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Markedly Different Effects of Monovalent Cations on the Efficiency of Gene Expression

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The effect of monovalent cations on a cell-free transcription–translation (TX–TL) system is examined using a luciferase assay. It is found that the potency for all ions analyzed here is in the order $Rb^+ > K^+ > Cs^+ > Na^+ \approx Li^+ > (CH_3)_4N^+$, where Rb^+ is most efficient at promoting TX–TL and the ions of Li^+, Na^+, and $(CH_3)_4N^+$ exhibit an inhibitory effect. Similar promotion/inhibition effects are observed for cell-free TL alone with an mRNA template.

1. Introduction

The potassium ion (K^+) is the most abundant cation in intracellular fluids. In contrast, Na^+ is much more abundant than K^+ in seawater. The natural content of monovalent alkali cations in seawater is reported as 0.00017 g L^{-1} for Li^+, 11 g L^{-1} for Na^+, 0.39 g L^{-1} for K^+, 0.00012 g L^{-1} for Rb^+, and 0.00 000 00012 g L^{-1} for Cs^+ (1.2×10^{-9}).[1] The reason why all types of living cells on Earth have evolved with an intracellular fluid rich in K^+ is a long-standing and unsolved problem in the life sciences. A number of studies have been performed to clarify the biological effects of K^+ relative to other monovalent cations. K^+ exhibits a significant effect on the structure and function of ribosomes[2–5] and also contributes to the stability of RNA structures,[6–8] both of which contribute to activating translation. Hempel et al.[4] examined the effect of monovalent cations on cell-free translation (TL) in rabbit reticulocyte lysates and reported that the optimal concentrations of the monovalent cations were ≈80 mM and had maxima with respect to TL efficiencies in the order $K^+ > Rb^+ > Cs^+ > Na^+ \approx Li^+$. Regarding the binding/association affinity of monovalent cations for DNA, several studies have reported the order $Li^+ > Cs^+ > Rb^+ > K^+ \approx Na^+$[9–11] or a similar ordering,[12,13] although the differences in affinity among the monovalent cations are not significant and depend on the experimental methods used. In contrast, an exactly reverse order of action for the binding affinity of the progesterone receptor to DNA binding was reported, and such reverse order was attributed to the competitive effect of monovalent cations in the receptor–DNA interaction.[14] Except for the unique behavior of Li^+, this ordering of the binding potential has been explained,[15,16] as monovalent ions with a smaller size of hydration, corresponding to a larger ionic radius,[17–19] may cause a larger Coulombic attractive interaction with negatively charged DNA. On the contrary, it was reported that stronger counter ion condensation,[20] as well as larger contraction of DNA minor groove,[21] is induced by monovalent cation with smaller size; $Li^+ > Na^+ > K^+ > Rb^+$. As a different proposal, Gebala et al. argued[22] that ion atmosphere occupancy around double-strand DNA is insensitive to the cation size across the alkali metal ions Na^+, K^+, Rb^+, Cs^+, except for a preference of around 25% for Li^+. Based on the hypothesis of counterion condensation, a number of studies have reported the specific manner of interaction of cationic counterions with DNA molecules as highly negative-charged polyelectrolytes, including the effect on higher-order structural changes.[23–25] Concerning polymer-and-salt-induced condensation of DNA (Ψ-condensation), Zinchenko et al.[26] performed measurements of single DNA molecules for the transition of higher-order structure from an elongated coil into a compact globule. The observation on the conformational transition was carried out on individual genome-sized DNA molecules (T4 GT7 DNA) in a crowded environment with polyethylene glycol, and the researchers found that monovalent ions promote the folding transition with the potentiality: $Na^+ > K^+ > Li^+ > Rb^+ > Cs^+$. Based on measurements of single DNA molecules, Hibino et al.[27] reported that the protective effect of monovalent cations against spermidine^{3+}-induced DNA compaction showed the following order: $Na^+ > Li^+ > K^+ > Rb^+ > Cs^+$. Similar ordering with $Na^+ > K^+ > Li^+ > Rb^+ > Cs^+$ was reported with regard to the potentiality of monovalent ions to cause deswelling for DNA gels collapsed by several condensing agents such as polyamines, multivalent metal cations, cationic surfactants, where the gels were prepared by crosslinking

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double-stranded DNA with ethylene diglycidyl ether.[28] It was also reported that the association in a nucleosome array reconstructed from oligomeric DNA was promoted by Na^+ rather than by K^+.[29] Fujii et al. found that for G-quadruplexes of oligomeric DNA, Na^+, and K^+ stabilize antiparallel and parallel conformations, respectively.[30] Regarding the interaction to RNA, several studies reported that Na^+ condenses more onto the RNA than K^+ and that this effect induces larger stability of RNA by Na^+ compared to K^+.[7,31,32] Further studies are needed to understand the extent to which monovalent cations affect actual biological processes in living cellular environment.

In the present study, we focus our attention on the effect of monovalent cations for transcription (TX) and translation (TL) reactions, as indispensable biochemical reactions in living cells. We report the ability of monovalent cations to promote or inhibit gene expression by using a cell-free expression system with a DNA or mRNA template. During the past several decades, cell-free gene expression systems have been developed as a useful in vitro model for shedding light on the fundamental mechanism of transcription–translation (TX–TL) in living cells and have been actively applied to various subjects in the biological and medical sciences. Several recipes for cell-free gene expression have been proposed based on extensive efforts to adjust the concentrations of various salts, small bio-species and macromolecules through a large number of trials to obtain larger quantities of expression products. It is becoming clearer that, in addition to key–lock type specific interactions, environmental parameters in the cytoplasm have a substantial effect on activity related to gene expression. For example, it has been reported that macromolecular crowding causes either the promotion or repression for both TX and TL through the in vitro experiments by using cell lysates.[33–35] It has also been noted that the divalent cations Mg^{2+} and Ca^{2+} and polyamines exhibit a rich variety of important effects on biological activities in living cells. Using a cell-free gene expression system prepared from cell lysates, we have found[36] that polyamine with a trivalent positive charge (3+) completely inhibits protein synthesis above a certain threshold concentration, accompanied by the tight compaction of DNA molecules. Below this critical concentration of 3+ ions to induce DNA compaction, the DNA exhibits a shrunken conformation and, as a result, gene expression activity is much higher than that in the usual cell lysate. Interestingly, the enhancement of gene expression with the addition of K^+ ions is about twice as great as that of Na^+, which is attributed to the greater depletion effect of polyamine binding to DNA in the presence of Na^+ as compared with K^+. A similar competitive effect of polyamine binding to DNA that is caused by divalent cations such as Mg^{2+} and Ca^{2+} has also been found.[37] As an extension of these earlier studies on the effects of environmental parameters such as monovalent and multivalent cations on the conformation of genome-sized DNA as well as its genetic activity, in the present study, we focus on the effect of monovalent cations on gene expression with a TX–TL system, by comparing with the TL reaction.

2. Results

We have performed experiments to observe how gene expression (TX–TL) changes the activity depending on the concentration of added monovalent cations, through the adaption of cell-free in vitro luciferase assay with the TnT (Rabbit Reticulocyte Lysate) T7 Quick Coupled Transcription/Translation System. **Figures 1** and **2** show the relative luminescence intensity as a marker of protein synthesis through gene expression (TX–TL) at various concentrations of alkali metal cations, Li^+, Na^+, K^+, Rb^+, and Cs^+, together with tetramethyl ammonium cation ($(CH_3)_4N^+$). Li^+, Na^+ and $(CH_3)_4N^+$ tended to suppress TX–TL activity. In contrast, K^+, Rb^+, and Cs^+ increased TX–TL activity, with maxima at ≈20 mM. This effect decreased as the concentration increased above 20 mM, and TX–TL activity disappeared almost completely at concentrations of >100 mM. The promotional effect on TX–TL at 20 mM followed the order $Rb^+ > K^+ > Cs^+ > Na^+ \approx Li^+ > (CH_3)_4N^+$ (Figure 2). We note that the expression level with 20 mM Rb^+ was ≈1 order of magnitude larger as compared with the control without addition of any monovalent cation. It is also noted that Li^+ caused inhibition around 20 mM, then recovered the TX–TL activity around 50 mM, and finally led to complete inhibition at higher concentrations.

We also evaluated the effect of monovalent cations on TL alone by modifying the luciferase assay to include an mRNA encoding luciferase as the template. **Figures 3** and **4** show that Li^+, Na^+, and $(CH_3)_4N^+$ have a tendency to suppress TL activity. In contrast, K^+, Rb^+, and Cs^+ increased TL activity with increases in their concentration up to 20 mM; further increases resulted in a decrease in TL activity. When we compared the TL activity at 20 mM, the strength of the promotion effect followed the order $Rb^+ > K^+ \approx Cs^+ > Na^+ \approx Li^+ > (CH_3)_4N^+$ (Figure 4). Thus, the order of the effect on TL was essentially the same as that on TX–TL, although the promotional effect at a concentration of ≈20 mM was greater for TX–TL (Figures 1 and 2) as compared to TL alone (Figures 3 and 4). Similar to the concentration dependence on the TX–TL activity, Li^+ exhibits inhibition, recovery and finally complete inhibition accompanied by the increase of its concentration.

In order to gain insight into the effects of the physicochemical properties of monovalent cations on the activity of gene expression, **Figure 5** shows how the ionic radius (a,b) and the hydration energy (c,d)[38,39] correlate with TX–TL activity (a,c) and TL activity (b,d), respectively. Here, it is noted that both the ionic radius and hydration energy follow the ordering of monovalent cations in the Hofmeister series,[40] which is a well-known physico-chemical property of ions in aqueous solutions. In the figure, the TX–TL and TL activities are the experimental data with the addition of 20 mM of monovalent cations. Figure 3a,b clearly indicates that the strength of the promotion effect on gene expression (both TX–TL and TL) does not linearly correlate with neither ionic radius nor hydration energy, indicating that the promotion effect does not follow the ordering in the Hofmeister series. In other words, there exists a suitable size of the ionic radius and also hydration energy as in Rb^+, for the efficiency of gene expression both for TX–TL and TL.

3. Discussion

As mentioned above, in the present study the following points have been clarified: (1) Rb^+, K^+, and Cs^+ promote both TX–TL

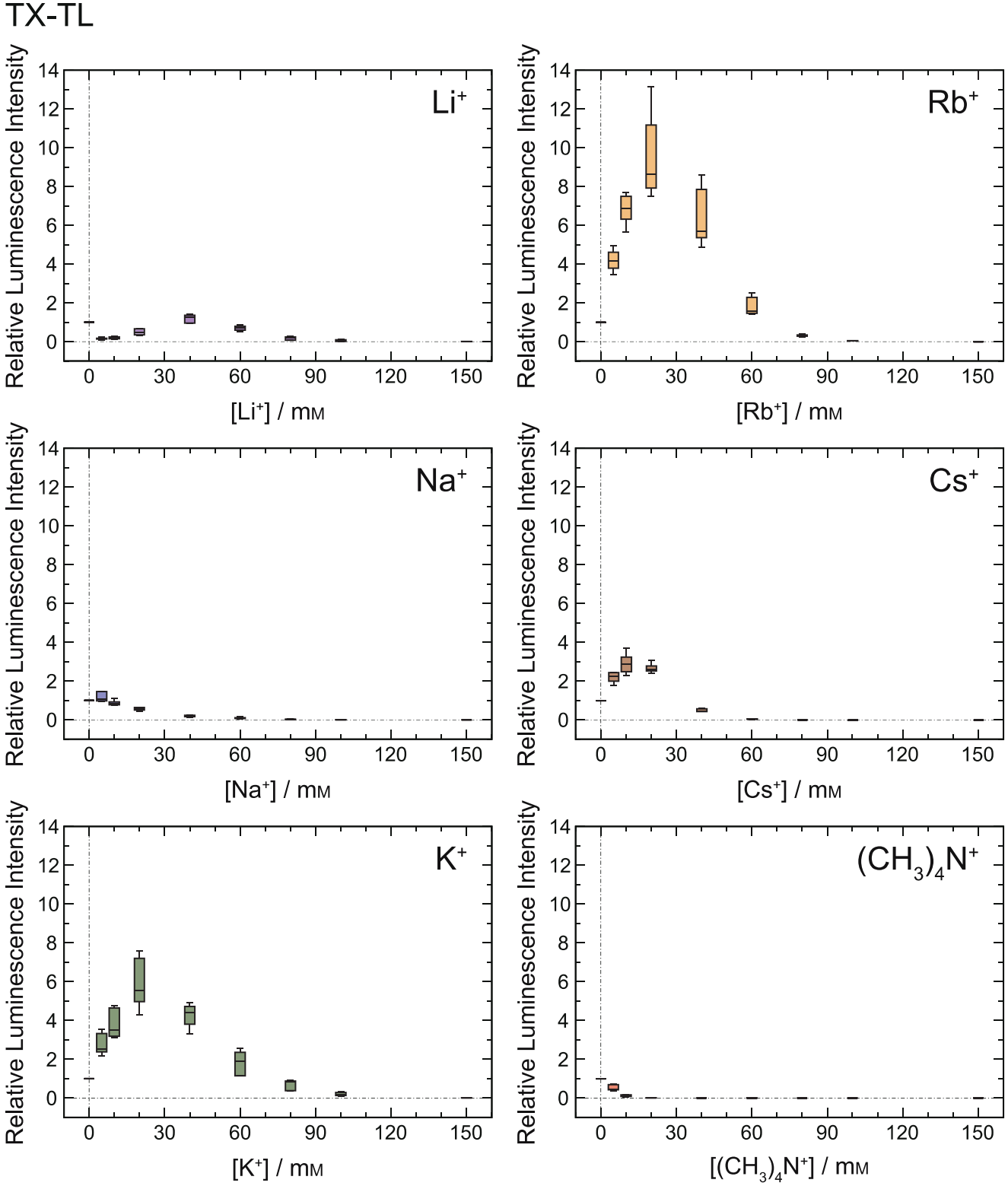
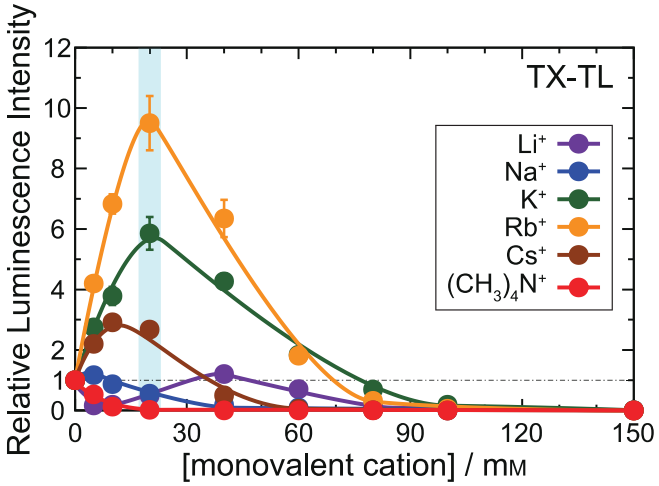


Figure 1. Box-and-whisker plot of relative luminescence intensity as a marker of gene expression (TX–TL) efficiency versus the concentration of monovalent cations added to a cell-free gene expression mixture. The boxplots show the median and the interquartile range for each condition with whiskers extending to the furthest value within 1.5 times the edge of the interquartile range (6 data points). The luminescence intensity was normalized to the control condition (i.e., without the addition of any monovalent cations to the reaction mixture). Luminescence was measured after 90 min of incubation at 30 °C. The concentration of DNA (Luciferase T7 Control DNA) was fixed at 0.3 μm in nucleotide unit. The concentrations of Na^+ and K^+ in the control condition were 18 and 33 mM, respectively (see Table S1, Supporting Information).



p-value at 20 mM

Rb^+, K^+	K^+, Cs^+	Cs^+, Na^+	Na^+, Li^+	Li^+, $(CH_3)_4N^+$
<0.01	0.02	<0.01	0.6	0.02

$Rb^+ > K^+ > Cs^+ > Na^+ \approx Li^+ > (CH_3)_4N^+$

Figure 2. Comparison of the effect of monovalent cations on the gene expression (TX–TL) efficiency based on the results shown in Figure 1. Data presented as mean ± standar error of mean (SEM), 6 data points, and *p*-values are calculated using a two-way Welch's *t*-test. The significance was defined as $p \leq 0.05$.

and TL at ≈20 mM and then tend to depress this activity with further increases in their concentrations. (2) This positive effect is absent for Li^+, Na^+, and $(CH_3)_4N^+$. (3) Among the monovalent cations, the effects on TX–TL and TL follow the order $Rb^+ > K^+ > Cs^+ > Na^+ \approx Li^+ > (CH_3)_4N^+$. (4) TX–TL and TL activities are most enhanced for Rb^+, suggesting the existence of a most suitable ionic size and hydration effect on these genetic reactions. (5) Li^+ shows non-monotonous effects for both TX–TL and TL, namely inhibition, recovery, and complete inhibition as a function of its concentration.

More than a couple of decades ago, Hempel et al.[4] reported results concerning the effect of monovalent cations Li^+, Na^+, K^+, Rb^+, Cs^+, NH_4^+, and $(CH_3)_4N^+$ on the efficiency of cell-free TL in a rabbit reticulocyte lysate. By adapting the time period of the cell-free TL reaction to 3.5 h as the fixed parameter, Hempel et al. observed the promotive effect of TL for the following ions with the ordering; $K^+ > Rb^+ > NH_4^+ > Cs^+$. On the other hand, Li^+, Na^+, and $(CH_3)_4N^+$ exhibited inhibition in a monotonous manner with the increase of ion concentration and there was almost no change in the inhibitory activities among them. Such experimental trends on the effect of TL reaction are similar to those reported in the present study, except that 1) the ordering of K^+ and Rb^+ is opposite, and 2) that there is a non-monotonous effect of Li^+ with inhibition and promotion at lower and larger concentrations being different from Na^+ and $(CH_3)_4N^+$. The authors discussed these effects of K^+ and Rb^+ in relation to their weak hydration because of their relatively large ionic radii as compared with Na^+. This was explained as a working hypothesis: "the interaction of weakly hydrated monovalent cations with weakly hydrated surfaces of the ribosome promotes efficient TL in vitro."[4] In our observations, the positive effect of K^+ and Rb^+ on TX–TL is much larger (more than twice) than that on TL. This suggests that the promotions by K^+ and Rb^+ are attributed to both TX and TL. It is expected that various nonspecific interactions among biochemical species, such as nucleic acids, enzymes, substrates, polyamines, and so on, that are present in the reaction mixture are involved in this effect. For example, we recently found that polyamine has a biphasic promotion/inhibition effect on cell-free gene expression depending on its concentration, and concluded that such an effect is mainly attributed to changes in the higher-order structure of DNA.[41–43] In addition, we found that K^+ enhances gene expression much more strongly than Na^+.[36] Through the measurements of the difference in binding of Na^+ and K^+ to DNA in the presence of a fixed amount of polyamine, it became clear that Na^+ exhibits a larger inhibitory effect of polyamine binding to DNA than K^+. This effect is attributable to the stronger binding of Na^+ to phosphate groups of DNA because of its smaller ionic radius. Actually, it has been revealed that acceleration of gene expression for polyamine concentrations smaller than the critical value to induce DNA compaction is caused by the larger binding degree of polyamines, owing to a decrease of the effective negative charge through the enhanced binding of polyamines accompanied by the parallel ordering of DNA segments. In relation to this, it was observed that the promotion of gene expression in the presence of a suitable amount of polyamine and also K^+ exhibits a good correlation with the appearance of shrunken (not compact) conformations of template DNA.[36] It is to be noted that interactions of polyamines with DNA and also with RNA are rather nonspecific and that polyamines are ubiquitous in the medium of living cells. In other words, nonspecific and abundant chemicals existing in the cell medium, such as monovalent cations and polyamines, have a substantial effect on biological activity, including gene expression, in addition to a rather complicated network with a number of key–lock interactions in biochemical reaction network. To confirm the presence of polyamines in the intrinsic medium of our TX–TL and TL experiments, we have performed a chemical analysis (see Table S1, Supporting Information). Our reaction mixture contained 1.97 mM spermidine^{3+} and 0.032 mM spermine^{4+}. The concentrations of Na^+ and K^+ were 18 and 33 mM, respectively, before the addition of the monovalent cations to the reaction mixtures.[36] It is, thus highly probable that the notable differences in the effects of monovalent cations on gene expression are attributable at least partly to their tuning effect on the manner of interaction of

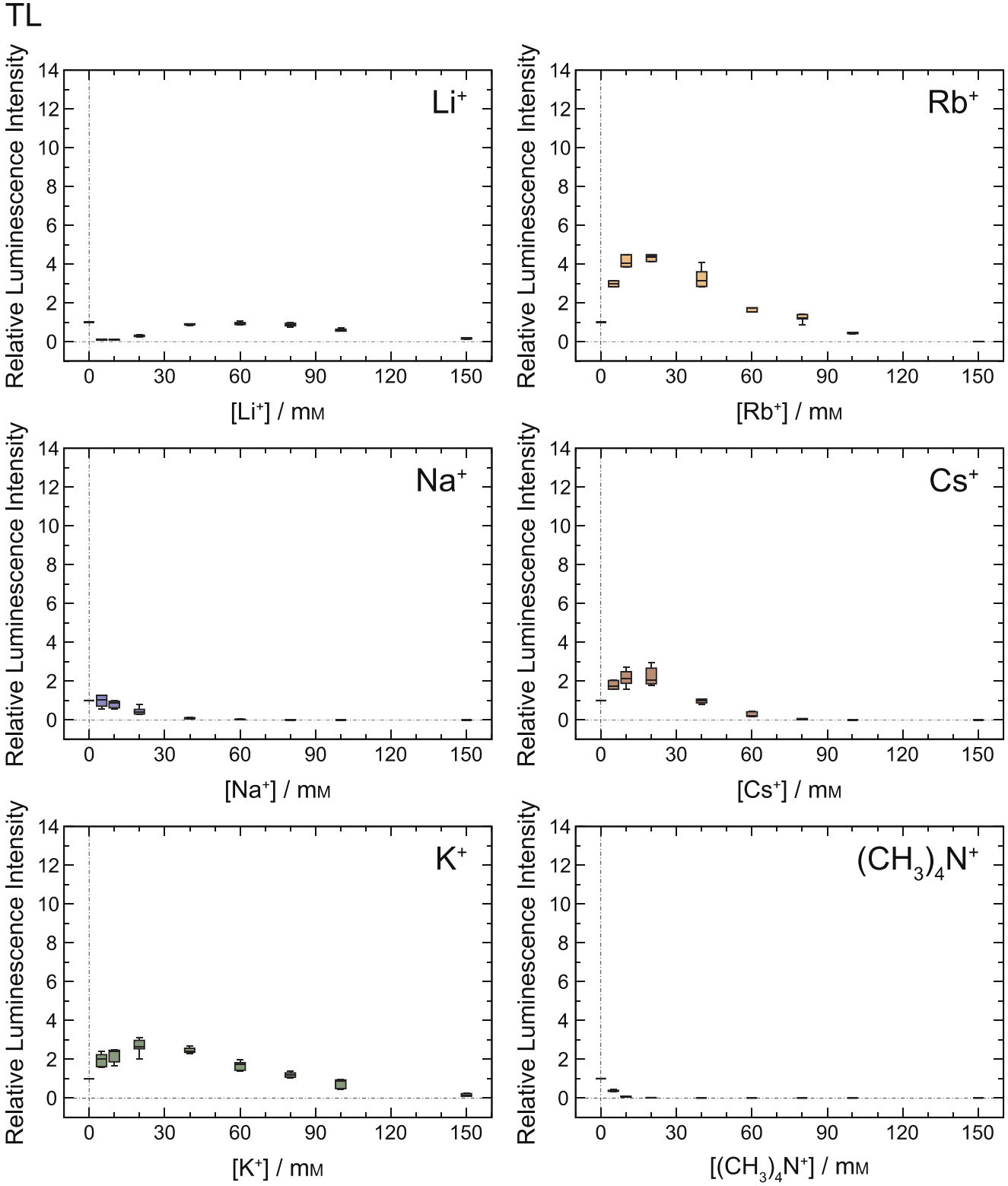
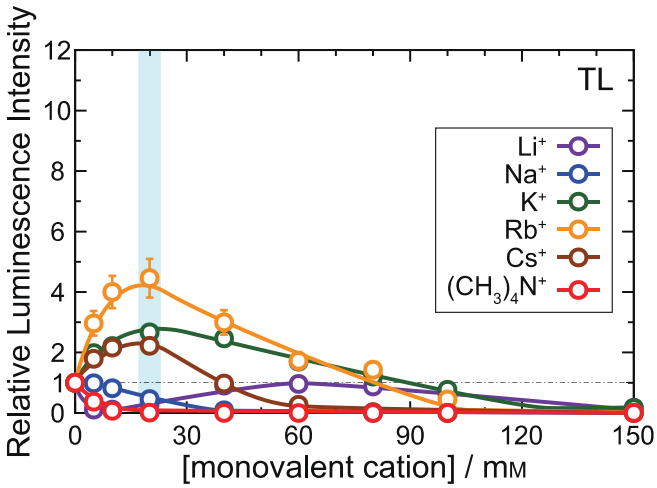


Figure 3. Box-and-whisker plot of relative luminescence intensity as a marker of TL efficiency versus the concentration of monovalent cations added to a cell-free gene expression mixture. The boxplots show the median and the interquartile range for each condition with whiskers extending to the furthest value within 1.5 times the edge of the interquartile range (6 data points). The luminescence intensity was normalized to the control condition (i.e., without the addition of any monovalent cations to the reaction mixture). Luminescence was measured after 60 min of incubation at 30 °C. The concentration of mRNA (Luciferase Control RNA) was fixed at 0.3 μm in nucleotide unit. The concentrations of Na^+ and K^+ in the control condition were 18 and 33 mM, respectively (see Table S1, Supporting Information).



p-value at 20 mM

Rb^+, K^+	K^+, Cs^+	Cs^+, Na^+	Na^+, Li^+	Li^+, $(CH_3)_4N^+$
0.037	0.115	<0.01	0.1	<0.01

$Rb^+ > K^+ \approx Cs^+ > Na^+ \approx Li^+ > (CH_3)_4N^+$

Figure 4. Comparison of the effect of monovalent cations on the TL efficiency based on the results shown in Figure 3. Data presented as mean ± SEM, 6 data points, and *p*-values are calculated using a two-way Welch's *t*-test. The significance was defined as $p \leq 0.05$.

polyamines with DNA and RNA. Here, it may be of scientific value to mention the possible effect of anionic species, such as phosphate, ATP/ADP, etc. In relation to this, it was reported that the higher-order structure, as well as TL activity, of DNA is sensitively dependent on the concentrations of NTP (ATP, CTP, GTP, UTP) and also of RNA.[44,45] The non-monotonous effect of Li^+ is also interesting. Among the chemically available monovalent cations, lithium is the smallest and sodium the next smallest. Past literatures[46,47] described competing effect of Li^+ for access to sodium sites in living cellular systems, including the effect to the association of magnesium-ATP. As the biochemical reactions both in TX and TL are regarded as complex system with various substrates and enzymes, the specific effect Li^+ is attributable to the complexity in the biological

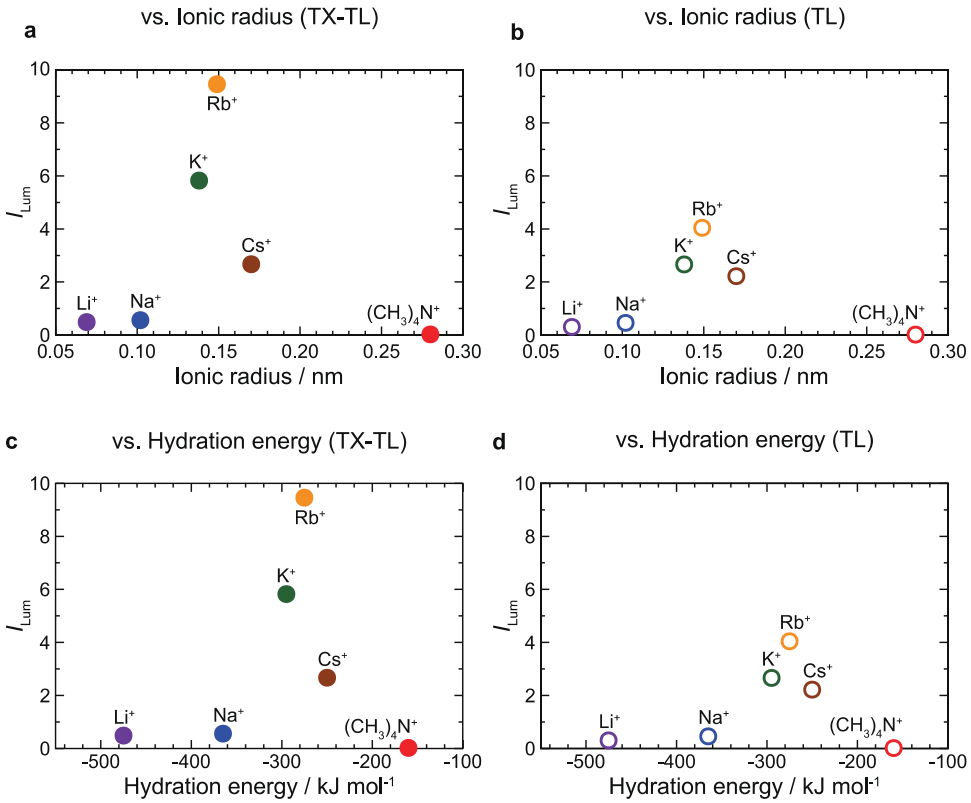


Figure 5. Effect of changes in the ionic radius and hydration energy of cations on TX–TL and TL. a–d) Changes in the efficiency of gene expression (TX–TL) (a,c) and TL alone (b,d) with the addition of 20 mM monovalent salts. Cations are plotted relative to their (a,b) ionic radius and (c,d) free energy of hydration.

reacting system with various substrates and enzymes in TX and TL.

It is known that Rb^+ has a similar biochemical nature to that of K^+ and is easily exchanged with K^+ in biological systems.[48–50] Here we showed that, at least for TX–TL and TL reactions in cell-free extracts, Rb^+ had the greater potential for promoting relevant and important biochemical reactions. It may be interesting to examine the effect of Rb^+ on other biological reactions, and to make clear whether the promotive effect of Rb^+ is higher than that of K^+ in actual biological systems. Such future studies may provide insights into why living organisms on Earth have adapted to K^+-rich medium conditions after a long history of evolution.

4. Conclusion

Monovalent cations had significant effects on both cell-free TX–TL and TL systems. The activities of TX–TL and TL follow this order from high promotion to inhibition: $Rb^+ > K^+ > Cs^+ > Na^+ \approx Li^+ > (CH_3)_4N^+$. Both TX–TL and TL were most enhanced by Rb^+, suggesting that this cation has the most appropriate ionic size and hydration effect for these genomic reactions. From the viewpoint of both biotechnology and the biomedical sciences, it would be useful to establish the most effective experimental conditions for cell-free gene expression. In addition, further research is needed on the importance of small cation species with respect to the essential and indispensable bioreactions of life.

5. Experimental Section

Materials: Lithium chloride (LiCl), sodium chloride (NaCl), potassium chloride (KCl), rubidium chloride (RbCl), cesium chloride (CsCl), and tetramethylammonium chloride ($(CH_3)_4NCl$) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Plasmid DNA (Luciferase T7 Control DNA, 4331 bp) containing both the gene encoding luciferase and the promoter region of T7 RNA polymerase was purchased from Promega (Madison, USA). The mRNA (Luciferase Control RNA), which is uncapped in vitro-transcribed RNA containing a 30-base poly(A) tail, was used for the production of luciferase and was purchased from Promega.

Luciferase Assay for Gene Expression: A cell-free in vitro luciferase assay was carried out with the TnT (Rabbit Reticulocyte Lysate) T7 Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's instructions (instruction with movie of technical procedure: www.promega.jp/resources/protocols/technical-manuals/0/tnt-quick-coupled-transcriptiontranslation-system-protocol/, pdf of the manual: www.promega.jp/-/media/files/resources/protocols/technical-manuals/0/tnt-quick-coupled-transcription-translation-systems-protocol.pdf?rev = feefdae9889c4cf99491809d81723fc3&la = en) and also to the past reports.[36,41–43] For the assessment of TX–TL, plasmid DNA encoding firefly luciferase was used as the template. The concentrations of monovalent cations in the reaction mixture were 18 mM for Na^+ and 33 mM for K^+ (see Table S1, Supporting Information). The pH of the reaction mixture that was measured by pH meter LAQUA F-72 (HORIBA Advanced Techno, Co., Ltd., Kyoto, Japan) was 7. The reaction mixture was incubated for 90 min at 30 °C on a Dry Thermo Unit (TAITEC, Saitama, Japan). For the measurement of TL, the uncapped mRNA was used and was incubated for 60 min under essentially the same conditions as in the TX–TL reaction. In Figure S1 (Supporting Information) the time courses of luminescence intensity both for TX–TL and TL are shown. Based on the observed curves, the above-mentioned time periods were adapted for the reaction mixtures under the conditions that the reactions were well progressing and did not reach the rate-slowing stage. The concentrations of plasmid DNA and mRNA in the starting reaction medium were both fixed at 0.3 μm in nucleotide unit. The expression levels of luciferase were evaluated after the addition of luciferin as a luciferase substrate (Luciferase Assay Reagent, Promega) by detecting the emission intensity at ≈565 nm using a luminometer (MICROTEC Co., Chiba, Japan).

Statistical Analysis: Both gene expression experiments, TX–TL (Figures 1 and 2) and TL (Figures 3 and 4), were carried out as independent three reactions and each reaction was measured twice; 6 data points at each experimental condition. Data analyses were performed for the relative luminescence intensities normalized to the control experimental condition (without the addition of any monovalent cations to the reaction mixture). For the box plots shown in Figures 1 and 3, the lower and upper box boundaries indicate the 25th and 75th percentiles, respectively, the line inside the box is the median, and the lower and upper error lines indicate the maximum and minimum data points that are included inside the 1.5 times area of the box boundaries. Data shown in Figures 2 and 4 were expressed as means ± SEM, with 6 data points, and *p*-values are calculated using a two-way Welch's *t*-test for each combination. In all cases, significance was defined as $p \leq 0.05$. Statistical analysis was performed using IBM SPSS Statistics (International Business Machines Corporation (IBM), Armonk, New York, USA).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords

cell-free gene expression, luciferase assay, monovalent cations, transcription–translation

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