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DIFFERENTIAL GENE EXPRESSION ACTIVITY AMONG SPECIES-SPECIFIC POLYPYRIMIDINE/POLYPURINE MOTIFS IN MU OPIOID RECEPTOR GENE PROMOTERS

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Abstract

The mu opioid receptor (MOR) is the principle molecular target of opioid analgesics. An appropriate understanding of MOR gene expression across species is critical for understanding its analgesic functions in humans. Here, we undertake a cross-species analysis of the polymorphic polypyrimidine/polypurine (PPy/u) motif, a key enhancer of MOR gene expression. The mouse PPy/u motif is highly homologous to those of rat (67%) and human (83%), but drives reporter gene expression tenfold and fivefold more effectively than those of rat and human, respectively. Circular dichroism profiles of PPy/u oligonucleotides from different species showed that they are primarily different in structure. Conformational studies of reporter plasmids using confocal Raman spectra, S1 nuclease and restriction enzymes demonstrated that the structural difference is the result of changes in the phosphodiester backbone. Furthermore, these conformational disparities produce differences in torsional stress, as shown by topoisomerase II relaxation and activation of different levels of gene expression under hypertonic conditions. This study demonstrates that homologous PPy/u motifs adopt unique species-specific conformations with different mechanisms and activities for gene expression. We further discuss how structural aspects of transcription regulatory elements, rather than the sequence itself, are significant when studying functional gene expression regulatory elements.

Keywords

Gene expression; Homologous PPy/u Motifs; Mu opioid receptor; Spectroscopy; Topoisomerase

1. Introduction

Opioid receptors mediate the diverse functions of endogenous opioid peptides and the opioid alkaloid derivatives of morphine, including analgesia, reward, autonomic reflexes, and endocrine/immune regulation (Loh and Smith. 1990). Since their initial identification in the

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mammalian brain and in the guinea pig intestine in 1973, the three major types of opioid receptors (μ , κ , and δ) have been found to be widely present in other vertebrate species, including amphibians and fish (Snyder and Pasternak, 2003; Dreborg et al., 2008). Among the receptor types, the μ opioid receptor (MOR) is the major molecular target of the highly addictive and clinically used analgesic drugs such as morphine, heroin, methadone and fentanyl (Matthes et al., 1996).

The levels of MOR expression differ in discrete brain regions (Uhl et al., 1999). Also, individual humans and mouse strains differ in their responses to painful stimuli and to opiate drugs (Mogil, 1999; Korostynski et al., 2006). Although no single study has proposed a mechanism to account for such differences, some have suggested a role for MOR expression (Doyle, et al., 2006). For example, quantitative trait loci mapping identified that C57BL/6J and DBA/2J mice with differences in morphine preference have single nucleotide polymorphisms in the promoter. However, the mechanism by which such a single nucleotide change might influence gene expression and morphine preference remains largely unknown.

To date, MOR gene expression studies have largely been performed on the mouse, assuming one of the basic tenets of comparative genomics: that conservation in the nucleotide sequence of the *cis*-acting regulatory elements of the promoter across closely related species translates to a similar function and molecular mechanism for gene expression (Hardison, 2003). In support of this presumption, DNA–protein binding studies such as gel shift assays showed that many transcription factors generally are not discriminatory with respect to their short binding sequences, often 6–20 nucleotides (Manke et al., 2008). Such results support biochemical studies showing that the binding energies of sequence-specific transcription factors to nearly identical *cis*-acting regulatory DNA sequences do not differ significantly (Stormo and Field, 1998). Therefore, it is assumed that simple primary DNA sequence comparisons provide insight into the molecular mechanism of *cis*-acting elements across species.

In contrast, there is growing evidence to question this assumption. A recent comparative genomics study reported species-specificity in biological phenomena that could be attributed to variations in the homologous sequence in the promoter, rather than differences between the regulators or nuclear environment. With the help of DNA topography, the structural profile of genomes across species showed that phenotypes are associated more with the three-dimensional molecular structure of DNA than with the nucleotide sequence (Wilson et al., 2008). Thus, sequence similarity in *cis*-acting elements does not automatically signify similarity in function and mechanism. In practice, even minor variations in the sequences of homologous *cis*-acting elements could be functionally significant during gene expression.

These studies motivated us to verify our assumption that the mechanism of MOR gene expression in the mouse functions similarly in humans and in the mouse's closely related species, the rat. For biopharmacological purposes, it is important to select a cross-species model for MOR gene expression that is an appropriate reference for the mechanism of the human MOR. We therefore undertook a comparative investigation of the gene expression mechanisms of mouse, rat, and human by focusing on the key transcription regulatory sequence PPy/u in the MOR gene promoter.

The PPy/u motif in mouse (−333 to −308 in the exon 1 proximal promoter) strongly activates the MOR gene promoter and contains multiple binding sites for the transcription factors Sp1 and Sp3, and for single-strand binding proteins (Ko and Loh, 2001). PPy/u motifs are very common in the eukaryotic genome, comprising as much as 0.4–0.5% of the human genome (Behe, 1995; Schroth and Ho, 1995). Under conditions of negative supercoiling, the PPy/u motif has special physicochemical properties that facilitate a number

of polymorphic non-B DNA conformations reactive to S1 nuclease, including the well-studied H-DNA and G-quadruplex structures (Mills et al., 2002). Formation of specific non-B isoforms, or stabilization of these structures once formed, has been implicated in the differential regulation of transcription (Belotserkovskii et al., 2007). Because the mouse PPy/u motif is very reactive to the conformational-sensitive S1 nuclease, it has been presumed to adopt a single-stranded DNA conformation (Ko and Loh, 2001). However, because S1 nuclease also cleaves DNA with unusual torsion angles in the phosphate backbone in most non-B DNA (Mooren et al., 1990), it is possible that mouse PPy/u could adopt other non-B DNA conformations susceptible to cleavage by S1 nuclease.

This study uses structural and gene expression criteria to examine the role of conformation and torsional stress on the transcriptional activation mechanisms of the PPy/u motif across species. Besides establishing organismal differences in the role of PPy/u sequences in MOR gene expression, it provides a theoretical background and an experimental framework to differentiate gene expression mechanisms, especially with regard to homologous regulatory sequences in the promoter that might contain different conformational information at the nucleotide level.

2. Materials and Methods

2.1. Recombinant luciferase reporter gene plasmid constructs

A pLPP-mouse construct of the mouse MOR gene (pL340/300) containing the upstream regulatory sequence −341 to −300 bp (relative to the translation start site at +1) has been described previously (Ko and Loh, 2001). To generate constructs containing rat (Kraus et al., 1995) and human (Wendel and Hoehe, 1998) MOR PPy/u motifs and the human c-Myc NHE III₁ sequence (also known as the CT-element) (Postel et al., 1991), double-stranded oligonucleotides (Fig. 1A) were cloned into the pGL3-basic plasmid digested with SalI and HindIII (Promega). The mutant mouse constructs pLPP-TCSM1 and pLPP-TCSM2 were generated by substituting GGGGG and AAAAA, respectively, for the CTTCT sequence of the PPy/u motif before cloning into the SalI and HindIII sites of the pGL3-basic plasmid. To generate the constructs pLPP-TOM and pLPP-TOR, oligonucleotides containing mouse and rat MOR and topo sequences (GAAAAAAGACTTAGAAAAAATTTT) (Busk, et al., 1987) connected by EcoRI restriction sites were cloned into the SalI and HindIII sites of the pGL3-basic plasmid. The sequences of all constructs were verified using Applied Biosystems 1377 DNA sequencers using RVprimer3 (Promega).

2.2. Preparation of supercoiled plasmid DNA

Plasmids were propagated in XL-1 blue *Escherichia coli* cells (Stratagene) and purified using a Midi kit (Qiagen) according to the manufacturer's instructions, except that the incubation time in alkaline lysis buffer was reduced to several seconds. Following precipitation, DNA was dissolved in 10 mM Tris-Cl, pH 8.5. DNA was checked for conformation by running on agarose gels and for purity by measuring the O.D._{260/280}. In most experiments, the native superhelical density of the DNA was distributed around −0.05.

2.3. Circular dichroism (CD) scanning

Oligonucleotides were purified by HPLC or polyacrylamide gel electrophoresis and dissolved in CD buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 50 mM NaCl) at a final concentration of 200 μM. Equal amounts of complementary oligos were annealed at a final single-stranded oligo concentration of 50 μM. The annealing mixture was heated for 5 min at 95°C, and then cooled to 25°C at 0.016°C/s in a PCR machine. The annealed oligos were diluted with the CD buffer or absolute ethanol to specific concentrations just before measurement. CD spectra were recorded using a Jasco J-815 spectrometer equipped with a

Peltier temperature control system (Jasco; Tokyo, Japan). The final concentration of the annealed oligonucleotides in the cuvette was 6.25 μ M. The cuvette-holding chamber was flushed with a constant flow of dry N₂ gas to avoid condensation on the cuvette surface. Spectral data were obtained from 200 to 330 nm with a 1-s response time and a 1- or 1.75-nm bandwidth using a 0.1-cm path-length quartz cuvette at 30°C. Each spectrum is the average of three individual scans and is corrected for the spectrum of the buffer. A 42-mer oligonucleotide derived from the pGL3-basic plasmid sequence (Promega) was used as a B-form DNA control.

2.4. Cell culture, transfection, and reporter gene assay

NS20Y cells (the C1300 murine neuroblastoma clone of the A/J mouse strain) were grown in Dulbecco's modified Eagle medium with 5% inactivated fetal bovine serum and 1% L-glutamine in an atmosphere of 5% CO₂ and 95% air at 37°C. Mouse embryonal sarcoma P19 cells derived from a C3H/He strain mouse (ATCC) were cultured in α -minimal essential medium supplemented with 7.5% newborn calf serum and 2.5% fetal calf serum. Cells were plated at a density of 1×10^5 cells/well in 6-well plates. Twenty-four hours later, cells were transfected with construct plasmids using Effectene (Qiagen) according to the manufacturer's instructions. For reporter gene assays in hypertonic solutions, cells were maintained for 3 hours in isotonic growth medium following transfection, washed twice with the same medium, and then cultured for 24 h with hypertonic growth medium (i.e., isotonic growth medium supplemented with 70 mM sucrose or 30 mM NaCl). To correct for differences in transfection efficiency, a one-fifth molar ratio of the pCH 110 plasmid (Amersham Biosciences) containing a β -galactosidase gene under the SV40 promoter was included in each transfection and used for normalization. Luciferase and β -galactosidase activities were determined as described by the manufacturers (Promega and Tropix, respectively). All transfection experiments were repeated four or more times with similar results, using plasmids prepared independently at least twice. Each normalized value represents the average of at least three independent determinations.

2.5. Nuclease S1 and restriction enzyme assays

Supercoiled plasmid constructs were digested with S1 nuclease (Promega) in a volume of 18 μ L S1 buffer for 15 min at 37°C. The S1 nuclease reaction was quenched with stop buffer (500 mM Tris, 125 mM EDTA), followed by heating for 10 min at 70°C. The resulting S1-digested plasmids were recovered by precipitation and digested further with XbaI. The products were resolved by agarose gel electrophoresis. For restriction enzyme assays, supercoiled plasmid DNA was digested with XbaI or HpaI for 45 min at 37°C. The cleavage efficiency was consistent between independent preparations of the supercoiled plasmids.

2.6. DNA relaxation assay

One μ g of supercoiled plasmid constructs were digested with 1.5–6 units of human topoisomerase II α (TopoGEN, Inc.) in a volume of 20 μ L topoisomerase II buffer (50 mM Tris-Cl [pH 8.0], 150 mM NaCl, 10 mM MgCl₂, 5 mM ATP, 0.5 mM dithiothreitol, 30 μ g/ml BSA) for 0–30 min at 37°C. The reactions were terminated by adding gel loading buffer containing 2% sodium dodecyl sulfate and the resulting relaxed products were analyzed by electrophoresis through horizontal 1% agarose gels at 1 V/cm for 20 h in TAE buffer. Gels were stained with ethidium bromide and photographed. The relaxation efficiency was consistent between independent preparations of the supercoiled plasmids.

2.7. Raman spectroscopy

Raman measurements of all DNA constructs were conducted using a Witec alpha 300 R confocal Raman microscope equipped with a UHT200 spectrometer and a DV401 CCD

detector (Ulm, Germany). Supercoiled DNA was dissolved in phosphate-buffered saline to a final concentration of 20 $\mu\text{g/L}$. An aliquot (4.5 μL) of the supercoiled DNA solution was placed on a 100-nm-thick Au-coated glass substrate and aligned under a 10X objective (NA = 0.3). Wet paper towels were placed around the objective to prevent evaporation of the DNA solutions. An argon ion laser (Melles Griot, CA) with 514.5-nm excitation and 50-mW maximum output power was used for excitation. The radiant power of the laser on the sample was adjusted to be $< \sim 5$ mW. Under these conditions, no changes were observed in the Raman spectra with prolonged laser illumination (> 30 min) of the DNA samples. All spectra were recorded in the 600–1600 cm^{-1} range, with a spectral resolution of ~ 1 cm^{-1} . Spectra shown are accumulated averages of forty exposures of 10 s each.

3. RESULTS

3.1. Species-specific polymorphisms in the secondary structure of homologous MOR PPy/u motifs

Cross-species analyses of MOR PPy/u motifs showed that the mouse PPy/u motif is highly homologous to those of the rat and human (67% and 83%, respectively; Fig. 1A). To correlate sequence similarity with structural similarity, we performed comparative CD analyses of the MOR PPy/u homologs. CD, which measures the absorption of circularly polarized light by DNA, can distinguish different DNA conformers because they absorb left-handed and right-handed polarized light differently (Jaganathan and Ivanisevic, 2008). Control oligomers derived from the pGL3-basic vector showed a conformation characteristic of the conservative profile of canonical B-form CD spectra with an ellipticity maximum at 273 nm and an ellipticity minimum at 250 nm. In CD spectra of the MOR PPy/u motifs of rat, human and mouse, the ellipticity minima shifted significantly to 240 nm. However, in contrast to the identical ellipticity minima, the ellipticity maxima differed between rat/human and mouse, at 265 and 275 nm, respectively (Fig. 1B; Table 1).

MOR PPy/u spectra were also compared with that of c-Myc NHE III₁, which has a PPy/u sequence known to adopt either triplex (H-DNA) or G-quadruplex conformations (Postel et al., 1991). The secondary structures of the rat and human PPy/u sequences were closer to that of human c-Myc NHE III₁ than to that of the mouse PPy/u motif, suggesting that these sequences might adopt similar secondary structures (Fig. 1B; Table 1). Given that the positive and negative peaks of DNA CD spectra are attributable to base-pair geometry and helicity, respectively (Jaganathan and Ivanisevic, 2008), these results suggest that the mouse PPy/u motif could form H-DNA or G-quadruplex-like structures with a base-pair geometry different from that of rat or human.

A previous study showed that the PPy/u motif activates the proximal promoter of the mouse MOR in a structure-sensitive manner and that alteration of the non-B DNA structure greatly reduced expression of the luciferase reporter gene (Ko and Loh, 2001). To examine if differences in CD spectra correlated with differences in transcriptional activity, we used a reporter gene assay in conjunction with PPy/u inserts in supercoiled DNA. Rat and human recombinant constructs expressed significantly less reporter gene (tenfold and fivefold lower, respectively) relative to the pLPP-mouse construct when expressed in mouse NS20Y cells; similar differences in were observed in mouse P19 cells (Fig. 1C).

The lower expression seen in cells transfected with the rat/human constructs coincided with a more pronounced shift in the positive peak of the CD spectra to a shorter wavelength (265 nm, i.e., similar to that of c-Myc NHE III₁), whereas the smaller shift produced by the mouse construct (to 275 nm) correlated with higher levels of expression. Furthermore, although the CD spectra of the human and rat PPy/u motif constructs showed similar wavelengths, they differed with respect to amplitude, and this difference coincided with a

threefold difference in gene expression activity. Taken together, these results suggest that changes in the PPy/u motif reflected in their CD spectra correlate with their transcriptional activity.

3.2. The sequence-specific structure of homologous PPy/u motifs is a primary determinant of gene expression

To confirm the relationship between structure and gene expression, we examined the effect of the mouse PPy/u sequence on non-B DNA structure. We first focused on the pentanucleotide CTTCT, because the repeat of this sequence has been reported to form H-DNA-like DNA structures (Belland, 1991). Substituting these bases in the MOR PPy/u motif with GGGGG (TCSM1) or AAAAA (TCSM2) specifically shifted the negative and positive elliptical peaks of the CD spectra, respectively (Fig. 2B; Table 1). This suggests that the CTTCT sequence is critical for the mouse PPy/u motif's non-B DNA structure, and that its secondary structure is sequence-specific. Transient transfection of NS20Y or P19 cells with the mutated mouse PPy/u reporter construct pLPP-TCSM1 (which shows altered helicity in the PPy/u oligonucleotide) nevertheless expressed the reporter gene at levels similar to those seen with wild-type PPy/u. In contrast, reporter gene expression was nearly tenfold lower in cells transfected with the pLPPT-CSM2 mutant (which has a different base-pair geometry in the PPy/u oligonucleotide), relative to cells transfected with the wild-type mouse motif (Fig. 2C).

To eliminate the possibility that the above results were caused by alternative transcription factors binding to the motif, we examined the effect of the topoisomerase I sensitive-topo sequence on MOR PPy/u motifs (Busk, et al., 1987). Attaching the topo sequence to the mouse and rat MOR PPy/u motifs (TOM and TOR, respectively) shifted the CD spectra of both to more closely resemble that of the topo oligonucleotide (Fig. 2D; Table 1). If reporter gene expression is regulated primarily by transcription factors, the topo sequence should have similar effects on luciferase activity in the mouse and rat. However, the topo sequence increased expression by the mouse MOR PPy/u motif, but decreased that by the rat motif (Fig. 2E). This opposite effect strongly suggests that the topo sequence drives different structural changes in the mouse and rat motifs. Taken together, these results suggest that the difference in gene activation activity between rat/human and mouse is caused by conformational differences related to base geometries.

3.3. MOR PPy/u motif polymorphism differentially influences phosphodiester backbone conformation under supercoiling

A previous study of the mouse PPy/u motif showed that adopting a conformation reactive to S1 nuclease is critical for transcriptional activation (Ko and Loh, 2001). Rat/human and mouse PPy/u inserts with different base-pair geometries might react differently to S1 nuclease. Supercoiled plasmids with recombinant PPy/u inserts were cleaved sequentially with S1 nuclease and XbaI, followed by agarose gel electrophoresis. The same fragments (1.7 and 3.1 kb) appeared in all plasmids tested (Fig. 3B), suggesting that differences in base-pair geometry do not correlate with S1 sensitivity. It is tempting to conclude that all the PPy/u inserts adopt the same S1 nuclease-sensitive non-B conformation in supercoiled DNA. However, S1 nuclease also cleaves DNA with unusual torsion angles in the phosphate backbones in most non-B DNA (Mooren et al., 1990). It is therefore possible that rat/human PPy/u motifs could adopt a different conformation from that of mouse and consequently differentially affect reporter gene expression.

Interestingly, the pGL3-basic control vector produced apparent multiple cleavage fragments in the agarose gel, indicative of nicking by S1 nuclease (Fig. 3B). In contrast, supercoiled plasmids with the PPy/u inserts did not show these fragments. It is possible that the inserts

significantly changed the secondary conformation of the existing S1-sensitive sites. Consequently, these sites might no longer be susceptible to S1 nuclease hydrolysis. We therefore examined the tertiary structure of the supercoiled DNA conferred by the PPy/u inserts using restriction enzymes. As expected, the recombinant plasmids showed different reactivities to XbaI: The supercoiled pLPP-mouse plasmid was significantly more resistant to XbaI digestion than the other constructs (Fig. 3C).

We expanded the restriction enzyme assay to test other sequences in supercoiled plasmids. Of particular interest was the HpaI restriction site within the poly(A) signal sequence, because the mouse MOR promoter is functionally contingent on the poly(A) signal (Wu et al., 2005). The pLPPy/u-TCSM1 was significantly more resistant to HpaI cleavage than either the wild-type pLPP-mouse or pLPP-TCSM2 mutant constructs (Fig. 3E). In agreement with these results, the pLPPy/u-TCSM1 also was significantly resistant to S1 nuclease, as shown by the decreased intensity of the 3.1- and 1.7-Kb fragments and the slow conversion of the open circular nicked band (Fig. 3D). This might account for the higher level of luciferase activity seen in pLPPy/u-TCSM1 relative to pLPPy/u-TCSM2 (Fig. 2C). Taken together, these results suggest that MOR pPy/u motifs globally influence the conformation of the phosphodiester backbone of supercoiled DNA. Indeed, the conformation of the phosphodiester backbone has been shown to affect recognition and catalysis by restriction enzymes (Thorogood et al., 1996). Furthermore, the rat/human PPy/u motif in supercoiled DNA appears to be different from that of the mouse with respect to the mode of changing the tertiary structure of supercoiled DNA.

3.4. Species differences in the Raman structural signature of phosphodiester backbone substates in supercoiled DNA containing the MOR PPy/u motif

Based on the above studies, we examined the conformation of the phosphodiester backbone at the nucleotide level. The phosphodiester backbone can adopt two predominant configurations: B_I (gauche⁻/gauche⁻ range) and B_{II} (gauche⁻/trans range). These conformations influence the position of the phosphate groups with respect to the grooves of the double helix and contribute to sequence-specific protein binding (Isaacs and Spielmann, 2001). We used Raman spectroscopy to obtain information about both the local and global structure of large DNA molecules in physiological solutions (Serban et al., 2002). The global conformations of all the supercoiled DNA with MOR PPy/u motifs were similar to each other, as reflected by the Raman markers for canonical B-form DNA at approximately 682, 732, 790/831, and 1095 cm⁻¹ (Fig. 4). However, the spectral bands at 926 and 1422 cm⁻¹ were particularly interesting. These bands likely result from small net changes in the average phosphodiester torsions α/ζ with supercoiling from B_I to B_{II} (Serban et al., 2002). The difference in intensity between pLPP-human and -mouse (Fig. 4, b-a) was significantly higher than that seen between pLPP-human and -rat (Fig. 4, a-c), suggesting that pLPP-human constructs have a distribution of B_I and B_{II} more similar to that of pLPP-rat than to pLPP-mouse. The Raman profiles of supercoiled plasmids present direct evidence that homologous MOR PPy/u motifs confer different conformations over a long tract in supercoiled DNA.

3.5. Differences in torsional stress levels in polymorphic MOR PPy/u motifs

To understand how species-specific PPy/u motifs inducing different phosphodiester backbone conformations can affect gene expression, we tested the torsional stress of supercoiled plasmids using a topoisomerase II assay. The supercoiled plasmids were progressively more relaxed by topoisomerase II with increasing amounts of the enzyme (Fig. 5A) and time of reaction (Fig. 5B). However, pLPP-rat/human relaxed more quickly than did pLPP-mouse. Because topoisomerase relaxation activity is influenced by torsional stress (Liu and Wang, 1987), these results support the notion that human/rat and mouse MOR PPy/

u motifs confer different superhelicity-dependent conformations to the supercoiled plasmids. This strongly suggests that species differences in the MOR PPy/u element confer different superhelicity-dependent conformations that could contribute to species-specific patterns of gene activation.

3.6. Conformation changes in the MOR PPy/u motif induced by dehydration

To further understand how different MOR PPy/u motifs differentially mediate gene expression, the motifs were tested for the ability to being hydrated. Changes in CD spectra reflect conformational changes caused by dehydration. MOR oligonucleotides in 40% ethanol showed no significant change in their ellipticity intensity or spectra (Fig. 6, upper). However, 80% ethanol significantly changed both the ellipticity intensity and spectra of all motifs tested, with the mouse showing the greatest change, the rat the least, and human in-between, suggesting that mouse is the most flexible, and the rat the least flexible, with respect to hydration/dehydration-induced conformational changes (Fig. 6, lower). This also suggests that rat and human MOR PPy/u motifs respond to physiological stimuli different than mouse MOR PPy/u motif. Interestingly, the CD spectrum of the rat motif in ethanol is similar to that of c-Myc NHE III₁, suggesting that the rat PPy/u element might act through the same molecular mechanism by which c-Myc NHE III₁ controls c-Myc gene expression.

3.7. The effect of dehydration on gene expression induced by the MOR PPy/u motif

The above results suggest that the MOR PPy/u motifs might mediate physiological signaling by inducing conformational changes. To test this, we examined reporter gene expression in NS20Y and P19 cells transfected with different MOR PPy/u motifs cultured in hypertonic sucrose or hypertonic NaCl media. Hypertonic sucrose generally increased reporter gene expression, but hypertonic NaCl had no apparent effect. In all cases, the results were more pronounced in the P19 cells (Fig. 7). Notably, in NS20Y cells incubated in hypertonic sucrose, pLPP-rat and pLPP-human luciferase activity was 80% and 40% higher than that of pLPP-mouse, respectively (Fig. 7). In contrast, this difference between rat/human and mouse was not observed in P19 cells. These results again suggest that rat and human MOR PPy/u motifs respond to physiological stimuli differently than does the mouse MOR PPy/u motif.

4. DISCUSSION

The molecular mechanisms of MOR gene expression have largely been studied in mouse, assuming one of the basic tenets of comparative genomics: that homologous sequences conserved across closely related species have similar functions and mechanisms during gene expression (Hardison, 2003). This presumption is also the basis for using animal models such as mice and rats to study human biology and disease, including MOR pharmacology. Surprisingly, this study reveals that this might not be accurate with respect to MOR gene expression: The molecular mechanism for gene expression activated by the MOR PPy/u motif of human is more similar to that of the rat than the mouse.

CD profiles showed that the underlying molecular basis for the differences in gene expression between rat/human and mouse MOR PPy/u motifs lie in their different nucleic acid geometries. Further analyses of mouse and rat MOR PPy/u composite plasmids showed that adding a flanking structure-sensitive topo sequence induced species-specific conformational changes as well as changes in reporter gene expression. The adoption of different conformations is surprising, because the sequences are highly homologous. However, these results strongly suggest that the structure and function of DNA structural motifs such as PPy/u do not tolerate subtle variations in the nucleotide sequence. For example, substitution of the CTTCT nucleotides in the mouse PPy/u sequence both

produced S1 nuclease resistance (pLPP-TCSM1) and decreased reporter gene activity (pLPP-TCSM2).

Superhelicity is important for transcription (Liu and Wang, 1987). Negative supercoiling affects transcription by promoting the formation of nucleoprotein structures and by altering the twist of DNA. In this study, polymorphic MOR PPy/u motifs were relaxed by topoisomerase II at different rates, with supercoiled plasmids containing mouse PPy/u relaxing more slowly than those with either rat or human motifs. This suggests that the mouse motif impedes relaxation by topoisomerase II, resulting in elevated gene expression. Several other studies have shown that non-B DNA structures, such as triplex or G-quadruplex, act as a buffer for superhelicity or transmit superhelicity to neighboring DNA sequences (Sun and Hurley, 2009). In this regard, the c-Myc promoter is particularly well-studied (Brooks and Hurley, 2009). The c-Myc promoter has two critical transcription elements, the far upstream element (FUSE), and NHE III₁. NHE III₁ displays a different secondary structure in single-stranded DNA templates than when it is incorporated into a supercoiled plasmid. Both elements are controlled by transcriptionally generated negative supercoiling *in vivo* and *in vitro*. The supercoiling *in vivo* is not relieved by topoisomerase I or II and directs the melting of the FUSE, which in turn binds to transcription factors.

Raman spectroscopy studies showed that the different MOR PPy/u motifs in supercoiled plasmids differentially influence the distribution of the two predominant phosphodiester conformations, B_I and B_{II}. The pattern of the B_I/B_{II} phosphodiester substates plays a role in groove opening/closing and in turn can modulate protein–DNA interactions (Isaacs and Spielmann, 2001). We hypothesize that species-specific MOR PPy/u motifs differentially influence the conformation of the phosphodiester backbone of supercoiled DNA and that the resulting difference in the B_I/B_{II} substates in plasmid DNA exert different effects on the superhelicity and the efficiency of transcription.

The biological significance of the different effects that rat/human and mouse PPy/u motifs have on the sensitivity of superhelicity with respect to gene expression *in vivo* remains to be determined. It should be noted that recent comparison studies of the genome showed changes not only at the level of nucleotide sequence but also structure (Brooks and Hurley, 2009; The Rat Genome Sequencing Project Consortium, 2004). According to those studies, evolutionary change has occurred roughly three times faster in rodents than in primates since the two lineages diverged approximately 80 million years ago (Parker et al., 2009). These studies also showed that the molecular shape of DNA is under selection during evolution, suggesting that DNA conformation plays a significant role in cross-species differences (The Rat Genome Sequencing Project Consortium, 2004; Yang et al., 2004). Most notably, the three-dimensional molecular structure of functional noncoding DNA differs in transcription factor binding affinity and phenotype across species. Consistent with this observation, in contrast to the ~20% difference between rodent and human sequences, the MOR PPy/u motifs of human, chimpanzee, orangutan and monkey differed in only one nucleotide: position –309 in the human sequence (i.e., the underlined C in Fig. 1A). However, PPy/u motifs altered at this nucleotide alone showed no variation in their CD spectra relative to the human PPy/u motif (data not shown). We therefore propose that the rat MOR promoter (especially its PPy/u motif) underwent rapid evolutionary change relative to the mouse's not only at the level of its nucleotide sequence but also at the level of DNA conformation, and that the primate sequence maintained this structural constraint during subsequent evolution. Future studies on the effects of sequence variations in the motif on transcriptional activity could well contribute to our understanding of how MOR gene expression is selectively regulated during evolution.

Gene expression in eukaryotes is the result of a coordinated multi-step process involving distinct chromatin modification and remodeling complexes that control the proper recruitment of transcription factors and assembly of the preinitiation complex (Janga et al., 2008). This study shows that the mechanism of MOR gene activation by the PPy/u motif in humans is more similar to that of the rat than the mouse. Although this finding can by no means be extended generally, this study presents a few points of significance. First, raman spectroscopy can be used to reveal the structural contexts of gene expression mechanisms and DNA geometries that might have a global or local impact on the interactions between transcription factors and structural regulatory sequences. Second, and more broadly, investigators should be aware that considering sequence information alone can overlook mechanisms that can manifest as the result of sequence-induced variations in the structure of canonical and noncanonical B-DNA. Indeed, variations in local and global DNA helix structure can significantly influence the indirect readout in sequence-specific protein–DNA recognition by governing the spatial organization of bond-forming atoms and the ability of DNA to change conformation on protein binding (Heddi et al., 2008).

Abbreviations

MOR	mu opioid receptor
CD	circular dichroism
PPy/u	polypyrimidine/polypurine
NHE III₁	c-Myc nuclease hypersensitive element III ₁

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Fig. 1. Differences in the secondary structure of vertebrate MOR PPY/u homologs and gene activation. (A) The mouse MOR PPY/u sequence (−341 to −300) and its corresponding 5′-flanking sequences compared with those of the rat and human MOR genes. Differences from the mouse sequence are shown in red. The C nucleotide in the human sequence (underlined) differs among primate species (human, chimpanzee, orangutan and monkey). The human c-Myc nuclease hypersensitive element (NHE) III₁ sequence is shown as a control for non-B form DNA. (B) CD spectra of PPY/u motif oligonucleotides. Oligomers were analyzed after duplex formation. The control sequence (used as a reference for B-form DNA) is a DNA fragment of the same length derived from the pGL3-basic vector. The spectral bandwidth is 1 nm. (C) Recombinant luciferase reporter constructs containing a PPY/u insert (pLPP-) derived from the MOR of the indicated species or the PPY/u from the c-Myc NHE III₁ were transfected into neuronal NS20Y or P19 cells. pGL3-basic, which contains no enhancer/promoter, was used as negative control. Luciferase activities are expressed as *n*-fold relative to the activity of pGL3-basic, which was assigned a value of 1.0. The data are means ± S.D. of three independent experiments, with at least two different plasmid preparations.

Fig. 2.

Expression from mutant and composite MOR PPY/u constructs. (A) Nucleotide sequences of mutated mouse PPY/u motifs (TCSM1 and TCSM2) and topo sequences. (B, C) CD spectra and reporter gene expression of TCSM1 and TCSM2. Luciferase activities are expressed as *n*-fold relative to the activity of pLPP-mouse, which was assigned a value of 1.0. (D, E) CD spectra and reporter gene activity of the mouse PPY/u composite oligonucleotides pLPP-TOM and pLPP-TOR in NS20Y cells. Luciferase activities are expressed as *n*-fold relative to the activity of pLPP-mouse and pLPP-rat, which were assigned activities of 1.0, respectively. Transfection, CD spectral and data analyses were performed as described in Fig. 1.

Fig. 3. Enzymatic analyses of conformational differences. (A) Schematic representing the region accessible to S1 nuclease (PPy/u motif) and the restriction enzyme cleavage sites. Fragments of 1.7- and 3.1-kb can be derived from the plasmid constructs by sequential digestion with S1 and XbaI. The PPy/u and poly(A) signals are indicated by gray boxes. (B, D) S1 nuclease reactivity of supercoiled DNAs of wild-type (B) and mouse mutant (D) PPy/u motif inserts. Supercoiled plasmids were treated with increasing amounts S1 nuclease (0–3 units), followed by digestion with XbaI. The 1.7- and 3.1-kb bands result from sequential cleavage by S1 nuclease and XbaI. (C, E) Comparison of the phosphodiester backbone conformations of supercoiled plasmids. Supercoiled plasmids with wild-type (C) or mouse mutant (E) PPy/

u motif inserts were digested with XbaI (C) or HpaI (E). OC: open circular DNA; L: linearized DNA; SCC: supercoiled circular DNA; M: DNA marker.

Fig. 4.

Raman structural signatures in supercoiled DNAs. Raman spectra ($600\text{--}1600\text{ cm}^{-1}$, 514.5-nm excitation) of solutions of supercoiled plasmids with the PPy/u homologs. Raman bands of pLPP-mouse (a), pLPP-human (b) and pLPP-rat (c). Spectra a and c were normalized to the pLPP-mouse integrated intensity at 1092 cm^{-1} , which is assigned to the PO_2^- symmetric stretching mode of the polydeoxynucleotide phosphate groups. Frequencies (in wave numbers/cm) of key representative Raman markers of DNA are labeled in upper panel. Intensity is expressed in arbitrary units. Each spectrum represents the average of 40 exposures of 10 s duration. Three independent experiments showed essentially identical results; representative results are shown. Computed difference spectra are in the lower panel. b-a: pLPP-human vs. pLPP-mouse; b-c: pLPP-human vs. pLPP-rat; a-c: pLPP-mouse vs. pLPP-rat. Differences in intensity reflect differences in the population of B_I and B_{II} substates.

Fig. 5. DNA relaxation assay. The ease with which supercoiled DNA with inserted PPy/u motifs is relaxed by topoisomerase II was monitored by the disappearance of supercoiled bands and the generation of relaxed (i.e., open circular) bands. (A) Dose-response curve using 1.5–4.5 units of topoisomerase II for 30 min. (B) Time-course (0–30 min) using 6 units of topoisomerase II. Three independent experiments for each assay exhibited essentially identical results; representative results are shown. OC/R: open circular DNA; SCC: supercoiled circular DNA. Lane 1: DNA marker.

Fig. 6.

Conformation of MOR PPy/u motifs in ethanol solutions. CD spectra in ethanol solutions were recorded immediately after mixing absolute ethanol into the annealed oligonucleotides to the indicated final concentrations. Control spectra were obtained in CD buffer. The graph shows the change in positive ellipticity between the control and 80% ethanol in the 250–300-nm spectral range.

Fig. 7. Differential activity of MOR PPy/u reporter genes in response to hypertonicity. Reporter genes (described in Fig. 2) were transfected to (A) NS20Y and (B) P19 cells in isotonic growth medium. After 3 hours of incubation, cells were incubated with isotonic growth medium or hypertonic medium (isotonic growth medium supplemented with 70 mM sucrose [hypertonic sucrose] or 30 mM NaCl [hypertonic NaCl]). Luciferase activities are expressed as *n*-fold relative to the activity of isotonic growth medium, which was assigned a value of 1.0. The data shown are means $\pm$ S.D. of three independent experiments, with at least two different plasmid preparations.

Table 1

Comparison of CD spectral features.

Sample	$\lambda_{\min}^*$	$\lambda_{\max}^*$	$\lambda_{\text{crossover}}^*$
	nm	nm	nm
pGL3-basic	250	275	260
Mouse PPy/u	240	275	250
Rat PPy/u	240	265	250
Human PPy/u	240	265	250
c-Myc NHE III ₁	240	265	248
TCSM1	245	275	255
TCSM2	240	270	252
TOM	249	256	278
TOR	247	262	281

* $\lambda_{\min}$ and $\lambda_{\max}$ represent the wavelengths at which the rotational strength of the spectra reach their greatest negative and positive values, respectively. $\lambda_{\text{crossover}}$ is the inflection point between the negative and positive peaks at which ellipticity is 0.