Published in final edited form as:

Gene. 2011 November 1; 487(1): 52–61. doi:10.1016/j.gene.2011.07.026.

THE POLYPYRIMIDINE/POLYPURINE MOTIF IN THE MOUSE MU OPIOID RECEPTOR GENE PROMOTER IS A SUPERCOILING-REGULATORY ELEMENT

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Abstract

The mu opioid receptor (MOR) is the principle molecular target of opioid analgesics. The polypyrimidine/polypurine (PPy/u) motif enhances the activity of the MOR gene promoter by adopting a non-B DNA conformation. Here, we report that the PPy/u motif regulates the processivity of torsional stress, which is important for endogenous MOR gene expression. Analysis by topoisomerase assays, S1 nuclease digests, and atomic force microscopy showed that, unlike homologous PPy/u motifs, the position- and orientation-induced structural strains to the mouse PPy/u element affect its ability to perturb the relaxation activity of topoisomerase, resulting in polypurine strand-nicked and catenated DNA conformations. Raman spectrum microscopy confirmed that mouse PPy/u containing-plasmid DNA molecules under the different structural strains have a different configuration of ring bases as well as altered Hoogsteen hydrogen bonds. The mouse MOR PPy/u motif drives reporter gene expression fortyfold more effectively in the sense orientation than in the antisense orientation. Furthermore, mouse neuronal cells activate MOR gene expression in response to the perturbations of topology by topoisomerase inhibitors, whereas human cells do not. These results suggest that, interestingly among homologous PPy/u motifs, the mouse MOR PPy/u motif dynamically responds to torsional stress and consequently regulates MOR gene expression *in vivo*.

Keywords

Gene expression; Homologous PPy/u motifs; Mu opioid receptor; Supercoiling-regulatory element; Topoisomerase Running head; Structural/functional characteristics of MOR PPy/u motifs

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1. INTRODUCTION

Mu opioid receptor (MOR) mediates 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). In addition, recent studies which challenge the classical model of MOR function reveal a novel role for MOR: regulating the death of normal and neoplastic cells in response to apoptosis-inducing agents (Polakiewicz et al., 1998; Suzuki et al., 2003). MOR agonist morphine is the analgesic of choice for moderate and severe cancer pain (Mercadante et al., 2005).

Polypyrimidine/polypurine (PPy/u) motifs occur frequently in the eukaryotic genome (comprising as much as 0.4–0.5% of the human genome; Behe, 1995; Hoyne et al., 2000; Schroth and Ho, 1995) and are clustered preferentially in genes that are highly expressed in brain and pseudoautosomal regions (Bacolla et al., 2006). Analysis of the positional preferences of the asymmetric purine-rich and pyrimidine-rich sequences of PPy/u motifs on the two DNA strands shows that the purine-rich sequence is more prevalent in the sense strand than in the antisense strand, suggesting evolutionary selection for the conservation of strandedness (Van Dyke, 2005). The PPy/u motif in the mouse MOR gene is located at positions –333 to –308 of the exon 1 proximal promoter and strongly activates the MOR gene (Ko and Loh, 2001). Homologous PPy/u motifs are present in the human and rat MOR gene promoters, but analysis by circular dichroism (CD) suggests that the homologous MOR PPy/u oligonucleotides adopt different DNA helical conformations (Choe et al., 2011). Indeed, such sequence-dependent polymorphism of DNA conformation is an important determinant of species-specific DNA transactions, including transcription (Vuillaumier et al., 1997; Zhao et al., 2010).

PPy/u motifs can form unusual non-B structures (e.g., triplexes, sticky DNA, single-stranded DNA, and composite quadruplex/i-motif assemblies) (Bacolla and Wells, 2004). The transition from B to non-B DNA secondary structures occurs by localized melting or unwinding of duplex DNA and is facilitated by negative supercoiling stress generated naturally during DNA transactions. Two different mechanisms have been proposed to account for the role of a non-B conformation of the PPy/u motif for transcription: First, the secondary structure of the PPy/u motif under supercoiling stress acts as an efficient transcription factor binding site for transcription factors such as single-stranded DNA-binding protein (SSB) (Rothman-Denes et al., 1998). Second, according to a DNA structural transmission model, the formation of the non-B conformation within the PPy/u motif disturbs the dissipation of the torsional stress along the surrounding DNA by buffering wave-like torsional stress or by providing a preferential cleavage site for topoisomerases (Antony et al., 2004; Sheridan et al., 1999). Consequently, undertwisted or overtwisted DNA downstream and upstream affects the topology of chromatin fiber and the indirect readout of the DNA sequences in sequence-dependent and-independent transcription-factor-DNA interaction, which relies on shape and deformability of DNA.

Although the effect of supercoiling on PPy/u conformation has been well understood in plasmid and oligonucleotide DNA, there is no direct evidence showing that supercoiling controls endogenous MOR gene expression through inducing non-B DNA conformation in a native chromosome environment. A previous study revealed that supercoiled plasmids with homologous MOR PPy/u motifs are differentially relaxed by topoisomerase II, suggesting that they differentially process the supercoiling and torsional stress (Choe et al., 2011). However, the mechanisms responsible for the differential effects remain to be explored. Here, we demonstrate that the mouse MOR PPy/u motif functions as a supercoiling regulatory *cis*-acting element for topoisomerases, which contributes to the expression of the MOR gene in response to DNA transactivation accompanying changes in torsional stress.

This is the first study demonstrating how PPy/u motifs mediate the dynamic processivity of supercoiling through adopting a non-B conformation *in vivo*. The possible biological significance of this finding is discussed.

2. MATERIALS AND METHODS

2.1. Reagents

A 14-mM stock solution of camptothecin (Sigma) was prepared in dimethyl sulfate; further dilutions were performed with media immediately before use. Irinotecan (19 mM; TopoGEN) was dissolved in water.

2.2. Recombinant luciferase reporter gene plasmid constructs

Constructs containing MOR PPy/u motifs in a sense orientation have been described previously (Fig. 4A) (Choe et al., 2011). Constructs containing MOR PPy/u motifs in an antisense orientation (pBMPPy/u-AS), or the human c-Myc nuclease hypersensitive element III₁ (NHE III₁) sequence (a CT-element) (Choe et al., 2011) in sense and antisense orientations (pBNHE III-S and pBNHE III-AS, respectively) were prepared by cloning double-stranded oligonucleotides (Fig. 1A) with restriction sites into pGL3-Basic plasmid digested with SalI and HindIII (Promega). The sequences of all constructs were verified by the Advanced Genetic Analysis Center at the University of Minnesota on Applied Biosystems 1377 DNA sequencers using RVprimer3 (Promega).

2.3. Preparation of supercoiled plasmid DNA

Plasmids were propagated in *endA*⁻ XL-1 Blue *Escherichia coli* (Stratagene) or DH5 α (Invitrogen) cells and purified using a Midi or Mini 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 and assessed for its conformation by electrophoresis on agarose gels and for purity and quantity by measuring the O.D._{260/280} using a NanoDrop spectrophotometer (Thermo Scientific). In most experiments, the native superhelical density of the DNA (except for the pBMPPy/u-AS plasmid) was ~ -0.05 .

2.4. Determination of plasmid DNA abundance

A single colony of *Escherichia coli* DH5 α cells from freshly streaked Luria Bertani (LB) agar plates was cultured at 37°C for 8 hr, followed by measuring the O.D.₆₀₀ to determine the cell density. The same number of cells was inoculated into new LB medium and grown for 12 hr. The plasmid was extracted using a Mini-prep kit (Qiagen), and DNA concentration was calculated by measuring the O.D._{260/280}.

2.5. Cell culture, transfection, and reporter gene assay

NS20Y cells (the C1300 neuroblastoma clone of the A/J mouse strain) were grown in Dulbecco's modified Eagle medium with 5% inactivated fetal calf serum and 1% L-glutamine in an atmosphere of 5% CO₂ and 95% air at 37°C. Human neuronal NMB cells were grown in RPMI 1640 medium with 10% heat-inactivated fetal calf serum in an atmosphere of 5% CO₂ and 95% air at 37°C. Mouse embryonal sarcoma P19 cells were purchased from ATCC and cultured in -Minimal Essential Medium supplemented with 7.5% newborn calf serum and 2.5% fetal calf serum. The procedure to differentiate P19 cells has been described previously (Chen et al., 1999). Briefly, cells were grown in Petri dishes in Minimal Essential Medium containing 5% heat-inactivated fetal bovine serum and 0.5 μ M all-*trans* retinoic acid for four days, with fresh medium and reagents added after the first two days. Aggregated cells were dissociated using DNase I and trypsin/EDTA and plated in

tissue culture dishes in the absence of retinoic acid. Cytosine arabinose (10 μ M) was added to the culture medium 24 h after plating to maintain only postmitotic neuronal cells and to eliminate proliferating glial cells. Following three days of incubation, cells were treated with topoisomerase inhibitors.

For transfection, cells were plated at a density of 1×10^5 cells/well in six-well plates. Cells were transfected 24 h later with construct plasmids using Effectene (QIAGEN) according to the manufacturer's instructions. 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 assayed according to the manufacturers' instructions (Promega and Tropix, respectively). All transfection experiments were repeated at least four times with similar results, using plasmids prepared independently at least twice. Normalized values are the averages of at least three independent determinations, and error bars represent standard errors of the mean.

In a separate series of experiments, 24 h after plating at a density of 1×10^5 cells per plate in six-well plates, cells were treated with various concentrations of camptothecin or irinotecan. After 1 h of incubation, cells were washed with medium twice and cultured for an additional 5–8 h. Harvested cells were subjected to RT-PCR.

2.6. RT-PCR

Total RNA was isolated using TRI reagent according to the supplier's (Molecular Research Center) protocol and analyzed by RT-PCR using the following primers (Choi et al., 2007): Mouse MOR (GenbankTM Accession no. AB047546), 5'-CATGGCCCTCTATTCTATCGTGT-3' (sense, located at exon 3) and 5'-CAGCGTGCTAGTGGCTAAGG-3' (antisense, located at exon 4); mouse β -actin (GenbankTM Accession no. NM_007393), 5'-ATATCGCTGCGCTGGTCGTC-3' (sense, located at exon 2) and 5'-TCACTTACCTGGTGCCTAGGG-3' (antisense, located at exon 3); human MOR (GenbankTM Accession no. NM_000914), 5'-GATCATGGCCCTCTACTCCA-3' (sense, located at exon 1), 5'-GCATTTCGGGGAGTACGGAA-3' (antisense, located at exon 1); human β -actin (GenbankTM Accession no. NM_001101), 5'-CATGTACGTTGCTATCCAGGC-3' (sense, located at exon 4), 5'-CTCCTTAATGTCACGCACGAT-3' (antisense, located at exon 4). RT-PCR was performed using the QIAGEN OneStep RT-PCR kit. The same amount of total RNA (0.2 μ g) was used from each sample. PCR was performed as follows: 50°C for 30 min and 95°C for 15 min; 33 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min. After cycling, the reaction was extended for 10 min at 72°C. Similar reactions were performed using primers for β -actin as an internal RT-PCR loading control, except the number of cycles was reduced to 22. PCR products were electrophoresed in a 2% agarose gel and quantified using ImageQuant version 5.2 (Amersham Bioscience). The DNA sequences of the PCR products were confirmed by restriction digestion.

2.7. Nuclease S1 and restriction enzyme assays

Supercoiled plasmid constructs were digested with S1 nuclease (Promega) in 18 μ l of 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 for 45 min at 37°C. The cleavage efficiency was consistent between independent preparations of the supercoiled plasmids.

2.8. Determination of strand specificity

The pBMPPy/u-AS plasmid was run in 1% low-melting agarose gel and the nicked DNA form was isolated. The nicked strand was identified by sequencing the double-stranded template with two primers that covered the PPy/u nucleotides: sense primer (4802–4821) 5'-CTAGCAAAATAGGCTGTCCC-3' and antisense primer (131–153) 5'-TATGTTTTTGGCGTCTTCCA-3'. Sequencing was conducted by the Advanced Genetic Analysis Center at the University of Minnesota on Applied Biosystems 1377 DNA sequencers.

2.9. Atomic force microscopy (AFM)

Ten μl of supercoiled DNA (0.3 ng/ μl in TE buffer containing 2 μM MgCl_2) were spotted on fresh mica for 2 min. The specimens were rinsed thoroughly with deionized water and argon-dried. The samples were imaged in air at ambient conditions using a NanoScope II MultiMode (Veeco, Santa Barbara, CA) atomic force microscope operating in tapping mode. Super-sharp silicone tips (Silicon-MTD Ltd., Moscow, Russia) with a resonance frequency of ~ 246 KHz were used at a scanning rate of 1–2 Hz.

2.10. DNA relaxation assay

One μg of plasmid DNA was catalyzed with 0–3 units of human topoisomerase II α (TopoGEN) in 20 μl of topoisomerase II buffer (50 mM Tris-Cl [pH 8.0], 150 mM NaCl, 10 mM MgCl_2 , 2 mM ATP, 0.5 mM dithiothreitol, 30 $\mu\text{g}/\text{ml}$ bovine serum albumin) for 0–30 min at 37°C. The reactions were terminated by adding 2 μl of gel loading buffer (10% sodium dodecyl sulfate, 0.025% bromophenol blue, 50% glycerol). 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. Band intensities were measured by ImageJ (National Institutes of Health, Bethesda, MD, USA). Intensities were measured along a line drawn vertically down the middle of each gel lane; the average of three measurements was plotted. The relaxation efficiency was consistent between independent preparations of the supercoiled plasmids.

2.11. Raman spectroscopy

Raman measurements of all DNA constructs were conducted using an alpha300 R confocal Raman microscope equipped with a UHT200 spectrometer and a DV401 CCD detector (WITec, Ulm, Germany). Supercoiled DNA was dissolved in phosphate-buffered saline to a final concentration of 20 $\mu\text{g}/\mu\text{l}$. An aliquot (4.5 μl) of the supercoiled DNA solution was placed on a 100-nm-thick gold-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 (CVI Melles Griot, Carlsbad, 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 <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. The MOR PPy/u motif and the relaxation of supercoiling

Supercoiling is a process that is highly regulated by cells and occurs when the DNA is subject to some form of structural strain (Kanaar and Cozzarelli, 1992). PPy/u motifs contain an excess of polypyrimidine and polypurine bases in each strand of the DNA duplex.

This bias in base composition produces duplex DNA strands with unique conformations such as the composite pyrimidine-rich G-quadruplex and purine-rich i-motifs, triplexes, and single-stranded conformations (Htun and Dahlberg, 1988; Sun and Hurley, 2009). Thus, depending on its orientation and position, each strand could be subject to different torsional stress and induce different helical distortion to the surrounding DNA sequences. To assess how sensitively the MOR PPy/u elements respond to the change in torsional stress *in vivo*, homologous MOR PPy/u motifs were inserted into a promoterless pGL3-Basic reporter vector in both sense and antisense orientations (Fig. 4A), and the plasmids' native topological conformations were examined by agarose gel electrophoresis (Fig. 1). Supercoiled DNA was predominant in the pGL3-Basic control plasmid, with residual amounts of the nicked and open circular DNA typical of negatively supercoiled plasmids isolated from bacteria (Fig. 1B). Interestingly, the pattern for the antisense mouse PPy/u construct (pBMPPy/u-AS) differed noticeably from that of the sense construct: The dominant band in the pBMPPy/u-AS sample was nicked DNA, whereas the upper bands presumably were multimeric DNA catenanes (Fig. 1B). In contrast, the orientations of the rat and human MOR PPy/u motifs and the c-Myc PPy/u motif NHE III₁ had no effect on the electrophoretic mobility of their constructs, suggesting that they have similar topologies (Fig. 1B). Furthermore, bacteria transfected with the pBMPPy/u-AS plasmid grew more slowly and produced an approximately sevenfold lower plasmid yield than did cells transfected with the sense plasmid (Fig. 1C). Constructs in which the mouse PPy/u motif was inserted into a pGL3-Promoter vector with an SV40 promoter (pPMPPy/u-S and pPMPPy/u-AS) showed no orientation-dependent differences in plasmid yield or conformation (Fig. 1B). In bacteria, the topological isomers (i.e., nicked and catenated DNA) seen in the pBMPPy/u-AS sample are replication intermediates and occur when DNA is not properly disentangled by topoisomerase (Martínez-Robles et al., 2009; Witz and Stasiak, 2010; Zechiedrich and Cozzarelli, 1995). Taken together, these results suggest that unlike the homologous human and rat PPy/u motifs, the mouse PPy/u motif responds differentially to different forms of torsional stress and consequently influences the relaxation activity of topoisomerase.

3.2. Confirmation of conformation by topoisomerase assay

To better understand how the mouse MOR PPy/u motif affects the biochemical process of DNA relaxation, plasmids were treated with human topoisomerase II. Topoisomerases bind to DNA and cut at one (topoisomerase I) or both (topoisomerase II) strands to release torsional stress that develops during DNA transaction events. Topoisomerases can easily knot/deknot, catenate/decatenate, and supercoil/relax DNA molecules *in vitro* (Mertz and Miller, 1983). Both pGL3-Basic and pBMPPy/u-S plasmid DNA were converted to relaxed topoisomers by topoisomerase II, whereas the dominant band of pBMPPy/u-AS DNA was converted to higher oligomeric conformers (Fig. 2). Given the fact that the conditions of the topoisomerase reaction were designed to decatenate catenanes like kinetoplast DNA (kDNA), this result differs from our expectation that topoisomerase II would convert the catenated plasmid DNA into relaxed, closed circular and nicked, open circular forms. The reason for this discrepancy can be ascribed to the difference in the topology of DNA substrates under the reaction conditions. Studies show that the DNA aggregation induced by condensing agents such as salt, polyethylene glycol (PEG), and polyamines significantly influences the modes of topoisomerase activity catenation and decatenation (Mertz and Miller, 1983). The aggregated DNA condensates undergo catenation, whereas disaggregation results in decatenation. Therefore, it is highly likely that pBMPPy/u-As DNA molecules adopt a disaggregated conformation under the reaction conditions tested. The results of the topoisomerase assay strongly suggest that the PPy/u motif, under different torsional stress, introduces an abnormal topological constraint globally or locally, the consequence of which is difficulty in the relaxation of supercoiling by topoisomerase.

3.3. AFM analysis of the topological structure of PPy/u-containing plasmid

To further characterize the physical and geometric features of pBMPPy/u-AS topological isomers, DNA was analyzed by atomic force microscopy (AFM) (Fig. 3). The structural features of the pGL3-Basic control plasmid and pBMPPy/u-S were consistent with the general features of B-form DNA, having the tightly twisted geometry typical of conventional plectonemic supercoiled DNA (Lyubchenko and Shlyakhtenko, 1997). In contrast, the most pronounced structural feature of pBMPPy/u-AS was the lack of supercoiling and the interlocked structure of single and multiple rings of DNA, with multimeric DNA catenanes (Fig. 3C), suggesting that the topological species are the product of incomplete segregation following replication rather than incomplete replication. Another notable feature of the pBMPPy/u-AS plasmid is that most of the DNA occurs in a loose geometry, with rosette-like tertiary supercoiling. However, given that pBMPPy/u-AS DNA is predominantly nicked monomeric DNA (Fig. 1), the presence of many larger compact globular structures suggests that the pBMPPy/u-AS plasmid likely intertwines with itself depending on the conditions. This result is consistent with the topoisomerase analysis showing that pBMPPy/u-AS undergoes catenation with topoisomerase II. In summary, the AFM analysis of the topological structure of pBMPPy/u-AS led us to conclude that mouse PPy/u element is a topological barrier to the relaxation of supercoiled DNA by topoisomerases.

3.4. Mouse MOR PPy/u is the topoisomerase nick site

To locate the nicked site of the pBMPPy/u-AS plasmid, we performed S1 nuclease mapping. DNA was cleaved with S1 nuclease, which preferentially cleaves opposite a nick in duplex DNA (Chaudhry and Weinfeld, 1995) and opens the DNA. The linear DNA was treated with XbaI, the site of which is unique in the DNA (Fig. 4A), and the resultant fragments indicate the nick site. This analysis used low concentrations of S1 nuclease and XbaI because high concentrations can introduce breaks in double-stranded DNA and produce nonspecific fragments (Choe et al., 2011). The pGL3-Basic control plasmid did not generate fragments (Fig. 4C), suggesting an absence of nicks and single-stranded regions (Fig. 4C). Although *XbaI* treatment of both pBMPPy/u-S and -AS generated linear bands at the same position (5 kb) (Fig. 4B), the band was more pronounced in the pBMPPy/u-AS sample, confirming that the major species in this plasmid is nicked circular DNA. S1 nuclease and *XbaI* treatment generated two fragments of 1.7 and 3.1 kb in pBMPPy/u-S and pBMPPy/u-AS (Fig. 4C), and the intensity of both bands increased with more S1 nuclease. These results suggest that the nick is located ~1.8 kb from the *XbaI* site, which is very likely at or near the PPy/u cloning site. In contrast, altering the orientation had no apparent effect when the PPy/u element was inserted in the pGL3-Promoter vector (Fig. 4D). Run-off sequencing shows that the nick site in pBMPPy/u-AS is in the purine-rich strand (Fig. 5). This data, with the S1 nuclease mapping of pBMPPy/u-AS, suggests that the site nicked by topoisomerase is near the plasmid's PPy/u motif. Topoisomerase relaxes the supercoiled plasmid in two steps: cleavage and religation. Whereas cleavage activity is unaffected by the sequence composition or context, religation can be influenced by both, including the sequence's orientation and position (Koster et al., 2008). Thus, the major species of pBMPPy/u-AS (Fig. 1) likely results from resistance of the plasmid to the religation activity of topoisomerase, i.e., the plasmid is cleaved by the topoisomerase, but not religated.

3.5. Raman structural signature of pBMPPy/u-AS

The vibrational and rotational frequencies of a variety of DNA functional groups can be identified by Raman spectroscopy (Serban et al., 2002). Thus, Raman analysis can reveal information regarding the local structures of nucleotide bases and the phosphodiester backbone structure. Plasmids with PPy/u motifs were examined by confocal Raman microscopy to determine the molecular basis for the pBMPPy/u-AS plasmid's religation

resistance (Fig. 6). Consistent with known spectra, supercoiled (pBMPPy/u-S) and nicked circular (pBMPPy/u-AS) plasmids with the PPy/u motif had bands diagnostic of B-form DNA at approximately 682, 732, 790/831, a broad band at 835, and 1095, and 1420 cm^{-1} (Serban et al., 2002). This suggests that the global conformations of plasmid DNA molecules containing PPy/u motifs are significantly similar to each other and to pGL3-Basic (control) DNA. However, a comparison of the spectra for supercoiled pBMPPy/u-S and nicked circular pBMPPy/u-AS plasmids yielded several significant differences, particularly in the 1245–1490 cm^{-1} range (Fig. 6, lower panel). Specifically, the broad Raman band at 1245 cm^{-1} — characteristic for the ring-stretching motions of cytosine, thymine, and guanine (Grajcar and Baron, 2001) — was present in the pBMPPy/u-AS plasmid, whereas there was no apparent difference between the supercoiled control pGL3-Basic and pBMPPy/u-S plasmids. Secondly, the peak at 1476 cm^{-1} — diagnostic of Hoogsteen hydrogen bond formation involving the guanine N7 site (White and Powell, 1995; Miura and Thomas, 1995) — also differed between the pBMPPy/u-S and -AS plasmids. These differences suggest that the mouse PPy/u motif put under the structural strain in the antisense orientation perturbs the ring configuration of the bases and changes Hoogsteen hydrogen bond formation, which might account for the inhibition the religation of topoisomerase.

3.6. Orientation dependence of gene activation by the MOR PPy/u motif

We tested the ability of PPy/u-AS topological isomers to affect gene activation. Reporter genes with PPy/u motifs were transfected into mouse neuronal NS20Y cells (Fig. 7). Interestingly, using the mouse motif, the antisense construct expressed fortyfold less reporter gene activity than the sense construct. In contrast, orientation had no significant effect on reporter gene expression in cells transfected with human or rat PPy/u motifs (data not shown) or with the control NHE III₁ motif (Fig. 7). Similar differences in luciferase activity were also observed in transfected P19 cells (data not shown). In contrast, consistent with a previous study (Ko and Loh, 2001), the orientation of the PPy/u motif had no effect on reporter gene activity when inserted in pGL3-Promoter vectors (Fig. 7). Thus, we conclude that the topological environment of the plasmid containing the mouse MOR PPy/u element is important for transcriptional activity.

3.7. The effect of topological perturbation on MOR gene expression

The relaxation mechanism of topoisomerase is conserved evolutionarily (Corbett and Berger, 2004; Roca, 1995). Because the mouse PPy/u motif strongly influenced the relaxation activity of topoisomerase in bacteria, we determined if the mouse PP/u motif can contribute to MOR gene expression in response to changes in supercoiling. MOR gene expression in response to topoisomerase I poisons camptothecin (CPT) and irinotecan (Iri) was examined in mouse NS20Y and P19 cells and human NMB cells, all of which can express MOR. CPT and Iri bind to topoisomerase I/DNA complexes and inhibit religation; the remaining nick removes superhelical tensions within the chromosome. Exposure to CPT significantly increased MOR transcripts in NS20Y (Fig. 8A) and P19 (Fig. 8B and 8C) cells, but not in NMB cells (Fig. 8D). Interestingly, Iri did not strongly activate MOR gene expression in NS20Y (Fig. 8A) and differentiated P19 (Fig. 8C) cells. However, higher concentrations of Iri enhanced MOR gene expression in undifferentiated P19 cells (Fig. 8B) after 5 h in drug-free medium to levels comparable to those of CPT-treated cells after 8 h (lane 9). Given the fact that CPT and Iri influence the topology of chromosomes by inhibiting religation transiently or permanently, the differential effects of topoisomerase poisons on MOR gene expression seen between mouse and human cells and differentiated and undifferentiated mouse P19 cells suggest that the process of religation is different between mouse and human cells and differentiated and undifferentiated P19 cells. In light of the above observations that pBMPPy/u-AS topological isomers could be the result of the defect in religation in bacteria (Fig. 1B), we conclude that the mouse PPy/u element in the

promoter is important for endogenous MOR gene expression in response to topological perturbation, whereas the human PPy/u motif is not. We propose a mechanism underlying how the efficiency of religation regulates supercoiling-driven gene expression in the discussion.

4. DISCUSSION

PPy/u elements are a conformation-dependent, cis-acting element in promoters. To affect transcription, either as topological buffers or transcription factor binding sites, they need to adopt a non-B DNA structure. Superhelical stress is a key driving force for such transitions. However, despite considerable speculation and accumulation of data using oligonucleotides and plasmids, evidence that the supercoiling-dependent conformational transition of PPy/u elements is important for gene expression *in vivo* has never been shown.

The topological state of the chromosome is known to affect the transcription activity of many promoters. The activity of some promoters significantly decreases when DNA is relaxed, while the activity of others is unaffected or even enhanced (Drlica, 1992). The present study shows, for the first time, that the mouse MOR PPy/u motif is a key *cis*-acting element that mediates superhelical stress *in vivo*, suggesting that the mouse MOR gene is regulated by the alteration in the topology and geometry of the chromosome. We propose that the mouse MOR PPy/u element acts as a DNA damage-induced transcription signal. Furthermore, our study shows that homologous rodent and human MOR PPy/u motifs process superhelical tension differently, suggesting that PPy/u motifs in the MOR gene promoter are species-specific. It will be interesting to determine whether the PPy/u motif is widely employed in biological systems as a structural *cis*-acting element for species-specific gene expression. In this regard, there is a study showing that the PPy/u motif of the cystic fibrosis transmembrane conductance regulator gene is species-specific (Vuillaumier et al., 1997).

Our previous study showed that the mouse MOR PPy/u motif activated a pGL3-promoter-based reporter gene in both sense and antisense orientations, suggesting that this sequence is a generally orientation-independent transcription enhancer (Ko and Loh, 2001). However, the study did not explore whether the PPy/u motif adopts a non-B DNA conformation in its antisense orientation. One objective of the current study was to determine how the motif could produce the same level of transcription activation, regardless of orientation. What prompted this question is that polypyrimidine-rich and polypurine-rich strands in the duplex DNA of the PPy/u motif, which have distinctive physicochemical properties, adopt distinctive secondary structures. For example, the duplex DNA of the PPy/u element of c-Myc NHE III₁ adopts purine-rich G-quadruplex and pyrimidine-rich i-motif secondary conformations (Sun and Hurley, 2009). This fact led us to hypothesize that the MOR PPy/u motif conformation is subject to regulation by different structural stresses and consequently induces different helical distortion to the surrounding DNA sequences in a stress-dependent fashion. As a consequence of the change in local and global topology, the transcription activity of the MOR PPy/u element changes, depending on its position in duplex DNA strands. In support of this idea, for example, the human U1 gene HU1-1 TC/AC₁₈ adopts two different conformers of triplex and single-stranded conformations (H-y3 and H-y5), depending on the orientation of the half mirror sequence (Htun and Dahlberg, 1988). In the present study, the PPy/u motif was inserted in both orientations in two different plasmids to cause the various forms of structural strain. The mouse PPy/u motif in the sense orientation had no effect on the ability of either pGL3-Basic or pGL3-Promoter plasmids to activate transcription (Fig. 7), nor on the ability of the plasmids to adopt non-B DNA conformations (Fig. 1). In contrast, this motif in the antisense orientation produced differences in conformation and transcription activation, depending on the plasmid into which the motif

was inserted (Fig. 1B). Homologous rat and human MOR PPy/u motifs showed no difference in conformation and reporter gene activity, regardless of orientation and position. These results strongly support our hypothesis that homologous MOR PPy/u elements under different structural strain differentially process the topology of supercoiled DNA.

The most striking finding of this study was that the mouse MOR gene is expressed in response to the relaxation of supercoiling by topoisomerase I poisons CPT and Iri (Fig. 8). Many studies have reported that non-B DNA is the preferential site of topoisomerase cleavage (Antony et al., 2004). However, because these studies were carried out *in vitro*, it was not clear that non-B DNA is the preferential site for topoisomerases *in vivo*. The result that plasmid pBMPPy/u-AS, in which mouse MOR PPy/u element is under antisense structural strain, is composed exclusively of nicked and catenated topological isomers strongly indicates that the MOR PPy/u element is the site for topoisomerases. Furthermore, our interpretation that pBMPPy/u-AS topological isomers are the result of inhibition of religation suggests that mouse and human cells differentially regulate MOR gene expression in response to supercoiling. That is, although human and mouse PPy/u elements are the preferential cleavage sites for topoisomerase, the mouse MOR PPy/u motif is not a favorable structure for religation. In contrast, religation is efficient at the human MOR PPy/u motif. The existence of diverse topological isomers of pBMPPy/u-AS suggests that the mouse MOR PPy/u element could drive DNA transactions such as DNA damage repair, while the human MOR PPy/u element is normally religated without activating DNA damage-coupled transcription. Indeed, DNA damage in cells induces many genes whose products facilitate DNA repair (D'Onofrio et al., 2011). Poly(ADP-ribose) polymerase (PARP) is a nuclear protein that recognizes DNA lesions and protects cells against the genome-destabilizing effects of DNA damage. As a mechanism for this protection, PARP activates numerous genes involved in this process. Recent studies showed that PARP binds to complexes of topoisomerase 1 inhibitor and stalled topoisomerase I and facilitates resealing of DNA strand breaks, resulting in the activation of genes such as p53 and p21. MOR has been shown to protect against apoptosis in lymphocytes (Suzuki et al., 2003); it will be worth investigating if PARP is involved in MOR gene expression. We propose that the mouse MOR PPy/u element could act as a DNA damage site and that this DNA damage could activate the mouse MOR gene. Although numerous studies have attempted to detect non-B DNA *in vivo*, to the best of our knowledge, their presence has yet to be reported. We believe this is the first report of a naturally occurring mammalian PPy/u motif adopting a non-B conformation capable of perturbing the relaxation activity of topoisomerase *in vivo*.

How might the mouse PPy/u motif affect the relaxation process of topoisomerase? The nicked and catenated DNA molecules produced by the pBMPPy/u-AS plasmid are common intermediate products of basic biological processes such as DNA replication, chromosomal segregation, recombination, and topoisomerase relaxation. Topoisomerase separates supercoiled replication intermediate DNA molecules in two sequential steps: cleavage and religation. Whereas non-B DNA conformations are preferred cleavage sites for topoisomerase, the orientation and position of triplex-forming oligonucleotides profoundly affects the religation process (Antony et al., 2004). These facts strongly suggest that the PPy/u motif facilitates the disentangling of replication intermediate catenanes, resulting in supercoiled DNA. However, the predominance of the nicked DNA species in the pBMPPy/u-As plasmid suggests that structural strain such as in the antisense orientation alters the topology of the DNA such that the religation of nicked DNA is affected, but not plasmid cleavage. We believe that this perturbation of the religation process by pBMPPy/u-AS is not a factor of chemical interactions *per se*, but rather the result of a lack of torque (torsional stress). The torque present in the DNA drives the rotation of the nicked strand around the other strand and is sensitive to the mechanical properties of DNA (Koster et al., 2005). Indeed, studies have shown that PPy/u sequences influence flexibility and curvature globally

and locally by absorbing the negative superhelical density downstream (Kouzine et al., 2008). In conclusion, we propose that the mouse PPy/u motif under the antisense structural strain stiffens the DNA, resulting in a decrease in torque.

The discovery that abnormalities in DNA structure can drive genetic instability has changed our understanding of the genetic mechanisms of some human diseases (Bacolla and Wells, 2004). PPy/u motifs are common in the eukaryotic genome, with some genes having a purine-rich template and a pyrimidine-rich nontemplate strand, and others vice versa. Although this study demonstrates that the mouse MOR PPy/u motif perturbs the conformation of plasmid DNA, it also points to a broader molecular mechanism by which non-B DNA conformations present in chromosomal DNA control biological processes including replication, recombination, and genome organization. Supporting this idea, DNA transaction processes are always linked to changes in the topological states of the chromosome (Kanaar and Cozzarelli, 1992). As our results show, non-B DNA can be prone to being nicked and resist religation. The unrepaired DNA breakage could lead to irreversible chromosome damage and consequently to genetic and epigenetic diseases including cancer.

The MOR agonist morphine is the first line opioid recommended by the World Health Organization (WHO) to relieve the moderate to severe pain that often accompanies treatment with anticancer drugs such as topoisomerase inhibitors (Mercadante et al., 2004). Future studies on the mechanism by which the PPy/u motif controls MOR gene expression in response to changes in superhelicity and DNA damage will contribute to our understanding of how MOR gene expression is selectively regulated by various DNA-interacting drugs during chemotherapy.

Acknowledgments

This work was supported by the National Institutes of Health [DA-00546, DA-01583, DA-05695] and the A&F Stark Fund of the Minnesota Medical Foundation.

Abbreviations

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

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Fig 1.

Effect of orientation and position on the electrophoretic mobility and yield of plasmid. **A.** The mouse MOR PPy/u sequence (MPPy/u) (–341 to –300) of the sense strand DNA and its corresponding 5′-flanking sequences compared with those of the rat (RPPy/u) and human (HPPy/u) 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 NHE III₁ sequence serves as a control for non-B form DNA. **B.** Gel-electrophoretic analysis of plasmid DNA. Upper: Electrophoretic mobility of plasmids in pGL3-Basic (i.e., without an enhancer/promoter; pB) containing a PPy/u insert from mouse (M), rat (R), or human (H), or the PPy/u from the c-Myc NHE III₁ (NHEIII) in sense (-S) or antisense (-AS) orientations. Lower: Electrophoretic mobility of plasmids in a pGL3-promoter vector (i.e., containing the SV40 promoter) containing a PPy/u insert from mouse in sense (pPMPPy/u-S) and antisense (pPMPPy/u-AS) orientations. Plasmid DNAs were resolved by 1% agarose gel electrophoresis and stained with EtBr. C: catenanes; NC: nicked circular DNA; SCC: supercoiled circular DNA. **C.** Bar chart of plasmid isolation from XL-1 Blue *E. coli* cells. Cells were grown overnight in 1.5 ml of LB medium and then the plasmid was extracted using a QIAGEN Miniprep kit. The data shown are means ± S.D. of three independent experiments, with at least two different plasmid preparations.

Fig 2.

DNA catenation assay. The ability of topoisomerase II (0–3 units) to relax supercoiled DNA in which rodent and human PPY/u elements inserted into pGL3-basic vector was monitored by the disappearance of supercoiled bands and the generation of relaxed bands. The intensity of topological isomers of pBMPPy/u-AS plasmid (lanes 8–10) was measured and plotted as described under *Materials and methods*. Pixel in the plot is from top to bottom bands. C: catenanes; NC: nicked circular DNA; R: relaxed DNA; SCC: supercoiled circular DNA. Three independent assays showed essentially identical results; a representative result is shown.

Fig 3.
Atomic force microscopy of plasmid DNA molecules with the PPy/u inserts in pGL3-Basic vector (*A*) in sense (*B*) and antisense (*C*) orientations. Upper: low resolution; Lower: high resolution.

Fig. 4.

Enzymatic mapping of the nick site. (A) Schematic showing the region accessible to S1 nuclease (i.e., the PPy/u motif) and the cleavage sites of restriction enzymes. The PPy/u and poly(A) signals are indicated by gray boxes. (B) Restriction digestion of the supercoiled (pBMPPy/u-S) and open circular (pBMPPy/u-AS) plasmid DNA of the mouse MOR PPy/u motifs with increasing amounts of XbaI (0–0.6 units). (C, D) S1 nuclease digestion of the supercoiled (pBMPPy/u-S) and open circular (pBMPPy/u-AS) plasmid DNA of the mouse MOR PPy/u motifs. The plasmid DNAs in pGL3-Basic (C) and pGL3-Promoter (D) 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. Three independent assays showed essentially identical results; representative results are shown. L: linear DNA; M: DNA ladder.

Fig. 5. Chromatograms showing run-off sequencing. *A*. pBMPPy/u-S plasmid was sequenced using sense and antisense primers as a positive control for the ability of polymerase to read through a polypyrimidine•polypurine-rich template. *B*. The major species of pBMPPy/u-AS DNA form excised from the agarose gel was sequenced. Arrows indicate direction of DNA synthesis. The PPy/u sequences are underlined. The drop in peak signal in the chromatogram indicates where the DNA polymerase runs off the template at the nicked site.

Fig. 6.

Raman structural signatures of plasmids containing the PPy/u motif. Raman bands of (a) pGL3-Basic, (b) pBMPPy/u-S and (c) pBMPPy/u-AS plasmids are shown. Spectra b and c were normalized to the pGL3-Basic integrated intensity at 1092 cm^{-1} , which is assigned to the PO_2^- symmetric stretching mode of the polydeoxynucleotide phosphate groups. Labels indicate representative Raman markers of DNA. Upper panel: Frequencies (in wave numbers/cm) of key bands. Intensity is expressed in arbitrary units. Each spectrum represents the average of 40 exposures of 10-s duration. Three independent experiments for each assay exhibited essentially identical results; representative results are shown. Lower panel: Computed difference spectra. Spectrum d = spectrum b – spectrum a; spectrum e = spectrum b – spectrum c. Differences in intensity reflect differences in the local distribution of different conformational populations.

Fig. 8.

Camptothecin- and irinotecan-induced expression of the MOR gene. Exponentially growing NS20Y (A), undifferentiated and differentiated P19 (B & C, respectively) and NMB (D) cells were treated with the indicated concentrations of camptothecin and irinotecan for 1 h, followed by washing with medium. Cells replenished with drug free-medium were incubated for an additional 5 or 8 h. MOR transcript levels in treated and untreated cells were determined by RT-PCR and agarose electrophoresis, using β -actin as an internal RNA loading control for each sample. Control samples consisted of untreated cells harvested at 5 h (5C) and 8 h (8C) (lanes 2 and 8, respectively). Lane 1, DNA molecular weight ladder. CPT, camptothecin; Iri, irinotecan; P19-U, undifferentiated P19 cells; P19-D, differentiated P19 cells.