Developmental Biology **233,** 329–346 (2001)
doi:10.1006/dbio.2001.0242, available online at http://www.idealibrary.com on IDEAL®

Tracing Transgene Expression in Living Zebrafish Embryos

Reinhard W. Köster and Scott E. Fraser[1]

Division of Biology and Beckman Institute, California Institute of Technology, Pasadena, California 91125

Ectopic expression by injection of plasmid DNA is rarely used in zebrafish embryos due to a low frequency of cells expressing a transgene of interest at detectable levels. Furthermore, the mosaic nature of ectopic expression by plasmid injection requires the direct detection of transgene-expressing cells. We have used the transcriptional activator Gal4-VP16 to amplify transgene expression in living zebrafish embryos. In comparison to conventional expression vectors, Gal4-VP16-amplified expression results in a significant higher number of cells which express a transgene at detectable levels. The Gal4-VP16-activator and the Gal4-VP16-dependent transgene can be placed on a single expression vector. Using tissue-specific regulatory elements, we show that expression of a Gal4-VP16-dependent transgene can be reliably restricted to muscle, notochordal, or neuronal tissues. Furthermore, Gal4-VP16 can drive the expression of two or more transgenes from the same construct resulting in simultaneous coexpression of both genes in virtually all expressing cells. The reported expression system works effectively not only in zebrafish embryos but also in *Xenopus* embryos, chicken, mouse, and human cultured cells and is thus applicable to a broad variety of vertebrates. The high frequency of transgene expression together with the linked coexpression of more than one transgene opens the possibility of easily monitoring the behavior of individual transgene-expressing cells in real time by labeling them with the fluorescent reporter GFP. The combinatorial nature of the expression system greatly facilitates changing the tissue-specificity, the transgene expressed, or the cell compartment-specific GFP reporter, making it simpler to address a gene's function in different tissues as well as its cell biological consequences.

Key Words: **transgene; Gal4-VP16; UAS; zebrafish; GFP; tissue-specific.**

INTRODUCTION

In recent years, zebrafish has become a widely used model organism for studying vertebrate development. One of its major advantages is the almost complete transparency of the zebrafish embryo, which permits individual cells and subcellular structures to be followed directly during their developmental program. For example, single cell movements in gastrulating zebrafish embryos can be analyzed by time-lapse imaging (Concha and Adams, 1998). Double-dye labeling of synaptic partners, such as Mauthner neurons and primary motoneurons, allows axonal outgrowth and growth cone dynamics to be monitored during synaptogenesis (Jontes *et al.*, 2000). Even the rapid changes in intracellular calcium levels of the Mauthner neurons have been successfully imaged during zebrafish escape response, coupling behavior with neuronal activity of individual neurons in living embryos (O'Malley *et al.*, 1996).

Aside from its transparency, the easy accessibility of zebrafish for genetic methods has made it a powerful genetic model organism to analyze embryogenesis. The numerous mutant strains that have resulted from genetic screens (Haffter *et al.*, 1996; Driever *et al.*, 1996) are a valuable tool to address gene functions (e.g., Picker *et al.*, 1999; Gritsman *et al.*, 1999). For example, in the BMP-signaling pathway, several mutants have already been identified. These strains carry mutations in genes at different hierarchical levels of the BMP-signal cascade, ranging from the ligands to cytoplasmic signal transduction components (Hild *et al.*, 1999; Dick *et al.*, 2000; Schmid *et al.*, 2000). This allows both the specific contributions of each component of the pathway to be examined as well as their interactions or potential redundancy by making use of double (Dick *et al.*, 2000; Schmid *et al.*, 2000) and even triple mutants (Shimizu *et al.*, 2000).

[1] To whom correspondence should be addressed. Fax: +1 (626) 449-5163. E-mail: sefraser@caltech.edu.

0012-1606/01 $35.00

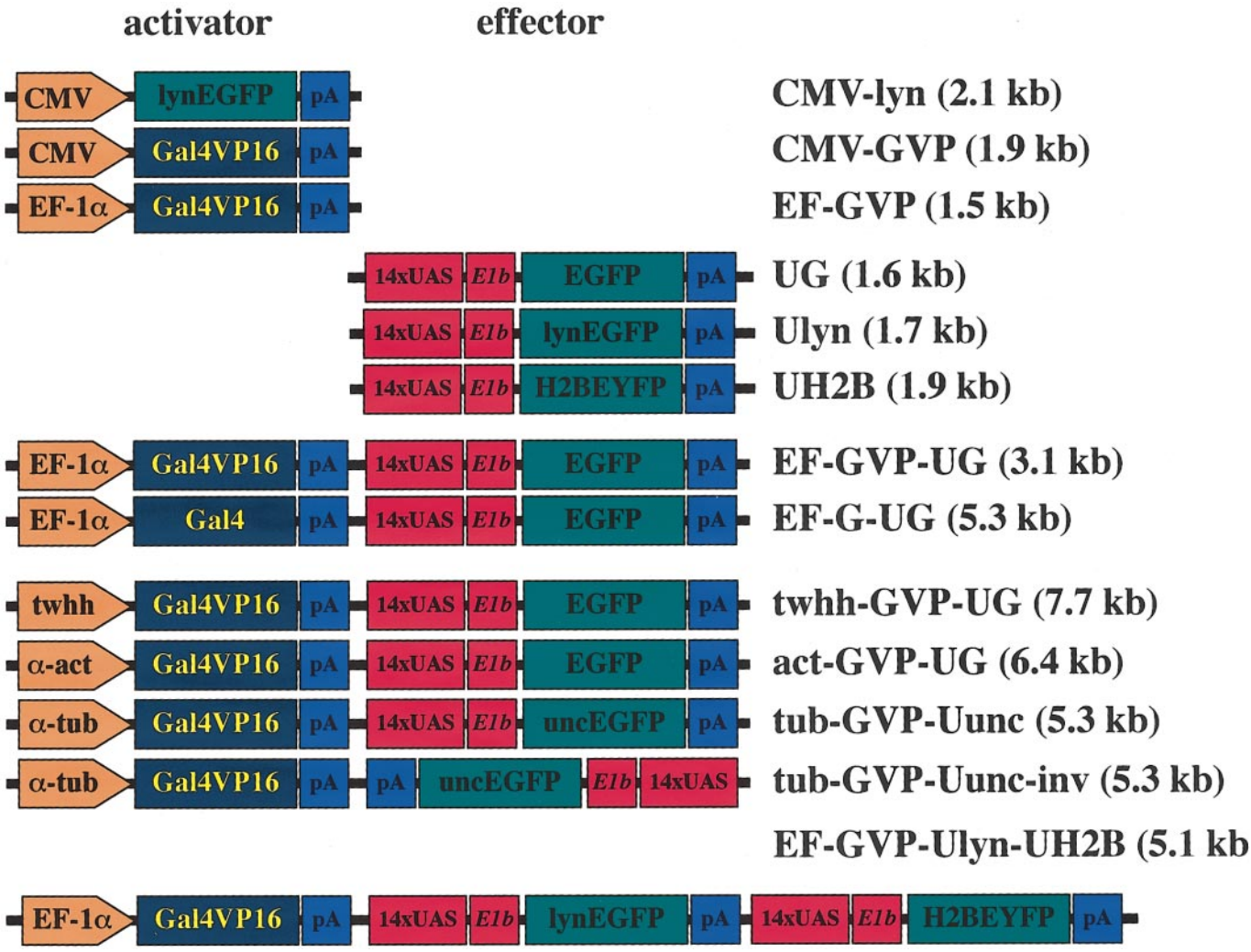

FIG. 1. Schematic representation of constructed injection vectors. Gal4- or Gal4-VP16-containing activator units are shown on the left, effector units on the right reflecting also their 5-prime to 3-prime order in the respective constructs. On the right following the schematic drawings, abbreviations for the respective constructs and their actual insert sizes are given. All inserts have been cloned into the pBluescriptII SK plasmid (Stratagene) with the exception of CMV-lyn and CMV-GVP, which were cloned into the pCS2+ vector, consisting mostly of pBluescriptII sequences (Rupp *et al.*, 1994). Similar functional sequences are shown in the same color (e.g., enhancer/promoter in orange, EGFP variants in green). Abbreviations: CMV, cytomegalovirus; EF-1α, elongation factor 1α; EGFP, enhanced green fluorescent protein; pA, SV40 polyadenylation site (from pCS2+), see also text. For construction details see also Materials and Methods.

An effective technique for transient transgenesis is needed for several reasons: (1) to explore the function of genes with no identified mutant strain by gene overexpression or perturbation; (2) to test consequences of gene overexpression to further characterize identified mutant strains; (3) to study the effects of regionally or temporally perturbing gene expression levels; (4) to rescue mutants in a tissue- and stage-specific manner; and (5) to effectively map *cis*-regulatory control elements for tissue-specific expression. mRNA-injections into single blastomeres at the one- or two-cell stage are very powerful but offer no spatio-temporal control of transgene expression; furthermore, the stages of expression are limited because injected mRNA is somewhat rapidly degraded. In principal, the injection of plasmids could complement these drawbacks of mRNA injection. They are stable over weeks in fish embryos (Winkler *et al.*, 1991). The injected plasmid gets distributed in a mosaic manner to the descendants of the injected cell. Therefore, ectopic expression by DNA injection allows one to study the phenotypic behavior of a transgene-expressing cell or cell cluster in a largely unaffected, wild-type environment. Moreover, gene expression from injection plasmids can be regulated spatio-temporally making use of identified tissue-specific regulatory elements. Despite these

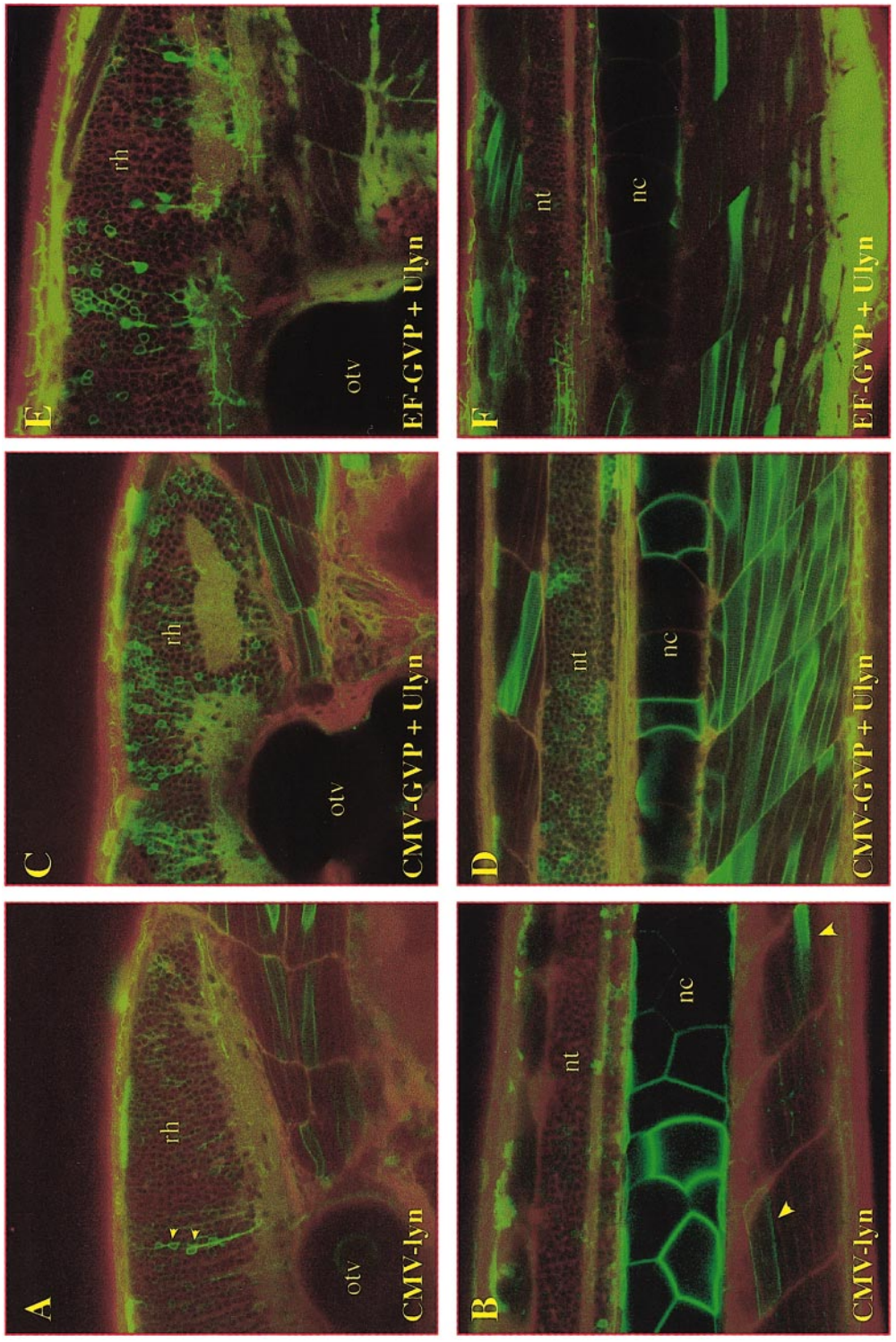

FIG. 2. Gal4-VP16-mediated expression in comparison to conventional DNA-construct expression. Lateral views of the hindbrain (A, C, E) and trunk (B, D, F) of CMV-lyn-injected (A, B), CMV-GVP/Ulyn-coinjected, and EF-GVP/Ulyn-coinjected embryos at 3 dpf. Pictures represent pseudocolored composites of single optical sections using confocal microscopy. EGFP-expressing cells are displayed in green. Embryos where counterstained with Bodipyceramide TexasRed (Molecular Probes) displayed in red to visualize overall morphology. Note the precise membrane-localization of the lynEGFP-fluorescence in expressing cells (e.g., neuron in A or muscle cells in D). Abbreviations: nc, notochord; nt, neural tube; otv, otic vesicle; rh, rhombencephalon.

potential advantages, injection of plasmid DNA is rarely used in fish embryos due to the very small number of cells that eventually express the transgene of interest at detectable levels.

To make plasmid DNA injection suitable for transient ectopic expression to address developmental questions, two goals have to be achieved: (1) The frequency of cells expressing transgenes at detectable levels has to be increased in order to raise the probability that the tissue of interest contains transgene expressing cells and (2) transgene-expressing cells must be easily detectable, ideally without sacrificing the injected specimen. This would take advantage of the transparency of the zebrafish embryo and allow the behavior of transgene-expressing cells to be studied *in vivo* in real-time. A potential solution can be adapted from the *Drosophila* Gal4-UAS-system (Fischer *et al.*, 1988; Brand and Perrimon, 1993), in which stable transgenic lines expressing the transcriptional activator Gal4 under control of an endogenous promoter (Gal4-driver/-activator lines) can be crossed to transgenic lines (Gal4-effector lines) which carry a transgene of interest under the control of Gal4-binding sites (UAS, upstream activating sequences). The progeny of this cross express the transgene strongly in a Gal4-dependent manner (Fischer *et al.*, 1988; Brand and Perrimon, 1993). This meets both of our challenges as expression is amplified via Gal4 and in principal more than one effector can be expressed at the same time. A direct application of the *Drosophila* approach by creating stable transgenic lines of the Gal4-UAS-system in zebrafish (Scheer and Campos-Ortega, 1999), while promising, shows only weak expression. Furthermore, the generation of stable transgenic lines is both space and time consuming.

Here, we apply an activator/effector system to the creation of transient transgenics in zebrafish. We find that the transcriptional activator Gal4-VP16, a fusion of the yeast Gal4-DNA-binding domain with the herpes simplex virus transcriptional activation domain VP16 (Sadowski *et al.*, 1988), is capable of driving GFP-expression in a strong manner in zebrafish embryos. Gal4-VP16-mediated expression significantly increases the frequency of detectable GFP-expressing cells most likely by increasing the expression level. Furthermore, activator and effector units can be placed on a single-injection construct, and the use of tissue-specific enhancers driving Gal4-VP16 results in tissue-specific transgene expression. To address our second goal, the easy detectability of transgene-expressing cells, we asked whether Gal4-VP16 can simultaneously drive the expression of two UAS-dependent transgenes from a single DNA construct in the same cell *in vivo*. We find that this approach results in strong coexpression of two transgenes in virtually all transgene-expressing cells. This allows one to easily trace transgene-expressing cells *in vivo* by virtue of their coexpression of the fluorescent reporter GFP. Interestingly, this expression system is applicable to a broad variety of established vertebrate model organisms.

MATERIALS AND METHODS

Maintenance of Fish

Raising, maintaining, and spawning of adult zebrafish were performed as described (Westerfield, 1995; Kimmel *et al.*, 1995).

Construction of Plasmids

CMVlyn. The lynGFP-coding region from pCDNA3-lynEGFP (Teruel *et al.*, 1999) was cloned as a *Hin*dIII-blunt/*Xba*I fragment into *Stu*I/*Xba*I-cut pCS2+ vector (Rupp *et al.*, 1994).

CMV-GVP. The coding region of the Gal4-VP16 fusion was isolated from pPC97-VP16 (obtained from David Stillman; Sadowski *et al.*, 1988) as a *Hin*dIII/*Not*I fragment, blunted and cloned into *Stu*I-cut pCS2+ (Rupp *et al.*, 1994).

EF-GVP. A *Bam*HI/*Not*I-blunt Gal4-VP16-pA-fragment was isolated from CMV-GVP and cloned into *Bam*HI/*Eco*RV-cut pBluescriptII SK (Stratagene) to obtain pBGal4-VP16pA. pBGal4-VP16pA was *Bam*HI-cut and blunted to insert a blunted *Xho*I/*Hin*dII fragment (ca. 500 bp) of the *Xenopus* EF-1α-promoter isolated from XEX-76/eGFP (Dynes and Ngai, 1998).

UG. An *Eco*RI-blunt/*Apa*I EGFPpA fragment was isolated from pCSEGFP and cloned into *Xho*I-blunt/*Apa*I-cut pBluescriptII SK (Stratagene) to obtain pBEGFP. This was subsequently *Sal*I-blunt/*Hin*dIII-cut to insert a 14-mer of UAS Gal4-binding sites (Rorth, 1996) fused to the fish basal promoter *E1b* (Scheer and Campos-Ortega, 1999) as a *Hin*dIII/*Sma*I fragment (ca. 500 bp).

Ulyn. An *Eco*RI/*Not*I lynEGFPpA-fragment was isolated from CMVlyn and cloned into the blunted *Sal*I-site of pBluescriptII SK (Stratagene). Subsequent digestion with *Cla*I-blunt/*Hin*dIII allowed the insertion of 14xUAS*E1b* (see above) as *Hin*dIII/*Sma*I fragment.

UH2B. The coding region of a histone 2B-EYFP-fusion (H2BEYFP; Kanda *et al.*, 1998; and R. Lansford, unpublished results) was cloned as *Xho*I/*Not*I-blunt fragment into *Xho*I/*Xba*I-blunt-cut pCS2+ (Rupp *et al.*, 1994). Out of this vector, the H2BEYFPpA fragment was released as *Xho*I/*Apa*I fragment and cloned into *Xho*I/*Apa*I-digested pBluescriptII SK (Stratagene) to obtain pBH2BEYFPpA. pBH2BEYFPpA was *Hin*dIII/*Sal*I-blunt-digested to introduce 14xUAS*EIb* (see above) as *Hin*dIII/*Sma*I fragment.

EF-GVP-UG. pBGal4-VP16pA (see cloning of EF-GVP above) was *Bam*HI-digested and blunted to insert a blunted *Xho*I/*Hin*dII fragment (ca. 500 bp) of the *Xenopus* EF-1α-promoter isolated from XEX-76/eGFP (Dynes and Ngai, 1998). From this pBEF-1αGal4-VP16pA plasmid, the EF-1αGal4-VP16pA fragment was released by *Hin*dIII/*Not*I digest, blunted, and introduced into *Eco*RV-cut UG (see above).

EF-G-UG. The Gal4 coding region from pBSGal4 was isolated as a blunted *Hin*dIII fragment and cloned into the blunted *Xba*I site of pCS2+ (Rupp *et al.*, 1994). Subsequent digestion with *Eco*RI/*Not*I-blunt released the 3.1-kb Gal4pA fragment to be cloned into *Eco*RV/*Eco*RI-cut pBluescriptII SK (Stratagene). Into this vector, a *Xho*I/*Hin*dII fragment (ca. 500 bp) of the *Xenopus* EF-1α-promoter isolated from XEX-76/eGFP (Dynes and Ngai, 1998) was introduced into the blunted *Bam*HI site to obtain pBEF-1αGal4. The effector cassette 14xUAS*EIb*EGFPpA was isolated from UG (see above) as *Hin*dIII/*Apa*I fragment and cloned into *Hin*dIII/*Apa*I-digested pBEF-1αGal4.

twhh-GVP-UG. The effector cassette 14xUAS*EIb*-EGFPpA from UG (see above) was isolated as *Hin*dIII/*Apa*I fragment and cloned behind Gal4-VP16pA in *Hin*dIII/*Apa*I-cut pBGal4-VP16pA

(see EF-GVP above). Subsequently, the Gal4-VP16pA-14xUAS*EIb*-EGFPpA module was released as a blunted *Sac*II fragment and cloned into *Bam*HI/*Xho*I-cut pCS-twhh-*β*-gal-vec, replacing the coding region of the *β*-galactosidase gene (Du *et al.*, 1997).

act-GVP-UG. A Gal4-VP16pA fragment was isolated by *Cla*I-blunt/*Not*I digestion of CMV-GVP (see above) and cloned into *Eco*RI-blunt/*Not*I-cut *α*-p-SK-vector, which contains ca. 3.9 kb upstream regulatory sequences of the muscle-specific *α-actin* gene from zebrafish (Higashijima *et al.*, 1997). Subsequent digestion with *Not*I-blunt/*Sac*II allowed us to clone the effector cassette 14xUAS*EIb*-EGFP, isolated as *Sma*I/*Sac*II fragment from UG (see above), behind *α*act::Gal4-VP16pA.

tub-GVP-Uunc/tub-GVP-Uunc-inv. A Gal4-VP16pA PCR fragment was obtained using the primer 5′-TTGGATCCG-ATGAAGCTACTGTCTTCTATCG-3′ and 5′-AGCGCGCAATT-AACCCTCACTAAAGG-3′ on CMV-GVP (see above) as template. Subsequent digestion of this fragment with *Bam*HI/*Not*I allowed the fusion of the Gal4-VP16 open reading into the reading frame of the second exon of goldfish *alpha1-tubulin* in −1696*α*1TIpEGFP vector (Hieber *et al.*, 1998), which had been digested with *Bam*HI/*Not*I. Subsequently, the *α*tubulin-Gal4-VP16pA fragment was released as blunted *Eco*47III/*Not*I fragment and cloned into the blunted *Xba*I site of pBluescriptII SK (Stratagene). In this pBtub-GVP-vector, Gal4-VP16 is expressed under the control of regulatory elements from the neuron-specific *alpha1-tubulin* gene from goldfish. To generate the Uunc-effector cassette, a blunted unc76:EGFPpA fragment from XEX-76eGFP (Dynes and Ngai, 1998) was isolated by *Hin*dIII/*Bgl*II digest and cloned into the blunted *Xho*I site of pBluescriptII SK (Stratagene). A *Hin*dIII-blunt/*Sma*I 14xUAS*E1b* fragment (see above) was subsequently inserted into the blunted *Sal*I site in front of uncEGFPpA. The effector cassette 14xUAS*EIb*-uncGFPpA was released as a *Pst*I/*Apa*I fragment, blunted, and cloned behind *a*tubGal4-VP16pA into the blunted *Pst*I site of pBtub-GVP. Depending on the orientation of the Uunc-effector cassette, the obtained vectors were assigned as tub-GVP-Uunc or tub-GVP-Uunc-inv (see also Fig. 1).

EF-GVP-Ulyn-UH2B. The effector cassette 14xUASEIb-H2BEGFPpA from UH2B (see above) was isolated as a blunted *Hin*dIII/*Apa*I fragment and cloned into the blunted *Xho*I site of Ulyn (see above). Subsequently, *Eco*RV digestion allowed the activator cassette EF-1*α*Gal4-VP16pA to be cloned as a blunted *Hin*dIII/*Not*I fragment from EF-GVP (see above) in front of both effector cassettes.

Further structural details on the plasmids are available upon request.

Microinjection and Embedding of Embryos

Capped Gal4-mRNA was transcribed from EF-G-UG as described (Wormington, 1991) by using a cap structure analog (New England Biolabs).

Plasmid DNA was prepared by using the EndoFree plasmid kit (Qiagen) or the Concert plasmid purification kit (Gibco BRL). Subsequently, plasmid DNA was subjected to gel electrophoresis and further purified using the GenecleanII kit (Bio101). Circular plasmid was injected into single cell-stage embryos at a concentration of 2.5 pg/embryo (30 ng/*μ*l) in 100 mM KCl containing 0.02% Phenol Red. Injected embryos were raised in 30% Danieau solution/1% penicillin/streptomycin to the desired stage. To block pigmentation, phenylthiourea was added to Danieau medium when the embryos reached the 10-somite stage (ca. 14 hpf). Injection of linearized plasmid DNA gave no significant difference in expression frequency or intensity.

To demonstrate that comparable amounts of EF-GVP-UG and EF-G-UG were injected (see Fig. 4), 3.3 *μ*l, which is equivalent to 100 ng of plasmid DNA, from the individual injection solutions were subjected to a *Sac*II/*Xmn*I-restriction digest after having performed the single cell injections. These digests release the activator/effector inserts from the pBluescriptII SK-vector backbone. Subsequent gel electrophoresis displayed an equivalent intensity of both insert and vector backbone fragments, showing that equivalent amounts of plasmids were used in the respective injection solutions.

To permit imaging, embryos were dechorionated with forceps, anesthetized with 0.05% Tricaine as described (Westerfield, 1995) and mounted on a coverslip in 1.2% low-melting agarose (Sigma). To visualize the overall morphology for confocal microscopy, injected embryos were counterstained by soaking in 0.001% BodipyCeramideTR (Molecular Probes), which labels the extracellular matrix and cell membranes.

Xenopus embryos were injected with 50 ng/*μ*l of plasmid DNA into one blastomere at the two-cell stage and raised to the desired stage.

RNA in Situ Hybridization

RNA *in situ* hybridization was performed as described (Hauptmann and Gerster, 1994).

Cell Culture

Zebrafish embryos were dechorionated at 36 h postfertilization (hpf), anesthetized with 0.05% Tricaine, transferred into a microcentrifuge tube, and overlaid with culture medium (L15/1% L-glutamine/1% penicillin/streptomycin/10% fetal bovine serum, FBS). Subsequently, the embryos were dissociated by pipetting them up and down, and the cell suspension was plated onto culture dishes that had been coated with 0.5% gelatin. Primary zebrafish cells were incubated at 28°C in culture medium. Within 24 h, cells attached to the coated bottom of the culture dish and started to develop characteristic morphologies. The vast majority of cells differentiated into fibroblasts as judged by morphological criteria. Rarely, single melanocytes and neurons could be identified. Primary zebrafish cells were lipofected with plasmid DNA by using Effectene Transfection Reagent (Qiagen) according to the manufacturer's instructions. Cells were analyzed around 18 h after transfection. Bodipy-ceramide-TR (Molecular Probes) counterstaining was performed by adding the dye to the culture medium in a 1:100,000 dilution for 15 min.

Primary cultures of chicken embryo fibroblasts (CEF) were cultivated at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS)/1% penicillin and streptomycin (Morgan and Fekete, 1996). For lipofection of CEF cells with plasmid DNA, lipofectamine was used according to the manufacturer's instructions (Gibco BRL).

Mouse 3T3 cells (American Type Culture Collection catalog no. CRL-1658) were cultured in DMEM containing 10% fetal calf serum (FCS)/1% penicillin and streptomycin/1% L-glutamine. Lipofection with plasmid DNA was performed by using the Superfect Transfection Reagent (Qiagen) according to the manufacturer's instructions (Gibco BRL).

Human 293 GPG cells (Ory *et al.*, 1996) were cultured in DMEM containing 10% FCS/1% penicillin and streptomycin/1% L-glutamine/1% nonessential amino acids. For lipofection of 293 GPG cells with plasmid DNA, Superfect Transfection Reagent

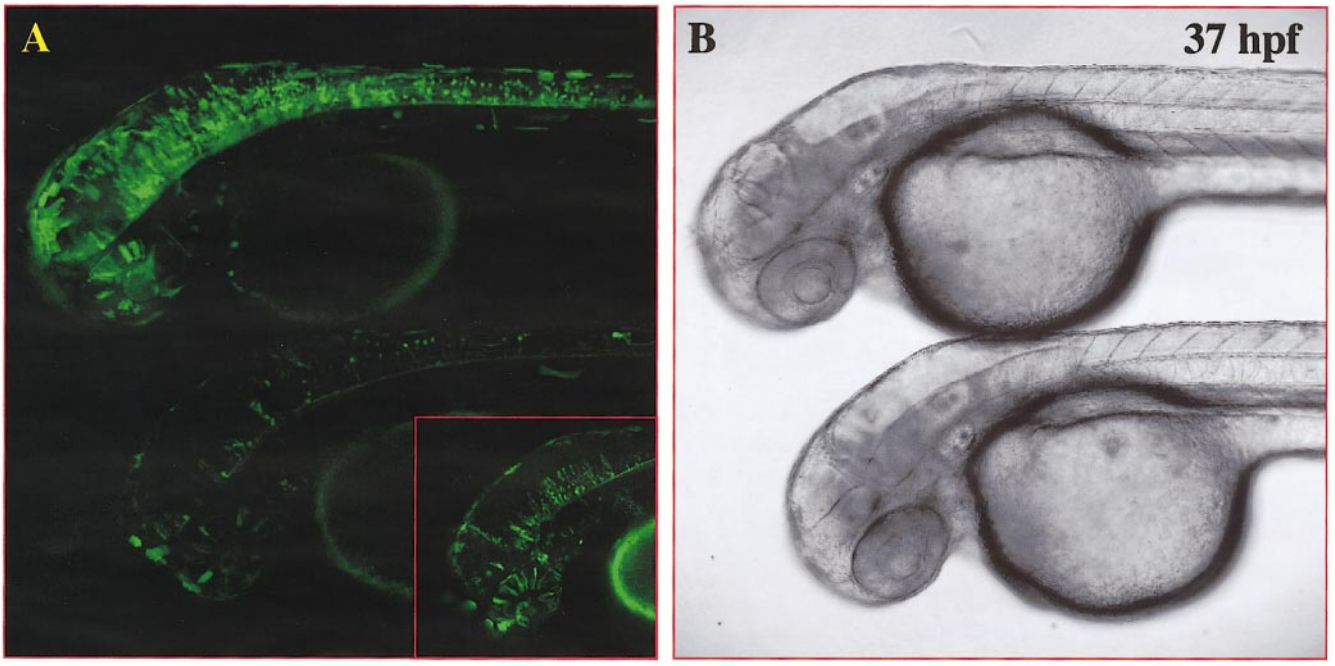

FIG. 3. Combined Gal4-VP16-activator/effector constructs result in strong EGFP expression in numerous cells of DNA-injected embryos. Single optical confocal microscopy section (lateral view) focusing on the nervous system of injected embryos at 37 hpf. Two embryos were embedded together in low-melting agarose to allow direct comparison of EGFP-expression levels and number of EGFP-expressing cells in a single optical section. Fluorescent (A pseudocolored in green) and transmitted light (B) images were taken at the same focal plane without moving the embryos. (A, B) EF-GVP-UG-injected embryos of the same batch. EGFP expression in EF-GVP-UG-injected embryos can vary strongly in intensity and frequency from a high number of embryonic GFP-expressing cells (A, upper embryo) to very few EGFP-expressing cells (A, lower embryo). Higher laser power and increased contrast, however, revealed a higher frequency of EGFP-expressing cells in the lower embryo than judged by the initial conditions (see A, inset).

(Qiagen) was used according to the manufacturer's instructions (Gibco BRL).

Optics

Whole-mount *in situ* hybridization patterns were analyzed by using a Roche ProgRes digital camera attached to a Zeiss Axioplan microscope. Transfected cells were analyzed with a Zeiss Axiovert epifluorescence microscope. Laser confocal microscopy images were obtained by using a Zeiss LSM 410 laser-scanning microscope. Subsequently, images were processed and pseudocolored in Photoshop 5.0 (Adobe).

RESULTS

Gal4-VP16-Mediated Expression Leads to Strong and Frequent Transgene Expression in Living Zebrafish Embryos

Conventional expression vectors were compared to Gal4-VP16-mediated expression with regard to their potential to drive transgene expression after injection into one-cell-stage zebrafish embryos. The conventional vector CMVlyn (Fig. 1) encodes a membrane-localized variant of the enhanced Green Fluorescent Protein (EGFP; Clontech) adding a membrane attachment site from the human lyn receptor tyrosine kinase to the EGFP N terminus (Teruel *et al.*, 1999). Expression is driven by the cytomegalovirus enhancer (CMV), which leads to strong expression in most vertebrate cells (e.g., Winkler *et al.*, 1991; Okada *et al.*, 1999). At 3 days postfertilization (dpf), strong EGFP expression, scored by confocal microscopy in living embryos, could be observed in only a small number of scattered cells (Fig. 2A, hindbrain neurons; Fig. 2B, muscle cells, notochord).

To test the relative strength of Gal4-VP16-mediated expression, two types of plasmids were generated: activator constructs containing the transcriptional activator Gal4-VP16 (Sadowski *et al.*, 1988) under control of different ubiquitous or tissue-specific enhancers/promoters; effector constructs containing a 14-mer of optimized Gal4-binding UAS sites (Rorth, 1996) followed by a synthetic basal promoter (*E1b* derived from the carp *β-actin* gene; Scheer and Campos-Ortega, 1999) and various versions of *egfp* (Fig. 1). In the first test, ubiquitous activator plasmids CMV-GVP and EF-GVP were coinjected into single-cell-stage zebrafish embryos with the Ulyn effector plasmid (Fig. 1).

Strong membrane-localized fluorescence could be observed in numerous cells of all germ layers in a much higher frequency than achieved with CMVlyn-injections (compare Fig. 2A with Figs. 2C and 2E and Fig. 2B with Figs. 2D and 2F). In the nervous system, Gal4-VP16-mediated expression resulted in both a much higher number of GFP-expressing cells and a stronger expression in comparison to CMV-lyn (compare Figs. 2A, 2C, and 2E; the hindbrain of the CMV-lyn injected embryo displays only two to three strong expressing neurons; see arrowheads). The extent of the mosaicism varied strongly between injected embryos from the same batch (see also below, Figs. 3A and 3B). Embryos injected with the Ulyn effector construct alone displayed no expression of lynEGFP (or, in rare cases, very weak expression in a very low frequency). This indicates that expression of the Ulyn effector is dependent on Gal4-VP16; the basal promoter *E1b* is not capable of driving expression on its own. Thus, Gal4-VP16-mediated expression leads to a substantially higher frequency of detectable transgene expressing cells in transient transgenic zebrafish embryos than conventional expression vectors.

Gal4-VP16-Effector and -Activator Can Be Placed on the Same Expression Construct

To ask whether the high frequency of Gal4-VP16-amplified transgene expression is maintained when the activator and the effector cassettes are localized on a single expression vector, we cloned the EF-1α-Gal4-VP16-activator fragment directly in front of the 14xUAS-*E1b*-EGFP-encoding effector cassette in UG to obtain EF-GVP-UG (Fig. 1).

Similar to the coinjection experiment, EF-GVP-UG-injected embryos showed widespread EGFP fluorescence beginning at 5 h postfertilization (not shown) and displayed an intense EGFP fluorescence in the cytoplasm of numerous cells at 37 hpf (Figs. 3A and 3B). The intense EGFP fluorescence lasted over several days to weeks (longest analyzed embryo: 14 days) and seemed not to decrease over time. Again, the nervous system displayed a high frequency of strongly transgene-expressing cells. The number of EGFP-expressing cells in injected embryos from the same batch varied from almost none to an estimated 5–10% of the total number of embryonic cells (Figs. 3A and 3B, compare upper and lower embryos). These findings indicate that, in zebrafish, Gal4-VP16 is able to initiate and maintain expression of Gal4-dependent genes even if the activator and effector cassettes are positioned very close by.

Expression of the Gal4 and Gal4-VP16 Activators Is Tolerated during Zebrafish Embryogenesis

Strong transcriptional activators are capable of causing nonspecific promoter squelching (Gill and Ptashne, 1988) and can lead to retarded or malformed embryogenesis (Argenton *et al.*, 1996). When high injection concentrations (4.2 pg/embryo, 50 ng/μl) were used, some of the EF-GVP-UG-injected embryos indeed developed malformations (e.g., bent body axis or clusters of necrotic cells). However, malformation development did not correlate with EGFP expression levels. To address in more detail whether Gal4-VP16 expression interferes with normal embryonic development, EF-GVP-UG was injected at a moderate concentration of 2.5 pg/embryo (30 ng/μl) at the single-cell stage and the embryos were scored for malformations around 36 hpf. From six consecutive injection experiments, the vast majority, 91.6% (175/191), of EF-GVP-UG-injected embryos developed indistinguishable from wild-type embryos; 7.9% (15/191) developed morphological defects of varying severity (Fig. 4). A total of 98.9% (187/189) of the EF-GVP-UG-injected embryos were able to hatch out of their chorion, indicating that most malformations were not so severe as to prevent these embryos from hatching. This rate of defects is not significantly different from that obtained from injecting a weaker activator EF-G-UG in which Gal4-VP16 is replaced by the commonly used yeast endogenous Gal4. Of 189 EF-G-UG-injected (2.5 pg/embryo, 30 ng/μl) embryos, 94.2% (178/189) developed indistinguishable from wild-type embryos; 4.8% (9/189) displayed morphological defects; and 99.5% (186/187) were able to hatch (Fig. 4).

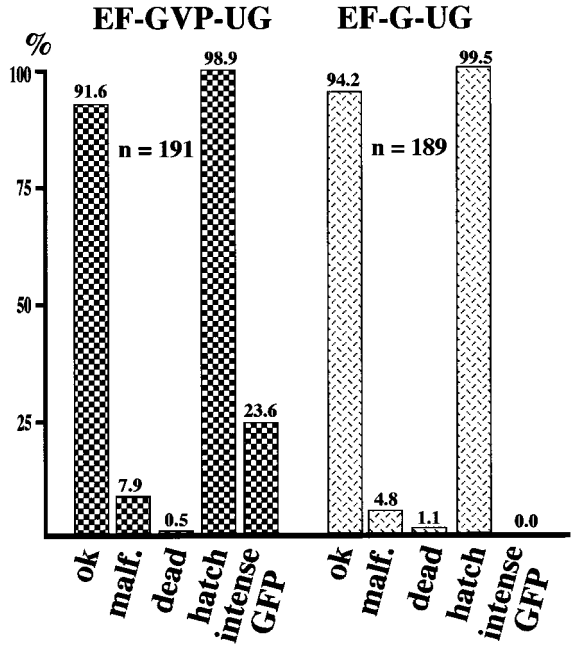

FIG. 4. Comparison of embryonic morphology and EGFP-expression levels between EF-G-UG and EF-GVP-UG-injected zebrafish embryos. Embryos from six consecutive injection experiments were visually scored around 36 hpf for correct embryonic development and EGFP fluorescence. The respective percentages of embryos displaying embryonic development indistinguishable from wild-type embryos, defective embryogenesis, as well as EGFP intensity are represented by vertical columns (EF-GVP-UG-injected embryos, columns in bricks; EF-G-UG-injected embryos, columns in diagonal stripes).

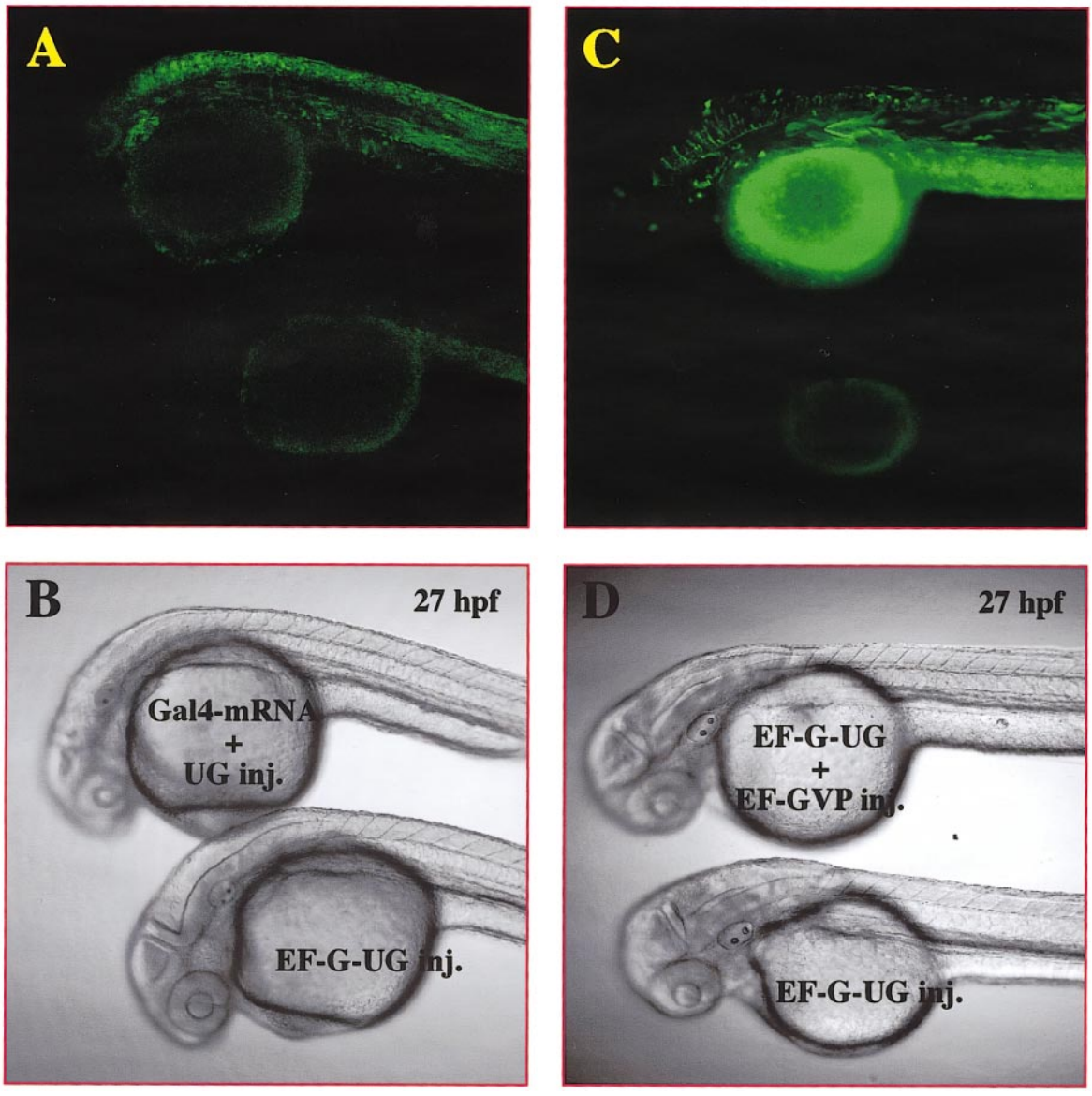

FIG. 5. Gal4 is a weaker transcriptional activator in zebrafish embryos than Gal4-VP16. (A–D) Single optical confocal microscopy sections (lateral view) of injected embryos at 27 hpf. Two embryos were embedded together in low-melting agarose to allow direct comparison of GFP-expression levels and number of GFP-expressing cells in a single optical section. Fluorescent (A and C, pseudocolored in green) and transmitted light (B, D) images were taken at the same focal plane without moving the embryos. (A, B) While Gal4-mRNA/UG-coinjected embryos display a broad variety of EGFPexpression, including embryos with a high frequency of EGFP-expressing cells (upper embryo), EF-G-UG-injected embryos show no or very dim EGFP fluorescence in a few cells (lower embryos). In contrast to EF-GVP-UG-injected embryos (see Fig. 3A, lower embryo), increase in laser power and contrast did not reveal an increase in the frequency of EGFP-expressing cells in EF-G-UG-injected embryos (not shown). (C, D) EF-GVP/EF-G-UG-coinjected embryos display a broad variety of EGFP expression, including embryos with strongly expressing cells in a high frequency (upper embryos), while EF-G-UG-injected embryos show no or very dim EGFP fluorescence in a few cells (lower embryo).

EF-GVP-UG- and EF-G-UG-injected embryos showed dramatic differences in the intensity and frequency of EGFP expression. A total of 23.6% (45/191) of EF-GVP-UG-injected embryos displayed high levels of EGFP expression in numerous cells distributed over the entire body (Fig. 3A, upper embryo). In contrast, EF-G-UG-injected embryos displayed no or only a dim EGFP fluorescence in the yolk and few cells inside the embryonic body (Figs. 5A and 5C, lower

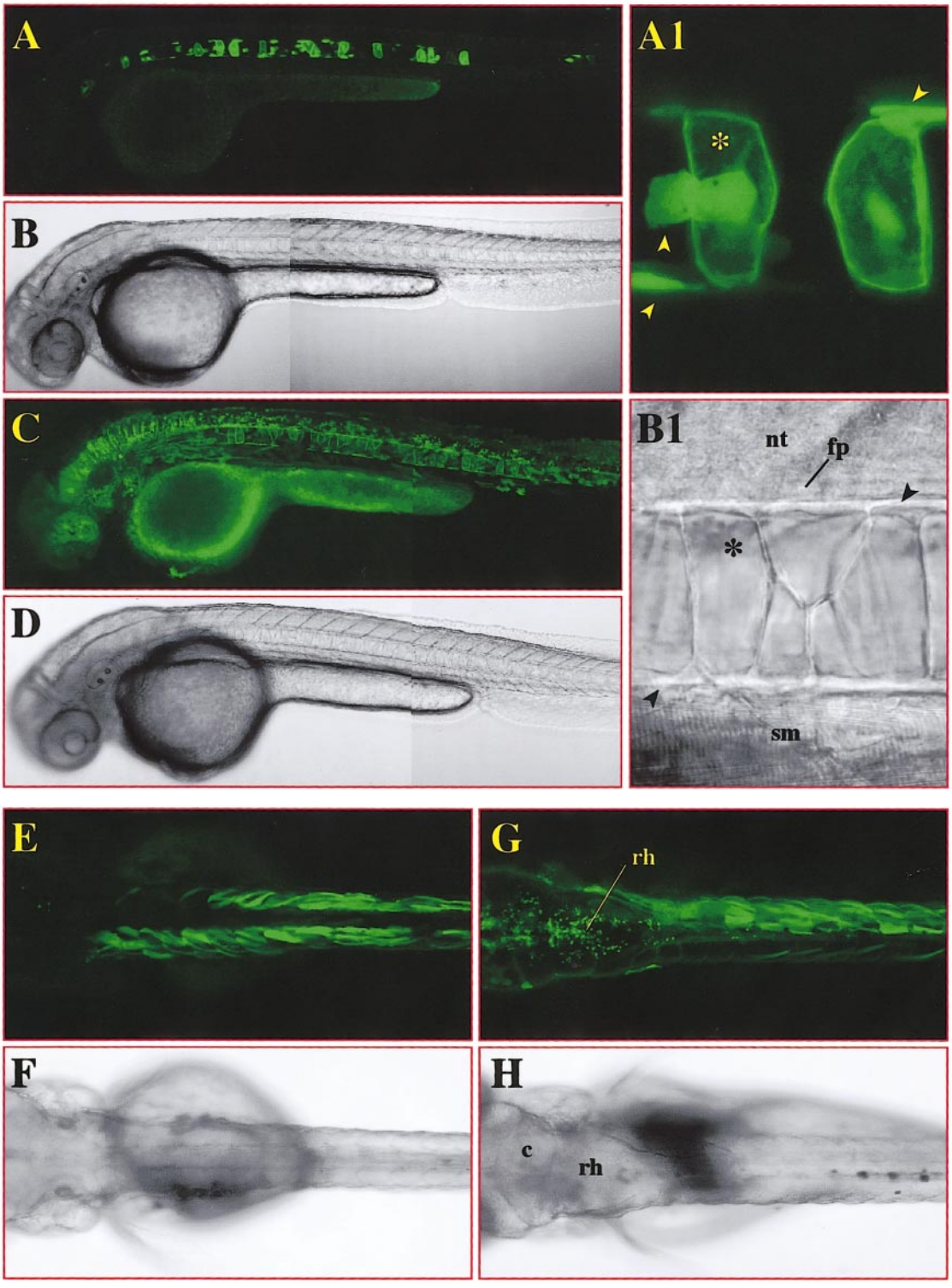

FIG. 6. Gal4-VP16-activator/effector constructs can drive expression in different mesodermal tissues. (A–D) Lateral views of injected zebrafish embryos at 27 hpf; images were taken by using confocal microscopy. Pictures represent merged stacks of 10 individual optical sections covering 90 μm in depth. (A, B) Same twhh-GVP-UG-injected embryo imaged with fluorescent (A, EGFP fluorescence pseudocolored in green) and transmitted light (B) without moving the specimen. (A1, B1) Magnification of notochordal region of images in A and B, respectively, to demonstrate EGFP fluorescence in vacuolated cells (*) in the center of the notochord and notochordal sheath cells

embryos). None of the 189 EF-G-UG-injected embryos expressed EGFP at levels comparable to EF-GVP-UG-injected embryos.

This lack of strong EGFP fluorescence in EF-G-UG-injected embryos is not due to the failure of the EF-G-UG vector. First, mRNA *in situ* hybridization showed that Gal4 is expressed in EF-G-UG-injected embryos (not shown). Next, Gal4-mRNA (11.5 pg/embryo, 140 ng/μl), using EF-G-UG itself as a template for transcription, coinjected with the effector plasmid UG (2.5 pg/embro, 30 ng/μl; Fig. 1) resulted in variable but strong EGFP fluorescence, ranging from almost no cells (not shown) to a high frequency of brightly fluorescent cells (Fig. 5A, upper embryo; Fig. 5B, brightfield). This indicates that the activator Gal4 from the EF-G-UG plasmid is capable of driving UAS-mediated GFP expression if Gal4 is expressed in very high amounts. EGFP fluorescence levels in Gal4-mRNA/UG coinjected embryos did not reach the intensities obtained with EF-GVP-UG injections, reflecting the stronger activation potential of Gal4-VP16 over Gal4. Finally, EF-G-UG was coinjected with the activator EF-GVP (2 pg/embryo, 25 ng/μl, each; Fig. 1). These coinjected embryos displayed a much stronger EGFP fluorescence (Fig. 5C, upper embryo) than EF-G-UG-injected embryos (Fig. 5C, lower embryo; Fig. 5D, brightfield). The strong and high frequency of EGFP expression in the coinjected embryos suggests that Gal4-VP16, expressed from the EF-GVP-vectors, can transactivate EGFP expression from the EF-G-UG vector. Combined, these findings show that both the effector cassette of EF-G-UG and the activator cassette are functional.

Tissue-Specific Enhancers Can Direct Gal4-VP16-Amplified EGFP Expression

To address whether tissue-specific enhancers can be used to mediate Gal4-VP16-amplified expression in distinct tissues of transient transgenic zebrafish embryos, the EF-1α-promoter in EF-GVP-UG was exchanged with various tissue-specific enhancer/promoter elements. Regulatory elements from the *zebrafish tiggy winkle hedgehog* (*twhh*) gene, reported to drive expression in the notochord (Du *et al.*, 1997), were used to create twhh-GVP-UG (Fig. 1). At 12 hpf, when scored by confocal microscopy, the twhh-GVP-UG-injected embryos displayed expression of EGFP in a very small number of cells (not shown). By 37 hpf, strong expression of EGFP was observed almost exclusively in the notochord (Figs. 6A and 6B), in both the characteristically vacuolated notochord cells (Figs. 6A1 and 6B1, star) and the flat sheath cells surrounding the notochord (Figs. 6A1 and 6B1, arrowhead). Whether the few cells expressing EGFP at earlier stages are notochordal precursors is currently being addressed by time-lapse analysis. Rarely, a few cells (1–5) outside the notochord expressed EGFP (not shown). These cells could be found in all germ layers and showed no indication for a preferential confinement to a particular tissue. The regional expression of twhh-GVP-UG was not due to a nonrandom distribution of the injected DNA. Coinjection of twhh-GVP-UG, together with the ubiquitously expressing activator EF-GVP, resulted in strong EGFP-expression in tissues of all germ layers, such as the nervous system, muscle cells, and in the yolk syncytial layer (Figs. 6C and 6D).

Similarly, a Gal4-VP16-UAS-EGFP module was cloned behind the muscle-specific $\alpha-$*actin*-enhancer/promoter element (Higashijima *et al.*, 1997) to create the construct act-GVP-UG (Fig. 1). Almost all of the act-GVP-UG-injected embryos showed strong expression of EGFP in a very high frequency in the muscles of the trunk at 3 dpf (Figs. 6E and 6F). Notochord cells almost never expressed EGFP. Outside the mesoderm, significant EGFP expression was only observed in the nervous system, varying from almost no expression (Figs. 6E and 6F) to a significant number of EGFP-positive cells in the central nervous system (Figs. 6G and 6H, rhombencephalon).

Neuronal-specific expression was directed with the α-*tubulin* enhancer fragment from goldfish (Hieber *et al.*, 1998). In addition, the EGFP-coding region was replaced by the unc76:GFP fusion to create tub-GVP-unc (Fig. 1). This unc76:EGFP fusion protein has recently been reported to be localized predominantly in neuronal processes of differentiating zebrafish neurons optimizing the visualization of outgrowing axons (Dynes and Ngai, 1998). In contrast to the very few neurons that express GFP in embryos that had been injected with a construct directly driven by the α-*tubulin* enhancer (-1696α1TIpEGFP; Fig. 7A) (Hieber *et al.*, 1998), tub-GVP-unc-injected embryos displayed intense EGFP-fluorescence at 3 dpf. The expression was predominantly localized in the nervous system (Figs. 7B and 7C), and was mosaic ranging from few labeled neurons to a high number of uncGFP-expressing neurons. In many cases,

(arrowhead) surrounding the vacuolated cell type. (C, D) Same twhh-GVP-UG/EF-GVP-coinjected embryo imaged with fluorescent (C, EGFP fluorescence pseudocolored in green) and transmitted light (D) without moving the specimen. Note intense ubiquitous EGFP expression in comparison to notochord-specific twhh-GVP-UG-injected embryo shown in (A). This indicates that injected twhh-GVP-UG is distributed in cells over all germ layers but activates expression almost exclusively in the notochord. (E–H) Dorsal views of act-GVP-UG-injected embryos at 3 dpf; images were taken by using confocal microscopy and represent single optical sections. Pictures in (E, F) and (G, H) show the same embryo imaged with fluorescent (E and G, EGFP expression pseudocolored in green) and transmitted (F, H) light. Note almost exclusive EGFP expression in muscles of embryo shown in (E and F), while the embryo shown in (G and H) displays also expression in numerous cells of the hindbrain. Abbreviations: c, cerebellum; fp, floorplate; nt, neural tube; rh, rhombencephalon; sm, somitic muscles.

axon tracts were brightly labeled. The higher frequency of neuronal-specific EGPF expression in tub-GVP-unc-injected embryos (compare Figs. 7A, 7B, and 7D) offers the chance to easily perform time-lapse studies on clusters of neurons during embryonic development (R.W.K. and S.E.F., unpublished data). These findings show that neuronal-specific Gal4-VP16-activator/effector constructs (Figs. 7B and 7D) result in a much higher frequency of detectable transgene expression in the nervous system of zebrafish embryos in comparison to both uniform (Fig. 2A) or neural-specific (Fig. 7A) conventional vectors.

EGFP fluorescence in tub-GVP-unc-injected embryos could be observed outside the nervous system mostly in muscle cells and rarely in endodermal tissues (Fig. 7B). The uncEGFP-expressing cells appeared before the onset of axonogenesis. This suggests that the isolated *α-tubulin* enhancer fragment is lacking some repressive regulatory elements needed to recapitulate the complete spatio-temporal expression pattern of the *α-tubulin* gene. Restriction of the uncEGFP-fluorescence to neuronal cells became more striking with ongoing embryogenesis.

A possible complication of this approach for controlling expression of uncGFP from such a binary activator/effector construct is RNA-readthrough during transcription. To test this possibility, we inverted the effector cassette Uunc. Injection of this tub-GVP-Uunc-inv vector (Fig. 1) resulted in intense uncGPF-fluorescence that was predominantly restricted to the nervous system (Figs. 7D and 7E) similar to tub-GVP-Uunc-injected embryos. These findings indicate that transcription of the effector cassette is independent of RNA-readthrough but controlled by Gal4-VP16 binding and activation.

Gal4-VP16 Is Capable of Simultaneously Driving Expression of Two Effectors from the Same Construct

The activator Gal4-VP16 should be capable of driving the expression of two or more different UAS-effector cassettes. We tested this by cloning two different UAS-EGFP effectors behind the activator unit of the EF-GVP construct, resulting in a single expression vector, EF-GVP-Ulyn-UH2B (Fig. 1). The two effector units can be scored without confusion because they contain EGFP variants that are localized in different subcellular compartments. The lynEGFP localizes to the cytoplasmic membrane (e.g., Fig. 2B); H2BEYFP encodes a fusion of the human histone2B with the enhanced yellow fluorescent protein (Hein and Tsien, 1996) and localizes to the nucleus (see Figs. 9A–9C; Kanda *et al.*, 1998; and R. Lansford, unpublished results).

To obtain a simple analytical system, a primary zebrafish cell culture was established. First, the correct localization of the EGFP variants in zebrafish cells was confirmed by cotransfection experiments (for details, see legend to Fig. 8). Both variants were effectively sorted and led to precisely restricted subcellular fluorescence [nuclear-localized H2BEYFP in 94.2% (339/360) of fluorescent cells, Figs. 8A–8C; membrane-localized lynEGFP in 99.4% (358/360) of fluorescent cells; see Figs. 8D–8F]. H2BEYFP and lynEGFP expression/coexpression can be scored by three different criteria: the morphology (compare fluorescent bean-like nucleus; Figs. 8G and 8J with cytoplasmic membrane labeling including filopodia, Figs. 8I and 8L), the size and the brightness (nuclei are usually brighter, see Figs. 8H and 8K) of the fluorescent subcellular compartment. In two independent EF-GVP-Ulyn-UH2B-transfection experiments of cultured zebrafish cells, the vast majority (94.2%, 622/660) of fluorescent cells displayed coexpression of both lynEGFP and H2BEYFP. Membranous (3.0%, 20/660) or nuclear (2.7%, 18/660) fluorescence alone was only found in a small fraction of the fluorescent cells. This indicates that Gal4-VP16 can drive very efficiently the simultaneous expression of two UAS-dependent transgenes inside an individual cell.

To address whether coexpression of both effectors occurs also *in vivo*, zebrafish embryos were injected with EF-GVP-Ulyn-UH2B (Fig. 1) and were analyzed by confocal microscopy at 4 dpf. These embryos displayed intense fluorescence in a high number of cells all over the body (Fig. 9A). Numerous cells could be observed that showed fluorescence in both the nucleus (bright punctuate labeling) and the plasma membrane (weaker staining outlining the cell shape). Skin (Figs. 9B–9D) and muscle (Figs. 9E–9G) cells show a clear separation of membrane and nucleus and were thus analyzed in more detail at a higher magnification. Most of the cells analyzed showed coincident nuclear and membrane-localized fluorescence, highlighting the polygonal morphology of skin cells (Fig. 9C) or the syncytial nature of muscle cells containing more than one nucleus (Fig. 9E, see star). Thus, Gal4-VP16 is capable of reliably coactivating several UAS-dependent transgenes from a single construct in cultured cells and living embryos at a high frequency.

Gal4-VP16 Can Be Used to Drive Simultaneous Coexpression of Two Transgenes in a Wide Variety of Vertebrates

To address whether EF-GVP-Ulyn-UH2B can drive the simultaneous coexpression of both EGFP variants in vertebrate cells other than zebrafish, *Xenopus* embryos were injected into one blastomere at the two-cell stage with this vector. Injected embryos showed strong expression of the EGFP variants, as indicated by their intense fluorescence in a high number of cells. Furthermore, confocal microscopy of skin and muscle (Figs. 10A and 10B, stage 40/41) revealed that a high percentage of cells showed fluorescence confined to the plasma membrane as well as to the nucleus (Figs. 9A and 9B), indicating that lynEGFP and H2BEYFP are coexpressed in most of these amphibian cells.

To address whether Gal4-VP16-mediated coexpression of lynEGFP and H2BEYFP is also applicable to higher vertebrates, EF-GVP-Ulyn-UH2B was transfected into different cultured cell lines and EGFP fluorescence was scored at

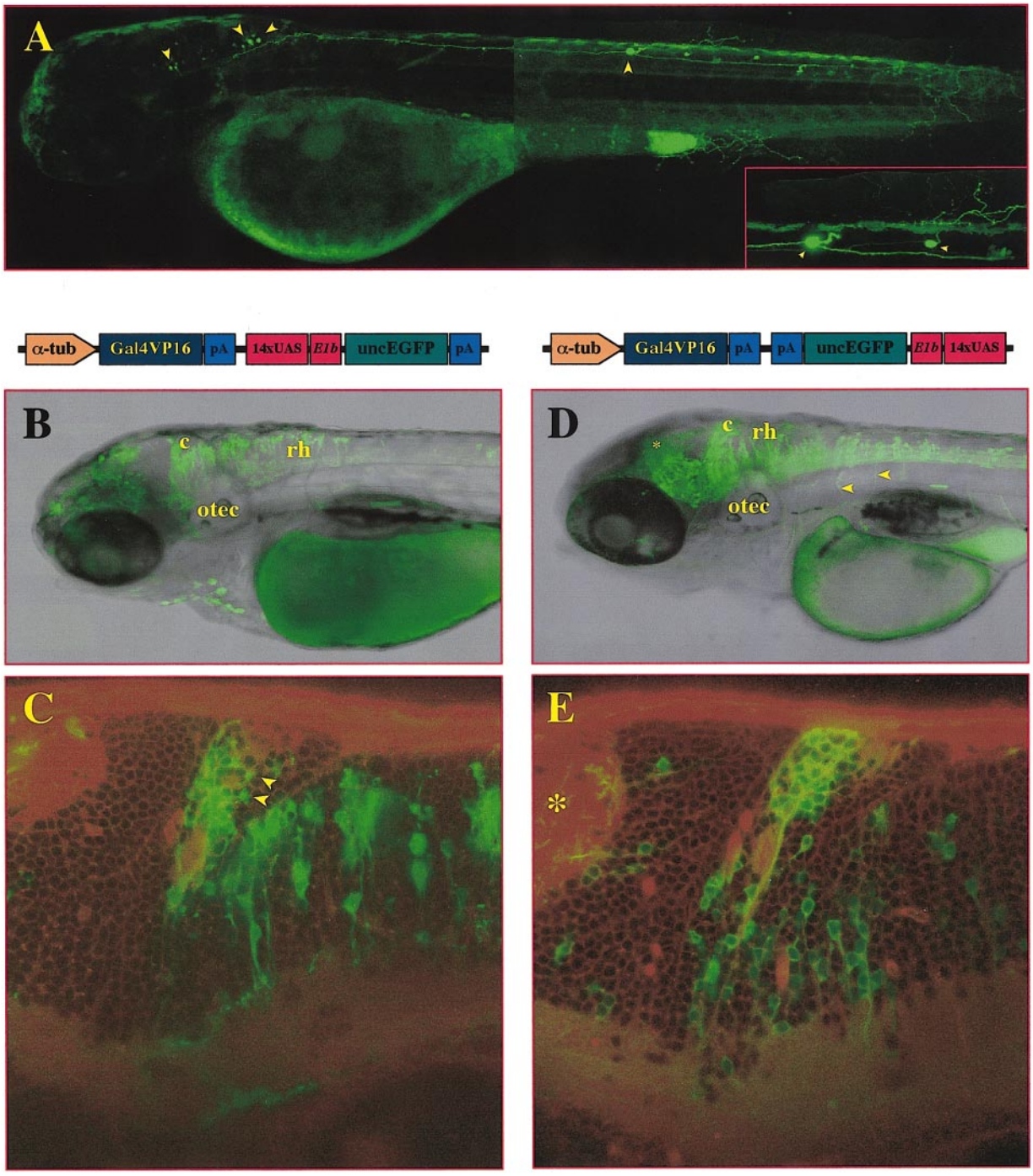

FIG. 7. Neural-specific expression in transient transgenic Gal4-VP16-activator/effector injected zebrafish embryos. Lateral views of −1696α1TIpEGFP- (A), tub-GVP-Uunc- (B, C), and tub-GVP-Uunc-inv-injected (D, E) embryos showing single optical sections which were taken at 2 (A) or 3 dpf (B–E) using confocal microscopy. Pictures represent pseudocolored composites with EGFP fluorescence displayed in green overlaid with either a transmitted light image (A, B, D) or Bodipyceramide-TexasRed counterstain to visualize overall morphology (C, E). (A) The lateral view of −1696α1TIpEGFP-injected embryo demonstrates that EGPF expression is almost exclusively confined to neurons (arrowheads). Only very few neurons show EGFP fluorescence at detectable levels. Diffusion of EGFP into the neuronal processes also visualizes axons, dendrites, and synaptic butons (see inset, magnification of EGFP-expressing neuron in trunk). (B, D) Overview of the head region of tub-GVP-Uunc- (B) and tub-GVP-Uunc-inv-injected (D) embryos, note the strong and high-frequent expression in the hindbrain and spinal chord. The tub-GVP-Uunc-inv-injected specimen (D) displays expression in the optic tectum demonstrating the linear arrangement of these neurons projecting into the tectal neuropil (*). Also, ventrally projecting axons leaving the spinal cord can be observed (e.g., arrowheads). (C, E) Higher magnification of the cerebellum (lateral views) of the injected embryos shown in (A) and (C). In the tub-GVP-Uunc-injected specimen (C), even tiny neurons (supposedly granule cells, arrowheads) and their processes can be visualized. In the tub-GVP-Uunc-inv-injected specimen (E), the predominant membrane localization of the unc76:EGFP-fusion protein can be seen highlighting the ventral projections (see arrowhead) of the labeled cerebellar neurons. Abbreviations: c, cerebellum; otec, optic tectum; rh, rhombencephalon.

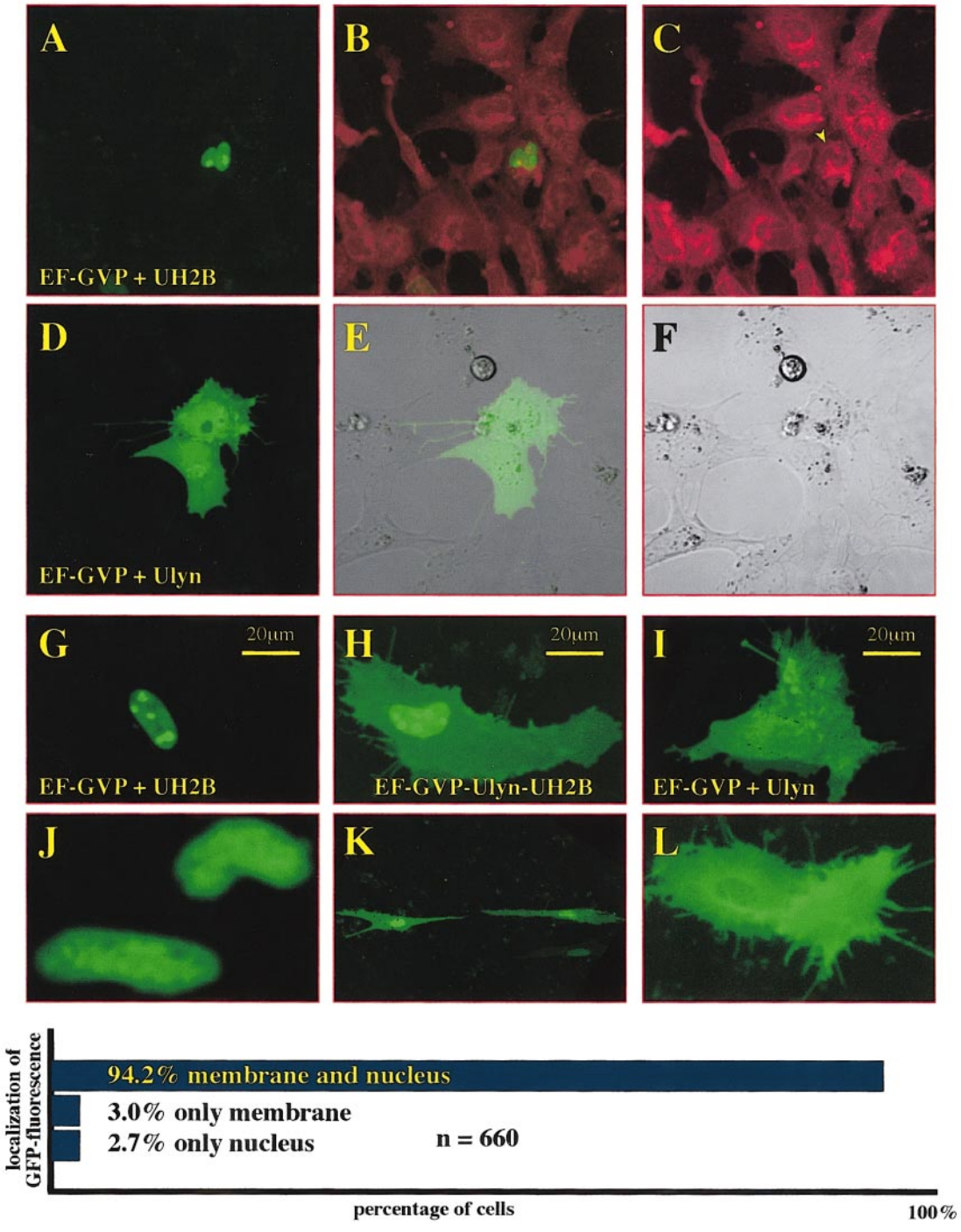

FIG. 8. Gal4-VP16-mediates coexpression of two UAS effectors in a very high frequency. (A–L) Confocal microscopy pictures of cultured primary zebrafish cells transfected with different Gal4-VP16 activator/effector constructs. (A–C) EF-GVP/UH2B cotransfected cells, images of H2BEYFP fluorescence (A, pseudocolored in green) and Bodipyceramide-TexasRed counterstained cells (C, pseudocolored in red) were overlaid (B) to confirm nuclear localization of the histone2B-EYFP-fusion protein. (D–F) EF-GVP/Ulyn cotransfected cells, fluorescent (D), and transmitted (F) light images were overlaid (E) to confirm the membrane localization and nuclear exclusion of the lynEGFP-fusion protein. Comparison of EF-GVP/UH2B cotransfected (G, J), EF-GVP/Ulyn cotransfected (I, L), and EF-GVP-Ulyn-UH2B-transfected cells (H, K) shows characteristic localization, size, and shape of the different EGFP-targeted organelles (pseudocolored in green) to allow scoring of simultaneous coexpression of both EGFP variants in individual and EF-GVP-Ulyn-UH2B-transfected cells. Scoring results in percentages are shown in the diagram below.

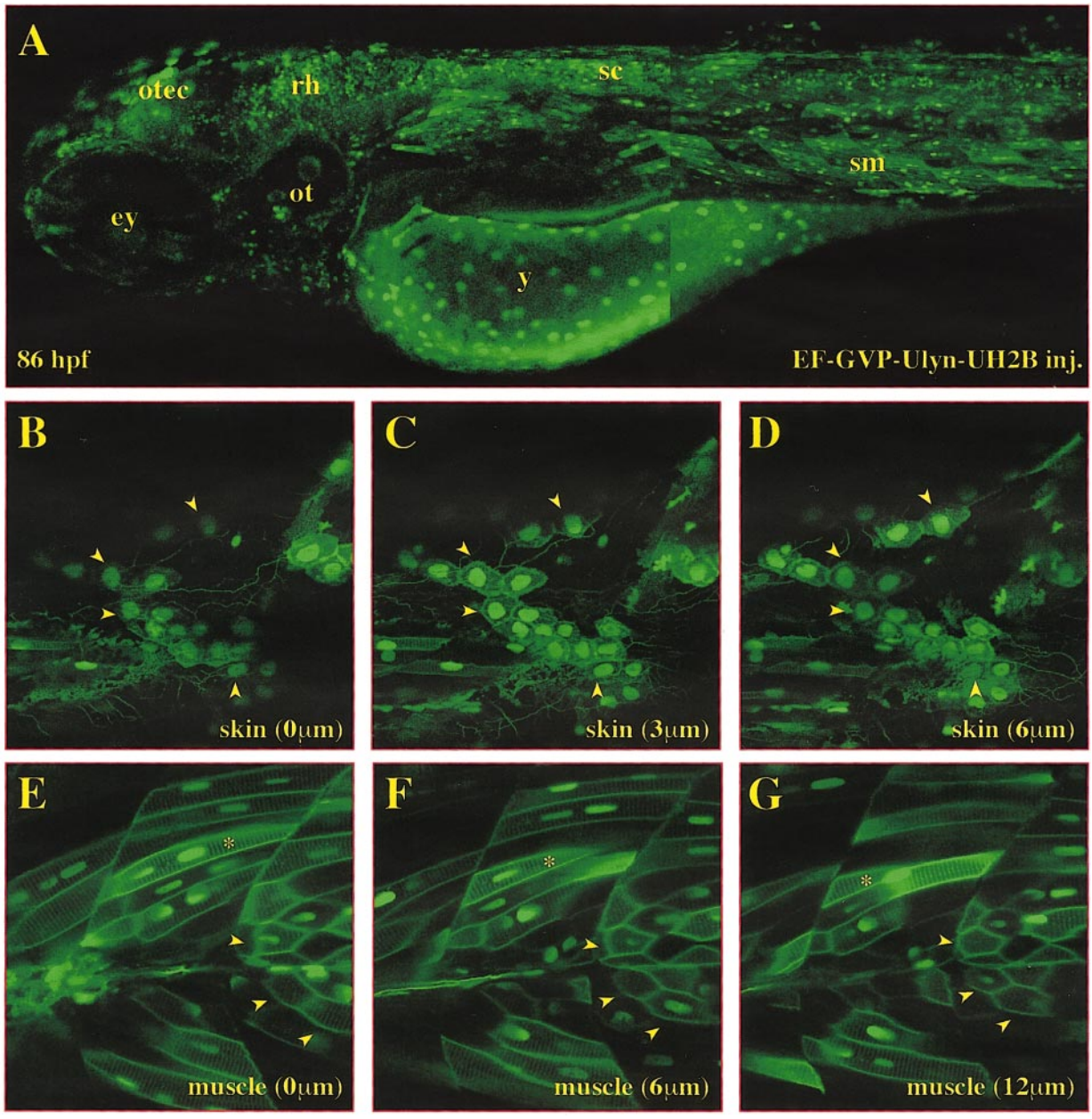

FIG. 9. Gal4-VP16 is capable of mediating coexpression of two UAS-dependent transgenes in individual cells of living zebrafish embryos. (A–G) Single optical sections through EF-GVP-Ulyn-UH2B-injected embryo at 86 hpf taken by confocal microscopy (EGFP fluorescence pseudocolored in green). (A) Lateral view of EF-GVP-Ulyn-UH2B-injected embryo, the high frequency of EGPF-expressing cells allows one to observe the overall morphology and to distinguish different tissues like nervous system, body muscles, and yolk syncytial layer. (B–G) Individual optical sections of stacks of pictures taken from the skin (B–D) or somitic muscles (E–G) of the injected embryo. Arrowheads mark same individual cells through these stacks to demonstrate that cells indeed coexpress both EGFP variants while scoring single individual optical sections might often underestimate the degree of coexpression. Note syncytial nature of muscle cells containing several nuclei (E*). Abbreviations: ey, eye; ot, otic vesicle; otec, optic tectum; rh, rhombencephalon; sc, spinal cord; sm, somitic muscles; y, yolk.

18 h posttransfection. Transfected chick primary embryonic fibroblasts (Fig. 10C), mouse 3T3 cells (Fig. 10D), and human 293GPG cells displayed simultaneous coexpression of lynEGFP and H2BEYFP in a high percentage of expressing cells [mouse 3T3-cells: coexpression in at least 77.8% (280/360) of fluorescent cells; weaker overall fluorescence

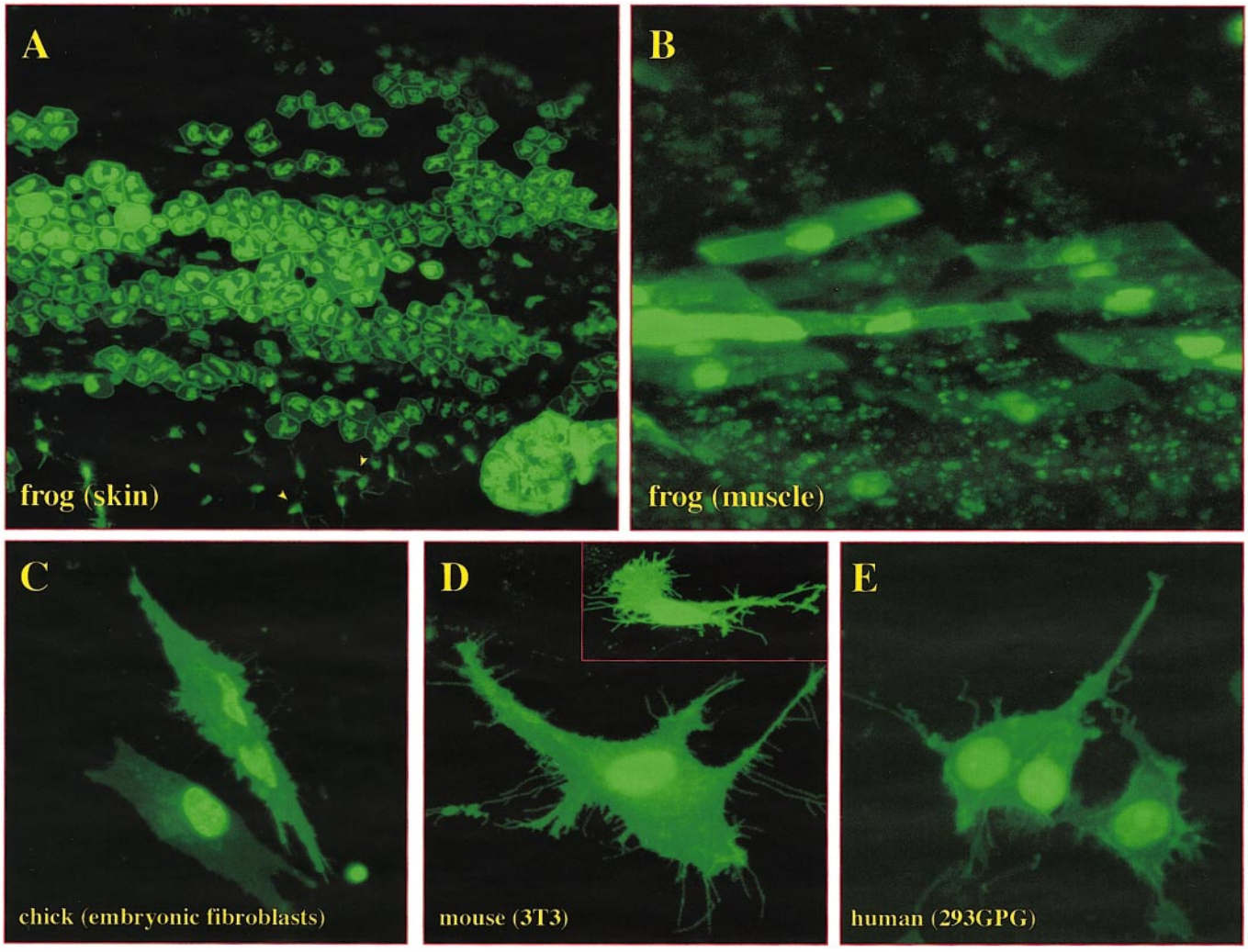

FIG. 10. Gal4-VP16-linked coexpression of two transgenes in different vertebrates. Single optical sections taken from skin (A) and muscle (B) tissue of EF-GVP-Ulyn-UH2B-injected *Xenopus* embryos (stage 40/41) using confocal microscopy. EGFP fluorescence was pseudocolored in green. Note the high percentage of nuclear and membrane fluorescence in individual cells clearly scorable in the polygonal skin cells; interspersed tiny neuronal or glia cells can be observed also (A, arrowheads). A high percentage of lynEGFP and H2BEYFP coexpression could also be found in EF-GVP-Ulyn-UH2B-transfected cultured chick embryonic fibroblasts (C), mouse 3T3 cells (D, the overexposed inset shows the intense labeling of filopodia by the modified lynEGFP variant), and human 293GPG cells (C). This indicates that the use of Gal4-VP16-activator/effector expression vectors is not restricted to zebrafish but can be used throughout all major vertebrate model organisms and is also applicable to human cells.

might have resulted in underscoring the degree of coexpression]. The overall intensity of EGFP fluorescence appeared to be weaker compared to the fish and amphibian cells. This is probably due to the fish-specific basal promoter *E1b* used in the effector cassettes driving Gal4VP16-mediated EGPF expression as well as to the *Xenopus*-derived promoter EF-1α-promoter. Replacement of these components with species-specific elements should result in higher expression levels. This demonstrates that Gal4-VP16-mediated coexpression of several transgenes can be efficiently used throughout a broad variety of vertebrates.

DISCUSSION

Here, we show a technique that answers the need for efficient, transient, mosaic, and traceable ectopic gene expression in zebrafish embryos by injection of Gal4-VP16-expression constructs. The frequency of this mosaic expression is high enough to lead to numerous transgene-expressing cells in particular tissues when tissue-specific regulatory elements are used. Moreover, Gal4-VP16 can drive expression of UAS-dependent transgenes contained on the same vector. In addition, we demonstrate the coexpres-

sion of several UAS-dependent transgenes from a single vector in individual cells. This will allow us to trace a transgene-expressing cell by the use of the fluorescent reporter EGFP and to observe any changes in behavior due to transgene expression.

Tissue-Specific Mosaic Transgene Expression in Zebrafish Embryos

The phenotype that results from the loss of expression in mutant zebrafish embryos largely reflects the first temporal function of the affected gene during embryonic development. Similarly, mRNA injections which lead to a ubiquitous ectopic expression are most informative of the early roles of the gene product of interest. Tissue-specific regulation of ectopic transgene expression or its dominant-negative forms opens the possibility to directly address a gene's function during any time point and tissue of interest given that a suitable enhancer is at hand.

We have shown that three different tissue-specific enhancer/promoter fragments from zebrafish led to almost exclusive (*twhh*) or predominant (*α-actin*, *α-tubulin*) expression of Gal4-VP16-amplified EGFP expression in the notochord, somitic muscles, and the nervous system, respectively. The tissue-specificity obtained with all three enhancers clearly demonstrates that the tissue-specific regulatory elements are not leaky but restrict Gal4-VP16, and thus transgene expression, precisely. Furthermore, injected embryos display tissue-specific EGFP fluorescence in a fairly large number of cells. Therefore, this method can be used in time-lapse studies or ectopic-expression experiments. While injected plasmids are stable and guarantee long-lasting expression, Gal4-VP16-mediated amplification ensures constant expression at the high levels often needed for expressing dominant-negative variants. An even more subtle regulation of expression levels can be obtained in the future by changing the number of UAS sites or separating the UAS sites spatially from the basal promoter (Chasman *et al.*, 1989).

Given the structure of the single vectors we injected, it is difficult to unambiguously exclude that the UAS-sites located 3′ to Gal4-VP16 lead to autoregulatory amplification of Gal4-VP16; however, some of our observations argue against this possibility. First, coinjection of Gal4-VP16 activator and effector constructs result in similar expression intensity and frequency than injection of the combined activator/effector constructs. Second, as most tissue-specific enhancers are often leaky to some extent in other tissues, autoregulation of Gal4-VP16-expression would render tissue-specific expression from combined activator/effector constructs impossible. Finally, the transactivation potential of the VP16 domain decreases with distance to the basal promoter (Chasman *et al.*, 1989), and we find that moving the UAS sites 2 kb away from the basal promoter does not change expression levels or frequency (tub-GVP-Unuc-inv compared to tub-GVP-Uunc). Thus, any possible autoregulation must be minimal and does not affect the usefulness of the combined activator/effector constructs for tissue-specific or temporal-specific expression.

The *α-tubulin* and *α-actin* enhancer drive low-level expression in muscle and neuronal tissue, respectively, suggesting that they (other than the *twhh*-enhancer) lack some repressive elements to restrict the expression exclusively to the target tissue. The amplification of expression, together with the high number of transgene-expressing cells, renders these constructs very sensitive in detecting subtle changes in promoter regulation. Thus, they offer a precise and fast analysis for the specificity of isolated regulatory elements.

The Transcriptional Activator Gal4-VP16 Drives Expression in a High Number of Cells Already at Low Concentrations

When expressed at high amounts, both the GAL4 (Gill and Ptashne, 1988) and the GAL4-VP16 (Sadowski *et al.*, 1988) activators can downregulate general transcription by binding and titrating out transcription factors (reviewed in Ptashne, 1988), a phenomenon called "squelching." Thus, any activator/effector approach must maintain a balance between maximal activation and minimal squelching.

We found that embryos were developmentally retarded in comparison to wild-type counterparts and develop malformations when injected with high amounts (4.2 pg/embryo, 50 ng/μl) of EF-GVP-UG (not shown). By determining the optimal concentration of injected Gal4-VP16 activator construct (2.5 pg/embryo, 30 ng/μl), we have reduced the problem of abnormal development to levels observed with constructs containing the commonly used Gal4 activator while maintaining the efficient expression of the effector. The observed levels of malformation for both constructs range around 5%, levels usually observed in injection studies. Fewer malformations were observed when tissue-specific promoters were used (expression of the Gal4-VP16 activator is initiated later when the plasmid is already distributed over more cells). Recently, a VP16 fusion has been used to efficiently rescue the zebrafish *schmalspur* mutant (Pogoda *et al.*, 2000). Thus, promoter squelching by the VP16-activation domain seems not to affect zebrafish embryonic development if the amount of Gal4-VP16 expression is properly controlled. Using this moderate injection concentration, EGFP is expressed in sufficient amounts and frequencies for imaging purposes (Fig. 4), allowing neuronal differentiation to be followed with single cell resolution *in vivo* by time-lapse recording (R.W.K., S.E.F., unpublished results). Stable integration of injected plasmid DNA often leads to the insertion of high copy numbers due to concatemer formation. Thus, these vectors may not be suitable for stable transgenic lines expressing Gal4-VP16.

Gal4V-P16-Mediated Expression Allows Imaging of Cellular Behavior under Wild-Type and Transgene-Expressing Conditions

The optical clarity and rapid development of the zebrafish embryo make it an ideal model organism to address real-time imaging questions. Detailed knowledge has been obtained with the injection of fluorescent dyes as labeling agent (Woo and Fraser, 1995; Shih and Fraser, 1995). However, they might not be suitable for long-time imaging purposes due to dilution and photobleaching of the dyes; furthermore, invasive labeling procedures might perturb the tissue of interest. Genetic labeling using EGFP as a vital dye can circumvent these problems, and the potential of labeling cells in a mosaic manner by the injection of EGFP-expression constructs has already been shown in an elegant paper describing the projection pattern of zebrafish olfactory neurons (Dynes and Ngai, 1998). Conventional expression vectors result in a low labeling efficiency, especially if the tissue of interest is small and questions during late zebrafish embryogenesis have to be addressed. The higher frequency and tissue specificity of Gal4-VP16-amplified EGFP expression facilitates the labeling of cells and the continuously high EGFP expression overcomes problems of photobleaching. Using these vectors, we have been able to follow a single cell over 3 days of embryonic development by continuous time-lapse imaging without a decrease in signal intensity (unpublished results).

The injection of expression plasmids into zebrafish embryos leads to a mosaic expression of the transgene. Thus transgene-expressing cells have to be identified ideally without sacrificing the specimen. We have shown that Gal4-VP16 vectors express two different effector cassettes simultaneously. Thus, EGFP coexpressed with any transgene of interest can serve as a reporter to detect transgene-expressing cells. This opens the possibility of imaging the behavior of transgene expressing cells in real time *in vivo*.

In principal, internal ribosomal entry sites (IRES) could serve the same purpose to link the coexpression of EGFP and a transgene. However, in fish cells, IRES-dependent cistrons seem to be expressed weakly and only in a few number of cells, probably depending on the tissue, upon plasmid injection into embryos (Köster *et al.*, 1996; Fahrenkrug *et al.*, 1999). If genes should be expressed at low levels, UAS-EGFP-IRES-transgene effector cassettes might be useful to achieve weak expression in a high number of cells.

One of the major strengths of these Gal4-VP16 imaging vectors is their modular character: transgene-containing effector cassettes and activator cassettes providing different tissue-specific Gal4-VP16-expression are easily exchangeable. Moreover, they can be combined with different modified EGFP variants being localized in different subcellular structures, such as chromatin or cytoskeleton. This allows one to address the consequences of transgene expression on a cell-biological level. The advent of the recent identified DsRed provides a second genetic dye easily separable from the EGFP fluorescence (Matz *et al.*, 1999; own observations) and will broaden the use of these vectors even more.

Taken together, the presented expression system offers an easy and fast way to simultaneously label transgene-expressing cells genetically by the coexpression of a reporter in a broad variety of vertebrates. Combined with the transparency and fast embryonic development of zebrafish embryos allowing time-lapse analysis, the presented Gal4-VP16-activator/effector constructs provide a powerful tool to follow a cell's response to the ectopic expression of a single gene with respect to behavior, fate, morphology, and cell—cell interaction.

ACKNOWLEDGMENTS

We thank Helen McBride for continuous discussions and helpful suggestions as well as Ying Gong for her help with zebrafish cell culture. We thank Jed Harmsen, Jennifer Wise, Jon Neri, and Arjun Menon for excellent technical assistance and animal care. We thank Emily Walsh and Didier Stainier for sharing results prior to publication. We thank Kang Shen/Tobias Meyer (pCDNA3-lynGFP), David Stillman (pPC97-VP16), Joseph Dynes/John Ngai (XEX-76/eGFP), Pernille Rorth (pBS 14xUAS), Nico Scheer/José Campos-Ortega (pBSUAS*EIb*Notch:intra, pBSGal4), Rusty Lansford (pLZRS.CAH2BEYFP), Virginia Hieber/Daniel Goldman (−1696α1TIpEGFP), Shin-ichi Higashijima/Goro Eguchi (α-p-SK), and Shao Jun Du/Randall Moon (pCS-twhh-β-gal-vec) for generously providing plasmids. This work was supported by grants from the Deutsche Forschungsgemeinschaft (to R.W.K.), the Human Frontier Science Program (to R.W.K.), the Beckman Institute, the National Institutes of Health (to S.E.F.), and the National Institute of Mental Health (to S.E.F.).

REFERENCES

Argenton, F., Arava, Y., Aronheim, A., and Walker, M. D. (1996). An activation domain of the helix-loop-helix transcription factor E2A shows cell type preference in vivo in microinjected zebra fish embryos. *Mol. Cell. Biol.* **16,** 1714–1721.

Brand, A. H., and Perrimon, N. (1993). Targeted gene-expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118,** 401–415.

Chasman, D. I., Leatherwood, J., Carey, M., Ptashne, M., and Kornberg, R. D. (1989). Activation of yeast polymerase-II transcription by herpesvirus Vp16 and Gal4 derivatives in vitro. *Mol. Cell. Biol.* **9,** 4746–4749.

Concha, M. L., and Adams, R. J. (1998). Oriented cell divisions and cellular morphogenesis in the zebrafish gastrula and neurula: A time-lapse analysis. *Development* **125,** 983–994.

Dick, A., Hild, M., Bauer, H., Imai, Y., Maifeld, H., Schier, A. F., Talbot, W. S., Bouwmeester, T., and Hammerschmidt, M. (2000). Essential role of Bmp7 (snailhouse) and its prodomain in dorsoventral patterning of the zebrafish embryo. *Development* **127,** 343–354.

Driever, W., Solnica-Krezel, L., Schier, A. F., Neuhauss, S.C.F., Malicki, J., Stemple, D. L., Stainier, D. Y. R., Zwartkruis, F., Abdelilah, S., Rangini, Z., Belak, J., and Boggs, C. (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* **123,** 37–46.

Du, S. J., Devoto, S. H., Westerfield, M., and Moon, R. T. (1997). Positive and negative regulation of muscle cell identity by

members of the hedgehog and TGF-β gene families. *J. Cell Biol.* **139,** 145–156.

Dynes, J. L., and Ngai, J. (1998). Pathfinding of olfactory neuron axons to stereotyped glomerular targets revealed by dynamic imaging in living zebrafish embryos. *Neuron* **20,** 1081–1091.

Fahrenkrug, S. C., Clark, K. J., Dahlquist, M. O., and Hackett, P. B. (1999). Dicistronic gene expression in developing zebrafish. *Mar. Biotechnol.* **1,** 552–561.

Fischer, J. A., Giniger, E., Maniatis, T., and Ptashne, M. (1988). Gal4 activates transcription in Drosophila. *Nature* **332,** 853–856.

Gill, G., and Ptashne, M. (1988). Negative effect of the transcriptional activator Gal4. *Nature* **334,** 721–724.

Gritsman, K., Zhang, J. J., Cheng, S., Heckscher, E., Talbot, W. S., and Schier, A. F. (1999). The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. *Cell* **97,** 121–132.

Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., van Eeden, F. J. M., Jiang, Y. J., Heisenberg, C. P., Kelsh, R. N., Furutani-Seiki, M., Vogelsang, E., Beuchle, D., Schach, U., Fabian, C., and Nüsslein-Volhard, C. (1996). The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. *Development* **123,** 1–36.

Hauptmann, G., and Gerster, T. (1994). Two-color whole-mount in situ hybridization to vertebrate and Drosophila embryos. *Trends Genet.* **10,** 266.

Hein, R., and Tsien, R. Y. (1996). Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr. Biol.* **6,** 178–182.

Hieber, V., Dai, X. H., Foreman, M., and Goldman, D. (1998). Induction of alpha 1-tubulin gene expression during development and regeneration of the fish central nervous system. *J. Neurobiol.* **37,** 429–440.

Higashijima, S., Okamoto, H., Ueno, N., Hotta, Y., and Eguchi, G. (1997). High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin. *Dev. Biol.* **192,** 289–299.

Hild, M., Dick, A., Rauch, G. J., Meier, A., Bouwmeester, T., Haffter, P., and Hammerschmidt, M. (1999). The smad5 mutation somitabun blocks Bmp2b signaling during early dorsoventral patterning of the zebrafish embryo. *Development* **126,** 2149–2159.

Jontes, J. D., Buchanan, J., and Smith, S. J. (2000). Growth cone and dendrite dynamics in zebrafish embryos: Early events in synaptogenesis imaged in vivo. *Nat. Neurosci.* **3,** 231–237.

Kanda, T., Sullivan, K. F., and Wahl, G. M. (1998). Histone-GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells. *Curr. Biol.* **8,** 377–385.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., and Schilling, T. F. (1995). Stages of embryonic-development of the zebrafish. *Dev. Dyn.* **203,** 253–310.

Köster, R., Götz, R., Altschmied, J., Sendtner, M., and Schartl, R. (1996). Comparison of monocistronic and bicistronic constructs for neurotrophin transgene and reporter gene expression in fish cells. *Mol. Mar. Biol. Biotechnol.* **5,** 1–8.

Matz, M. V., Fradkov, A. F., Labas, Y. A., Savitsky, A. P., Zaraisky, A. G., Markelov, M. L., and Lukyanov, S. A. (1999). Fluorescent proteins from nonbioluminescent Anthozoa species. *Nat. Biotechnol.* **17,** 969–973.

Morgan, B. A., and Fekete, D. M. (1996). Manipulating gene expression with replication-competent retrovirus. *Methods Cell Biol.* **51,** 186–218.

Okada, A., Lansford, R., Weimann, J. M., Fraser, S. E., and McConnell, S. E. (1999). Imaging cells in the developing nervous system with retrovirus expressing modified green fluorescent protein. *Exp. Neurol.* **156,** 394–406.

O'Malley, D. M., Kao, Y. H., and Fetcho, J. R. (1996). Imaging the functional organization of zebrafish hindbrain segments during escape behaviors. *Neuron* **17,** 1145–1155.

Ory, D. S., Neugeboren, B. A., and Mulligan, R. C. (1996). A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. *Proc. Natl. Acad. Sci. USA* **93,** 11400–11406.

Picker, A., Brennan, C., Reifers, F., Clarke, J. D. W., Holder, N., and Brand, M. (1999). Requirement for the zebrafish mid-hindbrain boundary in midbrain polarisation, mapping and confinement of the retinotectal projection. *Development* **126,** 2967–2978.

Pogoda, H.-M., Solnica-Krezel, L., Driever, W., and Meyer, D. (2000). The zebrafish forkhead transcription factor FoxH1/Fast1 is a modulator of Nodal signaling required for organizer formation. *Curr. Biol.* **10,** 1041–1049.

Ptashne, M. (1988). How eukaryotic transcriptional activators work. *Nature* **335,** 683–689.

Rorth, P. (1996). A modular misexpression screen in Drosophila detecting tissue-specific phenotypes. *Proc. Natl. Acad. Sci. USA* **93,** 12418–12422.

Rupp, R. A. W., Snider, L., and Weintraub, H. (1994). Xenopus-embryos regulate the nuclear-localization of Xmyod. *Genes Dev.* **8,** 1311–1323.

Sadowski, I., Ma, J., Triezenberg, S., and Ptashne, M. (1988). Gal4-Vp16 is an unusually potent transcriptional activator. *Nature* **335,** 563–564.

Scheer, N., and Campos-Ortega, J. A. (1999). Use of the Gal4-UAS technique for targeted gene expression in the zebrafish. *Mech. Dev.* **80,** 153–158.

Schmid, B., Fürthauer, M., Connors, S. A., Trout, J., Thisse, B., Thisse, C., and Mullins, M. C. (2000). Equivalent genetic roles for bmp7/snailhouse and bmp2b/swirl in dorsoventral pattern formation. *Development* **127,** 957–967.

Shih, J., and Fraser, S. E. (1995). Distribution of tissue progenitors within the shield region of the zebrafish gastrula. *Development* **121,** 2755–2765.

Shimizu, T., Yamanaka, Y., Ryu, S. L., Hashimoto, H., Yabe, T., Hirata, T., Bae, Y., Hibi, M., and Hirano, T. (2000). Cooperative roles of Bozozok/Dharma and Nodal-related proteins in the formation of the dorsal organizer in zebrafish. *Mech. Dev.* **91,** 293–303.

Teruel, M. N., Blanpied, T. A., Shen, K., Augustine, G. J., and Meyer, T. (1999). A versatile microporation technique for the transfection of cultured CNS neurons. *J. Neurosci. Methods* **93,** 37–48.

Westerfield, M. (1995). "The Zebrafish Book. " Univ. of Oregon Press, Eugene, OR.

Winkler, C., Vielkind, J. R., and Schartl, M. (1991). Transient Expression of foreign DNA during embryonic and larval development of the medaka fish (*Oryzias latipes*). *Mol. Gen. Genet.* **226,** 129–140.

Woo, K., and Fraser, S. E. (1995). Order and coherence in the fate map of the zebrafish nervous-system. *Development* **121,** 2595–2609.

Wormington, M. (1991). Preparation of synthetic mRNAs and analyses of translational efficiency in microinjected xenopus oocytes. *Methods Cell Biol.* **36,** 167–183.

Submitted for publication January 3, 2001
Revised February 8, 2001
Accepted February 11, 2001
Published online April 16, 2001