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Original article

Differential characteristics of mammalian and tick-derived promoters to trigger protein expression in transfected tick cell lines

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ABSTRACT

The transfection of plasmids into cell lines for the transient expression of exogenous proteins is a fundamental method for characterizing their functions, cellular localization and interactions. Currently, only a few reports on tick transfection systems and expression plasmids specifically constructed for tick cell lines have been published. In this study, the transcriptome of the tick cell line IDE8 was analyzed to screen for highly-expressed genes. The upstream sequences of these genes were selected as possible tick-derived promoters, and their promoter activity was evaluated using a luciferase assay. Four IDE8-derived sequences with promoter activity were identified, and the promoter activities of three common mammalian promoters, CMV, PGK and CAG, were studied and compared in the IDE8 and IRE/CTVM19 tick cell lines. In the two tick cell lines, the efficiency of the CAG promoter was considerably higher than that of CMV, PGK and the four newly-identified tick promoters. Additionally, time course experiments revealed that the protein expression driven by mammalian promoters reached peak levels on day 3, while the protein expression driven by our constructed tick-derived promoters reached peak levels on day 2 in tick cells. By comparing the transfection efficiency of three transfection reagents with different mechanisms in tick cell lines, we identified Effectene (with Enhancer, Qiagen) as the most effective reagent for tick cells. The findings of this study suggested that there are differences between tick and mammalian cell lines in their response to the transfection system. These findings will contribute to future studies on topics including tick protein function, tick genetic modification and tick-host-pathogen interactions.

1. Introduction

Ticks can transmit many pathogens, including viruses, bacteria and protozoa, between their different vertebrate hosts, including humans, due to their wide host range, global distribution and obligate hematophagous and multi-host developmental cycles (Jongejan and Uilenberg, 2004; Shi et al., 2018). With the extension of human habitats, rapid urbanization and climate change, the enzootic transmission cycle of ticks-pathogens-wildlife has increasingly mingled with the urban and peri-urban transmission cycle of ticks-pathogens-human/livestock, resulting in an upsurge in emerging and re-emerging tick-borne diseases (TBDs) (Merino et al., 2013). Since the identification of the first TBD, many devastating TBDs of humans or livestock have been identified, such as Crimean-Congo hemorrhagic fever, tick-borne encephalitis, tularemia, tick-borne rickettsioses, borreliosis, anaplasmosis, babesiosis, heartwater and theileriosis, which are yet to be eradicated or successfully controlled due to the lack of effective drugs and vaccines, thereby posing serious threats to public health and economic growth (Dantas-Torres et al., 2012; Estrada-Peña and de la Fuente, 2014). Although remarkable efforts have been dedicated to elucidating the pathogenesis of TBDs, the lack of knowledge regarding the natural biological and physical properties of ticks and the relationships between ticks and pathogens have limited current research. Conversely, owing to the availability of the complete genome sequences of several mosquito species and development of transfection techniques, remarkable advancements in mosquito control have been achieved in recent years (Carlson et al., 2006; Severson and Behura, 2012; Qasim et al., 2020). With the recent expansion of whole-genome sequencing of ticks (Gulia-Nuss et al., 2016; Cramaro et al., 2017; Jia et al., 2020; Guerrero et al., 2021), genetic manipulation will become one of the principal technologies for improving understanding of the biology of ticks in the post-genomic era.

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The availability of *in vitro* tick cell culture systems undoubtedly facilitates the investigation of host-tick-pathogen interactions (Bell-Sakyi et al., 2007, 2018). To date, cell lines derived from different tick species, including *Amblyomma* spp., *Rhipicephalus* subgenus *Boophilus* spp., *Dermacentor* spp., *Hyalomma* spp. and *Ixodes* spp., have been successfully established, thereby markedly facilitating the advancement of research on TBDs and the isolation of tick-related pathogens (Bell-Sakyi et al., 2018). To better understand the biological functions of ticks and interactions between ticks and tick-borne pathogens, it is necessary to establish effective gene manipulation methods. Compared to mammalian cell line systems, markedly fewer studies specific to gene manipulation of tick cell lines have been performed. Therefore, experiments involving tick cell lines have often been performed by referring to protocols established for mammalian cell lines. There are obvious differences between tick cell lines and mammalian cell lines in culture conditions, rate of propagation, and response to infection (Bayne, 1998; Senigl et al., 2006; Bell-Sakyi et al., 2007; Offerdahl et al., 2012; Bell--Sakyi et al., 2018). Therefore, the identification and development of heterologous protein expression plasmids and comprehensive studies on transfection systems in tick cell lines are required.

Most commercial expression plasmids are designed for mammalian cell lines with promoters such as CMV, SV40, and HSV-TK for mammalian cell lines, obtained from, respectively, human cytomegalovirus, Simian vacuolating virus 40 and herpes simplex virus. Another large group of promoters are derived from highly-expressed housekeeping genes of the organisms themselves, such as phosphoglycerate kinase (PGK) and human beta actin promoters for human cell lines (Nishijima et al., 2009). Most mammalian promoters are reported to have weak transcriptional activity in many insect cell lines (He et al., 2008; Qin et al., 2010). Therefore, it would be reasonable to construct an effective expression plasmid for tick cell lines based on tick-derived promoters. Here, we report the identification of four newly-identified tick-derived promoter regions using the embryo-derived *Ixodes scapularis* tick cell line IDE8 (Munderloh et al., 1994). We compared the expression efficiency of our tick-derived promoters with three widely-used promoters (CMV, PGK and CAG) in IDE8 cells and in the *Ixodes ricinus* embryo-derived cell line IRE/CTVM19 (Bell-Sakyi et al., 2007). We also systematically investigated the features of heterologous protein expression in tick cell lines, including the time course of protein production, transfection efficiency with three commercial transfection reagents, as well as appropriate ratios of plasmid and cell numbers, all of which reflected the obvious differences relative to mammalian cell transfection systems and ultimately indicated the intrinsic differences between tick and mammalian cells.

2. Materials and methods

2.1. Tick and mammalian cell culture

The IDE8 cell line was maintained using standard procedures (Munderloh et al., 1994) in sealed, flat-sided culture tubes (Nunc, Thermo Scientific, USA) with 3 mL L-15B medium supplemented with 10% tryptose phosphate broth (TPB, Sigma-Aldrich, USA), 5% fetal bovine serum (FBS, Invitrogen, Carlsbad, USA), 0.1% bovine lipoprotein (MP Biomedicals), 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. The IRE/CTVM19 cell line (Bell-Sakyi et al., 2007) was maintained at 28 °C in sealed, flat-sided tubes with 3 mL L-15 (Leibovitz) medium supplemented with 10% TPB, 20% FBS, 2 mM L-glutamine, and antibiotics as above. The tubes were incubated in ambient air at 30 °C, replenished with fresh culture medium every week, and subcultured every 15 days. Vero E6 cells (ATCC Cat. No. CRL 1586) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and incubated at 37 °C in an atmosphere of 5% CO_2.

Table 1
Primers used to amplify the regions of promoter candidates identified in the transcriptome of the *Ixodes scapularis* cell line IDE8.

Promoter	Primer	Sequences (5′-3′)
Pro1	F1	actccggccacgatgcgtccGATGAATCGCGCAGGCCTCGT
	R1	attgccaagcttgggctgcaTCGTCAAATCTCGCATGTTTG
Pro2	F2	actccggccacgatgcgtccTTCCATTCGAACTTGCTTAGA
	R2	attgccaagcttgggctgcaCGCGGCACGCGATTGGTTGAGAG
Pro3	F3	actccggccacgatgcgtccTGGCAAGTGCGTACCCTTAC
	R3	attgccaagcttgggctgcaGCATGCGTGCTATTTAGCTCA
Pro4	F4	actccggccacgatgcgtccACGCAATGCGCACGCGAAAG
	R4	attgccaagcttgggctgcaGCTGCGGCGACGGACAACCA
Pro5	F5	actccggccacgatgcgtccGGCGCAAAGCACCAAGTTCG
	R5	attgccaagcttgggctgcaCAATTGAAATTCCAGGGCTT
Pro6	F6	actccggccacgatgcgtccTCTGTGACGTCATTCGTTGC
	R6	attgccaagcttgggctgcaGCAGCGGGGGCTGGACTCCT
Pro7	F7	actccggccacgatgcgtccTTCACGTCATGCGCCCGTTC
	R7	attgccaagcttgggctgcaCAGAACACCACATCCGGGGTG
Pro8	F8	actccggccacgatgcgtccACTAATACAGTTTGGACAGG
	R8	attgccaagcttgggctgcaCCAGGAGATAAGCGGCGACG
Pro9	F9	actccggccacgatgcgtccTGCAAACTGTAAACCGCACA
	R9	attgccaagcttgggctgcaGGGTTCGCCACTCGCTGCTAC
Pro10	F10	actccggccacgatgcgtccCAAACGAGCTATCCGAGATGC
	R10	attgccaagcttgggctgcaACGAGAAAATCAGATGGTAA
Pro11	F11	actccggccacgatgcgtccGATGAATCGCGCAGGCCTCG
	R11	attgccaagcttgggctgcaTCGTCAAATCTCGCATGTTT
Pro12	F12	actccggccacgatgcgtccTACAGACGCACGGGCGTAGC
	R12	attgccaagcttgggctgcaTTTCGGCTTTCACAGGAAAA
Pro13	F13	actccggccacgatgcgtccAAATATATTTACACTGGAGA
	R13	attgccaagcttgggctgcaCCTTCCCTGATGTCCACAAA
Pro14	F14	actccggccacgatgcgtccAACATAATAAAATGTGTCTA
	R14	attgccaagcttgggctgcaTTGTCGTTGAATGAGTCACAG
Pro15	F15	actccggccacgatgcgtccATGCGCTTCGGCACACCCAC
	R15	attgccaagcttgggctgcaGATCTTCAGTACCTCTTCCTT
Pro16	F16	actccggccacgatgcgtccCGGCATTCGCTAAAACGTCG
	R16	attgccaagcttgggctgcaAGATACTTTCATTGAGCACA
Pro17	F17	actccggccacgatgcgtccAATCCGAAAGCCTGCCTTAC
	R17	attgccaagcttgggctgcaCCAAACTTATATGCGCCGGT
Pro18	F18	actccggccacgatgcgtccGTATAGTATCAGCAATACTA
	R18	attgccaagcttgggctgcaTGCTTCCAAATTCAAGATGA
Pro19	F19	actccggccacgatgcgtccCCACCCCAATCTTACCCGTC
	R19	attgccaagcttgggctgcaTCGGAGGAATACATGGCGCG
Pro20	F20	actccggccacgatgcgtccTCGGCGCAATCGGTTTTCTA
	R20	attgccaagcttgggctgcaGCAGACGAAATCGGCGTGCAA
Pro21	F21	actccggccacgatgcgtccAAATTCAAGGCGAAAAAGAA
	R21	attgccaagcttgggctgcaCTCTCCTTTGAACATTCATAG
Pro22	F22	actccggccacgatgcgtccTATATTTCCGACGTGGTGCC
	R22	attgccaagcttgggctgcaTAACCCAGGCCCGAGTCGGC
Pro23	F23	actccggccacgatgcgtccTGAATCGAAGCGTTTTCAAC
	R23	attgccaagcttgggctgcaTGCAAGCCGACAAGGAGGGC
Pro24	F24	actccggccacgatgcgtccATGCGCTTCGGCACACCCAC
	R24	attgccaagcttgggctgcaGATCTTCAGTACCTCTTCCT
Pro25	F25	actccggccacgatgcgtccATAGTTCAACTAGCACCGCT
	R25	attgccaagcttgggctgcaGGCAAGCGCTGCTGCGTGTC
Pro26	F26	actccggccacgatgcgtccGAGAGGGCAGAATTTCGAA
	R26	attgccaagcttgggctgcaGAGCAAAACAACGACCACACC
Por27	F27	actccggccacgatgcgtccTTTCAAAAGAGAATTAAAAAA
	R27	attgccaagcttgggctgcaTCCTCGAATTCTCGAAACGTT
Pro28	F28	actccggccacgatgcgtccACATACTATTCGGTACGACC
	R28	attgccaagcttgggctgcaCACGTGACTCGAAACCTCCT
Por29	F29	actccggccacgatgcgtccCCAGGACCTACAATGTCAGG
	R29	attgccaagcttgggctgcaTGATGCCACTCCAAGGGCAC
Pro30	F30	actccggccacgatgcgtccTGTTTGAATCCAAATGAAAC
	R30	attgccaagcttgggctgcaGCAACGAGGTCCGCGGACAA
Pro31	F31	actccggccacgatgcgtccCTCTCCCGGGTTTCGATCAG
	R31	attgccaagcttgggctgcaCTTCCCTGATGTCCACAAAG
Pro32	F32	actccggccacgatgcgtccTACCTAAGCGATGCTTCAGA
	R32	attgccaagcttgggctgcaAGCTCCTCAAAAAGCTCTTA
Pro33	F33	actccggccacgatgcgtccCGAATTTCCATGGCAACAGA
	R33	attgccaagcttgggctgcaATATAGCGCCGGGACGCCAG
Pro34	F34	actccggccacgatgcgtccTCGTCCTGATGTGGTCTTGC
	R34	attgccaagcttgggctgcaGGGGAAGGCCGCCACACAAG
Pro35	F35	actccggccacgatgcgtccCGACCGCGTTTATGCATCGT
	R35	attgccaagcttgggctgcaGTTGGCGATGCTGGTGATTG
Pro36	F36	actccggccacgatgcgtccGCTCCTTGCCGGAGCGAGGC
	R36	attgccaagcttgggctgcaGATGAGGACCTAGGGCCAGC
Pro37	F37	actccggccacgatgcgtccACACTATGTGCGCAGACTTA
	R37	attgccaagcttgggctgcaTCGGAGGAATACATGGCGCG

(*continued on next page*)

Table 1 (*continued*)

Pro38	F38	actccggccacgatgcgtccTTTGCCCACACCGGGTGTGG
	R38	attgccaagcttgggctgcaAATCTGACCGATGAGCCTGT
Pro39	F39	actccggccacgatgcgtccACGGCCACGTTTTTTCTAGC
	R39	attgccaagcttgggctgcaGTCCAGGACAGAGTCCACCA

2.2. Tick cell RNA preparation and sequencing

Total RNA was isolated from three independent confluent cultures of IDE8 cells using TRIzol reagent (Invitrogen, Carlsbad, USA) following the manufacturer's recommendations. The quality of the RNA was determined using an Agilent 2100 Bioanalyzer with an RNA 6000 Pico kit (Agilent Technologies, Santa Clara, USA). The three RNA samples were then each used for RNA library preparation and transcriptome RNA-seq sequencing, following the instructions of the Illumina Miseq 2000 platform.

2.3. Transcriptome and gene annotation analysis

The raw RNA-seq reads were filtered using the Trimmotic program to remove poor-quality reads and adapters. The program Bowtie2 (Version 1.1.2) was used as an assembler to align sequenced reads with the reference *I. scapularis* genome sequence (assembly GCF_016920785.1). The Cufflinks program (Version 2.2.1) was used to calculate gene abundance.

2.4. Cloning of possible promoter regions

Whole genomic DNA was extracted from the IDE8 cells using the PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The 1500 bp region upstream of the coding region of highly expressed genes was amplified via PCR using the primers listed in Table 1. Purified PCR products were used for luciferase reporter construction.

3. Construction of the luciferase reporters

The commercial plasmid pmriGLO (Promega, Madison, Wisconsin, USA), containing the firefly luciferase gene controlled by the human PGK promoter, was used as the backbone for construction of the recombinant plasmids pmir-CMV and pmir-CAG, in which the PGK promoter sequence was replaced by CMV and CAG promoters respectively, and the pmir-tickPro plasmids in which the PGK sequence was replaced with the sequences of newly-identified tick-derived promoters. The CMV promoter and the CAG promoter were cloned from the commercial plasmids pCAGGS-MCS and pCDNA3.1(+), respectively. PCR products were purified and inserted into the pmriGLO backbone using an infusion clone kit (One Step Cloning Kit, Vazyme, Nanjing, China). The recombinant plasmid sequences were verified by Sanger sequencing (Sangon Biotech, Shanghai, China).

4. Transient transfection

On the day before transfection, the tick and Vero E6 cells were seeded at 2×10^5 cells/well in 24-well plates with 0.5 mL culture medium and cultured under the corresponding conditions. Transfection with Lipofectamine 3000 (Invitrogen, Carlsbad, USA), Effectene (Effectene DNA Transfection; QIAGEN, Dusseldorf, Germany), and Entranster (Entranster-H4000; Engreen, Beijing, China) was performed according to the manufacturers' protocols, and the cells were harvested between one and four days later, depending on the purpose, and assayed for firefly luciferase activity.

Firefly luciferase activity was assayed using the Firefly Luciferase Reporter Gene Assay Kit (RG005, Beyotime, Shanghai, China), following the manufacturer's protocols. Data are presented as mean ± SD luciferase light units (llu), and the corresponding statistical charts were plotted using GraphPad Prism 7.04.

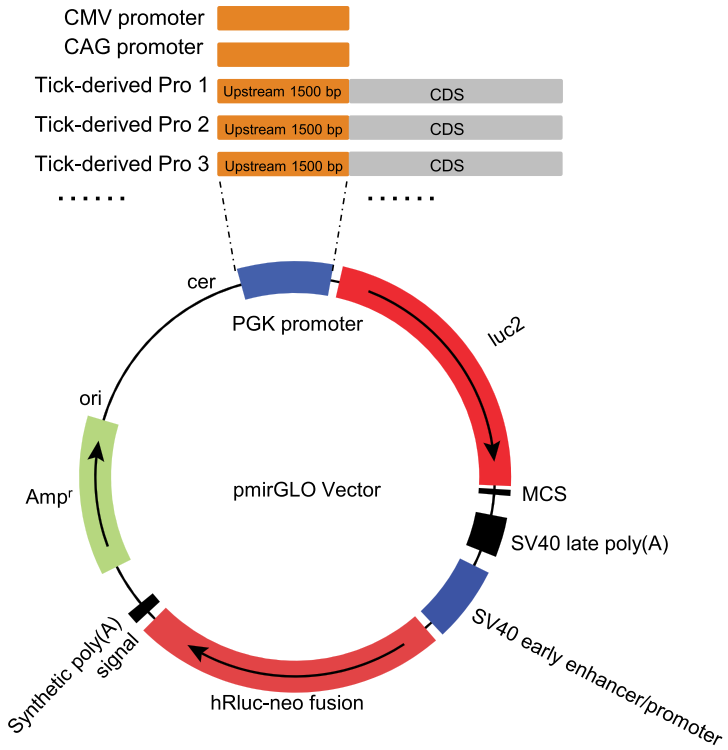

Fig. 1. Schematic diagram of construction of the recombinant plasmid pmirGLO containing mammalian promoters or tick-derived promoters. The red region labeled with luc2 represents the firefly luciferase gene. The blue region labeled with PGK promoter was replaced by CMV, CAG, or the tick-derived promoters (Tick-derived Pro). CDS: coding domain sequence.

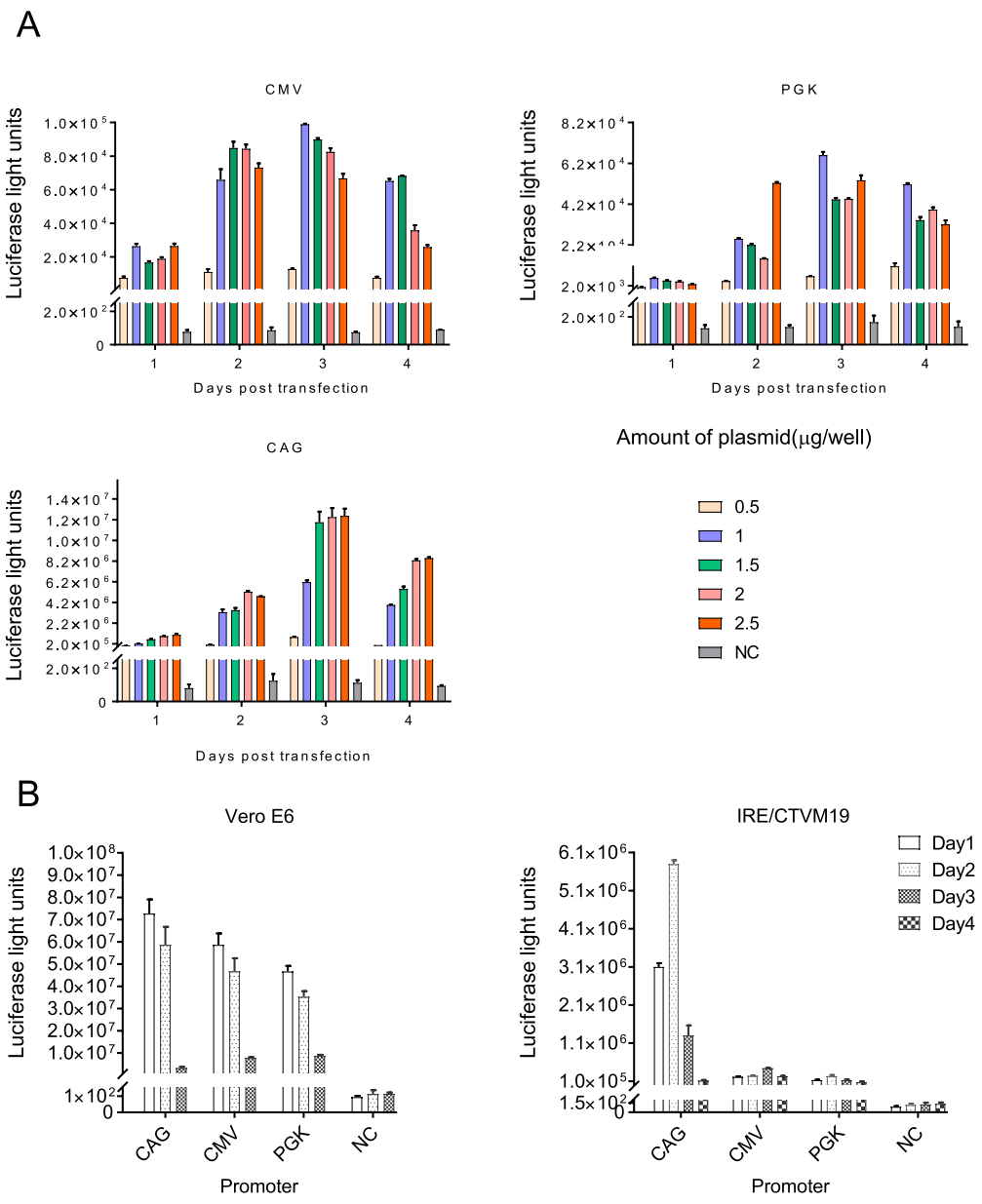

Fig. 2. Luciferase assay for the activity driven by commercial mammalian promoters in mammalian and tick cells. (A) The promoter activities of CMV, PGK, and CAG in the tick cell line IDE8 were assessed from day 1 to day 4 post-transfection. Transfection was performed with Lipofectamine 3000 using the plasmids in serial dilutions from 0.5 μg to 2.5 μg per well. (B) Comparison of protein expression in the time-course experiments guided by the CAG, CMV, and PGK promoters in Vero E6 and IRE/CTVM19 tick cells. A total of 1 μg/well plasmid was transfected with Lipofectamine 3000. Measurement was performed from day 1 to day 3 or day 4 post-transfection. All the data are presented from a representative experiment performed with three replicates; error bars are standard deviations of the mean. NC: negative control (wells transfected without plasmid).

5. Results

5.1. Activity of commercial mammalian promoters in tick cell lines

The pmirGLO dual reporter vector was used to generate recombinant plasmids pmirGLO-CMV and pmirGLO-CAG by replacing the PKG promoter with sequences of, respectively, the CMV and CAG promoters (Fig. 1), which were able to promote expression of the firefly luciferase gene (*luc2*). Cells of the *I. scapularis* cell line IDE8 transfected with the resulting plasmids using Lipofectamine 3000 were harvested to determine the firefly luciferase expression, by which the promoter activity of CMV and CAG was evaluated and compared with that of PGK.

The firefly luciferase assays demonstrated that all three mammalian promoters were able to drive expression of firefly luciferase in the IDE8 cell line (Fig. 2A). The CAG promoter showed the highest promoter activity with luciferase activity at 10^6 to 10^7 llu compared to that of CMV and PKG at 10^4 llu; however, when transfected at 1 μg/well, all of them showed considerably lower promoter activity in tick cells than the 10^7 llu achieved in Vero E6 cells (Fig. 2B). To optimize the transfection conditions, we performed serial dilutions of plasmid from 0.5 μg/well to 2.5 μg/well in a 48-well plate. The results revealed a dose-dependent increase from 0.5 to 1 μg/well and a slight decrease in luciferase activity over 1.5 μg/well (Fig. 2A). Therefore, we selected the 1 μg/well dilution for all subsequent transfection experiments.

The time course of luciferase expression driven by all three promoters revealed that the peak of protein expression was reached at approximately day 3 post-transfection in IDE8 cells (Fig. 2A) and at day 1 post-transfection in Vero E6 cells (Fig. 2B). The promoter activities of CMV, PGK, and CAG were also evaluated in the *I. ricinus* tick cell line IRE/CTVM19 (Fig. 2B). Based on the results, the three commercial mammalian promoters were able to drive expression of heterologous proteins in cells from *I. ricinus*, indicating the broad-spectrum feasibility of these three promoters in tick cells. Consistent with the results in IDE8 cells, the CAG promoter showed much higher promoter activity in IRE/CTVM19 cells than the CMV and PGK promoters. However, the pattern of the time course of protein expression was slightly different from that seen in IDE8 cells. For the CMV promoter, the peak was reached on day 3, while for CAG and PGK, the peak was reached on day 2.

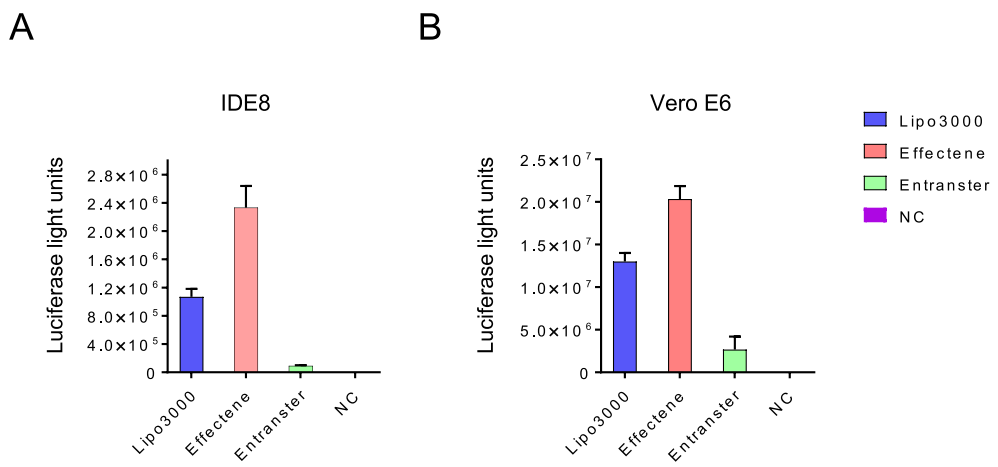

Fig. 3. Comparison of the efficiency of the transfection reagents Lipofectamine 3000 (Lipo3000), Effectene and Entrantser in tick and mammalian cells. A total of 1 μg/well plasmid was used for transfection into IDE8 (A) and Vero E6 (B) cells. Luciferase activity was measured on the third and first days post-transfection for IDE8 and Vero E6 cells, respectively. Data are presented from a representative experiment performed with three replicates; error bars are standard deviations of the mean. NC: negative control (wells transfected without plasmid).

Table 2
The candidate promoters predicted from transcripts highly expressed in IDE8 cell RNA using the Promoter 2.0 software.

Promoter	Length	PromoterScore	Gene
Pro1	1569	1.188	60S ribosomal protein L10
Pro2	1533	1.186	beta-tubulin
Pro3*	1333	1.175	hypothetical protein
Pro4	1615	1.159	eukaryotic translation initiation factor 2 gamma subunit
Pro5*	1628	1.122	ribosomal protein S24
Pro6	1603	1.096	ribosomal protein
Pro7	1531	1.074	40S ribosomal protein S11
Pro8*	1588	1.027	microsomal glutathione S-transferase
Pro9	1525	1	polyubiquitin-C
Pro10	1041	0.712	tubulin beta chain
Pro11	1557	0.705	tubulin beta chain
Pro12*	1571	0.697	ribosomal protein L4
Pro13	1582	0.694	60S ribosomal protein L9
Pro14	1585	0.693	hypothetical protein
Pro15*	1637	0.668	calreticulin
Pro16	1536	0.667	hypothetical protein
Pro17	1493	0.663	hypothetical protein
Pro18*	1550	0.563	ribosomal protein L17
Pro19	1602	0.663	hypothetical protein
Pro20*	1683	0.659	beta-tubulin
Pro21*	1565	0.658	60S ribosomal protein L10a-2
Pro22	1513	0.657	40S ribosomal protein S3A
Pro23	1669	0.648	ribosomal protein L39
Pro24	1586	0.643	tubulin alpha chain
Pro25	1529	0.64	40S ribosomal protein S18
Pro26	1549	0.628	elongation factor 1-beta
Pro27	1492	0.62	B-cell receptor-associated protein
Pro28	1560	0.61	ribosomal protein L11
Pro29	1536	0.607	40S ribosomal protein S12
Pro30	820	0.595	ribosomal protein L26
Pro31	1508	0.594	Hsp90 protein
Pro32	743	0.582	hypothetical protein
Pro33*	1626	0.58	elongation factor
Pro34	1707	0.58	protein disulfide-isomerase A6 homolog
Pro35	1659	0.579	alpha-tubulin
Pro36	1552	0.567	60S ribosomal protein L22
Pro37	1593	0.564	40S ribosomal protein S2
Pro38	1593	0.564	40S ribosomal protein S2
Pro39	1602	0.663	hypothetical protein

* Candidates were confirmed to have promoter activity by luciferase assay.

6. Transfection efficiency of different reagents

We compared three commonly-used transfection reagents with different mechanisms, employing IDE8 and Vero E6 cells as tick and mammalian cell models, respectively (Fig. 3). With the IDE8 cells, the Effectene reagent showed the highest transfection efficiency, approximately 3-fold higher than that of Lipofectamine 3000, while very low luciferase activity was seen with Entranster. With Vero E6 cells, Lipofectamine 3000 and Effectene had comparably high transfection efficiency, while Entranster resulted in markedly lower transfection efficiency. Based on our findings, subsequent transfections were conducted with Effectene reagent according to the recommendations for use at 1 μg/well to transfect cells seeded in a 48-well plate.

7. Identification of *I. scapularis* tick-derived promoters

As mentioned above, we confirmed the feasibility of three commercial mammalian promoters in IDE8 and IRE/CTVM19 tick cell lines; however, their promoter activity was remarkably lower in tick cells than that in mammalian cells. Therefore, we attempted to identify tick-derived promoters to construct a plasmid specific to the tick cell system, with high promoter activity. First, we performed three parallel transcriptome sequencing runs on IDE8 cells; the resulting data were deposited in the SRA database of NCBI (Accession No. SAMN19591046 to SAMN19591048). The assembly resulted in 11730, 36540, and 65379 annotated transcripts, accounting for 24%, 36%, and 29.6% of the total assembly, respectively. Genes with expression higher than the housekeeping gene *P450* were extracted for further GO analysis. The distributions of GO annotation amongst protein class were consistent among the three repeated RNA-seq libraries (data not shown), despite different amounts of assembly, indicating that our RNA-seq results were reliable. Further, our GO annotation analysis related to molecular function and biological process (Fig. S1) was generally in good agreement with tick transcriptome analysis from other studies, regardless of the tick species (Zivkovic et al., 2010; Karim and Ribeiro, 2015; Martins et al., 2021). These findings indicate that our data qualified for further tick-derived promoter identification.

The top 100 gene expression transcripts were selected for their persistently- and highly-expressed capacity. Based on the annotation of the whole genome sequence of *I. scapularis* in GenBank (GCF_016920785.1), the upstream 1500 bp sequence from the coding domain sequences of the top 100 genes were selected and assessed *in silico* as possible promoters. Thirty-nine possible sequences were predicted as promoter candidates based on the coverage of the promoter sequences (Table 2).

To evaluate the promoter activity of the 39 predicted promoter candidates, plasmids with each of the 39 promoter clones replacing the PGK promoter of pmiGLO were constructed and designated pmirGLO-tick plasmids. The luciferase assay showed that nine candidates were able to drive protein expression in IDE8 cells (Fig. 4), while the other 30 candidates did not show detectable promoter activity (data not shown). Moreover, only four of the nine candidates had promoter activity comparable to that of CMV and PGK, with luciferase activity at the 10^4 llu

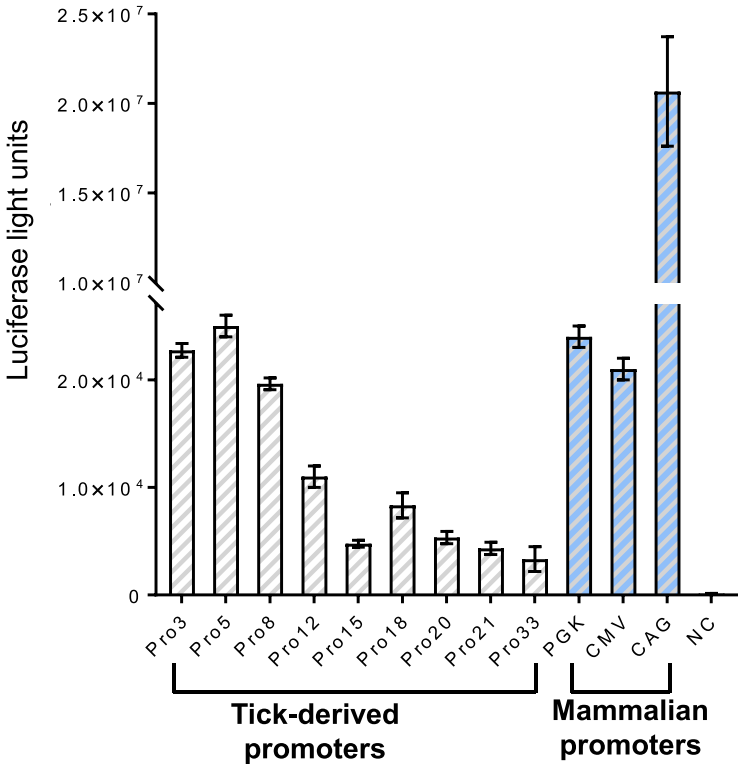

Fig. 4. Comparison of luciferase activity between tick-derived and mammalian promoters in IDE8 tick cells. A total of 1 μg/well of each plasmid was transfected with Effectene into the IDE8 cells and luciferase activity was measured on the third day post-transfection. Data are presented from a representative experiment performed with three replicates; error bars are standard deviations of the mean. NC: negative control (wells transfected without plasmid).

level, and all candidates had considerably lower activity than CAG. These results showed that the promoter activities of our four identified tick-derived promoters (tick-Pro3, tick-Pro5, tick-Pro8 and tick-Pro12) had almost the same promoter activity as the two commercial mammalian promoters (CMV and PGK) but did not outcompete promoter CAG in IDE8 cells.

The functions of the proteins encoded by the genes downstream of the tick-derived promoters tick-Pro3, tick-Pro5, tick-Pro8 and tick-Pro12 are hypothetical protein, ribosomal protein S24, microsomal glutathione S-transferase, and ribosomal protein L4, respectively (Table 2). Three of the four identified genes are housekeeping genes that are, theoretically, persistently expressed for the maintenance of cell survival. To investigate the time course of gene expression driven by our identified promoters, a luciferase assay was performed using both IDE8 and IRE/CTVM19 cells (Fig. 5). Tick-Pro3, tick-Pro8, and tick-Pro12 showed comparable efficiency between the two tick cell lines (Fig. 5A, C, and D), suggesting a wide promoter adaptability of these promoters among different *Ixodes* spp. tick cell lines. However, the promoter efficiency of tick-Pro5 was much lower in IRE/CTVM19 than in IDE8 cells, indicating that this promoter activity was restricted in the heterologous cells (Fig. 5B). As for the time-course of protein expression, the luciferase activity clearly increased during the first three days and was maintained until the fourth day with a small decrease observed, indicating that protein expression mainly began during the first day and high expression was maintained until the third day.

8. Discussion

Gene manipulation has had a long history of application for model organisms, such as mice and zebrafish (Shi et al., 2015; Hardesty, 2018), and insects such as silkworms (López-Ferber et al., 1995; Trochez-Solarte et al., 2019) and mosquitoes (Crampton et al., 1994). However, available gene transfer vehicles and corresponding comprehensive evaluation of transfection systems in tick cells were limited (Kurtti et al., 2008; Barry et al., 2013; Schnettler et al., 2014; Machado-Ferreira et al., 2015; Tuckow and Temeyer, 2015; Kusakisako et al., 2018). The commonly-employed commercial plasmids were originally designed for protein expression in mammalian cell lines, and the promoter activity in different organisms usually varies with cell type. In this study, we aimed to answer a series of basic questions on whether the available commercial mammalian transfection system was applicable to tick cells and whether any differences existed relative to the results obtained for mammalian cells when following the manufacturers' protocols for commercial transfection reagents. The commercial plasmid pmirGLO contains firefly luciferase controlled by the CAG promoter and was proved to function in the *I. scapularis* tick cell line ISE6 (Kusakisako et al., 2018). To evaluate promoter activity in other tick cell lines, we constructed a group of luciferase reporter plasmids based on the backbone of pmirGLO. Of the mammalian promoters investigated, the CAG promoter showed significantly higher promoter activity than the PGK and CMV promoters in both IDE8 and IRE/CTVM19 cell lines, which aligned with the previous report that employed the ISE6 cell line (Kusakisako et al., 2018). Such findings indicate that CAG may be a suitable promoter for tick cell lines when no tick-specific promoters are available. However, only three mammalian promoters were compared in tick cells in the present study, and previous studies have only examined a limited range of mammalian promoters (Tuckow and Temeyer, 2015; Kusakisako et al., 2018). Thus, promoters designed for other arthropods, with potentially better performance, should be considered and evaluated.

Transfection efficiency in tick cell lines may also be affected by the characteristics of different transfection reagents. A previous RNA interference study compared efficiency of six transfection reagents in a panel of tick cell lines including IDE8, ISE6 and IRE/CTVM19, and found only two that gave useful results (Barry et al., 2013). To further optimize the transfection system for tick cells, we evaluated three widely-used

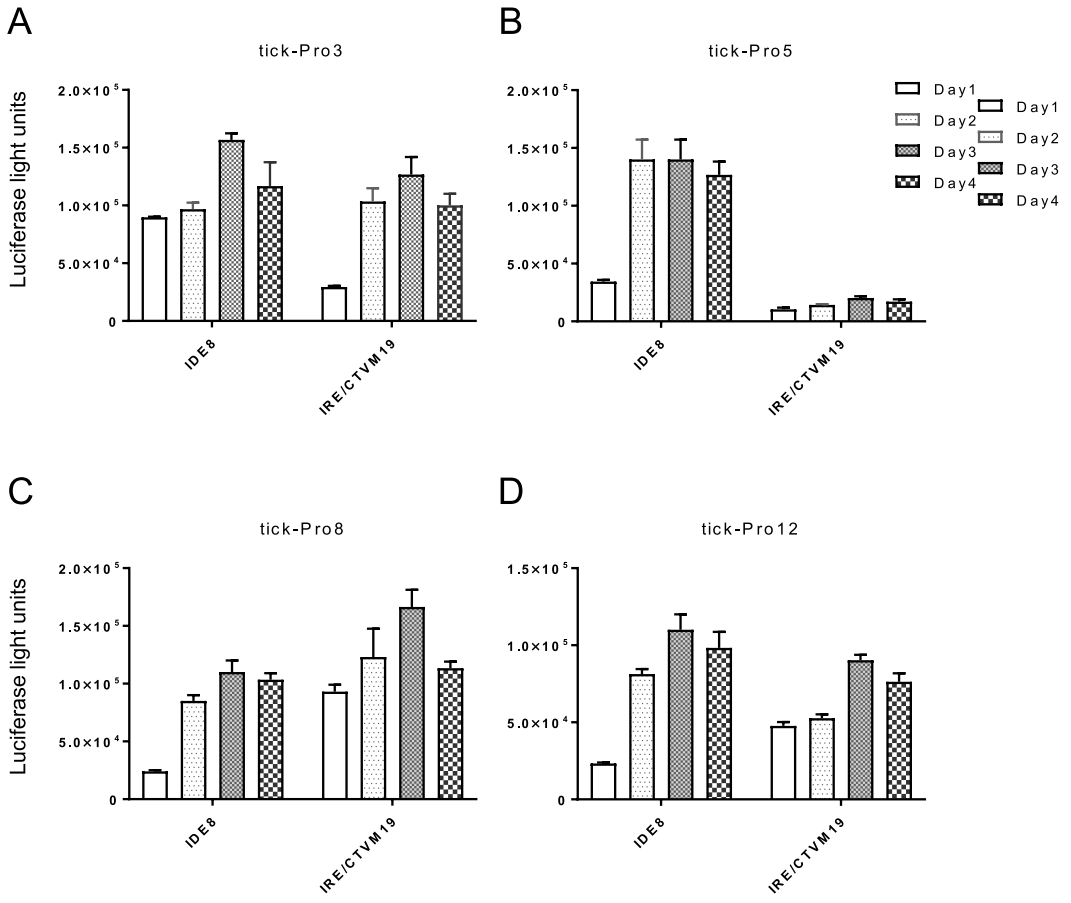

Fig. 5. Firefly luciferase activities driven by tick-derived promoters in IDE8 and IRE/CTVM19 cells. Luciferase activity driven by promoters of tick-Pro3 (A), tick-Pro5 (B), tick-Pro8 (C) and tick-Pro12 (D) was measured on days 1 to 4 post-transfection. Data are presented from a representative experiment performed with three replicates; error bars are standard deviations of the mean. NC: negative control (wells transfected without plasmid).

transfection reagents, which have different mechanisms: Lipofectamine 3000 contains cationic liposome nanoparticles which mediate the interaction of the negatively-charged nucleic acid and the cell membrane; Effectene is a non-liposomal lipid reagent in conjunction with a DNA-condensing enhancer suitable for DNA transfection of eukaryotic cells even in the presence of serum; and Entranster is composed of nanomolecular polymers, which can neutralize the negative charge on the surface of DNA plasmids, wrap the compressed molecular DNA into small DNA particles and carry molecular DNA into cells via endocytosis. Our results showed that for mammalian cells, Lipofectamine 3000 and Effectene have comparable efficiencies and supercede Entranster. Furthermore, both Lipofectamine 3000 and Effectene can exert activity in tick cells; however, Effectene was identified to be more efficient, while Entranster was not suitable for *Ixodes* spp. tick cell transfection. Effectene was also reported to be effective in transfection assays with *Rhipicephalus microplus*-derived cell lines (Tuckow and Temeyer, 2015). Our results reflected some differences in the physical and biological properties of tick and mammalian cells affecting their ability to receive exogenous DNA molecules. Therefore, one should exercise caution when performing tick cell transfection according to protocols designed for mammalian cells.

Constructing an effective promoter for gene manipulation in organisms is of fundamental importance. Usually, promoters from the target organism are the first choice for identification of promoters with high activity (Orr and Sohal, 1994; Asada et al., 2012). Owing to the availability of whole genome sequences, specific promoters for important genes have been identified for a number of model insects, such as the promoter of the *Drosophila* genes COPIA and ACT5C (Qin et al., 2010) and the promoter of the silkworm gene Chorion (Lecanidou and Papantonis, 2010). However, whole-genome sequencing of ticks has lagged behind that of insects, with few complete sequences available and with annotation incomplete or lacking (Gulia-Nuss et al., 2016; Jia et al., 2020). While multiple cell lines are now available from many of the tick genera and species of greatest medical and veterinary importance worldwide (Bell-Sakyi et al., 2018), as yet only a few of these cell lines have been exploited in studies on tick genetic manipulation (Esteves et al., 2008; Machado-Ferreira et al., 2015; Kusakisako et al., 2018). Thus, there has been a lack of expression vectors specific for ticks, which has hindered research on ticks and tick-related etiological studies. Accordingly, in the present study, we aimed to construct plasmids containing tick-derived promoters and evaluate their promoter activities in cell lines derived from two species of *Ixodes* ticks. Based on transcriptome analysis, the 1500 bp sequences upstream of the coding regions of the top 100 expressed genes were filtered and put into the Promoter 2.0 prediction server for promoter prediction. By replacing the original promoter region of pmriGLO, we constructed 39 luciferase reporter plasmids. According to the luciferase assay, four of the nine workable promoters displayed promoter activity comparable to that of PGK and CMV; however, their activity was significantly lower than that of CAG. May result some of the highly-expressed genes have been missed in our screening. And, we did not analyze the cis- and trans-regulatory elements in our identified promoter regions, which may contribute to or suppress promoter activity (Tuckow and Temeyer, 2015). Additionally, exploring elements with promoter activity from tick-borne viruses may be another promising method for identifying high effective promoters for ticks. Therefore, more improvements will be achieved

through further research.

The luciferase assay with IRE/CTVM19 suggested that the IDE8-derived promoters can also perform their intended tasks in cells derived from a different *Ixodes* species, indicating the possible high commonality of promoters among tick species as reported previously for a *Haemaphysalis longicornis* promoter in ISE6 cells (Kusakisako et al., 2018). Therefore, identifying promoters with high activity in one type of tick cell line will aid in the transfection of other tick cell lines. Recently, high-quality genomes of six species of ticks (*Ixodes persulcatus, Haemaphysalis longicornis, Dermacentor silvarum, Hyalomma asiaticum, Rhipicephalus sanguineus sensu lato* and *R. microplus*) were published (Nava et al., 2015; Jia et al., 2020), thereby markedly contributing to the improvement of our present work in identifying higher activity of tick-derived promoters, which may be applicable to many of the available tick cell lines.

In mammalian systems, it is generally recommended to collect or detect proteins from 24 h to 48 h post transfection (Pan et al., 2009; Lin et al., 2015). Our results from the time-course experiment on mammalian promoters in Vero E6 cells were consistent with previous findings, as the expression of foreign genes mainly began on the first day and continued to accumulate until the second day. In contrast, protein expression driven by these mammalian promoters was delayed in IDE8 and IRE/CTVM19 tick cells and mainly occurred during the second and third days, suggesting that foreign protein expression triggered by CMV, CAG and PGK in tick cells may be slower than in mammalian cells. This could indicate inefficient recognition between mammalian promoters and the tick transcription system, although the lower incubation temperature of the tick cells and their slower growth rate compared to mammalian cells (Bell-Sakyi et al., 2018) could also affect the rate of protein expression. The time course of protein expression driven by the identified tick-derived promoters presented a different pattern from that of the three heterologous mammalian promoters in tick cells, suggesting a potential for developing high promoter activity from tick-derived promoters specific for ticks.

In summary, we evaluated three commonly-employed commercial promoters constructed for foreign gene expression in mammalian cell lines and confirmed their feasibility in tick cell lines. The different transfection reagent preferences and patterns of gene expression via a time-course experiment indicated that mammalian and tick cells have essential discrepancies in their expression systems, thereby highlighting the need to perform a comprehensive investigation on the transfection system in tick cells, beside relying on prior experience with mammalian cells. In total, 39 *I. scapularis* tick-derived promoter regions were evaluated, four of which can be considered to have effective promoter activities in tick cell lines. The gene expression driven by the newly-identified tick promoter regions occurred earlier than that of the mammalian promoters in the two tick cell lines tested. Our study findings should enable further development of a genetic modification system for ticks. With in-depth whole-genome sequencing of ticks, tick-derived promoter identification will be markedly improved.

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Fig. S1 Transcriptomic analysis of total RNA extracted from IDE8 cells; distribution of genes with expression higher than the housekeeping gene *P450* across main GO terms. Proportions of genes assigned various GO terms under the molecular function category (A), and the biological process category (B).

Declaration of Competing Interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2022.101906.

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