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Transcriptional regulation of tubulin gene expression in differentiating trochoblasts during early development of *Patella vulgata*

Wim G. M. Damen*, Leo A. van Grunsven and André E. van Loon

Department of Experimental Zoology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

*Author for correspondence

SUMMARY

The expression of α- and β-tubulin genes during the early development of the marine mollusk *Patella vulgata* has been investigated. From the 32-cell stage onwards, an enhanced expression of both α- and β-tubulin mRNAs was detected in the primary trochoblasts. After one additional cleavage, these cells become cleavage-arrested and then form cilia. They are the first cells to differentiate during *Patella* development. Later, α- and β-tubulin mRNA is also found in the accessory and secondary trochoblasts. Together these three cell-lines form the prototroch, the ciliated locomotory organ of the trochophore larva. The early and abundant expression of tubulin genes precede and accompany cilia formation in the trochoblasts and provides us with an excellent molecular differentiation marker for these cells.

Apart from the trochoblasts, tubulin gene expression was also found in other cells at some stages. At the 88-cell stage, elevated tubulin mRNA levels were found around the large nucleus of the mesodermal stem cell 4d. In later stages, tubulin gene expression was detected in the cells that form the flagella of the apical tuft and in the refractive bodies.

An α-tubulin gene was isolated and characterized. A *lacZ* fusion gene under control of the 5′ upstream region of this tubulin gene was microinjected into embryos at the two-cell stage. The reporter gene product was only detected in the three trochoblast cell-lines at the same time as tubulin genes were expressed in these cells. Reporter gene product was not detected in any other cells. Thus, this 5′ upstream region of this α-tubulin gene contains all the elements required for the correct spatiotemporal pattern of expression.

Key words: in situ hybridization, α-tubulin, β-tubulin, *lacZ* fusion gene, cilia, molluscan development, differentiation marker

INTRODUCTION

Differential distribution of morphogenetic compounds plays a crucial role in the specification of the early cell fate in molluscan development. The first fully specified cells are the trochoblasts (Wilson, 1904). The trochoblasts soon stop dividing and differentiate into ciliated cells. Because of their differentiation early in development and their proposed cell-intrinsic developmental capacity (Wilson, 1904), they are an excellent model to elucidate the molecular mechanisms that underlie the process of specification and differentiation.

In our model system, *Patella vulgata*, the trochoblasts are the first differentiating cells. They form a transverse band of ciliated cells called the prototroch: the locomotory organ of the free-swimming larva. This prototroch originates from three different cell lines: the primary, the accessory and the secondary trochoblasts. At the 16-cell stage, one primary trochoblast is formed in each of the four quadrants of the embryo. These four stem cells divide only twice and form 16 trochoblasts. Almost immediately after their last division, differentiation characteristics become manifest in these cells: they develop cilia, become cleavage-arrested, reduce their communication via gap junctions and develop a band of filamentous (F-) actin (Wilson, 1904; Van den Biggelaar, 1977; Janssen-Dommerholt et al., 1983; Serras et al., 1990; Serras and Speksnijder, 1991; Damen and Dictus, 1994a). Isolated primary trochoblasts, taken from a 16-cell stage embryo, differentiate just as in the intact embryo: they also divide only twice, become cleavage arrested, develop cilia (Wilson, 1904), and a band of F-actin forms at the base of the cilia (Serras and Speksnijder, 1991). This shows that the primary trochoblasts contain cell-intrinsic developmental information for their differentiation. It is likely that this is the result of the segregation of regulatory molecules that control trochoblast-specific gene expression. This assumption is strenghted by the work of Janssen-Dommerholt et al. (1983), who showed that 8-cell stage embryos in which cytokinesis but not karyokinesis was blocked still develop cilia at the first quartet micromeres. The other cells that take part in the formation of the prototroch are the accessory and secondary trochoblasts, formed at the 64-cell stage. They divide only once and then form ten accessory and six secondary trochoblasts, respectively. The four quadrants do not contribute an equivalent number of accessory and secondary trochoblasts. In the D-quadrant, four accessory trochoblasts are formed, whereas in each of the three other quadrants only two accessory but addi-

tionally two secondary trochoblasts are formed (Damen and Dictus, 1994a).

First ciliation of primary trochoblasts is visible just after their last division at the asynchronous sixth cleavage (Damen and Dictus, 1994a). Between the fifth and sixth cleavage the overall activation of the embryonic genome starts (van Loon et al., 1994). Around that time, an enormous increase in the expression of both α- and β-tubulin genes is detected with northern blots (van Loon et al., 1994). Since tubulin is a major compound of cilia, this tremendous increase of tubulin mRNA might be correlated with the onset of ciliation of the trochoblasts.

We have studied the spatiotemporal expression pattern of both α- and β-tubulin genes in the early *Patella* embryo. The high level of tubulin gene expression in the early stages turned out to be restricted to the trochoblast cell lines. In order to gain insight into the regulation of the processes that play a role in the regional specification and the early differentiation of the trochoblast cells, we isolated and characterized one of the α-tubulin genes that is expressed in these cells. By placing a *lacZ* reporter gene under control of the regulatory part of this gene, we show that this sequence possesses elements sufficient for correct spatiotemporal expression. Although present throughout the whole embryo, the fusion gene is only expressed in trochoblasts.

MATERIALS AND METHODS

Chemicals

All restriction enzymes and modifying enzymes were purchased from Pharmacia (Milwaukee, WI) or Boehringer Mannheim (FRG). The Erase-a-Base system kit, pGEM3Zf(+) and pGEM5Zf(+) plasmid vectors, Sp6 and T7 promoter primers and λEMBL3A were obtained from Promega (Madison, WI), the T7 DNA sequencing kit from Pharmacia, and *Escherichia coli* β-galactosidase and Lucifer Yellow (K^+ salt) from Sigma (St Louis, MO). 5-Bromo-4-chloro-3-indolyl-galactopyranoside (X-gal), the digoxigenin labeling and detection kit, and the random primed labeling kit were obtained from Boehringer Mannheim. Radiochemicals ([α-^{32}P]dATP, α-^{35}S-dATP and α-^{35}S-dCTP) were obtained from Amersham (Buckinghamshire, England). The pNL vector containing the *lacZ* reporter gene was generously provided by Dr L. Gan, University of Texas (Gan et al., 1990).

cDNA and genomic clones

The α-tubulin-2 cDNA and the β-tubulin cDNA (pPDC301) derived from *P.vulgata* mRNAs are described by van Loon et al. (1994).

The α-tubulin-4 genomic clone was isolated from a genomic *P. vulgata* library in λEMBL3A (Frischauf et al., 1983). This library was constructed using the genomic DNA of the sperm of one male (van Loon et al., 1993) partially digested with *Sau*3AI. Fragments of 15-20 kb were ligated into the alkaline phosphatase-treated *Bam*HI-arms of λEMBL3A phage DNA. Genomic α-tubulin clones were isolated from this library using a ^{32}P-labeled α-tubulin-2 cDNA probe. A probe with only the 3′ untranslated region (UTR) of this cDNA (nt 1372-1473) hybridized to one of these isolated clones viz. the α-tubulin-4 clone. From this α-tubulin-4 clone a physical map was prepared (not shown) and a 2.2 kb *Sal*I-*Hin*dIII fragment, adjacent to the right arm of the lambda phage DNA containing the 5′ coding part and the 5′ upstream region of the gene was isolated. This fragment was made blunt with T4 DNA polymerase and subcloned blunt into the *Eco*RV site of pGEM5Zf(+). The 3′ coding and 3′ downstream part of the gene were localized on a 4.6 kb *Hin*dIII-*Hin*dIII fragment. This fragment was subcloned in the same way. A 0.3 kb *Hin*dIII-*Kpn*I fragment, a 0.45 kb *Eco*RI-*Eco*RI and a 1.6 kb *Eco*RI-*Apa*I fragment were isolated from this 4.6 kb *Hin*dIII-*Hin*dIII fragment, subcloned into the pGEM5Zf(+) cloning vector, and used to determine the nucleotide sequence of the 3′ part of the gene.

Nucleotide sequence analysis

The nucleotide sequence of the α-tubulin-4 gene was determined using the dideoxy method of Sangers et al. (1977). The reactions were performed using the T7 sequencing kit with [α-^{32}P]dATP, α-^{35}S-dATP or α-^{35}S-dCTP as radioactive label and Sp6 or T7 promoter primers. Successive deletion mutants were prepared using the Erase-a-Base Protocol. The data were analyzed with MacVector (IBI, New Haven, CT).

Southern blotting

A Southern blot was prepared as described previously (van Loon et al., 1993). The genomic DNA used was of the same individual as used for the genomic library.

Primer extension analysis

A synthetic 23-nucleotide primer (5′ dGAAGTGTAGTAGTAG-GTATCTAG 3′) complementary to the sense strand of the α-tubulin-4 gene (from nucleotide position +74 to +96) was used in the primer extension analysis. 1 μg of poly(A)$^+$ RNA (isolated 16 hours after first cleavage; van Loon et al., 1993) was denatured at 75°C in 50 mM Tris-HCl pH 8.3; 8 mM $MgCl_2$ and 10 mM DTT for 3 minutes. After cooling on ice, 8 units of RNAguard Ribonuclease Inhibitor (Pharmacia) and 30 ng of primer were added. For primer annealing, the mixture was incubated successively for 15 minutes at 42°C, 15 minutes at 37°C and 10 minutes at room temperature. Then the reaction mixture was made up to the following final concentrations: dATP, dGTP, dTTP (1 mM each); α-^{35}S-dCTP (10 μCi, 0.5 μM); 50 mM Tris-HCl pH 8.3; 8 mM $MgCl_2$; 10 mM DTT; 33 units RNAguard Ribonuclease Inhibitor; and 8 units Moloney Murine Leukemia Virus reverse transcriptase. After 3 minutes incubation at 37°C, unlabeled dCTP was added to a final concentration of 1 mM to ensure completion of the polymerization reaction and the incubation was continued for 30 minutes at 37°C. The reaction was stopped by adding 0.5 volume deionized formamide solution containing 10 mM EDTA, 0.3% xylenecyanol and 0.3% bromophenol blue. The reaction products thus obtained were subjected to electrophoresis on a 6% sequencing gel. The size of the reaction products was determined by parallel electrophoresis of the products of a sequencing reaction primed with the same 23-nucleotide primer.

Fusion of the promoter of the α-tubulin-4 gene to the *lacZ* reporter gene

A 1660 bp *Pal*I fragment, containing the 5′ upstream sequences, exon 1 (=the 5′ UTR + the ATG- codon), the intron and a small part of exon 2 up to the *Pal*I site (at +487), was isolated from the 2.2 kb *Sal*I-*Hin*dIII fragment subcloned in pGEM5Zf(+). To fuse this fragment in frame to the *lacZ* gene, the pNL vector was cut with *Bam*HI. This open *Bam*HI site was partially filled by T4 DNA polymerase using only dATP and dGTP as substrates. The protruding ends were removed by S1 nuclease to obtain blunt ends. The 1660 b.p. *Pal*I fragment containing the 5′ upstream region of the α-tubulin-4 gene was cloned into these partially filled and blunt *Bam*HI sites to construct an in frame fusion with the *lacZ* gene. The cloning was checked by sequencing the fusion to ensure that the *lacZ* gene was in frame with the start-ATG present in the tubulin fragment. This final construct was denominated pNL-α4-04.

Embryos

Embryos were obtained via an in vitro fertilization as described elsewhere (Serras and Speksnijder, 1991). Embryos used in the in situ hybridization experiments were dejellied at the required stage by a 1-2 minutes treatment in Millipore-filtered sea water, pH 3.9. Embryos

used for microinjection were dejellied in the same way just before the first cleavage. The developmental stage of the embryos is indicated as cell number or as time after first cleavage. In the in situ hybridization experiments groups of 200 synchronously (divergence less than 5 minutes) cleaving embryos were used, previously selected at the 1st, 2nd or 3rd cleavage. For the younger stages (younger than 4 hours after first cleavage), the developmental stage (cell number) of the embryo was confirmed by a Hoechst staining of the nuclei (Serras and Speksnijder, 1991) on 20-25 embryos. In the experiments with the reporter gene construct, the embryos were selected at the first cleavage and microinjected at the 2-cell stage. In order to obtain synchronously dividing groups of injected embryos, embryos in which the third cleavage started within an interval of 10 minutes were put together. For the denomination of the various trochoblasts the nomenclature of Heath (1899) is used.

In situ hybridization on whole embryos

Whole-mount in situ hybridizations were performed as described by Tautz and Pfeifle (1989) with some modifications. Dejellied *Patella* embryos were treated for 2 minutes with 0.025% Triton X-100 in sea water and then fixed for 15 minutes in 4% paraformaldehyde in 0.1 M Hepes, pH 6.9, 2 mM $MgSO_4$, 1 mM EGTA. One volume of methanol was added. Subsequently, the embryos were transferred into a solution of 90% methanol and 10% 0.5 M EGTA (ME) for 5 minutes. The embryos were then refixed and rehydrated, pretreated, hybridized and stained as described in the original protocol (Tautz and Pfeifle, 1989) with the following modifications. Hybridization solution (HS) was 50% formamide, 2× SSC, 2.5× Denhardt's solution, 0.1% Tween-20 and 100 μg/ml sonicated and denaturated salmon sperm DNA. The wash steps before the prehybridization were 10 minutes in 1:1 PBS-T:HS (PBS-T is PBS plus 0.1% Tween 20) and then 10 minutes in HS. The (pre)hybridization was at 42°C. The successive wash steps after hybridization were 1:1 PBS-T:HS for 15 minutes, 3:2 and 4:1 PBS-T:HS for 10 minutes each, and finally 2 times 10 minutes in PBS-T. The wash steps after antibody incubation were once for 5 minutes in PBS-T and three times 5 minutes in TMN-T (100 mM Tris-HCl, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl and 0.1% Tween-20). No Levamisole was used in the staining buffer. The embryos were dehydrated by successively washing in 30%, 70% and 2 times 100% ethanol, and cleared and mounted in methylsalicate (Merck, Darmstadt, FRG). Embryos were observed on a Zeiss Axiovert 35M microscope equipped with Differential Interference Contrast (DIC) optics. Photographs were taken on Kodak Ektachrome film (100 ASA).

In these in situ hybridization experiments, digoxigenin-labeled α-tubulin and β-tubulin cDNAs were used as probes. The complete 1473 bp α-tubulin cDNA insert and the 1223 bp *Bgl*II-*Eco*RI fragment of the β-tubulin cDNA insert were isolated from gel slices. These DNA fragments were cut with *Sau*3AI to obtain smaller fragments. After a phenol-chloroform extraction and ethanol precipitation, the DNA was labeled with digoxigenin using the digoxigenin-labeling kit as described by the supplier. As a control, the pGEM3Zf(+) plasmid was treated and used in the same way.

Microinjection of fusion gene DNA and detection of the reporter gene product

The fusion gene DNA or the *Escherichia coli* β-galactosidase was microinjected into one blastomere at the 2-cell stage in a 5 mM Hepes, pH 7.0 solution containing 0.45% Lucifer Yellow (LY). The injections were monitored using the coinjected LY as an injection marker. Occasionally LY could be detected in both blastomeres due to injection prior to completion of cytokinesis. Microinjections were performed as described elsewhere (Damen and Dictus, 1994a). *Hin*dIII linearized pNL-α4-04 DNA was injected together with five-fold excess sheared *Patella* sperm DNA. In the case of the *E. coli* β-galactosidase, the protein was injected in a 5 mM Hepes, pH 7.0 solution containing 0.45% LY.

At the desired stage, the embryos were fixed in Ca^{2+}- and Mg^{2+}-free sea water (CMFSW) containing 0.2% glutaraldehyde at room temperature for 15 minutes. Then the embryos were rinsed 2 times in color buffer [10 mM Na_2HPO_4, 150 mM NaCl, 1 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6$] for 5 minutes, rinsed once in color buffer + 3 mM 5-bromo-4-chloro-3-indolyl-galacto pyranoside (X-gal), and stained in color buffer + 3 mM X-gal at room temperature for several hours, or overnight.

To identify the labeled cells in the embryos, a gallocyanin counter staining was performed. The embryos stained for β-galactosidase were washed in CMFSW and refixed in Zenker's fixative for 45 minutes. After intense washing in water, the embryos were stained in a gallocyanin solution for 45-60 minutes (Van den Biggelaar, 1977) and washed again in water. Then, the embryos were dehydrated, mounted, and observed as described above for in situ hybridization.

RESULTS

Spatiotemporal expression of α- and β-tubulin genes in the trochoblast cells during early development

van Loon et al. (1994) have shown with northern blots that the amounts of both α- and β-tubulin mRNAs increase enormously shortly after the 32-cell stage in the *Patella* embryo. Which cells contribute to this increased expression of tubulin genes was not established. As the primary trochoblasts start ciliation around this time and as cilia contain microtubules, the increase of tubulin mRNA could be the result of a high expression level of tubulin genes exclusively in the primary trochoblasts. In order to determine whether this is indeed the case, the spatiotemporal expression of α- and β-tubulin genes was analyzed by using the in situ hybridization technique with non-radioactive probes. Identical patterns for α- and β-tubulin mRNAs were found at all stages. Therefore, only the pattern for β-tubulin mRNAs is shown in Fig. 1.

In 16-cell stage embryos (1 hour 55 minutes after first cleavage) no localized expression of tubulin mRNAs could be detected (Fig. 1A). In early 32-cell stage embryos (2 hour 25 minutes after first cleavage; this is about 20 minutes after the fifth cleavage) four groups of two cells each near the animal pole of the embryo were labeled in the nucleus (Fig. 1B). In each nucleus, the label was present in at least two intensively stained spots, presumably representing the nascent transcripts at the site of transcription. These cells were identified as the primary trochoblasts (the cells $1a^{21}$-$1d^{21}$ and $1a^{22}$-$1d^{22}$) because of the characteristic pattern of the labeled cells in the embryo and the expression patterns at later stages (see below). At the end of the 32-cell stage (3 hours after first cleavage, Fig. 1C), the amount of label had increased considerably, and was also present in the cytoplasm of the eight primary trochoblasts. At the onset of the sixth cleavage (40-cell stage; 3 hours 30 minutes after first cleavage) after the last division of the primary trochoblasts, the mRNAs for both tubulins were detected in four groups of four cells representing the primary trochoblasts (Fig. 1D). In 60-, 64-, and 88-cell stage embryos (4 hour, 4 hours 30 minutes and 5 hours after first cleavage respectively) the same labeling pattern is observed (Fig. 1E for 88-cell stage embryo, 60- and 64-cell stage embryos not shown) and the level of expression seems to increase steadily. In the 88-cell stage embryos, both tubulin mRNAs were also detected around a large nucleus positioned centrally in the embryo (Fig. 1F), presumably the nucleus of the mesodermal

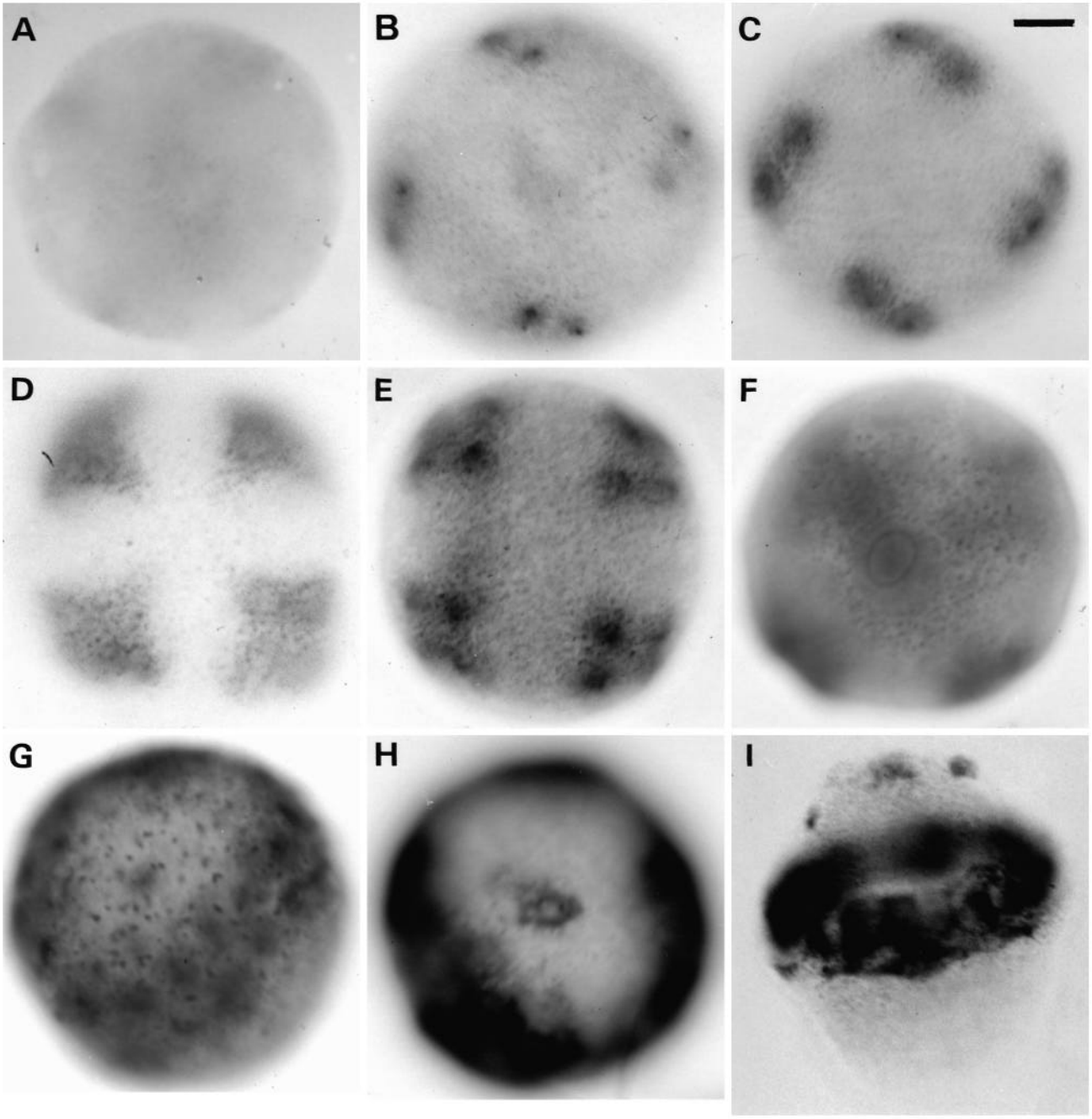

Fig. 1. Expression of tubulin mRNA during the early development of *Patella vulgata*. Whole-mount in situ hybridizations for β-tubulin in embryos of different stages. (A) 16-cell stage embryo, (1 hour 55 minutes after first cleavage). (B) Early 32-cell stage (2 hours 25 minutes after first cleavage), animal view. (C) Late 32-cell stage (3 hours after first cleavage), animal view. (D) 40-cell stage embryo (3 hours 30 minutes after first cleavage), animal view. (E) 88-cell stage embryo (5 hours after first cleavage), animal view. (F) 88-cell stage embryo, the plane of focus is on the large nucleus of 4d positioned centrally in the embryo, around which tubulin mRNA is present. The four groups of labeled cells out of focus are the primary trochoblasts. (G,H) Embryos of 7 hours 30 minutes after first cleavage: inbetween the quartets of primary trochoblasts a number of other positively reacting cells are visible. These are the accessory and secondary trochoblasts (G). Cells that will form the apical tuft also express tubulin mRNAs (H). (I) 22-hour old trochophore larva: the prototroch cells, the cells of the apical tuft and the refractive bodies express tubulin mRNAs. Scale bar, 30 μm.

stem cell 4d. At later stages (see blow) tubulin mRNA could no longer be detected in the progeny of 4d. At 6 hours and 15 minutes (not shown) and 7 hours and 30 minutes (Fig. 1G), stages beyond the 88-cell stage of which the exact cell number is not known, both α- and β-tubulin mRNAs were also detected in cells localized between the four groups of labeled primary trochoblasts. These cells were the accessory trochoblasts (the cells $1d^{1211}$, $1d^{1212}$, $1a^{1221}$-$1d^{1221}$ and $1a^{1222}$-$1d^{1222}$) and the secondary trochoblasts (the cells $2a^{111}$-$2c^{111}$ and $2a^{112}$-$2c^{112}$). These cells also become ciliated and contribute to the

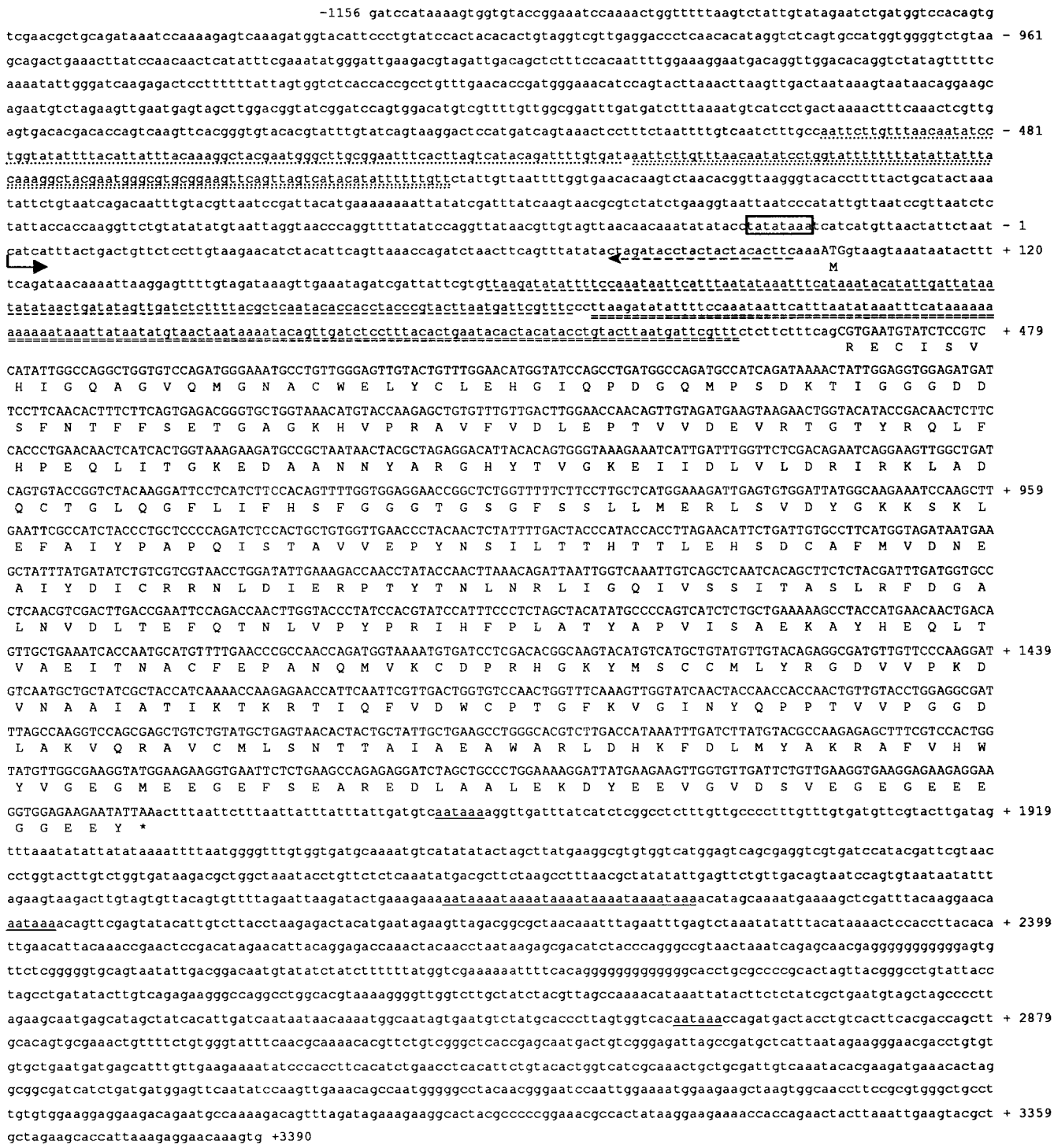

Fig. 2. The nucleotide sequence and deduced protein sequence of the α-tubulin-4 gene of *Patella vulgata*. Nucleotide sequence of the protein encoding part is shown in upper case letters. The 5′ flanking, 5′ untranslated region (5′ UTR), as well as the intron and the 3′ downstream sequences are presented with lower case letters. Numbering of the sequence is relative to the transcription start point (+1, marked with an arrow) as determined by primer extension (see Fig. 3); the position of the primer used in the primer extension analysis is indicated with a dashed arrow. The deduced amino acid sequence is shown below the nucleotide sequence using the one letter code; the TAA stop codon for translation is marked by an asterisk. The TATA-box is marked by an open box. The repeat sequences in the 5′ upstream part of the gene (from –501 to –406 and from –404 to –307) are underlined with a dotted-line (part 1) and a double dotted-line (part 2). The repeat sequences present in the intron (from +180 to +309 and from +313 to +450) are underlined with a dashed-line (part 1) and a double dashed-line (part 2). Putative polyadenylation signals present in the 3′ downstream sequences are underlined with a single-line. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X77618.

formation of the prototroch (Damen and Dictus, 1994a). Another group of larval cells, located at the animal pole of the embryo, also produces long cilia, the cilia of the apical tuft. These cells likewise accumulate tubulin mRNA at 7 hours 30 minutes after first cleavage (Fig. 1H). At 8 hours after first cleavage, both tubulin mRNAs were detected in a more or less double band of trochoblasts around the embryo (not shown). Finally, in the 22-hour old trochophore larva, α- and β-tubulin mRNAs were present in a single row of cells, corresponding to the single row of prototroch cells (Fig. 1I). These prototroch cells are trochoblasts that became repositioned in a single row of cells: the prototroch of the free-swimming trochophore larva (Damen and Dictus, 1994b). In this trochophore larva α- and β-tubulin mRNAs were also still present in the cells of the apical tuft and also in the two structures in the pretrochal ectoderm, the so-called refractive bodies (Wilson, 1904; Smith, 1935) (Fig. 1I).

Thus, high levels of tubulin gene expression precede and accompany cilia formation. The early and abundant expression of tubulin mRNAs in the trochoblasts provides us with an excellent molecular differentiation marker for these cells.

The sequence and organization of the α-tubulin-4 gene

To gain insight into the regulation of the tubulin gene expression in the trochoblasts, we decided to isolate one of the tubulin genes and to fuse its promoter to a reporter gene. From earlier observations it is known that the genome of *Patella* contains about 20 α-tubulin genes (A. E. van Loon, unpublished results). Therefore, we isolated five different α-tubulin clones from a genomic library using a ^{32}P-labeled α-tubulin-2 cDNA probe. To identify an α-tubulin gene which is expressed during development we hybridized these clones with the 3′UTR sequence of the embryonic α-tubulin-2 cDNA (van Loon et al., 1994). The 3′UTRs are specific for each of the different genes or classes of genes, as has been found also with actin genes (van Loon et al., 1993). One of these clones, α-tubulin-4, hybridized with the 3′ UTR sequence of the α-tubulin-2 cDNA. This indicates that the α-tubulin-4 gene is one of the members of the α- tubulin gene family that are expressed during early embryogenesis. This gene was subcloned and the nucleotide sequence was determined. The complete nucleic acid sequence and the deduced amino acid sequence of this gene are shown in Fig. 2.

The transcription start point of the gene (defined as position +1) was determined by primer extension (Fig. 3). It is located 99 basepairs (bp) upstream of the putative ATG start-codon. Therefore, the cloned α-tubulin-4 gene contains 1156 bp of 5′ upstream region. A TATA-box consensus sequence (Bucher and Trifonov, 1986) is present 30 bp upstream of the transcription start point. The gene contains one intron (359 bp, from +103 to +461) present between the first amino acid coding codon (the 'start'-ATG) and the rest of the amino acid coding part of the gene. Taking into account some minor deviations, the splice donor and splice acceptor site of the intron conform to the consensus sequences for these sites as defined by Mount (1982).

Two copies of a direct repeat (96-98 bp; 90% identical) are present in the 5′ upstream part of the gene (from –501 to –406 and from –404 to –307). Two copies of another direct repeat (130-138 bp) are present in the intron (from +180 to +309 and from +313 to +450). The copies of this repeat are 84% identical,

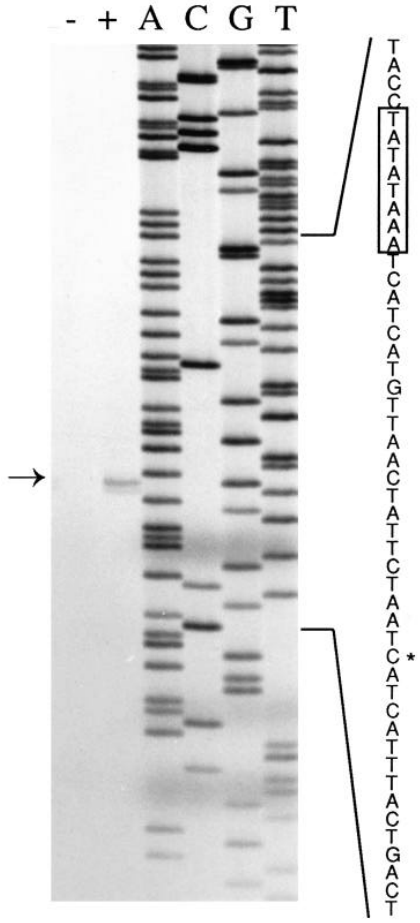

Fig. 3. Determination of the transcription initiation site of the α-tubulin 4 gene of *Patella vulgata* by primer extension analysis. The primer extension analysis was carried out as described in the Materials and Methods section; the (+) lane shows the extended products when the 23-mer oligonucleotide primer was used, the (–) lane shows the control reaction where the primer was substituted by H_2O. The arrow indicates the most prominent reaction product. The size of the primer extended products was determined by running the products of a sequencing reaction with the same primer next to the primer extension products. The sequence of the sense strand is shown next to the gel and the nucleotide corresponding to major reaction product in the primer extension reaction (+) is marked with an asterisk; the putative TATA-box-sequence is boxed.

the first 45 bp being 100% identical. There are several polyadenylation sites present in the 3′ part of the sequence, downstream of the stop codon, among them a stretch of 6 sites (from +2213 to +2143). Which of these signals is used is not known.

The deduced protein sequence of the α-tubulin-4 gene is 100% identical to the protein sequence deduced from the *P. vulgata* α-tubulin-2 cDNA as described by van Loon et al. (1994). At the nucleotide level, there are seven differences in the protein-encoding parts which do not affect the deduced protein sequence. The probably incomplete 5′ UTR (12 bp) and the probably incomplete 3′ UTR (106 bp) of the α-tubulin-2 cDNA match 100% with their corresponding sequences in the genomic α-tubulin-4 clone. This suggests that the α-tubulin-2 cDNA is a cDNA derived from a mRNA transcribed from the α-tubulin-4 gene. The occurrence of seven differences at the nucleotide level may be due to differences between individuals

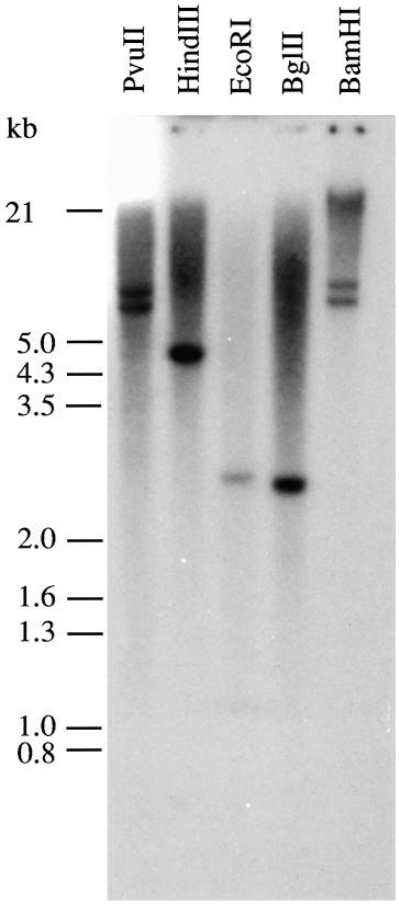

Fig. 4. Genomic analysis of the α-tubulin-4 gene. A Southern blot of genomic DNA that was digested with *Bam*HI, *Bgl*II, *Hin*dIII, *Eco*RI and *Pvu*II was probed with a fragment just downstream of the coding region of the α-tubulin-4 gene (446 b.p. *Mbo*II-*Taq*I fragment, from +1818 to +2263). The genomic DNA used in this Southern blot was from the same male as used for constructing the genomic library. This revealed the following hybridizing bands: *Pvu*II 5.7 kb and 6.2 kb; *Hin*dIII 4.6 kb; *Eco*RI: 2.6 kb; *Bgl*II: 2.5 kb and *Bam*HI: 5.9 kb and 6.4 kb.

used for the construction of the cDNA library and the genomic library, or to the existence of different alleles of this gene.

In a genomic analysis, DNA from the same individual used for the genomic library was digested with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, or *Pvu*II. Fragments were separated on a 0.6% agarose gel and a Southern blot was prepared. The DNA from one individual was used because the population from which the animals were collected is genetically heterogeneous, resulting in restriction polymorphism between different individuals (A. E. van Loon, unpublished data). The blot was probed with a 446 bp *Mbo*II-*Taq*I fragment located in the 3′ non-coding part (+1818 to +2263), directly downstream of the coding region of the gene (Fig. 4). In the *Bam*HI and *Pvu*II digest two bands were detected, in the *Bgl*II, *Eco*RI and *Hin*dIII digest one band was detected. The 4.6 kb band in the *Hin*dIII digest corresponds to the α-tubulin-4 4.6 kb *Hin*dIII fragment containing the 3′ part of the gene. The two bands in the *Bam*HI and *Pvu*II digests are possibly the result of a heterozygotic allele. If so, the α-tubulin-4 gene is a single copy gene, otherwise it is present in two copies.

Applicability of the *lacZ* reporter gene in *Patella* embryos

To monitor cell type-specific gene expression during trochoblast differentiation we wanted to use the *lacZ* reporter gene in the pNL vector (Gan et al., 1990). We therefore tested the embryos for endogenous β-galactosidase activity, intrinsic β-galactosidase activity of the pNL vector, stability of the β-galactosidase enzyme in the embryo, and the permeability of the fixed embryo for X-gal, the substrate for the β-galactosidase enzyme. Control embryos fixed and stained with X-gal do not show any detectable endogenous β-galactosidase activity. Nor do embryos that were injected at the 2-cell stage with pNL vector DNA, showing that there is no detectable β-galactosidase activity caused by the pNL DNA (Fig. 5B). Injection of *E. coli* β-galactosidase protein into one blastomere of a 2-cell stage embryo shows that the protein is stable and can be detected in all progeny of the injected cell (Fig. 5A). The progeny of the non-injected cell shows no β-galactosidase activity. Thus, the protein is stable in the embryo and the whole embryo fixed in 0.2% glutaraldehyde is accessible for the X-gal substrate.

The spatiotemporal expression pattern of the *lacZ* reporter gene under control of the regulatory region of the α-tubulin-4 gene

To monitor cell type specific gene expression, a 1660 bp fragment of the 5′ part of the α-tubulin-4 gene was fused to the *lacZ* reporter gene in the pNL vector (Gan et al., 1990) (Fig. 6). This α-tubulin-4::*lacZ* fusion gene, denominated pNL-α4-04, contains the 5′ upstream sequences (1156 bp), the first exon (5′ UTR and ATG-codon), the intron, and a small part of the second exon. The *lacZ* reporter gene in the pNL vector contains a SV40 T-antigen nuclear targeting signal and the SV40 T-antigen splice sites/poly(A) consensus sequence (Fig. 6A). Thus, in the pNL-α4-04 fusion gene the *lacZ* reporter gene is placed under control of the 5′ upstream elements of the α-tubulin-4 gene.

As fertilization rates of *Patella* oocytes may vary (from 1% - 95%), only 2-cell stage embryos were microinjected with the

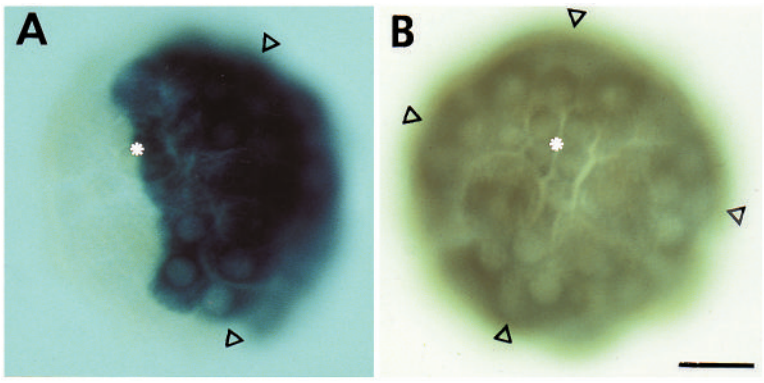

Fig. 5. Staining for β-galactosidase activity in control embryos. (A) A 64-cell stage embryo (animal view; 4 hour 30 minutes after first cleavage) that was injected with *E. coli* β-galactosidase into one of the blastomeres of the two-cell stage, shows β-galactosidase activity in all progeny of the injected cell. No counter staining with gallocyanin was done for this embryo, causing the part of the embryo that arises from the non-injected blastomere to be almost invisible after clearing the embryo in methylsalicate. (B) A 64-cell stage embryo (4 hour 30 minutes after first cleavage) injected with linearized (*Hin*dIII cut) pNL DNA at the two-cell stage shows no detectable β-galactosidase activity. 101 injected embryos were analyzed. In none of the embryos was β-galactosidase activity found. The asterisks indicate the animal pole of the embryos, the arrowheads point at the quartets of primary trochoblasts. Scale bar, 40 μm.

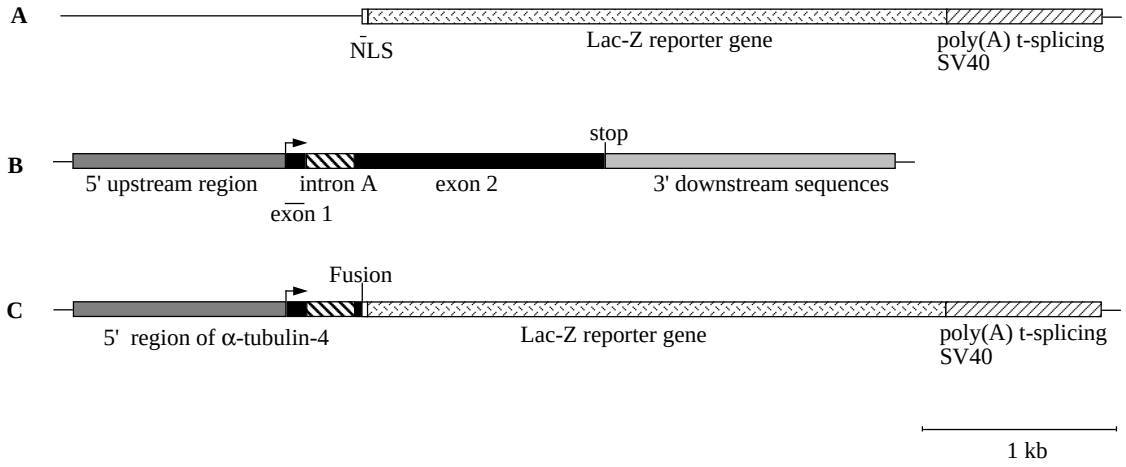

Fig. 6. Schematical representation of the construction of the α-tubulin-4::*lacZ* fusion gene. (A) The *lacZ* reporter gene as present in the pNL vector (Gan et al., 1990). (B) A schematic representation of the α-tubulin-4 gene of *Patella vulgata*. (C) Fusion of the 5′ part of the α-tubulin-4 gene to the *lacZ* reporter gene. For exact construction of the fusion gene, see text.

linearized pNL-α4-04 fusion gene DNA. The fluorescent dye Lucifer Yellow (LY) was coinjected with the DNA as a marker for injection. In a number of embryos LY, and thus DNA, spread to the non-injected blastomere, presumably via cytoplasmic bridges. Therefore, in these embryos, expression of the fusion gene could occur in only one half of the embryo, or throughout the whole embryo.

Microinjected embryos were reared to the desired stage, fixed, and assayed for β-galactosidase activity. Depending upon the stage at which the embryos were fixed, β-galactosidase activity was found in one or more trochoblasts in up to 31% of the embryos. In these embryos, the fusion gene was expressed in a varying number of trochoblasts. This is probably the result of a mosaic distribution of the microinjected DNA, as was also found in sea urchin (Gan et al., 1990; Hough-Evans et al., 1987, 1988).

After microinjecting the fusion gene DNA, β-galactosidase activity was detected for the first time at the 40-cell stage (3 hours 30 minutes after first cleavage) (Table 1). In these embryos a weak staining was detected only in some of the primary trochoblasts in two out of 126 injected embryos (1.6%). At the 60-cell stage (4 hours after first cleavage) β-galactosidase activity was only detected in one or more primary trochoblast cells in 10.5% of the injected embryos, whereas at the 64-cell stage (4 hours 30 minutes after first cleavage) stained primary trochoblast cells were observed in 24.3% of the embryos (Fig. 7A,B). In all these embryos (40-, 60-, and 64-cell stage) β-galactosidase activity was only detected in a varying number of primary trochoblasts (the $1a^{211}$-$1d^{211}$, $1a^{212}$-$1d^{212}$, $1a^{221}$-$1d^{221}$, and $1a^{222}$-$1d^{222}$ cells). In 43 of 381 injected embryos, one or more primary trochoblasts appeared to be positive. These 43 embryos contain a total of 43×16=688 primary trochoblasts. In 111 of these primary trochoblasts β-galactosidase activity was detected (Table 1). Prior to the 64-cell stage, β-galactosidase activity was never found in any cell other than a primary trochoblast, neither in a accessory or secondary trochoblast, nor in any other cell type.

In embryos that were injected with the fusion gene DNA at the 2-cell stage and fixed at 6 hours 45 minutes after first cleavage (a stage beyond the 88-cell stage of which the exact cell number could not be determined), β-galactosidase activity was not only present in primary trochoblasts but also in accessory and secondary trochoblasts (Fig. 7C-F, and Table 1). In 31.1% of the embryos (38 of 122 injected embryos) expression of the fusion gene was found in a varying number of trochoblasts. In these 38 embryos, a total of 51 primary, 18 accessory, and 10 secondary trochoblasts expressed the fusion gene. Also at this stage, no expression of the fusion gene has ever been detected in any other cell type.

From these experiments we conclude that the α- tubulin-4 gene is a gene that is expressed specifically in the trochoblasts. Thus, the sequences from −1156 to +487 in the 5′ part of the α-tubulin-4 gene are sufficient for a correct spatiotemporal expression in the primary, accessory, and secondary trochoblasts.

Table 1. Spatiotemporal expression of the *lacZ* reporter gene under control of the α-tubulin-4 promoter in *Patella vulgata* embryos

Stage (no. of cells)	Embryos scored	Embryos positive	(%) positive	Number of positive Trochoblasts			Other cells
				Primary	Accessory	Secondary	
40	126	2	1.6	6	0	0	0
60	152	16	10.5	34	0	0	0
64	103	25	24.3	71	0	0	0
$6h^{45}$	122	38	31.1	51	18	10	0
Total	503	81	-	162	18	10	0

The number and percentage of embryos expressing ('positive') the α-tubulin-4::*lac-Z* fusion gene in one or more cells at the different stages of development are indicated as well as the number of cells of the different cell types that are stained. $6h^{45}$: embryos analyzed at 6 hour 45 minutes after first cleavage, this is a stage beyond the 88-cell stage of which the exact cell number is not known. 40-cell stage: 3 hours 30 minutes after first cleavage; 60-cell stage: 4 hours after first cleavage, 64-cell stage: 4 hours 30 minutes after first cleavage.

DISCUSSION

Tubulin expression: a marker for trochoblast differentiation

In this study we analyzed the spatiotemporal expression of tubulin genes during the early development of the marine gastropod *P. vulgata.* Expression of (at least some of) the tubulin genes was detected in the three different cell-lines from which the prototroch arises. We showed that in the primary trochoblasts already at the 32-cell stage, i.e. before their last division, a considerable expression of both α- and β-tubulin genes could be detected. Other differentiation characteristics, such as the formation of cilia and a band of F-actin, first appear after their cleavage arrest (Serras and Speksnijder, 1991; Damen and Dictus, 1994a). Later in development, the accessory and secondary trochoblasts also express high levels of tubulin mRNAs. Apart from the different categories of trochoblasts, some other cells have been found to express elevated levels of tubulin mRNA: the ciliated cells of the apical tuft and the refractive bodies, and at the 88-cell stage the mesentoblast 4d.

The α- and β-tubulin subunits are both necessary for the formation of the tubulin heterodimers that polymerized into microtubules and constitute the major compound of cilia (Witman, 1990). Therefore it is not surprising that the spatiotemporal expression patterns for α- and β-tubulin genes are identical.

Tubulin expression in the mesodermal stem cell 4d

An explanation for the tubulin gene expression in the mesodermal stem cell 4d at the 88-cell stage is still lacking. An inductive interaction between the animal micromeres determines the 3D macromere, the founder cell of 4d (van den Biggelaar and Guerrier, 1979; Kühtreiber et al., 1988; Kühtreiber and Van Dongen, 1989). Serras and Speksnijder (1990) showed that the 3D macromere contains more F-actin than the other macromeres. After division of 3D only one of the daughter cells, 4D, inherits this high F-actin density. The other daughter cell, the mesodermal stem cell 4d, expresses tubulin mRNAs. Activation of the tubulin gene expression in 4d is likely the result of inductive processes.

Expression of the α-tubulin-4::*lacZ* fusion gene is restricted to trochoblasts

The early and strong expression of tubulin genes in the trochoblasts makes them an ideal marker to study the molecular mechanisms that underlie processes of early specification via differential distribution of morphogenic compounds and differentiation processes resulting from this distribution. As a first step to unravel these molecular mechanisms the 5′ part of one of the tubulin genes was fused to a *lacZ* reporter gene.

The *lacZ* reporter gene in the pNL vector is particularly well-suited for analyzing the spatiotemporal aspects of gene expression in *Patella* embryos. In combination with gallocyanin staining it is possible to identify exactly the cells that express the fusion gene. The α-tubulin-4::*lacZ* fusion gene is only activated in the different trochoblast cell lines. The possibility that the expression of the α-tubulin-4::*lacZ* fusion gene in the trochoblasts is the result of a high transcriptional activity in these early differentiating cells, can be ruled out. The expression of a fusion gene in which a β-tubulin promoter was fused to the *lacZ* reporter gene was not restricted to the trochoblasts (unpublished results, W. G. M. Damen). Therefore a putative high transcriptional activity did not result in trochoblast-specific expression of this construct and this β-tubulin promoter is thus regulated via a mechanism distinct from the one controlling the expression of the α-tubulin promoter. This is understandable as we do not expect that all of the 20 α- and β-tubulin genes (at least, A. E. van Loon, unpublished results) present in the genome of *Patella vulgata* to be expressed in the trochoblasts.

Thus, the 5′ part of the α-tubulin-4 gene (from −1156 to +487) contains all the elements required for a correct spatiotemporal expression of the α-tubulin-4::*lacZ* fusion gene. This implies that any cis-acting element that might be responsible for the expression of the α-tubulin-4 gene in the trochoblasts must be localized in this region. Identification of these regulatory sites should open up possibilities for identifying the regulatory molecules for the α-tubulin-4 gene expression. These determinants are supposed to be differentially segregated to the primary trochoblasts and probably also to the accessory and secondary trochoblasts. Experiments of Janssen-Dommerholdt et al. (1983) in which the cytokinesis but not karyokinesis was inhibited show that the morphogenic determinants for ciliation of the primary trochoblasts are initially segregated to the first quartet micromeres at the third cleavage, and, finally, during the fourth cleavage to the primary trochoblasts.

Thus, the regulation of the α-tubulin-4 gene expression in the trochoblasts may be regarded as a model system for the analysis of the relation between a prepattern of presumably maternal regulatory molecules and the spatiotemporal expression pattern of the embryonic genome.

A comparison of the results of the fusion gene injections with the in situ hybridization experiments reveals a seeming discrepancy between the two results from the temporal point of view. β-galactosidase, the protein-product of the *lacZ* reporter gene under control of the α-tubulin-4 regulatory sequences, could be detected for the first time about 1 hour after the first appearance of α-tubulin mRNAs in the primary trochoblasts. An explanation for this seeming discrepancy could lie in the different levels of gene expression that were assayed. In the in situ hybridizations the cells were screened for the presence of the transcription product, the tubulin mRNA, whereas in the fusion gene experiments the protein products of the reporter gene were visualized. The delay in expression of β-galactosidase may be due to the fact that the transcripts of the fusion gene have to be processed and translated before enzyme activity can be measured. Another possibility, might be that the α-tubulin-4 gene is one of the later genes of the α-tubulin gene family to be expressed in the primary trochoblasts.

Trochoblasts differentiation and cleavage arrest

Serras et al. (1990) assumed that the trochoblasts follow a characteristic sequence of events: (1) cleavage arrest, (2) reduction of gap junctional communication and (3) differentiation into ciliated cells. Our results show clearly that cleavage arrest does not precede the (initial) differentiation of the trochoblasts into ciliated cells, as the tubulin mRNA expression starts before cleavage arrest. Primary trochoblasts already express tubulin genes at the 32-cell stage prior to their last division at the transition of the 32-cell stage to the 40-cell stage. Primary tro-

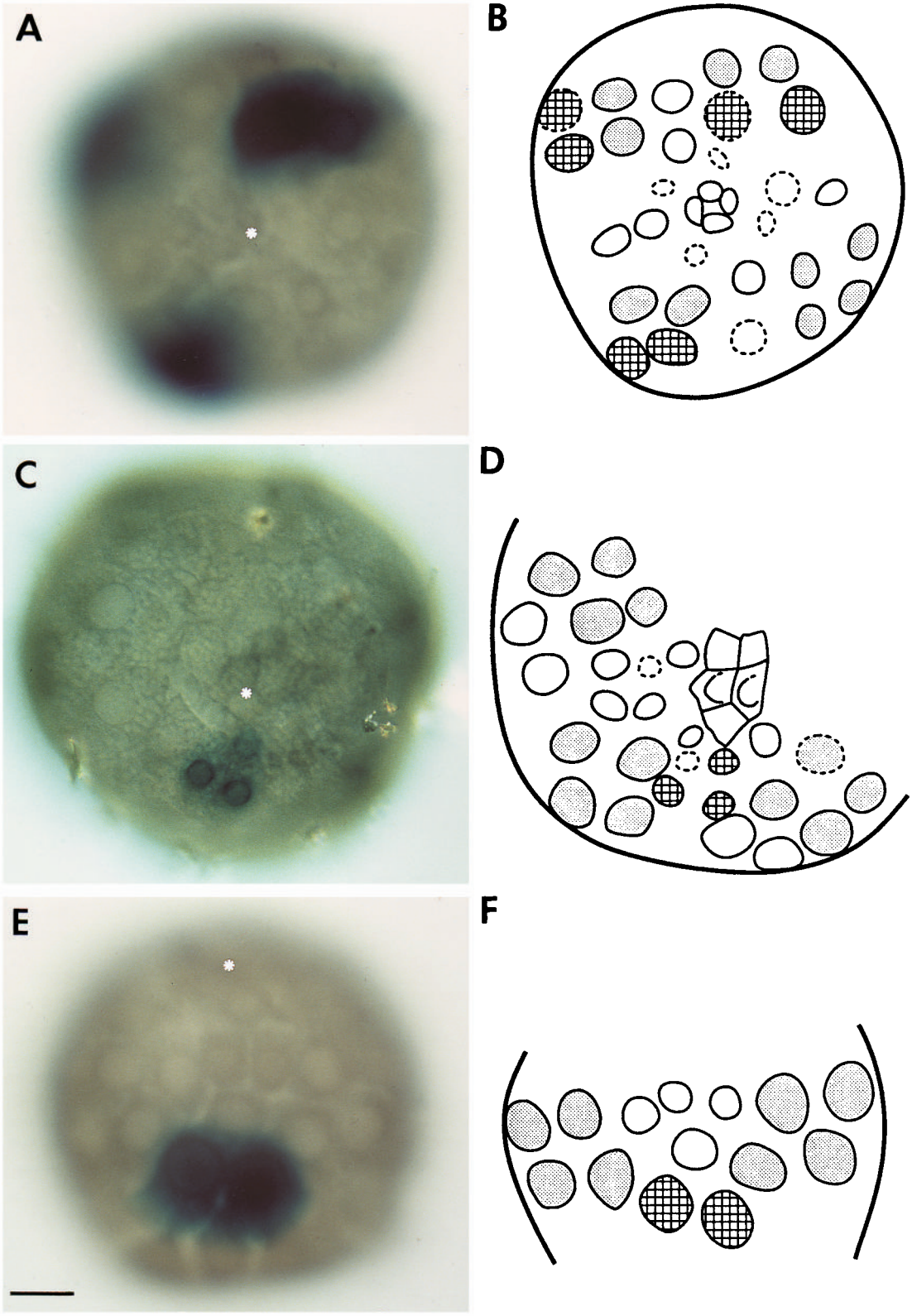

Fig. 7. Trochoblast-specific expression of the *lac-Z* reporter gene under control of the α-tubulin-4 promoter. Embryos were injected with the fusion gene pNL-α4-04 in one or both blastomeres at the two cell stage. They were analyzed for β-galactosidase activity at the desired stage. In order to identify the labeled cells, a gallocyanin staining was performed. The asterisks indicate the animal pole of the embryos. Scale bar, 30 μm. (A) Animal view of a 64-cell stage embryo (4 hours and 30 minutes after first cleavage) expressing the fusion gene in six primary trochoblasts in three quadrants. (B) Drawing of the animal side of this embryo, showing the position of the nuclei. The drawing is based on several optical sections of the embryo. Nuclei of primary trochoblasts are indicated by the stippled pattern, nuclei of the blue-stained primary trochoblasts are indicated by the cross-hatched pattern. (C) Animal view of an embryo of 6 hours and 45 minutes after first cleavage expressing the fusion gene in three accessory trochoblasts in the D-quadrant ($1d^{1212}$, $1d^{1221}$ and $1d^{1222}$). (D) Drawing of a part of the animal side of the embryo. Nuclei of primary trochoblasts are indicated by stippled pattern, nuclei of blue accessory trochoblasts are indicated by the cross-hatched pattern. (E) Lateral view of an embryo of 6 hours and 45 minutes after first cleavage, expressing the fusion gene in two secondary trochoblasts in the A-quadrant ($2a^{111}$ and $2a^{112}$). (F) Drawing of a part of this embryo, showing the position of the nuclei of the trochoblasts. Nuclei of primary trochoblasts are indicated by stippled pattern, nuclei of positive secondary trochoblasts are indicated by the cross-hatched pattern.

choblasts isolated from a 16-cell stage embryo differentiate as in the whole embryo: they divide two more times, become cleavage-arrested, and ciliate (Wilson, 1904). It remains to be determined whether tubulin gene expression and cleavage arrest of the trochoblasts are regulated by the same or different determinants.

Trochoblast differentiation in animals with spiral cleavage

Animals with spiral cleavage, like mollusks and annelids, form trochophores or trochophore-like larva. In the formation of the prototroch, primary, accessory and secondary trochoblasts are involved. The number of trochoblasts depends on the species but in all species at least the primary trochoblasts are formed. Therefore, we assume that during evolution the mechanism for the specification and the differentiation of these cells is conserved. This assumption is strengthened by the observations of Dorresteijn et al. (1993) on embryos of the annelid *Platynereis dumerillii*. They found expression of a protein in ciliated cells that was recognized by the monoclonal antibody 4D9. The antibody gave a strong reaction with the ciliated prototroch cells and a weak reaction with the ciliated apical tuft cells. This 4D9 antibody was raised against the engrailed protein of *Drosophila* (Patel et al., 1989). In the 5′ region of the α-tubulin-4 gene indeed some elements were detected (at –291, –192, –152, –92, and a number in the intron sequence) which show some resemblance to a binding site consensus sequence for engrailed (Desplan et al., 1988). It remains to be investigated whether these elements are indeed the cis-acting elements that play a role in activation of the tubulin genes.

As a conservative mechanism is supposed to act in the molecular specification of the trochoblasts in the different species with a spiral cleavage, it is likely that this is also the case for the regulatory molecules and the cis-acting elements of the genes that are activated during differentiation. The α-tubulin-4::*lacZ* fusion gene can be used as a tool for the analysis of prototroch formation in representatives of different phyla and classes.

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REFERENCES

Bucher, P. and Trifonov, E. N. (1986). Compilation and analysis of eukaryotic POL II promoter sequences. *Nucl. Acids Res.* **14**, 10009-10026.

Damen, P. and Dictus, W. J. A. G. (1994a). Cell-lineage analysis of the prototroch of the gastropod mollusc *Patella vulgata* shows conditional specification of some trochoblasts. *Roux's Arch. Dev. Biol.* **203**, 187-198.

Damen, P. and Dictus, W. J. A. G. (1994b). Cell-lineage of the prototroch of *Patella vulgata* (Gastropoda, Mollusca). *Dev. Biol.* **162**, 364-383.

Desplan, C., Theis, J. and O'Farrell, P. H. (1988). The sequence specificity of homeodomain-DNA interaction. *Cell* **54**, 1081-1090.

Dorresteijn, A. W. C., O'Grady, B., Fischer, A., Porchet-Henneré, E. and Boilly-Marer, Y. (1993). Molecular specification of cell lines in the embryo of *Platynareis*. (Annelida). *Roux's Arch Dev. Biol.* **202**, 260-269.

Frischauf, A.-M., Lehrach, H., Poustka, A. and Murray, N. (1983). Lambda replacement vectors carrying polylinker sequences. *J.Mol. Biol.* **170**, 827-842.

Gan, L., Wessel G. M. and Klein W. H. (1990). Regulatory elements from the related Spec genes of *Strongylocentrotus purpuratus* yield different spatial patterns with a *lacZ* reporter gene. *Dev. Biol.* **142**, 346-359.

Heath, H. (1899). The early development of *Ischnochiton*. *Zool. ahrb. Abt. Ontog. Tiere* **12**, 567-656.

Hough-Evans, B. R., Britten, R. J. and Davidson, E. H. (1988). Mosaic incorporation and regulated expression of an exogenous gene in the sea urc*Hin* embryo. *Dev. Biol.* **129**, 198-208.

Hough-Evans, B. R., Franks, R. R., Cameron, R. A., Britten, R. J. and Davidson, E. H. (1987). Correct cell-type-specific expression of a fusion gene injected into sea urchin eggs. *Dev. Biol.* **121**, 576-579.

Janssen-Dommerholt, C., Van Wijk, R. and Geilenkirchen, W. L. M. (1983). Restriction of developmental potential and trochoblast ciliation in *Patella* embryos. *J. Embryol. exp. Morph.* **74**, 69-77.

Kühtreiber, W. M. and Van Dongen C. A. M. (1989). Microinjection of lectins, hyaluronidase, and hyaluronate fragments interferes with cleavage delay in mesoderm induction in embryos of *Patella vulgata*. *Dev. Biol.* **132**, 436-441.

Kühtreiber, W. M., Van Til E. H. and Van Dongen C. A. M. (1988). Monensin interferes with the determination if the mesodermal cell line in embryos of *Patella vulgata. Roux's Arch Dev. Biol.* **197**, 10-18.

Mount, S. M. (1982). A catalogue of splice junction sequences. *Nuc. Acids Res.* **10**, 459- 472

Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B. and Goodman, C. S. (1989). Expression of *engrailed* proteins in Arthropods, Annelids, and Chordates. *Cell* **58**, 955-968.

Sanger, F., Nicklen, S. and Coulson A. R. (1977). DNA-sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.

Serras, F., Dictus, W. J. A. G. and Van den Biggelaar J. A. M. (1990). Changes in junctional communication associated with cell cycle arrest and differentiation of trochoblasts in embryos of *Patella vulgata. Dev. Biol.* **137**, 207-216.

Serras, F. and Speksnijder J. E. (1990). F-actin is a marker of dorsal induction in early *Patella* embryos. *Roux's Arch. Dev. Biol.* **199**, 246-250.

Serras, F. and Speksnijder J. E. (1991). F-actin localization during trochoblast differentiation in *Patella vulgata* embryos. *Development* **112**, 833-845.

Smith F. G. W. (1935) The development of *Patella vulgata. Phil. Trans. R. Soc. B.* **225**, 95-125

Tautz, D. and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**, 81-85.

Van den Biggelaar, J. A. M. (1977). Development of dorsoventral polarity and mesentoblast determination in *Patella vulgata. J. Morph.* **154**, 157-186.

Van den Biggelaar, J. A. M. and Guerrier P. (1979). Dorsoventral polarity and mesentoblast determination as concomitant results of cellular interactions in the mollusk *Patella vulgata. Dev. Biol.* **68**, 462-471.

van Loon, A. E., Goedemans, H. J., Daemen, A. J. J. M., Van de Kamp, A. J. and Van den Biggelaar, J. A. M. (1993). Actin genes expressed during early development of *Patella vulgata. Roux's Arch. Dev. Biol.* **202**, 77-84.

van Loon, A. E., Goedemans, H. J., Weijtens, M. E. M. and Daemen, A. J. J. M. (1994). Onset of transcription in *Patella vulgata* coincides with cell cycle elongation and expression of tubulin genes. *Roux's Arch. Dev. Biol.* (in press).

Wilson, E. B. (1904). Experimental studies in germinal localization: II. Experiments on the cleavage-mosaic in *Patella* and *Dentalium*. *J. Exp Zool.* **1**, 197-268.

Witman, G. B. (1990). Introduction to cilia and flagella. In *Ciliary and Flagellar Membranes* (ed R. A. Bloodgood), pp. 1-30. New York and London: Plenum Press.

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