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Highly-Restricted, Cell-Specific Expression of the Simian CMV-IE Promoter in Transgenic Zebrafish with Age and After Heat Shock

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Abstract

Promoters with high-levels of ubiquitous expression are of significant utility in the production of transgenic animals and cell lines. One such promoter is derived from the human cytomegalovirus immediate early (CMV-IE) gene. We sought to ascertain if the simian CMV-IE promoter (sCMV), used extensively in non-mammalian vertebrate research, also directs intense, widespread expression when stably introduced into zebrafish. Analysis of sCMV-driven expression revealed a temporal and spatial pattern not predicted by studies using the hCMV promoter in other transgenic animals or by observations of early F0 embryos expressing injected sCMV reporter plasmids. Unexpectedly, in transgenic fish produced by both integration of linearized plasmid or Tol2-mediated transgenesis, sCMV promoter expression was generally observed in a small population of cells in telencephalon and spinal cord between days 2-7, and was thereafter confined to discrete regions of CNS that included the olfactory bulb, retina, cerebellum, spinal cord, and lateral line. In skeletal muscle, intense transgene expression was not observed until well into adulthood (>2-3 months post-fertilization). One final unexpected characteristic of the sCMV promoter in stable transgenic fish was tissue-specific responsiveness of the promoter to heat shock at both embryonic and adult stages. These data suggest that, in the context of stable transgenesis, the simian CMV-IE gene promoter responds differently to intracellular regulatory forces than other characterized CMV promoters.

Keywords

CMV; simian cytomegalovirus; promoter; transgenic; danio rerio; zebrafish; heat shock; periglomerular cells; photoreceptors; pineal; cerebellum; lateral line; skeletal muscle; non-somitic muscle; appendicular muscle; delayed expression; late-onset expression

The promoter of the human cytomegalovirus immediate early (CMV-IE) gene has been used extensively to direct high-level gene expression in a variety of cultured cells and transgenic animals (Foecking and Hofstetter 1986; Miller and Rosman, 1989; Schmidt et al., 1990; Furth et al., 1994). In non-mammalian model systems such as *Xenopus*, the simian counterpart of this same promoter (sCMV) is frequently used to direct high-level gene expression in cells of

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the developing *Xenopus* embryo (Hoppler et al., 1996; Huang et al., 1999; Song et al., 1999; Matsui et al., 2000; Yoon et al., 2000; Werdien et al., 2001). The sCMV promoter is also active in embryos of the zebrafish, *Danio rerio*, and has been used to provide transient reporter gene expression in injected embryos (Hieber et al., 1998; Koster and Fraser, 2001; Kaley-Zylinska et al., 2002).

At a structural level, characterized CMV-IE promoters have many similarities. All DNA components required for maximal expression in host cells lie within approximately 0.8Kb of sequence composed of a large enhancer core and a TATA-containing basal promoter region (Boshart et al., 1985; Jeang et al., 1987; Alcendor et al., 1993; Sandford and Burns, 1996). Within the enhancer region, the sCMV and hCMV promoters share numerous binding determinants for known transcription factors; however, the number, composition, and location of these elements within the promoters differ significantly. For instance, the sCMV promoter contains eleven canonical cyclic-AMP Responsive Elements (CREs) within the core enhancer while the hCMV promoter displays only five CRE elements within the same region. Similarly, some elements are conserved in number and general location, but display subtle alterations in specificity. One example is three 21-bp repeats predicted to bind the transcriptional activators SP1 and YY1 within the hCMV promoter that in the sCMV promoter lack several bases required for the association with SP1 (discussed in Sandford and Burns, 1996). Indeed, when the structural comparison of CMV-IE promoters is extended to include the promoters from mouse and rat CMVs, the differences in promoter organization become much more pronounced even though it is generally assumed that these promoters function analogously in the cells of their host (Sandford and Burns, 1996).

Although the CMV-IE promoters (and many viral promoters in general) are very active and can provide higher levels of expression than the promoters of most animal genes, it has been clear now for many years that even in the context of the intact viral genome, these promoters are not equally functional in all cell types. For example, in one study examining CMV-IE promoter expression and viral replication using a variety of CMV types and target cells (Lafemina and Hayward, 1988), it was found that mouse and human CMV both can infect primary mouse fibroblasts, however, only the mouse viral IE promoter was expressed at high levels and supported viral replication. Similarly, human, simian, and mouse CMV can all infect primary human skin fibroblasts, but only the human and simian promoters direct high-level expression and resulting viral replication. It is of note that the rules of expression did not break-down explicitly along lines of species compatibility, though. Among the findings that illustrate the complexity of CMV-IE promoter responsiveness was the observation that both sCMV replication and the sCMV-IE promoter was active in undifferentiated human NT2/D1 teratocarcinoma cells while neither property was supported for hCMV. Interestingly, treatment of the NT2 cells with retinoic acid to differentiate them also rendered them able to support hCMV expression and replication. All of these results together confirm the idea that even though these promoters have similar structures and analogous functions, when introduced into a foreign environment, their activity may not be readily predicted.

Given the widespread utility of the hCMV promoter in the production of stable cell lines, and more directly, in the production of transgenic mammals, we sought to determine if the expression pattern of the sCMV promoter in transgenic fish was similar to hCMV promoter expression in transgenic mammals. Our data reveal that the sCMV promoter has a number of surprising properties that clearly differentiate it from the temporal and spatial expression pattern of hCMV promoter expression in transgenic mammals.

1. Results and Discussion

1.1 Production and characterization of transgenic zebrafish with sCMV-reporters

We used two methods to generate transgenic fish in which the sCMV promoter drives either dsRed or EGFP expression. For sCMV:dsRed transgenic fish we used a linearized pCS2+ vector that harbors the expression cassette to inject into single cell zebrafish embryos. This method often results in the insertion of plasmid concatamers. The other method takes advantage of the Tol2 transposase system to insert a single copy of the expression cassette, without flanking plasmid sequences, into the genome (Urasaki et al., 2006). Within 24-72 hours after plasmid injection, approximately 50% of the embryos displayed varying degrees of fluorescent reporter expression in multiple tissues (Figure 1A,B). Although there was a high degree of heterogeneity in the expressing population (likely due to variability in distribution of the unintegrated plasmid to daughter cells), recurring expression in a small population of cells in the rostral telencephalon, the eye, and occasional cells with the appearance of nerve fibers on the flanks or tail were observed. In addition to these tissues, nascent muscle fibers, and to a lesser extent, skin cells, were also observed (Figure 1A,B). Embryos with visible reporter expression were selected and grown to adulthood. Transgenic founders were identified by screening their F1 progeny for reporter expression at 2-14 days post-fertilization (dpf), and ultimately 11 sCMV:dsRed and 6 sCMV:EGFP F1 lines were isolated. Although there were some differences in reporter expression between lines (for example compare sCMV:EGFP lines 1 and 2 shown in Fig. 1E,F), the data below is focused on the general pattern shared by all lines. Most of the variations between lines were in the intensity of reporter expression. Lines sCMV:dsRed 2-1, sCMV:EGFP-1 and sCMV:EGFP-2 displayed the earliest and most intense fluorescence and were used for most analyses in this report.

In sCMV:dsRed 2-1, fluorescence microscopy and *in situ* hybridization showed the earliest expression was at 4 dpf in a population of cells within the anterior telencephalon (Figure 1 C,D). In contrast sCMV:EGFP lines showed reporter expression within the head, anterior telencephalon, spinal cord and trunk at 2.5 dpf (Figure 1E-F). By two weeks of age, a general pattern exemplified by line sCMV-dsRed2-1 in Figure 1G was established and included the interior of the eye, scattered cells of the rostral telencephalon, discrete populations within the cerebellum, brain stem, and spinal cord. With the exception of expression in skeletal muscle described in more detail below, the expression pattern observed at two weeks generally continued into adulthood. Further detail of reporter expression in CNS and muscle tissues is described below.

1.2 sCMV expression pattern in the nervous system

Whereas the hCMV promoter supported transgene expression throughout the mouse nervous system (Schmidt et al., 1990), stable dsRed expression from the sCMV promoter was restricted primarily to a small number of defined regions and cell types within the CNS. Expressing cells were found in discrete cell populations primarily within five regions: 1) olfactory bulb, 2) retina/pineal body, 3) cerebellum, 4) spinal cord and 5) brain stem/lateral line.

1.2.1 Olfactory bulb—In the olfactory bulb (OB), dsRed and EGFP reporter expression was initially observed bilaterally in a small population of cells with extensive arbors between 3-7 dpf that continued to express into adulthood (Fig. 2A-D). Histological examination of sections of 4-6 week old sCMV-dsRed-2-1 fish OB revealed that these cells resided primarily within the glomerular layer (GL) of the OB (Fig. 2C,D). The morphology of these cells suggested that they were neurons, and this was further confirmed by labeling with neuron-specific anti-HuC/D antibody (Fig. 2E). The location and morphology of these cells suggested that they might represent a subset of output neurons or interneurons within the glomerular layer (GL). Retrograde labeling of the medial and lateral olfactory tracts with the fluorescent dextran

microemerald revealed no back-labeling of the dsRed-positive population indicating that these cells were not output neurons such as mitral cells (Fig. 2 F-H). Immunocytochemical analysis (Fig. 2I) revealed numerous cells positive for the dopaminergic marker tyrosine hydroxylase (TH) that were also positive for dsRed. Not all TH-positive cells were dsRed-positive, and not all dsRed-positive cells were immunostained for TH, indicating that the dsRED-expressing population is a subset of periglomerular cells.

1.2.2 Retina/Pineal body—All sCMV:dsRed lines and all but one of the sCMV:EGFP lines showed expression in the retina (Figs. 1E-G). Retinal expression was detected as early as 2.5 dpf in sCMV:EGFP lines 1 and 2 (Fig. 1E,F), and by 4-10 dpf in sCMV:dsRed lines. This expression persisted into adulthood, although generally with diminished intensity. High-level reporter expression in the retina was restricted to the retinal layer harboring photoreceptors (Fig. 3A-C). The position of cells within the photoreceptor layer suggested that they are not light-sensing rod cells but are instead cone photoreceptors (Fig. 3C). In the sCMV-dsRed 2-1 line, the FRET43 antibody that specifically labels red and green double-cone photoreceptors (Larison and Bremiller, 1990) did not label dsRed+ cells but clearly labeled the non-dsRed population, indicating that the dsRed cells are UV/blue photoreceptors (Fig. 3D-G). Expression was observed strongly in both the cell body and the synaptic region located in the outer plexiform layer. In some dsRed lines, expression of the reporter in these photoreceptors was so strong that aggregates of dsRed protein, which typically functions as an oligomer at high concentration, were often observed in the photoreceptor somata. In the most highly expressing transgenic lines, such as sCMV:dsRed 2-1 and sCMV:EGFP2, expression in a subset of cells within the pineal body was first detected around 10-14 dpf and persisted into adulthood (Fig. 3H,I). The pineal body is a brain structure involved in the regulation of the circadian clock and is known to share characteristics of the eye, including the production of photoreceptor-like cells in most vertebrates including zebrafish (Allwardt and Dowling, 2001). Expression in the pineal body was more subject to line-to-line variation and was generally weak in lines with lower expression.

1.2.3 Cerebellum—Reporter expression in the cerebellum was not observed at high levels until approximately 3 weeks post-fertilization when it was observed in both cell bodies and fibers of a subset of cerebellar cells. In adults, the high level of expression in cerebellum is evident in fluorescent images of whole brain from sCMV-dsRed 2-1 fish crossed with α 1T-tubulin (α 1T)-GFP fish (Goldman et al., 2001). As shown in Figure 4A, α 1T expression of GFP appears as uniform low-level green fluorescence throughout all brain structures while intense sCMV-dsRed expression is observed in the cerebellum, pineal, and OB (Fig. 4A). Histological examination of dsRED expression at different levels of the cerebellum revealed expression in a subset of cells within both the granule layer and the Purkinje cell layer (Figs. 4B-D) and in numerous fibers. The cells within the Purkinje cell layer possess extensive arbors and may represent Purkinje cells themselves or one of a number of other support cell types. In lines with robust expression, bright fluorescence in the cerebellum could be visualized directly between 4-6 weeks, as in the sCMV:EGFP-2 fish shown in Figure 4E (yellow arrow). In lower expressing lines such as sCMV:GFP-1, cerebellar expression was not visible externally (Figure 4F).

1.2.4 Spinal Cord—Discrete neurons of the spinal cord were one of the earliest cell types observed to express the sCMV promoter in both sCMV:dsRed and sCMV:EGFP transgenic fish. These appeared as multiple bright points of fluorescent intensity at 2.5-10 dpf (e.g., see Figure 1E,F). With time, this intensity increased, and in 3-4 week old fish, numerous positive cells and fibers were observed (Fig. 4E-G). Many of the fibers appeared to originate from populations of cells that were spread throughout the spinal cord (Fig. 4E-I), many of which displayed large cell bodies that resided adjacent to the central canal and radiated thick fibers

to the periphery. One class of labeled cell appeared primarily to possess a large axon that traveled ventro-dorsally from near the central canal to the dorsal aspect of the cord (arrows in Fig. 4E-G and tissue sections in Fig. 4H,I). These strongly-positive large neurons co-labeled with neuronal markers and did not co-label with the radial glial marker ZRF1 (data not shown), although their axonal process frequently appeared to have migrated along radial glial processes.

1.2.5 Brainstem/Lateral Line—In sCMV:dsRed 2-1 transgenic fish, one area of intense transgene expression was in discrete nerves of the brainstem where numerous descending fibers can be observed by approximately two-weeks of development when the fish is both relatively mature and still relatively small and translucent (e.g. Fig. 1G). In the majority of sCMV-transgenic lines, however, high-level expression in the brainstem was more modest and was visible only by careful histological examination of tissue sections. One exception to this rule was fibers innervating both the anterior and posterior lateral lines (ALL and PLL, respectively) that are visible just under the skin by 2-3 weeks post-fertilization (Fig. 5A-C). Although all transgenic fish expressed in the lateral line, expression varied significantly from line-to-line. For example, fibers of the dsRed 13-5 line were easily visible over the extent of the flank all the way into the tail (Fig. 5A) while fibers of the dsRed 1-2 line were less intense (Fig. 5B) and were more representative of the sCMV:EGFP lines. In the sCMV dsRed 13-5 line, labeling of adult lateral line neuromasts with the vital dye DASPEI suggested that clusters of dsRed intensity at the end of the nerve were an accumulation of dsRED protein in the presynaptic terminal of afferent nerve fibers (Fig 5C).

1.3 sCMV expression in skeletal muscle

In addition to expression in discrete neuronal populations of the nervous system, the second major site of sCMV reporter expression was skeletal muscle. There were two notable observations of sCMV promoter activity in skeletal muscle: 1) The sCMV promoter was not active in muscle at high levels until later in adulthood, and 2) sCMV promoter activity initiated in a subset of muscle and progressed to include most skeletal muscle. Strong dsRed expression in muscle was observed in 6 out of 11 CMV:dsRed lines and 4 out of 6 sCMV:EGFP lines, and not prior to approximately three months of development. The most intense dsRed and EGFP fluorescence was observed in lines sCMV dsRed 2-1 and sCMV:EGFP lines 1 and 2 that displayed visible fluorescence in muscle over the entire flank by 5 months with highest expression in pectoral and jaw muscles (Fig. 6A-C, arrow points to pectoral muscle). A comparison of sCMV:dsRed fish line 2-1 in Figure 6A with similarly aged fish of lines 1-2 and 13-5 in Figure 5A and 5B shows the striking difference in the intensity of fluorescence in skeletal muscle, although inspection at higher magnification and on tissue sections indicated they expressed in a similar pattern. Sectioned sCMV:dsRed 2-1 fish showed that muscles driving all of the fins (including those proximal to the tail fin) expressed the transgene at high levels (Fig. 6D-E). As a group, these muscles are distinctive in that they represent appendicular muscle formed by the migration of myoblasts, as opposed to somitic muscle, derived directly as an extension of somites. Appendicular muscle also includes the muscles of the eyes, and examination of these muscles in sCMV:dsRed 2-1 revealed they also displayed strong reporter expression (Fig. 6G,H). In older adults, expression remained highest in appendicular muscles where small aggregates of dsRed protein could often be found. Expression progressed from appendicular muscle to other skeletal muscle of the trunk (Fig. 6D, green arrows). In this muscle, the expression clearly followed a distinct pattern, with some fibers expressing strongly and others not expressing visible fluorescence. Furthermore, muscle tissue along the most lateral aspects of the flanks never appeared to express the reporter protein strongly, suggesting that this muscle differs from much of the remaining skeletal muscle of the trunk.

1.4 sCMV promoter induction by heat shock

In the generation of sCMV-dsRed transgenic lines, positive F1 and F2 siblings were occasionally crossed resulting in the generation of homozygotes. In the case of line sCMV dsRed 13-5, it was observed that approximately 25% of progeny displayed dsRed expression at a dramatically higher level than would have been predicted for the expression of homozygotes. In addition, by 5 dpf, this highly-positive group displayed clear indications of malformation including small eyes, a shortened trunk, and edema culminating in death at 7-8 dpf (Fig. 7A, inset). Irrespective of the cause of lethality, it was evident that sCMV promoter activity was dramatically increased in dying embryos, and we speculated that if this increase was a response to cellular insult, we might be able to induce a similar effect with sublethal stimulus such as heat shock. As shown in Figure 7A-B, a non-lethal heat shock readily induced expression of fluorescent reporter in sCMV:dsRed transgenic animals. In addition, sCMV promoter activation was observed only when heat shock was applied later than 2-3 dpf (Fig. 7C-F). In sCMV:EGFP lines the same pattern was observed where heat shock after 2-3 dpf produced a dramatic increase in reporter gene expression (Figure 7 G,H). While both the cytotoxic insult in 13-5 homozygotes and heat shock of all sCMV-reporter lines thus far tested resulted in transgene induction, cold shock at 12°C had no observable effect on sCMV-reporter expression (data not shown).

Microscopic examination of retinal and brain sections from transgenic fish heat shocked at 3 dpf and harvested at 7 dpf confirmed observations with intact live fish that suggested that the increase was primarily due to the induction of weakly-positive cells to express at higher levels (Fig. I-L). In general, heat-shock induced expression appeared limited to the cell types that normally expressed the sCMV promoter, suggesting that cellular insult primarily influenced the amplitude - as opposed to the specificity - of response. In adult tissues that expressed a high level of transgene, heat shock did not appear to significantly induce additional expression; however, in transgenic lines with low basal expression in skeletal muscle, heat shock remained capable of inducing increased expression even in adults. Figure 7M shows that 10-week old sCMV:dsRed 2-1 fish with no exposure to heat shock display low level dsRED expression in appendicular fin muscle (band between white arrows in Fig. 7M) whereas clutchmates exposed to 10-minutes of 42°C heat shock (n=6) displayed dramatically increased positive fluorescence (Fig. 7N). In the same animals, dsRed expression in the lateral line nerves, where expression was already relatively intense, did not appear significantly augmented in the heat shock group (blue arrows in Figure 7M,N).

1.5 Summary and Conclusions

Several common aspects of sCMV promoter activity in stable transgenic fish were surprising and somewhat contrary to expectations, specifically: 1) the restricted spatial pattern of transgene expression that was accompanied by distinct temporal delays, 2) the virtual absence of expression during very early development, 3) intense expression in skeletal muscle only later in adulthood, and 4) changes in the expression pattern in response to heat shock. In general, the pattern of sCMV promoter expression appeared stable from generation-to-generation, through F2 in EGFP lines and F4 in dsRed lines. We created the sCMV:EGFP transgenic fish using the Tol2 transposase system to ensure the restricted expression pattern observed in sCMV:dsRed fish was not due to integration of DNA concatamers that may influence promoter activity. We also amplified the integrated transgene by PCR and used DNA sequencing to confirm no alterations within the promoter occurred and might explain the restricted pattern of expression.

As outlined in the Introduction, the sCMV promoter, like its human CMV counterpart, contains a highly repetitive enhancer core. Enhancer cores of this type are generally thought of as the “amplitude” controls for other promoter elements that govern properties such as tissue

specificity. Their repetitive nature provides them with a multiplicity of binding elements that respond to general and non-tissue restricted transactivators such as SP-family (Suske, 1999) and AP-1 family transcription factors (Hess et al., 2004). It is unlikely that the sCMV enhancer core is not recognized by members of these ancient transactivator families that are generally highly conserved across species (particularly within DNA-binding determinants). Furthermore, in some fish tissues, the sCMV promoter was expressed at very high levels, suggesting that basal promoter elements were intact and functional. This was further confirmed by PCR-based analysis and sequencing of integrated transgenes that indicated that the promoter-reporter cassette was full-length and intact (data not shown). Given this, we speculate that the restrictive pattern displayed by the sCMV promoter in zebrafish is the result of promoter silencing.

One observation that supports this notion is that while the temporal pattern of expression varied dramatically between the plasmid-injected F0 fish population and the stable transgenic F1 population, the spatial pattern was better-represented by injected embryos at very early times of development. For example, the periglomerular cell, photoreceptor, spinal cord, and skeletal muscle cell populations were all observed by 24-72 hpf in injected F0 embryos, but were not strongly observed in most transgenic F1s for days or weeks (nervous tissue), or for months (skeletal muscle).

It has long been established that virus-derived promoters can be rendered silent by passage to F1 progeny in the context of transgenic animals. The Moloney murine leukemia virus long-terminal repeat (MMLV LTR) promoter is fully competent to express in F0 animals but is silenced in F1 transgenic progeny of mammalian species such as mouse (Pannell et al., 2000), and more notably, in transgenic fish produced by retroviral infection (Lin et al., 1994). Ongoing experiments will determine whether the pattern of sCMV promoter expression in transgenic fish is due to similar chromatin silencing forces.

In order to begin to determine which individual binding determinants within the sCMV promoter control specific spatial and temporal characteristics, we have point-mutated multiple binding sites within the enhancer and basal promoter and looked for changes in the pattern of expression in injected early embryos. Preliminary findings suggest that mutation of any of three individual binding sites for MEF transcription factors (potentially involved in both CNS and skeletal muscle expression) had no observable impact on expression, suggesting either that these elements serve redundant purposes, have no significant role in expression, or are only important to promoter control in the context of stable integration and later in development. This latter possibility will only be determined by promoter analyses using stable transgenic lines generated from mutant promoters similar to those described in Senut et al. (2004). Ongoing studies with these and serial deletion constructs will ascertain the position of important control regions including the determinants for heat-shock responsiveness.

The multiplicity of positive and negative regulatory elements in the sCMV promoter likely contributes to the absence of an obvious pattern to sCMV promoter activity. Although the sCMV promoter is activated in only discrete populations of neurons within the CNS, we have yet to determine a clear linkage between the types of cells involved. For example, in the olfactory bulb, reporters are expressed in TH-positive periglomerular cells and in retina they are expressed in photoreceptors, but evidently not all TH-positive periglomerular cells nor all photoreceptor types. In addition, we were unable to correlate sCMV-reporter expression with excitatory or inhibitory neurons. For example, periglomerular cells are inhibitory dopaminergic interneurons (Goheen et al., 1996) and Purkinje cells of the cerebellum are inhibitory GABAergic neurons (Gabbott et al., 1986), while cerebellar granule cells (Drejer et al., 1986) and photoreceptors of the retina are excitatory glutamatergic neurons (Schmitz and Witkovsky, 1996).

Perhaps not coincidentally, one aspect of sCMV expression is shared by both CNS and skeletal muscle: intense expression is observed only in cells that appear to have reached advanced differentiation and matured, or “aged” significantly. By both in situ hybridization and visualization of either red or green fluorescence, expression from the sCMV promoter is not observed until approximately day 3. This tendency toward expression only after significant cellular maturation is also what is observed in skeletal muscle given that expression is only observed at high levels in multinuclear muscle fibers. In general, profound changes in gene expression after adulthood has been reached have been little considered outside of disease-induced changes and changes due to extreme age.

One of the most important findings of this report is that it demonstrates that promoters for an individual gene from different sources or species - in this case promoters of the IE genes of cytomegaloviruses adapted to different host organisms - will not necessarily perform identically. It may be that molecular determinants that influence efficient viral replication from host-species to host-species vary significantly, pushing CMV to mutate, adapt, and alter its genome in response. Although the fish described in this report do not display the ubiquitous high-level transgene expression initially predicted, they may still be of use to researchers exploring zebrafish biology. One unusual characteristic of the sCMV promoter in fish is the fact that it displays such a significant delay in expression. This could potentially be of use to researchers who hope to delay the high-level expression of a specific transgene until well-past the early stages of development. We are currently exploiting this property of the sCMV promoter to stably express reporter proteins with expanded polyglutamine tracts known to induce cytotoxicity when over-expressed during developmental stages in multiple animal and cultured cell models including the zebrafish (Miller et al., 2005). Another potentially useful property of the sCMV promoter expression pattern that capitalizes on its unique properties, is that it predictably expresses in only very discrete populations of cells that can vary even within a type – for example, not all periglomerular neurons, photoreceptors, or cerebellar granule cells, but only a subset that could provide for direct comparison to side-by-side controls for experimental purposes. Lastly, the sCMV promoter is not just heat-shock inducible, but also tissue-specific – a rare, and (as far as we can determine) undescribed combination in the fish that allows for added control of transgene expression by the researcher.

2. Experimental Procedures

2.1 Plasmids and transgenic fish production

The sCMV:dsRed and sCMV:EGFP plasmids were constructed by inserting the dsRed and EGFP ORFs into the sCMV promoter-containing vector pCS2+ (kindly provided by Dave Turner, University of Michigan) and then transferring the sCMV:EGFP expression cassette to the Tol2 vector pT2AL200R150G (Urasaki et al., 2006). Transgenic fish were produced using two methods: 1) by injection of linearized plasmid (pCS2+sCMV:dsRed) as described in Goldman et al. 2001, with plasmids at a final concentration of 20µg/ml, and 2) by Tol2 transposase-mediated integration of the sCMV:EGFP expression cassette as described in Urasaki et al., 2006. Injected eggs were analyzed for mosaic expression at 24-72 hpf using a Leica MZFLIII fluorescent dissecting microscope and identified mosaic animals were isolated and grown to adulthood. F0 animals were bred in groups of 4-6 fish and progeny examined between 2-15 dpf for expression of reporter transgenes. Visibly fluorescent animals were segregated and raised as founder lines. Transgenic lines were bred to wild-type fish and to transgenic siblings to perpetuate homo- and heterozygous lines. For double red-green transgenics pictured in Fig. 4, line sCMV:dsRed 2-1 was crossed with $\alpha 1T$:GFP line 2 fish as described in Goldman et al., (2001) and Senut et al., (2004). Genomic DNA from individual lines was subjected to PCR analysis and sequencing of the transgene promoter-reporter

expression cassette to verify that the integrated transgenes were uninterrupted and intact (data not shown).

2.2 Analysis of reporter expression

Transgene expression was analyzed in both live and 2% paraformaldehyde-fixed fish at timepoints described in the text. For histological sectioning, animals fixed overnight in 2% paraformaldehyde were subsequently cryoprotected by overnight immersion in 30% sucrose solution and then frozen at -70°C until processing. 8-12 μm sections were taken on a Leica CM3050S cryostat, mounted onto Fisher superfrost slides, and either stored at -20°C for additional processing or coverslipped and imaged as shown. Immunocytochemistry, tissue processing, and use of antibodies for HuC/D, TH, FRET43, and ZRF1 are essentially as described in Senut et al., 2004.

In situ hybridization was carried out using antisense digoxigenin-labeled probes as previously described (Gulati-Leekha and Goldman, 2006). DASPEI (2Di4Asp; Sigma D-3418) dye labeling was performed by immersing freely swimming fish in 10mM DASPEI in normal fish water for 5 minutes followed by transfer to >1 liter clean fish water for 20-30 min. to rinse away free dye prior to imaging.

2.3 Microemerald back-labeling of olfactory bulb output neurons

Adult zebrafish were anesthetized with tricaine, perfused transcardially with PBS, and brains were removed. The fluorescent dextran micro-emerald (3000MW, Molecular Probes, diluted 5mg/ml in PBS) was injected into the medial and lateral olfactory tracts of both olfactory bulbs using a pneumatic picopump. The brain was then transferred to a sterilized filter and incubated in artificial fish cerebrospinal fluid at 28.5°C and 1.5% CO_2 for approximately 4-6 hours before fixation in paraformaldehyde. They were embedded in gelatin, sectioned at $20\mu\text{m}$ on a cryostat, and viewed on a Nikon fluorescent microscope or a Zeiss LSM 510 confocal microscope.

2.4 Heat shock analyses

For initial heat-shock analysis, groups of 10-15 juvenile fish from lines described in the text were subjected to either a 1hr shock at 37°C or a 10-minute shock at 42°C to elicit the heat shock response. Adults were subjected to 42°C shock for 10 or more minutes and monitored until buoyancy decreased and the fish began to float near the bottom of the tank. At this point individual fish were removed and placed in 2 liters of fish water at 28°C . By looking for this behavioral/physiological response, variables in adult fish that could alter the intensity of the heat shock effect (e.g. body size) were minimized. For the time-course of heat shock response, embryos were shocked as described at approximately 12-16 hrs and then at 1 day intervals and monitored for induction of dsRed expression. In embryos shocked post 2-3-days, reporter fluorescence was observable within 24 hrs and reached maximal expression between 48-72 hrs. The induced level was maintained for 4-5 days in GFP animals and 7-10 days in dsRed animals.

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Figure 1.

A) Representative pattern of sCMV promoter-driven dsRed expression in a zebrafish embryo 3-4 days following injection of eggs revealing scattered cells throughout the embryo. Top view with brightfield overlay. **B)** As in (A), side-view fluorescent image. **C)** Top view of the head of control (left) and transgenic (right) embryos displaying early dsRed fluorescence in a small population of cells within the rostral telencephalon (arrows indicate cell, boxed region on control shows no positive signal). **D)** The same fish as in (C), processed for *in situ* hybridization and showing reporter mRNA expression in the transgenic fish (right) and a corresponding lack of signal in control siblings (left). Note: oval light-brown patches on both animals are pigment spots and not positive hybridization. **E)** Early (2.5dpf) EGFP expression in rostral

telencephalon and other scattered cells in the head, trunk, and spinal cord area of line sCMV:EGFP-1 fish. **F)** As in (E), for line sCMV:EGFP-2 that displayed higher expression in spinal cord by 2.5 dpf. **G)** Ventral view of the sCMV expression pattern in a 2-week old sCMV:dsRed 2-1 F1 transgenic fish that represents the general expression pattern observed in young sCMV transgenic fish. Stable transgenic animals after 7 dpf express most notably in the rostral telencephalon, within the eyes, and in discrete cells of the brain stem and spinal cord.

Figure 2.

A) dsRed fluorescence overlaid with a brightfield image of a 7dpf fish reveal the brightly fluorescent cell population in the olfactory bulb (OB) (arrows). **B)** The OB cell population in a fish from line sCMV:GFP-2 at 1 month post-fertilization. **C)** Horizontal section of the adult transgenic sCMV:dsRed 2-1 fish rostral telencephalon (TEL) and OB stained with the nuclear stain DAPI (blue) and showing fluorescent cell bodies and fibers in the OB (anterior left).

D) Horizontal section of the adult OB revealing dsRed cell bodies predominantly in the glomerular layer (GL) and superficial internal cell layer (ICL) with arbors extending into the olfactory nerve layer (ONL). **E)** Consistent with their neuronal morphology, dsRed-positive OB neurons colocalize with HuC/D immunoreactivity (yellow). Insets show individual green (HuC/D) and red (dsRed) channels. **F)** Retrograde labeling of olfactory bulb mitral cells (green) reveals dsRed-expressing cells are not mitral cells. **G,H)** Higher magnification image shows separate green (G) and red (H) channels. Arrows indicate the position of green-labeled mitral cells in both images. **I)** Antibody to tyrosine hydroxylase (green) colocalizes with a subset of dsRed-positive cells (arrows). Insets show individual channels for one double-labeled neuron. All images are of sCMV:dsRed line 2-1. Scale bars: A/B = 250 μ m; C = 150 μ m; D = 75 μ m.

Figure 3.

A) Horizontal section through the head of an adult transgenic fish showing the brain and adjacent eye with strong dsRed-expressing cells apparent in the retina (arrows). Blue is DAPI stain and anterior is up. **B)** Fluorescent cells appear as a ring (indicated by arrows) in parasagittal sections of retina. **C)** Cross-section of DAPI-stained retina revealing that fluorescent cells have the position and morphology of photoreceptors. **D-G)** Cellular analysis of reporter-expressing cells in the adult fish retina showing dsRed fluorescence (**D**), FRET43 immunolabeling (**E**), DAPI (**F**), and a merge of all three channels in (**G**). **H,I)** Low- (**H**) and high- (**I**) magnification images of dsRed-positive cells (red) and DAPI staining (blue) in horizontal sections of an adult transgenic fish brain showing the pineal body. In **H-I**, anterior is to the left. Scale bars: **A/B** = 500 μ m; **C** = 125 μ m; **D-G** = 50 μ m; **H** = 250 μ m; **I** = 100 μ m. All images are of line sCMV:dsRed 2-1.

Figure 4.

A) Fluorescent image of explanted whole brain from a sCMV:dsRed transgenic fish crossed with a $\alpha 1$ tubulin:GFP transgenic fish (sCMV:dsRed 2-1 X $\alpha 1$ T:EGFP-2) highlighting dsRed expression in the brain regions described in the text. Cerebellum (Cb), Optic tectum (OT), Pineal body (Pin), Telencephalon (Tel), and olfactory bulb (OB). Anterior is to the right in A-D. **B)** Horizontal section through the CNS of an adult sCMV:dsRed transgenic fish imaged at low magnification showing strong dsRed expression in multiple cells and fibers of the cerebellum. **C)** Higher magnification of the cerebellum and surrounding area with red reporter expression. ML – molecular layer, PCL – Purkinje cell layer, GL – glomerular layer, WM – white matter tracts. **D)** Close-up of the PCL reveals strongly ds-Red expressing cells with large

cell bodies and extensive arbors and smaller positive cells in the GL. **E)** Fluorescent image of the pattern of EGFP expression in a sCMV:EGFP-1 fish at 1 mpf. High-level expression is observed in the cerebellum (yellow arrow) and multiple cells of the spinal cord (white arrows) at this age. **F)** As in (E), for line sCMV:EGFP-2. **G)** Magnified image of fluorescent cells in the spinal cord of an sCMV:dsRed 2-1 fish (darkfield image overlaid). Red autofluorescence below the midline was obscured to permit better visualization of faint cells in the spinal cord. **H,I)** dsRed-expressing cells in the spinal cord in sagittal (H, rostral is left) and cross-sections (I) of the sCMV:dsRed adult fish. Non-red color in panel H is autofluorescence imaged to provide orientation, and blue fluorescence in (I) is DAPI nuclear stain. Arrowhead indicates the cell body of one of the strongly positive cells adjacent to the central canal (CC). Scale bars: A/B = 1 mm, C = 250 μ m; D = 100 μ m, E, F = 0.7 mm; G=0.5 mm; H = 250 μ m; I = 100 μ m.

Figure 5.

A) Fluorescent image of the flank of a sCMV:dsRed line 13-5 fish at 5 months showing expression in the anterior and posterior lateral line nerves (white arrows). **B)** Flank of a sCMV:dsRed line 1-2 fish age matched to the fish in A to show variation in the intensity of expression in the lateral line and appendicular muscle. **C)** The lateral line system in a two-month old sCMV:dsRed line 13-5 fish in three areas: head, trunk (flank), and tail. Lateral line fibers are shown with dsRed expression (red), the lateral line neuromast dye DASPEI (red/green in neuromasts only), and autofluorescence (blue – included to facilitate orientation). DASPEI stained hair cells of the neuromasts appear as bright dots in the green channel. Faint

green fluorescence in the fibers proximal to neuromasts in the 2Di4Asp panels is due to low levels of DASPEI back-labeling. Scale bars = 1 mm (anterior left in all images).

Figure 6.

A) Whole body fluorescent image of a sCMV:dsRed fish (line 2-1) at 5 months showing expression in the skeletal muscle and high-intensity fluorescence in areas proximal to fins such as pectoral fin muscles indicated by an arrow. **B,C)** Like (A), for lines sCMV:EGFP-1 and 2, respectively. **D)** Cross section of the adult sCMV:dsRed 2-1 fish trunk revealing lower transgene expression in general trunk muscle (green arrows) and a much higher level of expression in appendicular muscle such as muscles for dorsal (upper white arrow) and anal fins (lower white arrow). **E,F)** Higher magnification of expression in muscles driving the dorsal and anal fins respectively. **G,H)** Brightfield and fluorescent images (respectively) of explanted eyes and brain viewed from the ventral side showing dsRed-positive appendicular muscle controlling eye movement. Scale Bars = 1 mm except E/F = 0.5 mm.

Figure 7.

A) Heterozygotes of sCMV:dsRed line 13-5 displaying the typical low-level of dsRed expression at 1 dpf and a dramatically increased level of expression in a putative homozygote (inset) that also displayed an altered body morphology, edema, and decreased capacity for movement. **B)** A 7 dpf sCMV:dsRed 13-5 fish displaying dramatically increased dsRed fluorescence following heat shock three days earlier (compare to unshocked animal in 7A). **C-F)** Shown are 7 dpf sCMV:dsRed fish (line 13-5) heat-shocked at 16 hpf (**C**), 1 dpf (**D**), 2 dpf (**E**) or 3 dpf (**F**) and imaged at day 7. Note the increase red fluorescence in fish heat shocked at 3 dpf. **G-H)** sCMV:EGFP-1 fish unshocked (**G**) or shocked (**H**) show the same response as sCMV:dsRed lines. **I-L)** Cross-section of control (**I**) and heat shocked (**J**) fish retina and control (**K**) and heat shocked (**L**) fish brain stem revealing that induction remains largely confined to cell types with high-level expression at later ages. **M,N)** dsRed expression in a young adult sCMV-dsRed line 2-1 fish appendicular muscle of the pelvic fin in control fish (**M**) and fish subjected to heat shock and imaged one week later (**N**). In **M,N**, anterior is left. Scale bars: A-H = 500 μ m; I,J = 175 μ m; K/L = 200 μ m; M/N = 1 mm.