Cloning and Characterization of a Potassium-dependent Sodium/Calcium Exchanger in *Drosophila*

K. Haug-Collet,* B. Pearson,* R. Webel,* R.T. Szerencsei,‡ R.J. Winkfein,‡ P.P.M. Schnetkamp,‡ and N.J. Colley*

*Department of Ophthalmology and Visual Sciences, Department of Genetics, University of Wisconsin, Madison, Wisconsin 53706; and ‡Department of Physiology and Biophysics, Medical Research Council Group on Ion Channels and Transporters, University of Calgary, Faculty of Medicine, Calgary, Alberta T2N 4N1, Canada

Abstract. Sodium/calcium(-potassium) exchangers (NCX and NCKX) are critical for the rapid extrusion of calcium, which follows the stimulation of a variety of excitable cells. To further understand the mechanisms of calcium regulation in signaling, we have cloned a *Drosophila* sodium/calcium-potassium exchanger, *Nckx30C*. The overall deduced protein topology for NCKX30C is similar to that of mammalian NCKX, having five membrane-spanning domains in the NH_2 terminus separated from six at the COOH-terminal end by a large intracellular loop. We show that NCKX30C functions as a potassium-dependent sodium/calcium exchanger, and is not only expressed in adult neurons as was expected, but is also expressed during ventral nerve cord development in the embryo and in larval imaginal discs. *Nckx30C* is expressed in a dorsal–ventral pattern in the eye-antennal disc in a pattern that is similar to, but broader than that of *wingless*, suggesting that large fluxes of calcium may be occurring during imaginal disc development. *Nckx30C* may not only function in the removal of calcium and maintenance of calcium homeostasis during signaling in the adult, but may also play a critical role in signaling during development.

Key words: calcium • development • *Drosophila* • photoreceptor • signal transduction

In resting cells, intracellular calcium concentration is maintained at 10–100 nM, and can rise up to tens of μM during stimulation (Peretz et al., 1994b; Bootman and Berridge, 1995; Hardie, 1996b). The precise control of spatial and temporal profiles of calcium is essential for cellular function, and prolonged elevation of cytosolic calcium can be toxic, leading to cell death (Berridge, 1998; Berridge et al., 1998). Hence, proper calcium removal or sequestration after a transient rise in cytoplasmic calcium is vital to all cells. Calcium extrusion from cells is carried out by two classes of membrane proteins, ATP-driven calcium pumps and sodium/calcium exchangers (NCX).[1] The latter are thought to be particularly important in cells that handle a large flux of calcium across their plasma membrane, such as cardiac myocytes and many neurons, in-cluding vertebrate photoreceptors (for review see Blaustein and Lederer, 1999).

Exchangers function to lower and maintain intracellular calcium at or below 100 nM by using the transmembrane (TM) sodium gradient as an energy source. Of the two well-characterized families of mammalian sodium/calcium (Na^+/Ca^{2+}) exchangers, one family, NCX, utilizes a stoichiometry of $3Na^+/1Ca^{2+}$ and has three isoforms (Nicoll et al., 1996b; Philipson et al., 1996). NCX-type exchangers are expressed in a variety of tissues including heart, kidney, brain, as well as smooth and skeletal muscle (Nicoll et al., 1996b). The second family, called potassium-dependent sodium/calcium exchangers (NCKX), uses both the inward sodium gradient and the outward potassium gradient to extrude calcium at a stoichiometry of $4Na^+/1Ca^{2+},1K^+$ (Cervetto et al., 1989; Schnetkamp et al., 1989). NCKX-type exchangers appear to have a more limited tissue distribution, and have thus far only been characterized extensively in the outer segments of retinal rod photoreceptors (for reviews see Schnetkamp, 1989; Lagnado and McNaughton, 1990). Retinal rod NCKX1 has been cloned from several mammalian species and shown to be an NCKX after heterologous expression in HEK293 cells (Reiländer et al., 1992; Tucker et al., 1998; Cooper et al., 1999). It has been localized to the plasma membrane of

rod photoreceptor outer segments (Haase et al., 1990; Reiländer et al., 1992). Analysis of the results obtained by the various genomic sequencing projects demonstrate that NCKX homologues are encoded by eukaryotic and prokaryotic genomes (Wilson et al., 1994; Schwarz and Benzer, 1997). Although NCKX- and NCX-type exchangers display little overall amino acid sequence identity, they are thought to be evolutionarily related (Nicoll et al., 1996a; Schwarz and Benzer, 1997).

Calcium plays an important role in phototransduction in both vertebrates and invertebrates. Both utilize rhodopsin-mediated, G protein–coupled cascades, but with some important differences. Illumination of vertebrate rod photoreceptors results in the closing of cGMP-gated cation channels, and thus hyperpolarization of the photoreceptor cell (Stryer, 1986; Yau, 1994). A dark equilibrium between calcium influx via cGMP-gated channels and calcium extrusion via NCKX1 is disrupted, and net extrusion of calcium through NCKX1 results. Cytosolic calcium then falls from a dark value of 500–600 nM to a value of $<$50 nM in bright light (Gray-Keller and Detwiler, 1994; Sampath et al., 1998). This process mediates the process of light adaptation in both retinal rods and cones (Matthews et al., 1988; Nakatani and Yau, 1988a).

In *Drosophila*, calcium changes in the opposite direction (see Fig. 1). Phototransduction occurs via a phospholipase C–mediated cascade, and illumination triggers the opening of the cation-selective channels. The photoreceptors depolarize and intracellular calcium rises to tens of μM (Peretz et al., 1994a; Ranganathan et al., 1994; Hardie, 1995, 1996a,b; Ranganathan et al., 1995; Zuker, 1996). The mechanism that reduces calcium back to resting levels (100 nM) after illumination has not yet been identified, but may utilize an electrochemical exchanger which couples calcium release from the cell to the inward sodium gradient, as is the case in vertebrate rod photoreceptors (Yau and Nakatani, 1984; Schnetkamp, 1986; Lagnado et al., 1988; Lagnado and McNaughton, 1990). In fact, sodium/calcium exchange has been measured in *Drosophila* as well as in other invertebrate photoreceptors, and is also thought to be critical in light-adaptation (Lisman and Brown, 1972; Minke and Armon, 1984; O'Day and Gray-Keller, 1989; O'Day et al., 1991; Ranganathan et al., 1994; Hardie, 1995; Bauer et al., 1999).

To further understand the molecular basis of calcium homeostasis during signaling, we have cloned and characterized a *Drosophila* potassium-dependent sodium/calcium exchanger, *Nckx30C*. We show that it functions as an NCKX when expressed in High Five cells (an insect cell line). *Nckx30C* is distinct from *Calx*, an NCX-type exchanger in *Drosophila* (Hryshko et al., 1996; Ruknudin et al., 1997; Schwarz and Benzer, 1997; Dyck et al., 1998; Omelchenko et al., 1998). Both *Nckx30C* and *Calx* are expressed in the adult brain and in photoreceptor cells as well as during development. We hypothesize that these exchangers are not only critical to the proper functioning of the photoreceptor cells, and other cells in adult flies, but may also play critical roles in the removal of calcium generated during signaling processes in embryogenesis and in patterning of the imaginal discs of *Drosophila*.

Materials and Methods

Molecular Cloning and DNA Sequencing of Ncxk30C

A cDNA clone was constructed, corresponding to the bovine rod photoreceptor NCKX1 that encoded a fusion between the highly conserved TM1–TM5 and TM6–TM11 regions. The clone was ^{32}P-radiolabeled using the Prime-It II Random Primer Labeling Kit (Stratagene). We used this ^{32}P-radiolabeled DNA probe to screen a *Drosophila* genomic library (a gift of K. Moses, Emory University, Atlanta, GA, and G.M. Rubin, University of California, Berkeley, Berkeley, CA). The genomic library was screened according to the methods described in Sambrook et al. (1989). We obtained several genomic clones. A 1.3-kb EcoRI and XbaI genomic fragment was subcloned into pBluescriptII KS (Stratagene), ^{32}P-radiolabeled, and used to screen a *Drosophila* eye-enriched lambda ZAPII cDNA library (gift of C.S. Zuker, University of California, San Diego, San Diego, CA) (Shieh et al., 1989). A set of overlapping cDNAs encompassing the entire *Nckx30C* transcript was isolated and sequenced. No other cDNAs were identified. The full-length *Nckx30C* cDNA was constructed and the DNA sequence was determined by fluorescent-based sequencing methods (Applied Biosystems). The BLAST search program (Altschul et al., 1990) (http://www.ncbi.nlm.nih.gov/BLAST) and the CLUSTAL W multiple sequence alignment program were used for sequence similarity analysis (Thompson et al., 1994) (http://pbil.ibcp.fr/NPSA/npsa_clustalw.html). The sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number AF190455.

Functional Analysis

The full-length *Nckx30C* cDNA was cloned into the novel insect cell vector pEIA as an EcoRI fragment, and this was used for the stable transfection of High Five insect cells as described (Farrell et al., 1998). High Five cells (BTI-TN-5B1-4) derived from *Trichoplusia ni* egg cell homogenates were purchased from Invitrogen. These cells do not display endogenous exchanger activity.

Potassium-dependent sodium/calcium exchange activity was measured in High Five cells transformed with *Nckx30C* cDNA. The cells were loaded with sodium via the sodium-potassium ionophore, monensin, in a medium containing high sodium, 150 mM NaCl, 80 mM sucrose, 0.05 mM EDTA, and 20 mM Hepes, pH 7.4, according to the methods described for rod outer segments (Schnetkamp et al., 1995). The ionophore was removed and the sodium-loaded cells were washed with and resuspended in 150 mM LiCl, 80 mM sucrose, 0.05 mM EDTA, and 20 mM Hepes, pH 7.4. The cells were diluted 10-fold in media containing 80 mM sucrose, 20 mM Hepes, pH 7.4, and either 150 mM KCl, 150 mM NaCl, or 150 mM LiCl. ^{45}Ca uptake was initiated by addition of 35 μM CaCl$_2$ and 1 μCi ^{45}Ca. ^{45}Ca uptake in High Five cells was measured with a rapid filtration method with the use of borosilicate glass fibers over a time-course of 5 min as described previously (Schnetkamp et al., 1991b). The ice-cold washing medium contained 140 mM KCl, 80 mM sucrose, 5 mM MgCl$_2$, 1 mM EGTA, and 20 mM Hepes, pH 7.4.

Northern Analysis

Total RNA was prepared from the heads and bodies of 0–7-d-old *Drosophila w1118* and *eya* lines using the Ultraspec RNA isolation system (Biotecx). PolyA$^+$ RNA was isolated by affinity chromatography on oligo(dT) cellulose columns using the FastTrack 2.0 System (Invitrogen, Inc.). PolyA$^+$ RNA from third instar larvae and 0–24-h embryos (Canton S strain) was purchased from Clontech. PolyA$^+$ RNA (10 μg) from each sample was run on a denaturing 0.9% agarose gel for 3 h 130V/cm. The gel was stained with ethidium bromide and photographed on a UV transilluminator. The mRNA was transferred overnight by capillary action in 20× saline sodium citrate (SSC) to a positively charged nylon membrane (Nytran Plus), and fixed by UV cross-linking. The membrane was probed with [^{32}P]UTP antisense riboprobes (Maxiscript In Vitro Transcription kit; Ambion, Inc.). Four subclones of *Nckx30C* were constructed and used for riboprobe production: 1.3 kb BamHI-BamHI (5' end), 500 bp BamHI-ClaI (TM1–TM5), ClaI-ClaI (loop region), and ClaI-EcoRV (TM6–TM11) (see Fig. 4 A). The antisense and sense riboprobes were incubated with the membranes using the NorthernMax hybridization buffer (Ambion, Inc.), the membranes were washed with 0.2× SSC, 1% SDS at 65°C, and exposed to Kodak X-OMAT X-ray film (Eastman Kodak). All four antisense probes displayed the same labeling pattern. No signal was detected with the sense probes. A 250-bp mouse β-actin antisense riboprobe was

used as a control for the amount of RNA loaded (Maxiscript In Vitro Transcription kit; Ambion, Inc.).

In Situ Hybridization to Polytene Chromosomes

Polytene chromosome squashes (*w m f* strain) were prepared as described (Lim, 1993). A 1.3-kb EcoRI and XbaI genomic fragment was nick-translated using biotin-16-UTP (Boehringer Mannheim Corp.). Hybridization with the biotinylated probe was carried out in 600 mM sodium chloride, 1× Denhardt's, 50 mM magnesium chloride, and 50 mM sodium phosphate, pH 6.8, at 37°C overnight. Samples were washed with 2× SSC, 0.1% Triton X-100, and PBS, pH 7.2 (Lim, 1993). Hybrids were detected using Vectastain ABC (Vector Laboratories, Inc.). Labeling was identified at a single cytological position on chromosome 2L, 30C5-7.
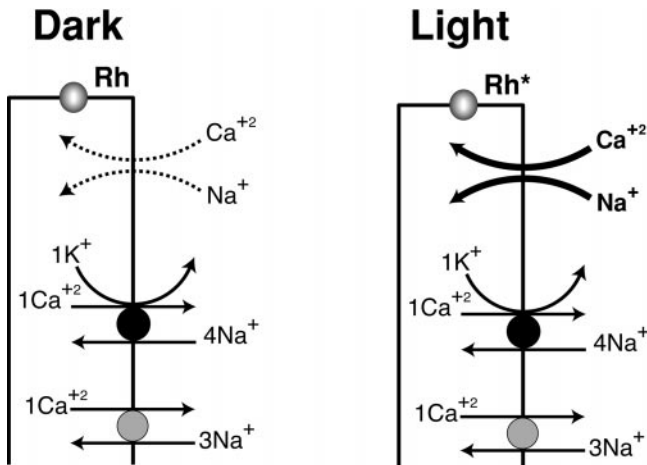
In Situ Hybridization

Heads from the *w1118* strain of flies were embedded in Tissue-Tek OCT Compound (Miles, Inc.) and frozen on dry ice. Eyes were fixed in 4% paraformaldehyde, infiltrated with 2.3 M sucrose, and frozen in liquid nitrogen according to methods described previously (Colley et al., 1991). In situ hybridizations were carried out on 14-μm head sections and 0.5-μm eye sections in 50% formamide, 5× SSC, 0.5 mg/ml sheared herring sperm DNA, 0.05 mg/ml heparin, 0.25% CHAPS, 0.1% Tween 20, 1 mg/ml yeast torula RNA, 1× Denhardt's, at 55°C overnight. Embryo and imaginal disc in situ hybridizations were carried out essentially as described in Panganiban et al. (1994). In brief, embryos (Canton S strain) were collected, dechorionated, fixed, and processed for in situ hybridization as described by Panganiban et al. (1994). Proteinase K was not used. Embryos were hybridized in 50% formamide, 5× SSC, 500 μg/ml sheared salmon sperm DNA, 0.1% Tween 20, 1 mg/ml glycogen. Hybridization was carried out at 55°C for 24–30 h. Probe concentrations were 90 ng/300 μl. The larval

Figure 1. The phototransduction cascade in *Drosophila*. Absorption of a photon photoactivates rhodopsin, leading to the opening of the cation-selective channels (Light). Extracellular calcium (Ca^{+2}) and sodium (Na^+) enter the cell via the light-activated channels, causing a depolarization of the photoreceptor cells. It is estimated that calcium levels rise from 100 nM to >10 μM upon light stimulation (Hardie, 1996b). Calcium entering through the light-sensitive channels is thought to play a key role in deactivation of the light response and light adaptation. Rapid removal of calcium from the photoreceptor cells is key to the recovery from the light response. Removal of calcium following light-stimulation may occur via NCKX (filled circle) and NCX (shaded circle). NCX utilizes a stoichiometry of $3Na^+/1Ca^{2+}$ to extrude calcium, and NCKX uses both the inward sodium gradient and the outward potassium gradient to extrude calcium at a stoichiometry of $4Na^+/1Ca^{2+},1K^+$. Broken arrows indicate that the cation influx through the light-sensitive channel does not occur in the dark (Dark). Rh, rhodopsin molecule; Rh*, photoexcited rhodopsin molecule. This figure is an adaptation of one drawn by Yau (1994).
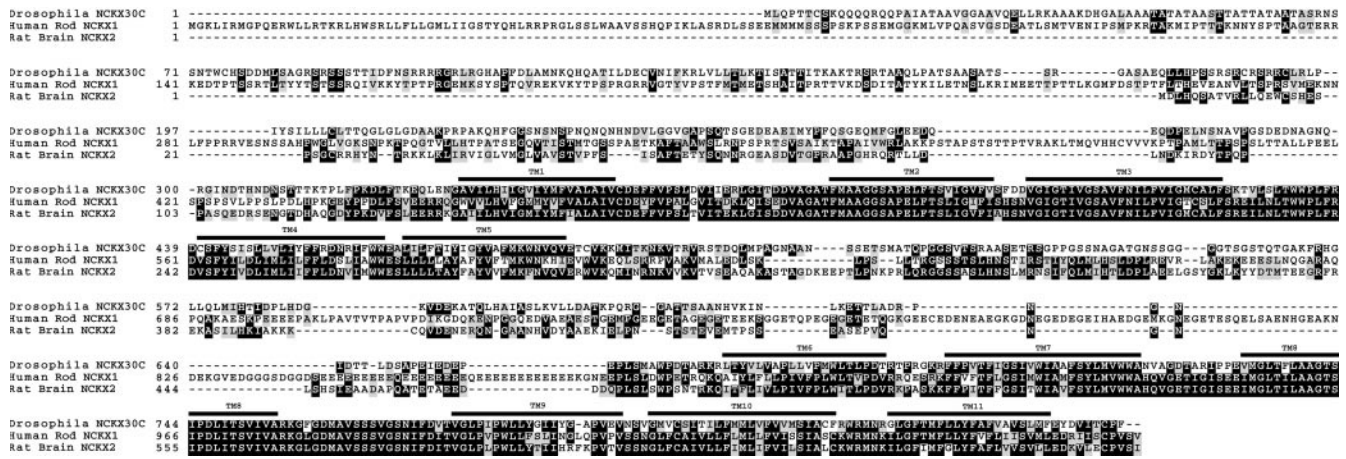
Nckx30C

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1    GAATTCCGAAGTGAAACGGAAAAGCCTGAAAAAAGGTGGCGTGAAAAATACCACAAGAGGCGGGGGTGTGTGAAAATCTA
81   GGGGAATAGCGCGCCCCGTGGAAATGGAAATGCCGAGGCTCCTGTTAGTCTCGCCAGCAGCGGATGTAGCAGCAACACCA
161  GCGGAAACAGCAACCTGAATCCGCATCCCGCATCCGGAACAGCAACCGGGCCCGCTCCCAATTTCTCCGCTCTCTCCGTTC
241  CGTGTTAATTGTTCATTTATGTCATAGTGTATATATAAAGTTGTGCGATACAAATGAATCGCCTAGATCTGACAGCAAGCGA
321  GAAACTGTAACCTAAACTCAGTCTAATCAAAAGCGTACAACGACAACAAACAGGTTTTGCAAGTGAAACGACATAACACCA
401  AGCCAACAAACCCAACAAACCCAAACAAACCGATCAAACAAGTAACCAACCAACAACATTAATCCAACGGGTCCA
474                                              ATG TTG CAG CCA ACA ACA
                                                 M   L   Q   P   T   T      6
492  TGC AGC AAA CAG CAA CAG CAG AGG CAG CAA CCA GCA ATC GCA ACA GCA ACA GTG GGT GGA
     C   S   K   Q   Q   Q   Q   R   Q   Q   P   A   I   A   T   A   T   V   G   G   26
552  GCA GCG GTC CAA GAG CTG CTT CGG AAA GCC GCA GCT AAA GAT CAT GGA GCC TTA GCA GCA
     A   A   V   Q   E   L   L   R   K   A   A   K   D   H   G   A   L   A   A   46
612  GCA ACT GCA ACA GCA ACA GCA ACA TCA ACA ACT GCA ACA ACT GCA ACA GCA GCA ACA GCA
     A   T   A   T   A   T   A   A   S   T   T   A   T   T   A   T   A   A   T   A   66
672  AGC CGC AAC AGC AGC AAC ACT TGG TGC CAC AGC GAC GAC ATA TCG TCC GCA GGC GAC AGC
     S   R   N   S   S   N   T   W   C   H   S   D   D   M   L   S   A   G   R   S   86
732  CGC AGC AGC AGC ACA ACG ATA GAC TTC AAT AGT CGC CGG CGG AGG GGG CGC CTA CGA GGC
     R   S   S   S   T   T   I   D   F   N   S   R   R   R   G   R   L   R   G   106
792  CAC GCC CCC TTT GAC CTG GCA ATG AAC AAG CAA CAC CAA GCT ACG ATA CTG GAC GAG TGT
     H   A   P   F   D   L   A   M   N   K   Q   H   Q   A   T   I   L   D   E   C   126
852  GTA AAT ATT TTC AAG CGC TTA GTA TTA TTA ACA TTA ACA ATA ACA TCA ACA ACA ACA ATA
     V   N   I   F   K   R   L   V   L   L   T   L   K   I   T   S   A   T   T   I   146
912  ACG AAA GCA AAG ACA AGA AGC AGA ACA GCA GCG CAG CTA CCA GCA ACA TCT GCA GCA TCT
     T   K   A   K   T   R   S   R   T   A   A   Q   L   P   A   T   S   A   A   S   166
972  TCT AGA TCA TCT CGT GGT GCC TCC GCG GAG CAA TTG CTC CAC CCA AGT AGC CGC AGT CGC
     S   R   S   S   R   G   A   S   A   E   Q   L   L   H   P   S   S   R   S   R   186
1032 TGC AGA AGT CGC CGC TGC CTC CGC CTG CCG ATC CAC AGC ATC CTG CTG CTC TGC CTA ACC
     C   R   S   R   R   C   L   R   L   P   I   H   S   I   L   L   C   L   T   206
1092 ACC CAG GGA TTG GGT CTG GGA GAT GCC GCC AAG CCC AGG CCG GCC AAG CAG CAT TTC GGT
     T   Q   G   L   G   L   G   D   A   A   K   P   R   P   A   K   Q   H   F   G   226
1152 GGC AGC AAC AGC AAC AGC CCA AAT CAG AAC CAG AAC CAC AAC GAT GTC CTG GGC GGA GTG
     G   S   N   S   N   S   P   N   Q   N   Q   N   H   N   D   V   L   G   G   V   246
1212 GGT GCC CCA TCC CAA ACG TCC GGC GAG GAC GCC GAG ATC ATG TAC CCG GAG CTC AAT TCC AAT
     G   A   P   S   Q   T   S   G   E   D   E   A   E   I   M   Y   P   E   L   N   S   N   266
1272 GGG GAG CAG ATG TTT GGC CTT GAG GAG GAC GAA CAG GAT CCG GAG CTC AAT TCC AAT
     G   E   Q   M   F   G   L   E   E   D   Q   E   Q   D   P   E   L   N   S   N   286
1332 GCA GTG CCC GGC TCC GAT GAG GAT AAT GCG GGC AAT CAA CGT GGT ATC AAT GAC ACG CAC
     A   V   P   G   S   D   E   D   N   A   G   N   Q   R   G   I   N   D   T   H   306
1392 AAC GAT AAC TCG ACC ACC ACG AAA ACG CCA CTA TTC CCA AAA GAT CTA TTC ACG AAA GAA
     N   D   N   S   T   T   T   K   T   P   L   F   P   K   D   L   F   T   K   E   326
1452 CAG CTT GAG AAC GGC GCT GTC ATC CTG CAC ATC ATT GGG GTG ATC TAT ATG TTT GTG GCG
     Q   L   E   N   G   A   V   I   L   H   I   I   G   V   I   Y   M   F   V   A   346
1512 CTG GCC ATT GTC TGT GAC GAA TTC TCC GTG CCT TCC CTT GAC GTA ATC ATT GAA AGG CTC
     L   A   I   V   C   D   E   F   S   V   P   S   L   D   V   I   I   E   R   L   366
1572 GGC ATC ACG GAC GAT GTG GCC GGC ACG ATT ATG GCG GCG GGT GGC AGT GCC CCC GAG
     G   I   T   D   D   V   A   G   T   I   M   A   A   G   G   S   A   P   E   386
1632 CTC TTC ACC AGT GTG ATT GGC GTT TTC GTG TCG TTC GAT GAT GTG GGC ATT GGT ACG ATC
     L   F   T   S   V   I   G   V   F   V   S   F   D   D   V   G   I   G   T   I   406
1692 GTT GGC TCC GCC GTG TTT AAC ATC CTG TTT GTG ATC GGA ATG TGC GCG TTC TTC TCC AAG
     V   G   S   A   V   F   N   I   L   F   V   I   G   M   C   A   F   F   S   K   426
1752 ACG GTC CTG TCG CTC ACC TGG TGG CCT TTG TTC CGC GAC TGC TCG TTC TAC AGC ATC AGT
     T   V   L   S   L   T   W   W   P   L   F   R   D   C   S   F   Y   S   I   S   446
1812 TTG CTG GTG CTG ATC TAC TTC TTC CGG GAT AAC CGC ATC TTC TGG TGG TGG AGG CTC ATC
     L   L   V   L   I   Y   F   F   R   D   N   R   I   F   W   W   W   R   L   I   466
1872 CTG TTC ACC ATC TAC ATC GGC TAC GTG GCC TTC ATG AAG TGG AAC GTC CAG GAG GAG ACG
     L   F   T   I   Y   I   G   Y   V   A   F   M   K   W   N   V   Q   E   E   T   486
1932 TGC GTC AAG AAG ATG ATT ACC AAA AAC AAG GTG ACT CGC GTC AGA AGT ACA GAT CAA CTT
     C   V   K   K   M   I   T   K   N   K   V   T   R   V   R   S   T   D   Q   L   506
1992 ATG CCA GCA GGT AAT GCG GCC AAC TCA TCG GAA ACA TCG ATG GCC ACC CAG CCG GGC GGC
     M   P   A   G   N   A   A   N   S   S   E   T   S   M   A   T   Q   P   G   G   526
2052 TCC GTA ACA TCG CGG GCG GCC AGT GCC GAA ACC CGC TCG GGT CCG CCC GGA TCG AGC AAT GGC
     S   V   T   S   R   A   A   S   A   E   T   R   S   G   P   P   G   S   S   N   A   546
2112 GGC GCC ACA GGT AAT AGC AGC GGC GGG GGC GGC ACC TCG GGT AGT ACT CAG ACC GGT GCC
     G   A   T   G   N   S   S   G   G   G   G   T   S   G   S   T   Q   T   G   A   566
2172 AAG TTC CGC CAC GGC CTG TTA CAG CTA ATG ATA CAG ATA GAT CCA CTA CAC GAT GGC
     K   F   R   H   G   L   L   Q   L   M   I   H   T   I   D   P   L   H   D   G   586
2232 AAA GTG GAC GAG AAG ACG ACG CAG CTG CAC GCC ATC GCC TCG CTG AAG GTG TTG CTG GAT
     K   V   D   E   K   A   T   Q   L   H   A   I   A   S   L   K   V   L   L   D   606
2292 GCC ACC AAA CCA CAG CGC GGT GGT GCC ACC ACC TCG GCG AAT CAC GTC AAG ATC AAT
     A   T   K   P   Q   R   G   G   A   T   T   S   A   A   N   H   V   K   I   N   626
2352 CTC AAG GAG ACC ACG CTC GCC GAT CGT CCC AAT GGG AAC ATC GAT ACC ACC CTC GAC TCG
     L   K   E   T   T   L   A   D   R   P   N   G   N   I   D   T   T   L   D   S   646
2412 GCG CCC GAA ATC GAG GAT GAG CCG GAA CCA CTG AGC ATG GCC TGG CCG GAT ACG ACG CGG
     A   P   E   I   E   D   E   P   E   L   S   M   A   W   P   D   T   T   R   666
2472 AAG CGG CTC ACC TAC GTC CTG GTG GCC AAG CGC TTC TTT CCG GTC ACC TTC ATT GGC TCC ATC GTG
     K   R   L   T   Y   V   L   V   A   P   L   V   P   M   W   L   T   L   P   686
2532 GAC ACG CGG ACG CCG CCG TGC GAG AAG CGC TTC TTT CCG GTC ACC TTC ATT GGC TCC ATC GTG
     D   T   R   T   P   R   G   K   R   F   F   P   V   T   F   I   G   S   I   V   706
2592 TGG ATA GCG GCC TTC TCC TAC CTG ATG GTG TGG TGG AAC GTG GCC GGC GAC ACA GCG
     W   I   A   A   F   S   Y   L   M   V   W   W   N   V   A   G   D   T   A   726
2652 CGC ATT CCG CCC GAG GTC ATG GGT CTG ACC TTC CTG GCG AGC ACC TCC ATT CCG GAC
     R   I   P   P   E   V   M   G   L   T   F   L   A   A   S   T   S   I   P   D   746
2712 TTG ATT ACC TCG GTG ATT GTG GCA CGC AAG GGA TTC GGC GAC ATG GCC GTA TCC AGT TCG
     L   I   T   S   V   I   V   A   R   K   G   F   G   D   M   A   V   S   S   S   766
2772 GTG GGC AGC AAC ATA TTC GAC GTG ACC GTG GGA CTG CCG ATA CCG ACC GTC TGC TCC ATC
     V   G   S   N   I   F   D   V   T   V   G   L   P   I   P   W   L   L   Y   Q   786
2832 ATC ATC TAT GGG CCC GTC GAG GTG GTG AAC TCG GTG GGC ATG GTC TGC TCC ATC ACC ATC
     I   I   Y   G   A   P   V   F   V   N   S   V   G   M   V   C   S   I   T   I   806
2892 CTG TTT ATG ATG CTG GTG TTC GTG ATG TCA ATC AGC TGC TTC CGC TCG GGC ATG AAC
     L   F   M   M   L   V   F   V   M   S   I   A   C   F   R   W   R   M   N   826
2952 AGG GGG TGC TTC ACC ATG TTC CTG TTG TAT TTT GTC GCC GTA TCG CTG ATG
     R   G   L   G   F   T   M   F   L   L   Y   F   A   F   V   A   V   S   L   M   846
3012 TTC GAA GAT GTG ATC ACG TGC TTT TAA
     F   E   Y   D   V   I   T   C   F   *   856
3045 ACAAGGGGCAGGCGCAAAGACAAAAGCGGGGCAATAGTTTACCGAGTTACCCGGCTGTCGCGGCCATCGAGCTACCGAGCCAG
3125 CAAGGATACCTGCCAAGGGATATCTGGATGCCGGCCGAGGACGAGACGAGGACCCCCGTCAGCCTCACACGCGAATTAGCTAA
3205 ATTATACACAAGCAAACTCTCAAAGTCTAATCAATTAATGGATGATAAACAAATTAATTAACAGTGGCAТCAACGTCTAACCTT
3285 ACAGACAGACGCACAGACATACAGAGAAACTCACTCACAGATACACACAAGCCACAACACATACACATGTAAAAGCGATA
3365 CTGAATTAACGAAATCGAAAACTTAACGTTAACAAAATGCGAGTTAGTTAGTGCCAGCGTTTCTTTTTCGAAAGACATTT
3445 ACTTTAAAGTGTTTAGCGCAGCTTTTTAACGCAAGCAGCCCCGTATCCTATCCTTAACTCTACGAATCCTTTGCTCCCAAC
3525 TATCGTATCTATCTGCGACCTAATTACCAAAACTAGCGAGAAAGGTAACACGACAATGGGCTTCCCAACCACTTACTAACTA
3605 TCTACCTAACGTTTGGGTTTTAACTACAAGCATTAAGAAACCAAGCATACCAGGCGGTATGTATCAACAAATATATATGAA
3685 TATCTCGGGCACAAGGCACCTATATCCTATATACGGTATGATATGATACTGTTACGACTTATACATATATATATCCACTCACAC
3765 TCCAAAAATTTGTGTGAAGCTCATTCGGTTCGATCGATCGAGCAGAAAGCCTTTCACTTGCGTGCTCTAACTAAGATTTC
3845 TCCTAAAAGCTTTATCCAGAGCCTATCTAAACCTTATCTAGTTTACGACTTATACAATAATACTTATATATCCACTCACAC
3925 GCACACTTACATGGCATACATATATATGACCTGTATTGATTAAGTGATAATTCGCGTACTTATTGAACAAGCAAGCAAG
4005 GCGAACAAATCGAAAACTCTCAAAGTTAGCTACAGTTGTACTCGGAATTCAACGCTAAATCGAAGCGAATGTAGGGCTTTA
4085 AGCAATTTGCTTACCAAGCAATTTGGCATACACCCAGACCAGATCATTATGATATGATTATCGACAACACTGATTCCAGTC
4165 CGATGCTAATTCCTAATTCTAATTCTAATTCCAATTCCAGTTATGATTATGATTTCTAG**CTGATCCCGTTTCTAGTCCTA
4245 ATTCTAATTCTGATTCATGTTGGCGTGTAGGAGGAGCAGAGTTTAAATTTAACTTTAAATTGAAGCCATCATTTATTATAG
4325 ACTACTTCAAATCCAAAACCAAAAGCCTAAGATAAAAAACGGAATTC
```

Figure 2. Complementary DNA and protein sequence. Numbers for nucleotides are indicated to the left and for amino acids to the right. TM domains are underlined.
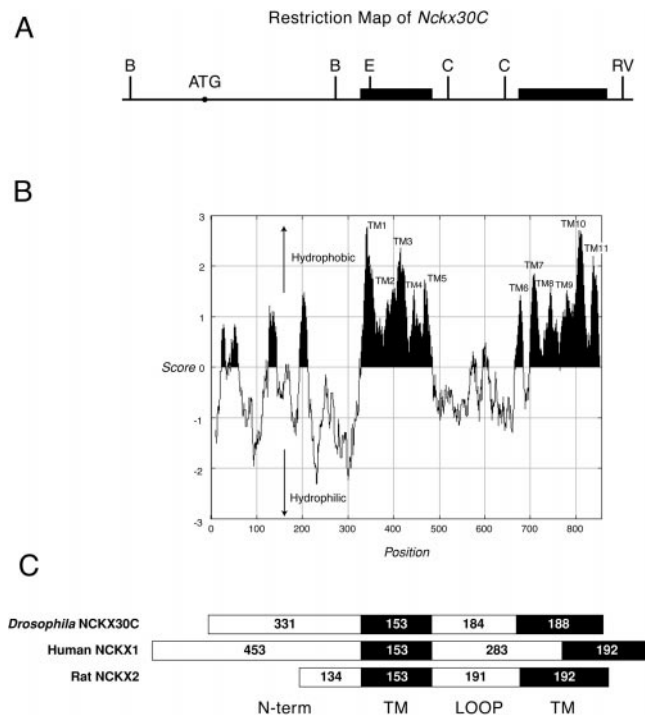
imaginal discs were dissected from crawling third-instar larvae, but left attached to the cuticle. The discs were fixed, permeabilized with proteinase K at 25 μg/ml for 3 min at room temperature, and postfixed. Probe concentrations were 120 ng/300 μl.

Digoxigenin-labeled sense and antisense riboprobes were generated by

Drosophila NCKX30C   1 --MLQPTTCKKQQQRQQPAIATAAVGGAAVQLLRKAAAKDHGALAAATATAAAHTATTATAATASRNS
Human Rod NCKX1   1 MGKLIRMGPQERWLLRTKRLHWSRLLFLLGMLIIGSTYQHLRRPRGLSSLWAAVSSHQPIKLASRDLSSEEHMMMMSSSPSKPSSEMGGKMLVPQASVGSEATLSMTVENIPSMPKRQAKMIPTTPKNNYSPTAAGTERR
Rat Brain NCKX2   1

Drosophila NCKX30C   71 SNTWCHSDDMLSAGRSRSRSSTTIDFNSRRRKGRLRGHAFPDLAMNKQHQATILDECVNIFKRLVLLELKHISATHITKAKTRSRTAHQLPATSAASATS-----SR------GASAEQLIUPSRSHGCRSRRKCLRLP--
Human Rod NCKX1   141 KEDTPTSSRTDTYYTSTSSRQIVKKYYTPTPRGEMKSYSPTQVREKVKYTPSPRGRRVGTYVPSTFMSMSGSHALFPRTTVKDSDITATYKILETNSLKRIMEETTFPTTLKGMPDSTPTFLTHEVEANVLTSPRSVMSKNN
Rat Brain NCKX2   MDLHQSATVKLQEWCSHRS--

Drosophila NCKX30C 197 -----------IYSILLLCLTTQGLGLGDAANPRPAKQHFGESNSNSPNQNQNHNDVLGGVGAPSQTSGEDEAEIMYEFPQSGEQMFGLEEUQ--------------------------EQDGELNSNAVEGGSDEDNAGNQ-
Human Rod NCKX1   281 LFPPRRVESNSSAHEWWLVGKSRDPKPQGTVLLHTPATSEGQWTISTMTGSSPAETKALTAAWSLRDPSPRTSVGSATKTATAIVWRLAKKPSTAPSTSTTTPTVRAKLTMQVVHHCCVVVKPTEAMLTHSSPSLTTALLPEEL
Rat Brain NCKX2   21 -------------SGCRRHYN--PRKKLRIRVIGLVMGLVMAVSSVPFS------ISAFFETYSQNNRGEASDVTGFRAAPGHRQRTLLU--------------------------------LNGRIRDYPFQE------

Drosophila NCKX30C 300 -RGINDTHNDQSTTTTKTPLPFPKDLFTKSQOLENGQAVALLHIIGVLYMFVALALIVCDEFFVPSLDVIQIRGIITDVAGAFMAAGSGAPELFTSLIGHPGVPVSFDDVGICATVGSAVFNIFVIGHCAHSFVTSLTAMNPLFR
Human Rod NCKX1   421 SRSSPSVLLPPSLPSDLPKEGYPDLFSVSERRQCHVVLBIVGMHDVFVALAIVCDEYFVPSLQVIQEKLGLSDDVAGATFMAAGGSAPELFTSLIGVFIGHSNVGIGTIVGSAVFNILFVIGTCSLFSREILNLTWWPLFR
Rat Brain NCKX2   103 -RASQEDRSERDLRHAQCDYPKDVFSLEERRKQAIILNVIGMIYMFIALAIVCDEFFVPSLQVITEKLGISDDVAGATFMAAGGSAPELFTSLIGVFIHAHSNVGIGTIVGSAVFNILFVIGHCALFSREILNLTWWPLFR

Drosophila NCKX30C 439 DVSFYSISHLVLCYYFRRDNRHFWNEALILFHIVIGILVAEMKWNNVQVETCVKKHIIEKHAVTRVRSTDQLMFAGNHAAN----SSETSMATQPQGSHFERAASETRSGPPGSSNAGATGNSSGG---QGTSGSTQTGAKFRHG
Human Rod NCKX1   561 DVSFYLLDLIMLILLFPLDVLHAVWNELLLLLAYVAFVVVEMKVNNKIEVVVKEQLSHKFVAKVAMALHSLUG------LPG--ILTRCGSSTSLHNSLMSNSHHPQLHIHTLDPIAEHVR--LAHKNKEEHSLNQGARAQ
Rat Brain NCKX2   242 DVSFYIVDLIMLIFPLDNVHWWESLLLLTAYFAYVVFMKFHNVVQVSRWVXQHITHRANKAVVKNTVSLAQAKASTGDKHEEPSLHNKPRLCRGGSSASLHNSLMRNSHFPQLHIHTLDPIAELGSYTEHLRCPVSV

Drosophila NCKX30C 572 LLQLMIHTHDPLHDG----------------KVDEKATQLHAIASLKVLLDDAHKPQRD---CAATSAANHVKIN--------LKEATLADR-P----------------N--------------------Q--N------
Human Rod NCKX1   686 POMKAESAGFEEEEPAKLPAVTVTPAPVPDIKGDQKENPCGQEDVAEKRSQEDVAERSGGGETQPFEGHCGETEWGGKGEECEDENEAEGKGDNEGEDEGEIHAEDGEMKGNEGETESQELSAENHGEAKN
Rat Brain NCKX2   382 EKASILHKIAKKK-------------------COVVDENERKN-GALANHVDYLAEKIRLPH---SRSTEVEMTPSS--------DASEPVQ

Drosophila NCKX30C 640 ------------------HDTT-LDSAPEIHDHP--------------------BPLEMAWHDTHARIRBLYVLVAMFLLVEHMNLLPLTRSTRPRGKRPFPVVUIGEIVMIAAASYDLVMWHANVAGDHARHPPHVMGLTILAAGTS
Human Rod NCKX1   826 DEKGVEDGGGSSDGGDSBEEEBEEEEBQSEBEEREEEESEQEEEEEEEEEEEEKGNEBEPLSLDMWPETROKQAIYFLPIVFPLNLTVPDVRKPASKKFFTFTFPGSIWIAMFPSYLMVWWAHQVGETIGISERIMGLTILAAGTS
Rat Brain NCKX2   444 ------------------LGHSHSAADAFQATRTAEED---------------DDQPLSLSWNSTHRKQIHFIVLPIVFPLNLTLPDVRKPDASKKFFTHTFPGSIWIAMFPSYLMVWWAHQVGETIGISERIMGLTILAAGTS

Drosophila NCKX30C 744 IPDLITSVIVARKGFGDMAVSSSVGSNIFDVTVGLFIDHLLAGTIIYG-AEVDNNSVGHVGSSTILIEGHHIVFVVMSLACFFRFRHRMRGHGFTTHFLLYFAIVAVSLAIFSVDVATCSF-
Human Rod NCKX1   966 IPDLITSVIVARKGLGDMAVSSSVGSNIFDITVGLFVPWLLFSLLHGLQDPVRYLVIGPLGCSIASCKWRMNKILGFTMPFLLYFAIVFIGSIASCKWRMNKILGFTMPFLLYFAIVFIGSIAL
Rat Brain NCKX2   555 IPDLITSVIVARKGLGDMAVSSSVGSNIFDITVGLFPLWLLYCIIHRFKPVTVSSNGLFCAIVLLFMHFVICSIALCKWRMNKILGFIMPQLYFAFLVVSVTLEDKVLECPVSV

Figure 3. *Nckx30C* encodes a novel *Drosophila* NCKX. CLUSTAL W alignment between *Drosophila* NCKX30C, human rod NCKX1, and rat brain NCKX2 (Tsoi et al., 1998; Tucker et al., 1998). Dark shading indicates identity and light shading indicates similarity. The 11 TM domains of NCKX30C are indicated by lines above each row.

in vitro transciption of five DNA templates as recommended by the digoxigenin/UTP supplier (Boehringer Mannheim Corp.). Five *Nckx30C* cDNA probes were used: 1.3 kb BamHI-BamHI (5′ end), 500 bp BamHI-ClaI (TM1–TM5), ClaI-ClaI (loop region), ClaI-EcoRV (TM6–TM11), and the full-length *Nckx30C* cDNA clone (see Fig. 4 A). *Calx* cDNA was provided by E. Schwarz (Columbia University, New York, NY) (Schwarz and Benzer, 1997). *Chaoptin* cDNA was provided by D. Van Vactor (University of California, Berkeley) and S.L. Zipursky (University of Califor-

nia, Los Angeles, Los Angeles, CA) (Reinke et al., 1988). After transcription, the reaction mix was treated with DNase and the riboprobes were hydrolyzed in carbonate buffer for 2 min at 80°C. All riboprobes were quantified by analysis in denaturing 0.8% agarose gels and by dot blot analysis. All detection steps were as described in Tautz and Pfeifle (1989). All *Nckx30C* antisense probes showed the same labeling pattern. No signal was detected with the sense probes.

Results

Isolation of a Drosophila NCKX

The NCKX-type exchangers are a novel group of calcium extrusion proteins. NCKX1 function was initially described in the outer segments of retinal rod photoreceptor cells (Fig. 1) (Cervetto et al., 1989; Schnetkamp et al., 1989). Antibodies directed to NCKX1 were used to screen a bovine retinal expression library, and NCKX1 cDNA was cloned and sequenced (Reiländer et al., 1992). A second isoform, NCKX2, was cloned from rat brain, and was shown on the basis of in situ hybridization to be widely expressed in various regions of the brain (Tsoi et al., 1998). To isolate *Drosophila Nckx30C*, we constructed a cDNA probe from the bovine rod photoreceptor NCKX1 that encoded a fusion between the highly conserved TM1–TM5 and TM6–TM11 regions. We used this probe to screen the *Drosophila* genomic library and obtained a 1.3-kbp EcoRI-XbaI fragment that corresponds to an EcoRI site at the 5′ end of the gene. The XbaI site is located in the intron. This 1.3-kbp genomic fragment was used to screen the cDNA library. A set of overlapping cDNAs encompassing the entire *Nckx30C* transcript was isolated and sequenced. The composite cDNA has a single open reading frame that encodes a protein of 856 amino acids (Fig. 2). We compared the derived amino acid sequence of *Nckx30C* with the human NCKX1 and rat NCKX2 (Fig. 3). There is 66 and 71% identity in two clusters of amino acids that corresponds to

Figure 4. Hydropathy plot of the conceptual NCKX30C protein. (A) Map of *Nckx30C* cDNA. The black boxes represent the TM regions of the deduced amino acid sequence. Cleavage sites for BamHI (indicated by B), EcoRI (indicated by E), Cla I (indicated by C), and EcoRV (indicated by RV) are shown. (B) Hydropathy plot of the conceptual NCKX30C protein, analyzed by the Kyte-Doolittle algorithm (Kyte and Doolittle, 1982). Hydrophobic regions are designated in black. (C) Domain structure of *Drosophila* NCKX30C, human rod photoreceptor NCKX1, and

rat brain NCKX2. Filled boxes represent the two clusters of TM segments (TM1–TM5 and TM6–TM11) that display high identity among the three sequences. Numbers represent the number of amino acids per segment.
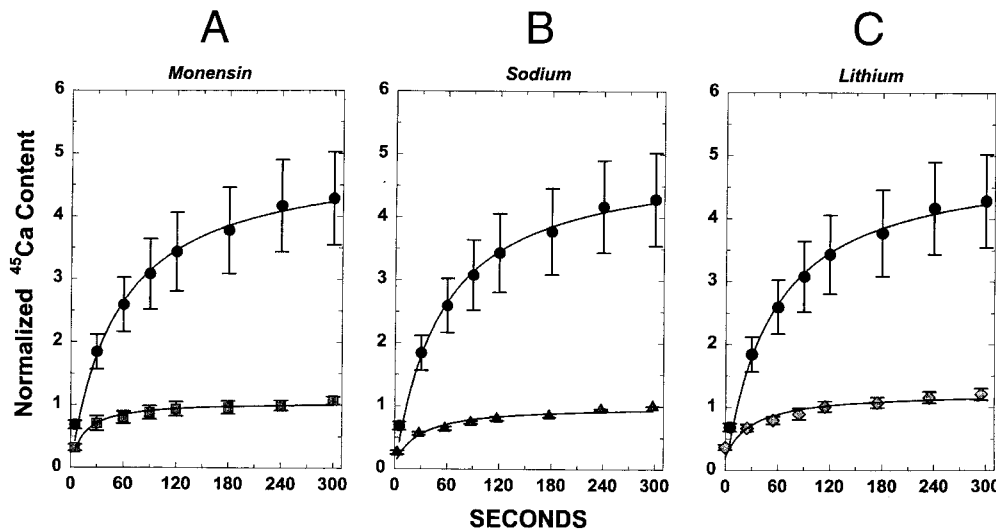
A Monensin
B Sodium
C Lithium

Figure 5. NCKX30C is an NCKX. Reverse exchange was measured as Na_{inside}-dependent ^{45}Ca uptake in High Five cells transformed with *Nckx30C* in the presence of external KCl (A–C, filled circles). Mean ± SEM results are shown in A–C (filled circles). (A) Reverse sodium/calcium exchange requires intracellular sodium. ^{45}Ca uptake was measured in KCl medium with 20 μM monensin present (shaded squares) or without monensin (filled circles). Monensin was added 30 s before addition of ^{45}Ca, and causes the release intracellular sodium to the external medium. Average values ± SEM are shown for 13 experiments conducted in KCl medium, and 4 experiments conducted in media containing KCl plus monensin. (B) Reverse sodium/calcium exchange is inhibited by extracellular sodium. ^{45}Ca uptake was measured in KCl medium (filled circles) or NaCl medium lacking potassium (triangles). Average values ± SEM are shown for 13 experiments conducted in KCl medium, and 10 experiments conducted in media containing NaCl. (C) Reverse sodium/calcium exchange requires extracellular potassium. ^{45}Ca uptake was measured in KCl medium (filled circles) or LiCl medium lacking potassium (diamonds). Average values ± SEM are shown for 13 experiments conducted in KCl medium, and 8 experiments conducted in media containing LiCl.

the predicted TM domains of human rod photoreceptor cell NCKX1 and rat brain NCKX2, respectively. A partial restriction map for *Nckx30C* is shown in Fig. 4 A. The hydropathy analysis confirms that *Drosophila* NCKX contains 11 hydrophobic regions that correspond to the 11 predicted TM helices in NCXK1 and NCKX2 (Fig. 4 B). As with NCKX1 and NCKX2, there is a large cytoplasmic loop located between TM5 and TM6 in *Nckx30C*. This large cytoplasmic loop displays very little amino acid identity with the corresponding cytoplasmic loops of NCKX1 or NCKX2 (Fig. 3). The number of amino acids that make up the hydrophilic loops and the two sets of membrane-span-

ning segments are shown in Fig. 4 C. One feature distinguishes NCKX30C from other members of the NCKX superfamily. Hydropathy analysis revealed that NH_2-terminal to TM1 is a region that may contain two additional membrane-spanning segments not observed in either NCKX1 or NCKX2. The correct identity of this NH_2-terminal sequence for *Nckx30C* was confirmed by 5′ rapid amplification of cDNA ends (RACE) starting with a primer corresponding to sequence of TM1, and by PCR using first-strand cDNA.

We cytologically mapped *Nckx* to 2L at 30C5-7 by in situ hybridization to polytene chromosomes, and based on the chromosomal location have named the gene *Nckx30C*.

NCKX30C Is an NCKX

We examined the functional properties of NCKX30C in High Five cells. The strategy for these experiments takes advantage of properties of both NCX and NCKX. First, NCX and NCKX are bidirectional in their ability to mediate both calcium efflux (forward exchange) and calcium influx (reverse exchange). The direction of transport is dictated by the direction of the TM sodium gradient. Normally, the inward sodium gradient removes calcium from the cell (forward exchange). However, when external sodium is removed, the outward sodium gradient drives calcium into the cell (reverse exchange). Therefore, we measured the properties of reverse exchange of NCKX30C by measuring ^{45}Ca uptake in sodium-loaded cells. We tested for NCKX activity by using three different manipulations of the cation gradient that are known to prevent NCKX activity. The cells were loaded with sodium via the sodium-potassium ionophore, monensin, in a medium containing high sodium, according to the methods described for rod outer segments (Schnetkamp et al., 1995). The ion-

Figure 6. Nckx30C encodes 8-kb and 10-kb transcripts. Northern blot analysis reveals two transcripts of 8 kb and 10 kb expressed in adult heads (H) and adult bodies (B). Mutant flies lacking eyes (*eya*) also express both transcripts, whereas only the lower 8-kb transcript is detected in embryos (E) and larvae (L). We loaded 10 μg of polyA$^+$ RNA in each lane. Identical results were obtained with four different radiolabeled riboprobes corresponding to various regions of the exchanger. Specifically, we used a 1.1-kbp BamHI-BamHI and 500 nucleotide BamHI-ClaI, ClaI-ClaI loop region, and ClaI-EcoRV (see Fig. 4 A).
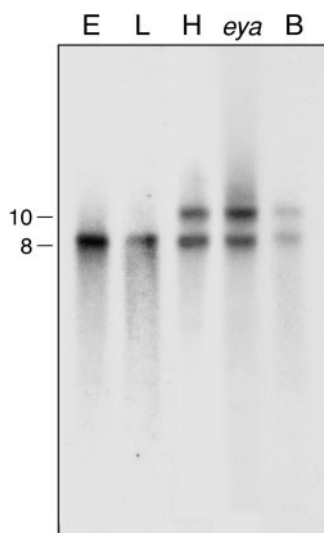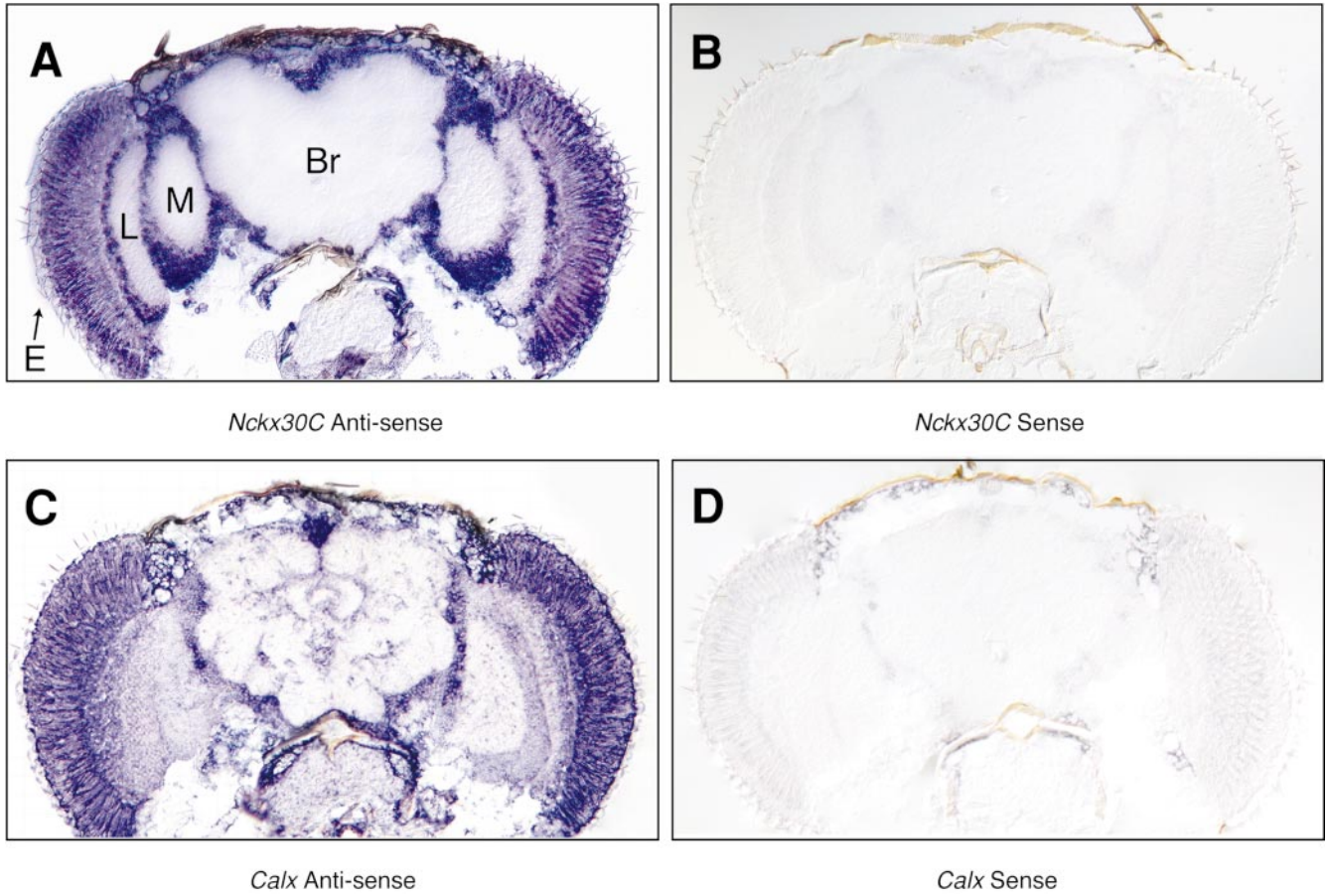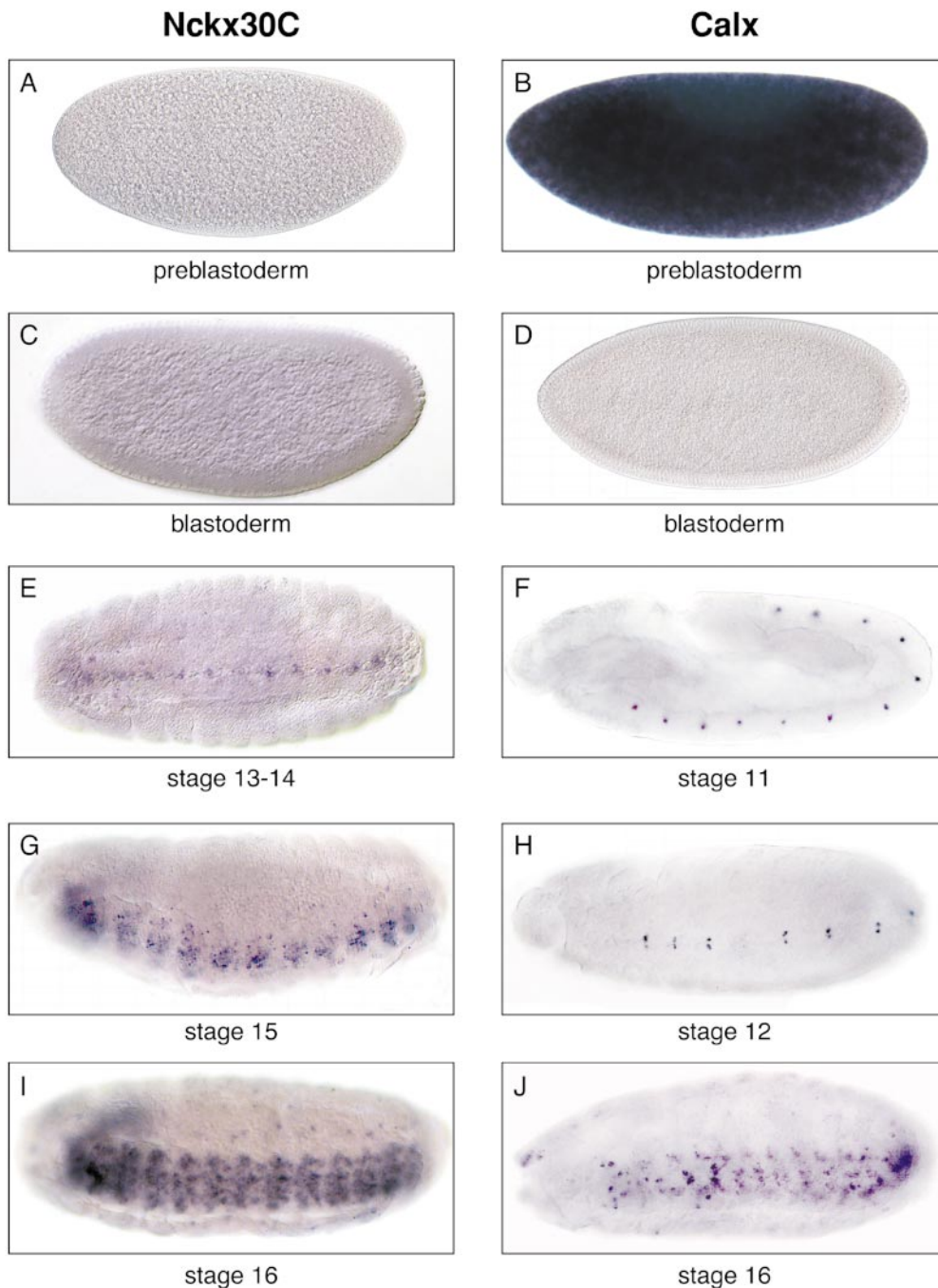
Figure 7. *Nckx30C* and *Calx* are expressed in the adult eye and the brain of *Drosophila*. (A) Shown are in situ hybridizations to 14-μm cryostat sections of adult heads hybridized with digoxigenin-labeled riboprobes. (A) Antisense riboprobe for *Nckx30C*; (B) sense probe for *Nckx30C*; (C) antisense riboprobe for *Calx*; and (D) sense riboprobe for *Calx*. E, eye; L, lamina; M, medulla; and Br, brain.

Figure 9.

Nckx30C

A
preblastoderm

C
blastoderm

E
stage 13-14

G
stage 15

I
stage 16

Calx

B
preblastoderm

D
blastoderm

F
stage 11

H
stage 12

J
stage 16
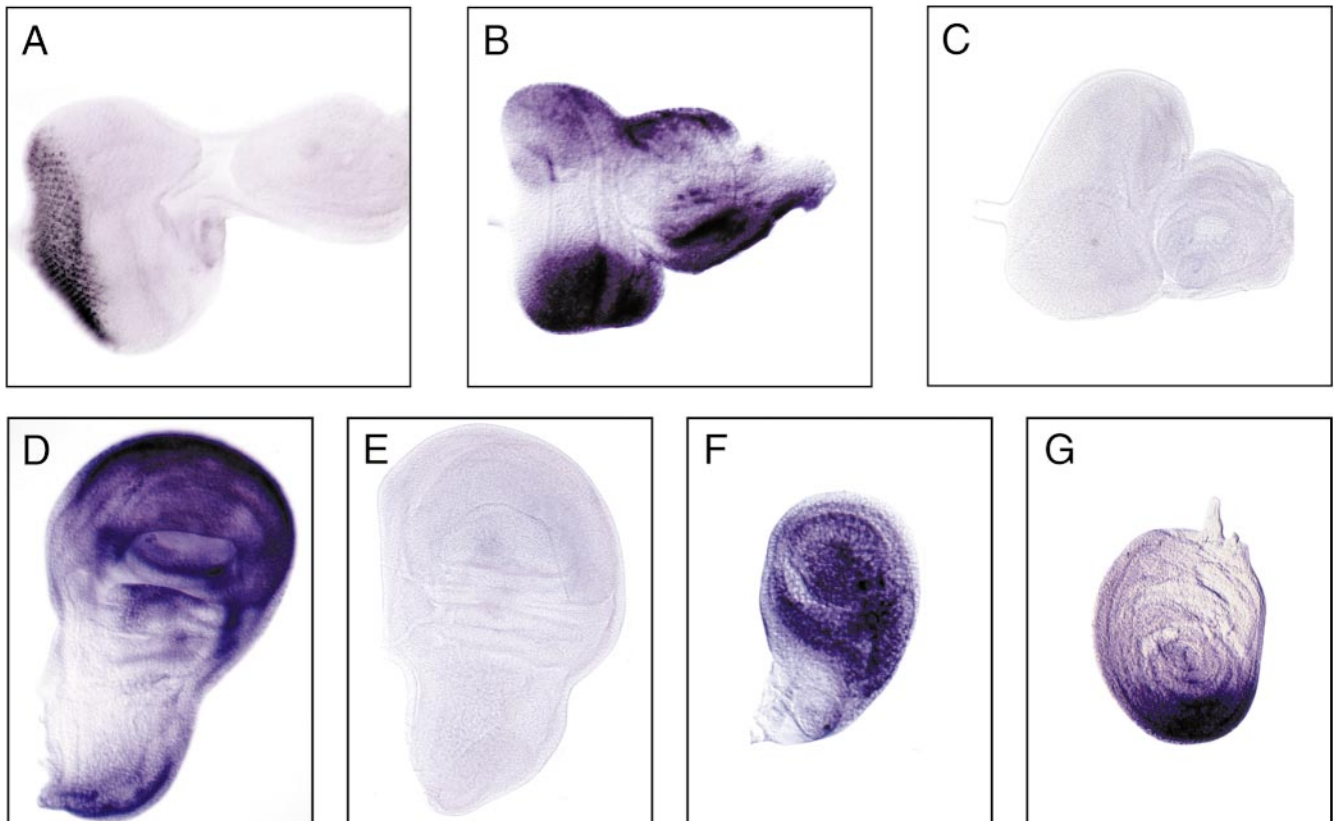
Figure 8. Nckx30C and *Calx* are expressed in the ventral nerve cord of the *Drosophila* embryo. Shown are in situ hybridizations to whole-mount wild-type embryos (Canton S strain) hybridized with digoxigenin-labeled antisense riboprobes for *Nckx30C* and *Calx.* (A) Lateral view, preblastoderm, *Nckx30C*; (B) lateral view, preblastoderm, *Calx*; (C) lateral view, blastoderm, *Nckx30C*; (D) lateral view, blastoderm, *Calx*; (E) ventral view, stage 13-14, *Nckx30C*; (F) lateral view, stage 11, *Calx*; (G) ventrolateral view, stage 15, *Nckx30C*; (H) ventral view, stage 12, *Calx*; (I) ventrolateral view, stage 16, *Nckx30C*; and (J) ventrolateral view, stage 16, *Calx.* Note the labeling of the developing ventral nerve cord. Control hybridizations with sense probes did not produce signals (data not shown). All embryos are orientated anterior to the left. In lateral views, all embryos are orientated dorsal side up.

ophore was removed and the sodium-loaded cells were washed with and resuspended in low sodium buffer as described in the Materials and Methods. The cells were diluted 10-fold in media containing 80 mM sucrose, and 20 mM Hepes, pH 7.4, and either 150 mM KCl, 150 mM NaCl, or 150 mM LiCl. ^{45}Ca uptake was initiated by addition of 35 μM CaCl$_2$ and 1 μCi ^{45}Ca. The first condition causes the release of internal sodium by the action of monensin, a sodium(-potassium) selective ionophore. Fig. 5 A shows that ^{45}Ca uptake by NCKX30C was strongly inhibited by the

Figure 9. Nckx30C is expressed in the third instar imaginal discs of larvae. Shown are in situ hybridizations to imaginal discs from wild-type larvae with digoxigenin-labeled riboprobes for *Nckx30C* and *chaoptin*. (A) Eye-antennal disc, antisense riboprobe for *chaoptin*; (B) eye-antennal disc, antisense riboprobe for *Nckx30C*; (C) eye-antennal disc, control sense riboprobe for *Nckx30C*; (D) wing disc, antisense riboprobe for *Nckx30C*; (E) wing disc, sense riboprobe for *Nckx30C*; (F) haltere disc, antisense riboprobe for *Nckx30C*; and (G) leg disc, antisense riboprobe for *Nckx30C*. Note that control hybridizations with sense probes did not produce signals. The eye discs are oriented posterior to the left and dorsal up. The wing and haltere discs are orientated with posterior to the left and ventral up. The leg disc is oriented ventral down.

addition of monensin, thus demonstrating a requirement for intracellular sodium for calcium transport.

A second property of both NCK and NCKX is that calcium uptake by reverse exchange is inhibited by high extracellular sodium (Schnetkamp et al., 1991a). This is thought to occur because sodium and calcium compete for a common binding site (Schnetkamp et al., 1991a). We demonstrate that ^{45}Ca uptake by NCKX30C is strongly inhibited by high extracellular sodium, consistent with its identity as an NCX (Fig. 5 B).

A critical distinction between NCKX and NCX is that calcium influx via NCKX requires the presence of potassium, and lithium cannot substitute for potassium (Tsoi et al., 1998). In contrast, calcium influx via reverse exchange in NCX is the same in media containing potassium or lithium (Tsoi et al., 1998; Cooper et al., 1999). ^{45}Ca uptake by NCKX30C requires potassium and was not observed in media containing lithium (Fig. 5 C). This result demonstrates that NCKX30C functions as an NCKX.

As negative controls, we subjected nontransformed High Five cells to the same ^{45}Ca uptake experimental protocols described above: ^{45}Ca uptake in media containing potassium was indistinguishable from that in media containing either sodium or lithium, showing that there is no endogenous NCX or NCKX activity in the cells (data not shown). The above results demonstrate that NCKX30C mediates potassium-dependent sodium/calcium exchange similar to that observed for NCKX1 and NCKX2 (Tsoi et al., 1998; Cooper et al., 1999).

Northern Blot Analysis

Northern blot analysis revealed an 8-kb transcript that is expressed in embryos and larvae (Fig. 6). In the adult, two transcripts of 8 kb and 10 kb were detected. Expression was detected predominately in the head. Transcripts present in the body may represent the thoracic ganglia or other components of the nervous system (Demerec, 1994). The 8-kb and 10-kb transcripts were also detected in *eya-1* mutant flies, which lack eyes, indicating that expression is not restricted to the eyes (Fig. 6).

Nckx30C and Calx Are Expressed in the Adult Eye and Brain of Drosophila

Previous work has identified a *Drosophila* NCX, *Calx*, which encodes a functional ortholog of the NCX-type exchangers (Hryshko et al., 1996; Ruknudin et al., 1997; Schwarz and Benzer, 1997). Expression patterns for *Calx* were reported in Schwarz and Benzer (1997). In situ hybridization analysis reveals that both *Nckx30C* and *Calx* are expressed in the adult nervous system. Fig. 7 A shows that *Nckx30C* is expressed in the photoreceptor cells as well as in the lamina, medulla, and optic lobes of the brain. Fig. 7 C shows that *Calx* is also expressed in the photoreceptors as well as in the brain. Analysis of many images did not reveal a convincing difference in expression patterns between *Nckx30C* and *Calx*. In situ analysis at higher resolution, on 0.5-μm cryosections, confirmed that expression is detected in the photoreceptor cells (data not shown). However, this does not exclude the possibility that the subcellular distribution of the proteins could be very different. No signal was detected with the sense probes (Fig. 7, B and D).

Nckx30C and Calx Are Expressed in the Developing Embryonic Nervous System

Beginning at embryonic stage 13–14, *Nckx30C* transcripts were detected in a discrete pattern of unpaired cells at the ventral midline of the central nervous system (Fig. 8 E). *Nckx30C* transcripts were not detected in the preblastoderm or blastoderm stages (Fig. 8, A and C). Unlike *Nckx30C*, *Calx* expression is robust in the preblastoderm before and during nuclear migration from the interior to the cortex (Fig. 8 B). Since this is before activation of zygotic transcription, *Calx* transcripts are likely to be maternally inherited. *Calx* transcripts disappear in the cellular blastoderm (Fig. 8 D) and then reappear in the stage 11 embryo (Fig. 8 F). By stage 12, *Calx* expression is noted in two cells per hemisegment, one on either side of the ventral midline (Fig. 8 H). In embryonic stage 15, *Nckx30C* expression was detected in several neurons within the ventral nerve cord (Fig. 8 G). By stage 16, when many neurons within the ventral nerve cord and the embryonic brain express *Nckx30C* (Fig. 8 I), *Calx* expression is restricted to a much smaller subset of neurons in the ventral nerve cord (Fig. 8 J). Outside the central nervous system, *Nckx30C* and *Calx* transcripts were observed in some cells, which may represent parts of the peripheral nervous system (Fig. 8, I and J).

Nckx30C Is Expressed in the Larval Imaginal Discs

Drosophila appendages develop from imaginal discs in a highly orchestrated series of events that divide the discs into distinct subregions during patterning. Retinal differentiation begins at the posterior end of the eye disc, which coincides with the dorsal–ventral midline (Treisman and Rubin, 1995; Heberlein et al., 1998; Treisman and Heberlein, 1998), and proceeds as a wave across the eye disc from posterior to anterior (Ready et al., 1976; Tomlinson and Ready, 1987; Tomlinson, 1988). The morphogenetic furrow is a dorsoventral indentation in the eye disc, with the region anterior to the furrow comprising actively dividing and unpatterned cells, and the region posterior to the furrow containing differentiating photoreceptor cells assembling into ommatidia. (Tomlinson, 1988; Ready, 1989; Banerjee and Zipursky, 1990). Fig. 9 A shows expression of *chaoptin*, which encodes a photoreceptor cell surface glycoprotein (Reinke et al., 1988), in photoreceptor cells located posterior to the furrow. *Calx* expression was not detected in any of the imaginal discs. *Nckx30C* transcripts were detected both anterior and posterior to the morphogenetic furrow in a dorsal–ventral pattern with no labeling in the midline (Fig. 9, B and C). This expression pattern is similar to but broader than that observed for *wingless* (*wg*), which is known to play an important role in dorsal–ventral patterning in the eye disc (Heberlein and Moses, 1995; Ma and Moses, 1995; Treisman and Rubin, 1995; Heberlein et al., 1998; Treisman and Heberlein, 1998). In vertebrates, one of the two apparently distinct Wnt pathways may act through a G protein–mediated phosphatidylinositol signaling cascade that results in an increase in intracellular calcium via inositol 1,4,5 trisphos-

phate (InsP$_3$)-sensitive stores (Moon et al., 1997; Slusarski et al., 1997a,b). It is intriguing to hypothesize that NCKX30C may be playing a role in modulating calcium in this patterning pathway.

Nckx30C is expressed strongly throughout most of the wing disc and throughout most of the haltere disc (Fig. 9, D–F). In the leg disc, *Nckx30C* is expressed in a ventral wedge in the cells that will give rise to the ventral and/or proximal structures (Fig. 9 G). This pattern of expression is similar to that observed for *wingless* in the leg discs (Baker, 1988; Couso et al., 1993; Theisen et al., 1994). Given what we know about the role of NCKX in retinal rod and cone photoreceptors, it is tempting to speculate that cells that express *Nckx30C* may be experiencing large and sustained rises in cytosolic calcium.

Discussion

We have cloned *Nckx30C* from *Drosophila* and have shown that it is expressed in the adult nervous system and also during development in the embryo and the imaginal discs. Heterologous expression in cultured insect High Five cells demonstrates that NCKX30C functions as an NCKX. *Calx*, a member of the NCX gene family, is distantly related to *Nckx30C* (Hryshko et al., 1996; Ruknudin et al., 1997; Schwarz and Benzer, 1997; Dyck et al., 1998; Omelchenko et al., 1998). Both NCX and NCKX function in sodium–calcium exchange in cells experiencing large calcium fluxes. In addition, both *Nckx30C* and *Calx* are expressed in *Drosophila* photoreceptors as well as in other cells, indicating that there may be multiple mechanisms for calcium efflux in these cells.

A puzzling finding is that the photoreceptors in *Drosophila* express both NCKX30C and Calx exchangers. The NCKX exchangers have several unique features that make them well-suited for calcium extrusion during phototransduction. Illumination of *Drosophila* photoreceptors activates the phototransduction cascade leading to the opening of the cation-selective channels and an increase in the intracellular calcium (Fig. 1). In both flies and vertebrate photoreceptor cells, sodium influx contributes substantially to the inward current, potentially leading to elevated [Na$^+$]$_{inside}$ (Coles and Orkand, 1985; Nakatani and Yau, 1988b; Hardie, 1996b; Gerster, 1997). As the cytosolic sodium concentration increases and reduces the TM sodium gradient, NCX exchangers, like Calx, are expected to reverse direction at much lower cytosolic sodium concentrations when compared with the potassium-dependent NCKX exchangers (Schnetkamp et al., 1991c). Therefore, the potassium-dependent exchangers will be better able to function in phototransduction under these conditions. The apparent redundancy could also be explained if the subcellular distribution of NCKX30C and Calx are very different, with each fulfilling distinct functions. A combination of immunocytochemistry with mutant analysis will assist in resolving these questions.

To date, the central focus of efforts in development of *Drosophila* has been on the identification of regulatory genes that control development, with the potential contribution of calcium signaling remaining largely unexplored. Notable exceptions include systems responsible for dorsal–ventral positional information, such as the dorsal pro-

tein in dorsal–ventral pattern information in the early embryo and the *wingless* pathway. It has been demonstrated that increased calcium can act as a second messenger in the signal transduction pathway leading to the nuclear localization of dorsal, a maternally inherited transcription factor (Kubota et al., 1993). Another signaling pathway that may utilize calcium is *wingless*. *wingless*, a member of the Wnt gene family, encodes a secreted glycoprotein that is involved in a complex variety of signaling events (Nusse and Varmus, 1992; Cadigan and Nusse, 1997). In vertebrate embryos, one of the two apparently distinct Wnt pathways can act through a G protein–mediated phosphatidylinositol signaling cascade, resulting in a release of intracellular calcium from inositol 1,4,5 trisphosphate (InsP$_3$)-sensitive stores (Moon et al., 1997; Slusarski et al., 1997a,b). Here, we demonstrate that *Nckx30C* is expressed in a pattern similar to, but broader than that observed for *wingless*. *Nckx30C* expression would indicate that, most likely, a substantial calcium flux is occurring in these cells. In this study, we show that exchangers are expressed during development, indicating that they may not only function in the removal of calcium and maintenance of calcium homeostasis during signaling in the adult, but may also play critical roles in signaling events during embryogenesis and patterning of imaginal discs. The isolation of *Nckx30C* in *Drosophila* permits a genetic analysis of the in vivo role of calcium in modulating signaling pathways in *Drosophila*.

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