* 1990). These features were observed in 6 vesicles incubated in 10 nM RA, 3 in 1 nM RA and 1 in 25 nM RA).

The second interesting morphological feature associated with retinoic acid incubation was the inversion of the direction of growth in the endolymphatic duct, which normally develops as a finger-like outpocketing of the dorsal vesicular wall but which in vesicles cultured with RA occurred as an invagination of the dorsal wall. Fig. 7 shows that the induction of the differentiation of the endolymphatic sac was also accelerated compared to normal development. The endolymphatic sac appears normally between days 6 and 7 of development *in vivo*. Fig. 7 also shows, however, that it was directed inwardly towards the vesicular cavity. The reversion of the endolymphatic duct was observed in 7 out of the 9 vesicles incubated in 10 nM RA. The fully developed endolymphatic sac could be detected in two of these experiments.

The tendency of the growing vesicle to polarize inwardly is also illustrated in Fig. 8 where the values of the internal and external vesicular surface areas (S_i, S_e) obtained morphometrically were plotted against RA concentration in the culture medium. During normal growth (serum-stimulated) S_i was always smaller than S_e, a relation that also applied for growth-arrested vesicles. Increasing concentrations of RA reversed this relationship S_i becoming larger than S_e. The inset of Fig. 8 shows the ratio S_i/S_e plotted *versus* RA concentration. It can be seen that the ratio S_i/S_e increased from about 0.7 in the absence of RA to 1.25 at 50 nM RA. Average values for S_i/S_e ratios were 0.77±0.03 and 1.33±0.07 for 10S and 10S+RA (50 nM), respectively (n=3). That is, at high concentrations of RA the internal surface had increased almost two-fold relative to the external surface, and the normal polarity of growth was reversed.

Retinoic acid inhibits mitogen-induced c-fos *expression*

Finally, we examined the possibility that the effects of RA were related to *c-fos* expression in the otic vesicle. Fig. 9A shows a Northern Blot analysis of otic vesicles at different developmental stages (indicated by the numbers). Levels of *c-fos* mRNA were undetectable in stage 12, they were just measurable in stage 18, increased in stage 21 and were again undetectable in stages 22 and 27. Values of optical density are plotted in Fig. 9B along with the cumulative cell number taken

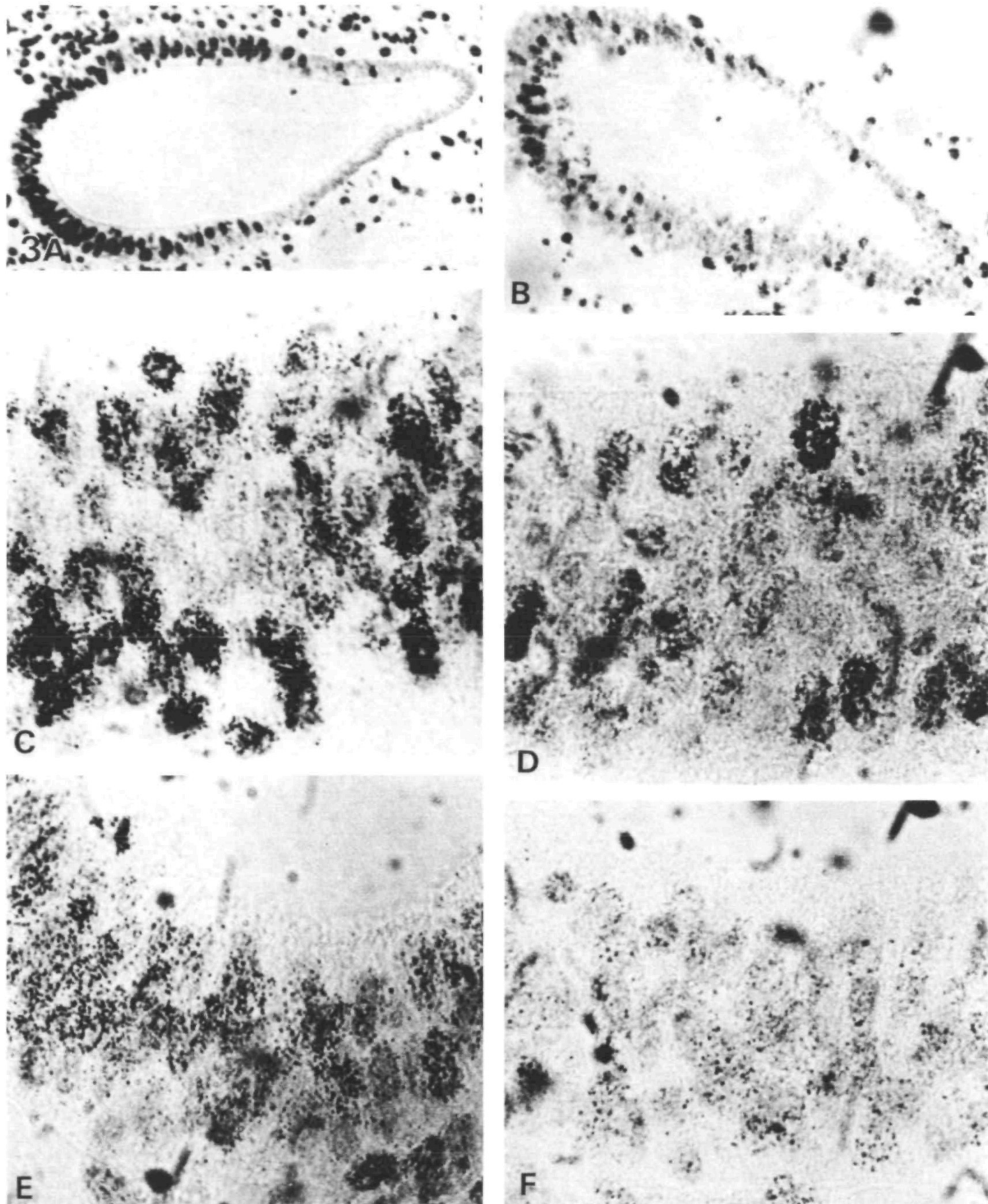

Fig. 3. Photomicrographs of 8 μm sections of otic vesicles processed for autoradiography. Quiescent vesicles were cultured for 24 h in 10 % FCS in the absence (A and C) and in the presence of 10 nM RA (B, D) and 25 nM RA (E). One vesicle incubated in serum-free is shown in F. Photographs at the bottom are enlargements of the vesicular wall corresponding to the vesicles shown above. Magnification: 75× for A and B, and 920× for the others.

from Giraldez *et al.* (1987) and normalized to the value in stage 21. Two other experiments gave a similar stage-dependent increase in the mRNA level of *c-fos*. The expression of *c-fos* appeared, therefore, transient and correlated to the proliferative phase of the development of the otic vesicle.

Incubation of growth-arrested vesicles with bombesin *plus* insulin for 20 min at concentrations that induced cell proliferation and vesicular growth, produced a strong induction of *c-fos* (Fig. 10A, lane B+I). RNA extracted from stage 21 otic vesicles was run in this particular experiment as a positive control (lane 21). In view of the anti-proliferative effect of retinoic acid on bombesin-induced cell proliferation, we examined the effect of RA on *c-fos* induction. Growth-arrested otic vesicles were incubated in the presence of bombesin *plus* insulin for 20 min in the presence of RA (25 nM). The result is shown in Fig. 10A (lane B+I+RA) and

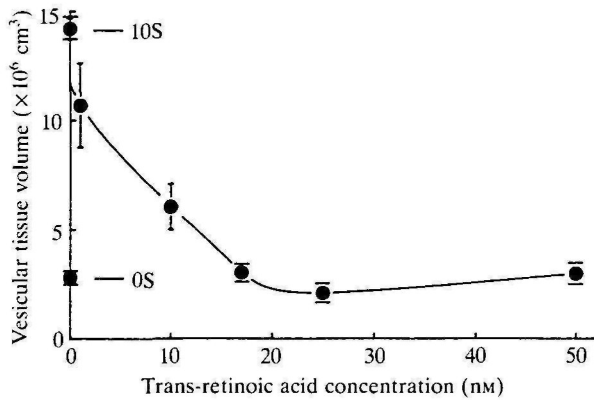

Fig. 4. Retinoic acid effects on epithelial tissue volume. Otic vesicles were cultured in the presence of 10 % FCS in the presence of different concentrations of RA. Values of epithelial tissue volume were obtained morphometrically and plotted against the concentration of RA in the incubation medium. Values are mean±S.E. of complete reconstructions of 3 different vesicles per condition.

demonstrates that the mitogen-induced increase in *c-fos* mRNA level was suppressed in the presence of retinoic acid even below the control value (0S). A semiquantitative analysis of these results is given in Fig. 10B, where normalized values of optical density are displayed.

Discussion

The results reported here show that retinoic acid inhibits cell proliferation, induces differentiation and positional changes, and suppresses *c-fos* induction. A crucial point is that the observed effects of retinoic acid on the otic vesicle are produced at concentrations ranging between 1 and 25 nM. These can be considered as 'physiological' concentrations since (1) these figures are within the range of concentrations measured in chick embryos by HPLC, 10–50 nM, (Thaller and Eichelle, 1987), and (2) they are close to the affinity of RA-receptors for RA, 0.6–30 nM (Giguere *et al.* 1987; Petkovich *et al.* 1987). This quantitative correspondence is important because it sets a constraint for any possible role of retinoic acid in normal development.

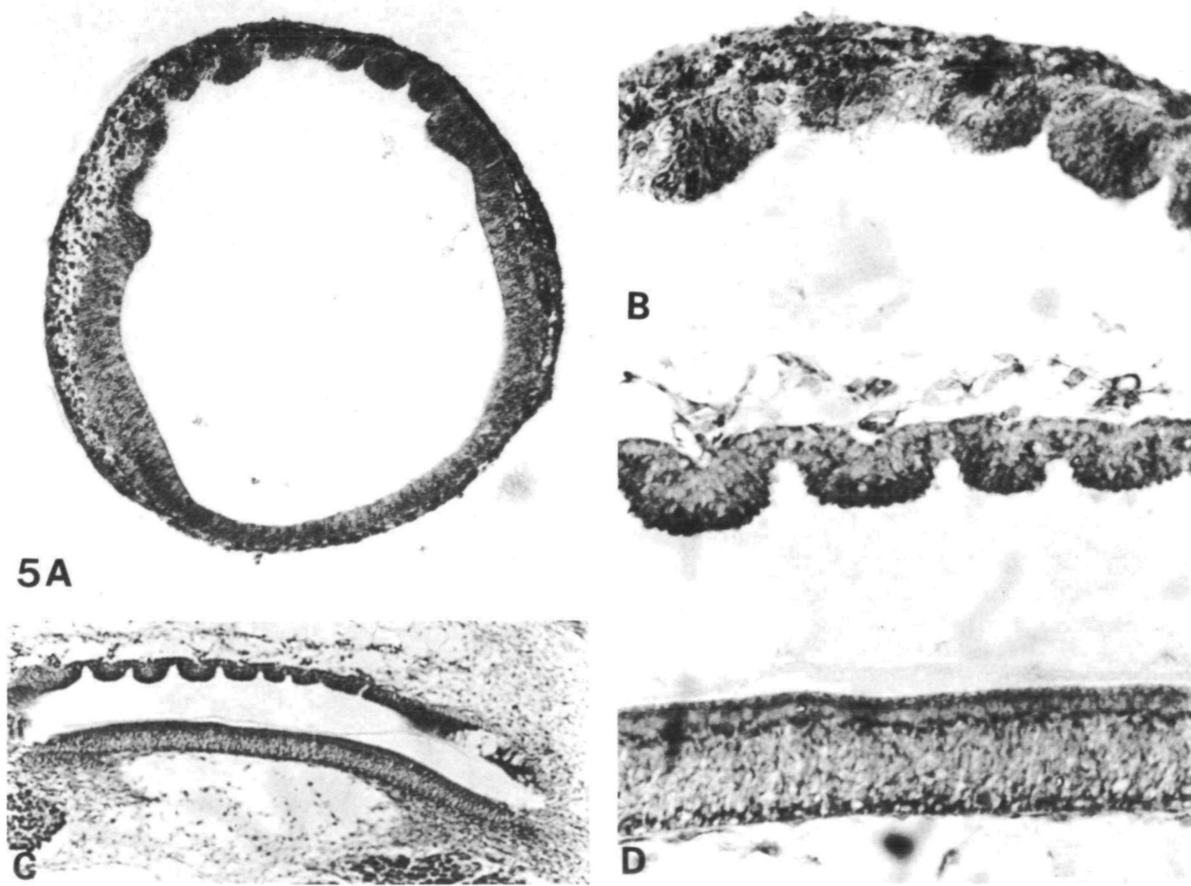

Fig. 5. Retinoic acid induction of the *tegmentum vasculosum*. Micrographs of 8 μm frontal sections of otic vesicles processed for conventional light microscopy. (A) Quiescent vesicles were incubated for 24 h with 10 % FCS in the presence of 10 nM RA (mgn.=150×). An enlargement of the upper part is shown in B (520×). To the right, C (100×) and D (460×) show the appearence of the cochlear duct after 5 days in culture with 10 % FCS.

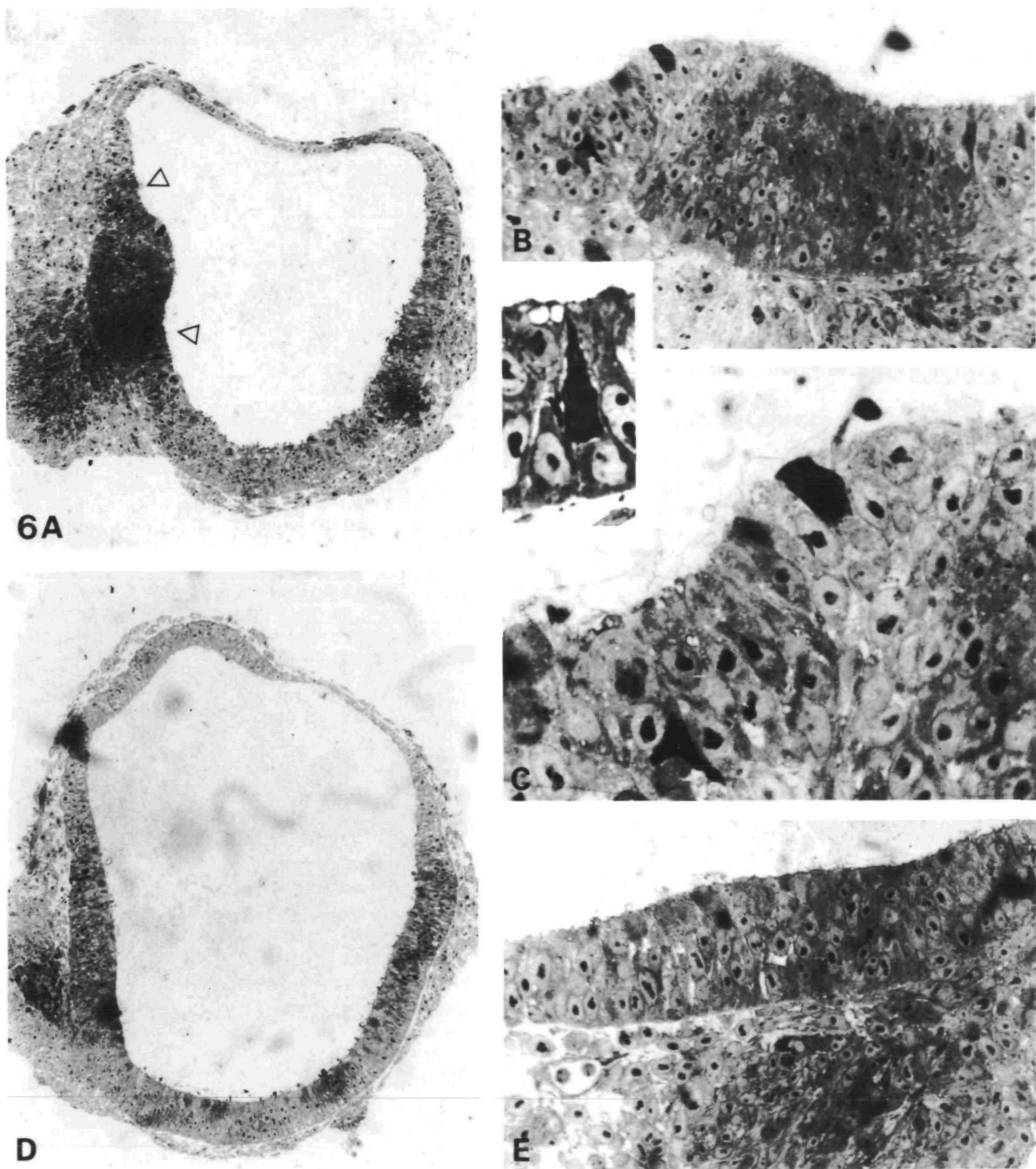

Fig. 6. Retinoic acid induction of differentiation to early sensory epithelium. (A) Low magnification of an otic vesicle cultured in 10% serum plus 10 nM RA (120×). An enlargement of the epithelium within arrows is shown in B (480×) and C (720×). The inset (800×) shows a typical sensory type 'dark cell'. D and E show cultures with 10% serum for comparison.

Evidence has accumulated during recent years that retinoic acid can operate as a morphogen during embryogenesis and that concentration gradients of RA modulate tissue pattern formation in the developing limb (see Summerbell and Maden, 1990 for review). The effects of retinoic acid on the otic vesicle can be taken as a more general extension of this property. Retinoic acid induced in the otic vesicle differentiated tissue organizations such as the *tegmentum vasculosum*, early sensory epithelium and endolymphatic sac, which normally appear much later in development. In view of the results presented here, the search for early

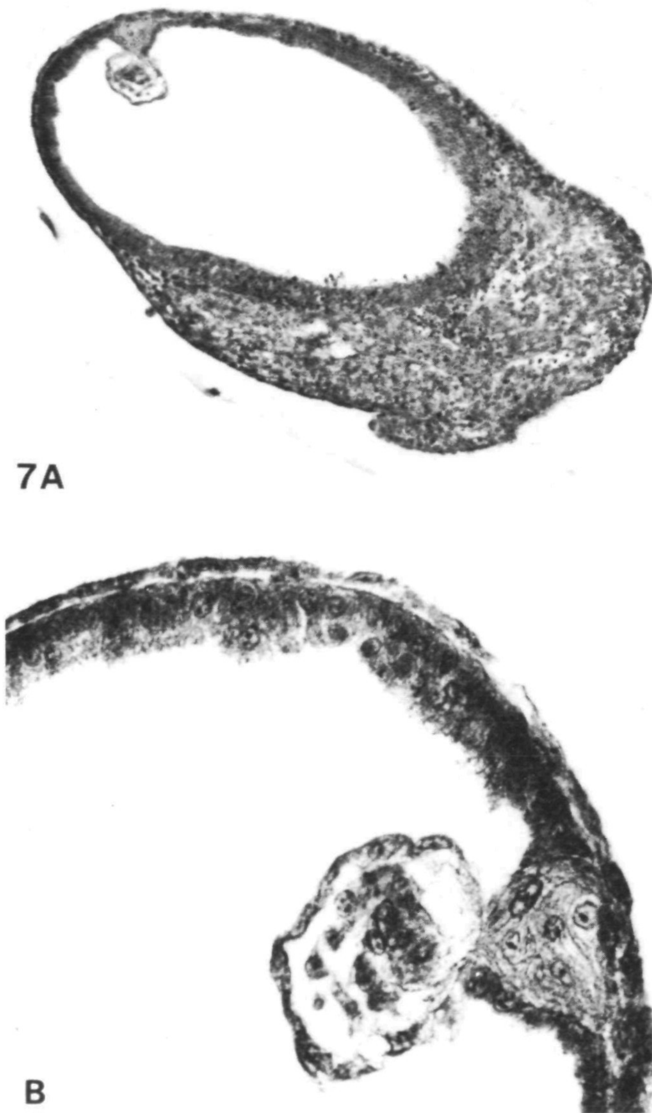

Fig. 7. Retinoic acid induction of a differentiated endolymphatic sac exhibiting reversed polarity. The photographs correspond to 8 μm paraffin sections of an otic vesicle cultured for 24 h in the presence of serum (10 %) and 10 nM RA. Magnification=100× and 800×.

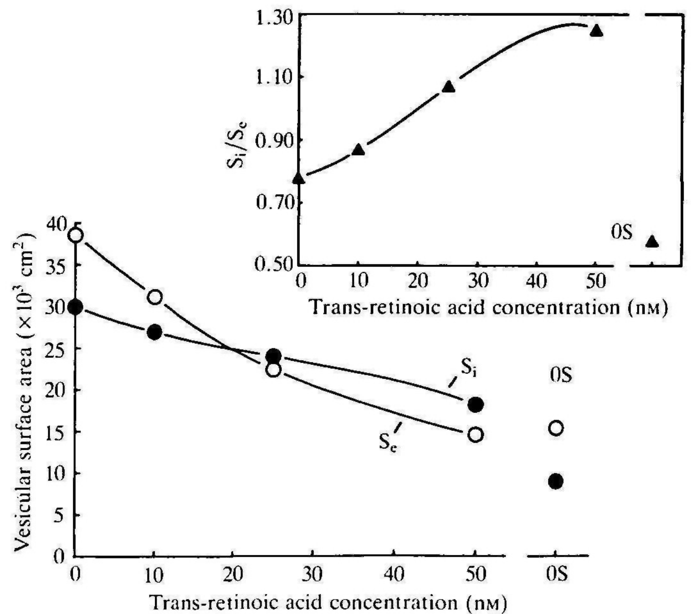

Fig. 8. Retinoic acid reduces vesicular surface area and reverses the polarity of growth. The internal surface area, S_i (●), and the external surface area, S_e (○), were plotted against the concentration of RA (nM) in the incubation medium. Measurements were done on experiments similar to those in Fig. 1A after processing for morphometric reconstructions from 8 μm sections (Giraldez *et al.* 1987). The ratio S_i/S_e *versus* RA concentration is shown in the inset graph.

molecular markers of differentiation for the specific cell types becomes imperative and current work is in progress in that direction. Our results show that, morphologically, the differentiation of secretory structures can be accelerated by retinoic acid to reach very advanced stages of differentiation in the 24 h incubation period of observation. This effect, although present, was less pronounced in the case of sensory epithelia where fully developed receptors were not detected. The origin of this apparent differential sensitivity to retinoic acid remains unknown. The induction of differentiation had not only the property of being physiological in terms of the appearance of the tissue, but also it was located in the 'correct' presumptive areas of the otic vesicle, i.e. those from which they would originate during normal development (Li *et al.* 1978). Whether this is related to the regional distribution of RA receptors or to the selective activation of cellular programme is unknown. The effect of RA reversing the normal polarity of the growth of the otic vesicle is perhaps more difficult to interpret, but may well reflect the ability of RA or RA-gradients to organize growth in three dimensions. It has to be considered that, although non-stationary, a gradient of RA was established in the cultures, which was normal to the plane of the epithelium and directed towards the vesicular cavity.

The effects of retinoic acid on the differentiation of vesicular tissue were associated with a strong inhibition of cell proliferation. That cells inhibited to proliferate were those of the vesicular epithelium with little contribution, if any, from the surrounding mesenchymal cells was confirmed by the parallel evolution of epithelial tissue volume and DNA synthesis measurements. Thus, as in normal development, RA-induced differentiation is associated with the interruption of cell division. The precocious appearance of differentiated structures could either be due to a direct activation of specific transcriptional programme by RA or to the induction of such activity as a result of the inhibition of cell proliferation. Differentiation may normally be incompatible with proliferative phases and the transition to differentiative periods could be then controlled by inhibition of cell proliferation by RA. These observations on the effects of RA on the early development of the inner ear, taken together, suggest a

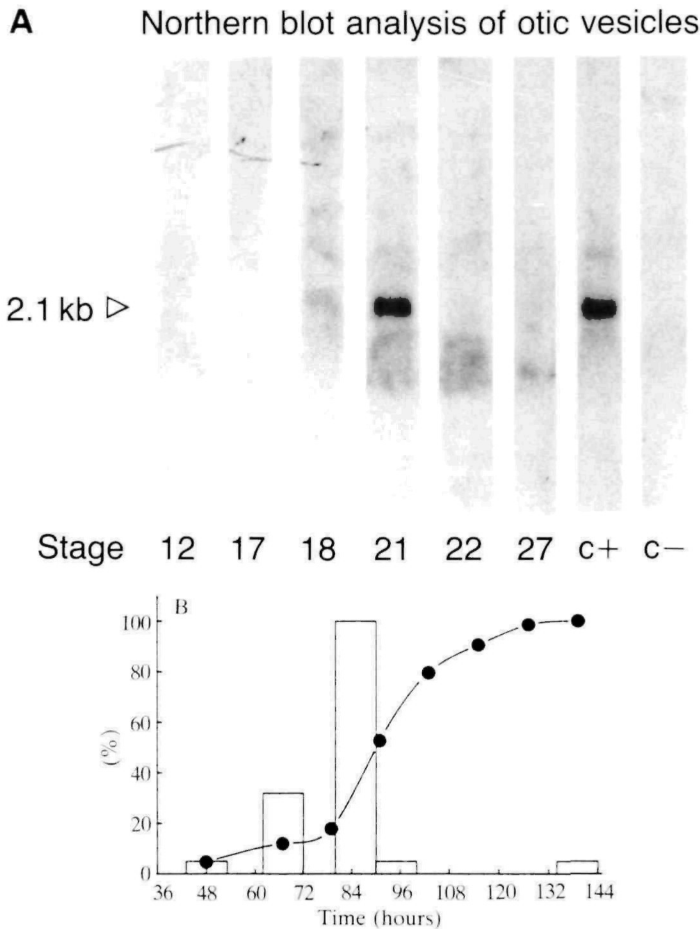

Fig. 9. (A) Northern blot analysis of isolated otic vesicles. Total RNA was purified from 50 otic vesicles for each stage and 20 μg of total RNA were loaded per lane. The numbers indicate the developmental stage. Lane labelled c+ was a positive control that consisted of RNA from extraembryonic membranes (Müller *et al.* 1982) and lane c− was RNA from L929 cells. (B) Densitometric values of different stages (bars) are plotted along with the cumulative increase in cell number (O) taken from Giraldez *et al.* (1987), against time and the corresponding developmental stage. Values were expressed as a percentage of the stage 21 value.

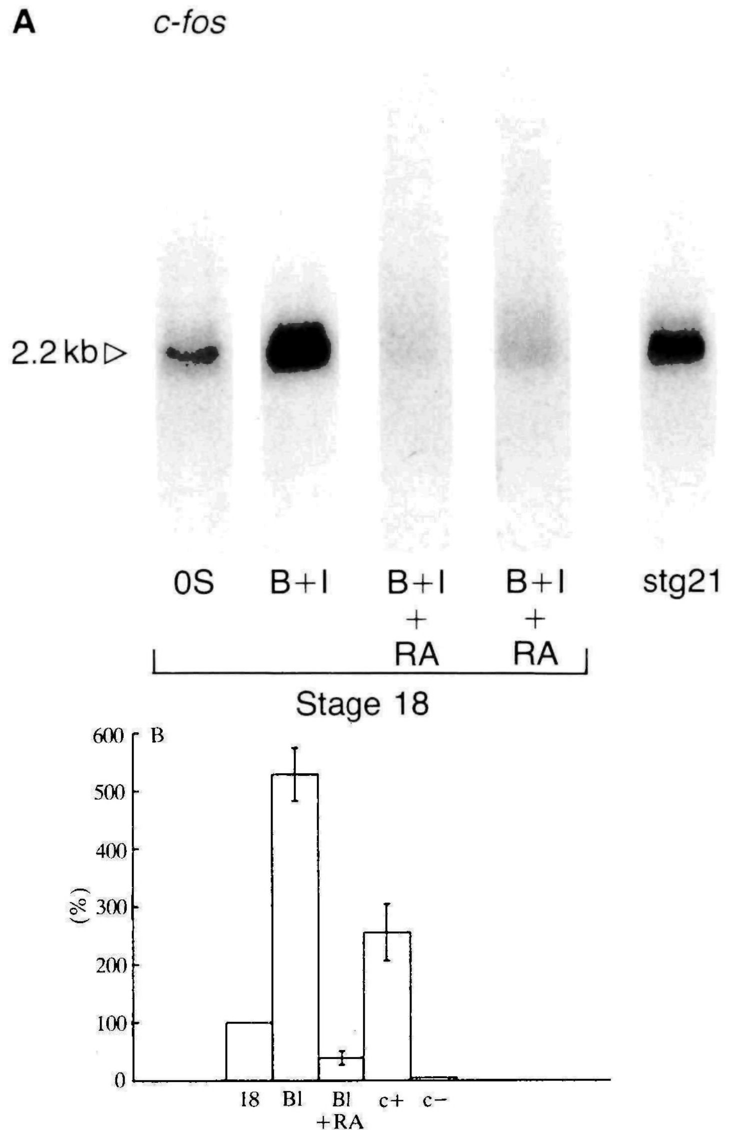

Fig. 10. (A) Effect of retinoic acid on mitogen-induced *c-fos* expression. Northern blot analysis was performed with 20 μg of RNA purified from 50 otic vesicles per condition. Lanes 18+BI and 18+BI+RA (duplicated) indicate RNA from vesicles in stage 18 incubated with 100 nM bombesin plus 5 μg ml^{-1} insulin for 20 min in the absence or in the presence of 25 nM RA, respectively. RNA from stage 21 (lane stg21) was run in parallel as a positive control. (B) Densitometric measurements of several experiments like in A are shown in part B, expressed as percentage of values in stage 18. Values are mean±S.E. of 4 (0S), 5 (B+I), 8 (B+I+RA), 5 (c+) and 4 (c−) measurements, respectively.

physiological role for this molecule in the regulation of the development of the otic vesicle. Retinoic acid could, therefore, represent a putative morphogen in the developing inner ear.

The *c-fos* proto-oncogene belongs to a group of rapidly induced genes encoding proteins that form complexes regulating transcription (see Herschman, 1989 and Curran, 1988). Several growth factors are known to induce *c-fos* gene and protein (Müller *et al.* 1984; Lau and Nathans, 1987; Almendral *et al.* 1988). Yet, the relationship between *c-fos* expression and the processes of cell proliferation and differentiation, as judged from studies on cultured cell lines, is still unclear. Expression of *c-fos* has been measured in mouse embryo extracts during days 1 to 10 of prenatal development (Müller *et al.* 1982) and, using *in situ* hybridization techniques, *c-fos* mRNA has been detected in certain areas of the nervous system in late stages of mouse development (Caubet, 1989). However, no functional relation to defined events during embryogenesis has been demonstrated. The results reported here show (1) a transient expression of *c-fos* throughout the development of the otic vesicle, (2) the rapid induction of *c-fos* by bombesin and (3) its inhibition by retinoic acid. The first point is of interest because it shows the constitutive expression of *c-fos* in

the otic vesicle and that it is stage dependent. Moreover, high levels of *c-fos* expression coincide with the most active period of the proliferative phase in the otic vesicle suggesting that the physiological regulation of *c-fos* is associated with the control of cell proliferation. This is also supported by the fact that a mitogen like bombesin is able to induce the expression of *c-fos* and, additionally, by the association between the inhibitory effects of RA and the suppression of *c-fos* induction. This indicates that *c-fos* expression may be a critical element for regulation of cell proliferation in the otic vesicle. Moreover, *c-fos* appears to be a target for retinoic acid. The developmental effects of RA, therefore, might be in part exerted *via* the regulation of the expression of *c-fos*.

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References

Adamson, E. D. (1987). Oncogenes in development. *Development* **99**, 449–471.

Almendral, J. M., Sommer, D., Macdonald-Bravo, H., Burckhardt, J., Perera, J. and Bravo, R. (1988). Complexity of the early genetic response to growth factors in mouse fibroblasts. *Molec. cell. Biol.* **8**, 2140–2148.

Astigiano, S., Sherman, M. I. and Abarzúa, P. (1989). Regulation and patterns of endogenous and exogeneous gene expression during differentiation of embryonal carcinoma cells. *Environ. Health Perspect.* **80**, 25–38.

Bernd, P. and Represa, J. J. (1989). Characterization and localization of nerve growth factor receptors in the embryonic otic vesicle and cochleovestibular ganglion. *Devl Biol.* **134**, 11–20.

Caubet, J. F. (1989). C-fos proto-oncogene expression in the nervous system during mouse development. *Molec. cell. Biol.* **9**, 2269–2272.

Curran, T. (1988). The fos oncogene. In *The Oncogene Handbook* (ed. E. P. Reddy and A. M. Skalka), pp. 307-325. Amsterdam: Elsevier.

Durston, A. J., Timmermans, J. P. M., Hage, W. J., Hendriks, H. F. J., Vries, N. J. de, Heideveld, M. and Nieuwkoop, P. D. (1989). Retinoic acid causes an anteroposterior transformation in the developing central nervous system. *Nature* **340**, 140–144.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. *Science* **240**, 889–895.

Geelen, J. A. (1979). Hypervitaminosis A induced teratogenesis. *CRC Crit. Rev. Toxicol.* **6**, 351–375.

Giguere, V., Ong, E. S., Segui, P. and Evans, R. M. (1987). Identification of a receptor for the morphogen retinoic acid. *Nature* **330**, 624–629.

Giraldez, F., Represa, J. J., Borondo, L. and Barbosa, E. (1987). Polarization and density of Na-pumps in the inner ear of the chick embryo during early stages of development. *Development* **100**, 271–278.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49–92.

Herschman, H. R. (1989). Extracellular signals, transcriptional responses and cellular specificity. *TIBS* **14**, 455–458.

Jetten, A. M. (1986). Induction of differentiation of embryonal carcinoma cells by retinoids. In *Retinoids and Cell Differentiation* (ed. M. I. Sherman) pp. 105–136. Boca Raton, Florida U.S.A.: CRC Press.

Lau, L. F. and Nathans, D. (1987). Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: coordinate regulation with c-fos or c-myc. *Proc. natn. Acad. Sci. U.S.A.* **84**, 1182–1186.

Li, C. W., Van De Water, T. R. and Ruben, R. J. (1978). The fate mapping of the eleventh and twelfth day mouse otocyst: An *in vitro* study of the sites of origin of the embryonic inner ear sensory structures. *J. Morph.* **157**, 249–268.

Miner, C., Represa, J. J., Barbosa, E. and Giráldez, F. (1988). Protein-kinase C is required during the early development of the inner ear in culture. *Roux Arch. devl Biol.* **197**, 294–297.

Mölders, M., Jenuwein, H., Adamkiewicz, T. and Müller, R. (1987). Isolation and structural analysis of a biologically active chicken *c-fos* cDNA: identification of evolutionary conserved domains in *fos* protein. *Oncogene* **1**, 377–385.

Morriss, G. M. (1972). Morphogenesis of the malformations induced in rat embryos by maternal hypervitaminosis A. *J. Anat.* **113**, 241–250.

Müller, R., Bravo, R., Burckhardt, J. and Curran, T. (1984). Induction of c-fos gene and protein by growth factors procedes activation of c-myc. *Nature* **312**, 716–720.

Müller, R., Slamon, D., Tremblay, J. M., Cline, J. M. and Verma, I. M. (1982). Differential expression of cellular oncogenes during pre and postnatal development of the mouse. *Nature* **299**, 640–644.

Petkovich, M., Brand, N., Krust, A. and Chambon, P. (1987). A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* **330**, 624–629.

Represa, J. J., Barbosa, E. and Giraldez, F. (1986). Electrical properties of the otic vesicle epithelium in the chick embryo. *J. Embryol. exp. Morph.* **97**, 125–139.

Represa, J. J. and Bernd, P. (1989). Nerve growth factor and serum differentially regulate development of the embryonic otic vesicle and cochleovestibular ganglion *in vitro*. *Devl Biol.* **134**, 21–29.

Represa, J. J., Miner, C., Barbosa, E. and Giraldez, F. (1988). Bombesin and other growth factors activate cell proliferation in chick embryo otic vesicles in culture. *Development* **102**, 87–96.

Sidell, N., Altman, A., Haussler, M. and Seeger, R. (1983). Effects of retinoic acid (RA) on the growth and phenotypic expression of several human neuroblastoma cell lines. *Expl Cell. Res.* **148**, 21–30.

Summerbell, D. and Maden, M. (1990). Retinoic acid, a developmental signalling molecule. *TINS* 13 4, 142–147.

Swanson, G. J., Howard, M. and Lewis, J. (1990). Epithelial autonomy in the development of the inner ear of a bird embryo. *Devl Biol.* **137**, 243–257.

Thaller, C. and Eichele, G. (1987). Identification and spatial distribution of retinoids in the developing chick limb bud. *Nature* **327**, 625–628.

Tickle, C., Alberts, B. M., Wolpert, L. and Lee, J. (1982). Local application of retinoic acid to the anterior margin of the limb bud mimics the action of the polarizing region. *Nature* **296**, 564–565.

Van de Water, T. R. (1984). *Developmental Mechanisms of Mammalian Inner Ear Formation* (ed. I. Berlin), pp. 49–107. San Diego, California: College Hill Press.

Whitehead, M. C. and Morest, D. K. (1985). The development of innervation patterns in the avian cochlea. *Neuroscience* **14**, 255–276.

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