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Construction of Cell-Type-Specific Promoter Lentiviruses for Optically Guiding Electrophysiological Recordings and for Targeted Gene Delivery

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Summary

It is often advantageous to identify and alter gene expression of specific cell populations within the brain. Currently, it is not possible to a priori identify specific cell types within the brain of rats for electrophysiological recordings, nor is it possible to routinely alter gene expression in specific cell types within the CNS of a variety of species. Here, we describe a general method for the relatively rapid screening of specific promoter activity in cell culture, in acute brain slice preparations, and in vivo. As an example, we describe the examination of an ~3 kb promoter region of the neuroactive peptide cholecystokinin (CCK) compared to the ubiquitous cytomegalovirus (CMV) promoter. We find a high degree of cell-type specificity in vivo using lentiviral approaches in rats and mice.

Keywords

Lentivirus; Electrophysiology; CCK; Cholecystokinin; Behavior; Neuroscience

1. Introduction

The potential for using lentiviral vectors in experimental therapeutics is very promising, owing largely to their ability to integrate into both dividing and postmitotic cells, and to achieve stable, long-lasting transgene expression (1). Additionally, in comparison to adeno-associated-based and sindbis-based vectors, lentiviral vectors allow for the packaging of larger inserts, opening the possibility for the use of larger promoter lengths to achieve gene expression in selected populations of cells (2–4). Achieving restricted expression would be beneficial to both basic science studies of different cell types interacting in complex biologic systems, and to the development of therapeutic gene-delivery systems that are active only within specific types of cells. Despite these potential benefits, the process of determining minimal promoter lengths to achieve biologically appropriate expression is made difficult by the inability to identify a priori enhancer and silencer regions within putative promoter

¹⁰An additional in vitro method for determining appropriate cell-type expression is primary cell culture from an area containing the cell type of interest. Appropriate cell-type expression can then be examined using dual-fluorescent in situ hybridization or double-labeled immunocytochemistry. This method is generally more difficult and time consuming than using HEK cells; however, it provides a better indication of appropriate and specific promoter activity. Of course, the virus should always also be examined for cell-type-specific expression in vivo using dual-labeled immunocytochemistry and/or dual fluorescent in situ hybridization if possible before proceeding with experiments.

regions, and by the possibility that regions of the 5' untranslated region (5' UTR) of a given gene may have an important bearing on its expression. In this context, the screening of putative promoters of varying lengths has largely depended on the selective expression of transfected promoter–reporter constructs in cultured cells, based on the notion that selectivity in vitro may indicate selectivity in vivo. However, this may not always be the case.

Here, we outline an alternative method of screening promoters for cell-type-specific expression in vitro and in vivo. As this method makes use of viral vectors to assess appropriate promoter expression, it offers several advantages over transgenic approaches, including lower cost, more rapid throughput and most importantly, the ability to use the resulting vectors in a variety of different species. We assess cell-type-specific expression using in vitro, acute brain slice electrophysiology and in situ hybridization methods.

2. Materials

2.1. Plasmid Design and Construction

1. Vector NTI Advance™ 10 (free for academic use from Invitrogen Corp., Carlsbad, CA), or equivalent sequence analysis program.
2. Species-specific genomic DNA or BAC DNA for amplifying the promoter region of interest.
3. Antisense and sense primers designed to amplify the promoter region of interest, with custom restriction sites incorporated into the original primers used for subcloning the promoter region in the lentivirus.
4. Any of the following TOPO® cloning vectors with electroporation or chemically competent cells (Invitrogen Corp.): pCR® 2.1-TOPO®, pCR® II-TOPO®, pCR®-blunt II-TOPO®, pCR® 4-TOPO®, or equivalent cloning vectors (see Note 1).
5. Phusion™ High Fidelity PCR Kit (Finnzymes cat.no. F-553S or F-553L).
6. dNTPs (dATP, dTTP, dGTP, dCTP, 100 mM each or 2.5 mM mix).
7. 0.2 mL PCR tubes.
8. Thermal cycler.
9. Restriction enzymes (e.g., *Bam*HI, *Eco*RI, *Cla*I) for restriction mapping and subcloning.
10. A Lentivirus packaging construct. We use pCMV-GFP-dNhe (a kind gift from Inder Verma, Salk Institute, La Jolla, CA). Other backbones are now available commercially.
11. Ampicillin (at 100 mg/mL) and kanamycin (at 100 mg/mL).

¹If you are using Taq polymerase, any of the T-A cloning vectors will be sufficient (e.g., pCR2.1, pCRII, pCR4); however, if you are using a proofreading polymerase, you will need to either add A-overhangs onto the PCR product or use a blunt cloning vector (e.g., pCRII-blunt-TOPO or equivalent).

12. 100-mm round Petri dishes.
13. Miller LB Broth.
14. Miller LB Agar plates: dissolve 37 g of LB Agar (amount may differ, see instructions on bottle) in 1 L of H₂O, autoclave, and allow to cool to ~50 °C. When cooled to 50 °C, add appropriate antibiotic (ampicillin and/or kanamycin) and pour ~15 mL into each 100-mm culture plate, cover and allow to cool. Store at 4 °C protected from light.
15. Gas burner.
16. Bacterial cell spreader.
17. Miniprep kit (Qiaprep Spin miniprep kit, Qiagen cat no. 27106 or equivalent) and Gel Extraction Kit (Zymoclean Gel DNA Recovery Kit™ Zymo Research Corp cat. no. D4002, or equivalent).
18. Vacuum manifold (Qiagen, QIAvac 24 plus Vacuum Manifold, 19413). Not essential, but recommended.
19. Antarctic Phosphatase (NEB cat. no. M0289S).
20. Agarose gel electrophoresis equipment.
21. Electroporator.
22. ElectroTen-Blue® electroporation competent cells (Stratagene cat. no. 200159).
23. T4 DNA ligase and buffer (NEB cat. no. M0202T).
24. Incubator set at 37 °C.
25. Shaking incubator.
26. X-Gal reagent (Gold BioTechnology cat. no. X4281C): dissolve 40 mg of X-Gal in 1 mL of dimethylformamide (Fisher Scientific cat. no. D119-1).
27. 14-mL BD Falcon round bottom culture tubes (Fisher Scientific cat. no. 14-959-45).
28. Sterile glycerol.

2.2. Assessment of Promoter Activity In Vitro

1. Tissue culture capabilities.
2. 293T Human embryonic kidney (HEK) cells (Invitrogen cat. no. R700-07) or equivalent.
3. 1× PBS (Invitrogen cat. no. 14025-092).
4. Trypsin–EDTA.
5. 100× Penicillin/streptomycin.
6. 24-well tissue culture plates.

7. Complete DMEM: Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker cat. no. 12-741F) with 10% fetal bovine serum (FBS) (Gibco/Invitrogen cat no. 26140-079) and 1× penicillin/streptomycin.
8. Transfection reagents: Lipofectamine or calcium phosphate (*see ref. 6 for calcium phosphate reagent setup*).
9. Fluorescent microscope.

2.3. Virus Production and Concentration and Titration

Tissue culture capabilities for working with lentiviral vectors (*see Note 2*).

1. Lentivirus packaging construct (*see Subheading 2.1, item 10*).
2. Lentiviral packaging vectors: pVSVG, p 8.9 (a kind gift from Inder Verma, Salk Institute or pMDL, pRev, and pVSVG (Invitrogen cat. no. K4975-00).
3. Endotoxin-free maxipreps (Endo-free Maxiprep kit, Qiagen cat no. 12632, or equivalent); resuspend DNA at 1 µg/µL.
4. Terrific broth (TFB): dissolve 25 g of LB Broth (amount may differ, *see instructions on bottle*) in 1 L of H₂O and add 5 g peptone, 5 g yeast extract, and 10 mL glycerol. Autoclave and allow to cool.
5. Avanti J-26 Series Centrifuge or equivalent (Beckman Coulter).
6. JLA-10.500 centrifuge rotor or equivalent (Beckman Coulter cat. no. 369681).
7. 500-mL centrifuge bottles or equivalent (Beckman Coulter cat. no. 361691).
8. *See materials and reagents #1–5 and #7–9 from Subheading 2.2.*
9. 150 mm tissue culture dishes.
10. 0.45 µm filter units, 500 mL capacity (Corning cat. no. 430773).
11. Conical bottom ultracentrifuge tubes (Beckman cat. no. 358126).
12. Round bottom ultracentrifuge tubes (Beckman cat. no. 326819).
13. Beckman SW28 rotor (or equivalent).
14. Beckman ultracentrifuge (or equivalent).

2.4. Virus Injections and In Vivo Assessment of Promoter Activity

1. Stereotaxic instrument (David Kopf Model 962 Dual Ultra Precise Small Animal Stereotaxic, or equivalent).
2. Microinfusion pump; Stereotaxically mounted (e.g., World Precision Instruments UMP 2) or stand alone (e.g., Harvard Apparatus PHD 2000) (*see Note 3*).

²Working with lentiviral vectors requires Bio-safety Level II containment. Although current lentiviral vectors have been constructed to be self-inactivating (SIN) as a safety feature, some residual transcription activity has been reported (5). Some institutions may require replication competence assays when using lentiviral vectors. Several chapters in this volume offer detailed descriptions of viral production and titrating, and an excellent protocol describing lentivirus production and concentration is available (6).

3. World Precision Instruments Nanofil 10 μ L syringe (WPI cat. no. NANOFIL) with 35G beveled Nanofil needle (WPI cat. no. NF35BV-2), or equivalent Hamilton syringe (*see* Note 3).
4. Sterile filtered 1% bovine serum albumin (BSA). BSA has been found by some to decrease molecular interactions that might lead the virus particles to “stick” to the injection apparatus.
5. 100% ethanol.
6. Wound clips or sutures.
7. Clippers for trimming hair prior to surgery.
8. Betadine or equivalent 10% providone-iodine solution topical antiseptic.

3. Methods

3.1. General Considerations

One of the most important considerations in the design of cell-type-specific promoter lentiviral vectors is the size and region of the promoter used to drive cell-specific expression patterns. Therefore, it may be advantageous to design several different size promoters keeping in mind that lentiviruses can accept inserts of up to ~8 kb and still successfully package virus particles. Another important consideration is to incorporate the region of the promoter containing the ATG transcription start sequence downstream of the stretch of the promoter to be tested. This promoter region will be inserted into the lentivirus packaging construct such that the ATG sequence of the original promoter remains in frame with the ATG transcription start sequence of the green fluorescent protein (GFP) sequence in the packaging construct. Incorporating the original ATG translation start site ensures that splice junctions near the ATG and possible cell-type control over translation initiation are likely to be conserved within the new construct.

One method for designing different size promoter regions is to examine regions of conservation within the promoter using the UCSC Genome Bioinformatics website's Genome Browser and incorporate regions of high conservation among different species into the region of the promoter to be cloned into the lentiviral vector. There may be several regions of high conservation along different points of the promoter. For instance, there may be regions of conservation at both 2.5 and 7 kb upstream of the ATG sequence. One would

³Numerous infusion methods are available for *in vivo* lentiviral injections; however, some consideration of the needs of your experiment is required before proceeding. The most common injection method is to use a Hamilton syringe attached to an infusion pump that is connected to a stereotactically mounted infusion needle or glass-pulled pipette via polyethylene (PE) tubing. This method generally works well; however, the connection between the Hamilton syringe and injection needle or glass pipette is frequently unreliable because air bubbles trapped within the system that often store the energy of the pressure injection and cause erratic infusions. Backfilling the syringe and PE tubing with mineral oil is generally a better method, but we find that although it works for most injections, it is still often unreliable when infusing lentiviral preparations. The type of injection apparatus used is often determined by the demands of the experiment. For instance, a glass-pulled pipette is the preferred method when conducting electrophysiological experiments following lentiviral infusions in order to reduce tissue damage. However, the viscosity of the lentivirus combined with the very small diameter tip often results in inconsistent injections. A compromise that appears to work well in our hands is to use a stereotactically mounted infusion pump with a WPI Nanofil syringe with a 35G beveled needle. The combination of the small diameter tip size reduces damage yet is large enough to produce consistent injections. For behavioral experiments, we often use a Hamilton syringe with a 26-G needle. This increases tissue damage but allows for very consistent injections because of the larger tip diameter.

then design two sets of primers to generate separate mini promoters of 2.5 and 7 kb incorporating the regions of conservation. Keep in mind, this may not always be possible, and may not always result in cell-type-specific expression patterns.

3.2. Plasmid Design and Construction

1. Evaluate your cloning strategy to assure that the ATG transcription start sequence within the promoter of interest will be in frame with the ATG sequence of the GFP coding sequence when cloned into the lentiviral packaging construct.
2. Design primers taking into account the above considerations and include custom restriction sites that will allow you to easily ligate your promoter into the lentiviral packaging construct. In the case of the pCMV-GFP-dNhe lentiviral construct, the restriction sites will be 5'-*ClaI* site on the sense primer and a 5'-*BamHI* site on the antisense primer (*see* Note 4).
3. Excise the CMV promoter from the pCMV-GFP-dNhe lentiviral construct using a *BamHI/ClaI* digest.
4. Following the digest, treat the reaction mix with Antarctic phosphatase to remove 5' phosphate groups from the DNA and prevent self-ligation.
5. Gel-purify the 6.8 kb virus backbone fragment minus the CMV promoter. When using a spin column kit for purification, elute the DNA in ddH₂O. Store at -20 °C until ready for use (*see* **step 15** below).
6. Meanwhile, set up a PCR reaction using the Phusion™ High Fidelity PCR Kit (or any equivalent kit) to amplify the promoter region of the gene of interest by following the instructions provided with the PCR kit (*see* Note 1).
7. Verify the size of your amplified fragments by running the reaction(s) on a 0.5% agarose gel and then gel purify the PCR fragment(s) of the correct size. When using a spin column kit for purification, elute the DNA in autoclaved ddH₂O.
8. Ligate the purified promoter PCR product into a TOPO or equivalent vector following the manufacturer's instructions (*see* Note 5).
9. Transform electrocompetent or chemically competent bacterial cells using the manufacturer's instructions.
10. Warm two plates per transformation in a 37 °C incubator while your cells are shaking. If you plan to use blue/white screening to identify positive transformations, spread 40 µL of X-Gal (40 mg/mL) on each plate and let warm for 15–30 min before plating.

⁴When determining your cloning strategy, it may be necessary to avoid using a *BamHI* restriction site on your primers (i.e., when there are *BamHI* sites within the promoter region or within the TOPO vector) in order to avoid partial digests. In this case, one may use enzymes with compatible ends (e.g., *BglII*), or if this is not possible, blunting the *BamHI* site on the lentivirus backbone and using a unique restriction site that leaves blunt-ends on your promoter.

⁵For large inserts (>1 kb), allow the ligation to incubate for 30 min at room temperature instead of the recommended 5 min.

11. Plate 50 and 150 μ L of each culture onto LB plates containing the appropriate antibiotic. Place the plates upside down in a 37 °C incubator and incubate overnight.
12. If you are using blue/white screening and TOPO vectors from Invitrogen, pick ten white colonies using a sterile pipette tip and inoculate 5 mL of LB in a 14-mL round bottom falcon tube. If you are using another manufacturer's vector, you may need to pick blue colonies. If you are not using blue/white screening, pick ten colonies and inoculate 5 mL of LB. In both cases, shake at 260 rpm overnight in a 37 °C shaking incubator.
13. Miniprep 1 mL of overnight culture from each colony and analyze using appropriate restriction enzymes. Send positive clones off for sequencing to confirm restriction digest analysis. Be sure to make bacterial stocks (1 mL of bacterial culture + 10% sterile glycerol) for each colony, and store in –80 °C.
14. Excise the promoter region from the TOPO clone with a *Bam*HI/*Cla*I digest. Run the digest on a 0.5% agarose gel and gel-purify the promoter fragment. Elute the fragment in ddH₂O and store in –20°C until use.
15. To ligate the cell-type-specific promoter region into the prepared lentiviral packaging construct from **step 5**, set up a reaction in a 10- μ L volume and use up to 7 μ L of insert DNA and 1 μ L of packaging construct DNA, plus 1 μ L of T4 DNA ligase and 1 μ L T4 DNA ligase buffer (including ATP). Bring up to 10 μ L with ddH₂O if you do not use 7 μ L of insert DNA. Incubate reaction at 16 °C for 4 h. Larger reaction volumes may also be used.
16. Transform the ligated product into ElectroTen[®] Blue cells according to the manufacturer's instructions.
17. Plate onto LB agar plates (50 and 150 μ L) containing 100 mg/mL ampicillin and 100 mg/mL kanamycin. ElectroTen[®] Blue cells are kanamycin resistant and the pCMV-GFP-dNhe lentiviral packaging construct is ampicillin resistant. Therefore, using both antibiotics selects for the appropriate constructs. Incubate plates upside down overnight in a 37 °C incubator.
18. Pick 10–12 colonies from the plates and inoculate into 5 mL of LB containing both ampicillin and kanamycin. Shake overnight in a shaking incubator at 260 rpm.
19. Miniprep 1 mL of overnight cultures and use appropriate restriction enzymes to determine positive clones. Send positive clones off for sequencing to confirm. Remember to make a bacterial stock (1 mL of bacterial culture + 10% sterile glycerol) and store at –80 °C.

3.3. Assessment of Minimal Promoter Activity In Vitro

Before production of active viral particles, the viral constructs should be transiently transfected into HEK 293T cells to determine if the cell-type-specific promoters will provide

for transgene expression and determine their level of expression compared with the CMV promoter.

1. Seed 1×10^5 293T HEK cells in a 24-well plate with 500 μ L of Complete DMEM. Grow cells to 90–95% confluent on the day of transfection.
2. Transiently transfect cells with the cell-type-specific promoter packaging construct using your method of choice (e.g., lipofectamine or calcium phosphate).
3. Also transfect control cultures with the pCMV-GFP-dNhe construct.
4. Incubate cells in a 37 °C incubator for 18–48 h then examine cultures for GFP expression and compare to expression from cells utilizing the CMV promoter (Fig. 1).
5. Be aware that in vitro GFP expression using a cell-type-specific promoter may not necessarily confer appropriate or robust expression in vivo (*see* Note 9).

3.4. Virus Production, Concentration, and Titration

1. Inoculate 5 mL of TFB with stock cells of the HEK-screened construct and grow at 37 °C overnight using appropriate antibiotic.
2. Using appropriate antibiotic, inoculate 500–1,000 mL of TFB with ~1 mL of the overnight culture from **step 1**.
3. Pour cultures into 500-mL or 1-L centrifuge bottles, balance precisely, and spin for 10 min in the Beckman or Sorvall centrifuge at the rate of 7,000 rpm at 4 °C (*see* Note 6).
4. Prepare plasmid DNA using Endotoxin-free Maxiprep kit (follow instructions with the kit) and resuspend DNA at 1 μ g/ μ L (*see* Note 7).
5. For viral production, our plasmid lentiviral transfer vector (LV-CCK-GFP) encoding GFP under the regulation of the CCK promoter and the lentiviral envelope and packaging vectors (p 8.9, pVSVG) were transfected into the HEK 293T cells using the calcium phosphate method. Cells were observed and supernatant was harvested on days 2, 4, and 6 following transfection, filtered, and then concentrated. Viral pellets were resuspended in PBS with 1% BSA, aliquoted, and stored at –80 °C (*see* Note 2).

⁹Examining minimal promoter expression in vitro using HEK cells is a first step at determining level and specificity of promoter activity. GFP or other gene expression driven by cell-type-specific promoters, in general, will express more poorly and/or more slowly than vectors using a CMV promoter. As noted above, this does not necessarily confer appropriate or robust expression in vivo. In other words, poor and/or slow expression in vitro using HEK cells does not indicate poor or inappropriate expression in vivo, nor does it indicate robust or appropriate expression.

⁶If necessary, the pellets can be stored frozen indefinitely at –20 or –70 °C.

⁷In our hands, Endotoxin-free Maxiprep kits do not always provide consistent plasmid DNA preparations. In addition, they are expensive for each preparation. We isolate our plasmid DNA using large-scale alkaline lysis and purify it using cesium chloride/ethidium bromide gradients. We then remove the ethidium bromide using *n*-butanol saturated with H₂O and remove CsCl by dialysis. This method provides milligram quantities of very pure plasmid DNA, is cheaper, and does not require much more actual bench time over the maxiprep kits. Excellent protocols are available in *Molecular Cloning* (7) as well as *Current Protocols in Molecular Biology* (8).

3.5. In Vivo Virus Injections

1. Anesthetize animal following approved animal care guidelines.
2. Shave the top of the head where the incision will be made.
3. Place the animal in the stereotaxic frame and scrub the shaved area with Betadine.
4. Preload the syringe with the 1% BSA for a few minutes and expel before loading the virus into the syringe. Be sure to load slightly more virus than needed for a typical injection (usually 2 μ L) (*see* Note 8).
5. After determining the location of bregma and appropriate coordinates for the injection, drill a small hole through the skull to dura. Verify that the needle is not clogged by expelling a small volume of liquid, and slowly lower the syringe to the appropriate depth below dura (lower the syringe to 0.1 mm below the desired depth and then raise it back to up to the appropriate depth).
6. Start the infusion pump. The typical volume used in our experiments is 2 μ L injected at a rate of 0.2 μ L/min. After the injection is finished, leave the needle in place for an additional 5 min to allow for diffusion of the virus, then slowly raise the needle.
7. Flush the syringe with ddH₂O between infusions to eliminate any debris and reduce the possibility of clogging.
8. Suture the incision and administer appropriate pain medication. Animals should recover for at least 2 weeks before assessment of in vivo GFP expression, as well as behavioral and/or electrophysiological experiments (Fig. 2), to allow for sufficient viral transduction and transgene expression.
9. Clean the syringe with 100% ethanol and ddH₂O before storing.
10. The cell-type specificity of the virus should be examined using in situ hybridization (Fig. 3) combined with in vivo virus injections (Fig. 4). If possible, dual-labeled fluorescence in situ hybridization for GFP and the gene of interest should be used in order to demonstrate co-localization of viral-induced GFP expression and endogenous gene expression. Immunocytochemistry may also be used to verify specificity before proceeding with electrophysiological and/or behavioral experiments. The plasmid packaging construct may also be used to generate transgenic mouse lines (Fig. 5).

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⁸Keep virus on ice at all times when not loaded into the syringe. If you are using the WPI Nanofil with the 35G needle for injection, because of the viscosity of the virus, it is easier to fill the syringe with the 26G needle, then draw the virus into the glass portion of the syringe, and then change to the 35G needle.

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Fig. 1.

GFP expression vectors. Here we describe the analysis of an ~3 kb promoter region of the neuroactive peptide cholecystokinin (CCK) compared to the ubiquitous cytomegalovirus (CMV) promoter. Before production of active viral particles, the viral constructs for CMV-GFP (**A**) and CCK-GFP (**C**) were transiently transfected into HEK293 cells to determine if the CCK promoter would provide for transgene expression, and determine its level of activity compared to the CMV promoter. Examination of cells 48 h posttransfection indicated that the CCK promoter was indeed able to generate detectable expression of GFP in HEK293 cells (**D**). The level of expression attained with the CCK promoter appeared to be quite high, although still far less than the expression of the CMV promoter-containing vector (**B**, **D**). This figure is adapted from Chhatwal et al. (9).

Fig. 2.

Using GFP-expressing lentivirus to record from rat interneurons. Acute brain slices were prepared from juvenile rats 12 days postinfusion of CCK-GFP into the CA1 region of the hippocampus (A–D). GFP fluorescence (B) was first used to identify a putative CCK interneuron within the slice (*arrow*), which was then located using normal brightfield illumination (DIC, *a*). The fluorescently identified cell was then patched and electrophysiologic recordings obtained (example trace shown in C). During the recording process, the cell was passively filled with 0.3% biocytin. Slices were then fixed overnight, resectioned at 75 μ m, and visualized using a streptavidin-Texas Red conjugate. This neuron

exhibited a voltage-dependent depolarizing sag in the voltage response to hyperpolarizing current injection (**C**). The amplitude and rate of onset of this rectification became more pronounced with increasing hyperpolarization, which is indicative of activation of hyperpolarization-activated nonselective cation channels. In the depolarizing direction, this neuron exhibited a rhythmic pattern of action potential firing, and a pronounced fast after-hyperpolarizing potential (fAHP) following each spike, which are properties similar to those previously reported for hippocampal CCK interneurons. The same CCK-GFP virus was also used to label rat BLA neurons, and to identify them for electrophysiologic recording (**E–G**). Confocal images of CCK neurons are shown at 60× magnification with *arrows* denoting fluorescing neurons. One of these putative CCK neurons (**E–G**, *left arrow*) was patched and recorded. During recording, this cell was filled with a biocytin and visualized (**F**, **G**) using the same post hoc technique as used in the hippocampus. This figure is adapted from Chhatwal et al. (9).

Fig. 3.

Endogenous CCK mRNA expression. CCK mRNA was examined using in situ hybridization, demonstrating high levels of expression within the hippocampal formation (low power **A**, high power **B**, **C**) and within the basolateral complex of the amygdala (**D**). Intense CCK mRNA expression is present within the CA3 subfield and within the interneuron rich region (polymorph layer of the dentate gyrus, PoDG) separating the granule cell layers of the dentate gyrus (GCL; **A**, **B**). Notably, the GCL lacks CCK mRNA + cell bodies. CCK mRNA is highly expressed in the CA1 and fasciola cinereum (fc; **A**, **C**). This figure is adapted from Chhatwal et al. (9).

Fig. 4.

Virally mediated GFP expression *in vivo* to examine promoter specificity. Virus encoding CCK-GFP was injected into the dentate gyrus of C57/BL6 adult mice (2 μ L/side over 10 min). Animals were sacrificed 10 days later and fluorescence was assessed on sectioned, fixed tissue. Examples from sections showing sparse (**A–C**) and dense expression (**D–F**) are shown above. **B**, **E** are Hoechst-stained photomicrographs of the same sections shown in (**A**) and (**D**), respectively. **c** and **f** are overlays of (**A**) and (**B**), and (**D**) and (**E**), respectively. We observed that GFP expression in various subfields of the hippocampus was generally similar to the patterns of expression seen previously. More specifically, we observed that the expression of GFP in the dentate was contained to the interneuron-rich regions in-between the pyramidal cell layers of the dentate, reflecting the expression patterns seen for CCK mRNA in the dentate gyrus (Fig. 3). The high level of GFP expression seen in these experiments suggests that the CCK promoter length used here may be suitable to drive selective expression of transgenes *in vivo*. Additionally, the high levels of expression generated by this promoter may make it useful in the generation of transgenic mouse strains. This figure is adapted from Chhatwal et al. (9).

Fig. 5.

Transgenic production with cell-type-specific viral vectors to examine promoter specificity. While the aforementioned viral vectors can be used to induce transgene expression in restricted regions, in many cases widespread transgene expression is desirable. To assess the suitability of using promoter lengths identified in the aforementioned manner for the production of transgenic mice, we subcloned the coding sequence for Cre-IRES-DsRed into the CCK-GFP vector. This new plasmid (CCID) was then linearized and microinjected into C57/BL6 embryos. These injections yielded three founders carrying the CCID gene, and these were subsequently mated with RosaLacZ reporter mice to assess transgene expression.

The patterns of Cre-mediated LacZ expression suggest that the CCK promoter was capable of driving Cre expression in the transgenic construct. The expression of LacZ varied widely among the three founder lines, although ubiquitous, nonselective expression was not observed in any of the lines. The variability of expression suggests that there may be significant silencing of transgene expression depending on the insertion site of the recombinant sequence, a commonly observed phenomenon in neural systems (this was especially true of the DsRed portion of the gene, which appeared to be completely silenced in all three transgenic lines). Animals from CCID line B showed the widest expression of LacZ, which was essentially identical to endogenous CCK gene expression (Fig. 4A, C), suggesting that they may be suitable for use in generating CCK-selective knockouts when crossed with animals containing floxed transgenes. This figure is adapted from Chhatwal et al. (9).