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.

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
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 GCC GGY GGY AAG AGT GGV AAR GA and 3′ TTG TGG ATG TGG GGR GGR ATG ACA CCW CCM CCM GCW A T. The amplified PCR product was cloned by means of the TOPO TA Cloning Kits (Invitrogen), and used to screen a cDNA library prepared in AZAP (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 SstIII, 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 (Invitrogen), and used to screen a cDNA library prepared in AZAP (Stratagene) from 3- to 15-h 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.ZI* from *Xenopus laevis*, which was published as “H2A.Z-like” (Juozaite 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