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Characterizing Mouse LINE-1 5’UTR Antisense Promoter Activities

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Characterizing mouse LINE-1 5’UTR antisense promoter activities

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Abstract

Mice are often the model of choice for genomic research because of their high similarity to the human genome and relatively easy maintenance. Thus, understanding the mechanisms and principles of their genome is vital to advancing our knowledge about human biology. Long interspersed elements type 1 (LINE-1s or L1s) are transposable elements within mammalian DNA. Promoter activities have been identified for the human L1 5' untranslated region (5'UTR) in both the sense and antisense orientation, but it was previously unknown if mouse L1 5'UTRs have antisense promoter activity. The goal of my project was to uncover mouse L1 5'UTR promoter activity in the antisense orientation. Various mouse L1 5'UTR promoters were designed in the antisense orientation. The promoter constructs were transfected into NIH/3T3 mouse fibroblast cells, and the promoter activity was quantitatively assessed by a dual-luciferase assay. The results showed promoter activity in the antisense orientation. Like the human genome, mouse L1s display both sense and antisense promoter activities revealing a further similarity between the two species.

Introduction

Long interspersed elements type 1 (LINE-1s or L1s) are abundant retrotransposons that populate mammalian genomes. The promoter activities of L1 retrotransposons reside in the 5' untranslated region (5'UTR)[1]. Specifically, the human genome is known to contain L1 promoter activity in both the sense and antisense orientations[2]. The antisense promoter activity affects as many as 4% of human genes by forming chimeric transcripts with neighboring genes[3]. However, the antisense promoter activities of mouse L1 5'UTRs have been previously uncharacterized.

In the mammalian genome, natural antisense promoters have a considerable role in conventional gene expression. The chimeric transcripts that they regulate are responsible for cellular processes such as vesicle-mediated transport, protein transport and modification, and mitotic processes[3,4]. The transcripts are also known to be involved in methylation of DNA, histone modification, and RNA modification[5]. Additional involvement in the genome is continually being discovered.

The laboratory mouse is an important animal model for studying the biology of L1 retrotransposons due to their high similarity to the human genome, relative ease of maintenance and high reproductive rates. Understanding mouse L1 promoter activity is critical for investigating the role of L1 in development and disease. For example, the activation of the L1 promoter sequence in tumor cells potentially increases the occurrence of oncogenic mutagenesis[6]. Additionally, an antisense L1 promoter is hypomethylated in tumor-bearing bladders but not in normal bladders, and its activation drives the expression of a truncated by constitutively active MET oncogene in bladder epithelium[7]. L1 promoter activity in the antisense orientation has also been reported to alter gene structure, activate genes, and silence genes in the

human genome[8]. The experiment may also lead to the application of L1 promoters in genetic engineering or gene therapy. Determination of mouse L1 5'UTR antisense activity broadens understanding of the mechanisms and characteristics of L1 promoters.

Methods and Results

Seven plasmid constructs containing sense oriented L1 promoter were used as the starting material in the experimental procedure: pLK049, pLK050, pLK051, pLK087, pLK088, pLK089, and pCH117 (Figure 1). They contain sense-oriented L1 promoters corresponding to mouse L1 A_I, Tf_I, Gf_I, A_II, Tf_II, Tf_III, and human L1 promoter, respectively. The promoter activity of the respective sense-oriented promoter has been previously measured in our lab by transfection into mouse NIH/3T3 cells followed by a dual-luciferase assay.

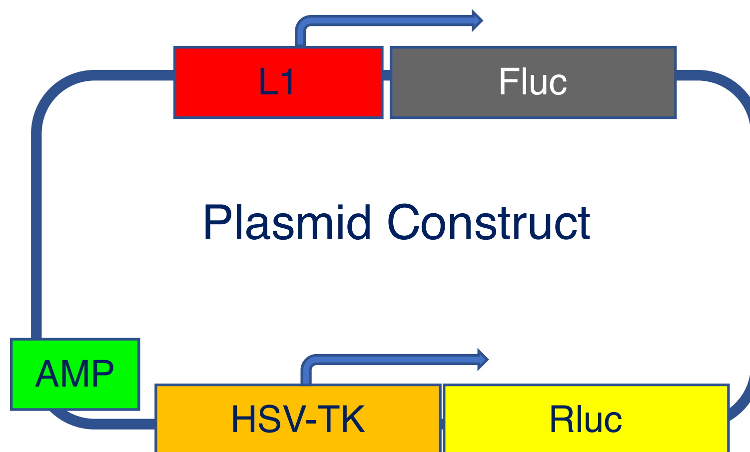

Figure 1. Dual-luciferase reporter constructs. The L1 promoter is located upstream of and drives the expression of the firefly luciferase (Fluc) and is variable in each of the seven plasmid constructs. The Fluc activity is indicative of L1 promoter activity. Similarly, a herpes simplex virus thymidine kinase (HSV-TK) promoter is present upstream of and drives the expression of Renilla luciferase (Rluc). The Rluc activity is used to normalize transfection efficiency. An ampicillin resistance gene (AMP) is also present on the plasmid construct.

A forward and a reverse primer were designed for each of the seven plasmids to be cloned. The reverse complement of the L1 5'UTR promoter region flanked by the two distinct SfiI sites was obtained from the parental plasmid. The reverse complement sequence was then inserted between the same two flanking SfiI sites (Figure 2). Primer sequences were derived from the resulting flanking regions (Table 1). An adenine nucleotide was added to the 5' end of the primers. Nucleotide length, % GC content, and melting temperature were recorded for each primer. Primers were ordered from Eurofins Genomics Company and resuspended in molecular biology grade water.

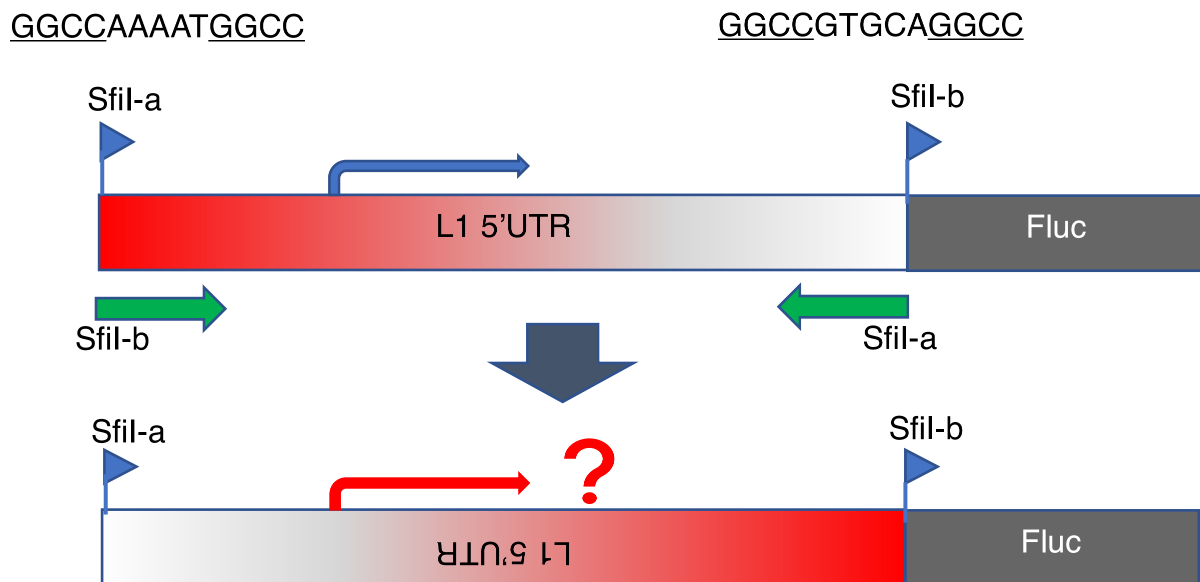

Figure 2. Primers for cloning L1 5'UTR in its antisense orientation. The L1 5'UTR is flanked by two heterotypic SfiI sites, denoted as SfiI-a and SfiI-b, with recognition sequences shown. The bent arrows show transcriptional direction. The green arrows show primers used to amplify the 5'UTR. Each has a SfiI site embedded in its 5' end but with the opposite SfiI site. Seven pairs of forward and reverse primers were synthesized, and each pair was specific to each of the seven promoters.

Table 1. Primers designed.

PCR Template	Primer Name	Primer Orientation	Primer Sequence
pLK049	WA1698	Forward	AGGCCTGACAGGCCGTGCCTGCCCCA
pLK049	WA1699	Reverse	AGGCCAAAATGGCCCTGGTAATCTCTGAAGCT
pLK050	WA1700	Forward	AGGCCTGACAGGCCGACAGCCGGCCA
pLK050	WA1701	Reverse	AGGCCAAAATGGCCCTGGTAATCTCTGGAGTTA
pLK051	WA1702	Forward	AGGCCTGACAGGCCTGAGAGCACGGGGG
pLK051	WA1703	Reverse	AGGCCAAAATGGCCCTGGCAATCTCTGGAG
pLK087	WA1704	Forward	AGGCCTGACAGGCCGCGCCTGCCCC
pLK087	WA1705	Reverse	AGGCCAAAATGGCCCTGGTAATCTCTGAAGCT
pLK088	WA1706	Forward	AGGCCTGACAGGCCGACAGCCGGCCA
pLK088	WA1707	Reverse	AGGCCAAAATGGCCCTGGTAATCTCTGGAGTTA
pLK089	WA1708	Forward	AGGCCTGACAGGCCGACAGCCGGCCA
pLK089	WA1709	Reverse	AGGCCAAAATGGCCCTGGTAATCTCTGGAGTTA
pCH117	WA1710	Forward	AGGCCTGACAGGCCGCTCTAGCCCTGG
pCH117	WA1711	Reverse	AGGCCAAAATGGCCCTTTGTGGTTTTATCTACTTTTG

Polymerase chain reaction (PCR) was done to amplify the selected promoter sequence in the antisense orientation. In a reaction tube 15 µL dH_2O, 5 µL 5x GXL buffer, 1.5 µL dNTPs, 1 µL reverse primer, 1 µL forward primer, 0.5 µL PrimeStar GXL DNA polymerase, and 1 µL of template plasmid were combined. The mixture was created for each plasmid. PCR cycled 30 times at 98 ºC for 10 s, 60 ºC for 15 s, and 68 ºC for 60 s. Gel electrophoresis of the PCR products was then performed using a 1% agarose gel containing ethidium bromide. The DNA band of each product was then examined with long-wave UV light (Figure 3), extracted, and purified using the QIAquick PCR purification kit protocol.

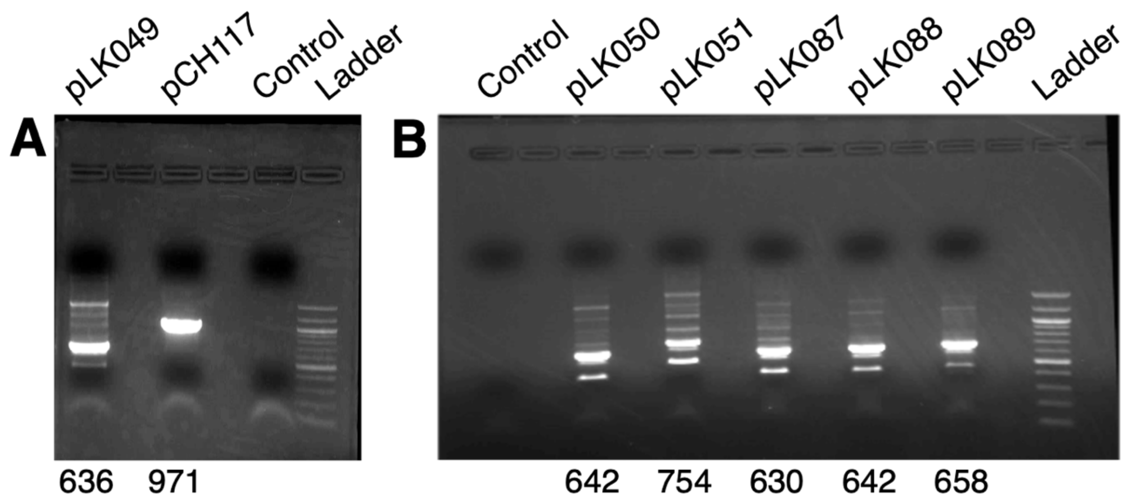

Figure 3. Gel electrophoresis of PCR products using (A) using pLK049 and pCH117 or (B) pLK050, pLK051, pLK087, pLK088, and pLK089 as templates. A 100 bp ladder was used as the DNA molecular weight standard. The expected amplicon sizes (bp) are indicated under the gel images. Control: no template control. The results of the gel electrophoresis confirmed correct fragment lengths, and the experiment continued. The DNA was then extracted and purified to prepare for the digestion and ligation procedure.

Plasmid pCH117 and the PCR products underwent a digestion procedure using SfiI restriction enzyme. Two reaction mixtures were created. The first mixture contained 19 µL dH_2O, 3 µL 10x NEB cutsmart buffer, 1 µL SfiI restriction enzyme, 1 µg (6 µL) pCH117, and 1 µL NheI-HF restriction enzyme and was incubated at 37 ºC for 2 hours and then at 50 ºC overnight. The second mixture contained 26 µL of the purified PCR product, 3 µL of 10x NEB cutsmart buffer, and 1 µL of SfiI restriction enzyme and was incubated overnight at 50 ºC. The result from the first reaction was a plasmid backbone without an L1 5'UTR promoter sequence. The backbone DNA then underwent a ligation reaction where the antisense L1 promoters were incorporated into the plasmid backbone sequence (Figure 4). In a reaction tube 4 µL dH_2O, 1.5 µL 10x T4 DNA ligase buffer, 1 µL backbone DNA, 7.5 µL purified PCR product, and 1 µL T4

ligase were combined. A no-insert control reaction tube was created with 11.5 µL dH_2O, 1.5 µL 10x T4 DNA ligase buffer, 1 µL backbone DNA, and 1 µL T4 ligase.

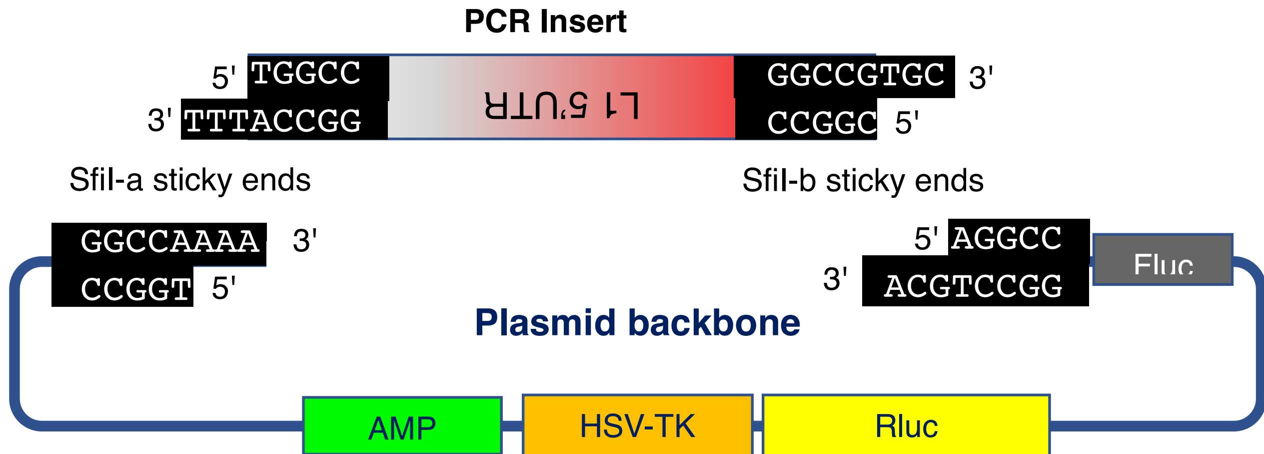

Figure 4. Restriction digestion and ligation into plasmid backbone. The SfiI digested antisense L1 5'UTR DNA fragment was ligated to the plasmid backbone containing compatible heterotypic SfiI-a/SfiI-b ends. Figure 3 shows the sequences of the sticky ends and the complimentary locations of ligation. The result of the ligation was constructs containing the L1 5'UTR in its antisense orientation relative to Fluc. The antisense plasmid construct counterparts for pLK049, pLK050, pLK051, pLK087, pLK088, pLK089, and pCH117 were named pJT01, pJT02, pJT03, pJT04, pJT05, pJT06, and pJT07 respectively.

The ligated plasmids (pJT01-pJT07) then underwent a transformation procedure. Each of the plasmid constructs was combined with TOP10 *E. coli* cells. The combination underwent a heat shock, and prepared LB-carbenicillin plates were inoculated with the bacteria containing the plasmid inserts. A control plate was inoculated with TOP10 *E. coli* cells transformed with a ligation reaction without 5'UTR inserts to evaluate the level of assay background due to self-ligation of plasmid backbones. The plates were incubated for 24 hours before being examined (Figure 5).

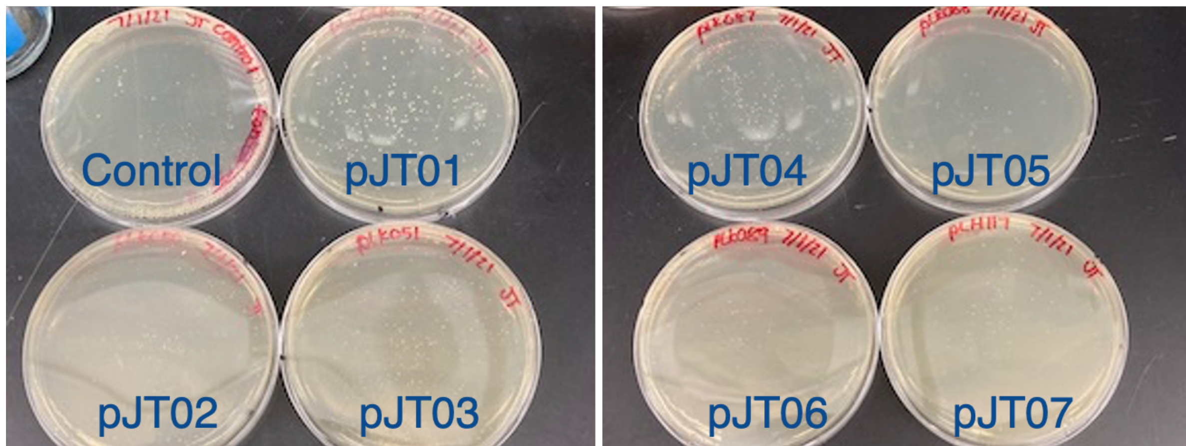

Figure 5. Transformation results of ligated plasmids into competent TOP10 *E. coli* cells. Colony growth is shown on the inoculated agar plates. The control included ligation product without PCR insert to serve as a negative control for transformation. Each plasmid contained an ampicillin resistance gene which allowed the bacteria to survive on the LB-carbenicillin agar plate. Colony growth after transformation confirmed plasmid uptake by the TOP10 cells.

Colonies from each plate were collected and cultured in liquid medium. The ZymoPURE plasmid kit was used to extract purified plasmids from the liquid culture. Three separate colonies for each antisense plasmid construct (pJT01-pJT07) were purified and digested with PvuII. PvuII was selected due to its sites of digestion relative to the antisense promoter sequences. PvuII cut the plasmid at a site directly before the SfiI-a site, in between the two SfiI sites, and directly after the SfiI-b site. A map for each antisense promoter plasmid under the condition of PvuII restriction enzyme digestion was generated using MacVector software (Figure 6). All colonies showed the predicted digestion pattern (Figure 7).

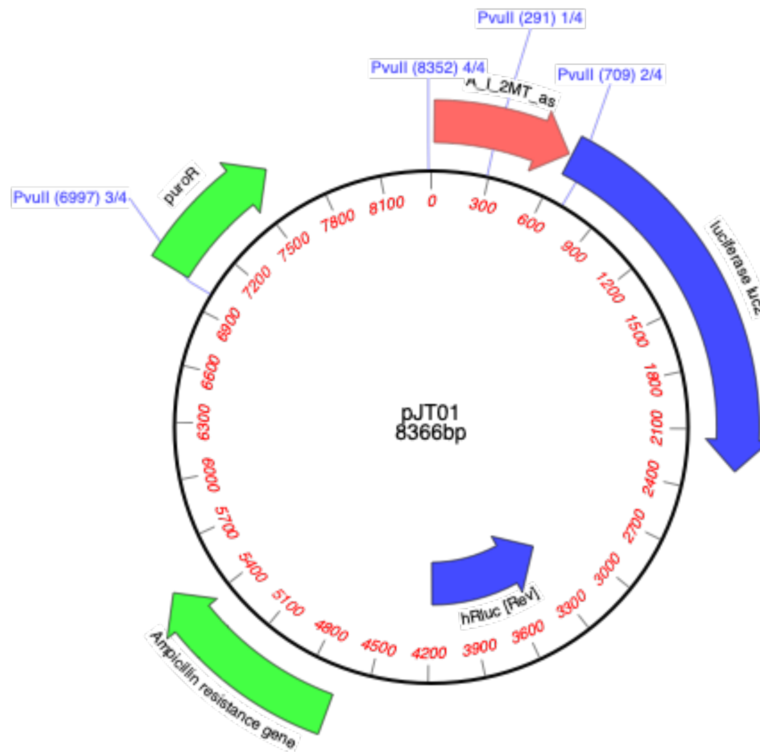

Figure 6. Schematic for screening plasmid clones with restriction enzyme PvuII. The predicted plasmid map for pJT01 using PvuII is displayed as an example. pJT01 was derived from pLK049 but the L1 5'UTR (red arrow) is now in antisense orientation relative to the firefly luciferase (the long blue arrow). There are four PvuII restriction sites (at the base pair locations of 291, 709, 6997, and 8352). Each plasmid had a generated model that was used to predict fragment lengths for a subsequent gel electrophoresis test.

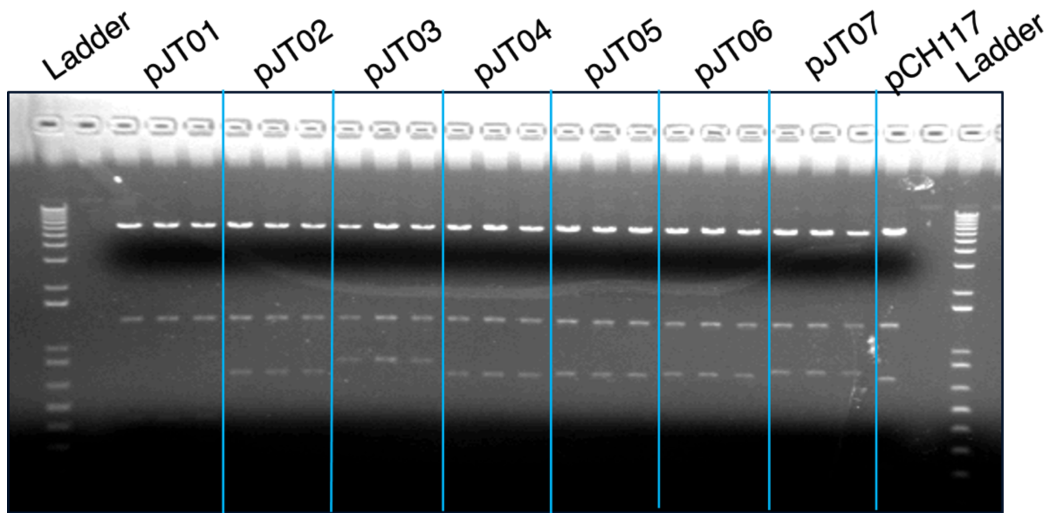

Figure 7. PvuII restriction enzyme digestion of purified plasmids. Three separate colonies for each antisense plasmid construct (pJT01-pJT07) were purified and digested with PvuII. A 1 Kb ladder was used on either side of the gel. pCH117 is the parental plasmid for pJT07 and was digested as a control. The fragment lengths corresponded with the generated MacVector predictions for each plasmid. Two clones for each plasmid were subsequently sent to Elim Biopharmaceutics Inc for sequencing verification of cloned antisense promoter sequences.

Mouse NIH/3T3 cells (mouse embryonic fibroblast cells) were cultured and maintained over 3 days until 70% confluency was reached. The plasmids then underwent the transfection procedure. The plasmids were prepared in a lipid complex and added to 100 μL of cultured cells at $2x10^5$ cells per mL in a 96-well plate. The plate was incubated for a 48-hour period. After incubation, the cells underwent dual-luciferase reporter assay protocol. Cell lysis buffer was added to each well with minimized light exposure followed by 15 minutes of incubation at room temperature. First, firefly luciferase activity was measured after Luciferase Assay Reagent II was added to the plate and used as a measure of the antisense promoter activity. Then Renilla luciferase activity was measured after Stop & Glo Reagent was added to the plate and used as the experimental control to normalize transfection efficiency. GloMax Multi Detection System

(Promega) was used for readings. The assay measured Renilla Luciferase activity. The results of the assay were compared to the results of the sense-oriented promoter activity results (Figure 8).

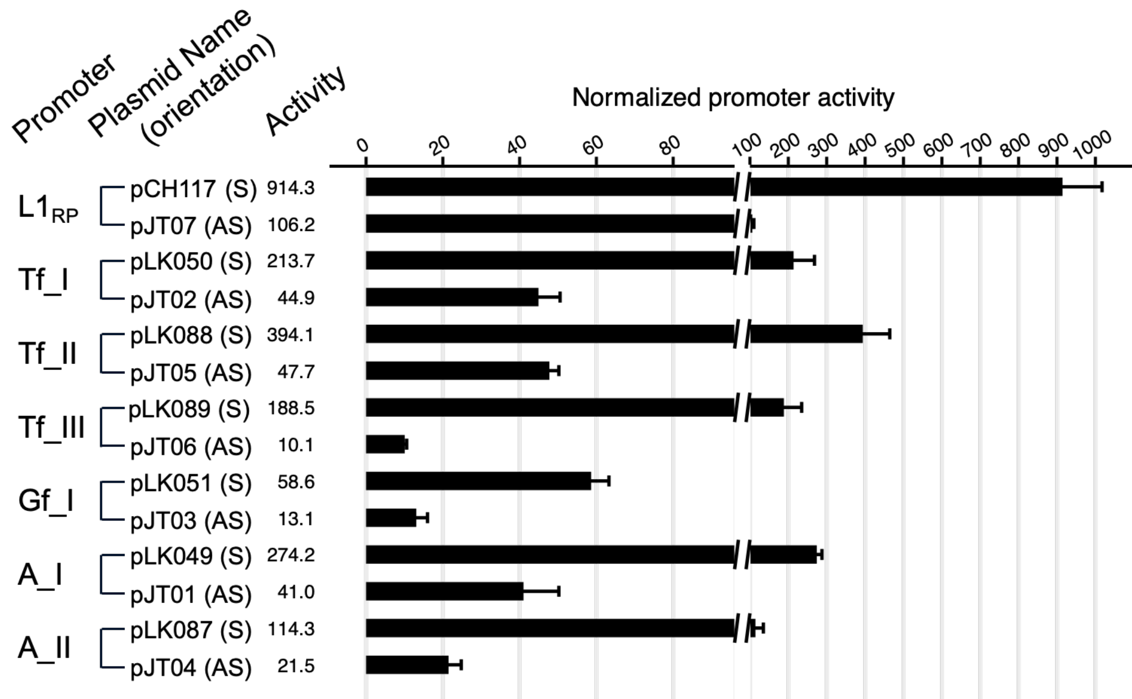

Figure 8. Results of dual-luciferase assays. Each plasmid was transfected into mouse NIH/3T3 cells, and a dual-luciferase assay was conducted two days later. Each sense-oriented plasmid (pLK049 to pCH117) was paired with its antisense clone (pJT01 to pJT07). The orientation of the L1 5'UTR in the plasmid is indicated in parentheses (S: sense; AS: antisense). Plasmid pCH117 was a positive control; pLK037 was a negative control (not shown), whose readout was normalized to 1, representing the assay background.

Discussion and Conclusions

Seven antisense promoter constructs were successfully cloned and tested in a dual-luciferase reporter assay. The results of the dual-luciferase assay show promoter activity in each of the seven antisense L1 5' UTR plasmid constructs. Antisense promoter activity is comparatively reduced from its sense-oriented counterpart. The antisense partner of the control human L1 5'UTR plasmid showed one-tenth of its sense counterpart's activity, which is

consistent with a previous report[9]. Results of the other plasmids varied slightly but were consistent in having antisense promoter activity.

Based on the results, it can be concluded that mouse L1 5'UTR display antisense promoter activity, confirming another shared characteristic between the human and mouse genomes. The information obtained from this experiment can be used in future genetic research projects using mice as comparative models. Future studies should focus on characterizing chimeric transcripts initiated from the antisense L1 promoter and their impact on neighboring gene expression.

Acknowledgements

Thank you to Dr. Wenfeng An for his expertise in L1 genetics research, guidance as a research mentor, and ceaseless patience towards a learning undergraduate student. Thank you to Karabi Saha for sharing her knowledge and skills in plasmid cloning techniques and to Simon Newkirk for his time in teaching essential laboratory practices.

Addendum

The findings from this project have been incorporated into a bioRxiv preprint (https://www.biorxiv.org/content/10.1101/2021.12.03.471143v1), for which J.T. is a co-author.

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