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**The location of the left-handedly curved DNA sequence affects
exogenous DNA expression *in vivo***

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Short title: Left-handedly curved DNA sequence and transgene expression

Abstract

The intranuclear disposition of a plasmid is extremely important for transgene expression. The effects of a left-handedly curved sequence with high histone affinity on plasmid expression were examined *in vivo*. A naked luciferase-plasmid was delivered into mouse liver by a hydrodynamics-based injection, and the luciferase activities were quantitated at various time points. The location of the left-handedly curved sequence determined the transgene expression, without affecting the amount of intranuclear exogenous DNA. The plasmid containing the curved sequence at the location that results in the exposure of the TATA box out of the nucleosome core showed the highest expression. These results suggest that sequences with high histone affinity could control transgene expression from plasmids *in vivo*.

Keywords: left-handedly curved DNA; histones; exogenous DNA; intranuclear disposition; hydrodynamics-based injection

(Introduction)

Plasmid DNAs are used for transgene expression with nonviral vectors in gene therapy and biotechnology, in either a naked or complexed form. Nonviral vectors are quite attractive, due to their excellent safety profile [1-5]. However, their low transgene expression efficiency, in comparison to that of viral vectors, is a major problem. To overcome this problem, control of intracellular DNA trafficking, particularly nuclear entry, has been studied in many laboratories.

In addition, the intranuclear disposition of the delivered plasmid is also a factor in efficient transgene expression [5]. The intranuclear disposition is also related to the transient transgene expression from the plasmid. Recently, the intranuclear disposition of a plasmid delivered into mouse liver by a hydrodynamics-based injection was examined [6]. The major reason for the transient transgene expression is the dramatic decrease in the expression efficiency from one copy of the exogenous DNA over time, and this phenomenon proceeds without promoter methylation. The decrease in the expression efficiency was also observed in cultured cells [7]. These results suggest that histones, which have an important function in chromosomal gene regulation, are involved in the phenomenon, since nucleosomes are formed on non-integrated plasmids [8]. The binding of histones limits the access of transcriptional factors to their recognition sites in the plasmid, and thus, the binding mode of histones to the plasmid would

affect transgene expression and its decrease. In addition, histone modifications could cause the decrease. Therefore, the interaction(s) between the plasmid and the histones are quite important for efficient transgene expression, and are one of the keys for controlling the intranuclear disposition.

We focused on DNA sequences that affect the histone binding. Previously, Nishikawa *et al.* reported that [CATGTTTTT]₄ (left-handedly curved DNA), with the appropriate combination of distance and spatial positioning, could activate a eukaryotic promoter when delivered in a naked form into cultured COS-7 cells [9]. They also showed that histones bound to the left-handedly curved DNA, and that the TATA box was exposed out of the nucleosome core when the curved DNA was located at appropriate positions [9]. This finding prompted us to examine the effects of this left-handedly curved sequence *in vivo*. In this study, we investigated the *in vivo* effects of this sequence on plasmids when delivered in a naked form into mouse liver. We found that the position of the left-handedly curved sequence affected the transgene expression by one order of magnitude, without altering the amount of the exogenous DNAs, suggesting the different accessibility of transcriptional factors to the plasmids *in vivo*. Similar results were observed when plasmids complexed with cationic lipids were delivered into mouse liver. These results indicate that the left-handedly curved sequence on plasmids also determined

transgene expression *in vivo*, and that controlled interactions between the plasmid and the histones are important for the intranuclear disposition.

Materials and Methods

Materials

Oligodeoxyribonucleotides were purchased from Sigma Genosys Japan (Ishikari, Japan) in purified forms. The pLHC4/TLN-6, pLHC4/TLN-16, pLHC4/TLN+47, and pST0/TLN-7 plasmids, containing the thymidine kinase (*tk*) promoter and the luciferase gene (Fig. 1) [9], were amplified in *Escherichia coli* strain DH5 α and purified with a Qiagen (Hilden, Germany) EndoFree Plasmid Mega kit.

Hydrodynamics-based injection

Plasmid DNA (20 μ g in 2 ml of saline) was injected into the tail vein of male six week-old Balb/c mice within 5 sec [10,11]. The livers were harvested from the injected mice at various time points, and the luciferase activity and the amount of the exogenous DNA were measured, as described below.

Luciferase activity

Livers were minced with scissors and homogenized completely in Lysis Buffer (100 mM Tris-HCl, 2 mM EDTA, 0.1% Triton X-100, pH 7.8). After centrifugation at 13,000 g for 10 min at 4°C, the supernatant was examined for luciferase activity, using a Luciferase Assay Systems kit (Promega, Madison, Wisconsin, USA).

Isolation of nuclear DNA and Quantitative PCR

Livers were homogenized in phosphate-buffered saline (PBS). After centrifugation at 2,500 g for 5 min at 4°C, the pellet was washed three times with PBS. The pellet was resuspended in DNA Lysis Buffer (100 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl₂, 0.5% (w/v) IGEPAL-CA630, pH 7.4) [12]. After centrifugation at 1,400 g for 5 min at 4°C, the pellet was washed three times with DNA Lysis Buffer. The intranuclear DNA was extracted with the SepaGene reagent (Sanko Jun-yaku, Tokyo, Japan).

Quantitative PCR (Q-PCR) was performed using an ABI 7500 real time PCR system, and SYBR-Green chemistry (Applied Biosystems, Norwalk, Connecticut, USA). A 100-ng portion of the recovered DNA was analyzed by Q-PCR. The luciferase gene in the mouse liver was detected using the following primers: Luc (+), 5'-GGTCCTATGATTATGTCCGGTTATG; Luc (-), 5'-ATGTAGCCATCCATCCTTGTC AAT.

Hydrodynamics-based injection of lipoplex

Plasmid DNA (50 μg) was mixed with 216.3 μl of Lipofectin Reagent (Invitrogen, Carlsbad, California, USA) in HEPES-buffered glucose (10 mM HEPES, 277.5 mM glucose, pH 7.4), and the mixture was incubated at room temperature for 15 min. HEPES-buffered glucose was then added to the mixture to a total volume of 2 ml. The DNA-lipid complex was injected as described above.

Statistical analysis

Statistical significance was examined by the Mann-Whitney test. Levels of $P < 0.05$ were considered to be significant.

Results

Luciferase-plasmids containing the sequence with high histone affinity.

Previously, Nishikawa *et al.* compared luciferase expression from various plasmids containing the left-handedly curved sequence ($[\text{CATGTTTT}]_4$) in the upstream region of the *tk* promoter, when they were electroporated into simian COS-7 cells [9]. The expression depended on the distance and the spatial positioning of the curved sequence. The luciferase expression was enhanced when the curved sequence was located

at appropriate positions, due to nucleosome formation at the curved sequence, resulting in the exposure of the TATA box in the linker region. The pLHC4/TLN-6 and pLHC4/TLN-16 plasmids were the best and second best ones in the above experiments, using COS-7 cells (Fig. 1). In contrast, the expression from the pLHC4/TLN+47 plasmid was the least efficient among the plasmids containing the left-handedly curved sequence, possibly due to the binding of the TATA box to the nucleosome adjacent to that formed at the curved sequence. We examined pLHC4/TLN-6, pLHC4/TLN-16, and pLHC4/TLN+47 as model DNAs in this *in vivo* study. We also examined the pST0/TLN-7 plasmid containing the straight instead of left-handedly curved sequence as a control (Fig. 1).

Effects of the left-handedly curved sequence on gene expression from plasmids delivered in a naked form.

A rapid, high-volume injection method (hydrodynamics-based administration) [10,11] was used for the delivery of naked plasmids to the livers of male Balb/c mice. This method enables the delivery of the plasmid into the nuclei of the liver, without the aid of cationic compounds, which could potentially affect the quantitation of the intranuclear exogenous DNA. The livers were harvested after 8, 24, 48, and 72 hr, and the luciferase activities were measured. The amounts of exogenous DNA at the same time points were examined by Q-PCR after isolation of the nuclei.

At 8 hr after injection, the luciferase expression from pLHC4/TLN-6 and pLHC4/TLN-16 seemed to be higher than that from pLHC4/TLN+47, although the difference was statistically insignificant, due to data variations, as often reported for hydrodynamics-based administrations (Fig. 2A) [10,11]. At 24 and 48 hr, the luciferase activity was significantly higher for pLHC4/TLN-6 than for pLHC4/TLN+47 (Fig. 2B and C). The expression from pLHC4/TLN-16 was comparable to that from pLHC4/TLN-6 at 8 and 24 hr, but was $\sim 1/2$ of that from pLHC4/TLN-6 at 48 hr (Fig. 2A-C). Importantly, the luciferase expression from pLHC4/TLN-6 was ~ 10 -fold more efficient than that from pLHC4/TLN+47 and pLHC4/TLN-16 at 72 hr. Thus, the expression levels were in the order of pLHC4/TLN-6 > pLHC4/TLN-16 > pLHC4/TLN+47 at the time points examined. These results are similar to those obtained with COS-7 cells [9]. The luciferase expression from pST0/TLN-7 was significantly lower than that from pLHC4/TLN-6 at 24 and 48 hr, and seemed lower at 72 hr (Fig. 2B-D).

We then quantitated the amounts of the exogenous DNAs by Q-PCR (Fig. 2E-H). The amounts of the DNAs were broadly equal at each time point, although the data are variable possibly due to variations of introduction efficiency of plasmid DNA by the hydrodynamics-based administrations, and we could observe no tendency for the amounts of the

DNAs. These results suggest that the location of the left-handedly curved sequence did not affect the amounts of plasmids in the nucleus.

Effects of the left-handedly curved sequence on decrease in expression

We then focused on the time courses of the luciferase expression and the amounts of exogenous DNA. As shown in Fig. 2A-D, the transgene expression was transient, and the luciferase activity decreased over time. The luciferase activities at 72 h were hundreds-fold less than those at 24 h for pLHC4/TLN-16 and pLHC4/TLN+47 (Table 1). In contrast, the decrease in the expression was smaller (1/56) for pLHC4/TLN-6. On the other hand, the amounts of these DNAs in the nuclei decreased similarly (Table 1). The amounts of the DNAs at 72 h were 12- to 20-fold less than those at 24 h. Thus, the decrease in the expression was one order of magnitude more rapid than the decrease in the amount of exogenous DNA for pLHC4/TLN-16 and pLHC4/TLN+47.

Ochiai *et al.* found that the transgene expression efficiency per copy of intranuclear plasmid rapidly decreased when the plasmid was delivered by the hydrodynamics-based administration method [6]. We calculated the expression efficiencies for the plasmids used in this study. The luciferase activities at 24 and 72 hr were divided by the amounts of the exogenous DNAs at the same time points. As shown in Table 1, the expression efficiencies, the ratios of the luciferase activities, and the

amounts of the exogenous DNA decreased from 24 to 72 hr. The expression efficiencies at 72 hr were 1/24, 1/15, and 1/19 of those at 24 hr for pLHC4/TLN-16, pLHC4/TLN+47, and pST0/TLN-7, respectively. Thus, the expression from the plasmid was suppressed over time. In the case of the luciferase-plasmid containing the cytomegalovirus promoter, the expression efficiency at 72 hr was 1/32 of that at 24 hr [6]. In contrast, the expression efficiency at 72 hr was only 1/2 of that at 24 hr, in the case of pLHC4/TLN-6. This result suggests that the inactivation of pLHC4/TLN-6 proceeded more slowly than that of other plasmids.

Luciferase gene expression from plasmids delivered as complexes with cationic lipids

Next, we delivered the plasmids as complexes with cationic lipids into mouse liver. The hydrodynamics-based administration was also used in this case. Plasmids (50 μ g) were mixed with Lipofectin reagent, and the luciferase expression in liver was monitored at 48 hr after injection.

As shown in Fig. 3A, the luciferase expression from pLHC4/TLN-6 was higher than that from the other plasmids at 48 hr. In contrast, the amounts of the exogenous DNAs were broadly equal (Fig. 3B). Thus, the effects of the sequence with high histone affinity were similar between the cases of lipoplexes and plasmid DNAs delivered in the naked form.

Discussion

The objective of this study was to examine the effects of the left-handedly curved sequence, with high histone affinity, on transgene expression from plasmids *in vivo*. Naked plasmids containing the sequence at different positions relative to the TATA box were delivered into mouse liver. As in the previous report using cultured cells [9], pLHC4/TLN-6 produced the luciferase protein most efficiently (Fig. 2A-D). The luciferase gene was expressed in the order of pLHC4/TLN-6 > pLHC4/TLN-16 > pLHC4/TLN+47 at every time point. Nishikawa *et al.* showed that a nucleosome was generated on the left-handedly curved sequence, and that the TATA box was located in the linker region for pLHC4/TLN-6 [9]. In the case of pLHC4/TLN-16, the TATA box was located at the edge of the nucleosome, making the access of the TATA box binding protein more difficult. The results obtained in this study also agreed with the findings of Nishikawa *et al.* Thus, the binding of histones to the left-handedly curved sequence seemed to affect transgene expression from plasmid DNA in the mouse liver. In addition, these results indicated that the location of the left-handedly curved sequence affected the transgene expression in non-dividing cells, such as mouse hepatocytes, as well as in dividing COS-7 cells.

Alternatively, the effects of the left-handedly curved sequence in mouse hepatocytes might be attributed to binding of transcription factor(s) to the sequence. If the binding of the factor(s) influences transcription efficiency depending on distance from the TATA box, the results shown above could be obtained. In addition, the left-handedly curved sequence bends the DNA and this might affect the luciferase expression.

The position effects of the left-handedly curved sequence were observed at 8 hr after the injection (Fig. 2A). The results may suggest that histones bound to the plasmids at least in part at 8 hr after their introduction. When we consider that the amount of the luciferase protein reflects the transcription efficiency at earlier time points, the binding of histones to plasmids may occur at an early stage after the administration. However, analysis on the histone binding is necessary for correct interpretation.

Liposomes are expected to be one of the vehicles for plasmids that will be used clinically in the near future. Liposomes have also been used as nonviral vectors in biological experiments. Thus, we examined the effects of the curved sequence on the expression from plasmids delivered in the lipoplex form *in vivo*. In general, transgenes are expressed mainly in the lung by the intravenous administration of lipoplexes, depending on the lipid composition [13-16]. Alternatively, the spleen is a transgene-expressing organ in the case of Lipofectin [17]. In this study, the hydrodynamics-based administration was also used for plasmid-Lipofectin

complexes, since we could deliver the complexes into the mouse liver, the same tissue examined for the naked plasmids. The luciferase gene was expressed in the order of pLHC4/TLN-6 > pLHC4/TLN-16 > pLHC4/TLN+47 as naked plasmids (Fig. 3). Thus, the sequence with high histone affinity affected the expression similarly for naked plasmids and lipoplexes. This result suggests that the histones bound to the plasmids delivered in the complexed form. We presently do not know the actual pathway(s) by which the DNA-lipid complexes are converted to the DNA-histone complexes in the mouse liver. In cultured cells, it is possible that the lipids are replaced directly by histones [18].

In conclusion, the left-handedly curved sequence at the appropriate position could enhance the *in vivo* expression of transgene delivered in naked and complexed forms. The ‘controlled intranuclear disposition’ of the delivered DNA is quite important for achieving practical gene therapy and efficient transfection [5]. Properly controlled interaction(s) of the plasmid with histones by the introduction of functional DNA sequence(s) would allow transcriptional factors to recognize their binding sites in the plasmid. The left-handedly curved sequence is one of the candidates of the functional sequences to control nucleosome formation on plasmids and transgene expression *in vivo*.

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FIGURE LEGENDS

Figure 1. Luciferase-plasmids used in this study. (A) Structure of the plasmid containing the left-handedly curved sequence and the *tk* promoter. LHC, left-handedly curved sequence; GC, GC box; CCAAT, CCAAT box; TATA, TATA box; *luc*, luciferase gene; *amp^r*, *E. coli* ampicillin resistance gene; *ori*, *E. coli* replication origin. (B) Distances between the 5'-end of the TATA box and the 5'-end of the left-handedly curved sequence. The pST0/TLN-7 plasmid containing straight sequence is also shown.

Figure 2. (A-D) Expression of the luciferase gene and (E-H) amounts of luciferase-DNA in the nucleus, examined by hydrodynamics-based injection of the naked plasmid. Plasmid DNA (20 μ g) was injected into the tail vein of male Balb/c mice (six weeks old). The livers were harvested, and the luciferase activities and the amounts of the exogenous DNA were measured. The values represent the averages of at least three separate experiments. A and E, 8 hr; B and F, 24 hr; C and G, 48 hr; D and H, 72 hr. White columns, pLHC4/TLN+47; gray columns, pLHC4/TLN-16; black columns, pLHC4/TLN-6; hatched columns, pST0/TLN-7. Bars indicate SE (standard error). (* P <0.05 and ** P <0.01).

Figure 3. Expression of the luciferase gene, examined by hydrodynamics-based injection of plasmid complexed with cationic lipids. Plasmid DNA (50 μ g) complexed with Lipofectin (charge ratio = 1:1) was injected into the tail vein of male Balb/c mice (six weeks old). The livers were harvested, and the luciferase activity was measured. The values represent the averages of at least three separate experiments. White columns, pLHC4/TLN+47; gray columns, pLHC4/TLN-16; black columns, pLHC4/TLN-6; hatched columns, pST0/TLN-7. Bars indicate SE (standard error). (* P <0.05).

Table 1. Decrease of luciferase activity and exogenous DNA from 24 to 72 hr after administration^a

Plasmid	Luciferase	Exogenous DNA	Ratio (luc/exogenous DNA) ^b
pLHC4/TLN+47	0.0070	0.0812	0.067
pLHC4/TLN-16	0.0023	0.0695	0.042
pLHC4/TLN-6	0.0177	0.0493	0.476
pST0/TLN-7	0.0121	0.1533	0.052

^aThe luciferase activity and the amount of exogenous DNA at 72 hr after administration were divided by those at 24 hr.

^bThe luciferase activities at 24 hr and 72 hr were divided by the amounts of exogenous DNA at the same time points. The quotient at 72 hr was divided by that at 24 hr.

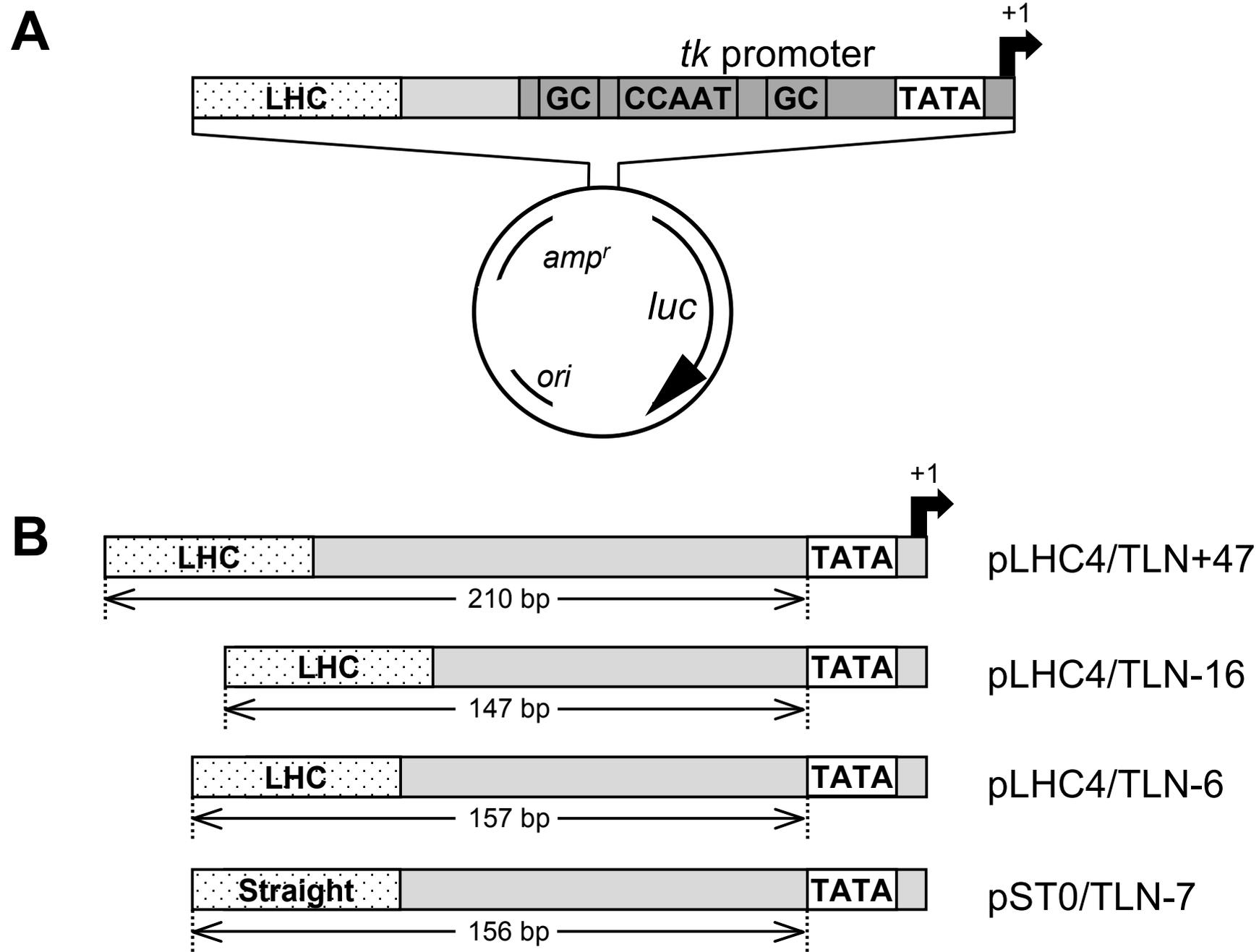


Figure 1

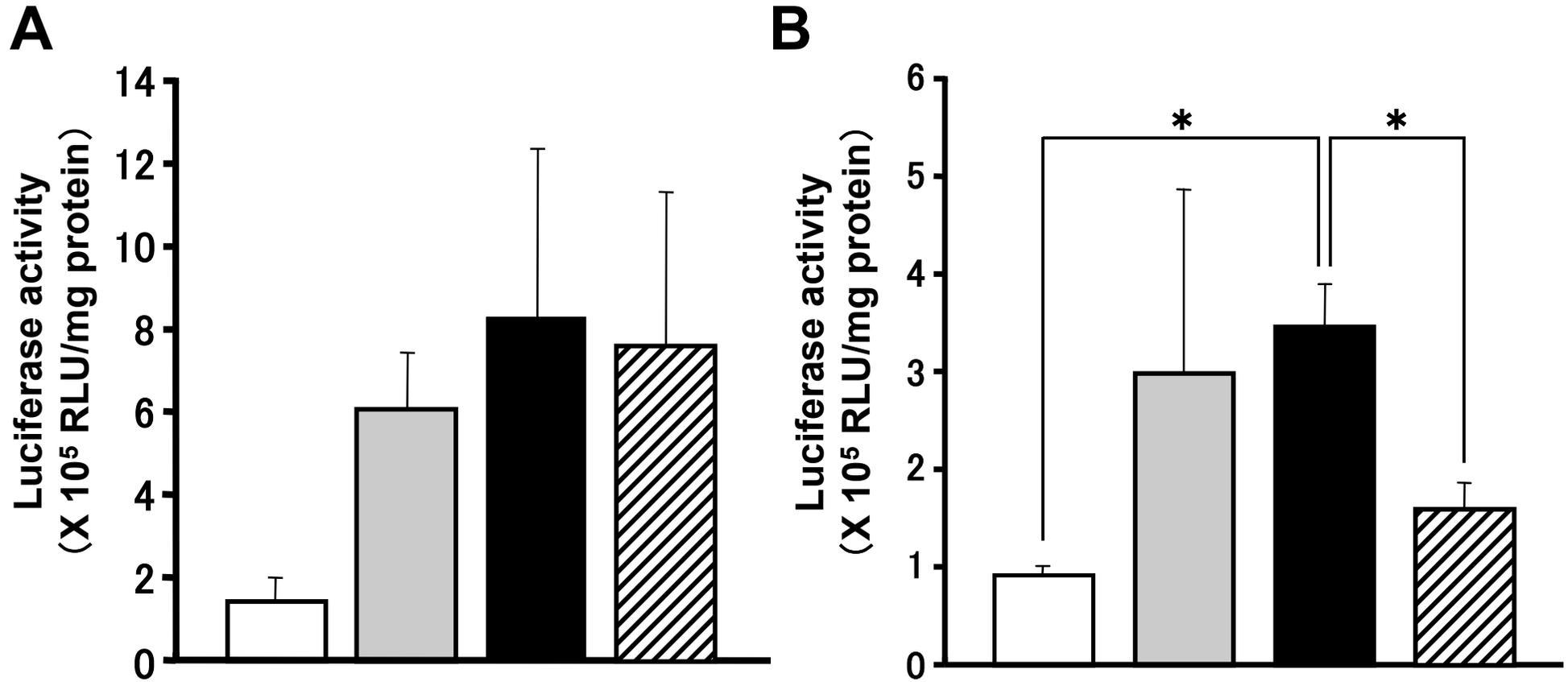


Figure 2

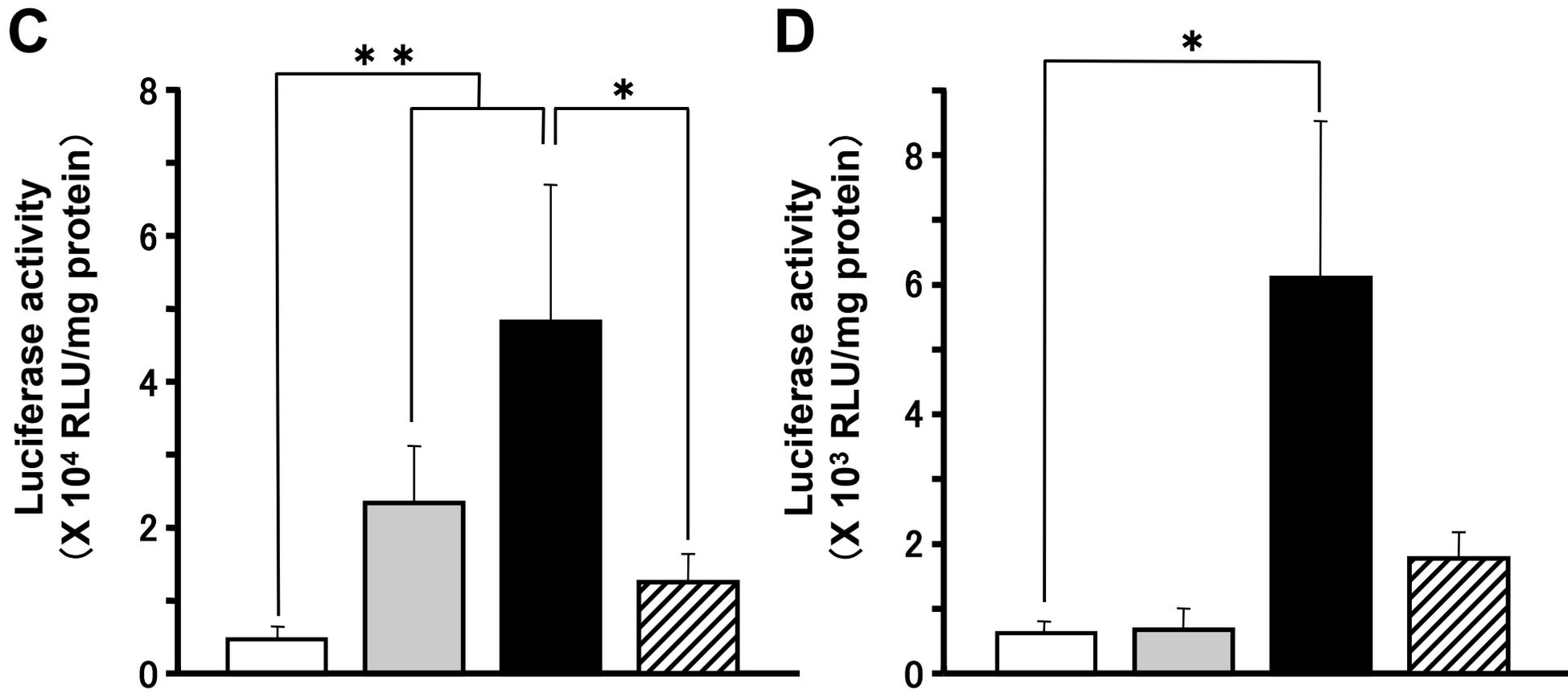


Figure 2

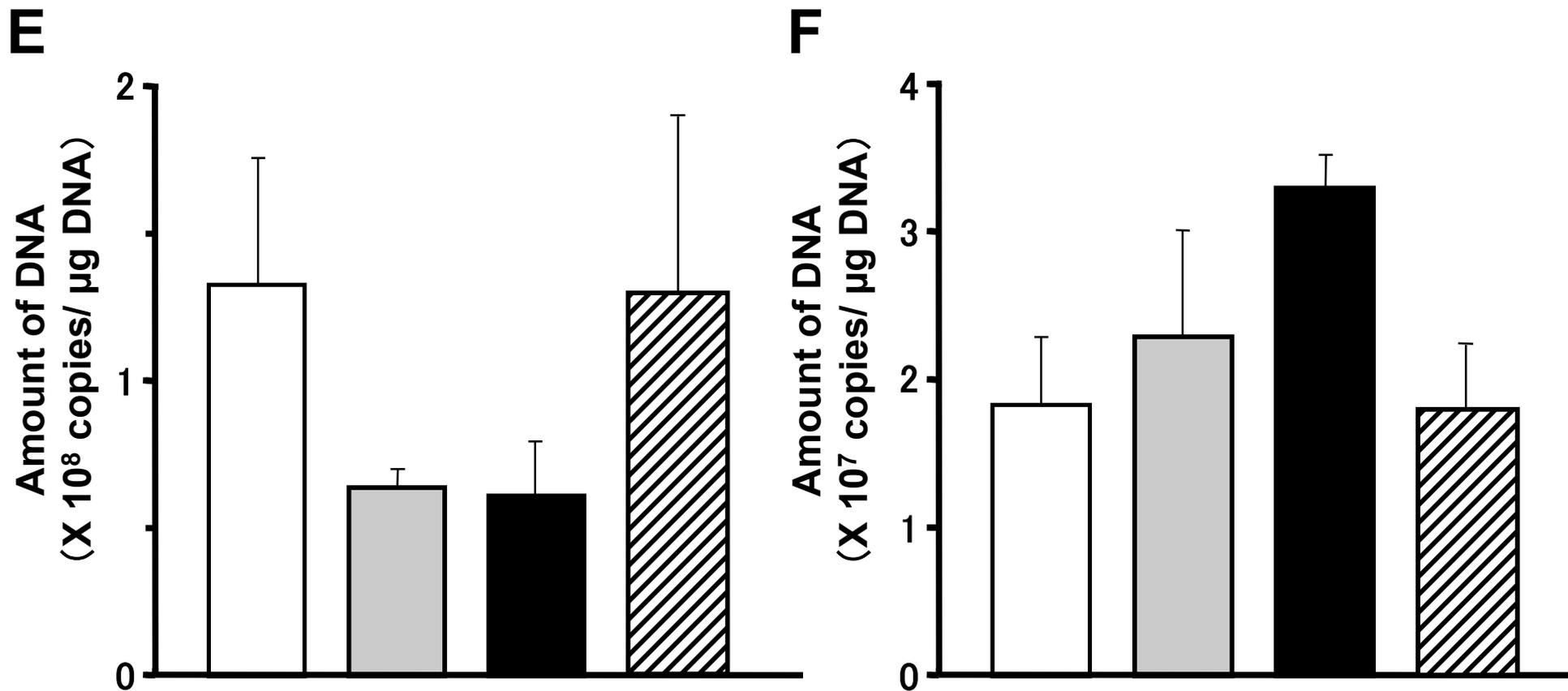
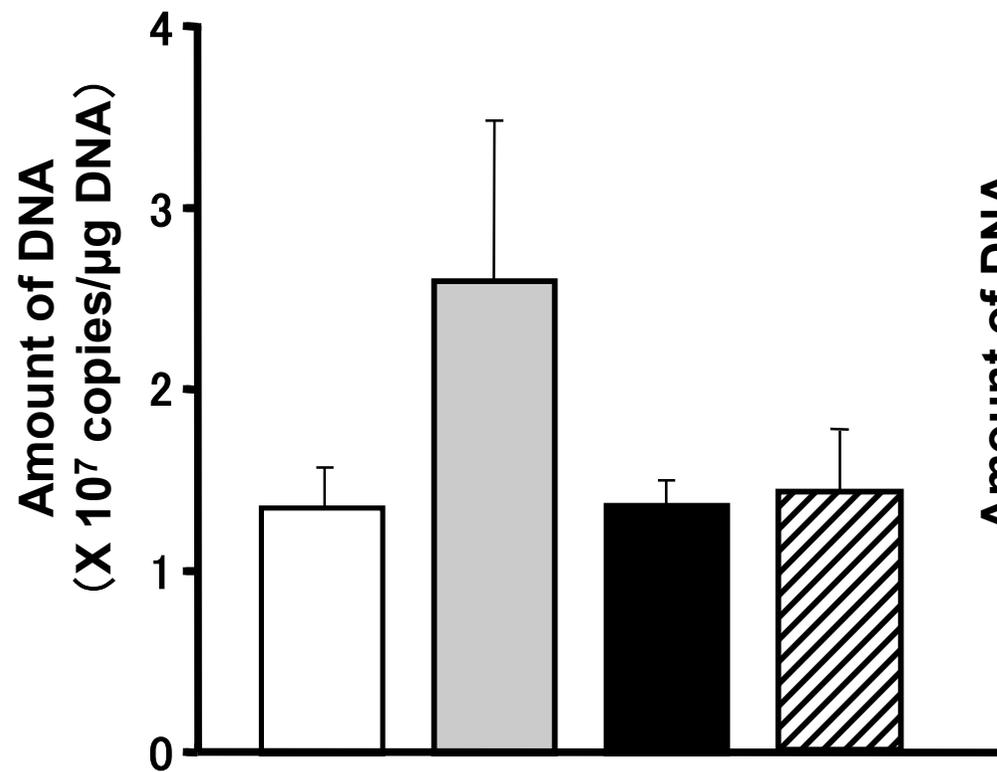
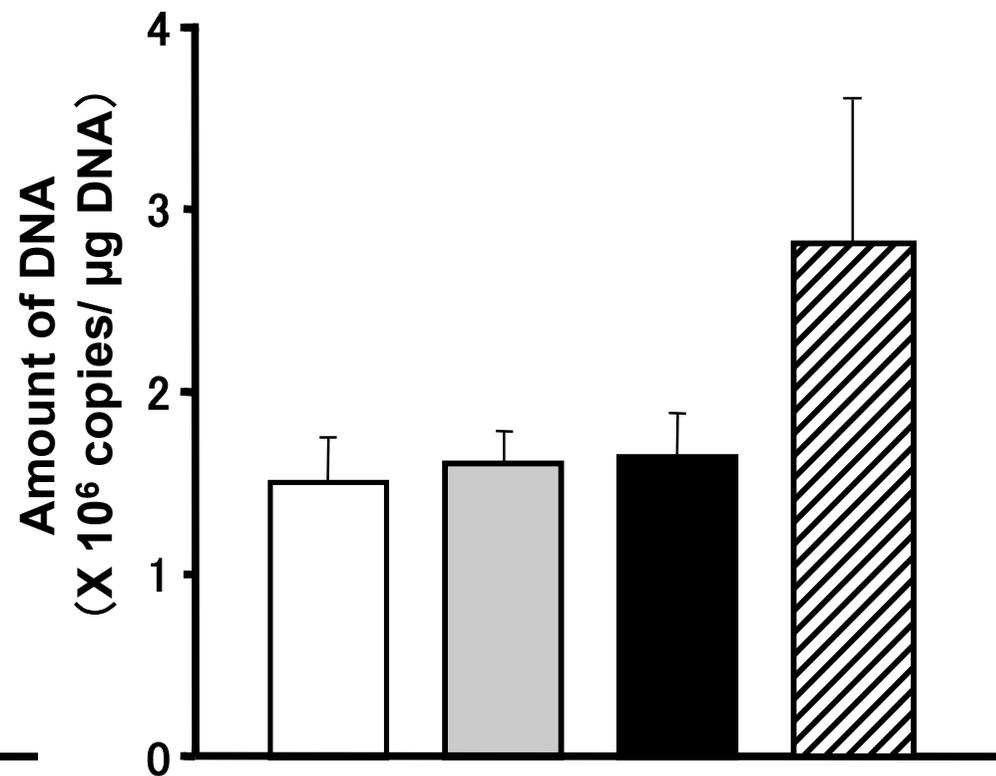


Figure 2

G**H****Figure 2**

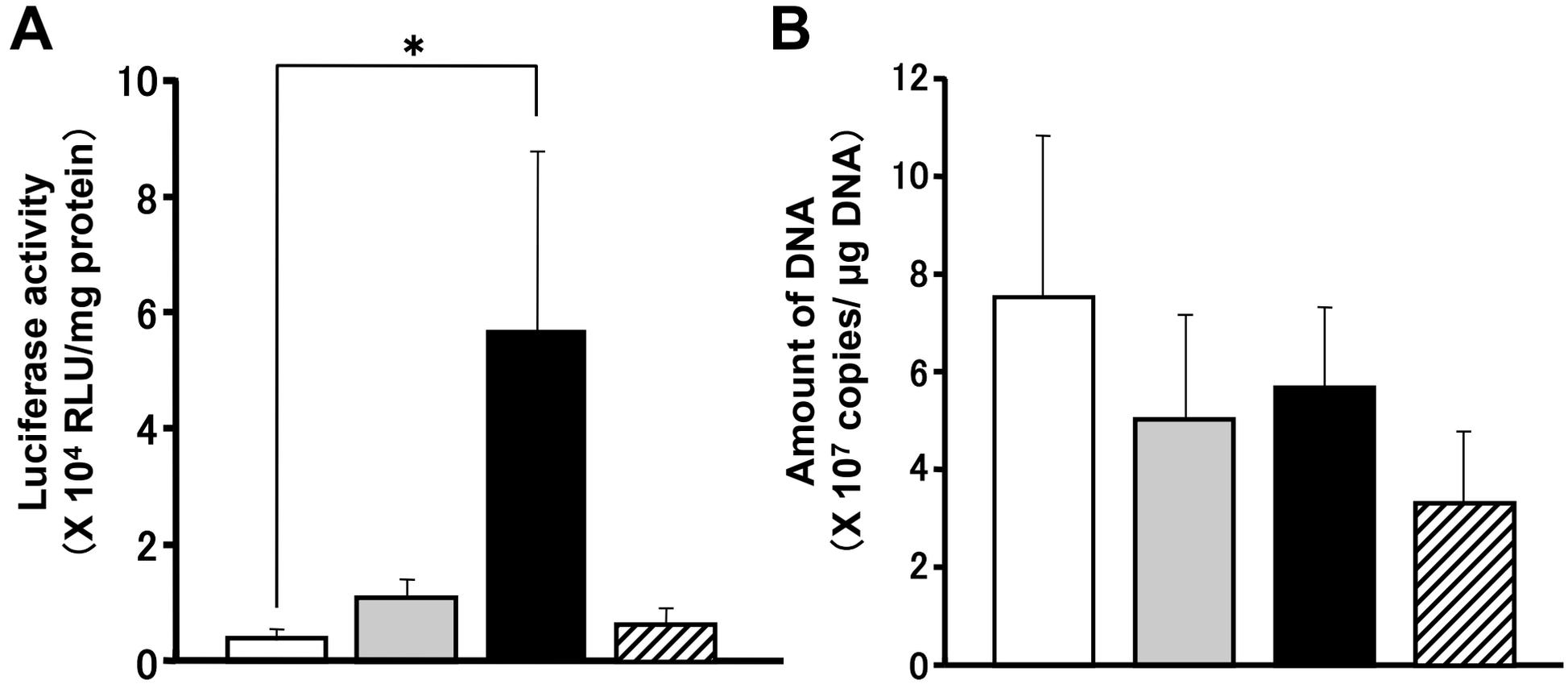


Figure 3