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Expression of *Xenopus* N-CAM RNA in ectoderm is an early response to neural induction

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Summary

We have isolated *Xenopus laevis* N-CAM cDNA clones and used these to study the expression of N-CAM RNA during neural induction. The results show that the first marked increase in N-CAM RNA levels occurs during gastrulation when mesoderm comes in contact with ectoderm and induces neural development. *In situ* hybridization results show that the early expression of N-CAM RNA is localized to the neural plate and its later expression is confined to the neural tube. Induction experiments with explanted germ layers show that N-CAM RNA is not expressed in ectoderm unless there is contact with inducing tissue. Together these results suggest an approach to studying how ectoderm is committed to form neural rather than epidermal tissue. Specifically, the data suggest that neural commitment is marked and perhaps mediated by the transcriptional activation of genes, like N-CAM, in the neural ectoderm.

Key words: N-CAM, induction, *Xenopus*, neurula, exogastrula, RNA.

Introduction

The separation of ectoderm into neural and epidermal lineages is an essential feature of vertebrate development. In amphibians, this separation occurs during gastrulation when ectoderm in contact with dorsal mesoderm forms the neural plate. While surrounding ectoderm gives rise to epidermis, the ectoderm of the neural plate forms the neural tube and eventually differentiates into the various cell types of the vertebrate nervous system.

Numerous studies have shown that prior to gastrulation all ectoderm of a blastula-stage amphibian embryo has the potential to give rise to either neural or epidermal tissue. During gastrulation, the ectoderm that comes in contact with invaginating dorsal mesoderm is induced to form neural tissue. Induction of ectoderm to form neural tissue was first shown by the now classic experiments of Spemann and Mangold in which mesoderm (an organizer) was transplanted from the dorsal side of one early gastrula to the ventral side of another (Spemann, 1938). As a result of this manipulation, the host ectoderm is induced by invaginating organizer tissue to form a second nervous system on the ventral side of the embryo (Gimlich & Cooke, 1983; Smith & Slack, 1983; Jacobson, 1984). It has also been shown that embryos will form epidermal but not neural tissue when gastrulation is disrupted. When embryos are stripped of the vitelline membrane and cultured in high salt, the mesoderm moves outward rather than into the blastocoel during gastrulation (Holtfreter, 1933). In these abnormal gastrulae or exogastrulae, the normal contact of mesoderm with ectoderm is prevented and consequently the ectoderm differentiates only into epidermis. Finally, if blastula ectoderm is isolated and placed in culture it gives rise only to epidermis. If cultured instead in contact with dorsal mesoderm, or treated with a wide variety of 'neuralizing' agents, ectoderm forms neural structures (Holtfreter & Hamburger, 1955). These and other studies have led to the view that the 'ground' state in the development of ectoderm is to form epidermal tissue. Induction directs ectoderm to form neural tissue and, as a result, it is considered to be one example of a primary mechanism for specifying cell fate in the vertebrate embryo.

At present it is not known what changes occur in ectoderm, in response to induction, that enable the formation of neural rather than epidermal tissue. One approach to this problem is to consider the question of when new gene expression occurs in ectoderm

following induction. It is possible that new neural specific gene expression does not occur until after the neural tube forms and differentiated cell types of the nervous system appear. The implication of this model is that the early steps in the formation of neural tissue utilize pre-existing mRNAs and new gene expression in these cells does not occur until cells differentiate into neurones and glia. This model is supported by the observation that the expression of many genes specific to the nervous system tends to occur relatively late in development. For example, glial acidic fibrillary protein and neurofilaments are first expressed after neural tube closure in association with neuro- or gliogenesis (Tapscott, Bennett, Toyama, Kleinbart & Holtzer, 1981; Godsave, Anderton & Wylie, 1986).

Another possibility is that new gene expression occurs as an immediate response to neural induction. The implication of this model is that even the initial stages of neural tissue formation require new gene products. Turning on the expression of these genes by induction enables the formation of neural rather than epidermal tissue. To support this second model, it is essential to identify genes that are first expressed at early stages of neural development. If such genes exist, then this model predicts that induction directly controls their expression. The final goal is to determine whether the expression of these genes causes ectoderm to form neural tissue.

One candidate for a gene expressed early in neural development comes from studies on the neural cell-adhesion molecule (N-CAM). N-CAM is a heterogeneous group of cell surface glycoproteins apparently encoded by one gene (Murray, Hemperly, Prediger, Edelman & Cunningham, 1986). These cell surface molecules are primarily expressed in the developing nervous system and are thought to mediate a variety of cell–cell interactions during neural development (Edelman, 1984). Antibodies against N-CAM stain the neural plate, notochord and myotomes, but not surrounding ectoderm in chick embryos (Edelman, Gallin, Delovee & Cunningham, 1983). Antibodies against *Xenopus* N-CAM show specific staining of the nervous system and this staining is dependent on induction (Jacobson & Rutishauser, 1986). Taken together the results from antibody staining in frogs and chickens suggest that N-CAM protein appears early in neural development. It is, however, not known precisely when the N-CAM gene is transcriptionally activated or whether this occurs in a restricted region of presumptive neural tissue.

We have isolated a cDNA clone for *Xenopus laevis* N-CAM in order to study the transcription of this gene during neural induction. Using a RNase protection assay to measure N-CAM RNA levels, we find a marked increase in N-CAM RNA during gastrulation. *In situ* hybridization to neural-plate-stage embryos shows that N-CAM RNA is localized exclusively to the neural plate and not expressed by surrounding epidermis. Finally, induction experiments show that N-CAM RNA expression does not occur in blastula ectoderm unless it is brought in contact with inducing tissue. We conclude from these results that N-CAM gene expression is one of the first responses marking neural tissue formation in *Xenopus* and that its expression in ectoderm is controlled by induction.

Materials and methods

Materials

Frogs were purchased from Xenopus 1 (Ann Arbor, MI). Eggs were obtained by injecting female frogs with mare serum gonadotropin (100 units, Sigma G4877) followed 2–6 days later by injection of human chorionic gonadotropin (1000 units, Sigma CG-10). Eggs were stripped and fertilized with minced testis. Embryos were raised in 0·1 × MBSH (Gurdon, 1977) at room temperature or, in some cases, at 17°C to slow the rate of development. Embryos were staged according to Nieuwkoop & Faber (1967).

Exogastrulae were generated by placing embryos in 1·1 × MBSH until they reached midblastula (stage 8). At this point the vitelline membrane was mechanically removed and the embryos were maintained in 1·0 × MBSH with penicillin/streptomycin (Gibco) in an agarose-coated Petri dish. Under these conditions, most embryos (>90 %) exogastrulate although there is some variation in the degree of displacement between the ectoderm and mesoderm/endoderm. The embryos that appeared to exogastrulate to the fullest extent were chosen for analysis.

Restriction enzymes, SP6 and T7 RNA polymerase, RNasin, and RQ1 DNase were all obtained from Promega Biotec. RNase A and RNase T1 were from Sigma. Klenow DNA polymerase was purchased from Bethesda Research Laboratories, reverse transcriptase from Life Sciences, *E. coli* DNA polymerase I, DNA ligase, *Eco*R1 methylase, and *Eco*R1 linkers (8-mers) from New England Biolabs. RNase H, T4 DNA polymerase, and S1 nuclease were purchased from Pharmacia. Radionucleotides were purchased from Amersham.

cDNA library construction and cDNA clone isolation

An early neurula (stage 17) cDNA library was constructed in λgt10 (Huynh *et al.* 1985) from poly(A)$^+$ RNA using the RNase H method (Gubler & Hoffman, 1983). Briefly, first-strand synthesis used AMV reverse transcriptase in the presence of actinomycin D (Sigma). Second-strand was synthesized using RNase H and *E. coli* DNA polymerase I. After a brief incubation with *E. coli* DNA ligase, the cDNA was treated with sufficient S1 nuclease to clip any hairpins formed during second-strand synthesis. Double-stranded cDNA was made blunt with T4 DNA polymerase, methylated with *Eco*R1 methylase, and ligated to *Eco*R1 linkers. Cleaving the linkers with *Eco*R1 and separation of the

cDNA from the linkers by Sepharose CL-4B chromatography, yielded size-selected cDNA which was ligated into the arms of λgt10 (molar ratio of vector: inset of 10:1). Recombinants were packaged as described (Huynh, Young & Davis, 1985) and amplified on C600 Hfl. The library contains 6×10^6 independent recombinant λ clones (before amplification) with an average size insert of 2·0 kb.

The stage-17 cDNA library was screened with the mouse N-CAM cDNA p1·3 (a kind gift of C. Goridis; Goridis *et al.* 1985) under conditions of low stringency using methods described previously (McGinnis, Levine, Hafen, Kuroiwa & Gehring, 1984; Weeks, Rebagliati, Harvey & Melton, 1985). Filters were hybridized overnight in 43·0 % formamide, 1·0 % SDS, 5 × SSPE, 5 × Denhardt's, 7·0 % dextran sulphate, and 100 μg ml^{-1} salmon sperm DNA at 37 °C and washed in 2 × SSPE, 0·1 % SDS at 37 °C for 4 h. The probe used in the screen was synthesized (Feinberg & Vogelstein, 1983) with random primers, Klenow polymerase, and the purified cDNA insert as template. Positive λ clones were detected with the mouse N-CAM probe at a frequency of 1 in 10^5. After plaque purification, the DNA from positive λ clones was isolated, digested with *Eco*R1, and the cDNA *Eco*R1 fragment was subcloned into the transcription plasmid vector pSP72 (Krieg & Melton, 1987).

An epidermal keratin cDNA, called Xek3, was isolated by screening a stage-14 library (constructed as described above) using a subtracted probe enriched for sequences expressed in normal embryos *versus* exogastrulae. Xek3 was one clone detected with this probe that was found by sequencing to be similar, but not identical to DG 81, an epidermal keratin cDNA described by Jonas, Sargent & Dawid (1985).

The cDNA clone AC100 which encodes a muscle-specific, cardiac actin (Dworkin-Rastl, Kelly & Dworkin, 1986) was a kind gift of T. Sargent.

Germ layer explants and induction assays

Animal and vegetal portions of blastulae (stage 8) were dissected and cultured in sterile 1 × MBSH in agar-coated Petri dishes. Explanted tissues were cultured at room temperature for about 18 h before RNA extraction and analysis.

RNA analyses

RNA was isolated from embryos using a proteinase-K digestion and phenol/chloroform method (Melton & Cortese, 1979) followed by sequential precipitation with 4 M-LiCl and ethanol. Poly(A)$^+$ RNA was isolated by two rounds of selection on oligo-dT cellulose (type III, Collaborative Research).

Northern blots were performed with 2·5 μg of poly(A)$^+$ RNA electrophoresed in agarose gels containing formaldehyde (Maniatis, Fritsch & Sambrock, 1982), blotted to Gene Screen (NEN) and crosslinked using published protocols (Rebagliati, Weeks, Harvey & Melton, 1985). Probes were synthesized using random primers as described above and hybridized overnight to blots under previously described conditions (Rebagliati *et al.* 1985). Blots were washed in 0·3 × SSPE, 0·1 % SDS and exposed to X-ray film with intensifying screens at −70 °C.

RNase protection assays were carried out as described previously (Melton, Krieg, Rebagliati, Maniatis, Zinn & Green, 1984). The probes used for these assays were derived from the following templates. For N-CAM, a 200 bp *Pvu*II/*Pst*1 fragment from N1 (see Fig. 1) was subcloned into the transcription vector pSP72 (Krieg & Melton, 1987), linearized with *Pvu*II and transcribed with T7 RNA polymerase. For the EF-1α probe, a 350 bp fragment from the EF-1α cDNA (Krieg & Melton, unpublished data) was subcloned into pSP72, linearized with *Pvu*II and transcribed with T7 RNA polymerase. For an epidermal keratin probe, the Xek3 cDNA was subcloned in pSP64 (Melton *et al.* 1984) in the anti-sense orientation, linearized with *Dde*1 and transcribed with SP6 RNA polymerase. All probes were purified by electrophoresis and elution from 4 M-urea/7 % polyacrylamide gels. Hybridization, RNase treatment and separation of protected fragments of the probe on gels were as described (Melton *et al.* 1984).

To quantify N-CAM RNA, the RNase protection assay was calibrated by performing the assay in parallel on known quantities of the sense-strand of N-CAM RNA, synthesized *in vitro*, as described (Harvey, Tabin & Melton, 1986).

In situ *hybridization*

In situ hybridization was carried out using published protocols with modifications (Cox, DeLeon, Angerer & Angerer, 1984; Melton, unpublished data). Briefly, embryos are fixed in acid/ethanol/chromium trioxide, washed in ethanol and embedded in paraplast. 8 μm sections are dried on slides and prepared for hybridization by mild digestion with proteinase K, treatment with 0·2 N-HCl, and incubation in acetic anhydride. Hybridizations are performed at 50 °C in 50 % formamide, 0·3 M-NaCl, 10 mM-Tris–HCl, pH 7·5, 5 mM-EDTA, 0·05 M-DTT, 1 × Denhardt's, 100 μg ml^{-1} tRNA, 10 % dextran sulphate, and ^{35}S-labelled RNA probe (0·1 μg ml^{-1}) for 10–16 h. Slides are washed in 2 × SSPE, 0·01 M-DTT at room temperature for 2 h, treated with RNase A (20 μg ml^{-1}) in 4 × SSPE at 37 °C for 30 min, and washed again in 2 × SSPE for 1 h at room temperature. A final wash is performed in 50 % formamide, 2 × SSPE at 65 °C for 45 min. Slides are then dipped in Kodak NTB2 emulsion and exposed for 3–5 days. Single-stranded RNA probes labelled with ^{35}S-UTP to a specific activity of 3×10^8 cts min^{-1} μg^{-1} were synthesized *in vitro* with SP6 or T7 RNA polymerase and hydrolysed to an average length of 50–100 bases. Complete details of this *in situ* hybridization protocol will be published elsewhere.

Muscle-specific probe was synthesized from a fragment of the cardiac actin cDNA, AC100, subcloned in pSP65. An epidermal keratin probe was synthesized from Xek3 cDNA subcloned into pSP64. The N-CAM probe was synthesized from either the N1 or N5 cDNA (Fig. 1) subcloned into pSP70 (Krieg & Melton, 1987).

Results

Isolation and characterization of Xenopus *N-CAM cDNAs*

A mouse N-CAM cDNA (Goridis, Hirn, Santoni, Gennarini, Deagostini-Baszin, Jordan, Kiefer & Steinmetz, 1985) was used to probe a λgt10 cDNA library constructed from *Xenopus* neurula (stage 17) poly(A)$^+$ RNA. The restriction maps for two of the clones, called N1 and N5, that were isolated are shown in Fig. 1. Sequence analysis of N1 (Kintner & O'Keefe, unpublished data) reveals extensive homology to the published sequence of chicken N-CAM (Hemperly, Murray, Edelman & Cunningham, 1986).

Northern blot analysis of poly(A)$^+$ RNA from staged embryos using the N1 or N5 cDNAs as hybridization probes detects four major RNA transcripts of approximately 9·5, 7·0, 4·0 and 3·8 kb (Fig. 2). This heterogeneous Northern pattern is not unexpected. In the chick embryo, two N-CAM transcripts of 7·0 and 6·0 kb are apparently derived from one gene by alternate splicing and these encode a 160 and $130\times10^3 M_r$ species of N-CAM polypeptides, respectively (Murray *et al.* 1986). In *Xenopus*, where four transcripts are detected (Fig. 2), and in mice, where up to five transcripts are detected (Gennarini, Hirsch, He, Hirn, Finne & Goridis, 1986), the situation is obviously more complicated. It is not yet known if all *Xenopus* transcripts are derived from the same gene, or if all encode functional N-CAM polypeptides. All the *Xenopus* transcripts detected by Northern analysis first appear at the neural plate stage (apparent in longer exposures but not shown in Fig. 2, lane 14) and increase in equal proportions to tadpoles (Fig. 2, lane 22) and later stages (data not shown).

In situ hybridization of N1 and N5 probes to late-stage embryos shows specific hybridization to the frog nervous system. An example of hybridization of N5 to a stage-22 embryo in cross section is shown in Fig. 3. Both N1 and N5 hybridize strongly to the neural tube, but do not hybridize over background to other tissues including epidermis, mesoderm and endoderm. Hybridization of N1 and N5 probes to longitudinal sections of stage-20 and tadpole embryos show specific hybridization to the embryonic nervous system with equal intensity along the anterior–posterior axis of the embryo (data not shown). Note

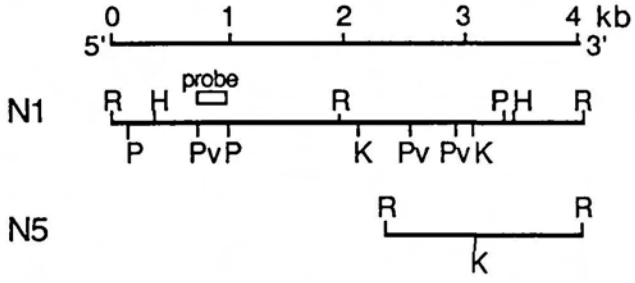

Fig. 1. Restriction maps for the N1 and N5 *Xenopus* N-CAM cDNAs. The *Pvu II*/*Pst*1 fragment of N1 marked by "probe" was used for RNase protection assays. This fragment is likely to be representative for all N-CAM transcripts because a labelled RNA probe synthesized from this fragment *in vitro* hybridizes on a Northern blot to the same four transcripts detected by the *Eco*R1 fragments of the N1 and N5 cDNAs. Moreover, the sequence of this fragment reveals that it contains one of the immunoglobulin-like repeats known from the sequence of chicken N-CAM to be present in the extracellular domain which is shared by all N-CAM polypeptides (Hemperly *et al.* 1986). The sites for *Eco*R1 (R), *Hin*dIII (H), *Pvu* II (Pv), *Pst*1 (P) and *Kpn*1 (K) are denoted.

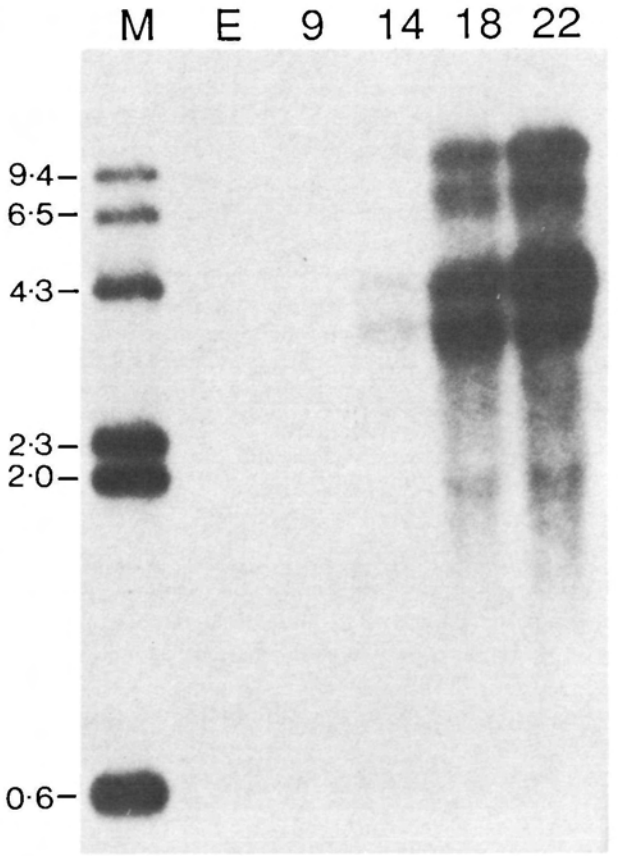

Fig. 2. Transcripts detected on Northern analysis by the N1 cDNA. 2·5 μg of poly(A)$^+$ RNA from eggs (E), blastula (stage 9), early neural plate (stage 14), neurula (stage 18), or early tadpole (stage 22) were electrophoresed in a formaldehyde agarose gel, transferred to Gene Screen and hybridized with radiolabelled N1 cDNA. An autoradiogram obtained by a 3-day exposure to preflashed X-Omat film is shown. An identical transcript pattern was detected when the N5 cDNA (Fig. 1) was used as the probe. The molecular weight markers (M) on the left refer to the size in kilobases of fragments produced in a *Hin*dIII digest of λ DNA.

that we do not detect any hybridization in the myotomes with the N1 or N5 cDNA probes. This is somewhat surprising since N-CAM is expressed in embryonic muscle in birds and mammals (Grumet, Rutishauser & Edelman, 1982; Sanes, Schachner & Covault, 1986). Control hybridizations show that epidermal keratin Xek3 hybridizes to epidermis (Fig. 3D) whereas muscle actin hybridizes to myotomes (Fig. 3E) as shown previously (Dworkin-Rastl *et al.* 1986).

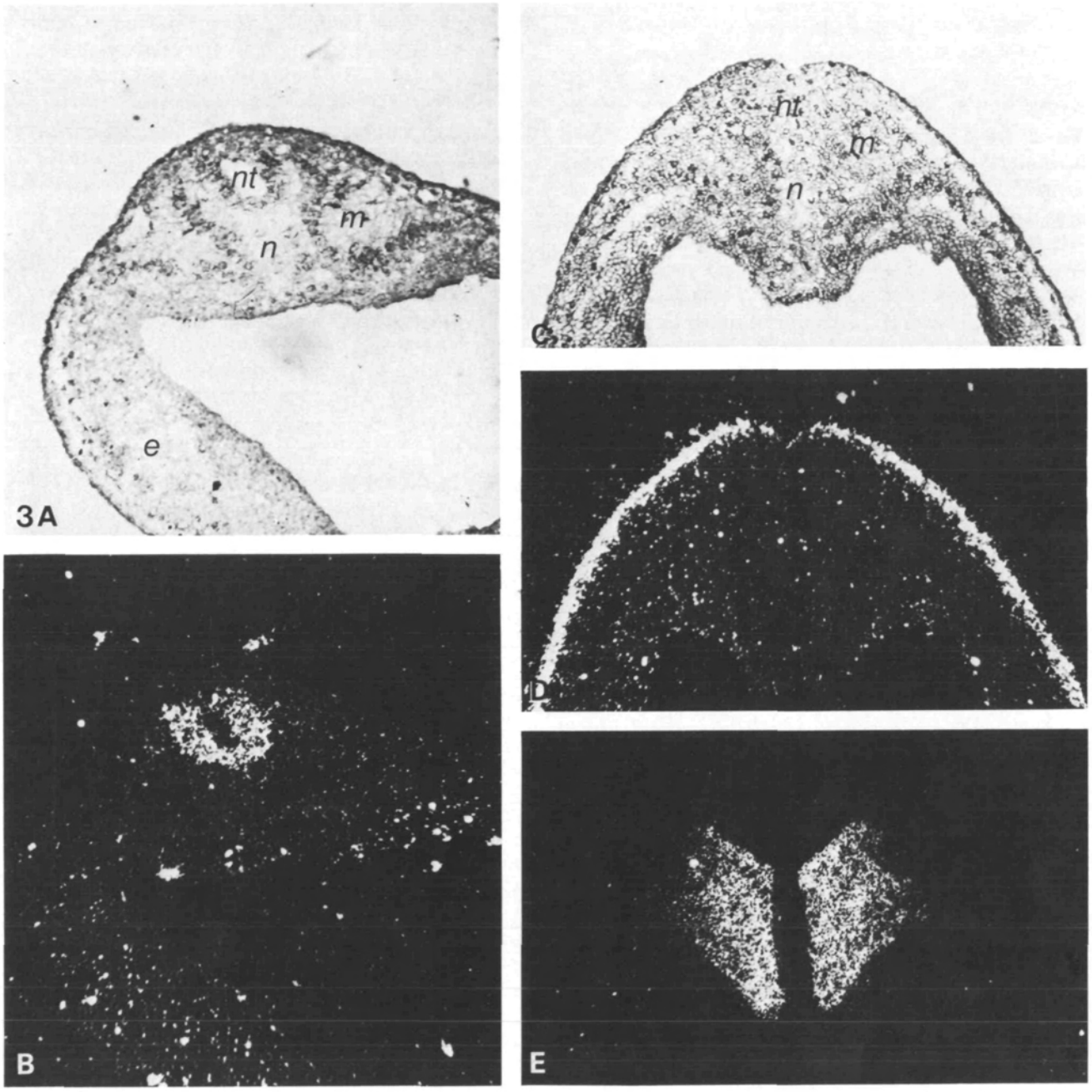

Fig. 3. *In situ* hybridization of N-CAM to the neural tube of a stage-22 embryo. A RNA probe synthesized from the N5 N-CAM cDNA was hybridized to a cross section of a stage-22 albino embryo. Albino embryos were used to avoid pigment granules which are indistinguishable from autoradiographic grains. Panel A shows a tissue section from the trunk region photographed under phase-contrast optics. The neural tube (*nt*), myotomes (*m*), notochord (*n*) and endoderm (*e*) are denoted. Panel B shows the same section under dark-field optics where autoradiographic grains appear white. The neural tube shows strong hybridization with the N-CAM probe. Panels C–E show a series of near adjacent transverse sections from a stage-20 albino embryo. Panel C shows a section photographed under phase-contrast optics with specific regions of the embryo denoted as in panel A. Panel D shows hybridization with an epidermal keratin Xek3 probe. Panel E shows hybridization with a muscle actin, AC100, probe. Hybridization is to epidermis in panel D and to the myotomes in panel E.

The partial sequence analysis and the transcript pattern detected in Northern blots lead us to conclude that N1 and N5 cDNAs encode *Xenopus* N-CAMs. Furthermore, the localization of the N1 and N5 RNA (hereafter referred to as N-CAM) in late-stage embryos by *in situ* hybridization shows that the expression of this gene is restricted to the embryonic nervous system.

Expression of N-CAM RNA in the neural plate

RNA transcripts for N-CAM first appear at the neural plate stage when assayed by Northern blots (Fig. 2). This appearance corresponds roughly with the developmental stage at which neural induction occurs. To determine where N-CAM RNA is expressed at this stage in development, neural-plate-stage embryos were sectioned and hybridized *in situ* with N1 and N5 probes. Examples of hybridization of N5 to a stage-16 embryo in cross section are shown in Fig. 4. The N-CAM probe hybridizes weakly, but definitely over background to the neural plate (Fig. 4C–F). Hybridization over background was not detected in surrounding ectoderm or in mesoderm or endoderm. The weak hybridization to the neural plate seen in Fig. 4D and F is to be expected given the low level of N-CAM RNA present in the embryo at this stage (1·0 pg, see Fig. 6) and the limits of detection for *in situ* hybridization (roughly 50 copies/cell). In contrast to the N-CAM probe, the epidermal keratin Xek3 probe shows reciprocal hybridization to the surrounding ectoderm. This probe does not hybridize to the ectoderm of the neural plate (Fig. 4A,B). Expression of Xek3 RNA in surrounding ectoderm is similar to the expression of other epidermal specific markers that have been studied including a peanut lectin (Slack, 1985) and two monoclonal antibodies (Akers, Phillips & Wessels, 1986; Jones & Woodland, 1986).

Interestingly, N-CAM hybridization at early stages of neural plate formation does not occur uniformly to all regions of the neural plate. Hybridization at early stages is concentrated in the deep ectodermal layer with low or undetectable hybridization to the superficial layer. In addition, hybridization is more concentrated in medial regions of the deep ectoderm with low or undetectable hybridization to the neural folds. This localization is particularly evident in the anterior neural plate of stage-16 embryos (Fig. 4E,F) and in most regions of a stage-14 neural plate (Fig. 5).

Two features of N-CAM hybridization are apparent from examining the hybridization of three different probes (N-CAM, Xek3 and muscle actin) to near adjacent sections of an early neural-plate-stage embryo. First, the hybridizations of the N-CAM and the epidermal keratin Xek3 probes do not overlap. While the Xek3 probe hybridizes to the superficial layer of surrounding ectoderm, the superficial layer of the neural plate ectoderm is not hybridized by the N-CAM probe (Fig. 5B,C). Second, N-CAM hybridization does not extend laterally the full width of the neural plate, but is concentrated in the medial portion (Fig. 5B). This medial location is adjacent to and approximately demarcated by the underlying region of mesoderm that hybridizes with the muscle actin probe (Fig. 5D). This result indicates that dorsal mesoderm expresses muscle actin long before myogenesis begins. Moreover, the data suggest that this dorsal mesoderm marks the region of overlying ectoderm that will express N-CAM.

From these data, we conclude that N-CAM RNA is localized to the neural plate and not to surrounding ectoderm or underlying mesoderm. In addition, early expression of N-CAM appears to be concentrated in deep ectoderm and, in that layer, to a medial portion in apposition to dorsal mesoderm.

Onset of the developmental expression of N-CAM RNA

The data described so far indicate that N-CAM RNA is expressed early in development in the ectoderm of the neural plate. To determine more accurately the time at which N-CAM RNA is first expressed during development, we have employed an RNase protection assay. The probe used in this assay is delineated in Fig. 1 and it detects all N-CAM transcripts in a Northern blot. RNA samples were prepared from eggs or embryos at regular intervals starting from stage 8, and assayed for levels of N-CAM RNA. As a control, these samples were also assayed in parallel for the level of EF-1α RNA. Transcription of the EF-1α gene increases precisely at the midblastula transition (MBT) and RNase protection assays for EF-1α transcripts can therefore be used to mark the MBT (Krieg & Melton, unpublished data). The EF-1α gene encodes an elongation factor required for translation and is presumably expressed at similar levels in all cells. The autoradiograms obtained from RNase protection assays were quantified by densitometer tracing and these values were compared to the values obtained from protection assays using known amounts of N-CAM or EF-1α RNA synthesized *in vitro*. The results (Fig. 6) indicate that N-CAM RNA levels markedly increase in development between stage 10 and 12. This increase comes about two stages after the MBT as marked by increase in the levels of EF-1α RNA. We conclude that N-CAM RNA expression increases after the MBT, during gastrulation, when morphogenetic movements bring mesoderm in apposition to ectoderm.

Surprisingly, the RNase protection assays reveal a low level of maternal N-CAM RNA (Fig. 6). Control

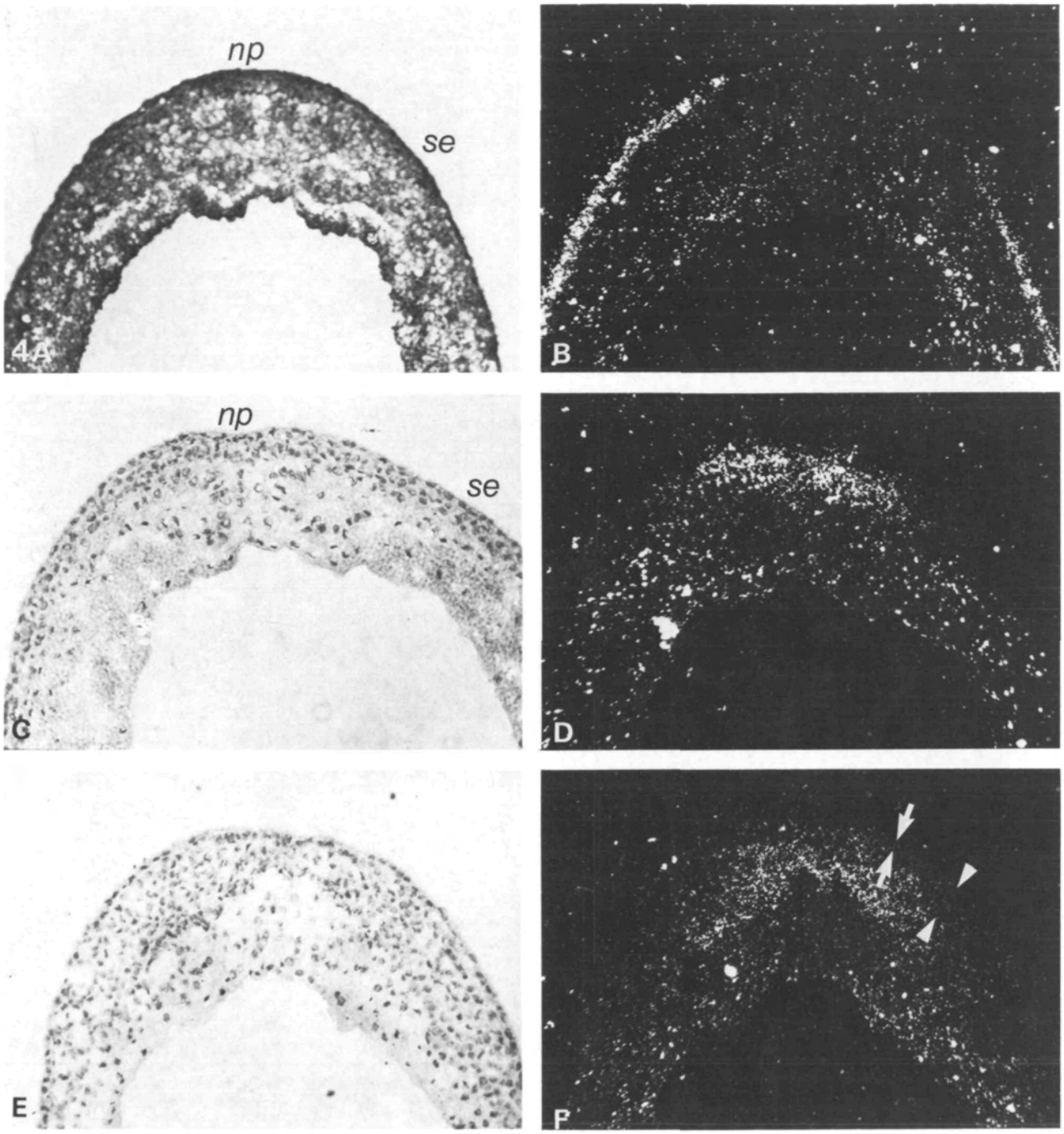

Fig. 4. *In situ* hybridization of a N-CAM probe to the neural plate. Transverse sections from a stage-16 albino embryo were hybridized with RNA probes synthesized from either the epidermal keratin Xek3 cDNA (A,B) or the N5 N-CAM cDNA (C–F). In each case a phase-contrast photograph (A,C,E) and dark-field photograph (B,D,F) is shown. B shows hybridization of an epidermal keratin Xek3 probe to a section from a more posterior region of the embryo. Note that hybridization occurs to surrounding ectoderm (*se*), but not to the neural plate (*np*) as labelled in the phase-contrast image shown in panel A. D shows hybridization of the N5 N-CAM probe to a section from a similar region of the embryo. Note hybridization to the neural plate (*np*), but not to surrounding ectoderm (*se*). F shows hybridization of the N5 N-CAM probe to sections from more anterior regions of a stage-16 embryo. Note that hybridization is in the neural plate, but not surrounding ectoderm. In addition, hybridization is concentrated in the medial, deep regions of the neural plate. Low or no hybridization is detected to the superficial ectoderm (arrows) or to lateral neural plate (arrowheads).

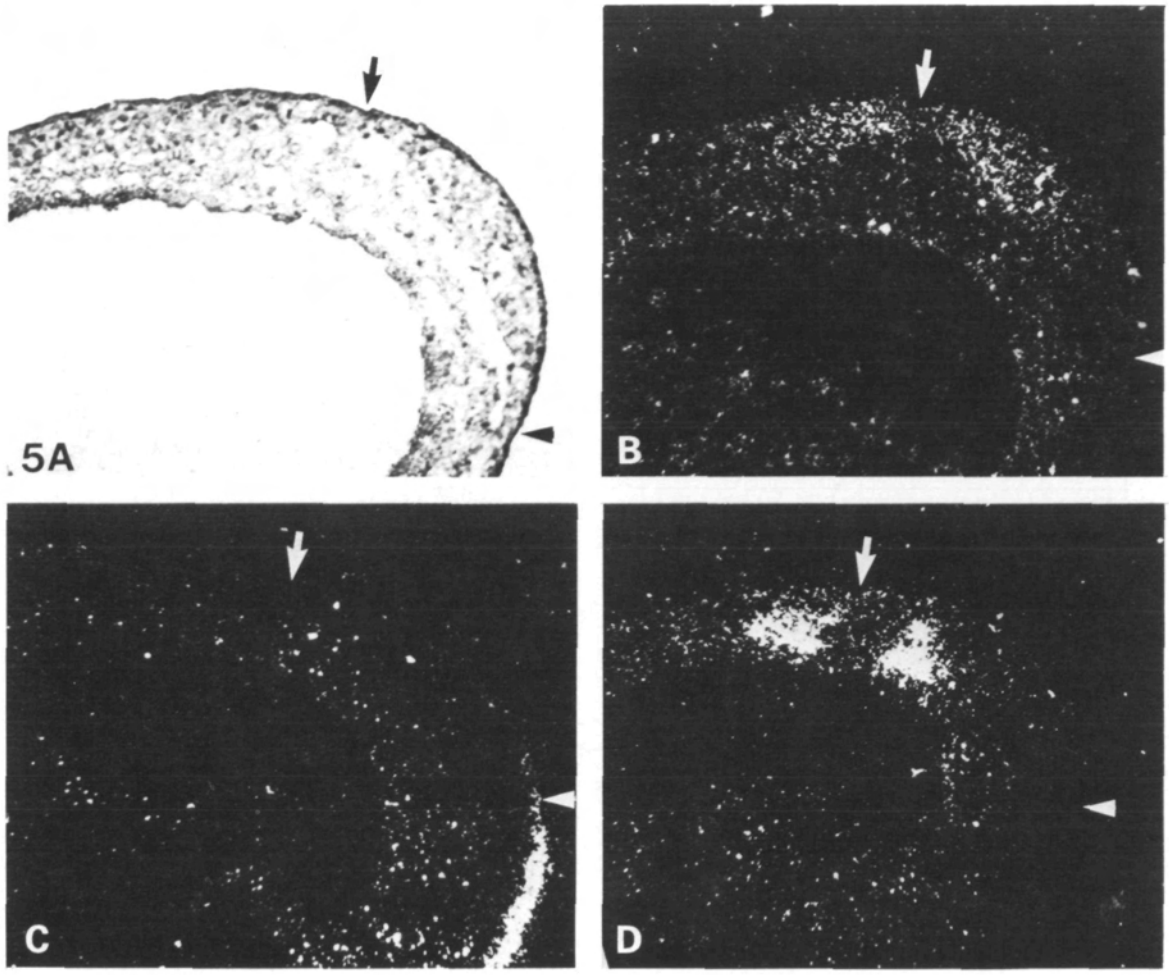

Fig. 5. *In situ* hybridizations of N1 N-CAM, muscle-specific and epidermal specific probes to a neural-plate-stage embryo. A series of transverse section from an early neural plate (stage 14) albino embryo were hybridized with RNA probes synthesized from the N1 N-CAM cDNA (B), the epidermal keratin Xek3 cDNA (C) and the muscle actin cDNA (D). A shows a section from this series photographed under phase-contrast optics. The dorsal midline is denoted with an arrow while the boundary between the neural plate and surrounding epidermis is denoted with an arrowhead. Note that N-CAM hybridization is concentrated toward the dorsal midline (arrow) and appears to be absent from lateral regions of the neural plate (arrowhead). Xek3 hybridization is to superficial ectoderm and stops at the boundary between the neural plate and surrounding ectoderm. Muscle actin hybridizes to the mesoderm. This dorsal mesoderm underlies the region of ectoderm expressing N-CAM RNA.

experiments with tRNA indicate that the maternal N-CAM RNA detected is neither a result of incomplete RNase digestion of the probe nor a low level contamination of the probe with sense-strand RNA. As stated previously, this low level of maternal N-CAM RNA is below the sensitivity of our *in situ* hybridization assay. Therefore, to determine whether the maternal RNA is localized, RNase protection assays were performed on samples prepared from animal, equatorial and vegetal sections from eggs (Weeks *et al.* 1985). These data (not shown) indicate that the low level of maternal N-CAM RNA is equally distributed along the animal–vegetal axis of the egg.

Induction of N-CAM RNA expression

Levels of N-CAM RNA increase during gastrulation and this increase is localized to neural plate ectoderm. To determine if this increase is dependent on induction, the expression of N-CAM RNA was measured in ectoderm cultured alone or in combination with inducing tissue. Ectoderm used in these experiments was isolated from stage-8 blastulae. For inducing tissue, the vegetal portion of a stage-8 blastula was chosen based on two criteria. First, this tissue does not give rise to any neural derivatives either *in situ* or when isolated in culture. Second, it has been shown that when endoderm is combined with ectoderm in culture, it induces a portion of

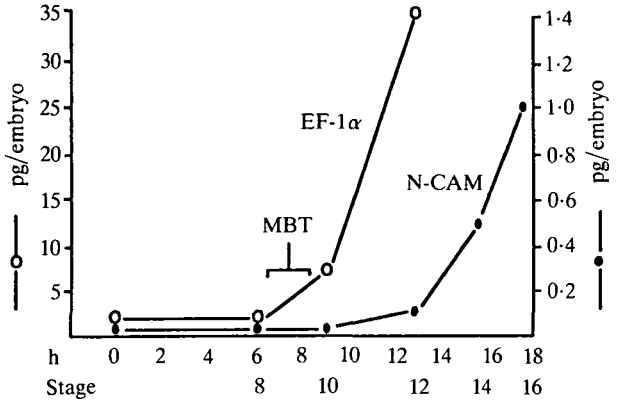

Fig. 6. Expression of N-CAM RNA in early development. RNA samples prepared from eggs, stage 8, 10, 12, 14 and 16 embryos were assayed by RNase protection for N-CAM and EF-1α RNA as described in Materials and Methods. The results were quantified by parallel assays performed using known quantities of sense N-CAM and EF-1α RNA synthesized *in vitro*. Note that EF-1α RNA increases and marks the MBT whereas N-CAM RNA first increases during gastrulation, between stages 10 and 12. The maternal level of N-CAM RNA (present in eggs through stage 10) is approximately 0·01 pg/embryo.

ectoderm to form dorsal mesoderm which, in turn, induces neural tissue (Nieuwkoop, 1973). While mesoderm could also have been used as inducing tissue, in practice it is difficult to obtain either pure mesoderm that is not contaminated with neural tissue or prospective mesoderm that, in explants, will not regulate to form neural tissue.

Ectoderm and endoderm were isolated from stage-8 embryos and cultured in isolation or in combination as diagrammed in Fig. 7. After approximately 18 h in culture, at the equivalent of stage 20, RNA samples were prepared and assayed by RNase protection for N-CAM RNA. The results show that ectoderm and endoderm cultured alone do not express detectable amounts of N-CAM RNA (Fig. 7 lanes A and V, probe: N-CAM). In contrast, ectoderm cultured together with endoderm expresses near control levels of N-CAM RNA (Fig. 7 lane A/V, probe: N-CAM). Although ectoderm cultured alone does not express N-CAM RNA, it does appear to express copious amounts of the epidermal keratin Xek3 RNA indicating that it has undergone epidermal differentiation (Fig. 7, lane A, probe: Epi.Ker.). Finally, the level of EF-1α RNA, used as a control for cell number and RNA recovery, appears to increase only marginally (two- to threefold) when ectoderm and endoderm are cultured together rather than alone. This difference is far too small to account for the >50-fold difference between N-CAM RNA levels in ectoderm cultured alone or in recombination with endoderm. We conclude that N-CAM RNA is not expressed in blastula ectoderm cultured alone, but is expressed when ectoderm is placed in contact with an inducing tissue.

To localize the expression of N-CAM RNA in these recombinates, ectoderm and endoderm were isolated from albino stage-8 blastulae and cultured together for 18 h. These recombinates were sectioned and hybridized with the N-CAM probe as well as muscle actin or epidermal keratin Xek3 probes. Representative sections from one recombinate hybridized with these three probes are shown in Fig. 8. The results show that these three probes hybridize to three distinct, nonoverlapping regions of the recombinate. The epidermal keratin Xek3 probe hybridizes to a relatively small portion on one surface of the recombinate (Fig. 8C,D). The N-CAM probe hybridizes to a central region of tissue (Fig. 8A,B) which is surrounded by two large blocks of tissues hybridized by the muscle actin probe (Fig. 8E,F). Hybridization of the same three probes to blastula ectoderm cultured alone for 18 h shows a background hybridization with both the N-CAM and muscle actin probes. In contrast, the epidermal keratin Xek3 probe hybridizes strongly not only to the surface, but also extensively to the internal regions of the ectodermal tissue (data not shown). The simplest interpretation of these results is that N-CAM is expressed in ectoderm induced to undergo neural differentiation, but not in the inducing tissue.

Expression of N-CAM RNA in exogastrulae

The role of induction in the expression of N-CAM by ectoderm was also examined in exogastrulae. As described in the Introduction, if an embryo is stripped of the vitelline membrane and cultured in high salt, it gastrulates incorrectly. Instead of invaginating into the blastocoel, the dorsal mesoderm moves outward to produce an embryo consisting of an ectodermal sack connected through a stalk of tissue to mesoderm and endoderm. Since the ectoderm of these embryos by morphological criteria differentiates solely into epidermis, exogastrulae have been described as neural deficient (Holtfreter & Hamburger, 1955). To determine if exogastrulae express N-CAM, RNA samples were prepared from exogastrulae grown to the equivalent of stage 20. These samples and those prepared by dividing exogastrulae into an ectodermal half and a mesoderm/endoderm half were assayed by RNase protection with a N-CAM probe. Unexpectedly, the results of this analysis show that exogastrulae express near control levels of N-CAM RNA (Fig. 7 compare lane E to lane N). Furthermore, this N-CAM RNA appears to be expressed solely in the ectoderm half with little or no expression in the mesoderm/endoderm half (Fig. 7 compare lane AE to VE).

The expression of N-CAM RNA in exogastrulae was localized more accurately by *in situ* hybridization of the N1 probe. Fig. 9 shows the results obtained when near adjacent sections of an albino exogastrula were hybridized with either the N-CAM, epidermal keratin Xek3 or muscle actin probe. The results indicate that N-CAM RNA is expressed primarily at the junction between the ectoderm and the mesoderm (Fig. 9C, arrowhead). A lower level hybridization with N-CAM was also apparent in the internal portion of the ectodermal sack (Fig. 9A, denoted by *ec*). The epidermal keratin Xek3 probe hybridizes to the entire surface as well as some internal regions of the ectodermal sack (Fig. 9B). The muscle actin

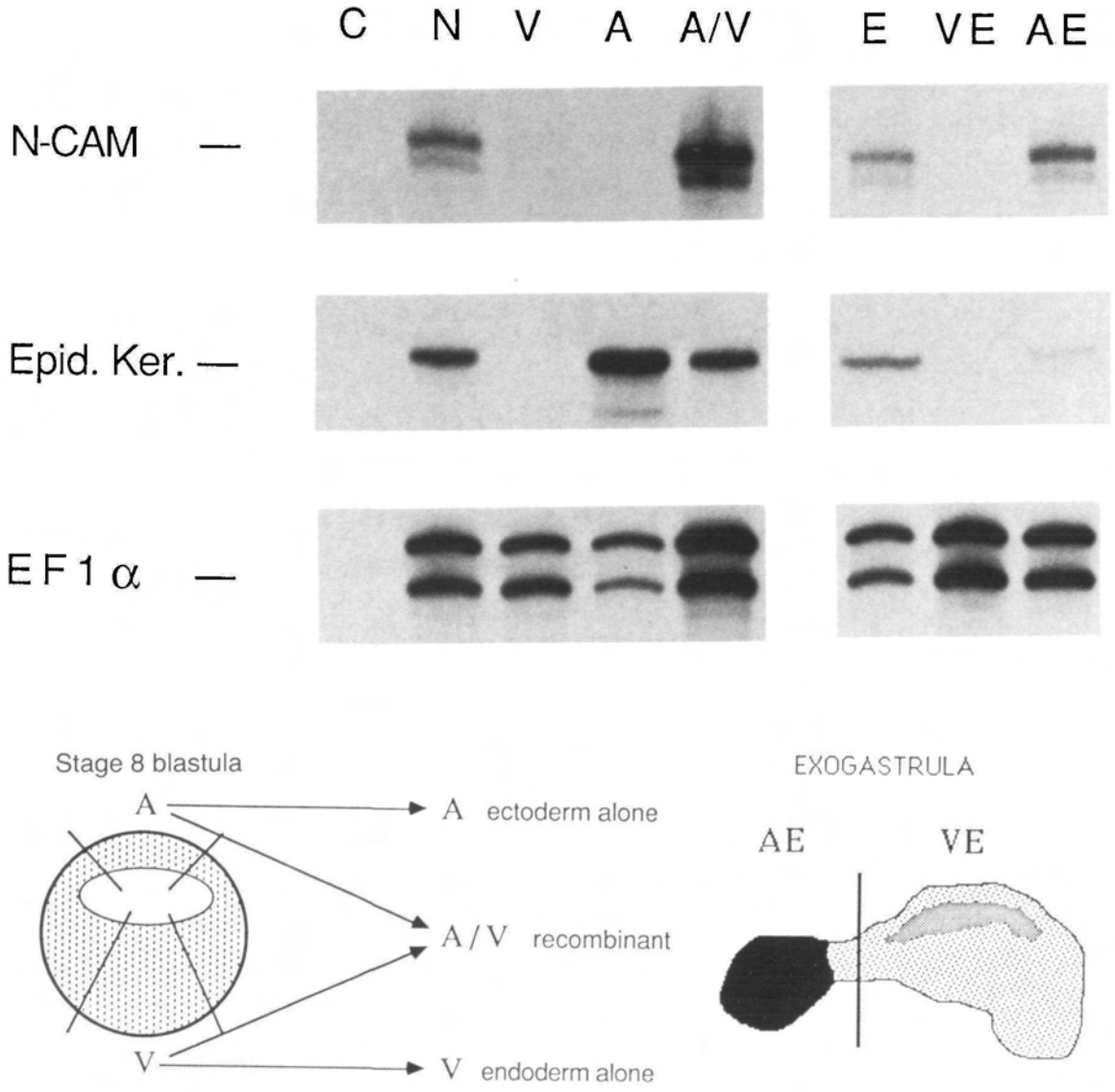

Fig. 7. Induction of N-CAM RNA in recombinates and exogastrula. Ectoderm from the animal pole (A) and endoderm from the vegetal pole (V) were isolated from a stage-8 blastula as diagrammed and cultured alone or in combination (A/V). After 18 h in culture, RNA samples were prepared and assayed by RNase protection with probes for EF-1α, N-CAM, or the epidermal keratin Xek3 RNA (Epi.Ker.). The EF-1α probe is used to control for cell viability and number (see text). Lane N shows the protected probe obtained from RNA samples of normal st 22 embryos while lane C shows the probe protected by a negative control, tRNA. Note that N-CAM RNA is not expressed in the animal (A) or vegetal (V) fragment when these are cultured separately, but is in the A/V recombinate. Xek3 RNA is not detected in the V sample, but is in both the A sample and the A/V recombinate.

Exogastrula were generated as described in Materials and methods and grown to the equivalent of stage 22. RNA samples were prepared from the total exogastrula (E) or from exogastrula divided into an ectodermal half (AE) and a mesodermal/endodermal half (VE) as shown. Samples were assayed as above. Note that N-CAM RNA is present at similar levels in exogastrula (E) and normal embryos (N). This N-CAM expression is concentrated in the ectodermal half (AE) of the exogastrula.

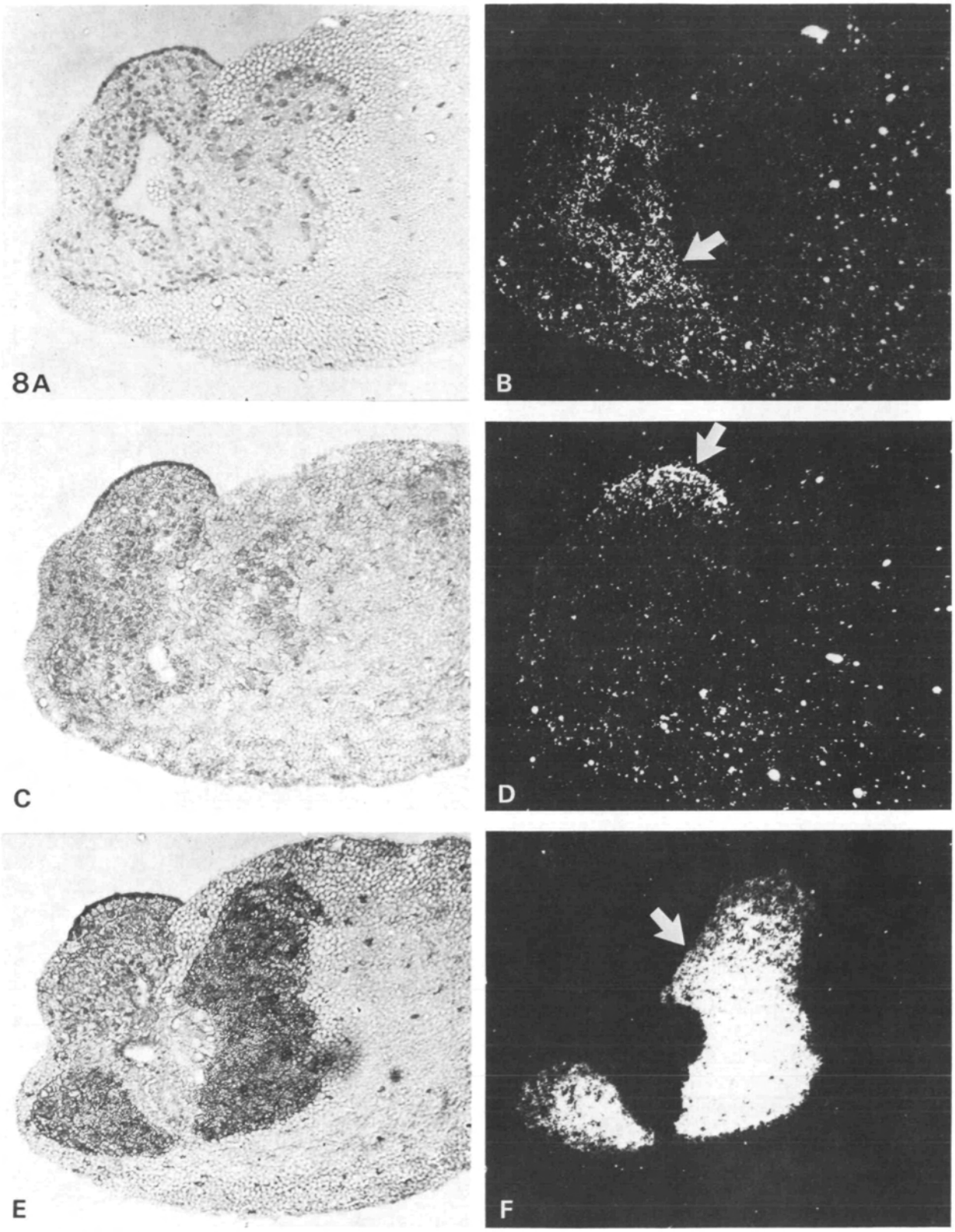

Fig. 8. Localized expression of N-CAM RNA in recombinates. The ectodermal and endodermal portion of stage-8 albino blastula were recombined as shown in Fig. 7 and cultured for 18 h. A series of transverse sections were hybridized with a N1 N-CAM probe (A,B), an epidermal keratin Xek3 probe (C,D) or a muscle actin probe (E,F). In each case a phase-contrast photograph (A,C,E) and dark-field photograph (B,D,F) is shown. White arrows point to N-CAM hybridization in B, epidermal keratin Xek3 hybridization in D, and muscle actin hybridization in F. Note the regions hybridized with these three probes do not overlap.

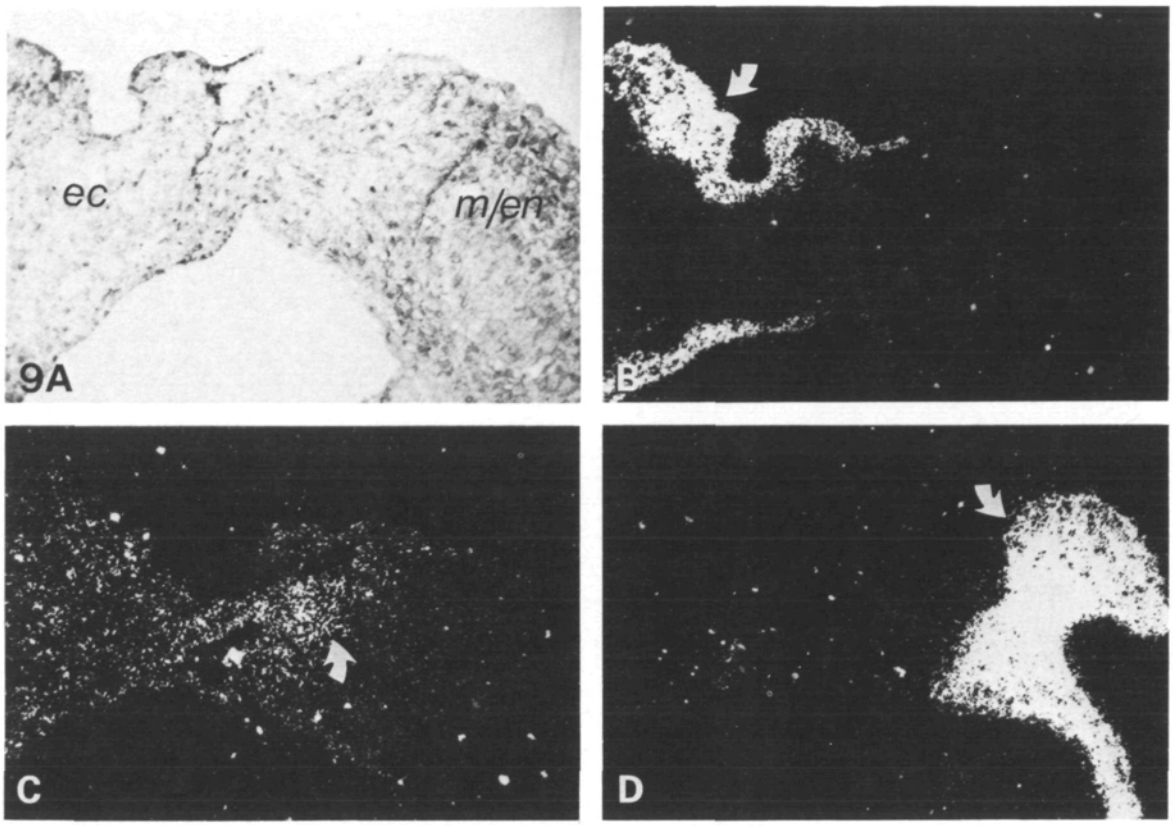

Fig. 9. A series of longitudinal sections from albino exogastrula were hybridized with the epidermal keratin Xek3 probe (B), the N1 N-CAM probe (C), and the muscle actin probe (D). A shows a photograph under brightfield optics of one section from this series. The ectodermal sack (*ec*) is to the left of the photograph and the mesoderm/endoderm (*m/en*) portion is to the right. Note that the epidermal keratin Xek3 probe hybridizes to the ectodermal sack (arrow in B) and that the muscle actin probe hybridizes to a block of tissue in the mesodermal half (arrow in D). The N-CAM probe hybridizes to the junction between ectoderm and mesoderm (arrow in C).

probe hybridizes to a large block of tissue present in mesoderm (Fig. 9D). We conclude that exogastrulae express near normal levels of N-CAM RNA and that expression occurs at the junction between ectoderm and dorsal mesoderm.

Discussion

The main conclusions from the results presented here are that the expression of N-CAM RNA marks a very early stage of neural development and that this expression in ectoderm depends on induction. The evidence for these conclusions is the marked increase in expression of N-CAM RNA during gastrulation as detected by a RNase protection assay and the localization of N-CAM RNA to the neural plate as shown by *in situ* hybridization. Isolation experiments show that N-CAM RNA expression in ectoderm depends on induction. Ectoderm cultured alone does not express N-CAM RNA whereas ectoderm cultured in combination with endoderm does express N-CAM RNA. *In situ* hybridization of a N-CAM probe to recombinates and exogastrulae shows that N-CAM RNA is localized to tissue in contact with dorsal mesoderm, the latter being identified by hybridization with a muscle-specific actin probe.

Using polyclonal antibodies raised against N-CAM from adult frog brain, Jacobson & Rutishauser have previously shown that N-CAM determinants first appears at stage 14 (early neural plate) in *Xenopus* development (Jacobson & Rutishauser, 1986). The results presented here are consistent with their findings in that we find the first appearance of N-CAM mRNA during gastrulation (stage 10–12, see Fig. 6), a few hours before the appearance of N-CAM protein. Moreover, our results and those of Jacobson & Rutishauser support the view that the early appearance of N-CAM mRNA and protein depends on an inductive interaction that occurs during gastrulation.

The induction of N-CAM RNA expression can be contrasted to the expression of muscle actin RNA in response to mesodermal induction (Mohun, Brennan, Dathan, Fairman & Gurdon, 1984; Gurdon, Fairman, Mohun & Brennan, 1985). The exact stage

in development when the cellular events of mesodermal induction occur is not known, but they may begin during the cleavage divisions (stage 6, Gimlich & Gerhart, 1983) and continue as late as early gastrula (stage 9, Gurdon *et al.* 1985). However, the expression of muscle actin does not occur until at least stage 12/13 (Gurdon *et al.* 1985) or 6–7 h after the inductive interaction. In contrast, the expression of N-CAM RNA appears to follow closely the contact of invaginating mesoderm with ectoderm. Indeed, N-CAM RNA appears within 1–2 h after gastrulation begins. This implies that the activation of N-CAM gene expression may be a direct and nearly immediate genetic consequence of induction.

Localized expression of N-CAM within the neural plate

In situ hybridization of N-CAM probes to the early stages of the neural plate reveals a striking localization of N-CAM RNA to medial portions of the deep ectodermal layer. One explanation for this localization is that it reflects the fact that different regions of the neural plate have different developmental fates. The superficial ectoderm of the neural plate gives rise to the ependymal layer of the neural tube. The medial portions of the deep ectoderm give rise to most of the central nervous system while lateral regions, or neural folds, give rise to the neural crest and therefore, the peripheral nervous system. The localization of N-CAM RNA at early stages may simply reflect the events that divide the ectoderm into these three separate lineages. Alternatively, the localization of N-CAM RNA within the neural plate may reflect a sequential order of induction. Induction, and N-CAM RNA expression, may occur first in deep ectoderm adjacent to inducing tissue and then spread laterally and up into the superficial ectoderm. Further experiments are needed in order to determine more accurately when the superficial layer of ectoderm expresses N-CAM RNA and if this expression is induced by underlying deep ectoderm or by a signal from dorsal mesoderm.

Expression of N-CAM in mesoderm

In situ hybridization indicates that N-CAM RNA is localized to the neural tube and is not expressed in mesoderm (Fig. 2B). This result is surprising given that N-CAM is known to be expressed in developing and denervated muscle in birds and mammals (Grumet *et al.* 1982; Sanes *et al.* 1986). One explanation is that N-CAM is expressed in *Xenopus* myotomal muscle, but that this expression is below the sensitivity of *in situ* hybridization. We have tried to determine if myotomes express N-CAM RNA using more sensitive means. Somites and neural tubes were dissected manually from stage-24 embryos and then assayed for N-CAM RNA using a RNase protection assay. When normalized for EF-1α RNA levels in each sample, the somite sample was found to contain approximately 100-fold less N-CAM RNA than the neural tube sample (data not shown). While this result might indicate a very low level of N-CAM RNA expression in myotomes it also could be explained by contamination of the somite sample with cells of the peripheral nervous system such as the sensory ganglia and/or Schwann cells of the innervating nerve sheaths. In other experiments. N-CAM RNA levels were determined by RNase protection in exogastrulae divided into mesoderm and ectodermal halves (see Fig. 7). The results show undetectable levels of N-CAM RNA in the samples prepared from the mesodermal half. Although, this result indicates that mesoderm does not express N-CAM RNA, it could also be argued that the expression of N-CAM RNA in myotomal muscle is abnormal as a result of exogastrulation. Finally, we have assayed N-CAM RNA in denervated and normal adult frog muscle, again by RNase protection. N-CAM RNA was not detected in either the denervated or normal adult muscle RNA samples. Taken together, our results indicate that if expression of N-CAM RNA does occur in mesoderm, it is far below (>50-fold) the level of its expression in the nervous system. It is not yet known why these results are at odds with the expression of N-CAM in developing and denervated muscle in higher vertebrates. One explanation is that it simply reflects a species difference in the way N-CAM is expressed. Another explanation is that *Xenopus*, in fact, has several genes for N-CAM and the cDNAs that we isolated correspond to a gene strictly expressed in a neural specific manner.

Expression of N-CAM in exogastrulae

In situ hybridization and RNase protection assays indicate that exogastrulae express near normal levels of N-CAM RNA. This observation indicates that exogastrulae are not entirely neural deficient in that part of the ectoderm that has taken one early step towards becoming neural tissue. These data highlight the fact that formation of a mature neural structure, like a neural tube, can be experimentally separated from early steps in neural induction such as the expression of N-CAM.

In situ hybridization reveals that most N-CAM RNA expression in exogastrulae occurs at the junction between ectoderm and mesoderm. This is consistent with the idea that dorsal mesoderm induces ectoderm to express N-CAM. A more difficult observation to explain is the expression of N-CAM RNA in exogastrulae in the deep ectoderm of the ectodermal sack (Fig. 9). Since isolated ectoderm does not express detectable levels of N-CAM RNA even when

assayed by RNase protection (Fig. 7), the expression of N-CAM RNA in the 'isolated' ectoderm of the exogastrula must have been induced by some mechanism. One possible mechanism is that induction spreads from induced ectoderm into the isolated ectoderm. Another possibility is that mesoderm begins to move inward before exogastrulating and this transient contact between ectoderm and mesoderm is sufficient for induction to occur.

N-CAM as a marker of neural induction

There are three reasons why the expression of N-CAM RNA in ectoderm soon after induction is significant. First, the use of N-CAM as a marker for neural induction may allow induction to be studied as a problem in gene regulation. The molecular nature of the signal produced by inducing mesoderm and the reception and response of ectoderm to this signal have been problems that have eluded analysis. Studying how induction turns on N-CAM RNA expression may allow one to trace back the mechanism necessary for transcriptional activation to events at the cell surface.

A second significant point is that N-CAM RNA expression provides a convenient assay for early steps of neural development. This point can be best illustrated by considering the example of the exogastrulae. Exogastrulae are considered neural deficient by the morphological criteria that they fail to form a neural plate/tube or generate other neural structures. The results with N-CAM reveal that at least the early steps of induction have indeed taken place. In many cases, it will be useful to score the early stages of neural development as marked by N-CAM RNA expression without requiring the later stages such as the ones that fail in a spatially disorganized exogastrula.

Finally, the results with N-CAM indicate that induced gene expression is an early response to induction. This result opens up the possibility that the initial response of ectoderm to induction is to express genes that favour the formation of neural rather than epidermal tissue. A test for the role of genes expressed early in neural development may come from experiments where manipulated genes are injected back and expressed in developing embryos (Krieg & Melton, 1985). For example, N-CAM could be indiscriminantly expressed in all ectoderm before induction or, alternatively, its expression could be delayed or blocked by anti-sense experiments (Melton, 1985; Weintraub, Izant & Harland, 1985). Interpretable phenotypes from such experiments may help determine the role of these genes in the specification of neural tissue in *Xenopus* embryos.

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