The mouse kappa opioid receptor (KOR) gene is constitutively expressed in mouse embryonal carcinoma P19 stem cells and suppressed by retinoic acid (RA) in cells undergoing neuronal differentiation. A negative regulatory element is located within intron 1 of the KOR gene, which contains an Ikaros (Ik)-binding site (GG-GAAgGGGAT). This sequence is an Ik-1 responsive, functionally negative element as demonstrated in the context of both natural KOR and heterologous promoters. The two underlined G residues of the second half-site are critical for Ik-1 binding and Ik-mediated repression of the KOR gene. RA induces Ik-1 expression within 1 day of treatment and suppresses KOR expression between 2 and 3 days. Overexpression of Ik-1 in P19 suppresses endogenous KOR gene expression, accompanied by increased binding of Ik-1 to the Ik-binding site and chromatin histone deacetylation on KOR promoters. It is proposed that in an RA-induced P19 differentiation model, RA elevates Ik-1 expression, which recruits histone deacetylase to intron 1 of the KOR gene and silences KOR gene promoters.

Opioid receptors are the molecular targets for analgesic compounds. From studying genetically altered animal models, we have begun to understand the roles of opioid receptors in animals, including a direct role in mediating the pharmacological action and certain side effects of morphine, as well as some behavior changes (1–6). Three opioid receptor genes have been cloned, and their genomic structures have been determined (7–9). The expression of opioid receptor genes in animals has been disclosed for the kappa opioid receptor (KOR) from developing animals, we have tested various hor- mones and opioid compounds in developing animals as well as an embryonal carcinoma cell model, P19, which constitutively expresses the KOR gene (18). We found retinoic acid (RA) to be a potent suppressor of the KOR gene in both animals and P19 cells. This study aims at understanding the mechanism of RA suppression of KOR gene expression. We now report the identification of an intronic silencer element that contains a binding site for the Ikaros 1 (Ik-1) transcription factor. Ik-1 is induced by RA in P19, and its overexpression in P19 results in suppressed KOR mRNA expression, accompanied by chromatin histone deacetylation on the KOR promoters. This study proposes a novel mechanism of targeted suppression of the opioid receptor gene by RA through Ik-1 binding to an intronic silencer, which renders histone deacetylation of the promoters.

EXPERIMENTAL PROCEDURES

Plasmid Constructs and Determination of Reporter Gene Activity in COS-1 and P19 Cells—The homologous reporters were constructed by inserting different KOR genomic DNA fragments (19) into a luciferase cassette lacking the promoter pGL3B (Promega, Madison, WI). K45 contained a KOR genomic fragment of 1320 bp from the BamHI site to the ATG codon. K18 and K19 were truncated from K45, generating reporters starting from the end of exon I (retaining intact intron 1) or deleted in intron 1, respectively. K20 was generated by removing intron I from its original position in K45 and inserting it in front of the BamHI site. K27 was made by deleting 76 bp (SpfI site) from K20. The heterologous reporters were constructed by fusing various KOR genomic fragments to either the TK (K38, K50, K49, and K48) or SV40 (K85) promoter. K38, K50, K49, and K48 each contained a KOR sequence of 324 to 15, 310 bp (271 to 271), 257 bp (271 to 271), and 149 bp (163 to 15), respectively. K55 contained a 128-bp KOR sequence (269 to 142). All of the nucleotide positions were numbered in relationship to the initiating codon, ATG. The expression vector of Ik-1 was a gift from Dr. S. T. Smale (20). Mutated reporters K97 (m1) and K99 (m2) were made by introducing these mutated sequences into the background of K45 by polymerase chain reaction (PCR), and K98 and K100 were made in the background of K85.

COS-1 cells were transfected using the calcium phosphate precipitation method as described (21). P19 cell were transfected using Superfect.

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The abbreviations used are: KOR, kappa opioid receptor; RA, retinoic acid; Ik, Ikaros gene; bp, base pair(s); PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; ChIP, chromatin immunoprecipitation; HDAC, histone deacetylase.
lipofection (Qiagen, Santa Clarita, CA). A specific reporter (0.5 μg) and 0.05 μg of cytomegalovirus-lacZ internal control were used in each transfection. Cells were harvested 48 h after transfection, and specific reporter gene activity, after normalizing to internal control and represented as relative luciferase units, was determined as described previously (21). All of the transfection experiments were carried out at least three times with duplicate or triplicate cultures to obtain the means and the standard errors of the means.

Analysis of Endogenous mRNA in P19 Cells—Two methods were used to examine endogenous Ik-1 expression in P19 cells, including reverse transcription-PCR (RT-PCR) and Northern blot analyses. P19 cells were transfected with either an Ik-1 expression vector (20) or an empty vector, pcDNA3 (Invitrogen, Carlsbad, CA) by Superfect lipofection. Twenty hours after transfection, total RNA was isolated, and KOR expression was detected with an established RT-PCR protocol (22). For RT analysis, P19 cells were treated with 1 μM all-trans-RA for 18, 24, or 48 h or for 3 days. The Ik-1 specific primers are 5′-GGGTTAAGGGGATTT-3′ (exon 4) and 5′-TCCTCTATTAGATG-3′ (exon 6) (20). Amplified KOR (730, 760, and 800 bp for isoforms a, b, and c, respectively) and Ik-1 (304 bp) fragments were detected on Southern blots and quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Actin-specific primers were included for internal control in each RT-PCR. For Northern blot analysis, RNA was isolated from P19 and separated on a denaturing agarose gel followed by blotting onto a nylon membrane and detection with Ik-1 cDNA probes.

Gel Shift Assay—The sequence of the wild type Ik element on intron 1 of the KOR gene is 5′-GGGGTAAAAGGGGATT-3′, and two mutants with mutations (underlined) introduced into either the first or the second repeat are 5′-GGGGTTAAAGGGGATT-3′ (m1) and 5′-GGGGTTAAGGGGATT-3′ (m2), respectively. The annealed oligos were end-labeled with [32P]dCTP by Klenow fragment. Ik-1 protein was synthesized in TNT reactions (Promega) from a T7 expression vector specific to the KOR promoter regions, i.e. 5′-GATGCACAGTAGCTT-3′ and 5′-GCAAGGAAGCAAGTGGTAGC-3′, and two mutants 5′-GATGCACAGTAGCTT-3′ (m1) and 5′-GCAAGGAAGCAAGTGGTAGC-3′ (m2), respectively. The annealed oligos were end-labeled with [32P]dCTP by Klenow fragment. Ik-1 protein was synthesized in TNT reactions (Promega) from a T7 expression vector (pSP73, Promega) that contained Ik-1 cDNA. DNA-protein interactions were detected as described (23). Briefly, 2 μl of TNT product were allowed to react at 4 °C for 15 min followed by the addition of DNA probes for 30 min at 4 °C.
gene suppression, as determined in the context of both homologous and heterologous promoters.

**Ik-1 Suppresses Endogenous KOR Expression in P19 Cells**—The data presented above showed that Ik-1 was a negative factor for KOR expression as determined in reporter assays. To further determine whether Ik-1 could suppress endogenous KOR expression in P19 stem cells, we then introduced Ik-1 expression vectors into P19 stem cells and analyzed the expression of endogenous KOR mRNAs 20 h following transfection, as shown in Fig. 4. The results revealed that all three of the KOR mRNA isoforms were suppressed, although to different extents, confirming that elevated Ik-1 expression down-regulated endogenous KOR gene expression for all three mRNA isoforms.

**RA Induces Ik-1 Expression in P19 Cells**—RA suppressed endogenous KOR gene expression in P19, following treatment for 2–3 days (Fig. 1), suggesting an indirect nature of the suppressive effect of RA. It was then predicted that RA treatment might induce regulatory components, such as Ik-1, responsible for KOR suppression. To examine such a possibility, we then determined whether Ik-1 could be induced by RA treatment in P19 cells using both RT-PCR and Northern blot analyses. Using specific primers in PCR that allowed Ik-1 to be amplified to a 304-bp fragment, it was found that Ik-1 mRNA expression was indeed elevated in RA treated cultures. As shown in Fig. 5, A and B, Ik-1 was induced by RA after 18 h of treatment, and the induction peaked at 24 h and then leveled off. Fig. 5C shows the Northern blot analysis of Ik-1 mRNA expression in untreated P19 cells (ctrl) and RA-induced P19 (RA, 24 h). The bottom panel shows the amount and integrity of these RNA preparations. Ik-1 mRNA was clearly induced by RA as demonstrated in both RT-PCR and Northern blot.

The experiments described above demonstrated that, in P19 cells, either RA or Ik-1 expression could suppress the expression of all three KOR mRNA isoforms. In addition, RA induced Ik-1 expression, preceding the suppression of KOR gene for ∼24 h. Therefore, Ik-1 could be a physiological mediator responsible for the suppression of the KOR gene by RA.

**Ik-1 Binds to the Putative Ik-binding Site on Intron 1 of the KOR Gene**—To examine whether the Ik response element was indeed an Ik-binding element, gel mobility shift experiments were performed as shown in Fig. 6A. The wild type Ik element was bound specifically by Ik-1 protein as evidenced by the competition of cold probes (lanes 2–6). We then mutated either one of the two repeats and tested these mutated sequences for Ik-1 binding in gel shift assays. Interestingly, mutation at the first repeat (m1, lane 7) did not affect Ik-1 binding, whereas mutation at the second repeat (m2, lane 8) abolished the specific binding. Furthermore, when an anti-Ik antibody was added into the reaction mixture, the shifted band diminished, indicating a disruption of Ik-DNA interaction by this antibody. In the presence of preimmune serum, the specifically retarded band remained the same (lane 10), supporting the specificity of the effect of antibody observed on lane 9. The gel shift patterns remained the same when nuclear extracts isolated from P19 transfected with the Ik-1 expression vector were used (data not shown).

To further confirm the biological activity of this Ikaros-binding sequence, the two mutations (m1 and m2) were introduced into KOR reporter K45 and the heterologous reporter K85, and Ikaros-mediated repression of these reporters was examined in COS-1 cells. Mutations on the background of K45 are designated as K97 and K99 for mutants 1 and 2, respectively. Mutations on the background of K85 are designated as K98 and K100 for mutants 1 and 2, respectively. As shown in Fig. 6B, mutant 1 had no effect on Ikaros-mediated repression of these reporters (K97 and K99), whereas mutant 2 abolished Ikaros-mediated repression (K98 and K100). This result confirmed that repression of reporters containing the Ikaros-binding sequence was indeed mediated by Ikaros binding to this sequence. Therefore, it was concluded that the putative Ik-bind-
ing sequence on intron 1 of the KOR gene was a functional Ik-1 binding site, which could mediate the suppression of KOR gene expression.

Ik-1 Expression Renders Histone Deacetylation on KOR Promoters—
Ik-1 has been shown to be associated with histone deacetylase (HDAC) complexes (24), which would silence target genes by deacetylating specific gene promoters. To determine whether Ik-1 binding to the KOR sequence in P19 could be enhanced by RA and whether Ik-1 expression would affect the acetylation status of histone proteins on the KOR promoters, ChIP assays were performed as shown in Fig. 7. The principle was based upon immunoprecipitation of acetylated chromatin, but not deacetylated chromatin, by a specific anti-acetylated histone antibody followed by PCR detection of the immunoprecipitated DNA sequences. If, as predicted, Ik-1 could bring the HDAC complexes to KOR intron I, which was in close proximity to promoters 1 and 2, KOR promoter regions would be deacetylated upon the overexpression of Ik-1. Thus, chromatin in KOR promoters would not be immunoprecipitated with the anti-acetylated histone antibody, and no KOR promoter DNA sequences could be detected by PCR. To examine this scenario, we first tested the histone acetylation status of KOR promoters in the presence or absence of Ik-1 in COS-1 cells, as shown in Fig. 7A. COS-1 cells were transfected with the KOR reporter containing both promoters 1 and 2, K45, in the presence or absence of Ik-1 expression vector. An anti-acetylated histone antibody was used to precipitate chromatin. Immunoprecipitated chromatin DNA was then detected by PCR with primers flanking either KOR promoter 1 (top panel) or promoter 2 (middle panel). It is obvious that only in the absence of Ik-1 were KOR promoter histones acetylated, and thereby immunoprecipitated, and the DNA sequences of these regions detected (lane 2). On the contrary, in the presence of Ik-1, these KOR

Fig. 3. The Ik-binding element of KOR intron 1 is a functional, repressive element. A, biological activities of Ik-binding sequence in the context of natural KOR promoters determined in COS-1 cells. Different KOR-luc reporter activities were determined in COS-1 cells cotransfected with a control (open bars) or an Ik-1 expression vector (filled bars). K45, K19, and K20 were the same as that shown in Fig. 2. K18 was deleted from K45 by truncating the 5’ upstream region that contains promoter 1 and leaving intron 1 intact. K27 was deleted from K20 by truncating the 5’ flanking sequence of the Ik-binding site. RLU, relative luciferase units. B, biological activities of the Ik-binding sequence in the context of heterologous promoters determined in COS-1 cells. The Ik-binding sequence was fused upstream to a reporter driven by SV40 promoter (Control), generating K85. Cells were cotransfected with the specific reporter and a control expression vector (open bars) or the Ik expression vector (filled bars). C, Ik-1 suppresses K85 reporter activities in a dose-dependent manner in COS-1 cells. Different amounts of Ik expression vector and a fixed amount of K85 reporter were cointroduced into COS-1 cells and specific reporter activities were determined as described for panel B.

Fig. 4. Suppression of endogenous KOR mRNA isoforms by Ik-1 expression in P19. Expression of KOR mRNA isoforms a, b, and c in P19 following transfection with a control vector (ctrl) or the Ik expression vector (Ikaros) for 20 h was detected by RT-PCR as described under “Experimental Procedures.”
sequences could not be detected (lane 1), indicating histone deacetylation on these KOR promoter regions presumably brought about by Ik-1 binding. For a control of antibody specificity, a nonspecific antibody (anti-TR2) was used in parallel reactions as shown in lanes 3 and 4. With this nonspecific antibody, no PCR products were detected, confirming the specificity of immunoprecipitation of acetylated chromatin detected in lane 2. Lanes 5 and 6 are positive controls of input, lane 7 shows a negative control reaction of water, and lane 8 shows a positive control using plasmid DNA for PCR. For the control of equal loading of reactions in the presence (lane 1) or absence (lane 2) of Ik-1, a nonspecific promoter, the cytomegalovirus promoter of the expression vector, was also examined, as shown on the bottom panel of this figure. On this nonspecific promoter, chromatin acetylation was the same for reactions in the presence (lane 1) or absence (lane 2) of the Ik-1 expression vector, confirming equal loading of these two reactions. These results support the effect of Ik-1 expression on the acetylation status of KOR promoters, i.e. Ik-1 expression brings about histone deacetylation of KOR promoters.

To provide evidence for changes in Ik-1 binding to the Ik-binding site of KOR in P19 cells following RA induction, a ChIP assay was conducted using an anti-Ik antibody to precipitate chromatin, as shown in Fig. 7B. P19 cells were transfected with K45 and treated with RA or vehicle for 24 h. Cells were lysed for the ChIP assay as described under “Experimental Procedures.” Anti-Ik antibody was used to precipitate DNA, and the precipitate was subjected to PCR with specific primers flanking the Ik-binding site. As shown in lanes 1 and 2, specific fragments were amplified only in the presence of anti-Ik antibody. Moreover, RA enhanced Ik-1 binding (lane 2) to the Ik-binding site as compared with an untreated culture (lane 1). In the absence of anti-Ik antibody, no PCR products were detected (lanes 3 and 4). Lanes 5 and 6 show input control, lane 7 shows water control, and lane 8 shows the plasmid control.

These data provide physiologically relevant evidence for RA induction of Ik-1 which acts as a negative regulator for KOR...
of Ik-1 to its intron 1 sequence and histone deacetylation in the three mRNA isoforms, accompanied by increased binding of P19 suppresses the endogenous KOR gene expression for all tyve DNA element as demonstrated in reporters driven by the gene, which can be bound specifically by Ik-1 protein. This Ik-binding element was identified in the first intron of this gene. Elevation of Ik-1 expression in these cells within 20 h. An Ik-1 expression renders histone deacetylated on KOR promoters, and therefore DNA is precipitated and detected (lane 7) shows the result of a nonspecific promoter. Input DNA (1 μl from a 20× dilution) from each lysate before immunoprecipitation (lanes 5 and 6) and K45 plasmid DNA (lane 8) were used as positive controls in PCR. Negative controls are shown in lanes 3 and 4 for reactions with a nontarget antibody and in lane 7 for water control. Ik-1 expression renders histone deacetylated on KOR promoters, and therefore no KOR-specific PCR products (140 bp for promoter 2 and 240 bp for promoter 1) are detected (lane 2). On the contrary, these promoter regions are acetylated in cultures expressing an empty expression vector, and therefore DNA is precipitated and detected (lane 1, bottom panel). The equal loading of lanes 1 and 2 is demonstrated in parallel reactions examining a nonspecific promoter as shown in the bottom panel. B, ChIP assay demonstrating Ik-1 binding to KOR DNA. Anti-Ik antibody was used in ChIP assay as described above, and the sequence flanking the Ik-1 binding site on the KOR gene was examined. Lanes 1 and 2 show the result of positive reaction using anti-Ik antibody. Lanes 3 and 4 show the negative results using preimmune sera to precipitate, lanes 5 and 6 are control of input, lane 7 shows a negative control of water, and lane 8 shows a positive control using plasmid DNA in PCR.

gene expression. The effect of Ik-1 is mediated by its binding to the Ik-1 element of KOR intron 1, which recruits HDAC and renders histone deacetylation of KOR promoter regions.

DISCUSSION

We present the first evidence for a negative regulation of KOR gene expression by RA and provide a molecular mechanism underlying this suppressive phenomenon. P19 stem cells express KOR constitutively at a basal level, and RA induces an elevation of Ik-1 expression in these cells within 20 h. An Ik-1 binding element is identified in the first intron of this gene, which can be bound specifically by Ik-1 protein. This Ik-binding element is a position-independent, functional, negative DNA element as demonstrated in reporters driven by the KOR promoters or heterologous promoters. Elevation of Ik-1 in P19 suppresses the endogenous KOR gene expression for all three of the mRNA isoforms, accompanied by increased binding of Ik-1 to its intron 1 sequence and histone deacetylation in the KOR promoter regions. This study is the first to demonstrate hormonal regulation of opioid receptor gene expression, which involves chromatin remodeling brought about by a negative transcription factor that is induced by RA.

The suppression of the KOR gene by RA does not occur until 2–3 days of treatment (Fig. 1), suggesting the indirect nature of RA suppression of the KOR gene. This hypothesis is further supported by the fact that within a 4-kilobase pair regulatory region of the mouse KOR gene, no typical RA response element can be found. Interestingly, Ik-1 is transiently induced by RA within 20 h, preceding the suppression of KOR by RA for ~24 h. This would leave enough time for Ik-1 to regulate the KOR gene. Because induction of Ik-1 expression occurs relatively early and levels off as culture is depleted of RA (Fig. 5), it is possible that the Ik-1 gene contains an RA response element. However, this possibility remains to be examined experimentally.

The presence of a negative element in the first intron of KOR gene is interesting. This intron also encodes the second promoter of the KOR gene, and therefore, it may potentially be involved in both the transcriptional control of promoter 2 and alternative splicing of transcripts initiated from promoter 1. The fact that the suppressive activity of this intron, particularly the Ik-binding element, is position-independent and is functional in the context of heterologous promoters would suggest that this intron is a common regulatory element for both promoters 1 and 2 of the KOR gene. Furthermore, because Ik binding renders both promoters 1 and 2 of the KOR gene deacetylated, a most probable hypothesis for HDAC action on this gene is a spreading model. Upon Ik binding, HDAC is recruited and spread along the regulatory region of KOR gene. Both promoters are thus deacetylated and tightly packed. However, the extent of the suppressive effects of Ik-1 on RNA variants transcribed from the two KOR promoters may vary because this sequence can also affect splicing of transcripts derived from promoter 1 (isoforms a and b). This hypothesis is supported by the results of overexpressing Ik-1 in P19, where the three isoforms are all suppressed but to different extents (Fig. 4). According to the DNA sequence, the KOR gene belongs to the housekeeping gene category because no TATA or CAAT boxes can be found; this is in agreement with the fact that KOR is constitutively expressed in P19 stem cells at a basal level. In our transgenic mouse model (17), KOR-lacZ reporter gene is also more widely expressed in early gestation stages before the birth of neurons. However, in older embryos, KOR-lacZ expression becomes restricted to specific areas of the central nervous system, primarily in sensory organ primordia. It is possible that the chromatin structure of the KOR gene is readily open in certain stem cells, such as P19 and those of young embryos, for a basal level of gene activity. When cells undergo differentiation such as being induced by RA, KOR expression is shut down in most cell lineages except specific mature KOR neurons. The silence of the KOR gene may be brought about by specific negative regulatory factors induced by RA, such as Ik-1 protein. However, it remains to be determined what positive factors are required to maintain or induce the expression of KOR gene in mature KOR neurons.

IkaroS transcription factors, also named LyF-1 (25), are found to be relatively restricted to the early stages of lymphoid cells (26). KOR expression is apparent in P19 stem cells, which constitute a pool of precursor cells for a variety of cell lineages. It is widely recognized that P19 is induced by RA to differentiate into various cell types including neurons. However, neurons, especially KOR-positive neurons, do not appear until the later stages of differentiation (18). It is conceivable that KOR-expressing stem cells will gradually change cell fate as various...
transcription factors are induced by RA. Ikaros could be one of these important factors that help to shape early cell differentiation processes and turn off certain genes such as the KOR gene. In fact, KOR expression has been detected in lymphoid cell lineages, such as R1.1, in addition to mature KOR neurons (19). Therefore, the suppression of the KOR gene by a lymphoid transcription factor is physiologically relevant. Important questions to be answered in the future would be why the KOR gene is active in undifferentiated cells and how the KOR gene is reactivated in mature KOR neurons after being suppressed during early stage of differentiation.

Recently, two reports have demonstrated the recruitment of mSin3A-HDAC complexes to a neurally restrictive silencer element that regulates more than 20 neuron-specific genes (27, 28). Our study demonstrates the presence of a novel gene silencer in the KOR gene intron 1, which is a target of a specific transcription factor, Ik-1. The repressive activity of Ikaros has first been suggested to involve its interaction with mSin3, which binds to histone deacetylases (24). Recently, it was shown that Ikaros can also repress gene expression by interacting with a repressor, CtBP, that acts in a HDAC-independent manner (29). Our current studies show increased Ik-1 expression in RA-induced P19, which renders the KOR gene suppressed and histone deacetylated in the KOR promoter regions, presumably brought about by recruiting HDAC complexes. Although our studies support HDAC-dependent action of Ikaros on KOR gene expression, it cannot be ruled out that other mechanisms may exist for Ikaros-mediated KOR gene regulation, such as a potential role of other repressors in KOR regulation. Our study is a first example of negative regulation of an opioid receptor gene by RA, mediated by a transcription regulator that can potentially cause histone deacetylation on the promoters and interact with other gene repressors.

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