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Abstract

Complete sexual development is not easily amenable to experimentation in hydra. Therefore, the analysis of gene function and gene regulation requires the introduction of exogenous DNA in a large number of cells of the hydra polyps and the significant expression of reporter constructs in these cells. We present here the procedure whereby we coupled DNA injection into the gastric cavity to electroporation of the whole animal in order to efficiently transfect hydra polyps. We could detect GFP fluorescence in both endodermal and ectodermal cell layers of live animals and in epithelial as well as interstitial cell types of dissociated hydra. In addition, we could confirm GFP protein expression by showing colocalisation between GFP fluorescence and anti-GFP immunofluorescence. Finally, when a FLAG epitope was inserted in-frame with the GFP coding sequence, GFP fluorescence also colocalised with anti-FLAG immunofluorescence. This GFP expression in hydra cells was directed by various promoters, either homologous, like the hydra homeobox cnox-2 gene promoter, or heterologous, like the two nematode ribosomal protein S5 and L28 gene [...]
Cnidarian and Bilaterian Promoters Can Direct GFP Expression in Transfected Hydra

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Complete sexual development is not easily amenable to experimentation in Hydra. Therefore, the analysis of gene function and gene regulation requires the introduction of exogenous DNA in a large number of cells of the Hydra polyps and the significant expression of reporter constructs in these cells. We present here the procedure whereby we coupled DNA injection into the gastric cavity to electroporation of the whole animal in order to efficiently transfect Hydra polyps. We could detect GFP fluorescence in both endodermal and ectodermal cell layers of live animals, and in epithelial as well as interstitial cell types of dissociated Hydra. In addition, we could confirm GFP protein expression by showing colocalisation between GFP fluorescence and anti-GFP immunofluorescence. Finally when a FLAG epitope was inserted in frame with the GFP coding sequence, GFP fluorescence also colocalised with anti-FLAG immunofluorescence. This GFP expression in Hydra cells was directed by various promoters, either homologous like the Hydra homeobox cnox-2 gene promoter, or heterologous, like the two nematode ribosomal protein S5 and L28 gene promoters, and the chicken ß-actin gene promoter. This strategy provides new tools for dissecting developmental molecular mechanisms in Hydra, more specifically the genetic regulations that take place in endodermal cells at the time budding or regeneration are initiated.

Key words: cnidaria, hydra, electroporation, transfection, GFP, ectoderm, endoderm, cnox-2 promoter, actin promoter, ribosomal gene promoters

INTRODUCTION

The early evolution of animal phyla can be seen as three major steps: first divergence of the Porifera (the sponges), then divergence of the Cnidaria and later divergence of the protostome/ deuterostome phyla. Hence, cnidarians including hydra, represent the most ancestral species in the metazoan evolution displaying nerve cell differentiation and bipolar morphogenetic processes resulting in the formation of two differentiated regions, one of them being responsible for the active feeding behaviour. The freshwater hydra displays a simple tube-shape form, named polyp, with at both extremities, differentiated structures, like a head at the apical pole, and a foot at the basal pole, that can attach to the substrate. Hydra is made up of two multifunctional epithelial-muscular layers separated by an extracellular matrix named the mesoglea. In addition to the two distinct epithelial cell lineages, the interstitial stem cells provide the nerve cells, gland cells, nematocytes, and the gametes when the animal follows the sexual cycle. Finally, hydra bud and regenerate all along their life, implying that the developmental programs that lead to the differentiation of a new axis including a new
complete head can be reactivated whatever the age of the animal.

It is now clearly established that many of the developmental genes involved in head and/or axis patterning in bilaterians, were already present in cnidarians and, according to the temporo-spatial regulations they exhibit, involved in apico-basal patterning (reviewed in Galliot, 2000). Furthermore, according to the dynamics of expression patterns observed during budding, regeneration, reaggregation, endodermal cells seem to play a key role in the organizer activity that develops during early patterning (Gauchat et al., 1998; Technau and Bode, 1999; Smith et al., 1999; Hobmayer et al., 2000; Mochizuki et al., 2000). Therefore, cnidarians and more specially hydra, provide interesting model systems to investigate basic developmental mechanisms already at work in the common ancestor of most animals (Galliot and Miller, 2000).

Beside the cloning of evolutionarily-conserved genes and the analysis of their expression patterns, functional tools that address the question of gene function were recently established in hydra, using either ds-RNA interference (Lohmann et al., 1999; Lohmann and Bosch, 2000) or antisense assays (Yan et al., 2000a; Leontovich et al., 2000; Yan et al., 2000b). In both cases, specific gene down-regulations were observed and phenotypes were obtained. However, the expression of reporter constructs in hydra polyps that is a prerequisite to the analysis of the genetic regulatory elements, was never reported. In order to characterise the regulatory sequences that direct expression of specific genes at the time morphogenetic events take place in hydra, we have established a transfection procedure that leads to the efficient expression of GFP reporter constructs in various hydra cell types. We have combined injection of plasmidic DNA into the gastric cavity to electroporation and obtained significant and reproducible GFP expression, in both layers and in epithelial and interstitial cell types under the control of both homologous and heterologous promoters.

**MATERIAL AND METHODS**

**Hydra culture and regeneration experiments:** *Hydra vulgaris*, Basel strain, were used for transfection. Cultures were maintained in hydra medium (HM, 1 mM NaCl, 1 mM CaCl₂, 0.1 mM KCl, 0.1 mM MgSO₄, 1 mM Tris pH 7.6) (Muscatine and Lenhoff, 1965) and fed 5 times a week with freshly hatched swimming *Artemia* nauplii. Animals were starved for 24 to 48 hours before transfection.

**Codon usage analysis:** The *Hydra vulgaris* codon usage (Galliot and Schummer, 1993) was updated (http://www.kazusa.or.jp/codon/) and defined by the analysis of 27 sequences corresponding to 13 726 residues. For each gene, the codon frequency was analysed with the GCG Wisconsin Package (Version 9.1) and the percentage of codons showing either a very low (lower than 10%) or a low (between 10 and 20%) representativity was calculated by referring to the hydra codon usage.

**Reporter constructs and DNA preparation:** The *cnox-2* promoter region was obtained by inverted PCR (Ochman et al., 1990) performed on *Chlorohydra viridissima* genomic DNA and the start site was mapped (Schummer, 1994; Mazet et al. in preparation). Two *cnox-2* promoter fragments, cx2-1000 (–790 / +158) and cx2-700 (–490 / +158), were inserted into the pUC19-GFP vector (kindly provided by G. Plickert), which contains the wt GFP coding region followed by the 3’UTR region of the nematode *unc54* gene but no specific localization signal.

In parallel, we used GFP reporter constructs kindly provided by the laboratory of Andy Fire (Fleenor et al., 1999). These constructs contain the S65C GFP variant coding region and four tandem copies of the SV40 T-antigen nuclear localisation signal (NLS) located at the N-terminus. In addition 3 different introns interrupt the GFP coding region. These constructs carry either the poll S5 ribosomal protein *rps-5* gene promoter or the poll L28 ribosomal protein *rpl-28* gene promoter. These constructs were named *Ce rps5* _GFP* and *Ce rpl28* _GFP_ respectively (Table 2). In the *Ce rps5* _FLAG-GFP_ construct, a 6His-FLAG sequence was inserted upstream to and in frame with the GFP coding sequence of the *Ce rps5* _GFP_ plasmid. Finally, we also used the pCAGGS _GFP_ reporter construct (Momose et al., 1999) where the EGFP coding sequence was placed under the control of the chicken β-actin promoter. Characteristics of all constructs are
listed in Table 2. These plasmidic DNAs were multiplied in DH5α E. coli, purified either by Wizard® Plus midipreps (Promega) or by EndoFree™ Plasmid mega preparations (Qiagen) and resuspended in H2O (5 µg/µl).

Transfection of hydra polyps: injection coupled to electroporation (EP): We tested several types of electroporators and obtained the best results with the Equibio Easyject Plus apparatus that delivers two independently modulated currents through two separate condensators. Double pulse further optimized the pore size and number of pores generated from the first pulse, the second is thought to electrophoretically move the DNA into the cell. Both pulses together have a reduced energy level, thus increasing cell survival. Nevertheless, we also obtained significant positive results with the Eppendorf Microporator. In both cases, the electrical conditions were relatively soft, in order to keep animals in healthy state, and no mortality was actually noted in the transfected animals. The transfection procedure was adapted from that described in (Momose et al., 1999). Hydra were first transferred from HM to MilliQ H2O for 25 minutes, then pretreated with 1.5% Bisolvon (Boehringer Ingelheim) diluted in H2O for 5 minutes to reduce the amount of mucus on the surface of the animal, extensively washed in large volumes of H2O, then placed into a 0.5 mm x 0.7 mm x 1 mm well previously molded in a 1.5% agarose dish. For injection, plasmidic circular DNA was loaded into the micropipette (Drummond Scientific Company, 6.6 µl). The micropipette was inserted through the mouth opening into the gastric cavity in direct contact with the endoderm.

The injection (Inject+Matic apparatus, Geneva), delivering about 50 nl, was performed before the EP was initiated. The well was then filled with 2.5 to 3 µl of the DNA solution (5 µg/µl) and the electroporation was immediately performed using two platinum electrodes; the anode (φ = 0.2 mm) was twice thinner than the cathode and both were held by a holder connected to the electroporater (Equibio Easyject Plus apparatus). Two successive pulses were applied at the following conditions: pulse 1: V= 200 Volts, C= 150 µF, R=99 ohms, t= 0.050 msec; pulse 2: V= 30 Volts, C= 150 µF, R=99 ohms, t= 14.8 msec. Hydra were then transferred in HM, stored in dark and examined every 24 hours. Mock hydra were injected and electroporated in the absence of any DNA. Except when indicated differently, animals were incubated prior to GFP detection in 1 µg/ml DAPI (4,6-diamidino-2-phenylindole-2-HCl) solution in HM for 30min in the dark. In order to immobilise animals, hydra were incubated in chilled 0.01% heptanol and 0.5% urethane solution in HM and kept on ice (Yan et al., 2000a). Fluorescence of the animals was screened on a Zeiss Axioplan2 microscope equipped with the X100-2 GFP-filter set (Omega, 475 nm excitation, 535 nm emission). In order to keep UV-induced fluorescence to a low level, animals were conserved in the dark in the course of the experiment and at the time of fluorescence capturing, UV excitation applied for the detection of DAPI staining was always used after GFP detection.

Cellular analysis of GFP expression: Cellular localisation or cell-type specificity of GFP expression was analysed after dissociating live hydra 48 hours after EP. For dissociation, hydra were macerated according to David’s method (David, 1973) or treated either with pronase (Greber et al., 1992) and cells were spread over gelatine-coated slides (0.5% gelation, 0.1%

For nuclear staining, slides were incubated for 2 minutes with DAPI (Roche) or TO-PRO-3 nuclear dye (Molecular Probes, 642 nm excitation, 661 nm emission) diluted in HM, 0.1 g/ml and 0.2 g/ml respectively, washed with HM, mounted in DABCO and sealed with nail polish. Pictures were captured on a Zeiss confocal laser scanning microscope (LSM 510) or on a Zeiss Axioplan2 microscope.

Immunocytochemistry and Western analysis:
Anti-GFP ab290 (Abcam) and anti-FLAG BioM2 (Kodak) antibodies were used at 1/1000 and 1/300 dilutions respectively on hydra cells obtained from macerated hydra as described above. Slides were treated according to (Soltermann et al., 1999) with minor modifications. For Western analysis, live animals were directly dissociated in Laemmli’s loading buffer, boiled for 3 minutes and loaded onto 12% PAGE. After migration, proteins were blotted onto Immobilon membranes (Millipore) subsequently treated according to the supplier instructions. ECL chemiluminescence (Amersham) was used for detection.

RESULTS

Choice of an efficiently-translated reporter construct
In order to analyse the possible consequence of the hydra codon usage (Galliot and Schummer, 1993) on the level of expression of transfected reporter constructs in hydra cells, we compared the frequency of used codons in four hydra genes and in several classical reporter genes that we wanted to use, to the codon usage currently defined in Hydra vulgaris (Table 1). For that purpose, we updated the analysis of the hydra codon usage and characterised for each of these sequences the rate of non-preferred codons that display a representativeness that is either very low, less than 10% (very rare codons) or low, between 10 and 20% (rare codons). We noted that in the sequences of the hydra genes that show a high level of expression, such as actin (Fisher and Bode, 1989) or collagen (Kurz et al., 1991), non-preferred codons (very rare plus rare) were represented in only 12.5% and 10% of the codons respectively. In contrast, over 30% of the codons present in the CAT, β-galactosidase or enhanced-GFP reporter coding sequences have a low representativity when analysed according to the hydra codon usage. This observation suggests that these genes cannot be efficiently translated and could explain why we observed only low levels of CAT activity or limited expression of human-optimised GFP and DsRed variants (EGFP, EBFP and DsRed1-N1) in hydra cells (FM, MM, unpublished data). The case of the luciferase...

Table 1: Abundance of rare codons in Hydra and reporter gene products.

<table>
<thead>
<tr>
<th>Gene products</th>
<th>Length (AA)</th>
<th>Number AAs encoded by very rare codons (&lt;10%)</th>
<th>% AA encoded by very rare codons (&lt;10%)</th>
<th>% AA encoded by rare codons (10-20%)</th>
<th>% AA encoded by non-preferred codons (0-20%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydra genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-collagen Hm</td>
<td>150</td>
<td>9</td>
<td>6</td>
<td>4</td>
<td>10</td>
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<tr>
<td>Actin Hv</td>
<td>377</td>
<td>28</td>
<td>7.4</td>
<td>5</td>
<td>12.5</td>
</tr>
<tr>
<td>Cnox-2 Hv</td>
<td>256</td>
<td>32</td>
<td>12.5</td>
<td>5.9</td>
<td>18.4</td>
</tr>
<tr>
<td>Cnox-2 Cv</td>
<td>257</td>
<td>39</td>
<td>15.2</td>
<td>7</td>
<td>22.2</td>
</tr>
<tr>
<td>Reporter genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFP wt</td>
<td>239</td>
<td>19</td>
<td>8</td>
<td>6.7</td>
<td>14.7</td>
</tr>
<tr>
<td>DsRed wt</td>
<td>226</td>
<td>31</td>
<td>13.7</td>
<td>12.4</td>
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<tr>
<td>Luciferase</td>
<td>550</td>
<td>99</td>
<td>18</td>
<td>1.1</td>
<td>19.1</td>
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<td>19.1</td>
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<td>62</td>
<td>27.3</td>
<td>23.8</td>
<td>51.1</td>
</tr>
<tr>
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<td>1024</td>
<td>307</td>
<td>30</td>
<td>10.7</td>
<td>40.7</td>
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<tr>
<td>EGFP</td>
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<td>73</td>
<td>30.4</td>
<td>20</td>
<td>50.4</td>
</tr>
<tr>
<td>EBFP</td>
<td>240</td>
<td>73</td>
<td>30.4</td>
<td>20.4</td>
<td>50.8</td>
</tr>
<tr>
<td>hrGFP</td>
<td>240</td>
<td>96</td>
<td>40</td>
<td>26.25</td>
<td>66.2</td>
</tr>
</tbody>
</table>

Gene products

Length

Number AAs encoded by very rare codons (<10%)

% AA encoded by very rare codons (<10%)

% AA encoded by rare codons (10-20%)

% AA encoded by non-preferred codons (0-20%)

Table 1: Abundance of rare codons in Hydra and reporter gene products.
sequence is also significant: despite an acceptable proportion of very rare codons, their absolute number is rather high (99 residues), providing an explanation for the absence of luciferase protein expression in hydra polyps (Brennecke et al., 1998). Finally, out of these commonly used reporter genes, the wild type gfp gene (isolated from jellyfish, a cnidarian species) displayed a favourable codon usage that corresponded more closely to the H. vulgaris codon usage. Consequently, GFP is likely one of the best

Figure 2. The nematode ribosomal protein S5 gene promoter directs GFP expression in live hydra. a-d) Live hydra 72 hours after Ce rps5_GFP EP showing a large proportion of endodermal cells expressing GFP on one side (a, arrows and b) and very few GFP expressing cells on the opposite (a, arrowheads and d). e-g) Live hydra 48 hours after Ce rps5_GFP EP showing colocalization between GFP-expressing cells and DAPI stained nuclei (arrows). h-j) Live hydra transformed with GFP-let858 (promoter-less construct) in the same conditions as in e-g. In this experiment DAPI was added to DNA at the injection time leading to a predominant staining of nuclei of endodermal cells. k-n) Live hydra 10 days after Ce rps5_GFP EP showing persistent GFP expression in cells of the body column, predominantly above the budding zone (white square magnified in l-n). In this animal, endodermal and ectodermal cells expressed both GFP while DAPI staining was predominantly detected in the ectoderm. GFP fluorescence: a (left panel), b, d, e, h, k (left panel), l. DAPI detection: a (right panel), c, f, i, k (right panel), m. Merged views: g, j. DIC view: n. The thin white line (b-d, l-n) indicates the position of the mesoglea between the ectodermal (ec) and endodermal (en) cell layers. Bars correspond to 500 μm (a, k), 100 μm (b-d) or 50 μm (e-n).
candidates among reporter genes to be efficiently produced in hydra cells.

**UV-induced fluorescence in Hydra cells**

Many cnidarian species display bioluminescence and GFP protein was originally discovered in a hydrozoan jellyfish named *Aequorea victoria* (Shimomura, 1998). We had noticed that hydra cells stained with DAPI exhibited GFP-like fluorescence when submitted to UV excitation. In order to analyze in further details this endogenous fluorescence, we exposed DAPI-stained live total hydra or DAPI-stained fixed hydra cells to UV for increasing periods of times ([Fig.1](#)). We did not detect any UV-induced fluorescence in live total hydra, even after 1 minute of UV exposure. In contrast, cells prepared from dissociated hydra displayed GFP-like nuclear signals when exposed to UV, the intensity of these signals being correlated with exposure time ([Fig.1f,g](#)). In the absence of UV exposure, no GFP-like nuclear fluorescence was noted in DAPI-stained fixed cells ([Fig.1e](#)). This UV-induced fluorescence was not observed when cells were stained with TO-PRO-3 (not shown). In order to avoid this GFP-like nuclear fluorescence, in all experiments described in this paper we
captured GFP fluorescence of DAPI-stained animals and DAPI-stained cells before any UV exposure.

Efficient GFP expression in Hydra polyps

We previously observed a rather variable efficiency of transformation of ectodermal cells when electroporation of whole hydra was performed in cuvettes. Moreover, we never detected any transformed endodermal hydra cells when using this way of transfection (Mazet, 1999). Therefore, in order to transform endodermal cells more efficiently and to improve the reproducibility of hydra polyps transformation, we coupled injection of plasmidic DNA into the gastric cavity to immediate electroporation of animals. For this procedure, each hydra was treated separately, being placed in a small well in an agarose dish, pre-filled with the DNA solution. Hence at the time of electroporation, both layers, endodermal and ectodermal were surrounded with the DNA solution. Using these conditions for DNA delivery, we were able to obtain large patches of GFP-expressing cells when GFP expression was driven either by two distinct C. elegans ribosomal promoters (Fig.2-4,
In several cases, hydra carrying evaginating buds (stage 3 and 4) were injected and electroporated. Two days after transfection, the newly formed bud exhibited a ubiquitous GFP expression in its endodermal layer (Fig. 5B), proving that developing buds are permissive for exogenous gene expression. In most experiments, GFP expression was transiently detected in whole hydra polyps 48 to 72 hours following electroporation. In fact we noted that this period of time corresponded to the highest observed level of GFP expression. Thereafter, the level of GFP expression slowly decreased, likely as a consequence of degradation and/or dilution of the exogenous plasmidic DNA. However, in several cases, we recorded GFP expressing cells 10 days after electroporation. In the animal depicted in Fig. 2b, Ce rps5_GFP expressing cells were detected in the budding area 48 hours after EP, and in the same location 8 days later. The persistence of GFP expression in the close vicinity of the budding zone, for at least 10 days in several animals, suggests that in such cases, stem cells might have been targeted. Unfortunately, these animals were submitted to numerous harmful examinations and eventually got destroyed after 10 days.

Thus, in contrast to the electroporation procedure where the animals were placed in cuvettes, we reached a high level of GFP expression in endodermal cells when animals were electroporated one by one directly placed between the 2 electrodes. However, with this procedure, we repeatedly noted that the GFP-expressing cells were more numerous in the endoderm than in the ectoderm.

**Comparison between the different GFP constructs**

In order to evaluate the reproducibility of the transformation procedure we used, we first calculated the number of experiments where GFP positive animals were detected (Table 3). Each construct was tested in at least 12 distinct independent experiments carried out in similar conditions over a period of one year and using in each experiment 2 to 10 animals per construct. We noted a very different rate of reproducibility between the different constructs, ranging from 88% with the Ce rps5_GFP construct down to 20% with the cx2-700_GFP construct. However, when we calculated the number of GFP positive animals detected in each positive experiment, we obtained a similar average rate for each of the constructs, i.e. approximately 50% of the animals expressing GFP 2 or 3 days after EP (Table 3). Similarly, we did not record any obvious difference in the spatial pattern of expression between the different constructs, except that GFP expression was never detected in the ectodermal layer when the two cnox2 constructs were transfected. Finally, in order to quantify the rate of GFP expressing cells

| Table 2: Characteristics of the GFP-constructs electroporated in Hydra. |
|---|---|---|---|---|---|
| Plasmids | Promoter | Reporter gene | NLS | introns in GFP | 5'-UTR | 3'-UTR | Reference |
| GFP-unc-54 | - | GFP wt | - | - | - | unc54 | this work |
| cx2-1000_GFP | Cv cnox2 (790 bp) | GFP wt | - | - | - | Cv cnox2 (1-158 bp) | unc54 | this work |
| cx2-700_GFP | Cv cnox2 (490 bp) | GFP wt | - | - | - | Cv cnox2 (1-158 bp) | unc54 | this work |
| pCAGGS_GFP | chicken β-actin | EGFP (S65->A, Y145->F) | - | - | rabbit β-globin polyA | rabbit β-globin polyA | (Momose et al., 1999) |
| GFP-let858 (pPD 122.34) | - | GFP (S65->C) | 4x | 4 | - | let-858 | (Fleenor et al., 1999) |
| Ce rps5_GFP (pPD 129.51) | Ce rps-5 (4000 bp) | EGFP (S65->C) | 4x | 3 | - | let-858 | (Fleenor et al., 1999) |
| Ce rpl28_GFP (pPD 129.57) | Ce rpl-28 (1458 bp) | EGFP (S65->C) | 4x | 3 | - | let-858 | (Fleenor et al., 1999) |
| Ce rps5_FLAG-GFP | Ce rps-5 (4000 bp) | 6His-FLAG-EGFP (S65->C) | 4x | 3 | - | let-858 | this work |
in GFP positive animals, we dissociated hydra 48 hours after transfection using the pronase dissociation method pronase dissociation method (Greber et al., 1992) and counted the number of GFP-expressing cells. We estimated that in transformed areas approximately 35% of the cells expressed GFP under the control of either the cx2-700 promoter or the nematode rps5 and rpl28 ribosomal promoters (data not shown).

**Cellular analysis of GFP expressing cells**

In order to characterize the sub-cellular localization of GFP fluorescence, we first searched for colocalization of DAPI nuclear signals and GFP fluorescence in live hydra. We readily detected colocalized signals when animals had been injected with DAPI at the time of electroporation and NLS-containing constructs were used (Ce rps5_GFP and Ce rpl28_GFP constructs) (Fig.2f-h). In addition, we confirmed this nuclear localization of the GFP signal by confocal analysis of the cells obtained after hydra maceration (Fig.4). In this latter case, the TO-PRO™ 3 dye was used for nuclear staining and colocalization with the GFP fluorescence was clearly observed. We also noted artefactual fluorescence in cytoplasmic vacuoles of endodermal epithelial cells (see Fig.4j-l). As expected the actin-GFP, the cx2-1000_GFP and the cx2-700_GFP constructs that do not contain any NLS, provided cytoplasmic fluorescent signals (Fig.6 and not shown). The distribution of the GFP signal was relatively uniform, with fluorescence spreading within the cytoplasm. In some cells, we observed intra-cytoplasmic GFP condensations that might be related to the formation of GFP crystals. It was actually demonstrated that at high concentrations, wt GFP protein can form dimers and crystals (Ward, 1998). In hydra macerated 2 days after electroporation, cell-type analysis of GFP-expressing cells showed that all cell lineages present in hydra can express GFP from heterologous as well as homologous reporter constructs: endodermal epithelial cells (Fig.4d,f; Fig.6A), ectodermal epithelial cells (Fig.7B) but also interstitial stem cells and their derivatives, i.e. nematoblasts, nerve cells, gland cells (Fig.4g,i; Fig.6 and 7).

**Colocalisation of GFP fluorescence and immunofluorescence**

In order to confirm that the GFP fluorescent signals observed in cells prepared from transformed macerated hydra were emitted by the GFP reporter protein, we first performed an immunocytodetection using an anti-GFP polyclonal antibody (Fig.7A). Colocalisation of cytoplasmic GFP fluorescence and anti-GFP immunofluorescence was scored in several cell types. We tested this anti-GFP polyclonal antibody on an immunoblot and could detect GFP in Ce rps5_GFP expressing animals (Fig.8). As a second control experiment, we inserted a 6His-FLAG sequence into the Ce rps5_GFP plasmid. (Ce rps5_FLAG-GFP, Table 2) and electroporated this construct in live hydra.

After 2 days, hydra were macerated and cells were processed for immunodetection with an anti-FLAG monoclonal antibody (Fig.7B). Colocalisation of nuclear GFP fluorescence and anti-FLAG immune-fluorescence was

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Ce rps5_GFP (nuclear)</th>
<th>Ce rpl28_GFP (nuclear)</th>
<th>pCAGGS_GFP (cytoplasmic)</th>
<th>cx2-1000_GFP (cytoplasmic)</th>
<th>cx2-700_GFP (cytoplasmic)</th>
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</thead>
<tbody>
<tr>
<td>number of positive experiments</td>
<td>23/26</td>
<td>8/15</td>
<td>11/17</td>
<td>9/20</td>
<td>4/16</td>
</tr>
<tr>
<td>% of positive experiments</td>
<td>88.5%</td>
<td>53.3%</td>
<td>58.3%</td>
<td>45%</td>
<td>25%</td>
</tr>
<tr>
<td>average rate of GFP-expressing animals in positive experiments (n)</td>
<td>40.5% (± 23.4) n = 23</td>
<td>47.9% (± 28.7) n = 8</td>
<td>45.6% (± 14.7) n = 11</td>
<td>36.1% (± 25.9) n = 9</td>
<td>39.2% (± 23.3) n = 4</td>
</tr>
</tbody>
</table>

*Table 3: Reproducibility and efficiency of expression of each transfected GFP construct.*
noted in various cell types, derived from epithelial as well as interstitial cell lineages.

**DISCUSSION**

**Heterologous promoters drive ubiquitous GFP expression in transfected Hydra**

We have established conditions whereby a strong and ubiquitous expression of GFP reporter constructs under the control of two nematode polymerase I-type ribosomal promoters as well as the vertebrate β-actin promoter was observed in both endodermal and ectodermal cell layers. Although with a lower reproducibility, the hydra cnox-2

**Figure 5.** GFP expression directed by the hydra cnox-2 promoter and the chicken β-actin promoter in live hydra 48 hours after EP. (A) Two distinct constructs, the cx2-1000 GFP (a-c) and the cx2-700 GFP (d-f) displayed strong expression in endodermal cells of the body column not observed in mock-transfected animals (g, h). Bar corresponds to 200 μm; cell layers are indicated as in figure 2. GFP detection (a, d, g); DAPI detection (b, e, h); merged (c, f). (B) Ubiquitous expression of the pCAGGS-GFP construct in endodermal cells of a just detached bud (i, k, m). Note the absence of GFP expression in the ectodermal cell layer stained with DAPI staining (j, l) and in mock electroporated hydra processed in the same experiment (n, o). Cell layers are indicated as in figure 2. Bars correspond to either 500 μm (i, j) or 25 μm (k-o).
GFP expression in transfected Hydra

When we compared the results obtained with the different constructs, we noted that the ribosomal *Ce rps5*\_GFP construct provided the highest rate of reproducibility, in fact GFP expressing animals were observed in almost 90% of the experiments. This high rate of reproducibility is likely promoter-
GFP expression in transfected Hydra

Most of the GFP-expressing cells were located throughout the body column of electroporated animals, corresponding to the region where the DNA solution had been injected. This distribution of GFP expressing cells suggests that this region is highly permissive for gene expression. In contrast, we rarely detected GFP-expressing cells either in the head, hypostome, tentacles, or in the foot regions. The absence of GFP expressing cells in apico or basal regions probably reflects the fact that these constructs were only transiently expressed, and/or that the regulatory elements involved in head or foot expression were missing or inactive in the constructs we tested.

Surprisingly, we observed an endodermal distribution of GFP expressing cells when the two cnox-2-GFP constructs were used; these cells were predominantly detected in the body column showing a similar spatial distribution when the cx2-700_GFP and the cx2-1000_GFP were transfected (Fig. 5). However, the endogenous cnox-2 transcripts were detected by mRNA in situ hybridisation analysis in the ectodermal cell layer along the body column and in the head region, but not in the endodermal cell layer (Gauchat et al., 2000). Consequently, we deduce from these data that the cx2-700 GFP and the cx2-1000_GFP constructs do not contain the full panel of regulatory elements that would drive the cnox-2 endogenous expression pattern.

GFP expression in the endodermal cell layer

In positive experiments and whatever the type of the construct, we recorded a transient GFP expression 2 to 3 days after EP in endodermal cells of the hydra adult polyp. In these regions where endodermal GFP expression was detected, a large proportion of the cells were transformed. In addition, GFP-expression was detected in tissues
involved in morphogenetic processes, e.g. budding. Newly formed buds that had been transfected at early stages displayed a ubiquitous GFP expression in their endodermal layer 2 days after EP. At early stages of budding, the communication between the parent and the bud is open and parental cells still migrate towards the evaginating bud (Otto and Campbell, 1977). Therefore two non-mutually exclusive explanations can be given for this observed GFP-pattern in newly formed buds. On one hand, the DNA solution might have reached the cavity of the developing bud at the time of injecting the parent, and endodermal cells of the bud were directly transformed upon electroporation. On the other, the parental cells of the body column might have been transformed at the time they were migrating towards the bud. In fact, it was demonstrated that cells from the parental body column migrate towards the bud up to stage 6, a stage when tentacle rudiments appear, mouth opening forms and communication between the parent and the bud ceases (Otto and Campbell, 1977). After migration, these cells upon mitotic division would have given rise to a high number of GFP-expressing cells in the newly formed bud. Both, direct and indirect transformation of the endodermal cells of the bud could occur in the same animal.

The targeting of endodermal cells is of primary interest for understanding patterning in hydra as these cells are supposed to carry the organizer activity detected by transplantation experiments during the early phase of regeneration (MacWilliams, 1983) and present at the time budding is initiated. In fact, in both contexts a transient wave of expression of evolutionarily-conserved regulatory genes was observed in endodermal cells located in the budding zone or the regenerating stump (Gauchat et al., 1998; Technau and Bode, 1999; Smith et al., 1999; Hobmayer et al., 2000; Mochizuki et al., 2000). Therefore the procedure that leads to a strong exogenous GFP expression in endodermal cells for several days after transfection, provides new functional tools, like overexpressing tagged hydra proteins under the control of the ribosomal S5 promoter, or altering endogenous gene expression by expressing antisense RNA or constructs that would lead to ds RNA interference. Moreover, we expect that the fine monitoring of live GFP expression will help the functional characterisation of the sequences that regulate gene expression during budding and regeneration.

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