Erectile dysfunction in mice lacking the large-conductance calcium-activated potassium (BK) channel
M. E. Werner, A.-M. Knorn, A. L. Meredith, R. W. Aldrich and M. T. Nelson
[Abstract] [Full Text] [PDF]

Frequency encoding of cholinergic- and purinergic-mediated signaling to mouse urinary bladder smooth muscle: modulation by BK channels
M. E. Werner, A.-M. Knorn, A. L. Meredith, R. W. Aldrich and M. T. Nelson
[Abstract] [Full Text] [PDF]

Smooth muscle and neural mechanisms contributing to the downregulation of neonatal rat spontaneous bladder contractions during postnatal development
Y.-K. Ng, W. C. de Groat and H.-Y. Wu
[Abstract] [Full Text] [PDF]
Urodynamic properties and neurotransmitter dependence of urinary bladder contractility in the BK channel deletion model of overactive bladder

K. S. Thorneloe,1 A. L. Meredith,2 A. M. Knorn,1 R. W. Aldrich,2 and M. T. Nelson1
1Department of Pharmacology, College of Medicine, University of Vermont, Burlington, Vermont; and 2Department of Molecular and Cellular Physiology and Howard Hughes Medical Institute, Stanford University, Stanford, California
Submitted 11 February 2005; accepted in final form 11 April 2005

Thorneloe, K. S., A. L. Meredith, A. M. Knorn, R. W. Aldrich, and M. T. Nelson. Urodynamic properties and neurotransmitter dependence of urinary bladder contractility in the BK channel deletion model of overactive bladder. Am J Physiol Renal Physiol 289:F604–F610, 2005.—Overactive bladder and incontinence are major medical issues, which lack effective therapy. Previously, we showed (Meredith AL, Thornloe KS, Werner ME, Nelson MT, and Aldrich RW. J Biol Chem 279:36746–36752, 2004) that the gene mslo1 encodes large-conductance Ca2+-activated K+ (BK) channels of urinary bladder smooth muscle (UBSM) and that ablation of mslo1 leads to enhanced myogenic and nerve-mediated contractility and increased urine frequency. Here, we examine the in vivo urodynamic consequences and neurotransmitter dependence in the absence of the BK channel. The sensitivity of contractility to nerve stimulation was greatly enhanced in UBSM strips from slo−/− mice. The stimulation frequency required to obtain a 50% maximal contraction was 8.3 ± 0.9 and 19.1 ± 1.8 Hz in slo−/− and slo+/+ mice, respectively. This enhancement is at least partially due to alterations in UBSM excitability, as muscarinic-induced slo−/− contractility is elevated in the absence of neuronal activity. Muscarinic-induced slo−/− contractility was mimicked by blocking BK channels with ibiotixin (IBTX) in slo−/− strips, whereas IBTX had no effect on slo+/+ strips. IBTX also enhanced purinergic contractions of slo−/− UBSM but was without effect on purinergic contractions of slo+/+ strips. In vivo bladder pressure and urine output measurements (cystometry) were performed on conscious, freely moving mice. slo−/− mice exhibited increased bladder pressures, pronounced pressure oscillations, and urine dripping. Our results indicate that the BK channel in UBSM has a very significant role in urinary function and dysfunction and as such likely represents an important therapeutic target.

Incontinence; bladder dysfunction; cystometry; K+ channel

Effective urine voiding requires the coordinated contraction of the urinary bladder detrusor smooth muscle and relaxation of the bladder outlet. In adults, this process is under voluntary control and reflects the integration of neural and smooth muscle mechanisms. The contractile function of smooth muscle involves the functional interplay of multiple processes, including neurotransmitter receptor activation, intracellular Ca2+ signaling, and ion channel regulation of membrane excitability. Failure at the level of neural regulation or smooth muscle contractile function can lead to irritative voiding symptoms and/or incomplete bladder emptying.

The urinary bladder is extensively innervated. Stimulation of parasympathetic nerves induces the corelease of the neurotransmitters adenosine triphosphate (ATP) and acetylcholine (ACh), which act directly on smooth muscle purinergic and cholinergic receptors, respectively, to induce contraction. Cholinergic signaling mechanisms appear to predominate under normal conditions in humans (25, 29), but purinergic pathways may contribute substantially (or predominantly), depending on the pathophysiological state of the tissue, experimental conditions, and species (1, 5, 7, 18, 19, 21, 25, 26, 30, 32–34).

The urinary bladder generates spontaneous phasic contractions that are triggered by action potentials (4). Parasympathetic nerve stimulation coordinates this myogenic activity, giving rise to forceful bladder contraction and micturition. The various phases of the urinary bladder smooth muscle (UBSM) action potential reflect the coordinated action of distinct ionic conductances. The upstroke of the action potential is attributable to Ca2+ entry through dihydropyridine-sensitive voltage-dependent Ca2+ channels (10, 13, 14, 20). The repolarization phase is mediated by the activity of both voltage-dependent K+ (Kv) channels and the large-conductance, calcium-activated potassium (BK) channels (13, 20, 31). Following the spike and repolarization, the UBSM action potential displays a prolonged after hyperpolarization (10, 13), which is mediated by apamin-sensitive small-conductance Ca2+-sensitive K+ (SK) channels (11, 15, 16) and likely Kv channels (31). Overexpression of the SK3 channel leads to an increase in bladder capacity and increased urine production, and suppression of SK3 channel expression leads to an increase in nonvoiding contractions (17).

The BK channel has a central role in UBSM function. BK channels are activated by membrane potential depolarization and by increases in cytosolic Ca2+ concentration (2, 9). BK channel activity is potently stimulated by ryanodine receptor-mediated Ca2+ sparks and may also be regulated by Ca2+ entry through VDCCs or P2X1Rs (15, 16). Activation of BK channels by Ca2+ sparks is attributable to local elevation of Ca2+ at RyR Ca2+ release sites. The repolarization and basal membrane potential of UBSM are regulated by activation of the BK channel (13). Phasic contractility of UBSM is enhanced by deletion of the β1-subunit of the BK channel (27). We recently demonstrated that ablation of the gene mslo1 for the poreforming subunit of the BK channel leads to the disappearance of BK currents in UBSM, an increase in phasic and nerve-evoked contractions, and an elevation of urination frequency (23). These results suggest that the loss of the BK channel is associated with overactive bladder and urinary incontinence.

In the current study, we examined the effects of deletion of mslo1 gene on urodynamic properties (bladder pressures and...
voided volumes) in freely moving mice. We also simulated the effects of parasympathetic nerve stimulation by applying muscarinic (carbachol) and purinergic (αβ methylene ATP) receptor agonists to UBSM strips. In the absence of the BK channel, urinary bladders demonstrated pronounced overactivity, an increase in intravesical pressure, pressure oscillations, and urine leakage. Electrical field stimulation elicited enhanced contractility at lower frequencies in UBSM strips from \( \text{Slo}^{+/-} \) mice, similar to the effect of blocking BK channels with iberiotoxin (IBTX) in strips from \( \text{Slo}^{+/+} \) mice. Consistent with the effects of BK channel deletion deriving, at least in part, from the lack of the channel in smooth muscle cells, muscarinic receptor-mediated contractions were enhanced in UBSM strips from \( \text{Slo}^{+/-} \) mice and demonstrated insensitivity to BK channel blockade with IBTX. We conclude that BK channels in UBSM have a key role in bladder function in vivo and that dysfunction of this channel leads to overactive bladder and incontinence.

**METHODS**

**Contractility studies.** Male and female mice were euthanized by a lethal injection of pentobarbital sodium (150 mg/kg ip) under the approval of the Office of Animal Care Management at the University of Vermont. Contractility experiments on urothelium-denuded UBSM strips were performed at 37°C, as previously described (17), using a MyoMed myograph system (MED Associates, Georgia, VT). Electrical field stimulation (EFS) of UBSM strips activates neurotransmitter release and UBSM contraction. Strips were contracted with an increasing frequency EFS protocol (frequencies of 0.5, 2, 3.5, 5, 7.5, 10, 12.5, 15, 20, 30, 40, and 50 Hz). Strips were then treated with iberiotoxin (IBTX; 100 nM) and the EFS protocol was repeated. UBSM EFS contractions were prevented by pretreatment with 1 μM tetrodotoxin (TTX).

**Fig. 1.** Nerve-evoked urinary bladder smooth muscle (UBSM) contractions in \( \text{Slo}^{+/-} \) and \( \text{Slo}^{+/-} \) mice. A and B: electrical field stimulation (EFS) of UBSM strips activates neurotransmitter release and UBSM contraction. Strips were contracted with an increasing frequency EFS protocol (frequencies of 0.5, 2, 3.5, 5, 7.5, 10, 12.5, 15, 20, 30, 40, and 50 Hz). Strips were then treated with iberiotoxin (IBTX; 100 nM) and the EFS protocol was repeated. UBSM EFS contractions were prevented by pretreatment with 1 μM tetrodotoxin (TTX). C: average contraction amplitudes for \( \text{Slo}^{+/-} \) and \( \text{Slo}^{+/-} \) UBSM strips at different frequencies of nerve stimulation. D: average EFS contraction amplitudes normalized to the 50-Hz maximum. E: frequencies required for 50% maximal contraction. *P < 0.05 vs. \( \text{Slo}^{+/-} \). #P < 0.05 vs. \( \text{Slo}^{+/-} \) + IBTX.
cal field stimulation (EFS) for 2 s was delivered with increasing frequency at 0.5, 2, 3.5, 5, 7.5, 10, 12.5, 15, 20, 30, 40, and 50 Hz. Stimulations were delivered with a 20-V amplitude, alternating polarity between pulses, 0.2-ms stimulation width, and at 3-min periods. These conditions evoke UBSM contractions that are completely eliminated by blocking neuronal sodium channels with 1 μM tetrodotoxin (14). In other experiments, increasing doses of the muscarinic agonist carbachol (CCh; 0.001–100 μM) at 12-min intervals were added to the bathing solution of UBSM strips pretreated for 12 min with tetrodotoxin (1 μM). IBTX (100 nM; Peptides International) was dissolved in water and added directly to the bathing solution 12 min before a second round of EFS or CCh dosing. Time control experiments were performed for EFS and CCh experiments in which no IBTX was added, providing the basis for which the effects of IBTX were measured. EFS contractions and CCh-induced phasic contractile activity were analyzed using MiniAnalysis (Synaptosoft).

Urodynamic measurements in conscious unrestrained mice. Cystometry of freely moving, conscious female mice was performed as described previously (17). We chose females to be used for cystometry to facilitate a comparison of our previous study (23) with the current study. Briefly, mice were anesthetized with isoflurane and a lower midline abdominal incision was made to expose the urinary bladder. A polyethylene catheter (PE-10) was inserted into the dome of the urinary bladder and routed subcutaneously to the back of the neck, where it was stored in a skin pouch. Following a 1-wk recovery period, the bladder catheter was exteriorized and the mouse was placed in a Small Animal Cystometry Lab Station (MED Associates) for urodynamic measurements. Sterile isotonic saline at room temperature was continuously infused into the bladder at a rate of 25 μl/min. An analytic balance beneath the wire-bottom animal cage measured the amount of urine voided during continuous bladder filling. Cystometrograms (CMGs) were measured over a period of 1 h, simultaneously recording intravesical (bladder) pressure, infused and voided bladder volumes. Urodynamic parameters from multiple CMGs measured from a mouse were averaged to provide a representative measure of bladder function. Pressure oscillations were defined as rises in bladder pressure that exceeded 5 mmHg. At the end of the experiment, the mice were euthanized with pentobarbital sodium (150 mg/kg ip) followed by decapitation.

Fig. 2. Carbachol-induced contractions of UBSM strips from Slo+/− and Slo−/− mice. A and B: contraction of Slo+/− and Slo−/− UBSM strips, respectively, to increasing concentrations of the muscarinic agonist carbachol (0.001–100 μM) in the presence of 1 μM TTX. Phasic contraction frequency (C) and amplitude (D) are increased in Slo−/− vs. Slo+/− UBSM strips. IBTX potentiates carbachol-induced phasic contractions in Slo+/− (E) but not Slo−/− (F) UBSM strips (*P < 0.03).
Results

Enhancement of nerve-mediated contractions of Slo−/− UBSM strips. Urine voiding (micturition) is caused by stimulation of parasympathetic nerves in the bladder wall. To explore the role of BK channels in nerve-mediated urinary bladder contraction, we used EFS (0.5–50 Hz) to selectively evoke neurotransmitter release in UBSM strips, thereby mimicking the excitation that occurs during micturition. Isolated UBSM strips from both Slo+/+ and Slo−/− mice respond to increasing EFS frequencies with increasing contractile force (Fig. 1A–C). UBSM strips from Slo−/− mice exhibit a very significant elevation in nerve-mediated contractile force at frequencies below 20 Hz (Fig. 1C). However, the maximal contractile force elicited in the same strips by 60-mM K+-induced membrane depolarization is not different [Slo+/+ 5.1 ± 0.7 mN (n = 11); Slo−/− 4.5 ± 0.6 mN (n = 20)]. Inhibition of BK channels in Slo+/+ strips with IBTX also enhances nerve-evoked contractions (Fig. 1A). These results demonstrate the dynamic range of nerve-mediated responses that are potentiated in Slo−/− strips and extend our previous report that 20-Hz-evoked contractions of Slo−/− UBSM strips are about threefold greater than those of Slo+/+ strips (23).

To examine the dependence of nerve-mediated contractile force on EFS stimulation frequency, evoked amplitudes were normalized to the maximal force elicited by a 50-Hz EFS.

UBSM strips from Slo−/− mice were more sensitive to nerve-evoked stimulation, exhibiting a lower half-maximal EFS frequency than strips of Slo+/+ mice [Slo−/− 8.3 ± 0.9 Hz (n = 20); Slo+/+ 19.1 ± 1.8 Hz (n = 11); P < 0.05]. IBTX left-shifted the half-maximal EFS frequency of Slo+/+ strips from 16 ± 2 to 10 ± 1 Hz (n = 6, P < 0.05), with no effect on Slo−/− UBSM strips (n = 11; Fig. 1B). The increased contractile force and lower half-maximal contraction frequency demonstrate the increased sensitivity of UBSM to nerve-mediated stimulation in the absence of BK channels.

Enhancement of muscarinic contractions in Slo−/− UBSM strips. Stimulation of parasympathetic nerves in the bladder releases both acetylcholine and ATP onto the smooth muscle cells to cause contraction (25, 29). To bypass the nerves, muscarinic receptors of Slo+/+ and Slo−/− UBSM strips were stimulated directly with CCh, while inhibiting neuronal activity with tetrodotoxin. Two cumulative concentration-response curves to CCh were obtained in each Slo+/+ and Slo−/− strip. This was performed with, or without (time control), IBTX addition before the second CCh concentration-response curve. A leftward shift in the CCh concentration-response curve was observed in Slo−/− strips compared with Slo+/+ strips [apparent Kd of 180 nM for Slo−/− strips (n = 8); apparent Kd of 730 nM for Slo+/+ strips (n = 9)].

Both the frequency and amplitude of phasic contractile events induced by CCh were significantly enhanced in Slo−/−
mice (Fig. 2, C and D). The increase in phasic frequency was significant (P < 0.05) at CCh concentrations above 0.01 μM, and the amplitude of phasic contractions was enhanced at concentrations above 0.1 μM in Slo−/− mice. IBTX mimicked the effect of knocking out the mSlo1 gene, increasing the amplitude of phasic contractions in Slo+/- strips (Fig. 2E). The effect of IBTX was observed at all concentrations of CCh when comparing time control (n = 13) vs. IBTX-treated (n = 15) strips. In the absence of mSlo1 gene product, application of IBTX had no effect on CCh-induced phasic contractions (Fig. 2F).

Inhibition of BK channels with IBTX potentiates purinergic UBSM contractility of Slo+/- but not Slo−/− mice. We investigated the role of BK channels in modulating purinergic contractions of UBSM. As ATP is readily hydrolyzed by the activity of extracellular ATPases, we utilized the nonhydrolyzable ATP analog α,β methylene ATP (αβmATP). αβmATP evoked a rapid, desensitizing contraction of UBSM strips from both Slo+/- and Slo−/− mice (Fig. 3), and as a result cumulative dose-response curves to increasing concentrations of αβmATP were not feasible. Therefore, we compared purinergic contractions elicited by αβmATP (10 μM), applied twice to each strip. Between applications of αβmATP, the strips were washed three times over 36 min to allow for the purinergic receptors to recover from desensitization. In time control experiments of both Slo+/- and Slo−/− strips, the second αβmATP contraction amplitude was reduced relative to the first (Slo+/- 81 ± 10% of control, Slo−/− 85 ± 13% of control; Fig. 3C). IBTX applied to Slo+/- strips

Table 1. Urodynamic properties of Slo+/- and Slo−/− mice

<table>
<thead>
<tr>
<th></th>
<th>Slo+/- (n = 8)</th>
<th>Slo−/− (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micturation</td>
<td>31.1 ± 1.5</td>
<td>32.3 ± 5.1</td>
</tr>
<tr>
<td>Threshold</td>
<td>18.9 ± 1.9</td>
<td>20.4 ± 5.8</td>
</tr>
<tr>
<td>Average</td>
<td>10.9 ± 0.7</td>
<td>14.5 ± 1.1*</td>
</tr>
<tr>
<td>Minimum</td>
<td>4.8 ± 0.4</td>
<td>6.5 ± 0.5*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Slo+/- (n = 8)</th>
<th>Slo−/− (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pressure Oscillations</td>
<td>Frequency, min</td>
</tr>
<tr>
<td>Slo+/- (n = 8)</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Slo−/− (n = 8)</td>
<td>2.6 ± 0.5*</td>
</tr>
</tbody>
</table>

| Slo+/- (n = 8) | Infused | 230 ± 29 |
| Slo−/− (n = 4)* | Voided | 98 ± 17 |
| Slo−/− (n = 4)* |        | 68 ± 13* |

Values are means ± SE. *P < 0.05, †4 of 8 Slo−/− mice where discrete voided volumes were measurable and remaining 4 mice demonstrated a continuous urine dripping (void volumes ~20–30 μl).

Fig. 4. Urodynamic profile of conscious, freely moving Slo+/- and Slo−/− mice during continuous infusion of saline into the bladder. A: cystometrograms from Slo+/- and Slo−/− mice demonstrating higher bladder pressure, increased pressure oscillations, and a reduced bladder capacity in Slo−/− mice. B: increased average and minimum pressures in Slo−/− mice. C: increased pressure oscillations in Slo−/− mice. D: reduction in infused and voided volumes in Slo−/− mice. Data shown are averaged from 4 of 8 Slo−/− mice in which distinct micturition events were discernable. The remaining 4 Slo−/− mice demonstrated a constant dripping during bladder filling, i.e., void volumes <30 μl (*P < 0.05).
before the second \(\alpha\beta\text{mATP} \) contraction potentiated the second contraction amplitude \((118 \pm 6\% \text{ of control})\), where as there was no effect of IBTX in \(\text{Slo}^{-/-} \) UBSM strips \((68 \pm 14\% \text{ of control})\); Fig. 3).

\(\text{Slo}^{-/-} \) mice have increased bladder pressures, increased frequency of pressure oscillations, and urine leakage. UBSM strips from \(\text{Slo}^{-/-} \) mice have increased phasic and nerve-evoked contractions and increased urination frequency (Figs. 1 and 2) (23). To examine the in vivo urodynamic consequences of the loss of the BK channel, cystometry was performed on conscious, freely moving mice. \(\text{Slo}^{+/+} \) mice demonstrated an urodynamic profile consistent with previous reports of wild-type mice recorded under identical conditions (Table 1) (17), a 200-\(\mu\)l bladder capacity and a low frequency of nonvoiding pressure oscillations (Fig. 4, A and C). In stark contrast, \(\text{Slo}^{-/-} \) mice demonstrated wild oscillations in bladder pressure (Fig. 4C). Furthermore, average and minimum pressures during filling were significantly increased (Fig. 4B; Table 1). Bladder capacity was reduced to 43% in \(\text{Slo}^{-/-} \) mice (Fig. 4D; Table 1). In four of eight of the \(\text{Slo}^{-/-} \) mice, distinct micturition/urination events were discernable. In these mice, infused and voided volumes were measured to be 98 \pm 17 and 68 \pm 13 \(\mu\)l, respectively \((n = 4)\). In the remaining \(\text{Slo}^{-/-} \) mice, a continuous dripping of urine \((20-30-\mu\)l voids) was recorded simultaneously with pressure oscillations (Fig. 4A). These data demonstrate that the increased myogenic and neurogenic contractile force, and increased sensitivity to nerve-mediated stimulation of isolated USBM strips, translates to in vivo bladder dysfunction.

**DISCUSSION**

The principal finding of this study is that, in vivo, in the absence of the BK channel, \(\text{Slo}^{-/-} \) mice exhibit heightened bladder pressures and a high frequency of pressure oscillations, accompanied by small volume urine loss and reduced bladder capacity (Fig. 4; Table 1). UBSM strips from \(\text{Slo}^{-/-} \) mice exhibit an increase in the sensitivity to nerve stimulation (Fig. 1) and to exogenously applied muscarinic receptor agonist (Fig. 2). In a very significant way, this study extends our previous findings (23), which demonstrated a lack of BK channels in UBSM, elevated basal phasic and nerve-evoked contractions at 20 Hz, and increased urination frequency.

BK channels play a central role in UBSM excitability and contractility. Block of BK channels with IBTX depolarizes the membrane potential, prolongs the action potential in UBSM (13), and causes a substantial increase in phasic contractions (12, 14, 27). Phasic contractions are thought to be myogenic in origin, with an elevation of phasic contractions translating to detrusor overactivity, elevated bladder pressures and pressure oscillations, and urine leakage (incontinence) (3).

The enhancement of nerve-evoked contractions by IBTX and in \(\text{Slo}^{-/-} \) mice in this study, and Meredith et al. (23), could be explained by the absence of the BK channel in the smooth muscle. Nerve-evoked contractions are mediated by the release of acetylcholine and ATP from varicosities. To examine further the role of the detrusor smooth muscle, effects of bath applied CCh and \(\alpha\beta\text{mATP} \) on smooth muscle contractility were assessed in the absence of nerve stimulation. Phasic contractions from \(\text{Slo}^{+/+} \) mice induced by CCh were enhanced by IBTX. This enhancement was mimicked by the loss of BK channels in \(\text{Slo}^{-/-} \) mice in the absence of IBTX (Fig. 2). Purinergic stimulation was difficult to assess because the receptors rapidly desensitize and therefore do not provide a steady measurable level of force. However, it was clear that \(\alpha\beta\text{mATP} \)-induced contractions of \(\text{Slo}^{+/+} \) responded significantly to IBTX, whereas strips from \(\text{Slo}^{-/-} \) did not (Fig. 3). Collectively, our results indicate that the loss of the BK channel in the urinary bladder smooth muscle can explain in vivo bladder overactivity and incontinence in \(\text{Slo}^{-/-} \) mice.

Our results, however, do not exclude a contribution from BK channels in other cell types within these urothelial denuded detrusor strips, such as parasympathetic nerves. Although BK channels have been identified in other types of parasympathetic nerves (6), it is not known whether they exist in the nerve fibers in these isolated UBSM strips. If present in nerve fibers, loss of BK channels could 1) increase nerve excitability, due to the loss of the hyperpolarizing \(K^{+} \) conductance; or 2) decrease nerve excitability by an inactivation of sodium channels resulting from a more depolarized basal membrane potential (28).

Our in vivo results are consistent with loss of BK channels from UBSM accounting for the bladder overactivity and incontinent phenotype observed in \(\text{Slo}^{-/-} \) mice; however, effects on sensory nerves or more central processes cannot be excluded.

Overactive bladder and urine incontinence are a complex, major health issue and yet are poorly understood. In humans, detrusor overactivity can lead to diminished functional capacity (24), a marked feature of \(\text{Slo}^{-/-} \) mice (Table 1, Fig. 4). Our results indicate that the BK channel has a central role in the regulation of urinary bladder function, and its dysfunction leads to overactive bladder and incontinence. Consistent with this, injection of \(h\text{Slo} \) cDNA into rat urinary bladder decreases bladder overactivity induced by partial outlet obstruction (8). The loss of the smooth muscle-selective BK channel regulatory subunit, the \(\beta_{1}\)-subunit, also increases urinary bladder contractility (27) and supports the importance of BK channel in UBSM function. Our results, reported in this study and Meredith et al. (23), provide compelling in vitro and in vivo evidence that the BK channel is an important target in the treatment of overactive bladder and incontinence.

**ACKNOWLEDGMENTS**

We thank Drs. G. Petkov, M. Werner, and W. Martin for comments on the manuscript and advice.

**GRANTS**

The work was supported by National Institutes of Health Grants DK-5R01-DK-053832 and IR01-DK-065947, Howard Hughes Medical Institute, Canadian Institute for Health Research, and Alberta Heritage Foundation for Medical Research.


**REFERENCES**


