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# Mouse κ-Opioid Receptor mRNA Differential Transport in Neurons

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## ABSTRACT

Three κ-opioid receptor (KOR) mRNA isoforms have been detected in different parts of the central nervous system. At the cellular level, three KOR mRNA isoforms are also differentially distributed in the axons and cell bodies of adult mouse trigeminal neurons, as well as in the processes and cell bodies of differentiated P19 neurons. To determine the molecular basis underlying differential distribution of KOR mRNA isoforms, a GFP-fused RNA binding domain, MS2, was generated and used to trace movement of KOR mRNA tagged with the MS2-binding sequence in living neurons of dorsal root ganglia and in differentiated P19 neurons. The 5′- and 3′-untranslated regions (UTRs) of KOR, either alone or in combination, are able to mediate transport of mRNAs to processes of P19 neurons and axons of dorsal root ganglia. The efficiency of mRNA transport mediated by each 5′-UTR of KOR varies among the three isoforms; isoform A is most efficient. This study demonstrates the biological activity of the UTRs of KOR mRNA isoforms in directing differential transport of mRNA in mammalian neurons.

Opioid receptors interact with opiate drugs and endogenous opioid ligands to affect pain sensation, consciousness, and autonomic functions. Three major opioid receptor types, μ, δ, and κ, have been defined (Goldstein and Naidu, 1989; Masabumi and Satoh, 1995) that belong to the super family of G-protein-coupled membrane receptors (Wei and Loh, 2002). For each gene, mRNA isoforms have been detected but only one type of protein is produced. However, pharmacological studies indicate existence of more than one subtype of each receptor (Goldstein and Naidu, 1989; Masabumi and Satoh, 1995), which remains to be elucidated at the molecular level.

κ-Opioid receptor (KOR) proteins were detected both pre- and postsynaptically (Drak et al., 1996; Shuster et al., 1999). Activation of presynaptic KOR proteins, together with muscarinic receptors, inhibited calcium-dependent glutamate release (Rawls et al., 1999). The mouse KOR gene produces three mRNA isoforms varied at 5′-untranslated regions (5′-UTRs). We have previously demonstrated differential distribution of these KOR mRNA isoforms in the nervous systems and retinoic acid-induced, differentiated neurons of P19, as well as variation in the stability and translation efficiency of these KOR mRNA isoforms (Wei et al., 2000; Bi et al., 2001). However, how the different isoforms of KOR mRNAs can be present in different parts of brain areas and in vitro differentiated neurons remains unknown. In particular, whether these alternatively spliced KOR mRNAs are differentially distributed in cell bodies and fibers, including axons, is unclear.

Compelling evidence has accumulated for mRNA transport/targeting in the dendrites of invertebrate and vertebrate neurons (Kleman et al., 1994; Mohr, 1999; Mori et al., 2000; Jansen, 2001; Job and Eberwine, 2001). The issue of whether mRNAs extend into the axonal compartment, particularly in vertebrate neurons, was debated until recently (Mohr and Richter, 2000). Evidence for axonal mRNA transport has been provided for structural proteins in growing axons of developing and injured neurons (Olink-Cous and Hollenbeck, 1996; Eng et al., 1999; Alvarez et al., 2000; Zhang et al., 2001; Zheng et al., 2001). Most recently, Brittis et al. (2002) provided direct evidence for local synthesis of the EphA2 receptor, a protein involved in axonal pathfinding, in the axons of vertebrate neurons. Consistent with this, intra-axonal protein synthesis plays a role in chemotactic guidance for axons of developing *Xenopus laevis* retinal ganglion and motor neurons (Campenot and Holt, 2001; Ming et al., 2002). Nevertheless, transport and translation of mRNAs coding for nonstructural components in axons of sensory neurons is uncertain.

With the identification of different KOR mRNA isoforms in animal tissues and in vitro differentiated neurons and the functional evidence for alternative use of different poly(A)

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**ABBREVIATIONS:** KOR, κ-opioid receptor; UTR, untranslated region; RT-PCR, reverse transcription-polymerase chain reaction; NLS, nuclear localization signal; SV40, simian virus 40; DRG, dorsal root ganglia; GFP, green fluorescent protein.

signals from the KOR gene, it is interesting to determine whether KOR mRNA isoforms are differentiated transported into various compartments of neurons. This study attempted to address the molecular basis underlying differential distribution of KOR mRNA isoforms and the functional significance of these untranslated sequences that varied among these isoforms. We presented evidence, in this study, for 1) differential distribution of KOR mRNA isoforms in adult trigeminal nerves and ganglion, and in cell bodies and processes of differentiated P19 neurons, 2) the biological activity of the untranslated KOR mRNA sequences in mRNA transport to axons of dorsal root ganglia and processes of P19 neurons, and 3) different efficiency of mRNA transport in P19 neurons, directed by the untranslated sequences of the three KOR mRNA isoforms.

## Materials and Methods

**Analysis of KOR mRNA.** The presence of KOR mRNA was demonstrated using an established, semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) as described previously (Wei et al., 2000; Bi et al., 2001). Amplified 730-, 760-, and 800-bp fragments represent KOR mRNA isoforms A, B, and C, respectively.

**Reporter Constructs.** A nuclear localization signal (NLS)-tagged MS2 (Bertrand et al., 1998) was fused to enhanced GFP-C1 (BD Biosciences Clontech), named K89. A 3.6-kilobase 3′-UTR of KOR genomic DNA was fused to three copies of MS-2 binding site, followed by a luc cDNA and then a KOR genomic fragment containing KOR promoters, exon 1, intron 1, and exon 2, named 5′K3′K. 5′K3′SV was made by replacing its 3′-UTR with SV40 polyA signal. 5′tk3′K was made by replacing the KOR promoter and its 5′-UTR with the thymidine kinase promoter. 5′tk3′SV was made by replacing 5′- and 3′-UTRs of 5′K3′K with tk and SV40 sequences, respectively. The reporters for different 5′-UTRs of the KOR mRNAs were made by replacing the 5′-UTR of 5′K3′SV with tk promoter followed by the 5′-UTR of KOR mRNA isoforms A, B, and C and named 5′K-A, 5′K-B, and 5′K-C, respectively.

**P19 Cell Culture, Transfection, and Two-Surfaced Culture Methods.** The procedure for inducing neuronal differentiation of a mouse embryonal carcinoma cell line P19 was described previously (Bi et al., 2001). Differentiated neurons were monitored with an anti-Tau antibody (Litman et al., 1993). The two-surfaced culture method (Torre and Steward, 1992) was used to grow differentiated neurons. Arabinosylcytosine (5 $\mu$M; Sigma Chemical, St. Louis, MO) was added after 2 days of culture to inhibit glial proliferation. The processes remained on the coverslips and cell bodies remained on the polycarbonate membrane after separating the two layers. Transfection was conducted with a calcium phosphate precipitation method at 4 to 14 days after plating. The distance of GFP traveled within neuronal processes was determined by measuring the distance of process that contained GFP signal, which was then divided by the length of that fully extended processes to obtained a relative (%) number. At least 10 processes were examined for each construct to obtain the average numbers.

**DRG Culture, Transfection, and Immunohistochemistry Methods.** DRG was collected from newborn rats according to the described procedure (Stucky et al., 1998, Zheng et al., 2001). The dissected DRG was placed in a growth medium (Ham's F12 mixture, 5% fetal bovine serum, 40 mM glucose) with collagenase, and incubated at 37°C for 30 min followed by repeated triturating with a Pasteur pipette. Cells were collected by centrifugation, treated with Trypsin-EDTA, and plated onto culture dishes coated with poly-D-lysine for 4 to 10 days. On day 3, arabinosylcytosine (5 $\mu$M) was added to inhibit growth of glia cells. Transfection was conducted as described for the P19 neuron cultures (Bi et al., 2001). Immunostaining with an anti-Tau antibody (Sigma) and Cy3-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was conducted as described previously (Bi et al., 2001).

**Fluorescence Video Microscopy.** A high-resolution fluorescence video microscope (Nikon Diaphot 300; Niko, Tokyo, Japan) was used to capture green fluorescent protein signals in live cultures placed in a closed chamber maintained at 37°C. To minimize photobleaching and phototoxicity, a computer-driven automatic shutter was used to minimize illumination. Time-lapse recording was conducted for a 100- to 800-ms exposure every 20 s.

## Results

**Differential Distribution of KOR mRNA Isoforms in Adult Trigeminal Nerve and Ganglion and p19 Neurons.** We previously demonstrated differential distribution of KOR mRNA isoforms A, B, and C in the nervous systems of the mouse (Wei et al., 2000; Bi et al., 2001). To further determine the distribution patterns of these KOR isoforms in neuron cell bodies and fibers, we first examined the adult trigeminal nerve and trigeminal ganglion, which can be separated. RNA was then collected from the dissected trigeminal nerve and ganglion and detected with an established RT-PCR procedure to differentially detect the expression of isoforms A, B, and C (Wei et al., 2000; Bi et al., 2001). As shown in Fig. 1A, all three isoforms are detected in both trigeminal ganglion and nerve, but the levels varied among the three mRNA isoforms (bottom). Isoform A is most abundant, particularly in the nerve. Isoforms B and C can also be detected in the nerve but at much lower levels.

To determine the expression of these isoforms in in vitro-differentiated neuron cultures, we then used the P19 neuronal differentiation model, which has been used as a model to examine KOR regulation in this lab (Bi et al., 2001; Hu et al., 2001; Wei and Loh 2002). To obtain pure population of neuron cell bodies and fibers, we employed a two-layered matrix

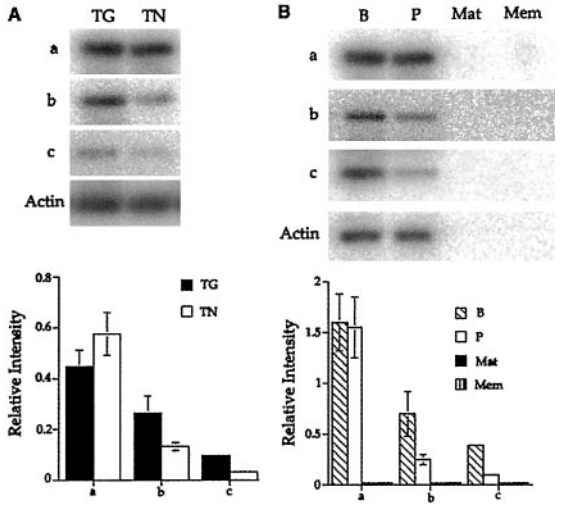


**Fig. 1.** RT-PCR analyses of KOR mRNA isoforms in trigeminal nerves. A, KOR mRNA isoforms a, b and c are differentially detected in cell bodies (TG) and axons (TN) of trigeminal nerves. Actin was included for a positive control. A statistical analysis of KOR mRNA isoforms distribution in trigeminal nerves from three independent experiments is shown at the bottom. B, KOR mRNA isoforms a, b, and c are differentially detected in cell bodies (B) and neuronal processes (P) of fully differentiated P19 neurons. Mat, Matrigel control; mem, nucleopore membrane control. Actin was included as a positive control. A statistical analysis of KOR mRNA isoforms distribution in P19 cell bodies and neuronal processes from three independent experiments is shown at bottom.

system (Torre and Steward, 1992) that allowed neuron fibers to extend to the bottom layer and cell bodies to remain on the top layer. After differentiation, P19 neurons were then plated on the matrix and allowed to undergo fully differentiation on the matrix. A pure population of neuron fibers was collected from the bottom layer and cell bodies were collected from the top layer. Hematoxylin and eosin staining was routinely used to confirm a lack of contaminating cell bodies in fiber preparations. RNA was prepared, followed by RT-PCR. As shown in Fig. 1B, all three isoforms are detected in both the cell bodies (B) and neuronal processes (P) of P19, but the expression levels vary. The matrix (Mat) and membrane (mem) used in preparing this culture are confirmed to be free of contamination. Similar to trigeminal preparation, isoform A is the predominant KOR message detected in P19 neuronal processes, whereas isoform C is expressed at the lowest level.

Therefore, KOR mRNA isoforms A, B and C can be detected in both the cell bodies and the processes of both primary sensory neurons and in differentiated P19 neurons. However, each isoform is differentially detected in both the cell bodies and neuron processes. The different levels detected in nerve processes and cell bodies would suggest specific mechanisms for differential transport of KOR mRNA isoforms into neuronal processes including the axons.

**Biological Activity of KOR mRNA UTR in Mediating mRNA Transport.** To determine whether KOR mRNA sequence contains functional transport signals for mobilizing mRNAs in neurons, we employed a strategy using GFP fused to a phage RNA binding domain, MS2, to trace mRNA tagged with the MS2-binding sequence. We first generated a GFP fused to an NLS-tagged MS2 (Bertrand et al., 1998), which was named K89 (Fig. 2A). Various KOR mRNA sequences were used to drive a luc cDNA and then tagged with three copies of MS2-binding sites. To first differentiate the biological role of 5′- and 3′-UTRs of KOR, four constructs were generated (Fig. 2A). The 5′K3′K construct contains the genomic sequences from both the entire 5′- and 3′-UTR of KOR, the 3′K3′SV contains only the 5′-UTR of KOR and the 3′-sequnce/poly(A) signal of SV40, the 5′tk3′K contains the thymidine kinase promoter in the 5′-end of luc cDNA and the entire 3′-UTR of KOR, and 5′tk3′SV uses the tk promoter and 3′-end of SV40 as a negative control. We first used P19 differentiated neurons as a model to explore the functional significance of these various KOR sequences in directing MS2-GFP (K89) in neuron cell bodies and their processes because the transfection efficiency of P19 neurons (20%) was much better than that of primary neurons. The differentiated P19 neuron cultures were transfected with K89, together with 5′K3′K, 5′K3′SV, 5′tk3′K, or 5′tk3′K, and GFP images were captured from live cultures.

As shown in Fig. 2B, GFP signals were detected in both cell bodies and processes of P19 cultures transfected with any of the three KOR constructs, 5′K3′K, 5′K3′SV, or 5′tk3′K, suggesting that all three KOR constructs were able to direct K89 translocation from the nuclei to the processes (Fig 2B, 5′K3′K, 5′K3′SV, and 5′tk3′K). To gain an insight into the efficiency of transport, the distance that GFP traveled in the processes was measured and presented as the percentage of the length of processes as shown in Fig. 2C. On the average, constructs 5′K3′K and 5′tk3′K transported with an equal efficiency in terms of the distance of transport, covering approximately 75% of the length of the fully extended processes. The 5′K3′SV was also able to direct mRNA into neuron processes, albeit at a much lower efficiency. Thus, KOR mRNA sequences, either the 5′-UTR, the 3′-UTR, or a combination of both, are able to direct mRNA transport from nuclei to neuronal processes in these in vitro differentiated neurons. Furthermore, although both the 5′- and 3′-UTRs of KOR mRNA are able to transport mRNA into neuronal processes, the 3′UTR of KOR sequence is apparently more effective than the 5′UTR in terms of the distance of transport.

However, differentiated P19 neuron culture is an artificial system and contains a mixture of different cell types. To validate the conclusion that the UTRs of KOR indeed were able to mobilize mRNA into neuron fibers, particularly the axons of sensory neurons, we then used primary DRG cultures in which neuron cell bodies and axonal processes could be better recognized. Although the cotransfection efficiency

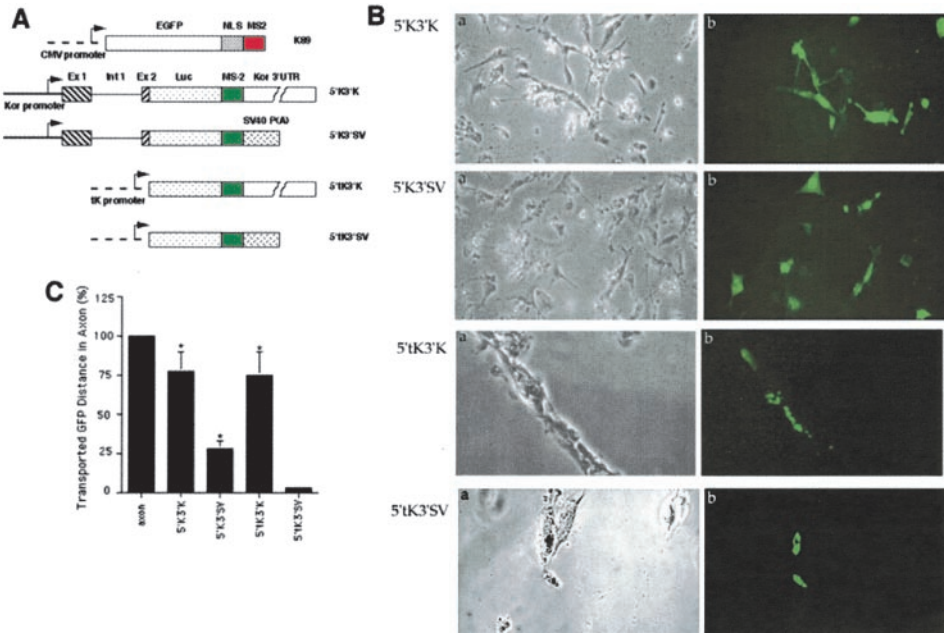


**Fig. 2.** Mobilization of MS2-GFP by the UTRs of KOR mRNA sequences into P19 neuronal processes. A, the maps of K89 (containing MS2/NLS-fused GFP) and four luc constructs directed by the 5′- and/or 3′-UTRs of KOR mRNA, tk, or SV40 sequences for controls. B, images of GFP mobilization, mediated by each luc construct, into P19 neuronal processes. C, a statistical analysis of the distance that GFP traveled in the processes for each luc construct. $P < 0.025$, $n = 10$.

of DRG (2%) was not as good as that of P19 (20%), we were able to successfully conduct this experiment as shown in Fig. 3. In this experiment, 5′K3′K was used because it was the most efficient construct in mediating GFP (K89) to neuron processes. As shown in Fig. 3b, GFP granules (5′K3′K construct) were seen in not only the cell bodies, but also the region of growth cones and the axon fibers leaving the soma (arrows). In the negative control (5′tk3′SV), GFP granules were only seen in the cell bodies (Fig. 3e, arrows). The morphology was better revealed in cultures stained with an anti-Tau (Litman et al., 1993) antibody (Fig. 3, a and d). The superimposed images were shown in Fig. 3, c and f. It is noted that GFP intensity is relatively weaker in these DRG cultures than in P19 cultures, probably because of the difference in the efficiency of the promoter used to express GFP. Nevertheless, it is confirmed that mRNA carrying the 5′- and 3′-UTRs of KOR indeed can be mobilized from the soma of DRG to the fibers, although the distance covered seems to be shorter than that seen in P19 neurons.

**Differential Ability of Various 5′-UTRs of KOR mRNA Isoforms in Mobilizing mRNA.** The 5′ sequence used in these constructs was derived from a KOR genomic fragment that was capable of producing all three 5′-UTRs of KOR mRNA isoforms A, B, and C. To dissect the 5′-UTR of isoforms A, B, and C, each specific 5′-UTR was fused to the MS2 binding site-tagged luc cDNA to generate 5′K-A, 5′K-B, and 5′K-C as shown in Fig. 4A. The ability of these constructs to mobilize K89 (GFP granules) into the processes was again monitored in P19 neurons. As shown in Fig. 4B, all three isoforms were able to mobilize nuclear GFP (control, b) to P19 neuronal processes (d, f, and h). This result is consistent with the finding that all three KOR mRNA isoforms can be detected in the axons of freshly isolated trigeminal neurons as well as P19 neuron processes (Fig. 1). To gain an insight into difference in their ability to mediate mRNA transport, the distance that GFP traveled was measured and the results were compiled as shown in Fig. 4C. The efficiency of transport was then examined by scoring the percentage of GFP-positive cells that showed GFP transport into neuronal processes as presented in Fig. 4D. By using this scoring method, it seemed that 5′K-A was most efficient, mediating mRNA transport to processes in approximately 20% of GFP-positive cells, and 5′K-B and 5′K-C constructs mediated mRNA transport in approximately 15% and 10% GFP-positive cells, respectively. Therefore, the 5′-UTRs of KOR mRNA isoforms contain functional signals to differentially direct mRNA into neuronal processes; isoform A is the most efficient. This is in agreement with the observation that isoform A, among the three isoforms, is the most abundant species detected in the axons of trigeminal nerves and P19 neuronal processes (Fig. 1).

To visualize mRNA transport directed by the KOR sequence in live cultures, a time-lapse video microscopy was conducted to record GFP mobilization in a live culture transfected with construct 5′tk3′K as shown in Fig. 5. From this recording, it is apparent that GFP granules, as shown in this recording and indicated with an arrow, indeed traveled along a P19 neuron fiber.

## Discussion

We provide evidence for differential distribution of mRNA isoforms of an opioid receptor, KOR, in the axons and cell

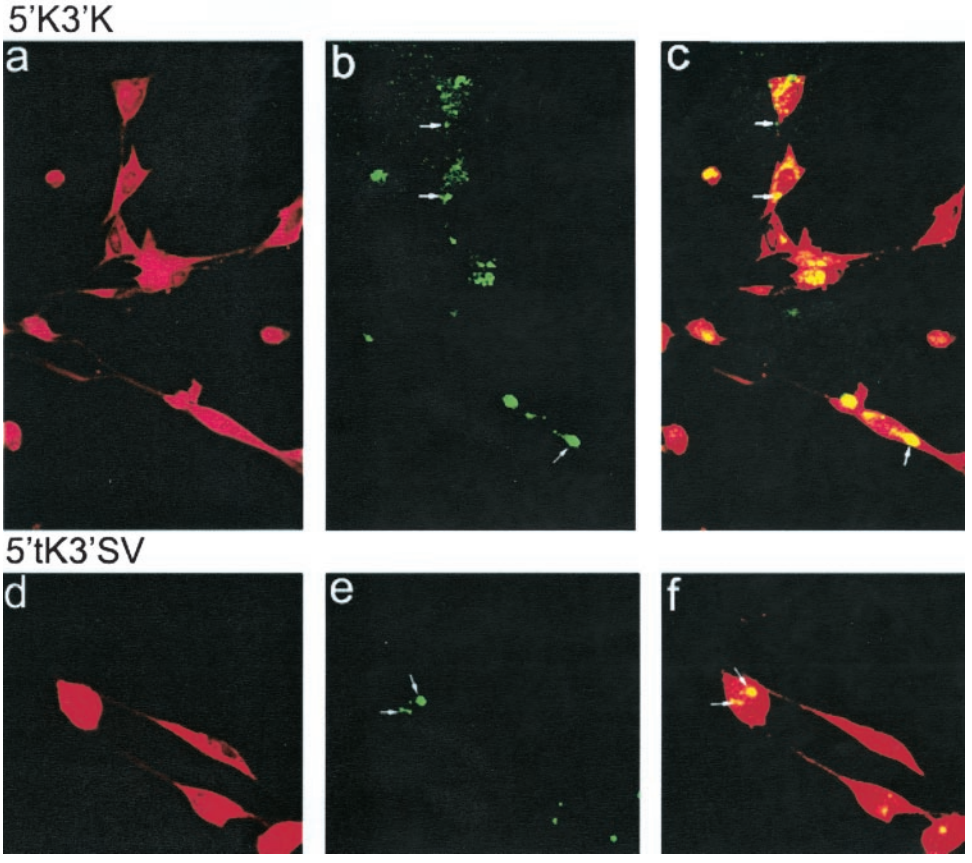


**Fig. 3.** Mobilization of MS2-GFP by the UTRs of KOR out of DRG soma. DRG cultures were cotransfected with 5′K3′K+K89 (top) or 5′tk3′SV+K89 (bottom). Cultures were observed, and GFP signals were captured 2 days after transfection. Shown for each set are the Cy3 images (Tau staining, a and d), GFP images (b and e), and the overlapped Cy3/GFP images (c and f). Several granules moving out of the nuclei and toward the processes for the 5′K3′K cultures are indicated by arrows. In the negative control (5′tk3′K cultures), granules were retained in the nuclei as indicated by arrows.

bodies of freshly isolated adult mouse sensory neurons, as well as in neuron processes and cell bodies of differentiated P19 neurons. Direct functional evidence is provided for the untranslated KOR mRNA sequences in mediating their transport into differentiated neuronal processes including axons of DRG. The various 5′-UTRs of KOR mRNA isoforms are able to mobilize mRNA into P19 processes with different efficiencies. The speed of KOR mRNA transport, mediated by its 3′-UTR, is calculated to be approximately 0.03 $\mu$m/s in P19 processes. This study demonstrated the biological significance of various 5′-UTRs of KOR mRNA isoforms in differential mRNA transport in neurons. This result suggested a potentially important pathway of producing KOR protein locally in different compartments of particular neurons.

Local protein synthesis in dendrites is well established, and the molecular signals mediating these events have been identified (Torre and Steward, 1996; Spencer et al., 2000; Job and Eberwine, 2001; Steward and Schuman, 2001). The signal for mRNA transport into dendrites is frequently mediated by the 3′-UTR of mRNA (Mohr, 1999; Mori et al., 2000; Hu et al., 2002). However, whether mRNAs are transported and nonstructural protein synthesis occurs in the axons of vertebrate neurons remains a highly debated issue (Job and Eberwine, 2001). It was believed that axons received their proteins from the cell bodies by anterograde transport. Only recently have studies begun to demonstrate local synthesis of structural proteins (Eng et al., 1999; Zhang et al., 2001; Zheng et al., 2001) in growing vertebrate axons (Zheng et al., 2001). Of most interest is the report by Ming et al. (2002) demonstrating the requirement of protein synthesis in resensitization of growth cones to chemotactic guidance, as well as that by Brittis et al. (2002), who provided strong evidence for localized protein synthesis of EphA2 receptor and regulation by a specific mRNA sequence. In this study, we have demonstrated not only the existence of, but also identified the mRNA regions for, differential transport of KOR in neurons.

The fact that the 5′-UTR isoforms of KOR mRNA are transported with varied efficiency suggests a biological significance of differential distribution of mature KOR mRNA isoforms in different brain regions. Although all three isoforms are able to travel a comparable distance (Fig. 4C), the efficiency of transporting GFP out of nuclei by each isoform varies (Fig. 4D). Isoform A is most efficient, followed by isoforms B and then C, consistent with the most abundant distribution of endogenous KOR mRNA isoform A in axons. Apparently, the 3′-UTR of KOR provides a common signal, in terms of the distance of transport, and it occurs at a speed of 0.03 $\mu$m/s in P19. The unique 5′-UTR of each isoform contributes to the efficiency of transporting mRNA out of nuclei. In this regard, it is interesting that the smallest isoform C encodes only 93 nucleotides, which can potentially form a limited number of secondary structures. Further systemic studies are required to address the regulatory role of these potential secondary structures and to determine the exact nucleotide sequences responsible for the intra-axonal transport processes. Another important task is to identify the *trans*-acting components that bind to these RNA sequences and the machinery responsible for mobilizing these messages into axons. Our previous study has demonstrated different stability and translation efficiency of these mRNA isoforms (Wei et al., 2000). Taken together, it can be concluded that, in addition to the well studied transcriptional regulation (Wei and Loh, 2002), the expression of KOR in different parts of neurons or in different brain areas is likely to be under

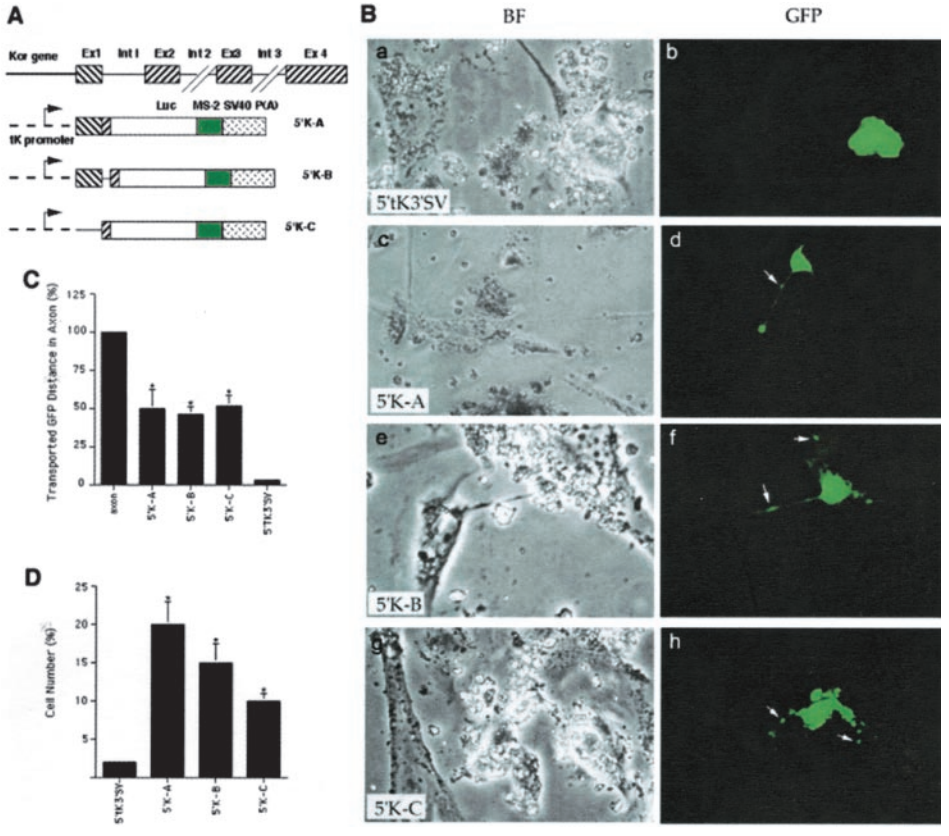


**Fig. 4.** Differential mobilization of MS2-GFP by the 5′-UTR isoforms A, B, and C of KOR mRNA into P19 neuronal processes. A, the KOR genomic structure and luc constructs of three 5′-UTR isoforms of KOR mRNA. B, images of GFP mobilization into P19 neuronal processes. BF, bright field images; GFP, GFP images. C, a statistical analysis of the distance that GFP traveled in the processes for each construct. $P < 0.025$, $n = 10$. D, a statistical analysis of GFP-positive cells that showed GFP mobilization into the processes for each construct. $P < 0.025$.

multilevel RNA-based regulatory events. The specificity of these RNA-based regulatory signals requires further study.

KOR immunoreactivity has been demonstrated in both pre- and postsynaptic membranes (Drak et al., 1996; Rawls et al., 1999). Furthermore, presynaptic KOR activation was shown to inhibit calcium-dependent glutamate release from the striatal synaptosomes (Drak et al., 1996), and a presynaptic mechanism was speculated for dynorphin, a putative endogenous ligand for KOR, to induce release of substance P from trigeminal primary afferent. The fact that different KOR mRNA isoforms are transported to neuronal axons with different efficiency suggests that the transport of KOR mRNA isoforms may play a regulatory role in presynaptic function of KOR protein. The production of several mature KOR mRNA isoforms encoding an identical amino acid sequence is intriguing. We have demonstrated different mRNA stability and translation efficiency among these mRNA isoforms (Wei et al., 2000). The present study provided further evidence for a role of these untranslated KOR mRNA sequences in directing differential transport to the remote parts of the neurons. It is tempting to speculate that the untranslated sequences of KOR mRNAs may play a role in specific physiological or pharmacological events by targeting or concentrating the receptors in neuronal subdomains, which may contribute to the distinction of KOR subtypes, such as KOR-1 and KOR-2, detected by pharmacological means.

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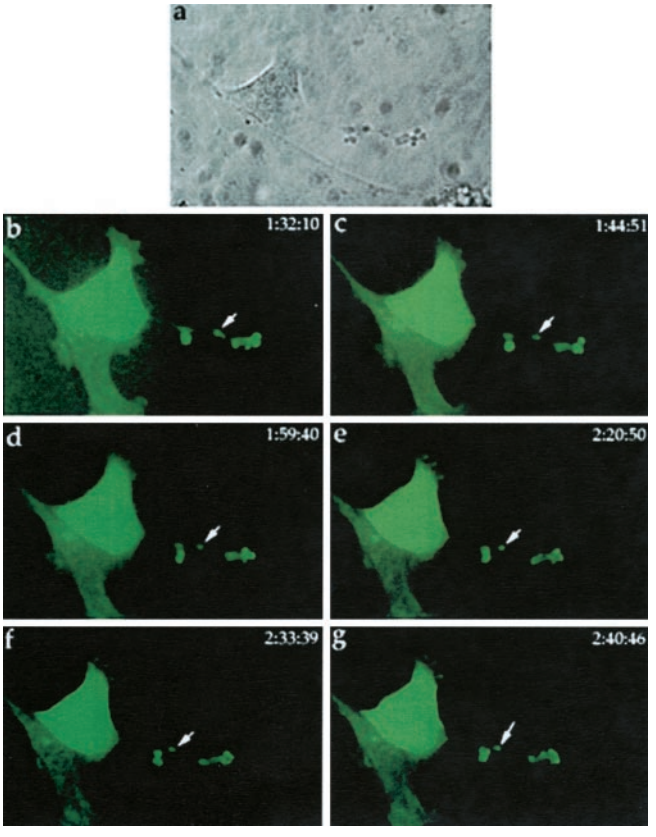


**Fig. 5.** Time-lapse images of MS2-GFP mobilization in P19 neuronal processes. a, bright field image; b–g, different time points of the recording for a duration of 1 h, 8 min, and 36 s.

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