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# Expression of the FGF-related proto-oncogene *int-2* during gastrulation and neurulation in the mouse

**David G.Wilkinson, Gordon Peters[1], Clive Dickson[2] and Andrew P.McMahon**

Laboratory of Developmental Biochemistry, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, [1]Imperial Cancer Research Fund Laboratories, St Bartholomew's Hospital, Dominion House, Bartholomew Close, London EC1A 7BE and [2]Imperial Cancer Research Fund Laboratories, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK

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**The proto-oncogene *int-2* has been implicated in the formation of mouse mammary-tumour-virus-induced mammary tumours. Analysis of the predicted coding sequence indicates that *int-2* is a member of the fibroblast growth factor family. Previous studies using Northern blot analysis suggested that normal expression of *int-2* may be confined to extra-embryonic endoderm lineages of embryonic stages of mouse development. We have used *in situ* hybridization and Northern blot analysis to examine directly *int-2* expression in embryo stem cells and in the developing embryo from early gastrulation to mid-somite stages. Complex patterns of accumulation of *int-2* RNA were observed in embryonic and extra-embryonic tissues. The data suggest multiple roles for *int-2* in development which may include migration of early mesoderm cells and induction of the otocyst.**

*Key words:* proto-oncogene/*int-2*/mouse development/*in situ* hybridization/embryonic expression/extra-embryonic expression

## Introduction

Considerable interest has arisen in the possible role of proto-oncogenes in mammalian development. A number of proto-oncogenes are temporally and/or spatially regulated in the developing mouse (for review see Adamson, 1987), suggestive of a normal role of these genes in the context of embryonic and fetal development.

One such gene, *int-2*, was originally identified as a common integration site for mouse mammary tumour virus (MMTV), and is implicated in mammary tumorigenesis in certain strains of mice (Peters *et al.*, 1983; Dickson *et al.*, 1984). Whereas *int-2* is not detectably expressed in normal mammary glands, transcripts accumulate in many mammary tumours as a consequence of proviral insertion 5′ or 3′ of the *int-2* gene (Dickson *et al.*, 1984; Moore *et al.*, 1986; Peters *et al.*, 1986). Insertion is found within the coding region, suggesting that a normal *int-2* protein is required for tumour formation. Recent observations demonstrated homology between *int-2* and members of the fibroblast growth factor (FGF) family (Dickson and Peters, 1987), other members of which are implicated in the formation of non-mammary human tumours (Delli Bovi *et al.*, 1987; Taira *et al.*, 1987). Although the roles of this family of proteins have only been partially defined, basic and acidic FGF have been implicated in endothelial cell proliferation and migration during angiogenesis (for reviews see Gospodarowicz, 1985; Folkman and Klagsbrun, 1987), and in mesodermal induction in *Xenopus* (Slack *et al.*, 1987).

Expression of *int-2* is highly restricted in normal cells with no transcripts identified in normal adult tissues to date (Dickson *et al.*, 1984; Jakobovits *et al.*, 1986). However, Northern blot analysis of RNA from mouse embryos demonstrated *int-2* transcripts in 7.5-day egg cylinders, which were no longer detected in early somite-stage embryos 24 h later (Jakobovits *et al.*, 1986). Thus, it would appear that a normal role exists in early mouse development for the *int-2* gene product. As *int-2* expression was also detected in differentiated embryonal carcinoma (e.c.) cells (Jakobovits *et al.*, 1986), it was proposed that expression seen *in vivo* at 7.5 days was probably confined to extra-embryonic endoderm surrounding the embryonic component of the egg cylinder since this endoderm (visceral endoderm) is developmentally related to the parietal endoderm cells formed on e.c. cell differentiation (Jakobovits *et al.*, 1986).

We have used *in situ* hybridization and Northern blot analysis to directly examine *int-2* expression in the conceptus. Our results reveal that *int-2* transcripts accumulate only within the parietal endoderm and not the visceral endoderm component of the extra-embryonic endoderm. In addition we identify striking temporal and spatial restriction in *int-2* RNA in the developing embryo from early gastrulation to early organogenesis stages. Embryonic *int-2* RNA accumulates in newly formed migrating mesoderm, neuroepithelial cells of the hindbrain adjacent to the otocyst and on the lower surface of the endodermally derived pharyngeal pouches.

## Results

### *int-2 probes*

*int-2* transcripts of 2.9, 2.7, 1.8 and 1.6 kb have been detected in RNA from mammary tumours and differentiated

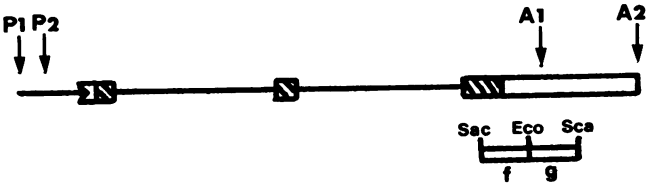


**Fig. 1.** Relationship between *int-2* probes and the *int-2* gene. The *int-2* gene is illustrated. Non-coding and coding exons are represented as open and hatched boxes respectively. The jagged 5′ box is to indicate that alternative upstream sequences are utilized as exons in transcripts initiated at the promoter regions P1 and P2 (Smith *et al.*, in preparation). Alternative poly(A) addition sites are indicated as A1 and A2. Sequences encompassed by the *int-2f* and *int-2g* probes are aligned below.

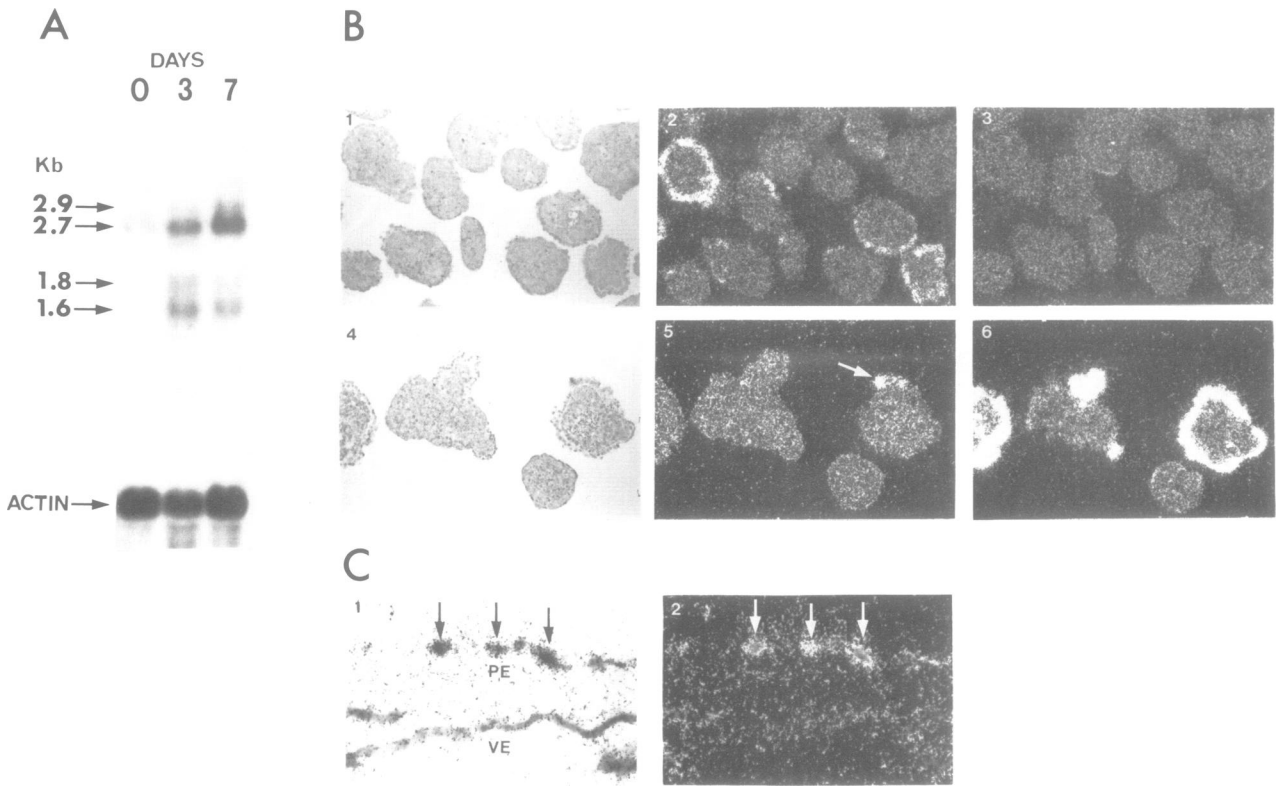


**Fig. 2.** *int-2* RNA accumulation in e.s. cells and extra-embryonic endoderm. (**A**) Northern blot analysis of e.s. cell RNA at 0, 3 and 7 days of differentiation, probed with $^{32}$P-labelled *int-2f* antisense RNA. Size of *int-2* transcripts (kb) is indicated. Hybridization of an actin coding probe to the same filter is shown below. (**B**) *In situ* hybridization of e.s. embryoid bodies. Sections of 3-day e.s. embryoid bodies were hybridized with $^{35}$S-labelled probes and, following autoradiography and staining, photographed under bright field (**1** and **4**) and dark field (**2, 3, 5** and **6**) illumination. *int-2* antisense (**1** and **2**) and sense (**3**) probes were hybridized to adjacent sections. To identify parietal and visceral endoderm-like cells, AFP (**4** and **5**) and SPARC (**6**) probes were also hybridized to adjacent sections. The arrow indicates AFP-expressing cells. (**C**) *In situ* hybridization to extra-embryonic endoderm. *int-2f* antisense probe was hybridized to sections of an 8.5-day conceptus. High power bright field (**1**) and dark field (**2**) photomicrographs of the extra-embryonic endoderm of the conceptus in Figure 5, 2 are shown. Arrows indicate *int-2* expression in parietal endoderm (PE) cells. VE, visceral endoderm.

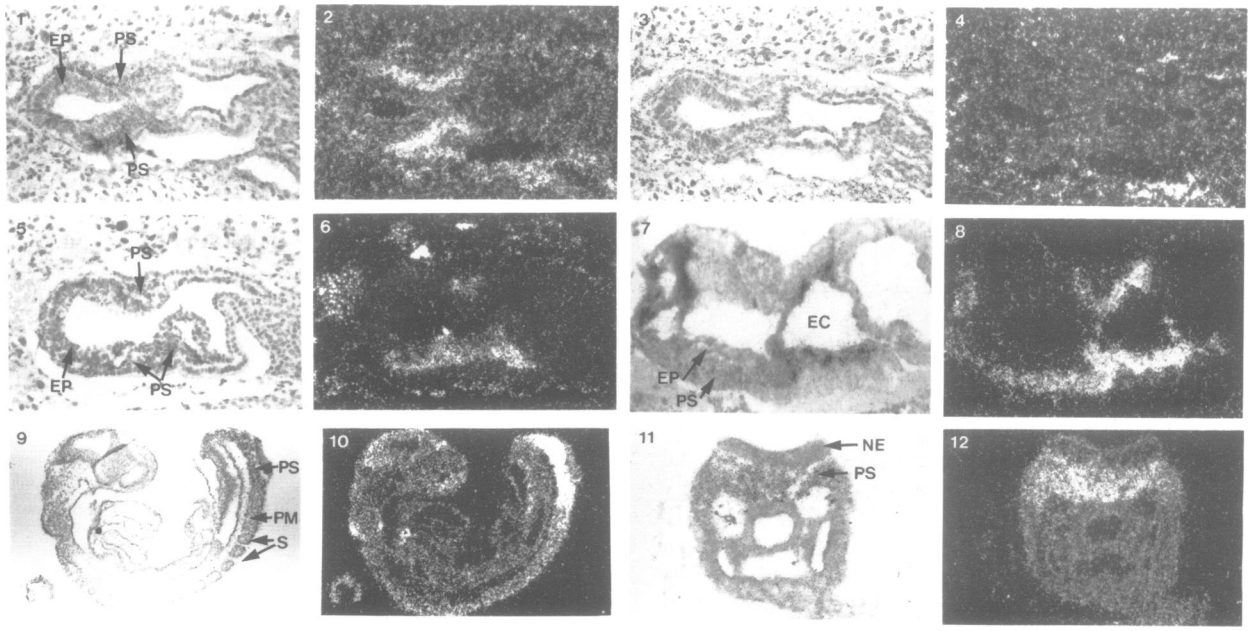


**Fig. 3.** *int-2* RNA in early mesoderm cells. *int-2* sense (**3** and **4**) and antisense (**1, 2** and **5–12**) probes were hybridized to sections of 7.5-day (**1–4**). 7.75-day (**5** and **6**), 8-day (**7** and **8**) and 9.5-day (**9–12**) *post coitum* embryos. *int-2f* and *int-2g* probes gave identical results (not shown). **Panels 1–10** represent longitudinal sections, whereas **panels 11** and **12** are a transverse section through the primitive streak. Bright field (**1, 3, 5, 7, 9** and **11**) and dark field (**2, 4, 6, 8, 10** and **12**) photomicrographs are presented. Arrows indicate the primitive streak (PS), epiblast (EP), exocoelom (EC), presomitic mesoderm (PM), somites (S) and neural ectoderm (NE). It should be noted that blood cells present in maternal decidual tissue in panels 2, 4 and 6 when viewed under dark ground illumination give the appearance of silver grains.

e.c. cells (Peters *et al.*, 1986; Jakobovits *et al.*, 1986; Smith *et al.*, in preparation). Mapping of promoter regions and poly(A) addition sites indicates that these four transcripts result from the use of alternative promoters and poly(A) addition sites but all contain an identical coding sequence (Smith *et al.*, in preparation). In this study we have used two probes, *int-2f* and *int-2g* (Figure 1). *int-2f* includes most of the third exon and some 3′ untranslated sequence while *int-2g* lies immediately 3′ of *int-2f* and includes only 3′ untranslated sequence. Both probes detect all four transcripts (Smith *et al.*, in preparation).

### *int-2 expression in extra-embryonic endoderm*

Previous work suggested that *int-2* is transcribed in both the visceral and parietal endoderm lineages of the extra-embryonic endoderm (Jakobovits *et al.*, 1986). We examined undifferentiated and differentiated embryo stem (e.s.) cells (Evans and Kaufman, 1981) for *int-2* transcripts by Northern blot analysis and *in situ* hybridization. Low levels of *int-2* transcripts were detected in undifferentiated e.s. cells and were found to accumulate significantly on differentiation (Figure 2A). All four transcripts were present but the 2.7- and 1.6-kb transcripts which share the same promoter (Smith *et al.*, in preparation) predominate. *In situ* hybridization using both *int-2g* (data not shown) and *int-2f* (Figure 2B) probes identified *int-2* RNA accumulation specifically in outer, flattened cells surrounding inner clumps of apparently undifferentiated e.s. cells (Figure 2B, 1−3). These outer cells morphologically resemble parietal endoderm. Hybridization of sections with probes for SPARC (Mason *et al.*, 1986) and alpha-fetoprotein (AFP; Gorin *et al.*, 1981), markers of parietal (Holland *et al.*, 1987) and visceral endoderm (Dziadek, 1978; Tilghman and Belayhew, 1982; Dziadek and Andrews, 1983) respectively, identified most outer cells as parietal endoderm (Figure 2B, 4−6). Few AFP-expressing cells were seen (Figure 2B,5).

To confirm and extend these results *in vivo*, *int-2f* and *int-2g* probes were hybridized to mouse embryo sections. *int-2* transcripts were detected in parietal but not visceral endoderm cells (Figure 2C). Taken together these results suggest that *int-2* RNA is present at low levels, probably $<1$ copy/cell, in pluripotent cells of the early post-implantation embryo. On differentiation of these cells, *int-2* RNA is specifically up-regulated in the parietal endoderm.

### *int-2 expression in early migrating mesoderm*

If *int-2* is not detectably expressed in visceral endoderm at 7.5 days, what is the source of *int-2* transcripts previously reported in egg cylinders at this time (Jakobovits *et al.*, 1986)? *In situ* hybridization revealed *int-2* transcripts in cells migrating through the primitive streak at 7.5 days (Figure 3, 1−4). Hybridization was only seen with antisense *int-2g* or *int-2f* RNA probes (Figure 3,1−4 and data not shown) and not with several control probes (data not shown) including α-cardiac actin (Minty *et al.*, 1982), β-h1 globin (Wilkinson *et al.*, 1987a), AFP (Gorin *et al.*, 1981) or *int-1* (Wilkinson *et al.*, 1987b). Thus hybridization was strand-specific and restricted to *int-2* probes.

Cells within the primitive streak are derived from the epiblast, a sheet of epithelial cells which are the sole progenitors of the fetus and which also contribute to extra-embryonic membranes of the amnion and yolk-sac (for review see Beddington, 1983). Cells detach from the epiblast and migrate into the streak between the epiblast and visceral

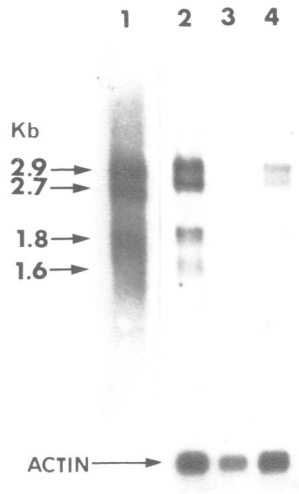


**Fig. 4.** Northern blot analysis of *int-2* RNA transcripts in microdissected regions of 9.5-day embryos. **Lane 1**, RNA from mammary tumours expressing *int-2*; **lane 2**, presomitic/primitive streak fraction; **lane 3**, somitic fraction; **lane 4**, head/visceral arch fraction. Size of *int-2* RNA species (kb) is indicated. Hybridization of an actin coding probe to the same filter is shown below.

endoderm to form mesoderm, as well as the definitive endoderm of the embryo (for reviews see Beddington, 1987; Snow, in preparation). Several studies indicate that cells occupying different positions in the primitive streak at 7.5 days contribute to different tissues of the embryo and surrounding structures. Thus cells at the posterior end are destined to form predominantly extra-embryonic mesoderm (Snow, 1981; Beddington, 1982; Tam and Beddington, 1987) which surrounds the exocoelomic cavity. At this time, *int-2* RNA was detected in these cells, as well as in more anterior regions of the primitive streak (Figure 3, 1−4). Looking at progressively later stages, some *int-2* expressing cells were seen moving from the primitive streak to extra-embryonic sites (Figure 3, 5 and 6). By 8 days *int-2* RNA was detected in the extra-embryonic mesoderm surrounding the entire exocoelomic cavity (Figure 3, 7 and 8), but on formation of a continuous mesodermal epithelium at 8.5 days *int-2* RNA is down-regulated (see Figure 5, 1 and 2). Thus *int-2* RNA accumulation in extra-embryonic mesoderm occurs only during the migratory period and decreases when epithelial association is established.

Throughout this period, and up until 9.5 days, *int-2* RNA was detected in cells within the primitive streak. In the embryo from 8.5 to 9.5 days these cells give rise to the mesoderm of the embryo. After leaving the primitive streak cells become loosely organized anteriorly into presomitic mesoderm and then paraxially to form epithelial blocks of cells, the somites. In the mesoderm of somite stage embryos, *int-2* RNA accumulation was confined to cells migrating through the streak (Figure 3, 9−12) and was not detected by *in situ* analysis in these more organized derivatives.

To examine the *int-2* transcription pattern, 9.5-day embryos were dissected into a somitic and presomitic/primitive streak fraction. As expected, *int-2* RNA was highly enriched in the presomitic/primitive streak region (Figure 4, lane 2). Four *int-2* transcripts were detected, which were identical in size to those seen in mammary tumours expressing *int-2* and in differentiated e.c. cells (Smith *et al.*, in preparation). Low levels of *int-2* RNA transcripts were also

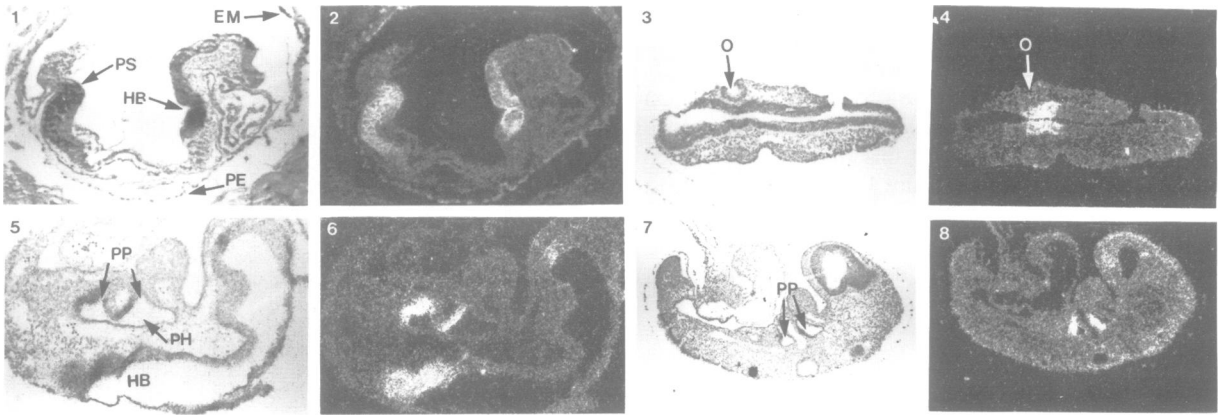


**Fig. 5.** *int-2* expression in neural ectoderm and embryonic endoderm. Sections of 8.5-day (**1** and **2**) and 9.5-day (**3**–**8**) embryos were hybridized with *int-2* antisense probes. The planes of sections are longitudinal (**1, 2** and **5**–**8**) or coronal through the hindbrain and upper spinal cord (**3** and **4**). Bright field (**1, 3, 5** and **7**) and dark field (**2, 4, 6** and **8**) photomicrographs are presented for each section. *int-2* RNA accumulates in the primitive streak (PS), hindbrain (HB) and parietal endoderm (PE) but is no longer present in extra-embryonic mesoderm (EM) at 8.5 days (**1** and **2**). At 9.5 days *int-2* RNA was detected in the hindbrain opposite the otocyst (O) (**3**–**6**) and in the pharyngeal pouches (PP) (**5**–**8**). The pharyngeal pouches appear as separate chambers in lateral sections (**7** and **8**), but their continuity with the pharynx (PH) is seen in more medial sections (**5** and **6**).

detected in the somitic fraction (Figure 4, lane 3), presumably reflecting a low residual expression of *int-2* in non-primitive streak mesoderm which is below the limit of detection by *in situ* hybridization.

### *int-2 expression in the hindbrain and pharyngeal pouches*

High levels of regionalized *int-2* expression were also observed in non-mesodermal cells in early (8.5 days) and mid-(9.5 days) somite stage embryos. *int-2* RNA was detected in the neuroepithelium of the developing myelencephalon of the hindbrain (Figure 5,1–6). Interestingly, this expression was restricted to cells adjacent to the developing otocysts (Figure 5,3 and 4), precursors of the inner ear. In addition, *int-2* RNA was also detected at 9.5 days in the endoderm of the pharyngeal pouches but restricted to the lower surface of the 1st and 2nd pouch (Figure 5,5–8). This expression was not detected in medial sections (data not shown) and is thus restricted to the outpocketings of the pharynx. Subsequently, *int-2* RNA was detected in the equivalent region of the 3rd pouch which forms at a later stage (data not shown). *int-2* RNA transcripts in dissected tissues which included both hindbrain and pharyngeal pouch regions were examined by Northern blot analysis (Figure 4, lane 4). Four transcripts with similar mobilities to those seen in primitive streak fractions (Figure 4, lane 2) were detected.

## Discussion

Our *in situ* hybridization and Northern blot analyses have revealed a complex pattern of *int-2* expression in mouse embryos from 7.5 to 9.5 days of development. Although our detection of *int-2* transcripts at later stages is in contrast to data of Jacobovits *et al.* (1986), this presumably reflects the greater sensitivity of the RNA probes used in the present study. Based upon our Northern blot analysis of e.s. cells, we suggest that *int-2* RNA may be expressed quite widely at low levels in early development (<1 copy/cell) and strongly up-regulated in specific cell types.

Although no biological properties have so far been attributed to the *int-2* protein, in the light of its identification as a member of the FGF family of proteins (Dickson and Peters, 1987), it would not be surprising if these encompassed properties already ascribed to other family members. While we cannot exclude roles for *int-2* in angiogenesis or cell proliferation, the spatial pattern of *int-2* expression does not correlate specifically with these events. However, two other properties, stimulation of cell migration (for review see Folkman and Klagsbrun, 1987) and tissue induction (Slack *et al.*, 1987), may be particularly relevant with respect to the sites of *int-2* accumulation observed in developing mouse embryos.

A role for *int-2* in cell migration during gastrulation is suggested by the correlation between *int-2* expression and the migration of mesoderm cells. High levels of *int-2* RNA were only detected in cells migrating through the primitive streak to embryonic or extra-embryonic sites. During this period these cells are essentially solitary, adopting only loose connections with their neighbours (Tam and Meier, 1982), in contrast to the epithelial epiblast from which they derive. *int-2* RNA is down-regulated on resumption of cell associations in the exocoelom and in the presomitic mesoderm which displays an early somitomeric organization (Tam *et al.*, 1982). Similarly, parietal endoderm cells which are highly active migratory cells accumulate *int-2* RNA, in contrast to the developmentally related visceral endoderm which forms an epithelial tissue. Thus, *int-2* may act as an autocrine factor maintaining and possibly initiating the migratory state of these cells. Such a role, if it exists, must be selective since other migratory populations of cells, notably the neural crest, do not accumulate *int-2* RNA. Moreover, expression of *int-2* in the epithelial endoderm of the pharyngeal pouches and neural ectoderm of the hindbrain seems unrelated to areas of cell migration.

While the possible significance of *int-2* expression in a portion of each pharyngeal pouch is not clear at present, the juxtaposition of the site of neural ectoderm expression and the developing otocysts is strongly suggestive. Several lines of evidence indicate that the neuroepithelium in this region is the source of an inductive signal, beginning at early somite stages and persisting to late head-fold stages, which is required for continued development of the otocyst (for review see Van de Water and Ruben, 1976). Thus, mutations which

disturb the normal spatial relationship between the neuroepithelium and the otocyst lead to the defective development of the otocyst and resultant abnormalities in the inner ear. In view of the inductive properties of other FGF-related proteins in *Xenopus* embryos (Slack *et al.*, 1987), the temporal and spatial coincidence in accumulation of *int-2* RNA in the neuroepithelium associated with otocyst induction is highly provocative. Clearly, a better understanding of the role(s) of *int-2* in both development and tumorigenesis awaits a thorough characterization of the biological properties of the *int-2* protein.

## Materials and methods

### *Isolation of embryo and e.s. cell RNA*

9.5 Day CBA/J mouse embryos (average 12–18 somites) were dissected free of decidual and extra-embryonic tissues in M2 medium (Quinn *et al.*, 1982) and cut into head, somitic and presomitic/primitive streak fractions. The head fraction included all tissues rostral of the 2nd visceral arch, the somitic fraction all tissues caudal of the 2nd visceral arch to the rostral boundary of the presomitic mesoderm, and the presomitic/primitive streak fraction all tissues of the most caudal region. RNA was isolated by the LiCl/urea method (Auffray and Rugeon, 1980). These fractions contained 44, 52 and 4% respectively of the embryo RNA. e.s. cells from the CCE cell line (Robertson *et al.*, 1986) were seeded in bacteriological culture dishes and RNA extracted 0, 3 and 7 days after seeding. RNA was extracted as above.

### *Northern blot analysis*

RNA (2.5 μg total RNA from embryo dissection, 20 μg total RNA from e.s. cells) was fractionated by electrophoresis on agarose gels containing formaldehyde (Maniatis *et al.*, 1982), transferred to Genescreen (New England Nuclear) and crosslinked to the membrane by UV irradiation (Church and Gilbert, 1984). $^{32}$P-labelled single-stranded *int-2* RNA probes (see below) were prepared by standard procedures (Melton *et al.*, 1984) and used at $5 \times 10^6$ c.p.m./ml to hybridize to filters overnight at 65°C in 60% formamide, 1 M NaCl, 50 mM Tris–HCl, pH 7.5, 10 × Denhardt's, 1% SDS, 0.1% sodium pyrophosphate, 10% dextran sulphate, 100 μg/ml yeast RNA. Washing of these filters was performed at a final stringency of 0.2 × SSC at 70°C. Following autoradiography, filters were washed at 80°C in 0.05% SDS to remove *int-2* hydridization and reprobed with $^{32}$P-labelled single-stranded actin RNA probes (Minty *et al.*, 1981) to verify similar loading of RNA in each lane.

### *In situ hybridization*

$^{35}$S-labelled single-stranded RNA probes were prepared by transcribing *int-2* genomic fragments, subcloned in pGEM vectors (Melton *et al.*, 1984; Cox *et al.*, 1984). Two non-overlapping sequences were used, a 532-bp *Sac*I–*Eco*RI fragment, *int-2f* and a 496-bp *Eco*RI–*Sca*I fragment, *int-2g*, which encompass residues 5741–6273 and 6274–6769 respectively of the *int-2* gene (Moore *et al.*, 1986). Preparation of embryo tissue sections and *in situ* hybridization were performed as described (Wilkinson *et al.*, 1987a).

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## Note added in proof

The work referred to throughout as Smith *et al.*, in preparation, is now in press: Smith,R., Peters,G. and Dickson,C. (1988) *EMBO J.*, in press.