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TECHNICAL NOTE

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A zebrafish histone variant H2A.F/Z and a transgenic H2A.F/Z:GFP fusion protein for in vivo studies of embryonic development

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Abstract We have generated transgenic zebrafish lines expressing a fusion of a histone variant, H2A.F/Z, to the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria*. Here, we describe the molecular cloning, partial characterisation and expression of the zebrafish *H2A.F/Z* histone gene, as well as the construction of the transgene and its transformation into the zebrafish germ line. No abnormality can be detected in transgenic fish expressing the H2A.F/Z:GFP fusion protein. The nuclear localisation of the fusion protein correlates with the start of zygotic transcription, in that it is present in the unfertilised egg and in the cytoplasm of cells after the first cleavages, being found in some nuclei after the seventh or eighth cleavage, whereas all nuclei from the 1,000-cell stage on, i.e. after midblastula transition, contain protein. In addition to these data, we present a few examples of the many possible applications of this transgenic line for developmental studies in vivo. Electronic supplementary material to this paper can be obtained by using the Springer LINK server located at http://dx.doi.org/10.1007/s00427-001-0196-x

Keywords Zebrafish · *Histone2A.F/Z:GFP* · Transgenesis · In vivo marker

Introduction

The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* is especially well suited for in vivo observations (Chalfie et al. 1994) as it has repeatedly proven to be innocuous for living cells, and in the course of the last few years GFP has become the marker of choice for analysing developing cells. The protein can be used to analyse the behaviour of cells in a large variety of developmental contexts. Thus, cell movements, like those that take place during gastrulation and when cells leave the neural crest, or cell growth and differentiation, for example pathfinding by axonal growth cones, can be followed, and qualitative and quantitative aspects of the pattern of cell divisions can be assessed. In addition to its use for in vivo observations, in studies with some organisms, e.g. the zebrafish (Amsterdam et al. 1995), *gfp* can be used in place of *lacZ* as a reporter gene while dissecting the regulatory region of genes to identify enhancer elements which drive specific gene expression temporarily and spatially (e.g. Meng et al. 1997; Higashijima et al. 2000).

Particularly useful for observations of developing cells in vivo is the fusion of *gfp* to a gene expressed in all cells of the body from early stages of development on. For the purpose of analysing mitotic patterns, the labelling of chromosomes with protein components of chromatin, e.g. histones, has been used several times. Sullivan et al. (1990) microinjected labelled histones and Kanda et al. (1998) used a plasmid encoding a histone-GFP fusion. In both cases, however, microinjection was used. An important disadvantage of microinjection is the inability to label all cells. A way of overcoming this drawback of microinjection would be to use a transgene stably inserted into the germ line.

Recently, Clarkson and Saint (1999) published the generation of a transgenic strain of *Drosophila*, which carries a fusion of the *gfp*-coding sequences to a histone gene of the H2A.F/Z class, *His2AvD*, as a marker for chromosomal studies. The *His2AvD* gene is particularly well suited for this class of studies, as the histone it encodes, although expressed in all cells, makes up only 5–15% of all H2A protein in a cell. Therefore, expression of the histone-GFP fusion protein should not affect cell physiology in any important manner. In fact, Clarkson and Saint (1999) demonstrated its innocuity by

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showing that the fusion protein can restore the gene function in the background of a mutation of the *His2AvD* gene.

Prompted by the observations in *Drosophila*, we have established transgenic lines of zebrafish carrying stable insertions of the zebrafish *H2A.F/Z* gene in fusion with the GFP-encoding sequence. Here, we describe cloning and organisation of the zebrafish homologue, germ-line transformation, and examples of a few of the many possible applications of these transgenic strains in the analysis of embryonic development of the zebrafish.

Materials and methods

Zebrafish embryos were obtained from spontaneous spawnings. Adult fish were kept at 28.5°C on a 14-h light/10-h dark cycle. The embryos were staged according to Kimmel et al. (1995).

Molecular cloning of *H2A.F/Z*

PCR using degenerate primers was performed on a cDNA library. The primers were designed using human, mouse, rat, bovine, rabbit, chicken and *Xenopus* sequences obtained from the *Entrez*-Browser (http://www.ncbi.nlm.nih.gov/Entrez) of the National Center for Biotechnology Information. The primers used were 5′ ATG GCW GGY GGY AAG GCT GGV AAR GA and 3′ TTG TGG ATG TGK GGR ATG ACA CCW CCM CCM GCW AT. The amplified PCR product was cloned by means of the *TOPO TA* Cloning Kits (Invitrogen), and used to screen a cDNA library prepared in λZAP (Stratagene) from 3- to 15-h zebrafish embryos (gift from C. Fromental-Ramain and P. Chambon, Strasbourg). The cDNA clones obtained were then used to screen the zebrafish genomic library "Easy-to-handle eukaryotic genomic libraries" (Mo Bi Tec, Göttingen). The accession number for the *H2A.F/Z* sequence is AF414110.

The generation of the plasmid encoding the H2AF/Z:GFP fusion protein is described in the Results section. DNA preparation and microinjection was according to Scheer and Campos-Ortega (1999). DNA fragments for injection were obtained by digesting plasmids with *Sst*II, which excises the insert fragment from the vector. Fragments were separated by electrophoresis on an agarose gel, recovered from the gel using the JETSORB Gel Extraction Kit (Genomed) and dissolved in distilled water. Approximately 5 nl DNA solution (about 50 ng/µl) containing 0.2% phenol red was injected into the cytoplasm of 1-cell zebrafish embryos. Injected, putative founder fish (G0) were crossed inter se and their progeny (F1) were screened with a fluorescence stereomicroscope [Leica-Stereomikroskop (*MZ FLIII*)] for GFP-mediated signals and by PCR. PCR conditions are described in Scheer and Campos-Ortega (1999). Animals which scored positive were raised to adulthood and crossed to wild type.

In situ hybridisation and histological methods

Hybridization of digoxigenin-labelled RNA probes to embryo whole-mounts was performed as described by Bierkamp and Campos-Ortega (1993). Digoxigenin-labelled probes were prepared using RNA labelling kits (Boehringer Mannheim). Anti-GFP antibody staining was according to Westerfield (1994). Confocal laser scanning microscopy was on a Zeiss LSM 400. Embryos of appropriate age were anaesthetised with MS222, immobilised and oriented in agarose; time lapse movies were made using a ×40 oil immersion objective on an upright Axiovert microscope.

Results and discussion

Cloning of a zebrafish *H2A.F/Z* histone variant

Using degenerate primers, a 347-bp DNA fragment was amplified by PCR. The sequence of this fragment showed great similarity to histone genes of the *H2A.F/Z* subfamily, an evolutionarily highly conserved representative of the H2A class (Thatcher and Gorovsky 1994; Jiang et al. 1998), already cloned for several other animal species, with the highest similarity to parts of the chicken *H2A.F* (Harvey et al. 1983) and human *H2A.Z* (Groitl et al. 1998). The amplified PCR fragment was used to isolate a full-size cDNA from a cDNA library. Four clones, IVE13, IVE14, IVE15 and IVE16, were isolated and sequenced. All four were found to contain an open reading frame comprising the entire coding sequence with a size of 384 bp. Two of the clones, IVE15 and IVE16, had a length of about 900 bp and 950 bp, respectively, and contained a polyA addition signal and the complete 3′UTR.

The encoding protein exhibits all features characteristic of members of the H2A.F/Z subfamily, such as the H2A signature sequences RAGLQFPVGR (Wu et al. 1986) from position 23 to 32 and LEYLTAEVLELAGNA (Jiang et al. 1998) from position 59 to 73. Sequence identity (Table 1) ranges from 100% (human and chicken) to 74% (yeast; see Table 1). In fact, the sequence of the predicted zebrafish protein is almost identical to that of members of the *H2A.F/Z* subfamily from other vertebrates, with the exception of *H2A.Zl* from *Xenopus laevis*, which was published as "H2A.Z-like" (Iuozalen et al. 1996). The coding regions of the human (Groitl et al. 1998), chicken and zebrafish genes show sequence identity of about 80% over a stretch of approximately 92% of the entire DNA coding region. The 5′ and 3′ untranslated regions of zebrafish *H2A.F/Z*, as well as the polyA tail, exhibit the same features characteristic for a number of subfamily members from mammals (Hatch and Bonner 1988). Even the H2A.F/Z proteins of invertebrates like *Drosophila* (van Daal et al. 1988; Clarkson et al. 1999) or the sea urchin (Ernst et al. 1987) are very similar to the zebrafish protein. The phylogenetic conservation of the H2A.F/Z subfamily suggests an important function for its members and, indeed, lack of the gene results in lethality in the case of *Drosophila* (van Daal and Elgin 1992) and in *Tetrahymena thermophila* (Liu et al. 1996).

The H2A.F/Z promoter

In order to express a H2A.F/Z:GFP fusion protein ubiquitously, we decided to use the promoter of the *H2A.F/Z* gene. To characterise the promoter region of the zebrafish *H2A.F/Z* gene, a dig-probe of IVE13 was used to isolate genomic fragments from a genomic DNA library. Eight fragments containing parts of the *H2A.F/Z* gene were isolated (IVE2 to IVE9). All eight fragments have a

Table 1 A comparison of the complete zebrafish H2A.F/Z protein sequence of 128 amino acids, to that of family members from other species. Alignment was with MegAlign Program from the DNA-Star Pack, Clustal method

Species	No. of residues	Identical residues	Identity (%)
Human 1 (Hatch and Bonner 1988)	128	125	98
Human 2 (Groitl et al. 1998)	128	128	100
Chicken (Harvey et al. 1983)	128	128	100
Mouse (Rocha et al. 1996)	128	125	98
Rat (Hatch and Bonner 1988)	128	125	98
Bovine (Hatch and Bonner 1988)	128	125	98
Rabbit (Jiang et al. 1998)	123	117	91
Drosophila (van Daal et al. 1988)	141	121	96
Sea urchin (Ernst et al. 1987)	125	117	91
Xenopus (Iouzalen et al. 1996)	128	115	90
Caenorhabditis elegans (*Caenorhabditis elegans* Sequencing Consortium 1998)	140	111	87
Arabidopsis (Callard and Mazzolini 1997)	136	100	78
Tetrahymena (White et al. 1988)	138	90	70
Saccharomyces pombe (Carr et al. 1994)	171	87	68
Saccharomyces cerevisiae (Jackson et al. 1997)	134	87	68

size of about 17 kb, and all have an identical 5′ region, but none contained the entire 3′ UTR. However, no attempt was made to complete the gene, as the 3′ UTR was dispensable for the synthesis of the H2A.F/Z:GFP fusion protein.

The genomic structure of the zebrafish *H2A.F/Z* gene was eventually deduced from clone IVE8. In zebrafish, *H2A.F/Z* has a size of about 5.2 kb and at least five exons. The human (Hatch and Bonner 1990) and chicken (Dalton et al. 1989) *H2A.F/Z* genes have five exons; the first three introns are at the same position as those of the zebrafish homologue. However, since the 3′ UTR was missing from our genomic clones, we could not establish whether the zebrafish gene has more than five exons. The analysis of the human *H2A.Z* promoter has shown that a region of 234 bp is sufficient to provide maximal promoter activity in a CAT assay (Hatch and Bonner 1995). The regulatory region of the *Drosophila H2AvD* gene appears to be very small as well, as a plasmid containing the coding region and approximately 600 bp in front of the start codon can rescue the mutant phenotype of flies with a deletion of the *H2AvD* gene (van Daal and Elgin 1992). Accordingly, we assume that the regulatory region of zebrafish *H2A.F/Z* has a similar size, as within 500 bp of the front of the putative transcription start site some promoter elements of those found in the human gene can be identified (Hatch and Bonner 1995).

The 3′ end of the zebrafish *H2A.F/Z* cDNA was amplified by PCR, cloned in a GFP vector, and the resulting insert was substituted for the 3′ end of a 7-kb deletion derivative [IVE8(SmaI)] of the IVE8 genomic fragment. The clone obtained includes 2 kb genomic DNA upstream of the transcription start site of *H2A.F/Z*. Sequencing of the 5′ end of the deletion clone showed a new transcription unit with similarity to mouse *Pur beta* (Kelm et al. 1997) and human *Pur alpha* (Bergmann et al. 1992). In addition, the *H2A.F/Z:GFP* clone comprises 2.5 kb genomic DNA with the coding region of *H2A.F/Z*, a 270-bp 3′ terminal fragment of the *H2A.F/Z* cDNA, fused in frame to a 1.4-kb GFP-SV40 polyA. Figure 1A shows the composition of the transgene. This plasmid was injected into wild-type zygotes and a fusion protein was found to be transiently expressed, as strong fluorescence was observed diffusely distributed in the injected embryos from about 50% epiboly on up to at least the 2.5-day-old larva. As expected, detailed analysis of the fluorescence demonstrated labelling of the chromosomes (data not shown).

Germ line transformation

The plasmid encoding the fusion protein H2A.F/Z:GFP was injected into wild-type zygotes, which were allowed to develop to adulthood. After reaching adulthood, pairs of injected animals were crossed inter se and embryonic progeny of each cross were screened for insertions of the plasmid both by GFP-mediated fluorescence and by PCR using *gfp*-specific primers. Progeny from a total of 70 injected animals were screened, and three different insertions [*Tg(H2AF/Z)*^{kca13}, *Tg(H2AF/Z)*^{kca37} and *Tg(H2AF/Z)*^{kca66}] were found. The transformants were used to establish zebrafish lines.

GFP-mediated fluorescence is visible in all cells of the body of the transgenic fish at all embryonic and larval stages that we have analysed. Expression of members of the subfamily correlates with transcriptionally active chromatin. Thus, the H2AvD:GFP fusion protein in *Drosophila* shows a clear nuclear labelling only from the ninth nuclear division on (Clarkson and Saint 1999), which corresponds to the MBT, i.e. to the beginning of zygotic gene transcription. However, protein is present in the cytoplasm during the first few nuclear divisions, but not in the nuclei. In the zebrafish, both maternal H2A.F/Z:GFP RNA and protein can be demonstrated in the cytoplasm of unfertilised eggs, which had been squeezed from the abdomen of the females, or in zygotes as well as in embryos with few cells, by in situ hybridisation (not shown) and either by GFP-mediated fluorescence or using anti-GFP antibody stainings

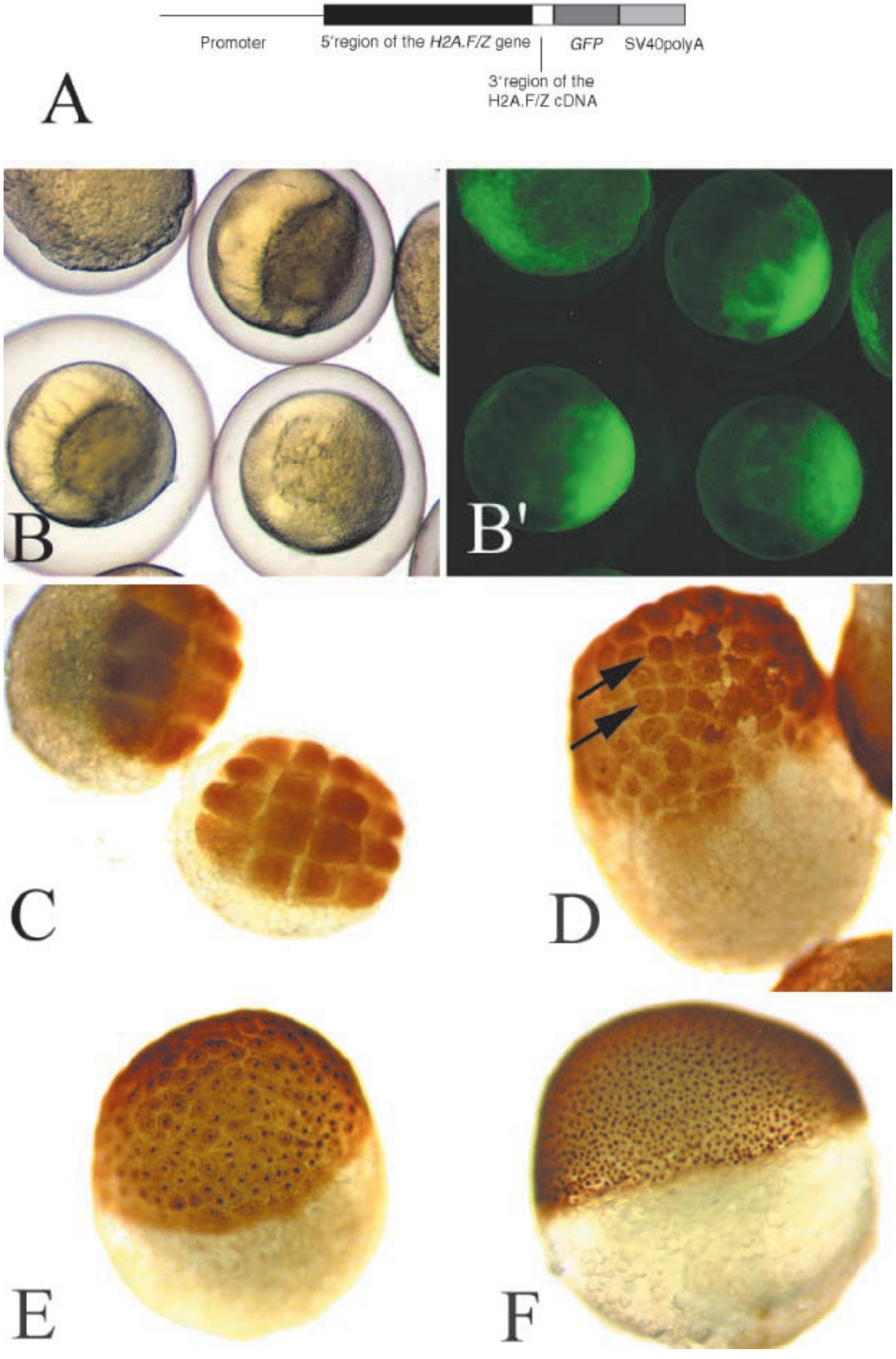

Fig. 1 **A** Structure of the *H2AF/Z:GFP* construct. **B** Several unfertilised eggs bserved with Nomarski optics and under light of 480 nm that have been squeezed from a female carrying insertion *Tg(H2AF/Z)*^{kca37} and insertion *Tg(H2AF/Z)*^{kca66}, to illustrate the presence of maternal protein. **C–F** Transgenic embryos stained with an anti-GFP (green fluorescent protein) antibody at the 8-cell and 16-cell stage (**C**), 250-cell stage (**D**), 1,000-cell stage (**E**) and at 35% epiboly (**F**). Notice that H2AF/Z protein is exclusively cytoplasmic in the early cleavage stages (**C**), but it is already visible in some of the nuclei at the 250-cell stage (*arrows*, **D**). From the 1,000-cell stage on, H2AF/Z is present in all nuclei (**E, F**)

(Fig. 1B, C–F). A nuclear localisation of the fusion protein can be detected from the 250-cell stage in individual nuclei (Fig. 1D), and from midblastula transition onwards, at the 1,000-cell stage, in all nuclei (Figs. 1E, F, 2, 3 and 4). Therefore, the H2A.F/Z:GFP protein is found in the zebrafish in single nuclei after the seventh cleavage, i.e. shortly prior to MBT (Kane and Kimmel 1993), and a little earlier than in the case of *Drosophila* (Clarkson and Saint 1999). From the 1,000-cell stage on, fluorescence is restricted to interphase nuclei or to the chromosomes of mitotic cells, being completely absent from the cytoplasm. Therefore, the fusion protein H2A.F/Z:GFP provides a ubiquitous nuclear marker. Expression of the H2A.F/Z:GFP fusion protein by the

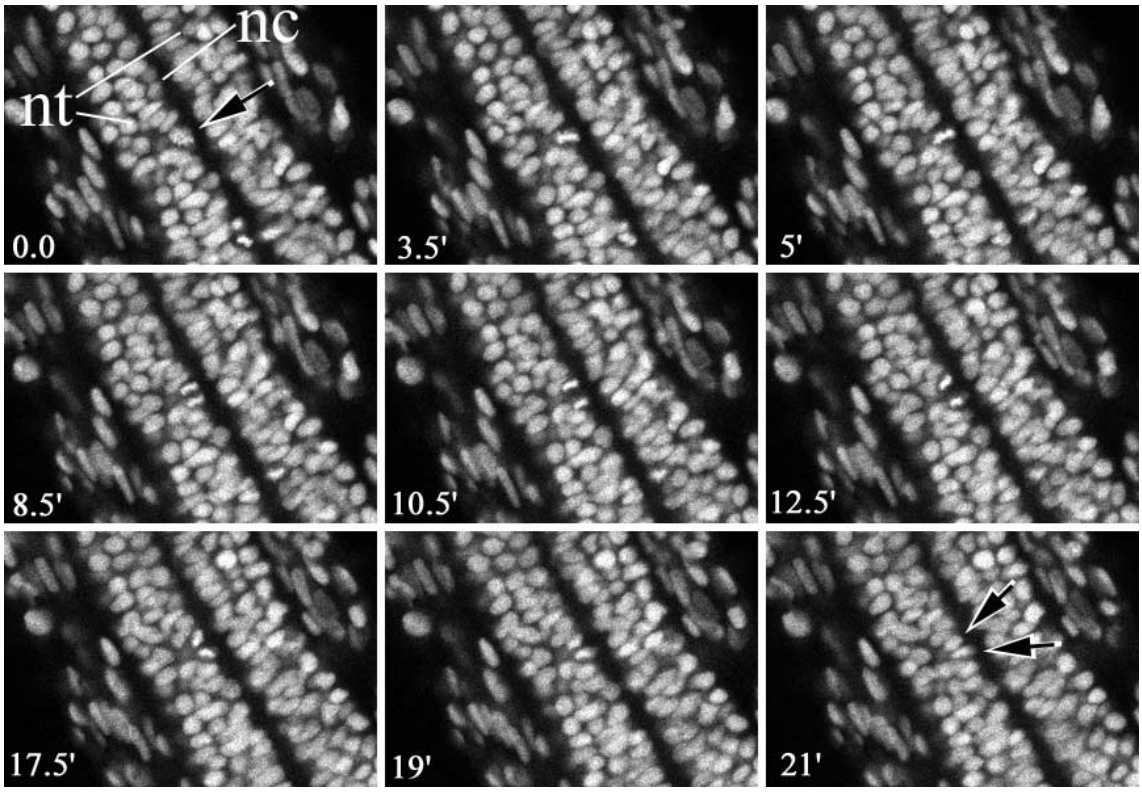

Fig. 2 A sequence of nine time-lapse photographs taken with the confocal microscope to illustrate a mitotic division in the neural tube. Each photograph shows an optical section of the neural tube (**nt**), which is obliquely oriented in the centre of each photograph. The neurocoel (**nc**) is the *dark stripe* in the middle. The first photograph of the series (*upper left corner*, labelled *0.0*) shows a cell in early prophase (*arrow*) located at the surface of the neurocoel. This cell completes mitosis within the following 21 min (*two arrows* in the photograph at the *lower right corner*)

transformants does not seem to affect either normal development or vitality and fertility. Individuals carrying the transgenes have been followed for a period of more than 1 year, and they appear to be completely normal, irrespective of whether they carry one, two or more copies of the transgene.

Use of the transgenic *H2A.F/Z:GFP* insertions for developmental studies

The intensity of fluorescence is relatively strong in animals carrying any one of the three insertions; however, expressivity is different in all three cases, suggesting that the insertions are subjected to chromosomal positional effects. Thanks to the transparency of the zebrafish embryo, developing cells can be easily observed and time-lapse films can be made with a confocal microscope. In time-lapse films, the differences in the expressivity of the insertions are particularly noticeable. Thus, fluorescence fades away within a few minutes in animals carrying insertion *Tg(H2AF/Z)*^{kca13}, whereas it persists over a period of several hours in animals carrying insertion *Tg(H2AF/Z)*^{kca37} or *Tg(H2AF/Z)*^{kca66}. It should be emphasised that the intensity and persistence of the signal can be increased by crosses between animals carrying *Tg(H2AF/Z)*^{kca37} and *Tg(H2AF/Z)*^{kca66} to raise the number of insertions in the genome and thus the amount of fusion protein eventually made in the progeny of those crosses. When the embryos carry two or more copies of the transgenic insertions, time-lapse films can be made with the confocal microscope over a period of 24 h or longer without a substantial reduction of the intensity of the signal.

The transgenic insertions *Tg(H2AF/Z)*^{kca37} and *Tg(H2AF/Z)*^{kca66} can be used for a variety of developmental studies in vivo. We would like to briefly refer to the analysis of mitotic patterns, particularly concerning spatial and temporal aspects of mitotic activity, with which we are currently concerned, and to cell movements. Thus, direct observation of the mitotic figures allows one to establish, for example, the existence of mitotic domains (Kane et al. 1992) similar to those described by Foe (1989) in *Drosophila* (not shown), to calculate the duration of the mitosis during specific developmental stages in particular organs, or to deduce the orientation of the mitotic spindle from the orientation of the metaphase plate. Thus, in cells of the neural tube at the level of the trunk at 22 hpf, the duration of mitosis, i.e. the time required to proceed from chromatin condensation at prophase to decondensation after telophase, was 21±3 min (Fig. 2). Observations of the developing retina between 24 and 30 hpf shows that cells divide in the marginal zone and in the outer layer (Fig. 3A–C), and that the orientation of the mitotic spindle is always paral-

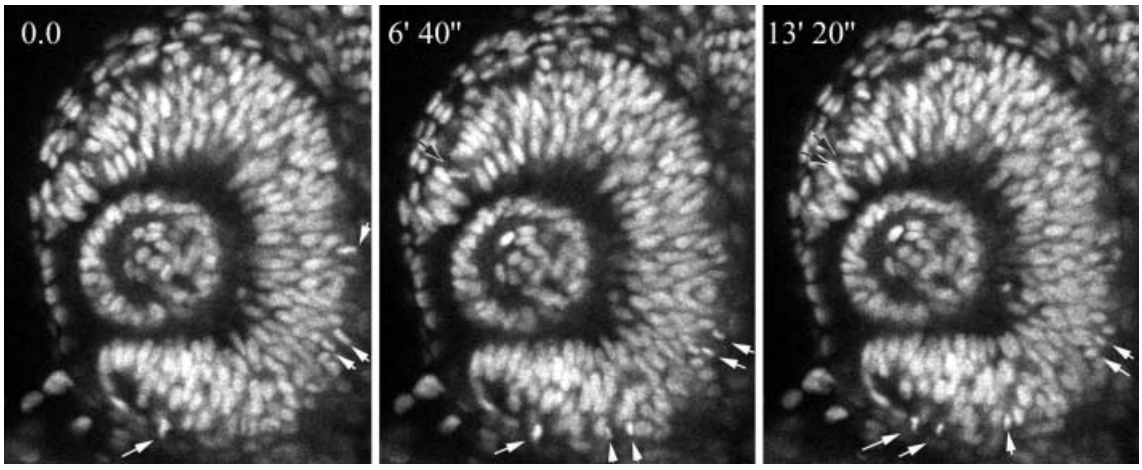

Fig. 3 A sequence of three time-lapse photographs taken with the confocal microscope to illustrate mitotic divisions in the retina (*arrows*). Notice that the orientation of the metaphase plate is always perpendicular to the choroidal face of the retina

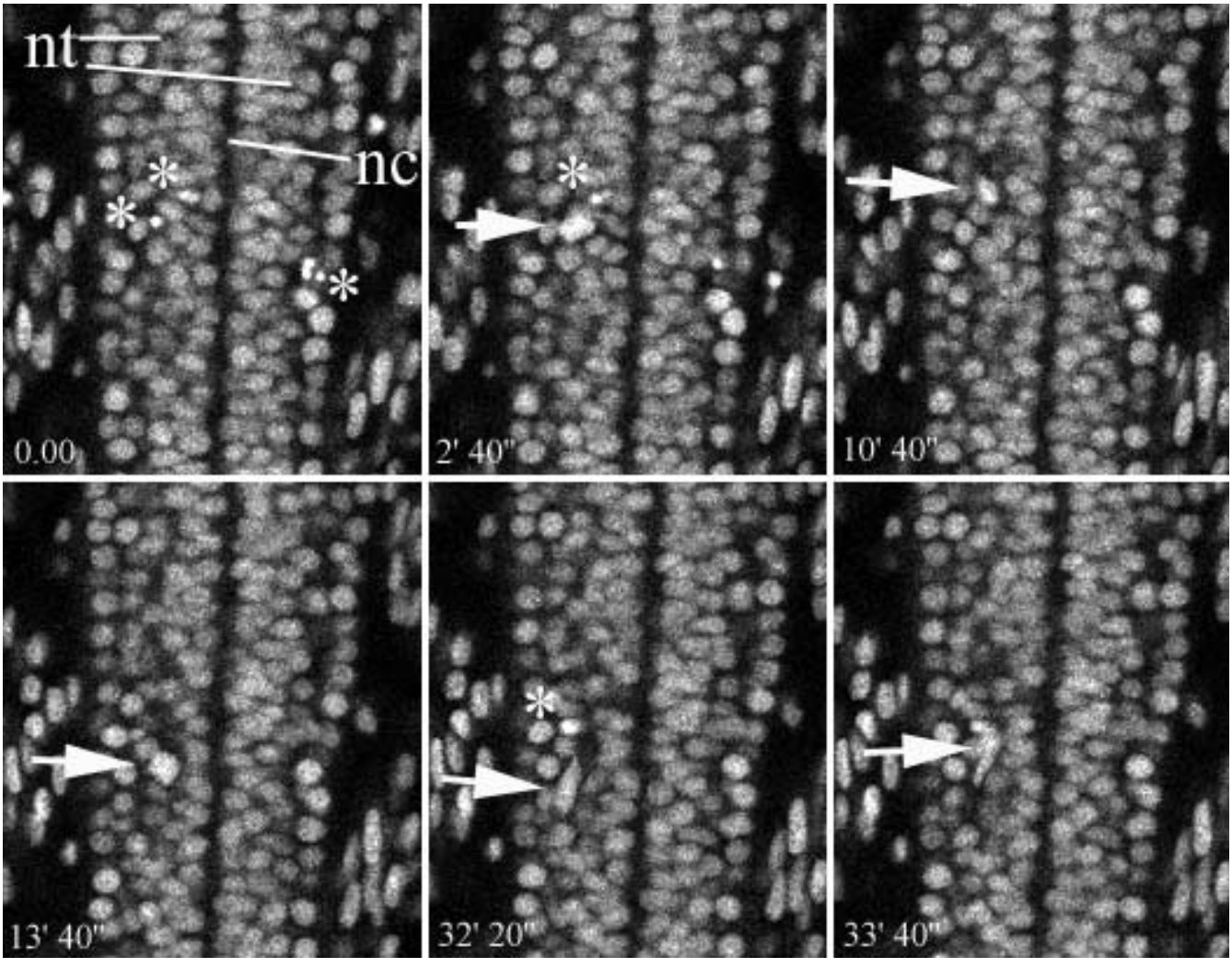

Fig. 4 A sequence of six time-lapse photographs taken with the confocal microscope to illustrate movements of a macrophage (*arrows*) to phagocytose apoptotic cells within the neural tube. Each photographs shows an optical section of the neural tube (*nt*, *nc* neurocoel). At time point *0.0*, two picnotic nuclei are visible to the left and another one to the right (*asterisks*). Within the following 2 min and 40 s, a macrophage appears to the left and phagocytoses one of the apoptotic cells. The macrophage stays in the same plane of focus for another 30 min to phagocytose another cell which undergoes apoptosis at 32 min 20 s'

lel to the choroidal face of the retina. A total of 71 mitoses were observed in this time in three different animals, 48 within the marginal zone and 23 outside this zone. All mitotic spindles were oriented either parallel to the plane of the neuroepithelium or slightly oblique to it, with an angle of up to 15°; none were oriented perpendicular to the neuroepithelium. The duration of mitosis in the retina during this time was 30±4 min, therefore longer than in the cells in the neural tube.

Other possible applications of this transgenic strain include the tracking of cell movements or to analyse the composition of cell lineages during embryogenesis (not shown). Movements of neural crest cells or of macrophages (Fig. 4) can be easily followed. This can be done directly in the transgenic animals or, even more easily, following transplantation of transgenic cells in wild-type hosts. Transplantation of transgenic cells into wild-type hosts, followed by investigation of the subsequent development of the transplanted cells, can also be used for cell lineage studies.

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