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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 and 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.

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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 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; Kalev-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 0.8 Kb 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 11 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

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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 h after plasmid injection, approximately 50% of the embryos displayed varying degrees of fluorescent reporter expression in multiple tissues (Fig. 1A and 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 (Fig. 1A and 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

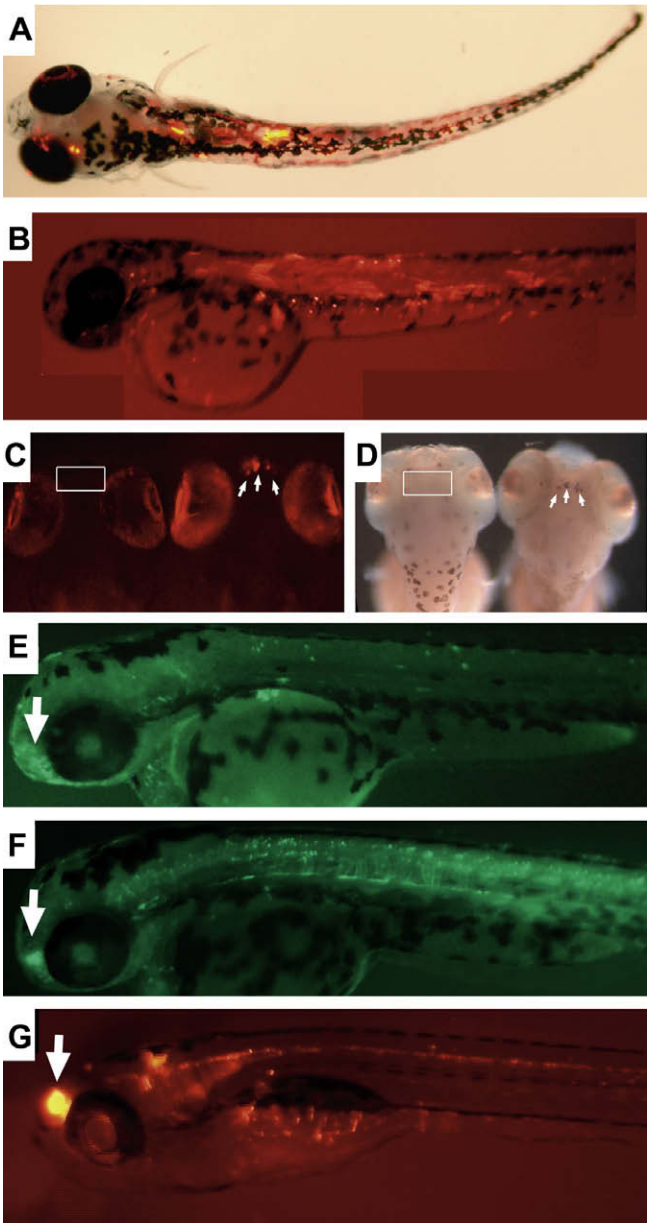

Fig. 1. (A) Representative pattern of sCMV promoter-driven dsRed expression in a zebrafish embryo 3–4 days following injection of eggs revealing scattered cells throughout the embryo. Top view with brightfield overlay. (B) As in (A), side-view fluorescent image. (C) Top view of the head of control (left 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 (2.5 dpf) 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.

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