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Regulation of Mouse κ Opioid Receptor Gene Expression by Different 3′-Untranslated Regions and the Effect of Retinoic Acid

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ABSTRACT

The mouse κ opioid receptor (*KOR*) gene uses two functional polyadenylation signals, separated by a distance of approximately 2.2 kilobases (kb) in the 3′-end of the gene. As a result, two major groups of KOR transcripts, with sizes of approximately 1.6 and 3.8 kb, respectively, are detected in mouse tissues and P19 cells. Utilization of different poly(A) of the *KOR* gene produces KOR transcripts of different mRNA stability, transcription efficiency, and regulatability. Retinoic acid specifically suppresses the expression of KOR transcripts using the second poly(A) in P19 cells. A putative transcriptional enhancer region is present within the second 3′-untranslated region (3′-UTR). It is concluded that alternative polyadenylation of the mouse KOR transcripts results in differential regulation of KOR expression at both transcriptional and post-transcriptional levels. A negative regulatory pathway for KOR transcription involves a putative enhancer region in its 3′-UTR. KOR mRNAs using the second poly(A) is more stable than that using the first poly(A).

Opiates and endogenous opioid peptides exert their pharmacological and physiological effects through binding to the opioid receptors. At least three types of opioid receptors, δ, μ, and κ, are present, each with a specific ligand-binding profile and a unique expression pattern (Goldstein and Naidu, 1989; Masabumi and Satoh 1995). The genes encoding all three types of opioid receptors have been cloned (Masabumi and Satoh 1995; Wei and Loh, 1996), which allows the molecular mechanisms underlying the regulation of the expression of these genes to be examined.

Most studies of opioid receptor gene regulation have been focused at the transcriptional level (Wei and Loh, 2002). However, it is known that RNA processing, mRNA stability, and translation efficiency are also important regulatory events. Our laboratory isolated the mouse κ opioid receptor (*KOR*) gene (Liu et al., 1995) and used it as a model gene for the examination of the molecular events involved in the control of opioid receptor expression (Lu et al., 1997; Wei et al., 2000; Hu et al., 2001). Of particular interest was the finding of two functional promoters used by the mouse *KOR* gene, as well as its mRNA variants derived from alternative splicing within its 5′-untranslated region (5′-UTR) (Lu et al., 1997). Subsequently, we provided evidence for not only transcriptional control of this gene (Hu et al., 2001), but also post-transcriptional regulation of this gene mediated by these different 5′-UTR sequences (Wei et al., 2000). In the examination of transcriptional control of the mouse *KOR* gene, we presented evidence for a primarily suppressive role for a differentiation agent, retinoic acid (RA), in the expression of the mouse *KOR* gene in mouse embryonal carcinoma cell line P19 (Bentley, 1999). The suppressive effect of RA was attributed, in part, to the induction of a negative transcription factor, Ikaros, that rendered deacetylation of the promoter regions of the mouse *KOR* gene, resulting in reduced transcription of the *KOR* gene in P19 cells (Hu et al., 2001).

In an attempt to examine the potential regulatory activity of the 3′-UTR region of the *KOR* gene, we first conclusively determined the sizes of the KOR mRNA species in Northern blot analyses. It was surprising that different sizes of KOR mRNA were detected in RNA samples prepared from animal tissues and P19 cells. Because the size difference among the KOR variants produced through alternative splicing at the 5′-UTR (approximately 94–216 bp) could not account for the difference in the size of KOR mRNA detected in animal tissues and cell lines (approximately 1.6 versus 3.8 kb), we therefore set up experiments to carefully examine the 3′-

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ABBREVIATIONS: KOR, κ opioid receptor; UTR, untranslated region; kb, kilobase(s); bp, base pair(s); RA, retinoic acid; RT-PCR, reverse transcription-polymerase chain reaction.

UTR region of the mouse *KOR* gene, which led to the discovery of different 3′-UTRs of these KOR transcripts.

It is known that post-transcriptional regulation, particularly RNA processing, involves a number of well orchestrated events, including RNA splicing, editing, and polyadenylation (Zhao et al., 1999). Furthermore, transcription and RNA processing can be coordinated (McCracken et al., 1997; Bentley, 1999; Minvielle-Sebastiz and Keller, 1999). The presence of KOR mRNA species varied at the 3′-UTR of this gene strongly suggested a potential role for this sequence in the expression of different KOR mRNAs. The purpose of this current study is to carefully determine the 3′-UTR sequence of the mouse *KOR* gene and to examine the potential regulatory events involving the 3′-UTRs of this gene. We demonstrate alternative poly(A) usage of the mouse *KOR* gene, which results in different mRNA stability, transcription efficiency, and regulatability. Therefore, alternative polyadenylation of the mouse KOR transcripts results in differential regulation of KOR expression at both transcriptional and post-transcriptional levels. RA seems to be able to elicit a negative regulatory pathway for the transcription of mRNAs using the second poly(A).

Materials and Methods

Northern Blot. Total RNA was isolated from mouse brain, spinal cord, and P19 cells using TRIzol reagent (Invitrogen, Carlsbad, CA) as described previously (Wei et al., 2000). Twenty micrograms of total RNA was loaded onto a formaldehyde denaturing gel and transferred to a nylon membrane. The membrane was hybridized to α-^{32}P-dCTP–labeled KOR cDNA probe or the probe containing different 3′-UTRs of the KOR sequence.

RT-PCR Reaction. Two micrograms of total RNA from brain, spinal cord, and P19 cells each was reverse transcribed by using Omniscript reverse transcriptase (QIAGEN, Valencia, CA) and an oligo(dT) as the primer. One fiftieth of the total RT product was used in the PCR reaction. The 5′ primer was specific to the end of exon II, 5′-TGGTCATGTTTGTCATC-3′. The 3′ primers were 5′-ATGATCAGGCTGGACAAG-3′ and 5′-GACCCAGTATTAGATGGA-3′, specific to the first and the second poly(A) signal, respectively. Southern blotting was used to analyze the PCR products, probed with either total KOR cDNA or specific 3′-UTR sequences.

Luciferase Reporters and Transfection of P19 Cells. A 1.3-kb *Bam*HI/*Nco*I genomic sequence containing the KOR promoter region and its translation initiation codon was subcloned into the *Bgl*II/*Nco*I sites of pGL3B (Promega, Madison, WI) to generate K45. From a genomic library, a 3.8-kb *Xba*I/*Xho*I fragment containing the 3′-UTR of KOR was obtained and subcloned into the reporter construct K45 (Hu et al., 2001) to replace the simian virus-40 poly(A) signal in the original reporter, resulting in the reporter construct K96. A *Hin*dIII and *Eco*RI partial digestion was conducted to generate deletions of KOR 3′-UTR from either the 5′- or the 3′-end of the 3′-UTR within K96. The resulting reporters with 3′ deletions were K113, K114, and K115, each with a 3′-UTR size of 2.4, 1.8, and 1.3 kb, respectively. The resulting reporter constructs with deletions from the 5′-ends were K121, K122, and K123, each containing 3, 2.6, and 2 kb of the 3′-UTR, respectively. K132 and K133 were generated by replacing the 3′-UTR sequence of K123 with two PCR fragments. The primers were 5′-CCGAATTCCCAGAAGCTTGTCCC-3′ and 5′-GTCCGCGGTAGATCATCGCTGAAG-3′ (for K132), and 5′-CCGAATTCTGTGCCTCTATTGTGC-3′ and 5′- GGCCGCGGAGGGATGTTCTTTACTG-3′ (for K133). K128 was constructed by replacing the 3′-UTR of K123 with an *Nco*I/*Sac*II fragment (approximately 200 bp) of the KOR genomic DNA fragment. Transient transfection of P19 cells was performed by using 0.1 pmol of one specific reporter construct together with 0.2 μg of *CMV-LacZ* reporter gene as an internal control. The cells were harvested 30 h after transfection.

Nuclear Run-on Assay. K96, K115, and K123 were each transfected into P19 cells with CMV-LacZ as an internal control. The nuclear extracts were prepared from the cells 20 h after transfection. One A_{260} unit of nuclear extract was used in each nuclear run-on reaction. Briefly, nuclear extract was mixed with 2× transcription buffer (10 mM Tris, pH 8.0, 5 mM $MgCl_2$, 0.3 mM KCl, 0.2 mM EDTA, and 1 mM dithiothreitol), 10 μl of 25 mM rNTP minus rUTP, 10 μl of 10 mM ATP, and 6 μl of [α-^{32}P]UTP, 1 μl of RNase Inhibitor (Promega; 40 units/μl), 1 μl of 1 M dithiothreitol and incubated at 30°C for 30 min. The reaction mixture was treated with 50 μl of DNase I solution (200 units of DNase I, 2.5% SDS, 100 mM Tris, and 50 mM EDTA) at 30°C for 10 min, followed by extraction with 250 μl of TRIzol (Invitrogen) reagent. The supernatant (400 μl) was transferred to a new tube and total RNA was precipitated by adding 300 μl of isopropanol, 20 μg of glycogen, and 40 μl of 3 M sodium acetate. The precipitated RNA was washed with 70% ethanol and resuspended in 100 μl of hybridization buffer. One million cpm of the RNA was used in hybridization. The luciferase, KOR, and LacZ cDNA were each blotted onto a nylon membrane using a slot-blot (Minifold II; Schleicher & Schuell, Keene, NH) for hybridization to the RNA probes. The membrane was incubated at 42°C for 72 h. The signal intensity was measured and analyzed using a PhosphorImager and Imagequan software (Amersham Biosciences, Piscataway, NJ).

RNA Stability. K115 and K123 were each transfected into P19 cells with or without RA treatment. Twenty-four hours after transfection, cells were treated with actinomycin D with a final concentration of 2 μg/ml for 3, 6, or 9 h. Cells were harvested and total RNA was prepared. After DNase I digestion, 2 μg of total RNA was used in a reverse transcription reaction (Invitrogen) according to the manufacturer's protocol. One-fiftieth of the total RT products were used in a PCR reaction using primers 5′- ATCAGCGATCTGGAGCT-3′ and 5′-TGGAAACGAACACCACGG-3′ to amplify the luciferase sequence. One two hundredth of total RT product was used to amplify β-actin (Wei et al., 2000) as the control. The PCR product was analyzed on a 1.5% agarose gel and subjected to Southern blot analysis using either luciferase or β-actin cDNA as the probe. The signal intensity was measured in a PhosphorImager and analyzed using Imagequan software.

Results

Characterization of the Mouse KOR 3′-UTR Sequence. The 3′-UTR of the mouse KOR was isolated from a genomic library with the probe prepared from exon IV of the mouse KOR cDNA (Liu et al., 1995). A genomic clone was isolated that spanned the entire KOR 3′-UTR sequence of the reported cDNA as well as a sequence extended from the end of the cDNA for approximately 3.7 kb. By sequence alignment, a number of potential polyadenylation signals that scattered in two regions were found in this sequence. The sequence was submitted to the Genome Data Base (accession number AF490606), and the potential poly(A) signals were highlighted as shown in Fig. 1.

Demonstration of Mouse KOR mRNA Variants Differed at the 3′-UTR. In our preliminary studies, KOR mRNA detected on the Northern blot of P19 cells seemed to be heterogeneous. Therefore, we performed Northern blot analyses of RNA isolated from mouse brain and spinal cord, as well as P19, with the mouse KOR full-length cDNA as the probe. As shown in Fig. 2A, the hybridizing signals on the Northern blot appeared in two major bands, approximately 1.6 and 3.8 kb, respectively. As shown in Fig. 2, B and C, both the 28S and 18S rRNA (Fig. 2C), and the β-actin signal (Fig. 2B) were intact and comparable among these three samples.

We have previously determined that three KOR variants could be generated from alternative splicing in the 5′-UTR, each with a 5′-UTR size of 94 to 216 bp. It is apparent that the difference of 2.2 kb between these two KOR-hybridizing bands detected on the Northern blot could not be accounted for by alternative splicing at the 5′-UTR. Therefore, it was speculated that the variation might have occurred at the 3′-UTR of the *KOR* gene.

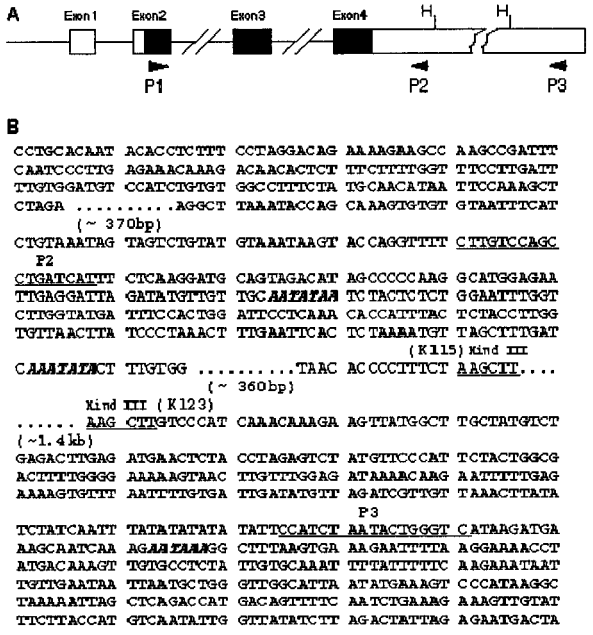

Fig. 1. Partial sequence of the 3′-UTR of the mouse *KOR* gene. A, the RT-PCR strategy to detect mRNA generated by using different potential polyadenylation signals. Primer P1 is located at the end of exon 2, and primers P2 and P3 are located in the 3′-UTR upstream of the poly(A) signals. The solid box indicates the coding regions of the mouse *KOR* gene, and the open box indicates the 5′- and 3′-UTRs of this gene. H, *Hin*dIII sites. B, a partial sequence of mouse KOR 3′-UTRs. The underlined sequences are the primers P2 and P3, and the two *Hin*dIII sites are as shown in A. The highlighted italic sequences are the potential polyadenylation signals.

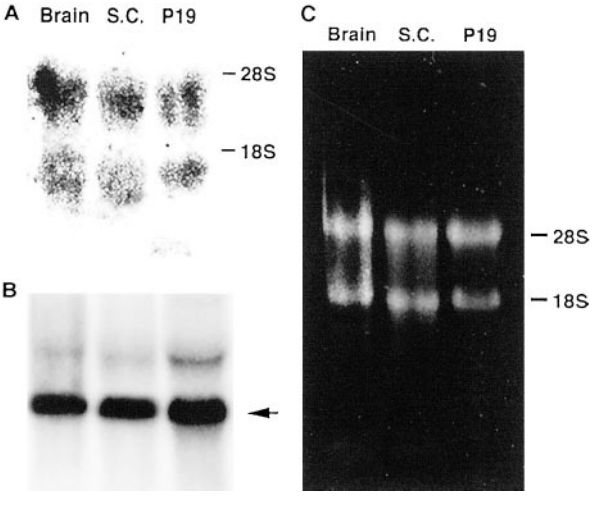

Fig. 2. Northern blot analyses of two major KOR transcripts. A, Northern blot probed with KOR cDNA; two major bands of approximately 1.6 and 3.8 kb are detected. B, Northern blot probed with β-actin. An arrow indicates the band of β-actin. C, a denaturing RNA gel stained with ethidium bromide, showing the integrity of these RNA samples. S.C., spinal cord.

To confirm further that the KOR-hybridizing signal on the Northern blot was from matured mRNA, RT-PCR was conducted by using an oligo(dT) as the primer for the reverse transcription reaction, followed by amplification with the help of a sense primer located in exon II and an antisense primer chosen according to the predicted poly(A) signal as shown in Fig. 1. The PCR product was then probed with KOR cDNA as well as the different 3′-UTR sequences, according to the predicted site of transcription termination. The results are shown in Fig. 3. The hybridizing bands were about 1.6 kb (Fig. 3, A and B) and 3.8 kb (Fig. 3, C and D), with probes prepared from either the KOR cDNA (Fig. 3, B and D) or specific 3′-UTR (Fig. 3, A and C). The lower bands in each PCR product could be caused by RNA secondary structure or alternatively spliced transcripts that have not been identified, particularly for a long PCR product like 3.8 kb. Nevertheless, the specificity of 1.6- and 3.8-kb fragments was confirmed by hybridization to KOR probe. The internal control β-actin is shown in Fig. 3E. This result suggests that transcription of the mouse *KOR* gene can be terminated at two major sites within its 3′-UTR. Site 1 is located at approximately 900 bp after the TGA termination codon, and site 2 is located approximately 2.2 kb further downstream from site 1. As a result, the mature KOR mRNA transcripts can be generated from either one of the two poly(A) sites at the sizes of approximately 1.6 and 3.8 kb, respectively.

Biological Activity of Alternative KOR poly(A) Signals. The presence of different sizes of poly(A)-positive KOR

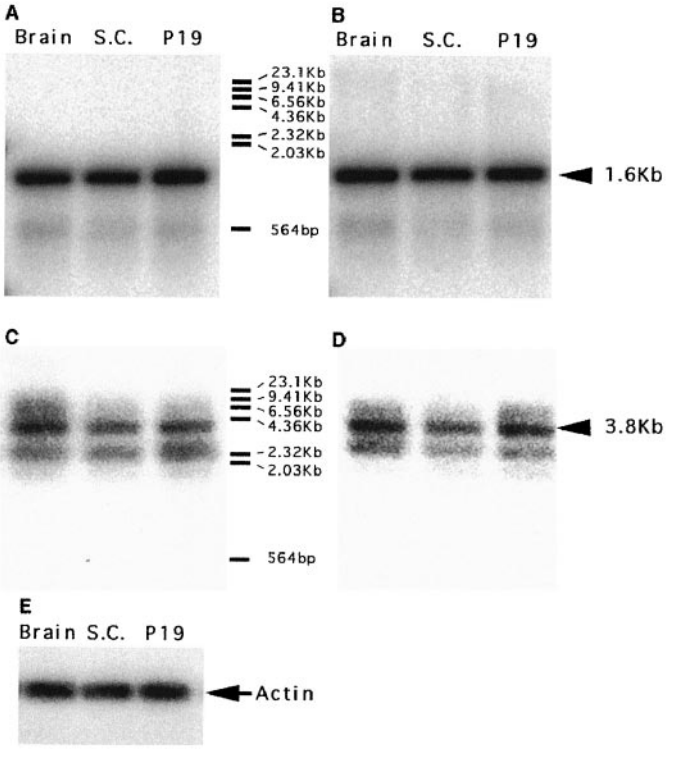

Fig. 3. RT-PCR to detect KOR mRNAs using two alternative poly(A) signals. A and B show the Southern blots of RT-PCR using primers P1 and P2 to amplify the mRNA using the first poly(A) signal. C and D show the results using primers P1 and P3 to detect the mRNA using the second poly(A) signal. A and C were probed with sequence specific to the 3′-UTR of transcripts using different poly(A) signal sequences. B and D were probed with the same KOR cDNA. E shows the RT-PCR result using the same RNA samples and probed with the β-actin cDNA probe. S.C., spinal cord.

RNA species suggested the possibility of alternative poly(A) usage. To determine whether these poly(A) sites are biologically functional, these sequences were tested in reporters lacking a poly(A) signal. A 3.6-kb genomic DNA sequence at the downstream end of the KOR coding region was first used to replace the simian virus-40 poly(A) signal of a reporter construct, K45 (Hu et al., 2001), which used the KOR promoter to drive a luciferase reporter. This new construct was named K96. Deletion mutants of this KOR 3′-UTR, at either the 5′- or the 3′-end, were then generated from K96 with the help of restriction endonuclease digestion (Fig. 4A). P19 stem cells, which expressed the *KOR* gene, were used in transient transfection assays to determine each specific reporter activity, together with an internal control LacZ reporter. As shown in Fig. 4B, all the constructs were able to activate the reporter except K128, which retained only the last 200 bp of the 3′-UTR sequence at the downstream end of the predicted second poly(A) site. Therefore, both predicted poly(A) sites are functional as evidenced from these positive reporter activities, with K113, K114, and K115 encoding the first poly(A) activity, and K132 and K133 encoding the second poly(A) activity. The relatively higher activity of K121, K122, and K123 was due to the activity of a potential enhancer (see the next section). It is concluded that the 3′-end of the mouse *KOR* gene encodes at least two functional poly(A) signals, separated by a sequence of approximately 2.2 kb.

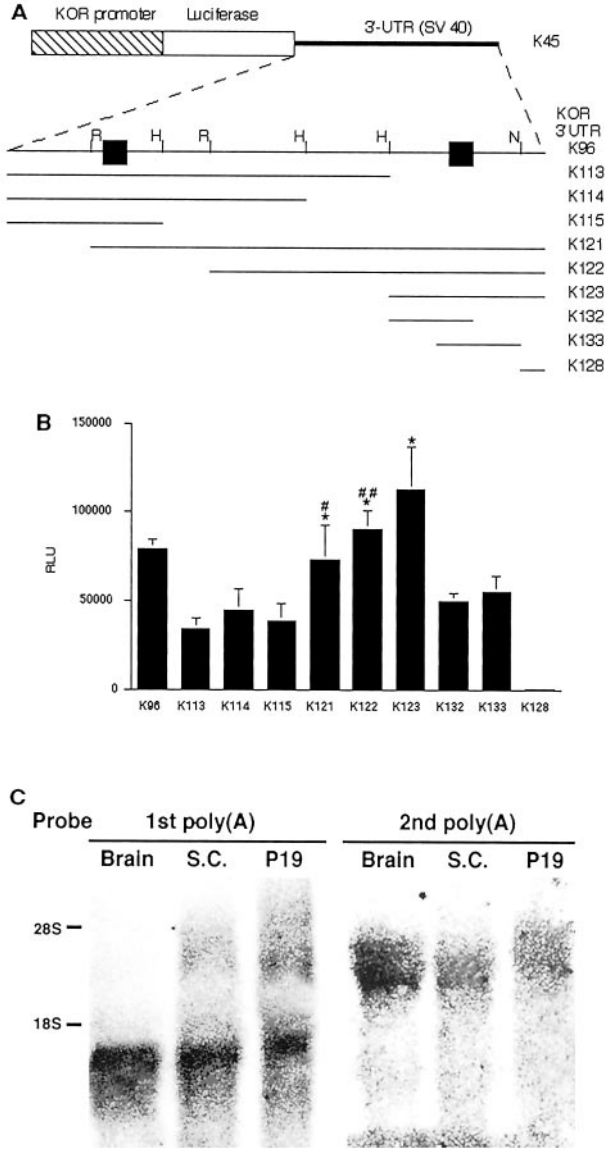

Fig. 4. Transient transfection assay to demonstrate the biological activity of the two poly(A) signals. A, the map of reporter constructs used in these transient transfection experiments. ▧, KOR promoter; □, luciferase reporter; ■, positions of potential poly(A) signal; solid lines, 3′-UTR sequences. R, *Eco*RII; H, *Hin*dIII; N, *Nco*I; SV 40, simian virus-40. B, relative luciferase activity of the reporter constructs. #, significant difference ($p < 0.05$) between K121 and K123; # #, no significant difference between K122 and K123; ⋆, significant difference ($p < 0.05$) comparing K115 to K121, K122, or K123. C, Northern blot with probes specific to the 3′-UTR of transcripts using either the first or the second poly(A) signal.

To further prove that these 3′-UTR sequences were indeed present in different KOR transcripts detected on the Northern blot (Fig. 2A), each sequence was used as the probe to reexamine the transcripts on the Northern blots. The sequence specific to transcripts using the second poly(A) detected transcript of 3.8 kb (Fig. 4C, right), whereas the sequence specific to transcripts using the first poly(A) detected transcript of 1.6 kb (Fig. 4C, left) in all these tissues. Therefore, it is concluded that the first poly(A) signal, as used in K113, K114, and K115, is responsible for the termination of transcripts of 1.6 kb, and the second poly(A) signal, as used in K123, K132, and K133, is responsible for the termination of the larger 3.8-kb KOR mRNA variant. This is in agreement with the result of RT-PCR shown earlier (Fig. 3).

The Effects of 3′-UTR on Transcription Efficiency of the *KOR* Gene. The finding that the reporters of the constructs using the second poly(A) signal (K121, K122, and K123) encoded significantly different activities suggested that factors other than polyadenylation were probably present in the sequence common to these constructs. Because transcription and RNA processing can be coupled, we then determined first whether the two poly(A) signals have different effects on the transcription rate. Nuclear run-on assay was carried out to examine the transcription efficiency of each reporter. P19 cells were transfected with the same molar amount of K96, K115, and K123 relative to the internal control lacZ reporter. Nuclear extracts were prepared at 24 h after transfection, and nuclear run-on assay was carried out as described previously (Sambrook and Russell, 2001). Figure 5A shows one representative set of the results and Fig. 5B shows a statistical analysis of results obtained from three independent experiments. It seemed that the signals of the two internal controls, the endogenous β-actin gene and the cotransfected LacZ reporter gene, exhibited no significant difference during the time of examination. Interestingly, the reporter carrying the first poly(A) signal, K115, seemed to be significantly less efficient in transcription ($p < 0.05$) compared with that carrying the second poly(A) (K121, K122, and K123). The construct carrying the entire 3′-UTR (K96) or the second poly(A) site (K123) was equally efficient in transcription.

The Effects of KOR 3′-UTR on KOR mRNA Stability. The alternative usage of poly(A) signals suggested potential regulation of KOR expression at the level of mRNA stability by the different 3′-UTRs. To test this possibility, we transfected P19 cells with two representative reporters, K115 [for the first poly(A)] and K123 [for the second poly(A)], and blocked transcription by actinomycin D. At different time points, RNA was collected and the expression level of these

reporters, as well as an internal control β-actin, was monitored by an established RT-PCR protocol in which cycles were controlled for amplifying specific messages within a linear range (Wei et al., 2000). PCR products were analyzed by hybridization with luciferase cDNA and β-actin, respectively. By including the internal control actin, which remained constant during the period of examination, accurate and reliable quantitation of specific messages in luciferase reporter could be obtained from which the half-life could be calculated. Figure 6A shows one of these Southern blots and Fig. 6B shows the statistical data. According to this experiment, mRNA using the second poly(A) signal (K123) has a half-life of 8.7 h, whereas mRNA using the first poly(A) (K115) has a half-life of 5.8 h. The disappearance of actin messages in both K115- and K123-transfected cultures exhibited no significant difference (Fig. 6B, #), assuring the significant difference in the half-lives of K115 and K123. Therefore, it is concluded that the mRNA using the second poly(A) is relatively more stable than that using the first poly(A).

The Effect of RA on *KOR* Gene Regulation, Mediated by the 3′-UTR of the *KOR* Gene. Our laboratory has previously shown that RA treatment suppressed *KOR* gene expression in P19 stem cells, which was partially attributed to the induction of a negative transcription factor, Ikaros, that silenced the two promoters of the *KOR* gene by recruiting histone deacetylases to these promoters (Hu et al., 2001). To determine whether RA has an effect on *KOR* gene regulation involving its 3′-UTR, we monitored the effects of RA on the steady-state level of the endogenous KOR messages by using probes specific to transcripts using either the first poly(A) or the second poly(A). P19 cells were treated with either vehicle or 1 μM RA for 48 h, and total RNA was prepared from both RA-treated and vehicle-treated cells. RNA was analyzed on a Northern blot with either KOR cDNA, or the first poly(A)- or the second poly(A)-specific probes as shown in Fig. 7A. The total KOR expression (probed with KOR cDNA) was suppressed, but only the higher band showed a significant reduction. That is further confirmed by the significantly reduced expression of transcripts using the second poly(A) signal. As predicted, the transcripts using the first poly(A) were not significantly affected by RA treatment at this time point.

In addition, the activity of reporters using either one of the two 3′-UTRs was determined in transient transfection (Fig. 7B). P19 cells treated with either vehicle or 1 μM RA were transfected with reporter K115 or K123 and a LacZ internal control. The reporter activity was assayed 36 h after transfection. The reporter activity of K115 was reduced by approximately 10% after RA treatment, whereas the reporter activity of K123 was decreased by almost 50%. Therefore, the suppressive effect of RA on K123 expression was much more dramatic than that on K115, with a statistically significant difference (t test, $p = 0.01$).

To determine whether transcription rate was a target of the suppressive effect of RA on the expression of KOR transcripts using the second poly(A), nuclear run-on experiments were performed as shown in Fig. 7, C and D. P19 cells were treated with vehicle or 1 μM RA, followed by transfection with reporters carrying the first poly(A) (K115) or that carrying the second poly(A) (K123), together with an internal

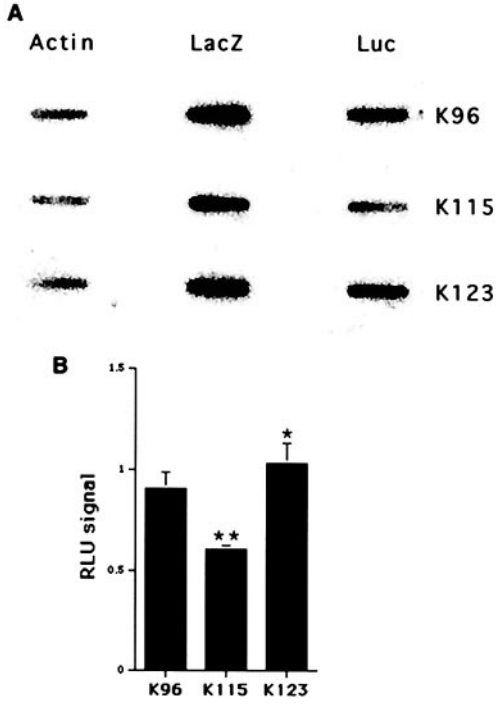

Fig. 5. Nuclear run-on assay to examine the transcription rates of reporter constructs with different 3′-UTR sequences. A, slot blot of the nuclear run-on assay. Whereas the transcription efficiency of β-actin and LacZ was relatively constant, the transcription efficiency of K115 was significantly lower than that of K96 and K123. Luc, luciferase. B, the statistical results of three independent nuclear run-on experiments. ⋆, no significant difference between K96 and K123; ⋆⋆, significant difference between K96 and K115 ($p < 0.005$). RLU, relative light units.

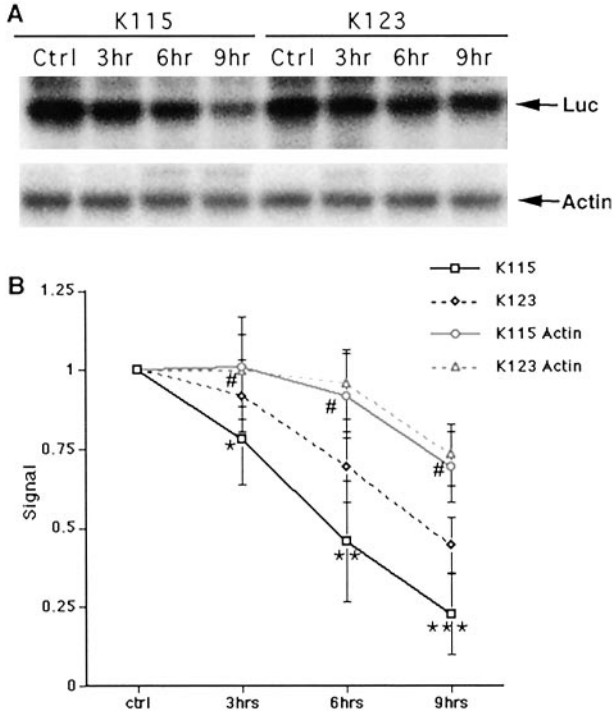

Fig. 6. RNA stability of KOR transcripts using different 3′-UTR sequences. A, Southern blot of RT-PCR products. After transient transfection, actinomycin D was added to determine the half-life of the specific reporter and β-actin mRNA. Ctrl, control; Luc, luciferase. B, the statistical results of three independent experiments. The calculated half-life of K115 mRNA is 5.8 h, whereas that of K123 is 8.7 h. #, no significant difference between actin messages; ⋆, no significant difference; ⋆⋆, significant difference ($p < 0.1$); ⋆⋆⋆, significant difference ($p < 0.05$).

control LacZ. The nuclear extract from each sample was prepared and used in the in vitro transcription. The in vitro synthesized RNA was used as the probe to hybridize the sequences of luciferase and LacZ immobilized to a nylon membrane. It seemed that the transcription rate of the control, LacZ, remained constant after the treatment. Interestingly, only the luciferase signal of K123, but not of K115, was significantly decreased. Therefore, it is concluded that RA selectively affects the transcription efficiency of specific KOR mRNA species by acting on the 3′-UTR of transcripts using the second poly(A) signal.

Discussion

This is the first study aiming at dissecting the regulatory pathway involving the 3′-UTR of the mouse *KOR* gene and the first report showing a suppressive effect of RA on gene transcription mediated by a 3′-UTR of the gene. The regulation of KOR expression mediated by the 3′-UTR of this gene occurs at both transcriptional and post-transcriptional levels. Two major groups of mouse *KOR* gene transcripts can be detected in both animal tissues and P19 cells, each sized approximately 1.6 and 3.8 kb, respectively. These variants are derived from the use of two alternative poly(A) signals at the 3′-end of the gene. The use of the first poly(A) signal generates the 1.6-kb transcripts and that of the second poly(A) signal generates the 3.8-kb transcripts. Both poly(A) signals are biologically functional, as evidenced by their ability to activate a poly(A)-less reporter expression. The 3.8-kb transcripts are more stable than the 1.6-kb transcripts, with a half-life of 8.7 h for the 3.8-kb transcripts and a half-life of 5.8 h for the 1.6-kb transcripts. RA exerts a suppressive effect specifically on the transcription of transcripts using the second poly(A) signal.

Alternative splicing has been observed in numerous genes including all three opioid receptor genes (Belkowski et al., 1995; Gaveriaux-Ruff et al., 1997; Pan et al., 1999, 2001). Our laboratory has previously demonstrated the presence of at least three mRNA variants derived from alternative splicing and promoter usage at the 5′-end region of the mouse *KOR* gene. However, the difference that resulted from alternative splicing within the 5′-UTR cannot account for the difference in the size of messages detected on Northern blots, raising a possibility of variation at the 3′-end of this gene. This study clearly demonstrates alternative polyadenylation of mouse *KOR* gene, which results in differential regulation of KOR mRNA variants that can be detected in both mouse tissues and P19 cells. The finding of an RA-regulated region in a specific 3′-UTR of the transcripts using the second poly(A) signal is particularly interesting. RA is known as a potent suppresser for *KOR* gene expression in P19 cells. Our previous study has focused on the role of RA on the regulatory activities encoded within the KOR promoters. This previous study has demonstrated an induction of a negative transcription factor, Ikaros, which recruits histone deacetylases to the KOR promoter region (Hu et al., 2001), thereby suppressing *KOR* gene transcription. This current study adds an additional role of RA on *KOR* transcription through its suppression on a 3′-UTR region.

Alternative splicing of opioid receptor genes has been demonstrated widely. The alternative splicing of the μ opioid receptor gene was shown to potentially produce different protein products (Pan et al., 2001). However, in the 3′-UTR region of the *KOR* gene, no open reading frame could be identified. Thus, these KOR mRNA variants seemed to encode the same protein product. The fact that KOR transcripts with alternative polyadenylation are differentially regulated, such as by morphogen RA, invites an interesting hypothesis for the biological or pharmacological significance of generating opioid receptor mRNA variants. It can be speculated that the production of mRNA variants may be for differential regulation of opioid receptor expression by different cells or at different physiological states. It remains to be determined whether they are differentially expressed in different types of

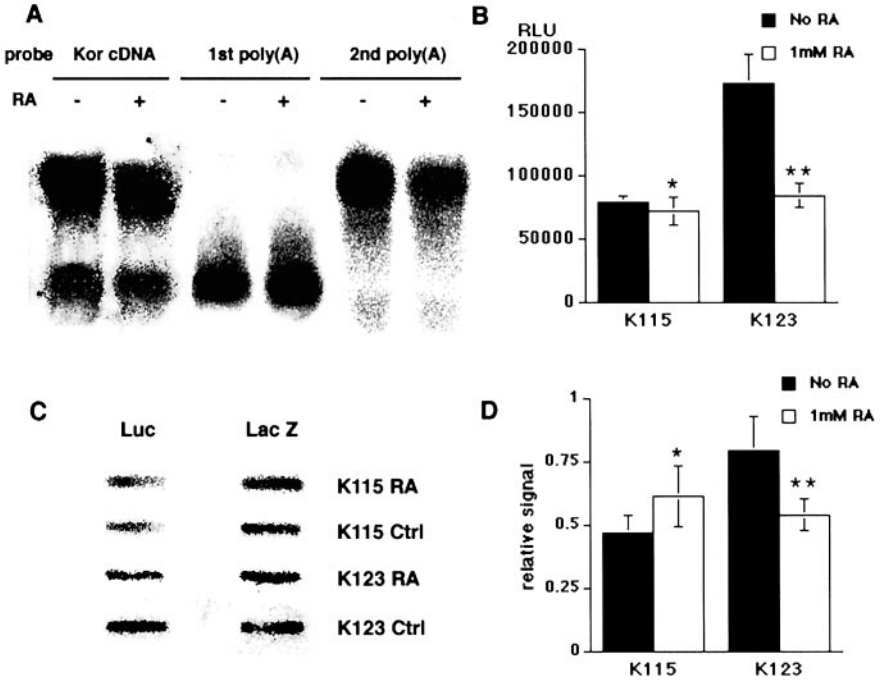

Fig. 7. The effect of RA on the transcription of KOR transcripts using the second 3′-UTR sequence. A, Northern blot of P19- and RA-treated P19 cells. RNA samples were hybridized with probe of either KOR cDNA or the sequence specific to the 3′-UTR of transcripts using either the first or the second poly(A) signal. B, reporter gene assay in transient transfection experiments. RLU, relative light units. C, nuclear run-on assays showing a suppressive effect of RA on the transcription of K123. Luc, luciferase; Ctrl, control. D, the statistical results of three independent nuclear run-on experiments. ⋆, no significant difference between control and RA treated for K115; ⋆⋆, significant difference ($p < 0.05$) between control and RA treated for K123.

neurons and what is the molecular mechanism underlying alternative polyadenylation of *KOR* gene.

This study presents the first example of alternative polyadenylation and demonstrates, for the first time, the functionality of alternative poly(A) signals in the studies of opioid receptor genes. It is noticed that multiple potential poly(A) signals are present in the 3′-end of the mouse *KOR* gene, although not all are conserved AAUAAA. However, it is known that only approximately 80% of the matured mRNAs use the conserved AAUAAA signal (Edwalds-Gilbert et al., 1997), and the strength of poly(A) signal is responsible for the amount of matured mRNA produced and exported (Edwalds-Gilbert et al., 1993). In the 3′-UTR region of the transcripts using the first poly(A) signal of the *KOR* gene, the potential poly(A) signals are AAUAUA. The less conserved poly(A) signal does not function as efficiently as the conserved AAUAAA (Sheets et al., 1990). Therefore, the use of less conserved poly(A) signals by the 1.6-kb transcripts may partially account for the lower reporter activity of that using the first poly(A) signal. It is known that the conserved carboxyl terminus of RNA polymerase II is required for efficient 3′-end processing of the transcripts (McCracken et al., 1997; Hirose and Manley, 1998). Furthermore, it is also known that factors for polyadenylation are recruited to the pre-mRNA by RNA polymerase II (Dantonel et al., 1997), which makes the processing steps more efficient. Therefore, it is tempting to speculate that the activity of the reporter using the 3′-UTR of the 3.8-kb mRNA transcripts is higher due to a combination of enhanced transcription and better RNA stability and polyadenylation.

The finding that alternative polyadenylation of KOR transcripts also affects mRNA stability is interesting. Several sequence elements are noticed that could potentially regulate the rate of mRNA turnover by interacting with specific RNA binding proteins. It will be interesting in the future to define these regulatory elements and identify proteins binding to these sequences.

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