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Translational Regulation of Rat Multidrug Resistance-associated Protein 2 (Mrp2) Expression is Mediated by Upstream Open Reading Frames (uORFs) in the 5' Untranslated Region (UTR)

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Abstract

Multidrug resistance-associated protein 2 (Mrp2/Abcc2), an organic anion transporter present in the apical membrane of hepatocytes, renal epithelial cells and enterocytes, is postulated to undergo translational regulation. Transcription of rat hepatic Mrp2 mRNA is initiated at multiple sites (-213, -163, -132 and -98 nucleotides relative to the Mrp2 ATG) and contains potential upstream open reading frames (uORFs) in the 5' untranslated region (UTR) starting at -213, -149 and -109 nucleotides. Ribonuclease protection assays demonstrated that transcription of the Mrp2 gene at the various initiation sites was tissue-specific, with the major initiation site in the liver and kidney being -98 and -132 nucleotides, respectively. In the jejunum, the primary and secondary initiation sites were -98 and -132 nucleotides, respectively, with the converse true in the ileum. The relative abundance of these Mrp2 transcripts expressed in tissues varied with age from birth to the adult. HepG2 transient expression assays and *in vitro* translation assays in which the 5' UTRs were fused with a luciferase reporter showed that the 5' UTR without any uORF (-98 nucleotide) expressed maximal luciferase activity compared to those with one (-132 nucleotides), two (-163 nucleotides) or three (-213 nucleotides) uORFs. Disruption of the uORF by site-directed mutagenesis at nucleotide -109 enhanced luciferase activity 2-3-fold, whereas disruption of the uORF at nucleotide -149 had little effect. We conclude that among the uORFs in the Mrp2 5' UTR, the uORF starting at nucleotide -109 likely plays an important role in the regulation of Mrp2 protein expression.

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The multidrug resistance-associated protein 2 (rat Mrp2 or human MRP2), a member of the ATP-binding cassette gene superfamily of transport proteins, is present in the apical membrane of hepatocytes, enterocytes and renal proximal tubules. Mrp2 protein mediates efflux of organic anions such as glutathione, glucuronide and sulfate conjugates against a concentration gradient from hepatocytes into bile. (Gerk and Vore, 2002; Jansen et al., 1987; Konig et al., 1999; Paulusma et al., 1996), and also contributes to bile flow by mediating the canalicular excretion of glutathione (GSH) (Ballatori and Truong, 1992).

Regulation of Mrp2 expression has been characterized primarily at the transcriptional level in rats and mice. Exposure to ligands for the nuclear receptors, such as Nrf2, CAR and PXR, increases Mrp2 protein expression in rat primary hepatocyte cultures (Kast et al., 2002), while studies in mice have shown that CAR and Nrf2 agonists, but not PXR agonists, increase Mrp2 mRNA expression (Maher et al., 2005; Wagner et al., 2005), supporting the importance of transcriptional regulation. However, in rats treated with pregnenolone-16α-carbonitrile (PCN), a PXR agonist, hepatic Mrp2 mRNA expression is unchanged, whereas Mrp2 protein expression is increased 2-3 fold (Johnson et al., 2002; Johnson and Klaassen, 2002; Jones et al., 2005). Ethinylestradiol treatment markedly decreases Mrp2 protein in rat liver, while Mrp2 mRNA remains unchanged (Trauner et al., 1997). Similarly, hepatic Mrp2 protein in the pregnant rat is significantly decreased by 50%, while Mrp2 mRNA is unchanged (Cao et al., 2001; Cao et al., 2002). Along the rat small intestine, Mrp2 protein is decreased by 90% in the distal ileum relative to that in the jejunum, whereas Mrp2 mRNA does not change significantly (Mottino et al., 2000). The inconsistency between the changes in Mrp2 mRNA and protein expression

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indicates that under some conditions, rat Mrp2 protein expression undergoes post-transcriptional regulation.

Post-transcriptional regulation of protein expression can occur through changes in mRNA stability, in the rate of protein degradation, or in the rate of protein synthesis. The minimal changes in Mrp2 mRNA expression in female control, pregnant and PCN-treated rats argue against significant differences in Mrp2 mRNA stability as likely to contribute to the mechanism of post-transcriptional translation of rat Mrp2 that could account for the 4-5-fold differences in Mrp2 protein expression among these groups. We recently showed that altered rates of hepatic Mrp2 protein degradation cannot explain the differences in its protein expression in control, pregnant and PCN-treated rats, whereas decreased and increased rates of Mrp2 protein synthesis were observed in pregnant and PCN-treated rats, respectively (Jones et al., 2005). In the present studies, we therefore focused on the potential mechanism for translational regulation of Mrp2 protein synthesis.

Accumulating evidence indicates that upstream open reading frames (uORFs) are important regulators of mRNA translation (Gray and Wickens, 1998; Morris and Geballe, 2000; van der Velden and Thomas, 1999), which can be explained by the ribosomal scanning model. Translation of the downstream main open reading frame (ORF) of a gene by ribosomes occurs through leaky scanning of any AUGs in the 5' untranslated region (5' UTR) when the sequence around the upstream AUGs is suboptimal (Kozak, 1986), or through reinitiation when the translation machinery is not dissociated from the mRNA chain after termination of translation of uORFs (Morris and Geballe, 2000). We identified four transcription initiation sites in rat hepatic

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Mrp2 cDNA that occur at -213, -163, -132, and -98, where the ATG of the Mrp2 coding gene is numbered +1, +2, and +3 (Jones et al., 2005) (FIG. 1A). In the present study, we fused these Mrp2 5' UTRs upstream of the luciferase reporter gene and investigated their effect on luciferase expression in HepG2 cells in transient expression assays and on the translation efficiency of the luciferase transcript in *in vitro* translation assays. We also used ribonuclease protection assay (RPA) to identify the transcription initiation sites in rat liver, kidney and small intestine, and during postnatal development. We found that these tissues utilize different Mrp2 transcription initiation sites, and that translation from these transcripts is greatly influenced by the presence of the uORF at -109 nucleotides.

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Materials and methods

Materials

α-^{32}P-UTP (800 Ci/mmol) and ^{35}S-methionine (1000 Ci/mmol) were obtained from Perkin Elmer Life and Analytical Sciences (Boston, MA). Unless otherwise noted, all other chemicals were of analytical grade and of cell culture grade from Sigma Chemical Co. (St. Louis, MO), Invitrogen™ life technologies (Carlsbad, CA), Roche Diagnostics (Indianapolis, IN) and Fisher Scientific (Pittsburgh, PA). Restriction enzymes were obtained from Invitrogen and Promega (Madison, WI).

Animals

Adult female Sprague Dawley rats whose weights were 215±25 g were obtained from Harlan Industries (Indianapolis, IN). The rats had free access to water and food and were maintained on an automatically timed 12-h light/12-h dark cycle. All experimental protocols involving animals were approved by the Institutional Animal Care and Use Committee of the University of Kentucky, and conducted following National Institutes of Health guidelines for the care and use of laboratory animals. In order to determine postnatal changes, female pups were removed at various times after birth, and tissues were immediately removed and frozen in liquid nitrogen until isolation of RNA.

Ribonuclease protection assay (RPA)

Postnatal and control (adult) rat liver, kidney, placenta, lung, and small intestine were removed immediately after decapitation and frozen in liquid nitrogen. Total RNA was isolated using TRIzol™ reagent (Invitrogen) following the manufacturer's instructions.

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The luciferase control vector (Invitrogen) was fused at cloning sites Hind III and BamHI with the Mrp2 5'UTR cDNA sequence starting at -1 to -214 relative to the ATG (numbered as +1, +2, and +3) of the *Mrp2* coding region. A double-stranded 280 bp fragment containing the T7 promoter and the *Mrp2* 5' UTR was purified after the fusion luciferase vector was digested with PvuII and BamHI. α-^{32}P-UTP labeled Mrp2 probe was prepared according to the instructions of MAXIscript T7 kit (Ambion, Austin, TX), using the 280 bp fragment as template. RPA was performed following the procedure of RPA III Kit (Ambion). Briefly, total RNA was incubated with the Mrp2 probe in the mixture containing 0.5M ammonium acetate and 2.5 volumes of ethanol. Following co-precipitation of the probe with total RNA after incubation at -80°C for 90 min, the RNA pellet was washed once with 75% ethanol and dissolved in hybridization solution. The hybridization reaction was incubated at gradient annealing temperature from 56°C to 36°C at the rate of 2°C per 2 hr. Single-stranded RNA was digested by RNAse A/T1 mix at 37°C for 1 hr. The fragments protected from RNAse digestion were identified by electrophoresis on 6% polyacrylamide, denaturing gels.

Plasmid construction

The Mrp2 5'UTR cDNA is shown in FIG. 1A. cDNA sequences of the wildtype Mrp2 5'UTRs (FIG. 1B), L, M1, M2, and S1, were PCR amplified using the forward primers TRF1, TRF2, TRF3, and TRF4, respectively (Table 1). The reverse primer for L and M1 PCR amplification was CONR. The reverse primer for amplifying M2 and S1 was T7R1. 5' UTRs cDNA were ligated upstream of the ATG of the firefly luciferase reporter gene into the pGL3 control vector (Promega) for transient co-transfection assays in HepG2 cells, and into the T7 control vector (Promega) for *in vitro* translation assays.

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cDNA sequences of the 5' UTRs, deL, deM, and S2 were PCR amplified using forward primers TRF1, TRF2, and TRF5, respectively, and the reverse primer TRRR that deleted 1 nucleotide from T7R1 (Table 1), resulting in uORF^{-109} being in-frame with the luciferase reporter gene ORF. These fragments were cloned into the T7 control vector (Invitrogen).

uORFs were disrupted by introducing a point mutation into start codons, ATG→AAG, using the corresponding wildtype constructs as templates. The point mutations of the nucleotides T at -148 and -108 to A were termed "a" and "b", respectively; and the mutation of the Kozak motif flanking the ATG at -109 was termed "c" (FIG.1B). All mutagenesis was performed according to the manufacturer's instructions of Quick-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). All the structures of plasmids were confirmed by DNA sequencing.

HepG2 cell transient co-transfection assays

HepG2 cells were cultured in DMEM/F12 (1:10 medium) supplemented with 10% charcoal-stripped fetal bovine serum (Hyclone Laboratories, Logan, UT), 3.58 mM glutamine, 55 μg/ml gentamycin, and 1 μg/ml insulin (Invitrogen). One day before transfection, culture medium was replaced by phenol red-free DMEM supplemented with 10% charcoal stripped FBS, glutamine and gentamycin. The plasmids (1 μg) were transfected by the ProFectin mammalian transfection system-calcium phosphate (Promega) into HepG2 cells together with 30 ng pSV40-Ren (Promega), used as an internal control for transfection efficiency. After 5-6 h incubation, the transfection medium was replaced with maintenance medium. Cells were harvested 24 h later for measurement of the firefly and *Renilla reniformis* luciferase activities by the Dual-luciferase

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reporter assay system (Promega). The firefly luciferase activity was normalized to *Renilla reniformis* luciferase activity.

In vitro translation assays

Each tested Mrp2 5'-UTR-luciferase construct was linearized by PvuII and SacI. Capped and α-^{32}P-UTP-labeled firefly luciferase transcripts that were fused with Mrp2 5'UTRs, were synthesized *in vitro* with the PvuII-SacI fragments as templates, according to the manufacturer's instructions of mMESSAGE mMACHINE T7 kit (Ambion). Transcription efficiency was quantified by scintillation counting of ^{32}P incorporation into RNA. The integrity and size of the luciferase transcripts were verified by formaldehyde-agarose gel electrophoresis. The luciferase protein was synthesized from the capped luciferase transcripts *in vitro*, according to the manufacturer's procedure of Rabbit Reticulocyte Lysate System (Promega). Briefly, 0, 2, 4, 10, or 20 ng of the luciferase transcript was added to a reaction mixture. The translation reaction was immediately incubated at 30 °C for 60 min and terminated by moving onto ice. The firefly luciferase activity was measured by the Luciferase Assay System (Promega) according to the manufacturer's instructions.

In vitro expression in Coupled Transcription/translation system

The TNT quick coupled transcription/translation system (Promega) was used to translate the luciferase protein using tested constructs as templates. According to the manufacturer's instructions, a reaction mixture (50 µl) containing 40 µl TNT Master Mix, 2 µl of ^{35}S-methionine was incubated at 30 °C for 90 min. The translated products were separated on 4-20% gradient

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denaturing SDS-PAGE and data processed using the STORM 840 Phosphoimager (Molecular Dynamics).

Data calculation

RPA bands were quantified by densitometry using Quantity One 1-D Analysis Software (Bio-Rad). Linear regression analysis was performed by Prism 4.0 (GraphPad Prism Software).

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Results

Identification of rat Mrp2 transcription initiation sites and their abundance in rat tissues by RPA

Four transcription initiation sites have been identified at -213, -163, -132, and -98 in the rat hepatic Mrp2 5' UTR (FIG. 1A) (Jones et al., 2005). We next investigated the transcription initiation sites in various rat tissues to determine if their use might be tissue-specific. Four transcription initiation sites were detected in rat liver, with the site at -98 as the primary site, and the site at -132 as the secondary site (FIG. 2). The transcription initiation sites in other tissues were different from that in the liver. In the placenta and kidney, the primary site was located at -132, while other sites were not detected. The primary site in the lung was at -98, while other transcription initiation sites were not detected. In the jejunum, the primary and secondary sites were located at -98 and -132, but were located at -132 and -98 in the ileum, respectively, while other sites were not detected.

We next investigated whether utilization of the transcription initiation sites might vary with age. In the liver, the ratio of expression of the transcript starting at -132 to the transcript starting at –98 was 0.73 ± 0.06 at Day 0, increased to 1.06 ± 0.10 at Day 10, and then decreased to 0.80 ± 0.03 in the adult (FIG. 3A). In the kidney, the ratio was similar at Day 0 and Day 6 (about 4), and increased to 13.4 ± 1.2 in adulthood (FIG. 3B). In the jejunum, the ratio was 1.2 ± 0.19 at Day 0, increased to 1.5 ± 0.02 at Day 20, and then decreased to 0.87 ± 0.1 in the adult (FIG. 3C). In the ileum, the ratio was very similar from Day 0 to adult hood (0.70 – 0.85) (FIG. 3D). The data indicated that the changes in expression of Mrp2 transcripts with age were relatively minor compared to those among the tissues.

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Effect of the rat Mrp2 5'UTR on expression of the luciferase reporter gene in transiently transfected HepG2 cells

In order to determine whether the various 5' UTRs differentially influenced Mrp2 protein expression, the effect of the Mrp2 5' UTR on expression of the firefly luciferase reporter gene was determined in transiently transfected HepG2 cells. Fusion plasmids were constructed by inserting the Mrp2 5' UTRs into the pGL3 control vector immediately upstream of the luciferase reporter gene. In addition, the wildtype 5' UTRs were altered by disruption of the uORFs to determine the influence of the uORFs on protein expression. Fusion plasmids were transiently co-transfected individually with pSV40-Ren into HepG2 cells, and firefly luciferase activity normalized to Renilla luciferase activity of cell extracts.

Compared to pGL3 control vector without any Mrp2 5' UTR (FIG. 4A), L decreased the luciferase activity by 60%, whereas L^{b} increased the luciferase activity 2-fold relative to L. L^{a+b} did not change luciferase activity relative to L^{b}. Similarly, the wildtype M1 expressed the lowest luciferase activity (25% of pGL3) (FIG. 4B). $M1^{b}$ increased the luciferase activity 3-fold, compared to M1, whereas $M1^{a+b}$ resulted in similar luciferase activity as $M1^{b}$. Taken together, these data implied that the $uORF^{-109}$ was more important in regulation of expression compared to the $uORF^{-149}$.

Effect of the rat Mrp2 5' UTR on the translation efficiency of the luciferase transcript by in vitro translation assays

Since the luciferase protein expression in HepG2 cells required both transcription and translation processes, we next investigated the influence of the various rat *Mrp*2 5' UTRs on the translation

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efficiency of mRNA. The capped luciferase transcripts were prepared using PvuII-SacI fragments as templates in which Mrp2 5' UTRs were located immediately upstream of the luciferase. The capped luciferase transcripts were added to the rabbit reticulocyte lysate to determine the effect of 5'UTRs on translation efficiency under conditions of linearity with respect to transcript concentration. Translation efficiency was calculated from the linear relationship between the luciferase activities and mRNA concentrations.

Translation efficiency of the capped luciferase transcripts with S1, M2, and L was 67-, 37-, and 15-fold higher than that of M1 (FIG. 5A). We next investigated contributions of the uORFs to the marked differences in translation efficiency of the various transcripts. L^b increased translation efficiency 3-fold, whereas L^a decreased translation efficiency 80%, compared to the wildtype L (FIG. 5B). Translation efficiency of wildtype M1 was 31-fold and 6-fold lower than that of $M1^b$, and $M1^a$ (FIG. 5C). Compared to the wild-type M2, $M2^b$ increased translation efficiency 4-fold, while disruption of the Kozak motif in $M2^c$ increased translation efficiency only 1.7-fold (FIG. 5D).

In vitro expression in Coupled Transcription/Translation system

The marked effect of the $uORF^{-109}$ on translation efficiency suggested that this uORF serves as a translation start site. To determine if translation could be initiated at $uORF^{-109}$, we inserted the *Mrp2* 5' UTRs into the T7 control vector in such a way that the $uORF^{-109}$ was in-frame with the luciferase ORF. When the plasmids containing deL and deM1 were used as templates, a peptide of a higher molecular weight was produced in the coupled transcription/translation system (Lanes 1 and 5, FIG 6). The higher molecular weight peptide was not produced when the plasmid

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containing S2 was used as template (Lane 3), or when the uORF^{-109} in deL and deM1 was disrupted (Lanes 2 and 4, FIG 6). Detection of a higher molecular weight peptide when wildtype plasmids containing the uORF^{-109} were used as templates indicated that the AUG at -109 can be used as an efficient translation initiation site.

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Discussion

This study shows for the first time that 1) the Mrp2 5' UTRs are involved in translational regulation of Mrp2 protein expression and 2) the uORF^{-109} has an inhibitory effect on translation. Disruption of the uORF^{-109} abolished the inhibitory effect of the wild type 5' UTRs (-213, -163, and -132) on translation of the luciferase reporter gene in HepG2 cell transient transfection assays (FIG. 4) and *in vitro* translation assays (FIG. 5B, C, D). These data suggested that translation was efficiently initiated at the uORF^{-109}, and was confirmed by demonstration of translation of a higher molecular weight peptide when this uORF was fused in-frame with the luciferase ORF (Fig 6). Thus, the absence of uORF^{-109} explains why the luciferase transcript containing S1-5' UTR was translated much more efficiently than were the luciferase transcripts containing L-, M1-, and M2-5' UTR (FIG 5). Recognition and translation initiation of ribosomes at the AUG^{-109} was also indirectly verified by disruption of the Kozak motif, since the mutation G***A/T***GAATG***G/T***A increased translation efficiency compared to the wildtype M2-5' UTR (FIG. 5D).

There are three upstream open reading frames (uORFs) in the 5' UTR of Mrp2. The first uORF, uORF^{-213}, is in-frame with the Mrp2 ORF, whereas uORF^{-109} and uORF^{-149}are out-of-frame with the Mrp2 ORF. The uORF^{-109} has a perfect Kozak motif, and overlaps the Mrp2 coding region by terminating at +60. Therefore, translation of the downstream *Mrp2* ORF must occur through partially leaky scanning of the uORF^{-109}, despite its perfect Kozak motif, not through reinitiation of ribosomes. That is, a portion of the ribosomes must bypass the AUG at -109 in order to translate the Mrp2 ORF. In addition to leaky scanning, there are other possible mechanisms for the uORF^{-109} to exert an inhibitory effect on translation of Mrp2 protein. As pointed out by

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Morris (Morris and Geballe, 2000), nascent peptides encoded by uORFs can mediate regulation through interfering with translation elongation or termination. As a result, ribosomes stall and translation of the downstream ORF is inhibited. These mechanisms have been identified with the peptide products of various prokaryotic and eukaryotic uORFs (Cao and Geballe, 1996; Lovett and Rogers, 1996). In the present study, we truncated the native uORF^{-109} at -1, by fusing it to the luciferase gene, so that the inhibitory effect of the uORF^{-109} could be exerted by competition for ribosomes with the downstream luciferase ORF, or by the sequence-dependent information in the first 36 amino acids of the nascent peptide. We are currently investigating whether the entire peptide of 56 amino acids encoded by the uORF^{-109} might mediate any sequence-dependent regulation of translation of Mrp2 protein.

Disruption of the uORF^{-149} alone (FIG 5B,C,D) or together with disruption of the uORF^{-109} (FIG. 4) showed a minimal effect on translation in *in vitro* translation assays or in transiently transfected HepG2 cells, indicating that the AUG at -149 is successfully bypassed by ribosomes, most likely because of the absence of the Kozak sequence. Since the AUG at -213 is the first codon at the 5' end of the Mrp2 messenger, and ribosomes poorly recognize AUG start codons close to the 5' end of a cDNA (Kozak, 1991), it is unlikely that the 40S subunits can recognize it and initiate translation. Therefore, we did not investigate this AUG start codon. Taken together, the data indicate that among the three AUG start codons identified in the Mrp2 5' UTR, only the AUG at -109 serves as an efficient translation initiation site and plays an important role in the translational regulation of Mrp2 protein expression.

The distance between the cap site and the initiation codon is very important for recognition of the uORF by 40S subunits. A study of mammalian S-adenosylmethionine de-carboxylase (AdoMetDC) shows that recognition of the single uORF in the AdoMetDC 5' UTR in nonlymphoid cells is increased by extending the space between the cap site and the upstream AUG codon from 14 to 47 nucleotides, leading to suppression of translation (Ruan et al., 1994). The distance between the cap sites and the AUG at -109 in L, M1, and M2 5' UTR are 104, 54, and 23 nucleotides, respectively. The order of suppression of translation of the four wildtype *Mrp2* 5' UTRs from high to low is M1, L, M2, and S1 (FIG. 5A). This implies that recognition and translation initiation of the AUG at -109 are most efficient when there are 54 nucleotides between the cap and the initiation site, as in the transcript M1, but less efficient when the distance is reduced to 23 nucleotides (i.e., M2) or increased to 104 nucleotides (L). It is likely that extending the distance from 23 to 54 nucleotides gives the 40S subunits the time needed to recruit additional translational factors, resulting in more efficient recognition. However, we cannot explain at this time why extending the distance from 54 (M1) to 104 (L) nucleotides impairs recognition, although potential secondary structures formed by the additional sequence could act to decrease translation efficiency.

We also showed for the first time that the transcription initiation sites were differentially used in the rat liver, kidney, small intestine, lung and placenta, and that their use varied with age in the liver, kidney and jejunum and ileum (FIG 2, 3). In the jejunum, the primary transcription initiation site was -98, with -132 as the secondary site, while in the ileum, the primary site was -132 and the secondary site was -98. Considering the fact that Mrp2 protein expression is decreased 90% in the distal ileum relative to that in the jejunum, with mRNA unchanged

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(Mottino et al., 2000), these data suggest that the uORF^{-109} exerts an inhibitory effect on translation of Mrp2 protein expression in the ileum. In the kidney, the transcription initiation site at -98 was barely detectable, with the site at -132 predominating. In the liver, the site at -98 was the primary site; however, the longer transcripts could all be detected. Low expression of Mrp2 protein in kidney may therefore be partially due to suppression of translation of Mrp2 by the uORF^{-109}. Further studies are needed to understand the basis and physiologic implications for use of multiple transcription initiation sites by *Mrp2*, and why their use varies among tissues, and to a lesser extent, with age. Our early studies demonstrated that use of transcription start sites in rat liver was not altered by treatment with PCN or in pregnancy, implying that this is a fundamental property of the gene that is not readily modified.

The function of the uORF in rat Mpr2 is not known. However, this feature is conserved in human MRP2, which also has an uORF with a perfect Kozak motif at -105 nucleotides; interestingly, the major transcription start site in human MRP2 occurs at -247 nucleotides (Tanaka et al., 1999), implying that the uORF in MRP2 could also be important in regulating MRP2 protein expression. Analysis of murine Mrp2 gene (Genbank AY905402) also indicates the presence of a uORF with a perfect Kozak motif at -110 nucleotides, however there is apparently no available information regarding Mrp2 transcription start site(s) in mice. The conservation of these uORF in rat, mouse and human does suggest a function that merits further investigation.

In summary, our present studies showed clearly that expression of various rat Mrp2 transcripts is tissue-specific. More importantly, the Mrp2 5' UTRs differentially influence translation since the uORF^{-109} has a marked inhibitory effect on translation. These data provide a new rationale for the

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high tissue expression of Mrp2 in liver and proximal intestine, since the primary transcription start of -98 nucleotides in these tissues lacks the inhibitory uORF^{-109}.

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FOOTNOTES

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Legends for Figures

FIG. 1. **A. The full length 5' UTR cDNA of rat Mrp2.** Transcription initiation sites that are underlined and in bold, and are located at -213, -163, -132, and -98, relative to the $A^{+1}TG$ which is the translation start codon of the rat Mrp2 gene. The shaded sequences represent the uORFs and their start codons ATGs are in bold. The $uORF^{-109}$ starts at -109; $uORF^{-149}$ starts at -149. The first uORF starting at -213 is in-frame; the $uORF^{-109}$ and $uORF^{-149}$ are out-of-frame with the Mrp2 ORF. The $uORF^{-109}$ terminates at +60 inside the Mrp2 coding region and has a perfect Kozak motif. The italicized nucleotides were mutated to disrupt the uORFs or Kozak motif. **B. Schematic representation of Mrp2 5'UTR-luciferase constructs.** The inserts are shown to be fused in the pGL3 vector or in the T7 control vector between the T7 promoter and the luciferase protein coding region. Wild type Mrp2 5' UTRs are shown as the bold lines to scale. The point mutations in the wild type 5' UTRs are listed in the table. a, mutation of the nucleotide T at -148 to A (disruption of the $uORF^{-148}$); b, mutation of the nucleotide T to A (disruption of the $uORF^{-109}$); c, mutation of both the nucleotides A at -112 and G at -106 to T (disruption of Kozak motif).

FIG. 2. **Identification of transcription initiation sites of the Mrp2 gene and the relative abundance in rat tissues by RPA.** The α-^{32}P-UTP-labeled Mrp2 probe of 280 nucleotides was synthesized, and contains the Mrp2 5' UTR from -1 to -214 and the T7 promoter. Total RNA was incubated with the Mrp2 radio-labeled probe. Following co-precipitation and hybridization, the single strand RNA was degraded by RNAse A/T1. The fragments protected from RNAse digestion were identified by electrophoresis on 6% polyacrylamide denaturing gel. Lane 1, Mrp2 probe (20 µg of yeast without RNAse treatment); Lane 2, 20 µg of yeast RNA; Lane 3, 10 µg of liver RNA; Lane 4, 80 µg of kidney RNA; Lane 5, 80 µg of placenta RNA; Lane 6, 80 µg of

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lung RNA; Lane 7, 40 µg of jejunum RNA; Lane 8, 40 µg of ileum RNA. A figure representative of 4 experiments is shown.

FIG. 3. **The relative abundance of transcription initiation sites of Mrp2 gene in various postnatal rat tissues and adult by RPA.** Rats were decapitated at day 0, 6, 10, 20, and adult. Liver, kidney, jejunum, and ileum were taken from rats and frozen in liquid nitrogen and stored at -80°C until total RNA isolation. Liver RNA (10 µg) and 20 µg of kidney, jejunum, and ileum RNA were used. Lane 1, Mrp2 probe (10 µg of yeast RNA without RNAse treatment); Lane 2, labeled RNA ladders (200nt, 150nt, 100nt); Lane 3, day 0; Lane 4, day 6; Lane 5, day 10; Lane 6, day20; Lane 7, adult. Ribonuclease protection assay representative of three similar experiments obtained at various time points is shown. The histograms represent the ratio obtained from densitometric band quantification of the expressed transcript starting at -132 to the transcript starting at -98. The data are represented as mean ± SD of three separate experiments.

FIG. 4. **Effect of the Mrp2 5'UTR on expression of the luciferase reporter gene in HepG2 cell transient co-transfection assays.** Mrp2 5'-UTR-luciferase constructs were co-transfected into HepG2 cells with pSV40-Ren. After 24 hours, the firefly and *Renilla reniformis* luciferase activities were measured. The effect of various 5' UTRs on luciferase expression is represented as the ratio of the firefly luciferase activity/*Renilla reniformis* luciferase activity. The assays were performed in triplicate. The data are represented as mean ± SEM and normalized to the Luc/Ren ratio for the pGL3 control vector.

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FIG. 5 **Effect of the Mrp2 5'UTR on translation efficiency of the luciferase reporter transcript by *in vitro* translation assays**. Mrp2 5'-UTR-luciferase constructs were linearized by restriction enzymes PvuII and SacI. The PvuII-SacI fragments were used as templates to synthesize the capped, Mrp2 5'UTRs-fused luciferase transcripts. The luciferase transcripts (0, 2, 4, 10, or 20 ng) were added to a rabbit reticulocyte lysate mixture. The translation reaction was incubated at 30°C for 60 min and terminated on ice. The firefly luciferase activity was measured. The linear lines represent the relationship of luciferase activity with respect to transcript concentration.

FIG. 6 ***In vitro*** **expression in Coupled Transcription/Translation system.** The rat Mrp2 5' UTR cDNA sequences were inserted into T7 control vector in a way that the uORF^{-109} was in-frame with the luciferase reporter reading frame. deL and deM1 contain the uORF^{-109} while S2 does not. The point mutation of AT^{-108}G→AAG disrupted the uORF^{-109} in deL and deM1, resulting in deL^{b} and deM1^{b} constructs. The TNT quick coupled transcription/translation system was used to express the luciferase protein *in vitro* using plasmids as templates. The translated products were separated on 4-20% gradient reductive SDS-PAGE. Data were processed using STORM 840 Phosphoimager. Lane 1, deL; Lane 2, deL^{b}; Lane 3, S2; Lane 4, M1^{b}; Lane 5: deM1.

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TABLE 1

Primers used for plasmid construction

Name	Sequence (5'-3')	cloning site
TRF1	5' ggaagcttatgtctgctcactggga 3'	HindIII
TRF2	5' ggaagcttattaagtcgtcaggatga 3'	HindIII
TRF3	5' tcaaagcttaggcctttaactgggctg 3'	HindIII
TRF4	5' ggaaagcttacggtgcactttaacatctg 3'	HindIII
TRF5	5' ggaagcttagaggaaaaagtaaaggag 3'	HindIII
CONR	5' acccccatggtaatgctctcctcgcgc 3'	NcoI
T7R1	5' ggggatccgaatgctctcctcgcgc 3'	BamHI
T7R2	5' tttggatccaatgctctcctcgcg 3'	BamHI
TRRR	5' ggggatccaatgctctcctcgcgc 3'	BamHI

Listed primers used for PCR amplication of rat *Mrp2* 5' UTR cDNAs are shown, with the cloning sites underlined.

A

-213

AT**G**TCTGCTCACTGGGATGACATAGAGTACAACATTCAGAGAAGTTAACT

-163 -148 -132

ATTAAGTCGTCAGG*A*T**G**AAAGGTCAGGAGGC**A**GGCCTTTAACTGGGCTGT

-108 -98

G*A*GAA*T***G**G*A*GAAAGC**A**CGGTGCACTTTAACATCTGCTTTCCCAGAGGAAA

AAGTAAAGGAGAAACAGTACAATCATAGAAGAGTCTTCGTAACAGAAGCG

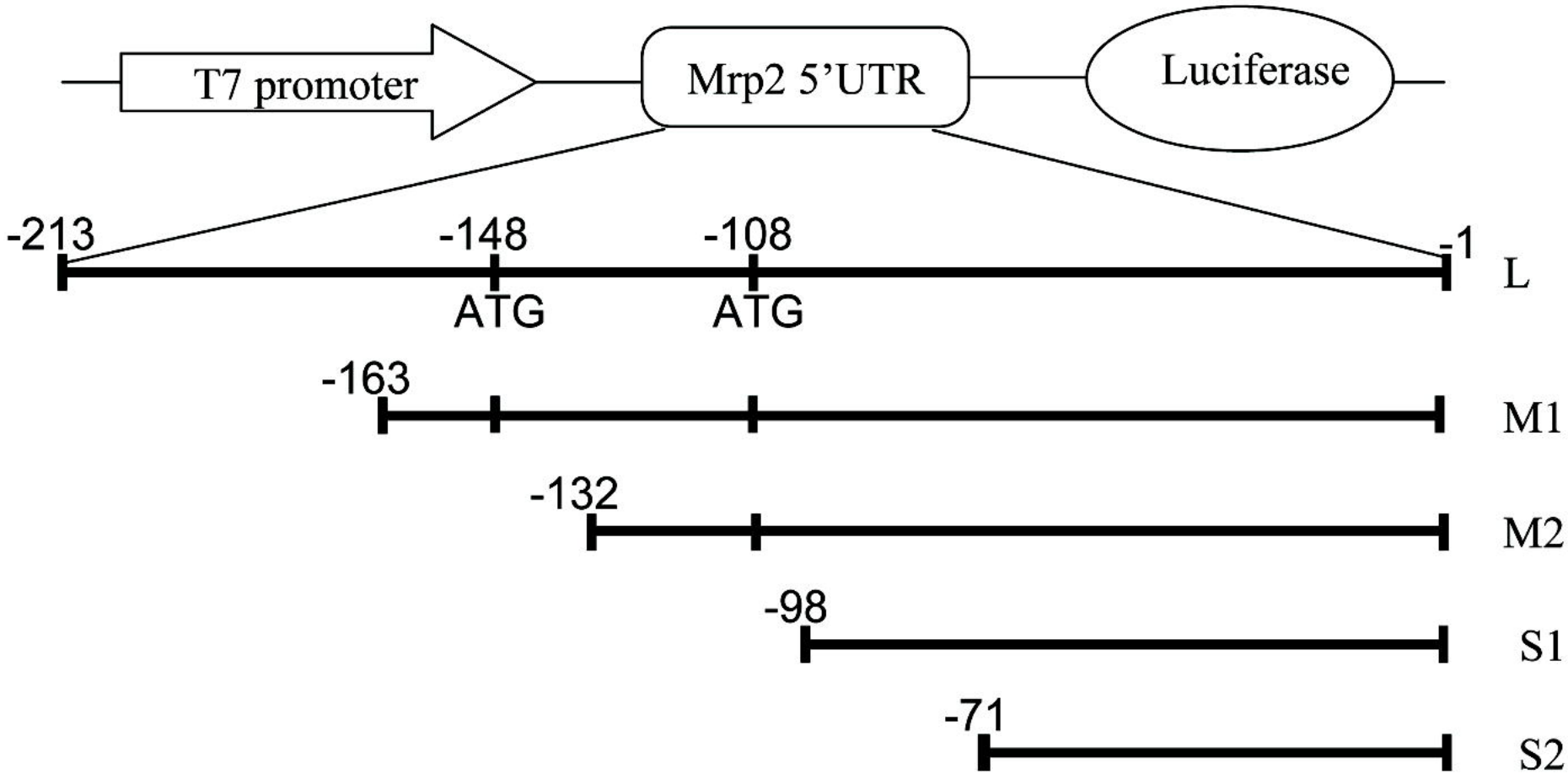
+1

CGAGGAGAGCATTATGGACAAGTTCTGCAACTCTACTTTTTGGGATCTCT

CATTACTGGA AAGTCCAGAG GCTGA...

Figure 1A

B



Mutations	-148 T	-108 T	Kozak Motif G*A*GAAT(-108)G*G*A
a	A	—*	—
b	—	A	—
c	—	—	G*T*GAAT(-108)G*T*A

"—" indicates no mutation

Figure 1B

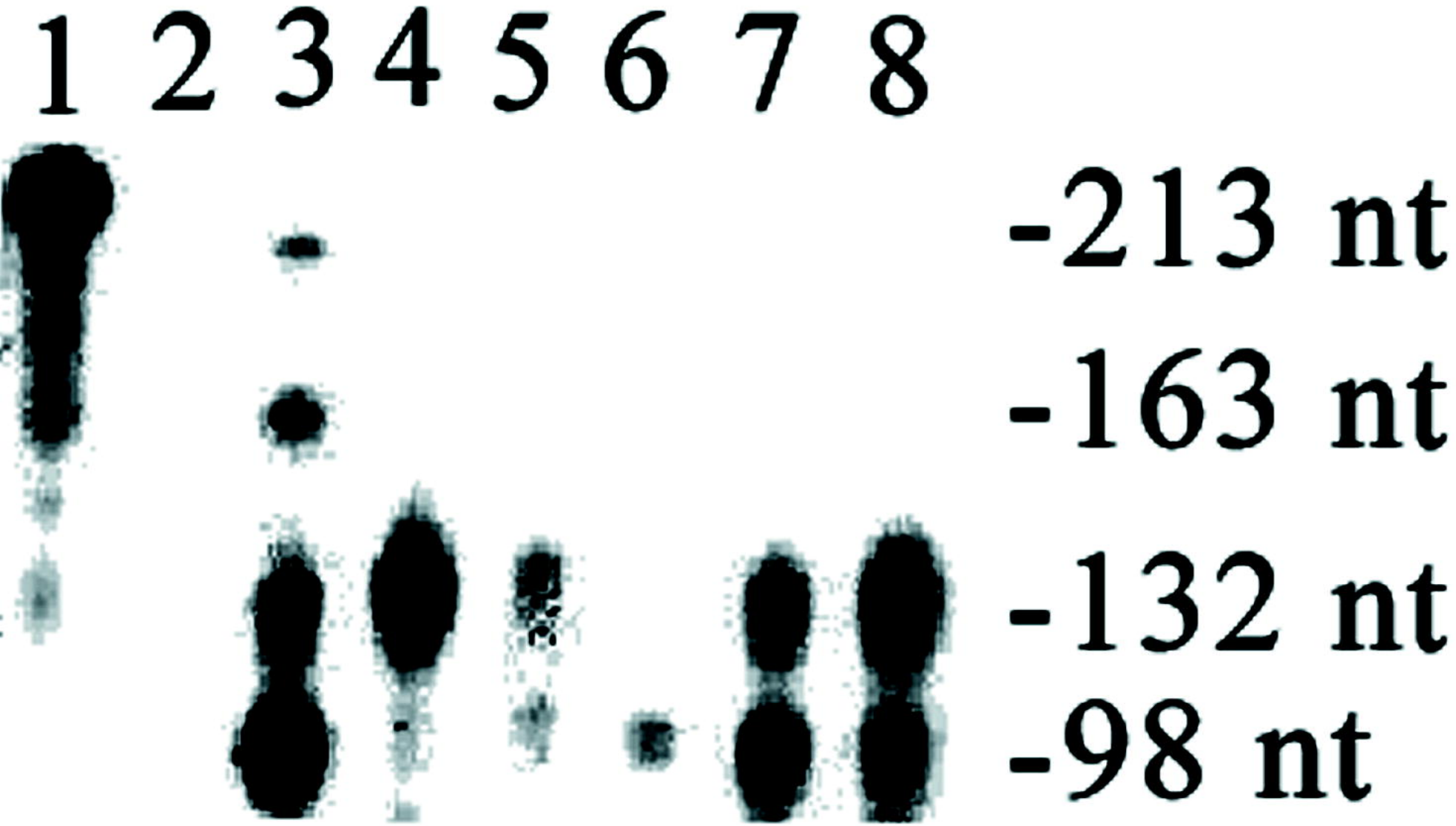
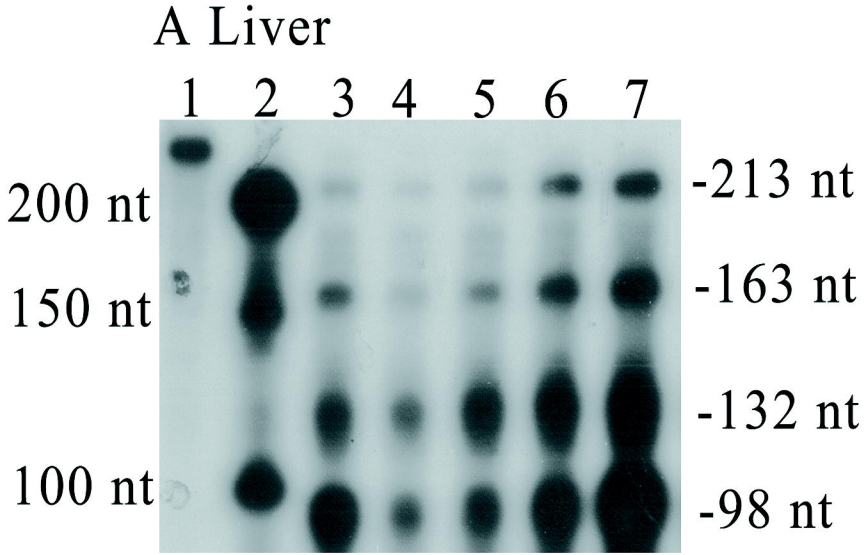


Figure 2



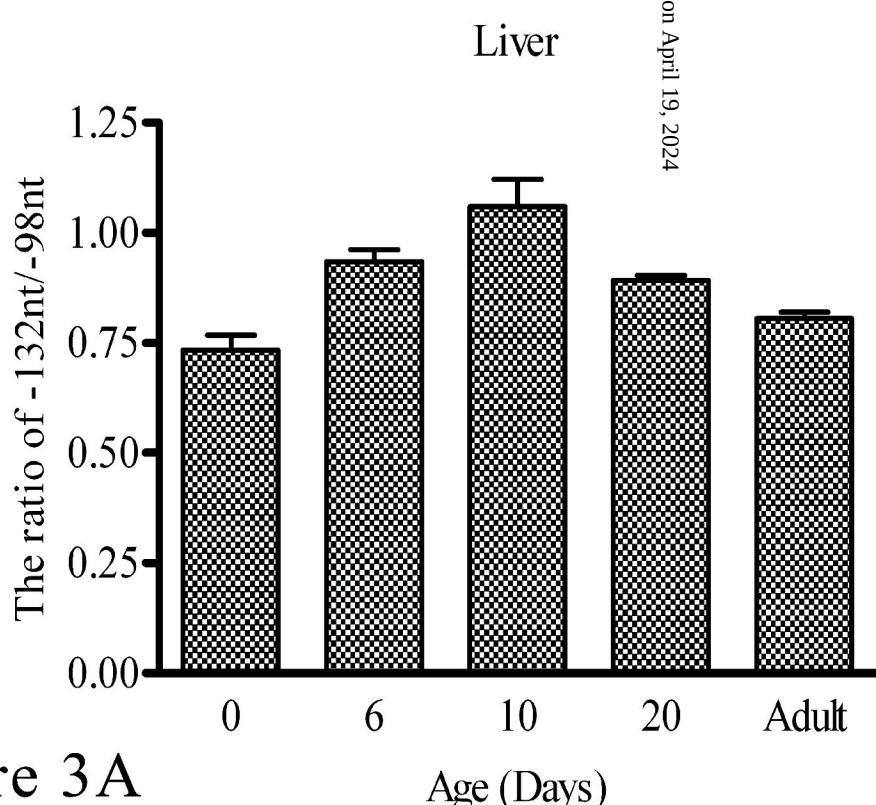
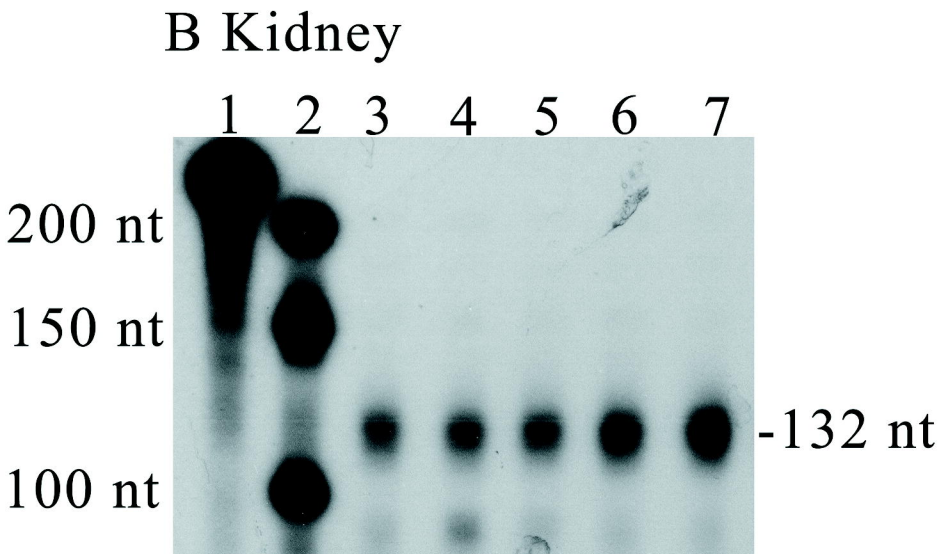


Figure 3A


B Kidney
1 2 3 4 5 6 7
200 nt
150 nt
100 nt
-132 nt

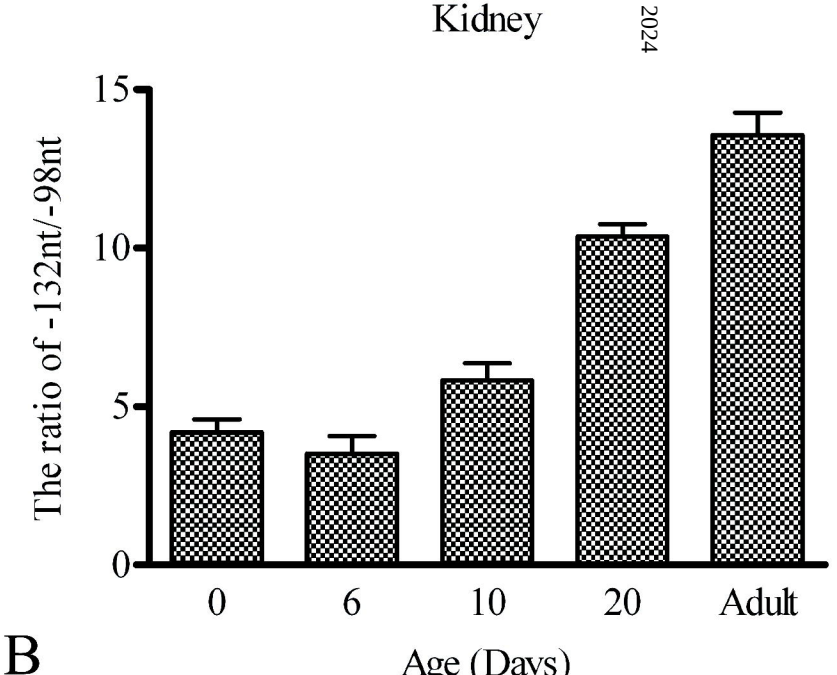
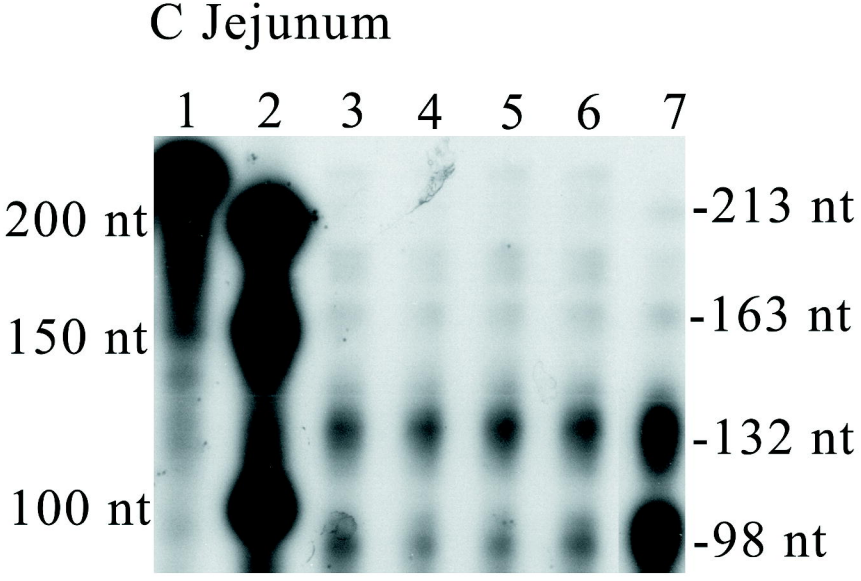

Kidney
The ratio of -132nt/-98nt
15
10
5
0
0
6
10
20
Adult
Age (Days)

Figure 3B



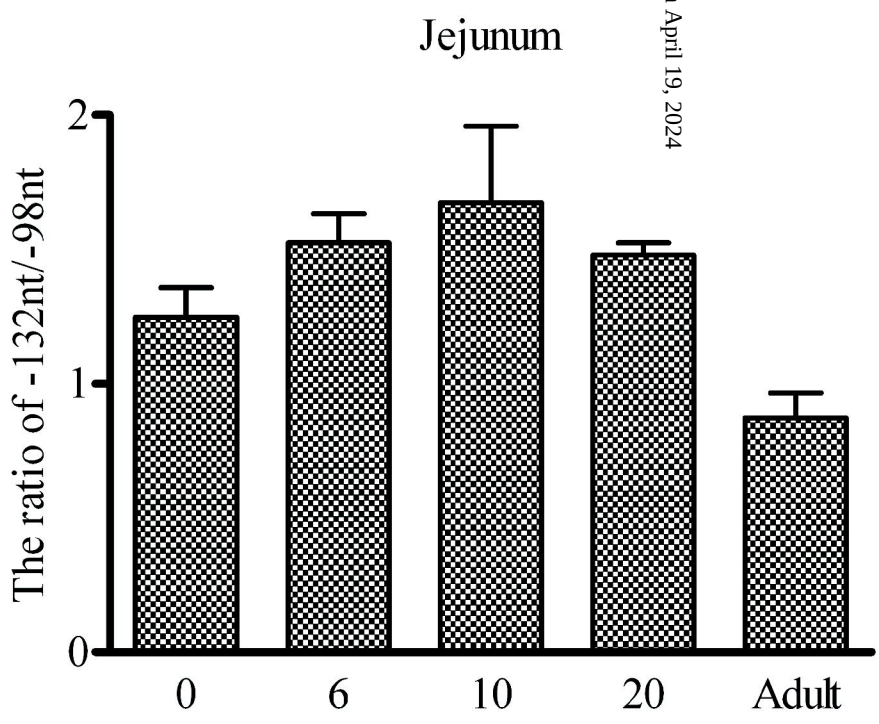
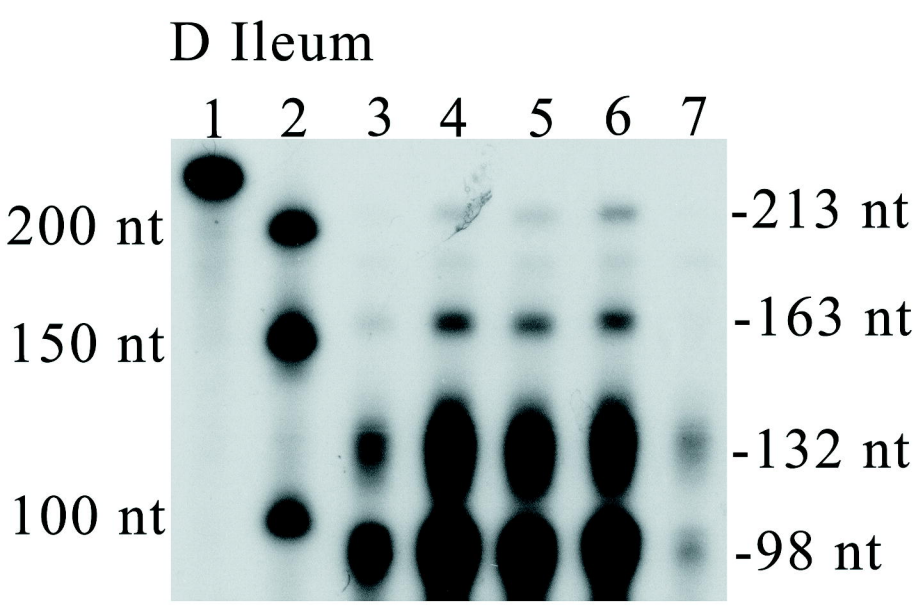


Figure 3C



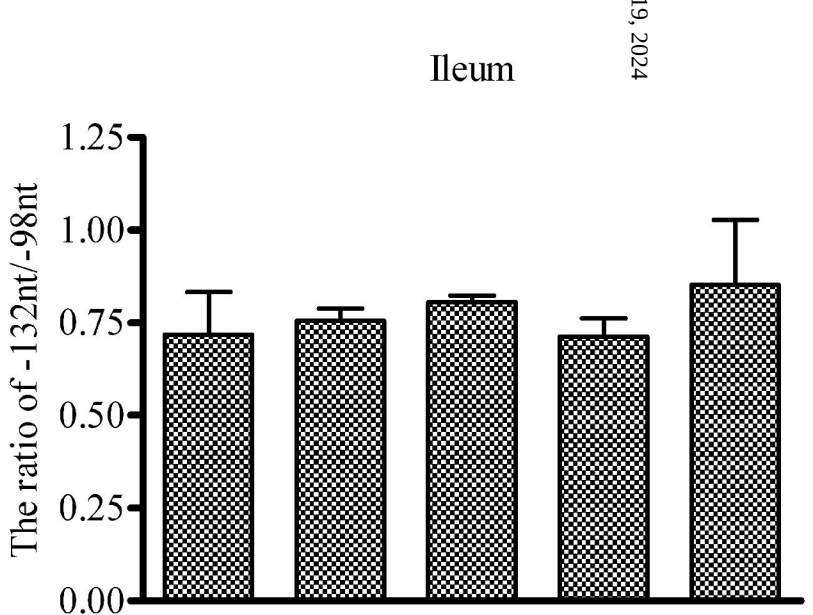


Figure 3D

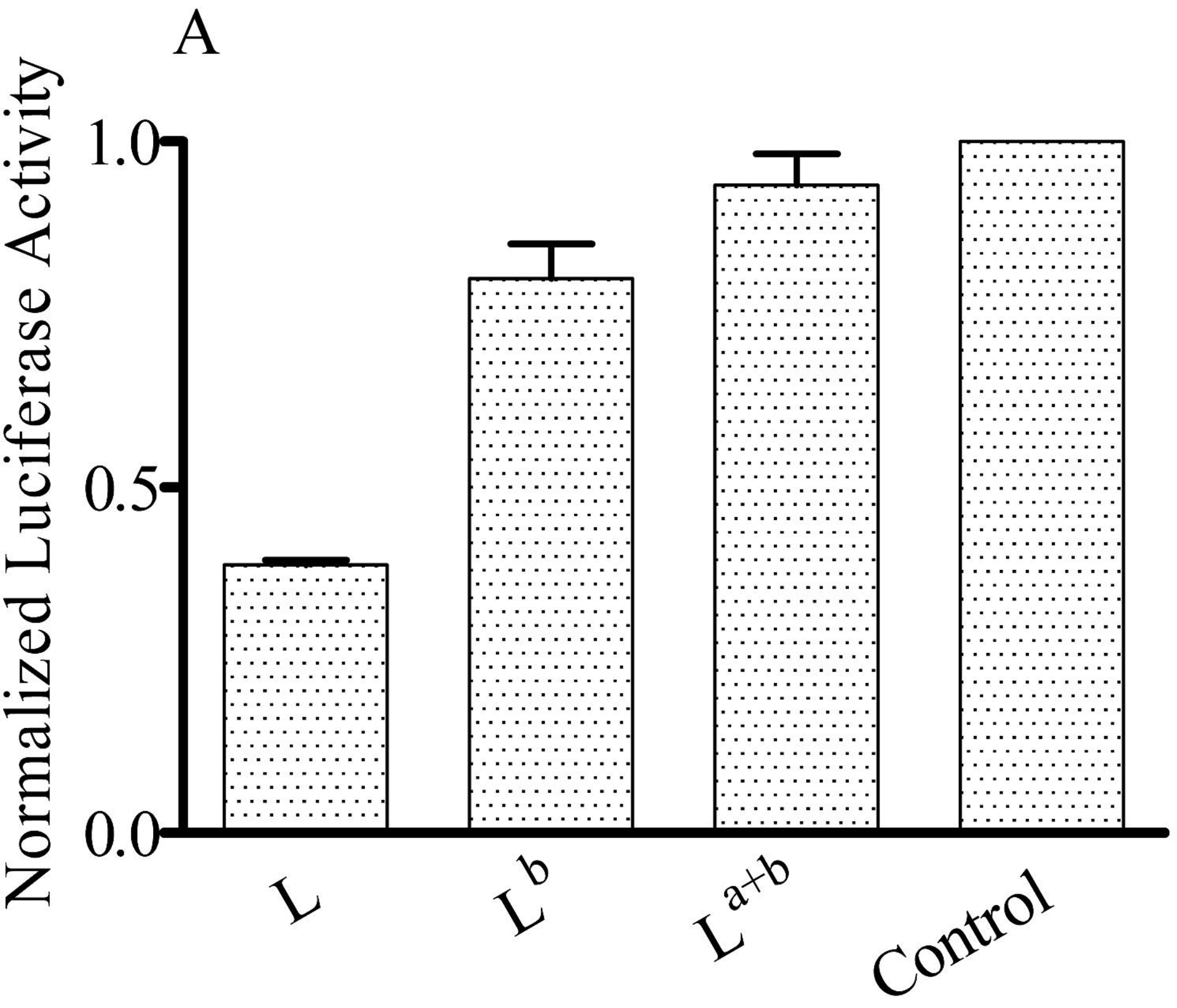


Figure 4A

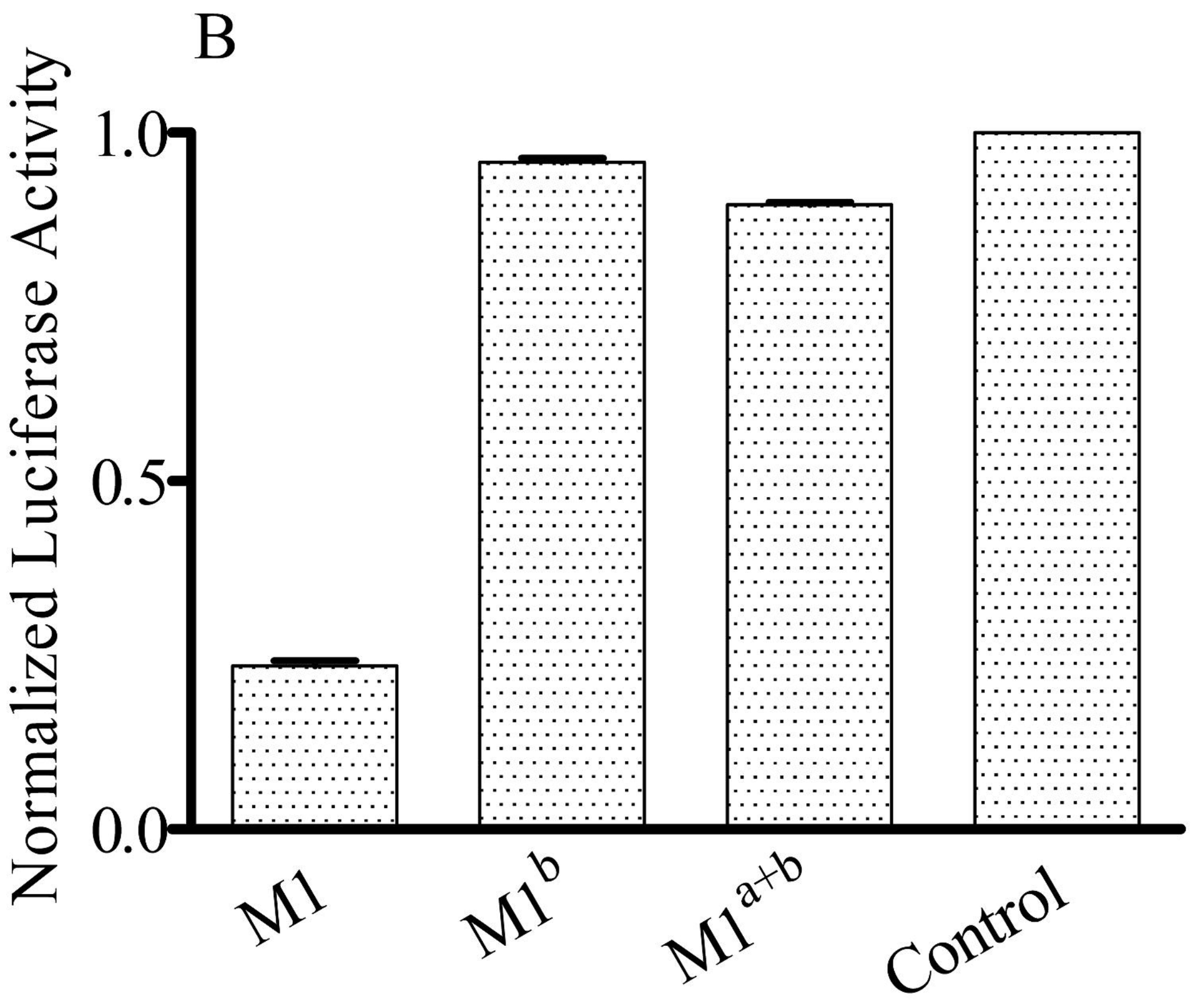

B
Normalized Luciferase Activity
1.0
0.5
0.0
M1
M1b
M1a+b
Control

Figure 4B

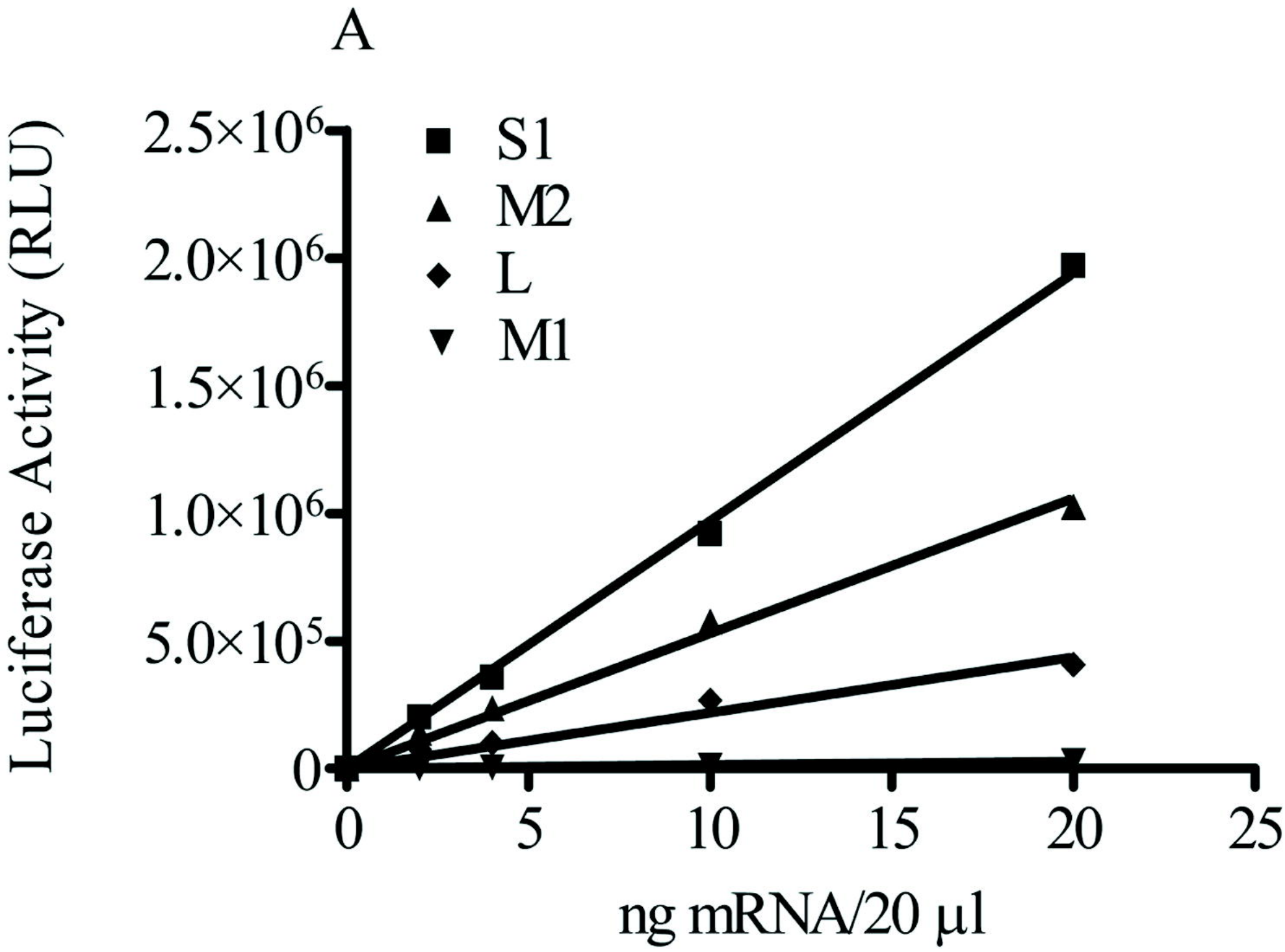

A
Luciferase Activity (RLU)
2.5×10^6
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0
S1
M2
L
M1
0
5
10
15
20
25
ng mRNA/20 µl

Figure 5A

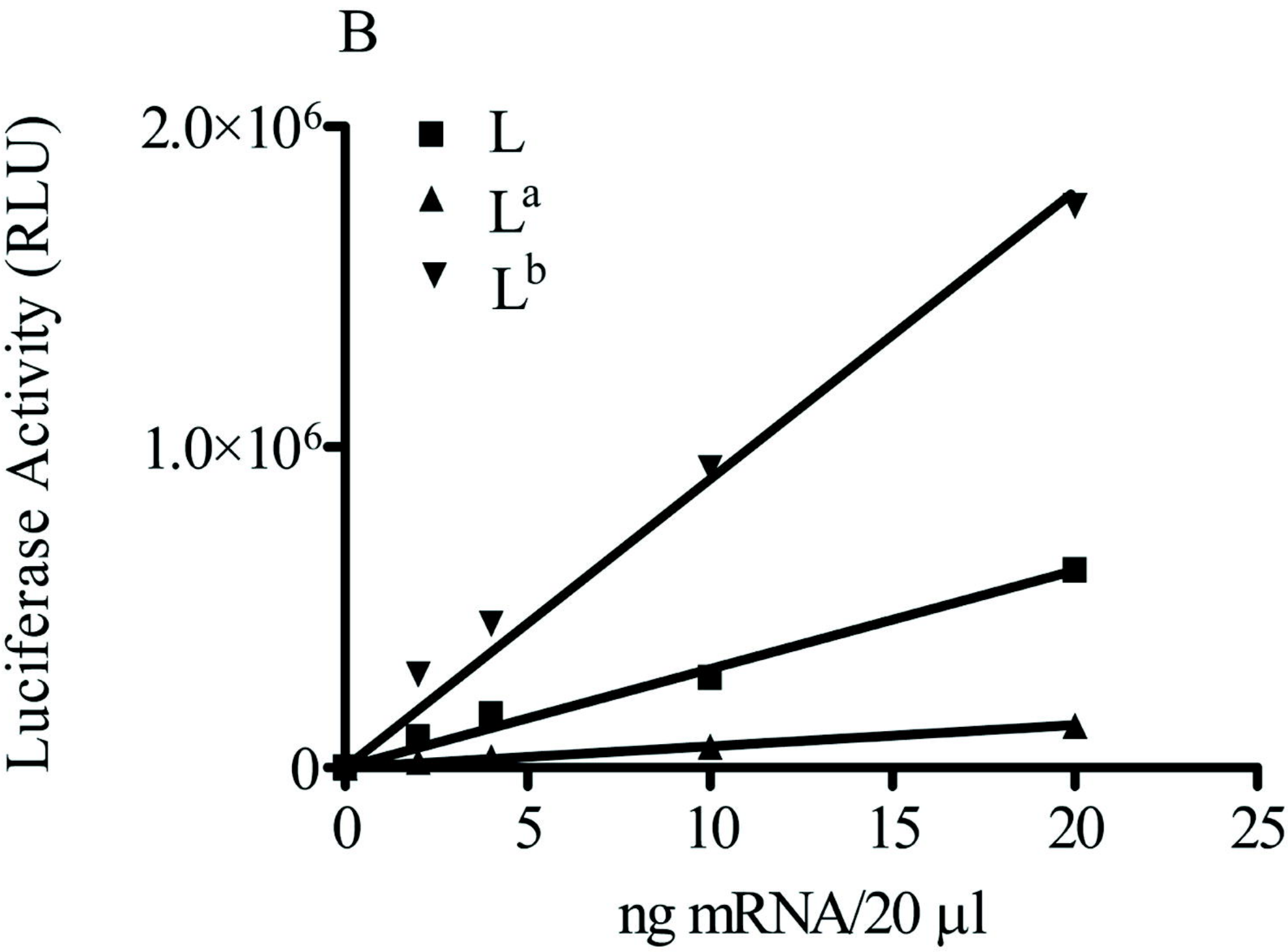

B
2.0×10^6
1.0×10^6
0
Luciferase Activity (RLU)
L
L^a
L^b
0
5
10
15
20
25
ng mRNA/20 µl

Figure 5B

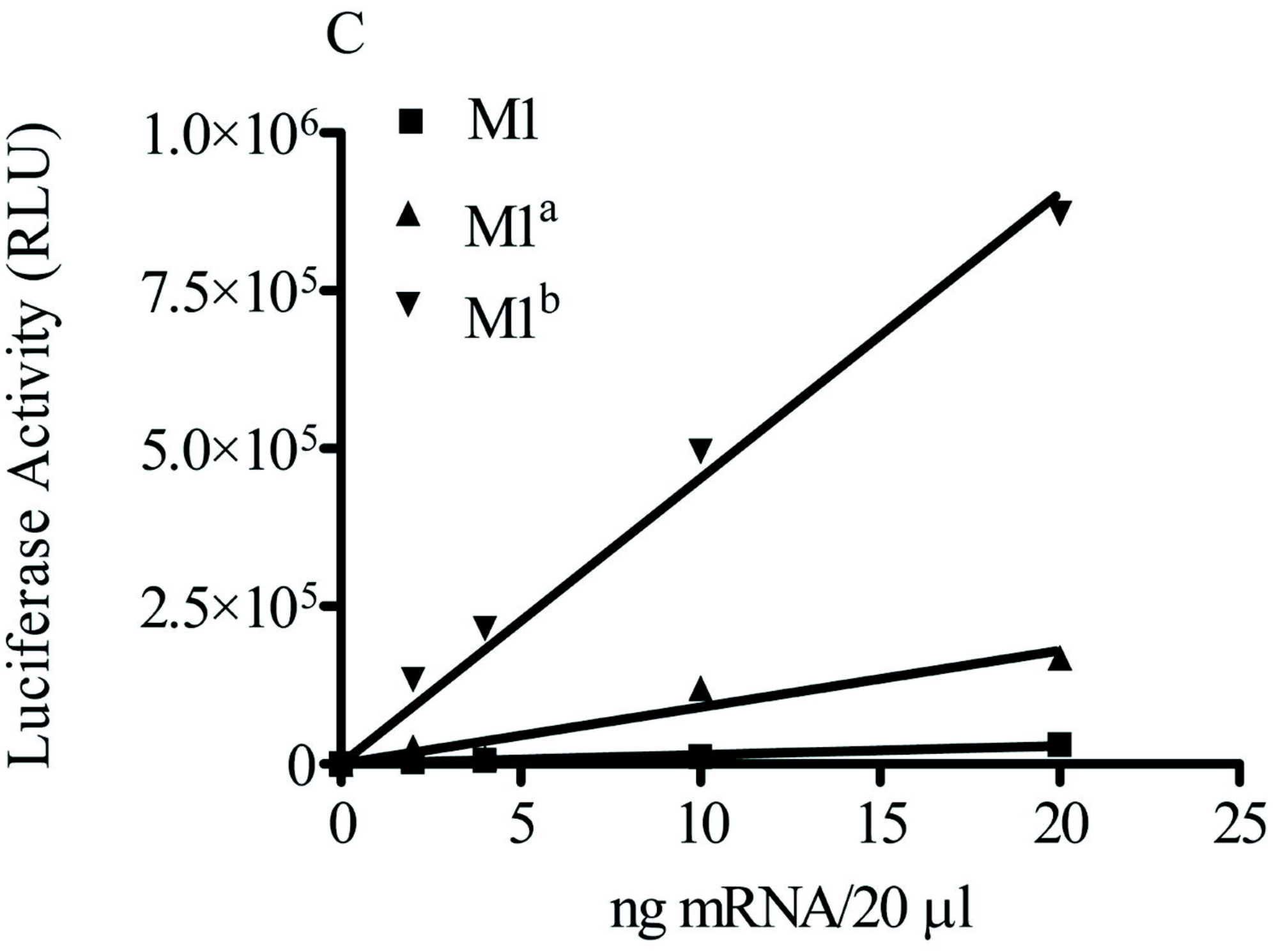

C
M1
M1a
M1b
1.0×10^6
7.5×10^5
5.0×10^5
2.5×10^5
0
Luciferase Activity (RLU)
0
5
10
15
20
25
ng mRNA/20 µl

Figure 5C

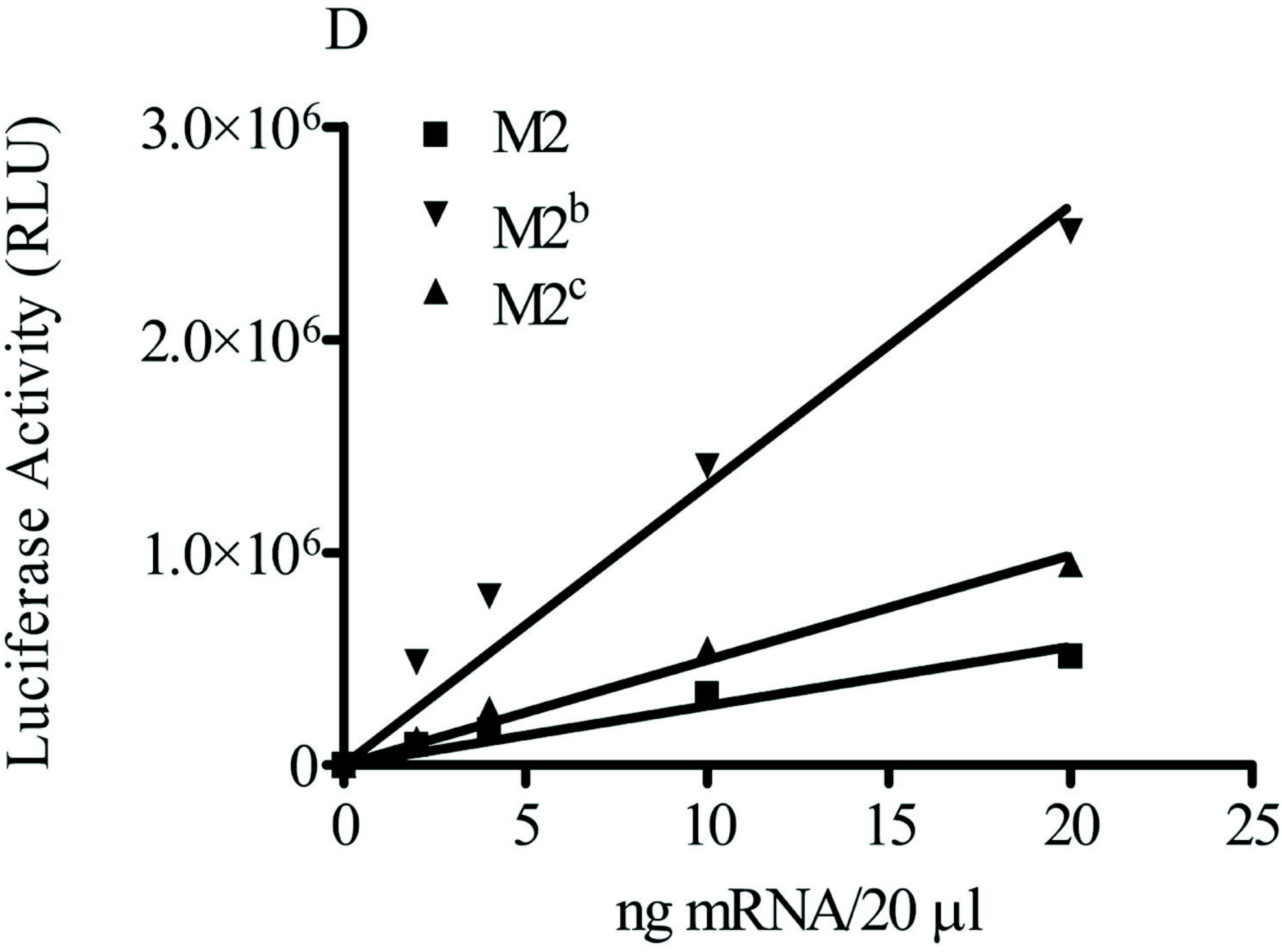

D
Luciferase Activity (RLU)
3.0×10^6
2.0×10^6
1.0×10^6
0
M2
M2^b
M2^c
0
5
10
15
20
25
ng mRNA/20 µl

Figure 5D

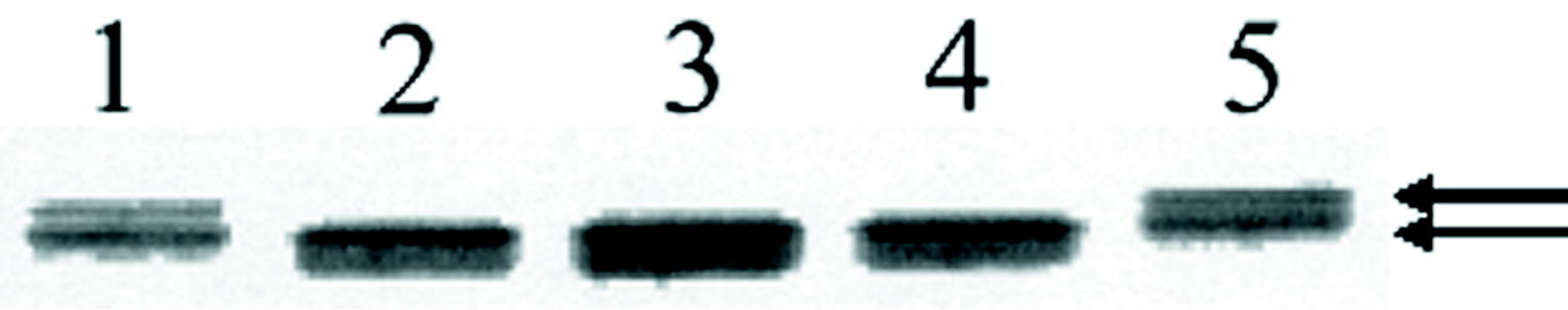


Figure 6