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Isolation of the *Xenopus* homolog of *int*-1/*wingless* and expression during neurula stages of early development

Jasprien Noordermeer, Frits Meijlink, Peter Verrijzer+, Frans Rijsewijk[1] and Olivier Destrée*

Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht and [1]Division of Molecular Biology, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

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ABSTRACT

We have isolated the *Xenopus* homolog (X*int*-1) of the mouse protooncogene *int*-1 from a neurula stage 17 cDNA library. The deduced protein sequence of X*int*-1 includes 371 amino acids. The X*int*-1 protein is more similar to the mammalian *int*-1 product (69%), than to the *Drosophila* counterpart of *int*-1, *wingless* (50%). X*int*-1 shares several characteristics of secreted proteins with the other *int*-1 homologs: it has a hydrophobic leader, multiple conserved potential N-linked glycosylation sites and is rich in cysteine residues. All 23 cysteines are conserved in the three proteins. X*int*-1 is transiently expressed during the neurula stages of early *Xenopus* development.

INTRODUCTION

The *int*-1 gene is a proto-oncogene that is activated in certain mouse mammary tumors by integration of the MMTV provirus in the host genome (1−3). Transcripts of this gene are found in mammary tumors, but no expression is detected in normal adult tissues, except for the testis of sexually mature mice (4). Expression of the *int*-1 gene during normal development is temporally and spatially regulated. Transcripts are found in murine embryos between day 9 and 14.5; *in situ* hybridization reveals that RNA accumulation is confined to certain regions of the neural plate and its derivatives (5). The murine *int*-1 product has characteristics of a secretory protein: it has a hydrophobic leader, four potential glycosylation sites and is rich in cysteine residues (2). The gene product enters the secretory pathway and is glycosylated at several sites (6). *Int*-1 is extremely conserved between the mouse and man: only 4 of the 370 amino acids are different (7).

A step forward in unraveling the function of the *int*-1 gene in normal development was the identification of the *Drosophila* homolog (8). Almost 55% of the amino acids are conserved between *Drosophila int*-1 (D*int*-1) and mouse *int*-1. D*int*-1 also has a hydrophobic leader, potential glycosylation sites and all 23 cysteine residues are conserved. These structural similarities suggest homologous functions of the two proteins. D*int*-1 is expressed during development, but transcripts are hardly detectable in adults. Interestingly, D*int*-1 turned out to be the known segment polarity gene *wingless* (*wg*) (8−10). Expression studies in developmental mutants show that *wg*/D*int*-1 belongs to a hierarchical network of genes that govern *Drosophila* development (reviewed in 11).

The present view on the function of *wg*/D*int*-1 is that it functions as an extracellular differentiation factor that contributes to the developing fate of neighboring cells by affecting their gene expression (12). There is no strong evidence on the function of the mouse *int*-1 gene, but it is surmised, that mouse *int*-1 also functions as an extracellular differentiation factor (reviewed in 13).

Recently another member of the *int*-1 family which shows a high similarity with the *int*-1 gene has been identified in man: 36% of the amino acid sequence is identical with that of *int*-1 (14). Expression of this *int*-1 related protein (*irp*) is not restricted to development.

We have initiated a study of the *int*-1 gene in *Xenopus laevis*. *Xenopus* is among the few vertebrate organisms of which embryos can be studied in very early development by tissue transplantation. Moreover, it has recently been shown that *Xenopus* offers great opportunities to analyse the effects of manipulation of gene activity by injecting sense RNA or antisense RNA of developmentally regulated genes into embryos (15, 16). Considerable knowledge exists with regard to cell lineage descendence in the *Xenopus* embryo in general (17) and specifically with regard to the central nervous system (18, 19). We expect that a detailed study of the *Xenopus int*-1 gene, its expression and regulation, will yield new information with regard to vertebrate pattern formation, particularly during neurogenesis.

In this report we describe the isolation of a cDNA clone containing the *Xenopus* homolog of the *int*-1/*wingless* gene. The amino acid sequence is highly conserved between *Xenopus*, mouse, man and *Drosophila*. Comparison of the *int*-1 and *irp*-sequences reveals several regions of functional interest that are virtually identical in all four genes. The *Xenopus int*-1 gene is transiently expressed during the formation of the central nervous system.

MATERIALS AND METHODS

Fertilization of Xenopus laevis eggs

Frogs were induced to ovulate by injection of 375 units Pregnyl (Organon). Eggs were fertilized *in vitro*. Development of the fertilized eggs was allowed to proceed at 16−23°C in 25% MMR (20). The embryos were dejellied using 2% cysteine-HCl pH 7.8. Staging of the embryos was carried out according to the normal table (21).

Screening of cDNA libraries

cDNA libraries of stage 17 (22) and stage 22−24 in λgt10, constructed by Dr. D.A. Melton, were screened with mouse *int*-1 cDNA probes. Two probes were used, the 0.6 kb *Fnu*D II-*Cla* I fragment from the 5′ end and the 1.5 kb *Cla* I-Bgl II fragment of the 3′ end of the mouse *int*-1 gene (23). Hybridization with random-primed DNA probes (24) was carried out at 42°C in 35% formamide, 5 mM EDTA, 1% glycine, 0.9 M NaCl, 50 mM sodiumphosphate pH 7.5, 0.1% Ficoll, 0.1% polyvinylpyrrolidone and 100 μg/ml sheared salmon sperm DNA. The filters were washed in 1×SSC, 0.1% SDS at 50°C. Positive clones were selected and rescreened.

DNA sequencing

For sequence analysis restriction fragments were subcloned in pGEM blue (Promega Biotec). Cloning and analysis were according to standard procedures (25). Nucleotide sequences were determined by the dideoxy chain termination method (26) using double stranded supercoiled plasmid DNA (27).

RNA isolation and hybridization

Total RNA was isolated as described (28). Embryos or oocytes were homogenized in guanidinium thiocyanate and RNA was pelleted through a CsCl cushion. Poly $(A)^+$RNA was selected on an oligo(dT)-column, fractionated by electrophoresis in 0.8% agarose slab gels containing formaldehyde (6.6% v/v) and transfered to nitrocellulose filters. Hybridization with random primed DNA was carried out at 42°C in 50% formamide, 20 Mm sodium phosphate pH 6.5, 4×SSC, 5×Denhardt's (25), 0.1% SDS, 2 Mm sodium pyrophosphate, 10% dextran sulphate and 100 μg/ml sheared salmon sperm DNA. The final wash was in 0.2×SSC, 0.1% SDS at 65°C.

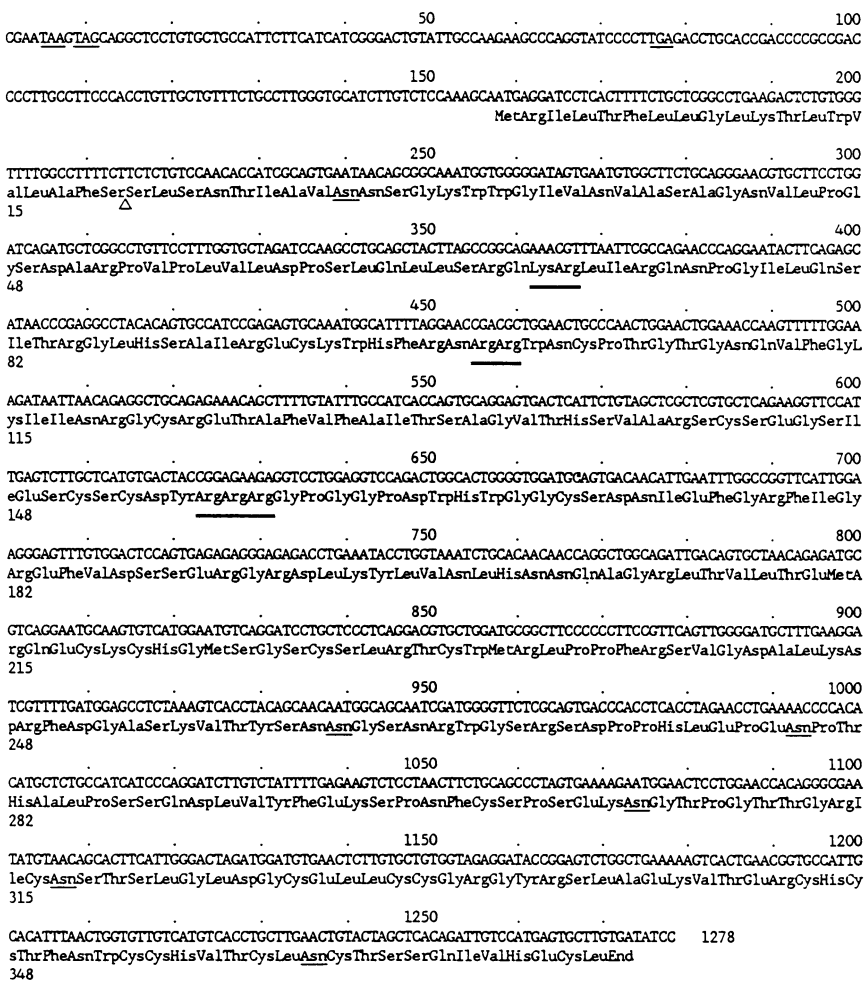

Figure 1 Nucleotide sequence of part of the *Xenopus int*-1 cDNA and the amino acid sequence deduced for the *int*-1 protein

The 5′ noncoding region, the coding region and part of the 3′ untranslated region of the isolated cDNA clone are shown. The longest open reading frame spans 1113 nucleotides and is preceded by three stop codons in the same frame. The six potential sites for N-linked glycosylation and the stop codons in the leader are indicated by thin underlining and possible sites for cleavage by proteases are indicated by fat underlining. An open arrowhead marks the potential signal peptide cleavage site.

RESULTS

Isolation and nucleotide sequence of the Xint-1 cDNA

To identify the *Xenopus* homolog of the mammalian *int*-1 gene, two cDNA libraries, one of mid (17) and one of late (22−24) neurula stage embryos, were screened with mouse *int*-1 cDNA probes under conditions of low stringency. Two probes, representing the 5′and 3′ ends of the coding region of the mouse *int*-1 gene (23), were used. One cDNA clone from the stage 17 library hybridized with the 5′ fragment of the mouse *int*-1 gene. This

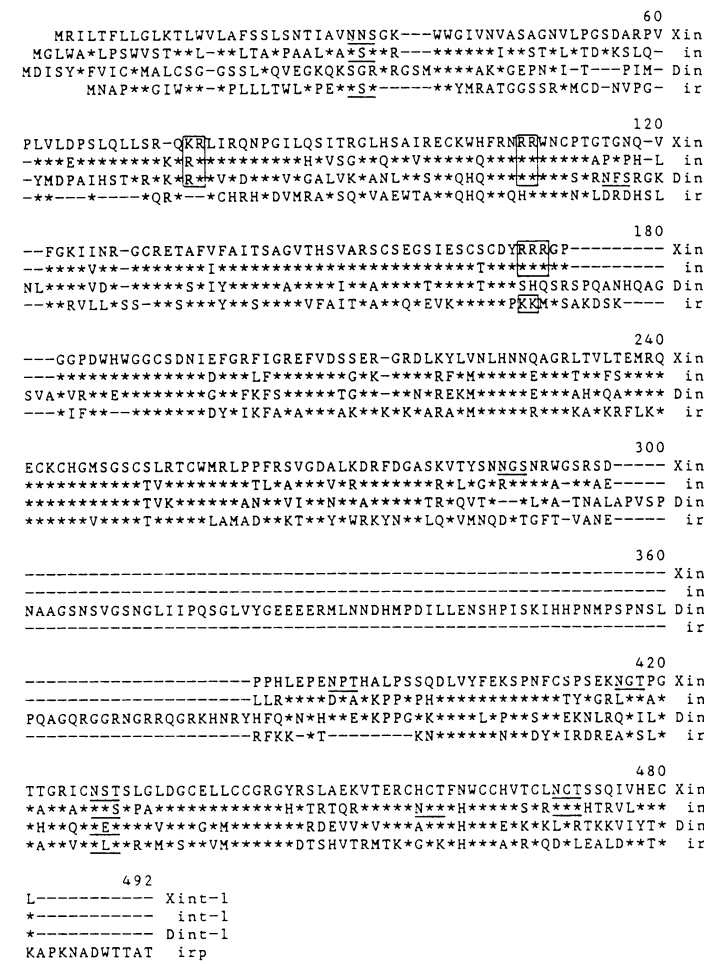

Figure 2 Comparison of the predicted amino acid sequences of the X*int*-1, mouse *int*-1 (2), D*int*-1/*wingless* (8) and *irp* (14) proteins

Horizontal lines are gaps introduced to align the four proteins. Conserved amino acids are indicated by an asterisk. Potential glycosylation sites are underlined, potential protease cleavage sites are boxed.

clone contains a *Xenopus* DNA insert of approximately 3.4 kb. The size of this cDNA corresponds with that of the transcript detected by RNA blotting (see below).

The nucleotide sequence of the 5′ untranslated region and the predicted coding region of the X*int*-1 gene are shown in Figure 1. The longest open reading frame, starting at the ATG codon at position 158, covers 1113 nucleotides, encoding a protein of 371 amino acids (41 kD). The nucleotides surrounding the start codon match the consensus sequence for initiation of translation (29). As three stop codons are located in the same frame more upstream, we assign the ATG at position 158 as the start codon for translation.

Comparison of the length of the transcript with the isolated cDNA shows that the length of the 3′ untranslated region is about 2.2 kb.

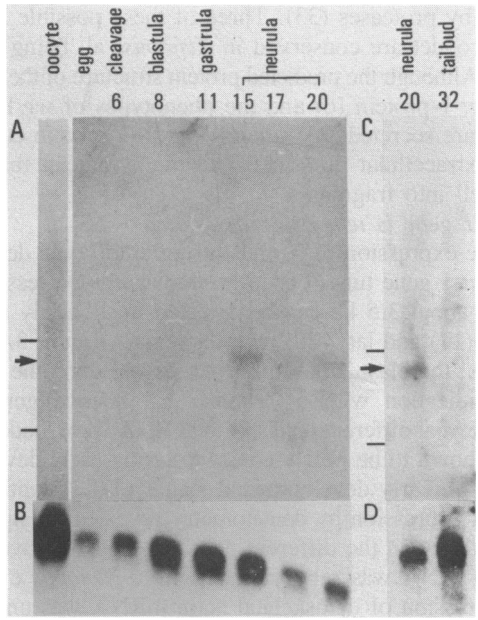

Figure 3 Expression of the X*int*-1 gene during embryonic development

RNA blot analysis was performed on total or poly $(A)^+$RNA from different stages of development of *Xenopus laevis* (21). Fifteen micrograms of total RNA (panels A/B) or 5 micrograms of poly $(A)^+$RNA (panels C/D) were layered in each lane. Blots were hybridized with random primed X*int*-1 cDNA. Bars indicate the size of the markers (2.0 and 4.5 kb) and an arrow marks the size of the single X*int*-1 transcript with an estimated length of 3.5 kb. Panel B shows rehybridization of the filter in A with the random primed H3 histone gene of *X.laevis* (35). Panel D shows rehybridization of the filter in C with the random primed cytoskeletal actin gene of *X.laevis* (36).

Characteristics of the predicted Xint-1 protein and similarity with int-1, wingless and irp

Analogous to the mouse and *Drosophila* counterparts, the predicted X*int*-1 protein has characteristics of a secretory protein: it has a hydrophobic leader, is rich in cysteine residues (23 out of 371 amino acids), contains several potential glycosylation sites and lacks a transmembrane domain (31). Using the weight matrix method described in ref. 32, we found that the most likely site for cleavage of the signal peptide is between amino acids 19 and 20.

The amino acid sequences of the *int*-1 and *irp* products are highly conserved between *Xenopus*, mouse, man and *Drosophila*: 69% of the amino acids of the X*int*-1 product are identical in the murine and human counterparts, 50% in the *wg*/D*int*-1 product and 40% in the human *irp* product. In addition 14, 16 and 15% of the amino acids in the respective proteins is structurally similar. *Wg*/D*int*-1 has an insert of 85 amino acids that is absent in the other proteins (see Figure 2). All of the cysteines in X*int*-1 are conserved in the mouse and *Drosophila int*-1 proteins and the *irp* product lacks one of the cysteines. Conservation is not restricted to particular regions of the coding sequence, but found over the entire length of the protein. Apart from the cysteines, some possible functional sites are conserved in all four proteins, potential Asn-linked glycosylation sites as well as potential protease cleavage sites. The X*int*-1 protein has six potential N-linked glycosylation sites, three of which are conserved in mouse and one in *Drosophila*.

Several double and triple basic residues in the *int*-1 protein have been pointed as poten-

tial sites for cleavage by proteases (33). Three of these possible protease cleavage sites of the murine *int*-1 product are conserved in *Xenopus*, all being located in hydrophilic regions of the protein. Although the predicted protein structure of the *int*-1 genes, the biosynthesis of the mouse *int*-1 protein (6) and the phenotypes of *wg*/D*int*-1 mutants suggest that the *int*-1 proteins are secreted (34), the mouse *int*-1 protein could not be detected on the cell surface or in extracellular fluids (6). This might indicate that the protein is rapidly cleaved outside the cell into fragments.

Expression of the int-1 gene is temporally regulated

We have examined the expression of X*int*-1 during embryonic development using RNA blot analysis. The X*int*-1 gene turns out to be transiently expressed during neurulation. A single transcript of about 3.5 kb can be detected in the early neurula (stage 15) and is still present in mid (17) and late (20) neurula stages (Figure 3A). No expression was found in oocytes, eggs, the blastula and gastrula stages, or in the tailbud stage (32−34) (Figure 3C). Rehybridization with a *Xenopus* H3 histone gene probe (Figure 3B) demonstrated that somewhat different levels of total RNA were loaded. The level of histone H3 mRNA has been shown to be nearly constant during early development, while being higher in oocytes than in early developmental stages (37). Quantification of the histone H3 and X*int*-1 mRNA expression by densitometry revealed no significant differences in the levels of X*int*-1 mRNA in the different neurula stages relative to the amount of H3 mRNA. The poly $(A)^+$RNA was rehybridized with a *Xenopus* cytoskeletal actin probe (Figure 3D). The expression of cytoskeletal actin mRNA was undetectable in the early stages of development but there was an increase in cytoskeletal actin mRNA expression in the tailbud stages, in accordance with the temporal change described before (36). No X*int*-1 transcripts could be detected in the tailbud stage.

DISCUSSION

Little is known about the function of the *int*-1 gene in early vertebrate development. In *Drosophila*, *wg*/*int*-1 is a member of a network of regulatory genes that direct development (12). Most likely *wg*/*int*-1 is an extracellular factor that interacts with receptors on neighboring cells, changing their pattern of gene expression (13). Support for this hypothesis comes from the observation that *wg*/*int*-1 is not cell autonomous (38) and that expression of the segmentation gene *engrailed* in adjacent cells is influenced by *wg*/*int*-1 expression (12).

The temporal expression patterns of the *int*-1 genes during early development of the mouse and *Xenopus* are similar. *Int*-1 expression was detected by RNA blotting from day 9 to day 12.5 of gestation (4). By more sensitive *in situ* hybridization it was demonstrated that *int*-1 transcripts continue to be present in a small subpopulation of cells until at least 14.5 days of gestation (5). In this period neurulation, somitogenesis and early organogenesis take place. We could not detect expression of X*int*-1 in eggs, blastula or in gastrula stages. The X*int*-1 transcripts are first detected at stage 15 of embryonic development, the early neural fold stage. In stage 20 embryos, when the neural folds are fused and the neural tube is almost closed, the X*int*-1 mRNA is still detectable. In stage 32−34 embryos X*int*-1 expression, is no longer found. This stage of development would be comparable in several aspects with the day 12 mouse embryo (39, 21).

In situ hybridization studies of the murine *int*-1 revealed that expression is restricted to specific regions of the neural plate and developing spinal cord and brain (5). We have not yet determined the spatial distribution of X*int*-1 transcripts, but expression of murine

and *Xenopus int*-1 is found during similar developmental stages.

The structural similarity with *wg/int*-1 and the fact that X*int*-1 and murine *int*-1 are expressed at the time when formation of the neural tube occurs suggest that X*int*-1 may be a differentation factor involved in neural development. In *Xenopus*, neurogenesis has been studied extensively. It is thought that predisposition of the ectoderm as well as induction by extracellular factors, having a position dependent role, are involved in neural induction (40). The homeobox containing gene XlHbox6 which is transiently expressed during neurulation is implicated in the predisposition of the ectoderm to form nerve in response to induction by the underlying mesoderm (40). The X*int*-1 product, having the structure of an extracellular protein and being transiently expressed during the formation of the neural tube, may be involved in establishing specific local differentiation within the neural ectoderm.

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*To whom correspondence should be addressed

+Present address: Laboratory for Physiological Chemistry, State University of Utrecht, Vondellaan 24a, 3521 GG Utrecht, The Netherlands

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