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## **A designed curved DNA sequence remarkably enhances transgene expression from plasmid DNA in mouse liver**

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Running title: Designed curved sequence and transgene expression

## Summary

The intranuclear disposition of plasmid DNA is extremely important for transgene expression. The interactions between the plasmid DNA and the histone proteins are one of the keys for controlling the disposition. In this study, the effects of a left-handedly curved sequence (20–40 repeated A•T tracts) on transgene expression from a plasmid were examined *in vivo*. A naked luciferase-plasmid with the curved sequence was delivered into mouse liver by a hydrodynamics-based injection, and the luciferase activities were quantitated at various time points. Interestingly, transgene expression was markedly increased by the addition of the curved sequence. An analysis of the nucleosome positions near the left-handedly curved sequence suggested that the sequence functions as an acceptor of the histone core and allows nucleosome sliding, resulting in transcriptional activation. These results suggested that the designed curved DNA sequences could control transgene expression from plasmid DNAs *in vivo*.

**Keywords:** left-handedly curved DNA; histones; nucleosome; exogenous DNA; intranuclear disposition

## INTRODUCTION

The development of techniques to express transgenes in living cells would benefit various biological, biotechnical, and biomedical (gene therapy) applications. Nonviral vectors are quite attractive, owing to their excellent safety profile.<sup>1-6</sup> However, low transgene expression efficiency is a major problem with nonviral vectors. Plasmid DNA is used for transgene expression with nonviral vectors, and its intranuclear disposition, as well as intracellular DNA trafficking, is an important factor in efficient and durable transgene expression.<sup>6</sup> To control the intranuclear disposition, many factors, including endogenous DNA binding proteins, should be considered.

Nucleosomes, complexes of DNA and histone proteins, are formed on non-integrated plasmid DNAs as well as the chromosomal DNA.<sup>7,8</sup> The nucleosome core particle consists of ~147 bp of DNA wrapped around a histone octamer, containing two molecules of H2A, H2B, H3, and H4. The binding of histones, in general, limits the access of transcriptional factors to their recognition sites in the plasmid DNA, and thus, the binding mode of histones to the plasmid DNA would affect transgene expression. Therefore, the interactions between the plasmid DNA 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 histone binding. Previously, it was shown that a designed, left-handedly curved DNA sequence, (TTTTT[CATGTTTTT]<sub>3</sub>, T4'), could activate a promoter in plasmid DNA, in cultured mammalian cells and mouse liver.<sup>9-11</sup> Recently, Sumida *et al.* found that longer versions of the left-handedly curved DNA sequence (connection of the T4' sequence in series, named T<sub>n</sub>, *n*=20–40, Fig. 1) markedly enhanced transgene expression in cultured cells.<sup>12</sup> These sequences would be quite interesting tools for controlling the intranuclear disposition, if they were also effective in animals. Thus, in this study, we examined the effects of T20–T40 on transgene expression in mouse liver. We investigated the *in vivo* effects of these sequences on plasmid DNAs delivered in a naked form by a hydrodynamics-based injection. We found that T36 markedly increased the transgene expression. Moreover, micrococcal nuclease (MNase) and DNase I analyses of the plasmid-histone complexes formed in the nuclei suggested that the left-handedly curved sequence acts as an acceptor of the histone core and allows nucleosome sliding, to facilitate the access of transcriptional factors to the promoter.

## RESULTS

## **Effects of the left-handedly curved sequence on transgene expression from plasmids in mouse liver**

Previously, Sumida *et al.* constructed various plasmids containing the left-handedly curved sequence in the upstream region of the thymidine kinase (*tk*) promoter, and compared the luciferase expression in cultured cells when the plasmid DNAs were delivered by electroporation.<sup>12</sup> The insertion of the T20, T24, T28, T32, T36, and T40 sequences (Fig. 1) markedly enhanced the luciferase expression. We examined these plasmids as model DNAs in this *in vivo* study, along with the pST0/TLN-7 (ST) plasmid containing the straight sequence, instead of the left-handedly curved sequence, as a control.

We used a rapid, high-volume injection method (hydrodynamics-based administration)<sup>13,14</sup> for the delivery of naked plasmid DNAs (20 µg) to the livers of male six week-old Balb/c mice. This method enables the delivery of the plasmid DNA into the nuclei of the liver, without the aid of cationic compounds, which could potentially affect the quantitation of the intranuclear exogenous DNA. First, we compared the luciferase expression for all plasmid DNAs, as shown in Fig. 1. The livers were harvested at 48 h after injection, and the luciferase activities were measured.

As shown in Table 1, the left-handedly curved sequence enhanced the luciferase expression. The luciferase activities for the plasmid DNAs

containing the curved sequence were one order of magnitude higher than that for the control (ST) plasmid DNA. Note that the apparent luciferase activity for the ST plasmid was close to the background level ( $\sim 0.3 \times 10^3$  relative light unit/mg protein) that is observed in mouse livers without injection: the expression was almost completely diminished for ST. In contrast, the luciferase activities for the plasmid DNAs containing the curved sequence were much higher than the background level.

We selected the T20 and T36 plasmids for further analysis, and examined luciferase expression at 8, 24, and 72 h, as well as 48 h, after administration. In addition, the amounts of exogenous DNA at the same time points were measured by quantitative PCR (Q-PCR) after isolation of the nuclei. The T28 plasmid was also used for comparison. At 8 h after injection, the luciferase expression from the ST plasmid seemed to be higher than that from the plasmid DNAs containing the curved sequence (Fig. 2). At 24 h, the luciferase activity for T36 was higher than that for the control plasmid, although the difference was statistically insignificant, due to data variations, as often reported for hydrodynamics-based administrations (Fig. 2).<sup>13,14</sup> At 48 h, the luciferase activity was significantly higher for T36 than that for ST (Fig. 2). Again, the apparent luciferase activity for the ST plasmid was close to the background level. Thus, the luciferase expression was maintained at 48 h for the T36 plasmid. At 72 h, higher luciferase expression was observed for all plasmid DNAs

containing the curved sequence, as compared to the control plasmid (Fig. 2). Thus, the curved sequences that we used in this study had the ability to enhance transcription *in vivo*. At 96 h, the expression was almost completely diminished for all plasmid DNAs (data not shown).

We then measured the amounts of the exogenous DNAs by Q-PCR (Fig. 3). When we compared the luciferase expression and the amounts of the exogenous DNAs at 72 h, for example, the highly increased luciferase expression for the T20, T28, and T36 plasmids could not be explained by the slight difference in the amounts of the DNAs. Thus, the left-handedly curved sequence improves transgene expression efficiency from plasmid DNAs in the nucleus. The amounts of the DNAs appeared to be slightly higher for all plasmid DNAs containing the curved sequence at 24 and 48 h. The averaged luciferase activities were divided by the averaged amounts of the exogenous DNAs at the same time points to calculate expression efficiencies. As shown in Fig. 4, the expression efficiencies from the T20, T28, and T36 plasmids were higher than those from the control plasmid at 48 and 72 h.

### **Effects on duration of transgene expression**

The transience of transgene expression is reportedly due to “silencing” (we use this term as a decrease in expression efficiency from a single molecule of plasmid DNA).<sup>15-21</sup> We focused on the time courses of the luciferase

expression, the amounts of exogenous DNA, and the expression efficiencies, by comparing them at 8 and 48 h after administration. The luciferase activity at 48 h was 2,000-fold less than that at 8 h for ST (Table 2). In contrast, the decrease in the expression was smaller for the plasmid DNAs containing the left-handedly curved sequence. In the case of the T36 plasmid, the activity at 48 h was only 29-fold less than that at 8 h. Regarding the amounts of these DNAs in the nuclei, the differences between 8 and 48 h were small (Table 2). The amounts of the DNAs at 48 h were ~5-fold less than those at 8 h for the T20, T28, and T36 plasmids, while the reduction was 15-fold for the control plasmid.

To calculate the expression efficiencies for the plasmid DNAs used in this study, the luciferase activities at 8 and 48 h were divided by the amounts of the exogenous DNAs at the same time points. As shown in Table 2, the expression efficiencies decreased from 8 to 48 h, as previously reported.<sup>19</sup> The expression efficiency at 48 h was 1/120 of that at 8 h for the ST plasmid (when the background was ignored). In contrast, the expression efficiency at 48 h was only 1/6 of that at 8 h, in the case of the T36 plasmid. This result indicated that the left-handedly curved sequence partially prevents the inactivation of the plasmid DNAs.

### **Comparison of histone binding to the promoter region**

The improved transgene expression from the plasmids prompted us to examine the histone binding to their DNA (nucleosome formation). We analyzed the binding of histones near the promoter by a chromatin immunoprecipitation (ChIP) assay.<sup>22,23</sup> This assay was performed by immunoprecipitating the histones that were crosslinked with DNA (after partial fragmentation of the DNA), followed by de-crosslinking and Q-PCR. An 87-bp region involving the TATA box and the proximal GC box was amplified. An anti-H3 antibody was used for the analysis of the histone binding to the DNA. We expressed the results of the ChIP analyses as pull-down/input ratios (%), the ratios of the amounts of exogenous DNA precipitated by the antibody to those of the total exogenous DNAs.

Interestingly, the pull-down/input ratio, corresponding to the ratio of the histone H3-bound form in the target DNA region, was significantly lower for T36 than the control (Fig. 5). This result suggested that fewer nucleosomes are formed near the promoter of the T36 plasmid DNA, as compared to those near the promoter of the ST plasmid DNA. Thus, the promoter of the T36 was open, consistent with the high transgene expression efficiency. Similarly, fewer nucleosomes seemed to be formed near the promoters in the T20 and T28 plasmid DNAs, as compared to those formed in the control plasmid.

### **Positioning of nucleosomes on and around the promoter**

We then analyzed the positions of the nucleosomes to examine the histone-plasmid DNA interactions in detail and to clarify the mechanisms underlying the increased expression. The T36 plasmid DNA was used for this analysis, as the expression from this plasmid was most efficient and the ChIP analysis indicated the open status of the TATA box in the T36 plasmid.

To determine how T36 activated transcription, at first, the potential sequence-specific localization (translational positioning) of the nucleosomes on the region containing the T36 sequence and the *tk* promoter was investigated by an indirect end-labeling analysis. Plasmid-containing nuclei were isolated, and were subjected to digestion with MNase, which cuts the linker regions between nucleosomes, and subsequently to the analysis (Fig. 6). The bands shown with white arrowheads indicate the sites that were digested by the enzyme in the naked state, but were fully or partially protected in chromatin (in this report, this term is used for histones bound to plasmid DNAs in the nucleus). The bands shown with black arrowheads indicate the sites that were newly or highly digested in chromatin. On the basis of these signal profiles and the distances between the black arrowheads, the nucleosome positions were deduced.

Regarding the chromatin formed on the control plasmid (“control chromatin”), several nucleosomes, referred to as I–V, were suggested to be

present, as indicated in the figure. The same nucleosomes seemed to lie on the corresponding regions in the T36-harboring chromatin (referred to below as “T36 chromatin”). Regarding the T36 chromatin, however, additional nucleosomes, referred to as **a**, **b** and **c**, were also suggested to be present (Fig. 6). This assignment was based on the results from the previous study, in which very similar digestion/protection patterns were observed in each of the T12, T20 and T28 regions in yeast minichromosomes and were caused by the high mobility of the nucleosomes on these curved DNA structures.<sup>24</sup>

### **Rotational settings of DNA in chromatin**

DNase I digestion of DNA in nucleosomes results in an average cleavage frequency of ~10 bp/turn. This is because the large enzyme, DNase I, can only access the surface of the nucleosomal DNA. When most of the nucleosomes formed on the same genomic region have almost the same structure; i.e., in the case where the rotational settings of the nucleosomal DNAs are almost uniform, then the cleavage sites become almost equal among the nucleosomes and generate a discrete pattern, the “10-bp ladder”.<sup>25</sup> We subjected the same nuclei fraction to DNase I digestion, to investigate the rotational setting of the DNA in the chromatin (Fig. 7). Except for the ST or T36 region, the control and T36-containing constructs share the same sequence (the shaded and striped regions in Fig. 7). For the

naked DNAs, this region was not necessarily digested in the same manner between the two constructs, indicating that the difference in the neighboring sequence caused this phenomenon. In the state of chromatin, the TATA-box-containing downstream region, that is, the striped region in Fig. 7, was differently digested between the two (compare lanes 5–7 with lanes 12–14), while the upstream region, the shaded region in the figure, generated almost the same digestion patterns (compare lanes 5–7 with lanes 12–14), indicating that the rotational settings of the DNA were dissimilar in nucleosome I between the two constructs, while they were essentially similar in nucleosome II.

A long, clear 10 bp ladder cleavage pattern with a rung spacing of 9 bp was observed for the T36 region of the T36 chromatin (lanes 12–14). However, a quite similar cleavage pattern was observed for the same region of the naked DNA (lanes 9–11). This indicates that the rotational phases of the T36 region did not change even on the histone cores, as compared with those of the naked DNA. In other words, these results suggest that the conformational change that occurred on the T36 region by nucleosome formation was only the compaction of the superhelical curvature to a supercoiled form. The upstream half of the shaded region formed a ~70 bp 10-bp ladder, which was linked in phase with the ~30-bp ladder in the ST region of the control chromatin and with the T36 region ladder of the T36 chromatin, respectively. Thus, the T36 chromatin generated an extremely

long 10 bp ladder, with a length greater than 300 bp. This kind of phenomenon occurs only when the following conditions are fulfilled: more than two nucleosomes are formed on the focused region of each plasmid, the nucleosomal DNAs are continuous in terms of the rotational setting (phase) and thus the DNase I cleavage sites emerge in phase with each other between nucleosomes, and the same rotational setting of the nucleosomal DNA is adopted by the major population of plasmids. This phenomenon can occur when histone core(s) can move within the cylindrical structure of superhelically curved DNA.<sup>24</sup> In such a case, the nucleosomes look as if they can slide, and thus this phenomenon is referred to as nucleosome sliding.

## DISCUSSION

One of the major purposes of this study was to examine the effects of the left-handedly curved sequence on transgene expression from plasmids *in vivo*. Previously, the short version of the left-handedly curved sequence (T4) was shown to have the ability to increase transgene expression from plasmid DNA in liver.<sup>10</sup> In the present study, longer versions of the curved sequence (T20–T40) were placed in the upstream region of the *tk* promoter (Fig. 1). We observed a one order of magnitude enhancement in transgene expression by the curved sequences in comparison to ST at 48 h (Fig. 2), with much more prominent effects than those induced by the T4 sequence

(only 3.5-fold enhancement compared to ST at 48 h).<sup>10</sup> This result agrees with the recent report on the influence of curved sequences in yeast and mammalian cells.<sup>12,24</sup> Thus, the superhelically curved sequence was indicated to be a useful sequence to increase transgene expression *in vivo*.

Another major purpose of this study was to understand the mechanism responsible for the enhanced transgene expression in mouse liver. A very long “10-bp ladder” was detected in the region of the nucleosomes II, *a*, *b* and *c* (Fig. 7). We can explain the mechanism underlying the T36-mediated transcriptional activation in terms of nucleosome sliding. Recently, we reported that nucleosomes slide on the T12, T20 and T28 segments, and this phenomenon can induce the activation of the downstream promoter in yeast.<sup>24</sup> The nucleosome sliding also seems to have occurred on the T36 region, which generated the nucleosome positions *a*, *b* and *c* (Fig. 6). This phenomenon could probably allow nucleosome II to transiently slide upward, which presumably provided a chance for TBP (TATA-binding protein) binding to the TATA box and/or Sp1 binding to the GC box, and allowed the subsequent transcription steps to proceed efficiently. The region spanning from the TATA box to the GC box, which comprises part of the nucleosome II DNA, generated different DNase I cleavage patterns between the control and T36 chromatins (Fig. 7). This difference may have been caused by the different extents of nucleosome II fluctuation between the two chromatins. The

nucleosome sliding also explains the open status of the region including the TATA box and the proximal GC box in the T36 plasmid, indicated by the ChIP analysis (Fig. 5). To prove the mechanism for the enhanced transgene expression in more detail, further studies including ChIP assays using anti-TBP and anti-Sp1 antibodies would be required.

We previously quantified luciferase activity and amount of luciferase mRNA in mouse liver after hydrodynamic injection. We observed that the activity correlated well to amount of the mRNA.<sup>19</sup> Thus, the influence of the curved sequence on luciferase activity would reflect that on transcription. However, the possibilities that the curved sequence affects mRNA stability and/or translation efficiency could not be excluded at this time. Experiments on these possibilities would reveal role(s) of the curved sequence in the increased transgene expression.

Nucleosome I comprised multiple populations of nucleosomes with different rotational settings of the DNA, in both the control and T36 chromatin, as judged from the absence of a 10-bp ladder (Fig. 7). Furthermore, the population ratios in the I-comprising nucleosomes were different between the control and T36 chromatin, as clearly detected in the different DNase I cleavage patterns between them. This difference was presumably caused by the variations in the dynamic properties of the upstream nucleosomes, as described above. At present, however, we cannot discriminate the nucleosome population that activated transcription in the

T36 chromatin from the other. Difference in the ratio of the “active” rotational settings may contribute to the enhanced transgene expression for T36, at least in part.

The 9-bp ladder (Fig. 7) is very unusual, but it was also detected in a previous study.<sup>24</sup> Therefore, the nucleosome structure that is formed on the T36 region must be defined. This study is now in progress.

The introduction of the superhelically curved sequences enhanced transgene expression. Interestingly, these sequences prevented the inactivation (silencing) of plasmid DNAs, as shown in Fig. 4 and Table 2. The expression efficiencies, defined as the quotient of luciferase activities divided by the amounts of the exogenous DNAs, declined quite slowly for the T20, T28, and T36 plasmids, as compared to that of the control plasmid. The reason(s) for the anti-silencing effect of the curved sequences cannot be explained at this time, since the actual mechanism(s) of the silencing has not been elucidated. Histones seemed to bind to plasmid DNA over time.<sup>21</sup> The left-handedly curved sequence could reduce the silencing of plasmid DNA by affecting the binding mode of histones, as shown in Figs. 5-7.

As shown in Fig. 2, expression-enhancing effects of the curved DNA were weak at 24 h and expression from the ST plasmid seemed higher than those from the T20, T28, and T36 plasmids at 8 h. Nucleosome positioning is a dynamic process and varieties of nucleosome positioning patterns could be present at early time points after exogenous DNA was

introduced. In this study, naked plasmid DNA was delivered by the hydrodynamic injection, and the plasmid DNA may not interact “properly” with histones at early time points. This may be one of the reasons for no (or weak) positive effects of the curved DNA at early time points.

We previously found that DNA fragments derived from the plasmid DNA existed in the nuclei within a 72-h time period.<sup>19</sup> To exclude undesirable effects of the DNA fragments, we performed the ChIP assay and the MNase and DNase I analyses using livers excised at 72 h postinjection.

In conclusion, the left-handedly curved sequence could enhance the *in vivo* expression of a transgene, possibly due to its function as a histone core acceptor that allows nucleosome sliding. In addition, the sequence could decrease the silencing of plasmid DNA. The “controlled intranuclear disposition” of the delivered DNA is quite important for achieving practical gene therapy and efficient transfection for biological and biotechnical experiments.<sup>6</sup> The interactions between the plasmid DNA and the histones seem to be one of the factors affecting the intranuclear disposition.<sup>10,11,26,27</sup> Thus, the control of the interactions of the plasmid with histones by the introduction of functional DNA sequences would be important. The left-handedly curved sequence described here is a candidate for such a functional sequence to control nucleosome dynamics on plasmid DNAs and transgene expression *in vivo*.

## **MATERIALS AND METHODS**

### **Materials**

Oligodeoxyribonucleotides were purchased from Life Technologies Japan (Tokyo, Japan), Sigma Genosys Japan (Ishikari, Japan), and Operon Biotechnologies (Tokyo, Japan) in purified forms. The pLHC20/TLN-6 (T20), pLHC24/TLN-6 (T24), pLHC28/TLN-6 (T28), pLHC32/TLN-6 (T32), pLHC36/TLN-6 (T36), pLHC40/TLN-6 (T40), and pST0/TLN-7 (ST) plasmids, containing the herpes simplex virus thymidine kinase (*tk*) promoter and the luciferase (*luc*) gene (Fig. 1),<sup>9,12</sup> were amplified in *Escherichia coli* (JM109) and purified with a Qiagen (Hilden, Germany) EndoFree Plasmid Mega kit.

### **Hydrodynamics-based injection**

Plasmid DNA (20 µg) in 2 ml of saline was injected into the tail vein of male six week-old Balb/c mice within 5 sec.<sup>13,14</sup> 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. All animal procedures were conducted according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee.

### **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).

### **Isolation of nuclear DNA and Quantitative PCR**

Livers were homogenized in phosphate-buffered saline (PBS). After centrifugation at 800 g for 5 min at 4°C, the pellet was washed three times with PBS. The pellet was resuspended in DNA lysis buffer (400 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.5% (w/v) IGEPAL-CA630, pH 7.4).<sup>28</sup> 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).

Q-PCR was performed using an ABI 7500 real time PCR system (Life Technologies, Carlsbad, California, USA), and SYBR-Green chemistry (Toyobo, Osaka, Japan). 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:  
 5'-dGGACCTATGATTATGTCCGGTTATG-3' and  
 5'-dATGTAGCCATCCATCCTTGTC AAT-3'.

**ChIP assay**

The ChIP assay was performed according to the methods of Eberhardy *et al.* and Scribner and McGrane,<sup>22,23</sup> with the following modifications. Seventy two h after the injection, the liver was excised from the mouse and dissected livers were minced with scissors and crosslinked with 1% formaldehyde for 30 min at room temperature. The crosslinking reaction was terminated by the addition of 0.125 M glycine. After centrifugation at 125 g for 5 min at 4°C, the pellet was washed with PBS. The crosslinked liver was then Dounce homogenized with a Digital Homogenizer (Iuchi, Osaka, Japan). After centrifugation at 1,750 g for 5 min at 4°C, the pellet was resuspended in ChIP lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 1% sodium dodecylsulfate, 1 mM phenylmethane sulfonyl fluoride (PMSF), 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, pH 8.0). The cell lysate was diluted with ChIP dilution buffer (16.7 mM Tris-HCl, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, 0.11% deoxycholate, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, pH 8.0) and was sonicated on ice with a Digital Sonifier (Branson, Danbury, Connecticut, USA) for twenty 30-s pulses (output, 20%). After centrifugation at 9,100 g for 10 min at 4°C, the chromatin was pre-cleared for 30 min at 4°C with rotation, using a Salmon Sperm DNA/Protein A

Agarose-50% slurry (Upstate Biotechnology, Lake Placid, New York, USA). The pre-cleared chromatin was aliquoted.

Immunoprecipitation was performed overnight at 4°C with rotation, using 2 µg of anti-H3 antibody (catalogue number 07-690; Upstate Biotechnology). After the overnight incubation, a 30 µl aliquot of Salmon Sperm DNA/Protein A Agarose-50% slurry was added, and the mixture was incubated for 2 h at 4°C with rotation. The beads were washed twice with low salt RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 0.1% deoxycholate, pH 8.0), once with high salt RIPA buffer (20 mM Tris-HCl, 500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 0.1% deoxycholate, pH 8.0), once with LiCl wash solution (10 mM Tris-HCl, 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% deoxycholate, pH 8.0), and finally twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The immune complexes were eluted with elution buffer (10 mM Tris-HCl, 300 mM NaCl, 5 mM EDTA, 0.5% SDS, pH 8.0) and were incubated for 6 h at 65 °C to reverse the crosslinking. After a treatment with proteinase K, the DNA was purified by phenol/chloroform extraction and ethanol precipitation.

The amounts of immunoprecipitated DNA were quantified by Q-PCR with the following primers: 5'-dTCTTGTCATTGGCGAATTCG-3' and 5'-dCCACACGCGTCACCTTAATA-3'. The precipitation ratio

(pull-down/input) was calculated by determining the amounts of the precipitated DNA relative to the input DNA.

### **MNase digestion-based analysis of chromatin structure**

Seventy two h after the injection, the liver was excised from the mouse and nuclei were prepared, as described by Nishikawa *et al.*<sup>9</sup> Aliquots of 100  $\mu$ l nuclei suspension ( $1 \times 10^7$  nuclei/ml) were incubated at 37°C for 1 min. Then, 0.3, 0.9, or 2.7 U of MNase was added, and digestion was performed at 37°C for 2 min. Naked DNA was digested with 0.1, 0.15, or 0.2 U of MNase. The samples were digested with BsrG I, which cuts at +553 (the translation start site of the *luc* gene is +1) within the *luc* gene in the plasmids. The products were separated on a 1.7% agarose gel prepared in 1 X TBE buffer, transferred onto a Hybond-XL membrane (GE-Healthcare, Little Chalfont, United Kingdom), and detected by a radioactively labeled probe (340 bp) corresponding to the region from +217 to +556 in the *luc* gene.

### **DNase I footprinting assay**

DNase I cleavage sites were detected according to the procedure described by Tanase *et al.*,<sup>29</sup> with slight modifications. Seventy two h after the injection, the liver was excised from the mouse. Nuclei suspensions were incubated with 2.7, 3.4, or 4.1 U of DNase I. Naked DNA was digested

using 0.001, 0.005, or 0.01 U of DNase I. Primer extension reactions were performed with the following primers: 5'-dTCTGCGTGTTCGAATTCGCC-3' for analysis of the T36 region and 5'-dGTCTTCCATTTTACCAACAG-3' for analysis of the *tk* promoter. After linker DNA ligation, PCRs were performed with the following sets of primers: 5'-dGCGGTGACCCGGGAGATCTGAATTC-3' and 5'-dAATTCGCCAATGACAAGACG-3' to detect signals in the T36 region; and 5'-dGCGGTGACCCGGGAGATCTGAATTC-3' and 5'-dTAAAGCCATGACAACCATT-3' to detect signals in the *tk* promoter region. The PCRs were performed under the following conditions: 95°C for 5 min; 20 cycles at 95°C for 30 sec, 58°C for 30 sec and 72°C for 2 min.

DNase I cleavage sites were detected using the following 5'-<sup>32</sup>P-labeled primers: 5'-dAAGACGCTGGGCGGGGTTTGTGTC-3' for detection of cleavage sites in the T36 region, and 5'-dGGAATGCCAAGCTTACTTAG-3' for detection of cleavage sites in the *tk* promoter region. The PCR conditions were as follows: 95°C for 5 min; 20 cycles at 95°C for 30 sec, 65°C or 60°C for 30 sec, and 72°C for 2 min. All samples were purified and resolved in 6% polyacrylamide-7-M urea gels.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## **ACKNOWLEDGMENTS**

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Table 1. Effects of the left-handedly curved sequence on luciferase gene expression, as examined by hydrodynamics-based injection of the naked plasmid DNA<sup>a</sup>

| plasmid | luciferase activity ( $\times 10^3$ RLU/mg protein) |
|---------|-----------------------------------------------------|
| ST      | 0.3 ( $\pm 0.2$ )                                   |
| T20     | 4.6 ( $\pm 7.2$ )                                   |
| T24     | 3.0 ( $\pm 1.7$ ) *                                 |
| T28     | 2.2 ( $\pm 1.8$ )                                   |
| T32     | 2.4 ( $\pm 1.5$ ) *                                 |
| T36     | 7.9 ( $\pm 3.7$ ) **                                |
| T40     | 2.3 ( $\pm 2.2$ )                                   |

<sup>a</sup>Plasmid DNA (20  $\mu$ g) was injected into the tail vein of male Balb/c mice (six weeks old). After 48 h, the livers were harvested, and the luciferase activities were measured. The values represent the averages ( $\pm$ SD) of at least four separate experiments. \* $P < 0.05$  and \*\* $P < 0.01$  versus ST. The data of ST, T20, T28, and T36 are also shown graphically in Fig. 2 (48 h).

Table 2. Effects of the left-handedly curved sequence on the intranuclear disposition of plasmid DNA<sup>a</sup>

| plasmid | luciferase activity  | amount of DNA        | expression efficiency |
|---------|----------------------|----------------------|-----------------------|
| ST      | $5.4 \times 10^{-4}$ | $6.6 \times 10^{-2}$ | $8.2 \times 10^{-3}$  |
| T20     | $2.4 \times 10^{-2}$ | $2.3 \times 10^{-1}$ | $1.0 \times 10^{-1}$  |
| T28     | $1.2 \times 10^{-2}$ | $1.7 \times 10^{-1}$ | $7.1 \times 10^{-2}$  |
| T36     | $3.4 \times 10^{-2}$ | $2.1 \times 10^{-1}$ | $1.6 \times 10^{-1}$  |

<sup>a</sup>The luciferase activity, the amount of exogenous DNA, and the expression efficiency at 48 h after administration were divided by those values at 8 h.

## FIGURE LEGENDS

**Figure 1.** Structure of the plasmid DNA containing the left-handedly curved sequence and the *tk* promoter. T4, TCAGTTTTT[CATGTTTTT]<sub>3</sub>; T4', TTTTT[CATGTTTTT]<sub>3</sub>; TATA, TATA box; GC, GC box; CCAAT, CCAAT box; *luc*, luciferase gene; *amp<sup>r</sup>*, *E. coli* ampicillin resistance gene; *ori*, *E. coli* replication origin.

**Figure 2.** Effects of the left-handedly curved sequence on luciferase gene expression, as examined by hydrodynamics-based injection of the naked plasmid DNA. Plasmid DNA (20 µg) was injected into the tail vein of male Balb/c mice (six weeks old). The livers were harvested, and the luciferase activities were measured, as described in the Materials and Methods section. The values represent the averages of at least five separate experiments. Bars indicate SD (standard deviation). \**P*<0.05 and \*\**P*<0.01 versus ST. The data of 48 h are from Table 1.

**Figure 3.** Amounts of luciferase-DNA in the nucleus, examined by hydrodynamics-based injection of the naked plasmid. Plasmid DNA was injected, the livers were harvested, and the amounts of the exogenous DNA were measured, as described in the Materials and Methods section. The

values represent the averages of at least five separate experiments. Bars indicate SD (standard deviation).

**Figure 4.** Expression efficiencies from plasmid DNAs containing the left-handedly curved sequence. The averaged luciferase activities (Fig. 2) were divided by the averaged amounts of the exogenous DNAs (Fig. 3) at the same time points to calculate expression efficiencies.

**Figure 5.** ChIP analysis of luciferase DNA in the nucleus. Plasmid DNA was injected, and the ratios of the amounts of exogenous DNAs precipitated by the anti-H3 antibody to those of the total exogenous DNAs were calculated, as described in the Materials and Methods section. Bars indicate SD (standard deviation). \* $P < 0.05$  versus ST.

**Figure 6.** Analysis of translational positions of nucleosomes on the upstream region of the reporter gene. The upstream structure of the transcription start site is illustrated on the right side of each gel. In each data set, the lanes labeled “N” and “Ch” indicate the MNase digestions of the naked DNA and the isolated nuclei (chromatin), respectively. Arrowheads indicate the following sites: white, fully or partially protected in chromatin; black, highly digested or specifically digested in chromatin; parenthesized black, digested more in chromatin than in naked DNA.

Position numbers are relative to the translation start site (+1). The ellipses indicate putative nucleosomes, as deduced from the band patterns.

**Figure 7.** Rotational positioning of DNA in chromatin. Nuclei harboring each construct were digested with DNase I, and the cleavage sites were analyzed as described in the Materials and Methods section. “N” and “Ch” indicate DNase I cleavage sites in naked DNA and chromatin, respectively. Putative “10-bp ladders” and the nucleosome positions shown in Fig. 5 are indicated on the left of each autoradiogram. The ST (A) and T36 (B) constructs have the same sequence in the shaded and striped regions. The chromatins formed on the former region generated almost the same digestion patterns between the two, while the latter chromatin region did not. The asterisk indicates the overlap points of the signals.



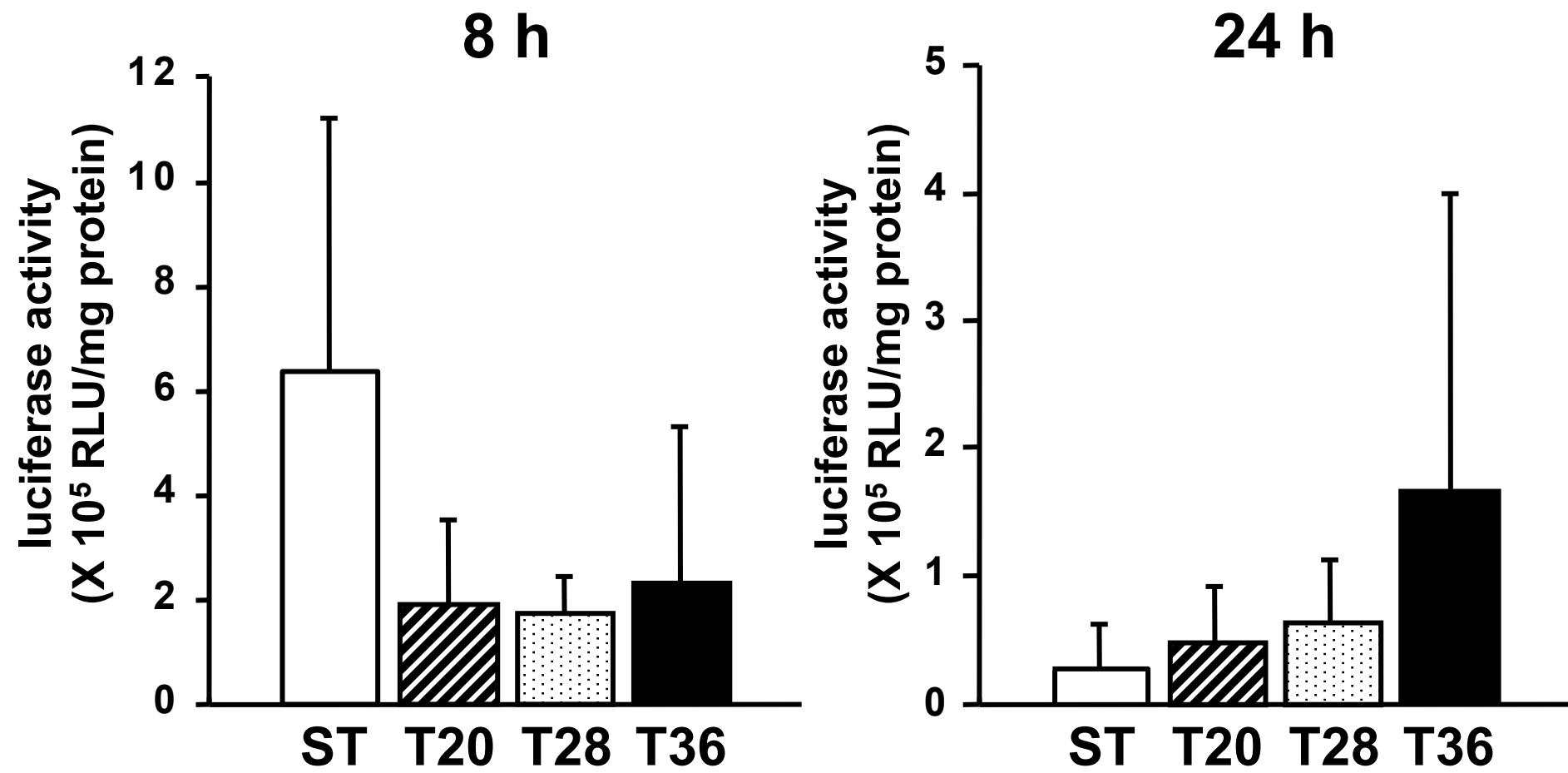


Fig. 2

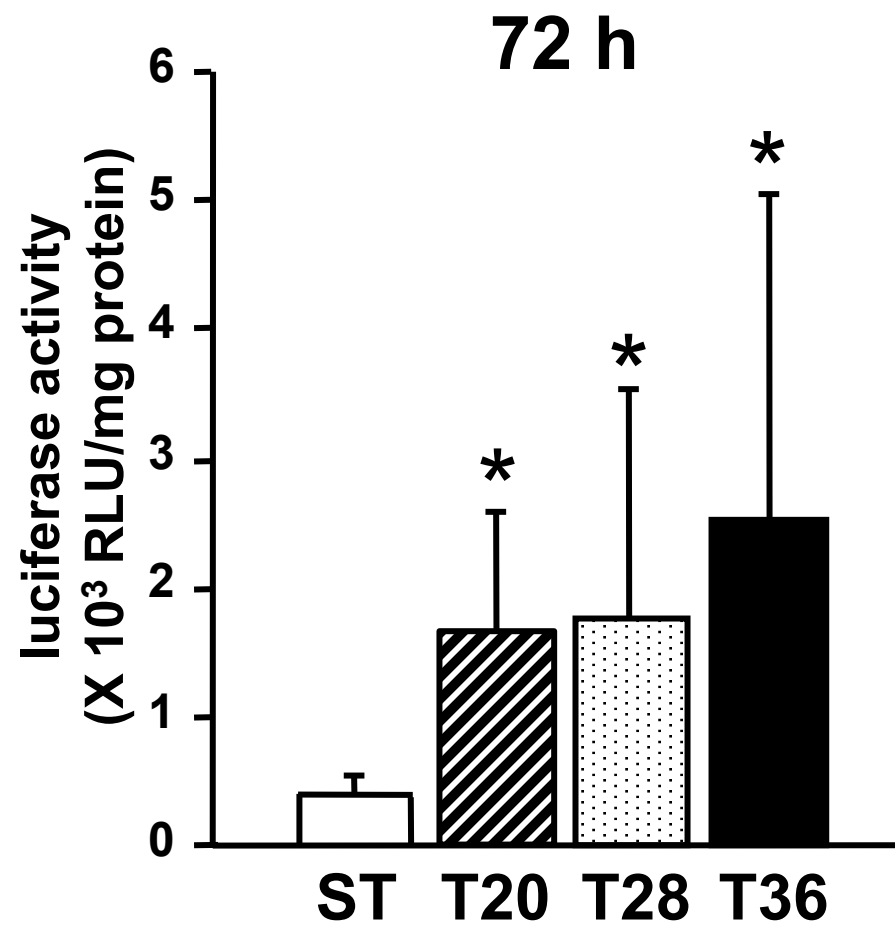
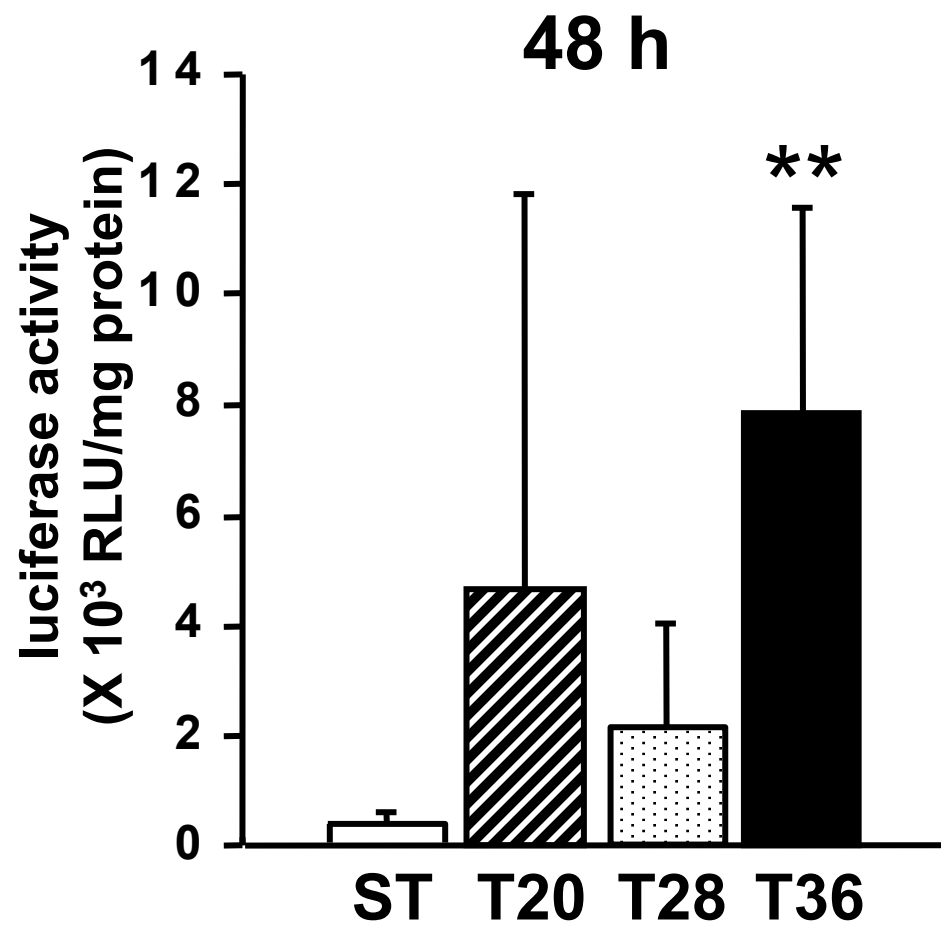
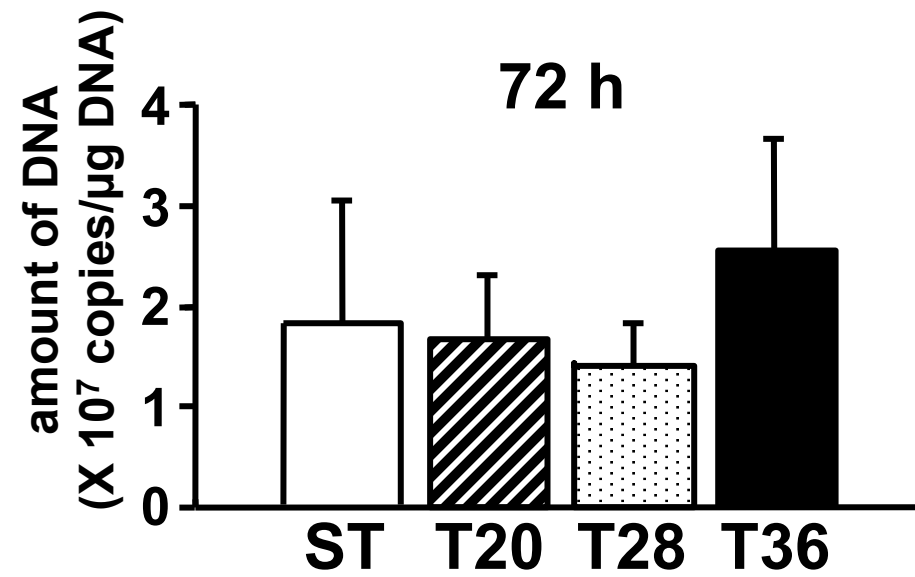
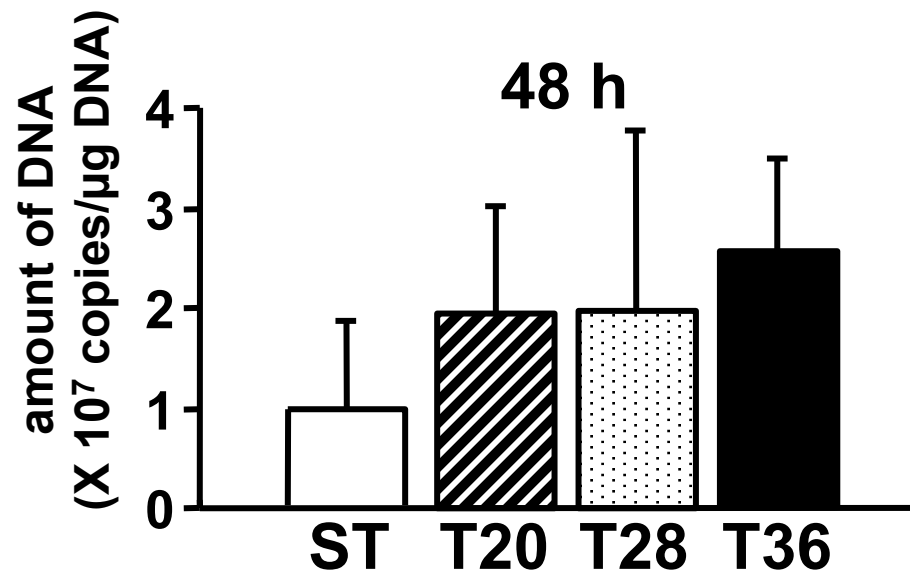
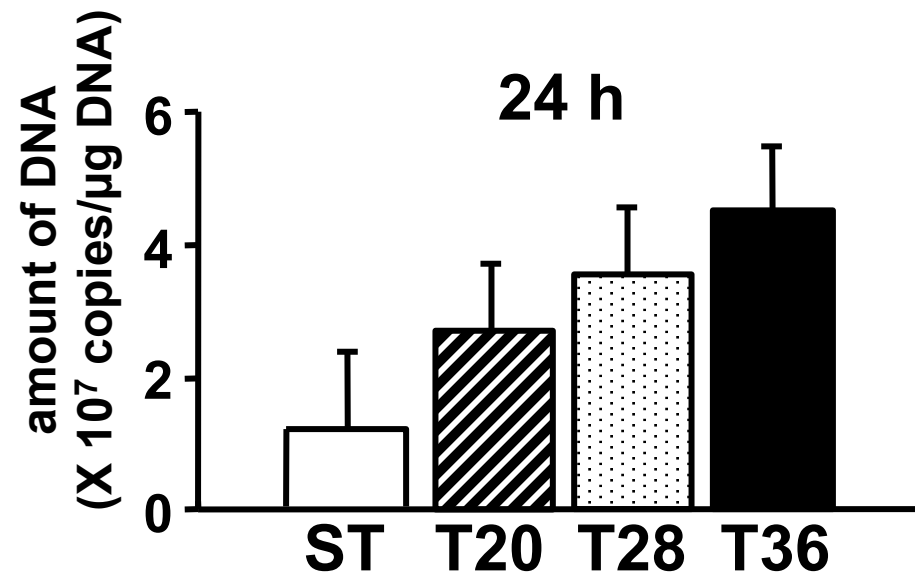
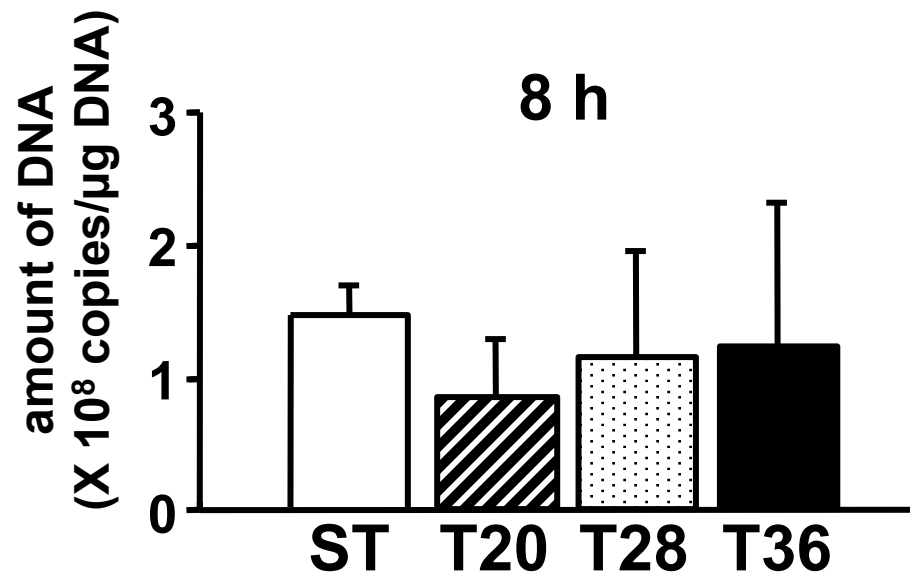
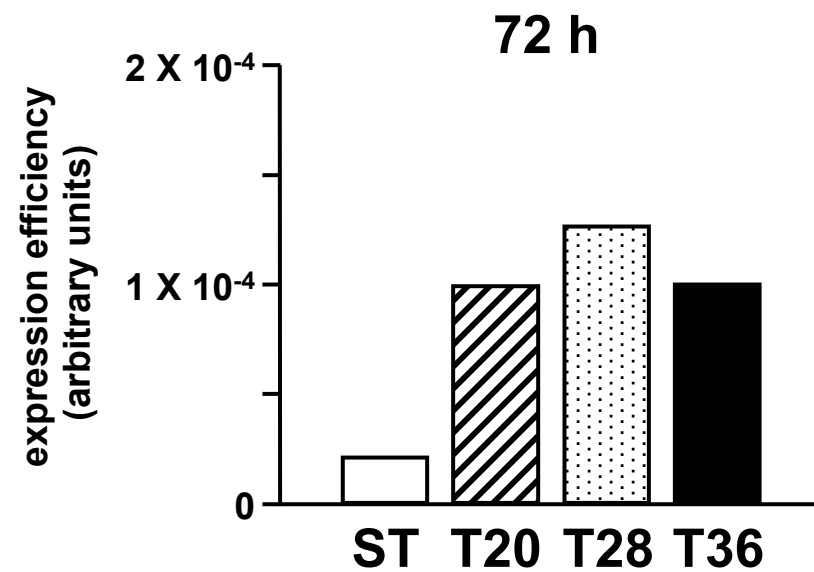
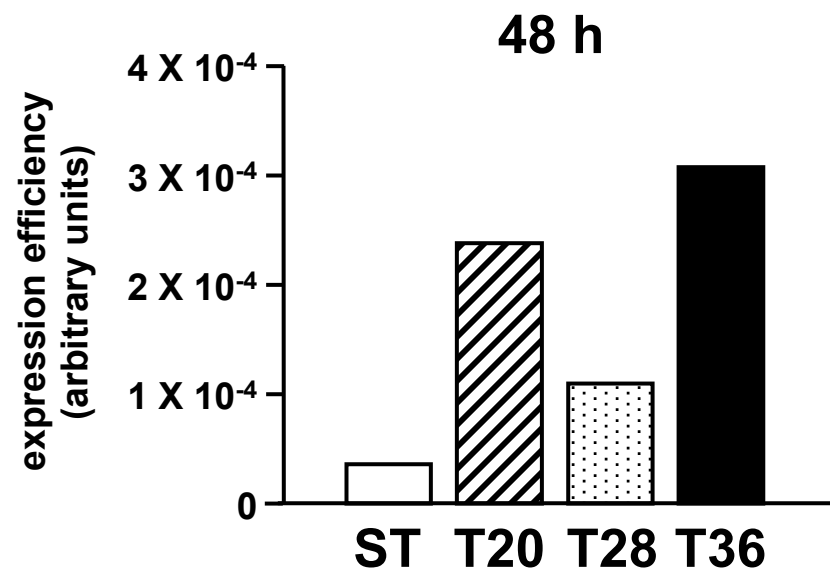
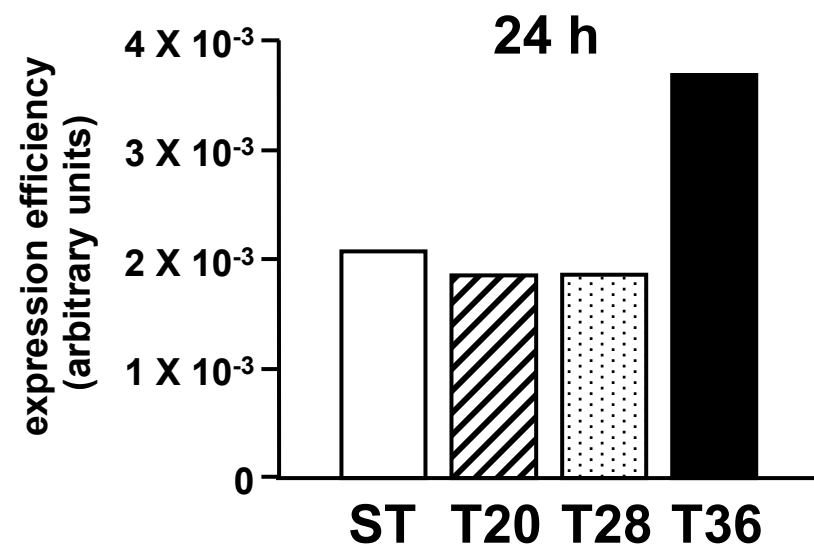
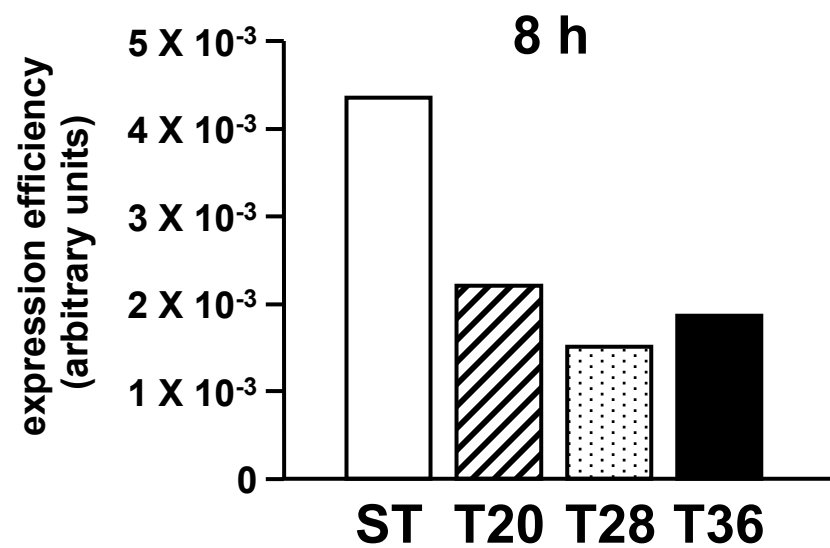


Fig. 2



**Fig. 3**



**Fig. 4**

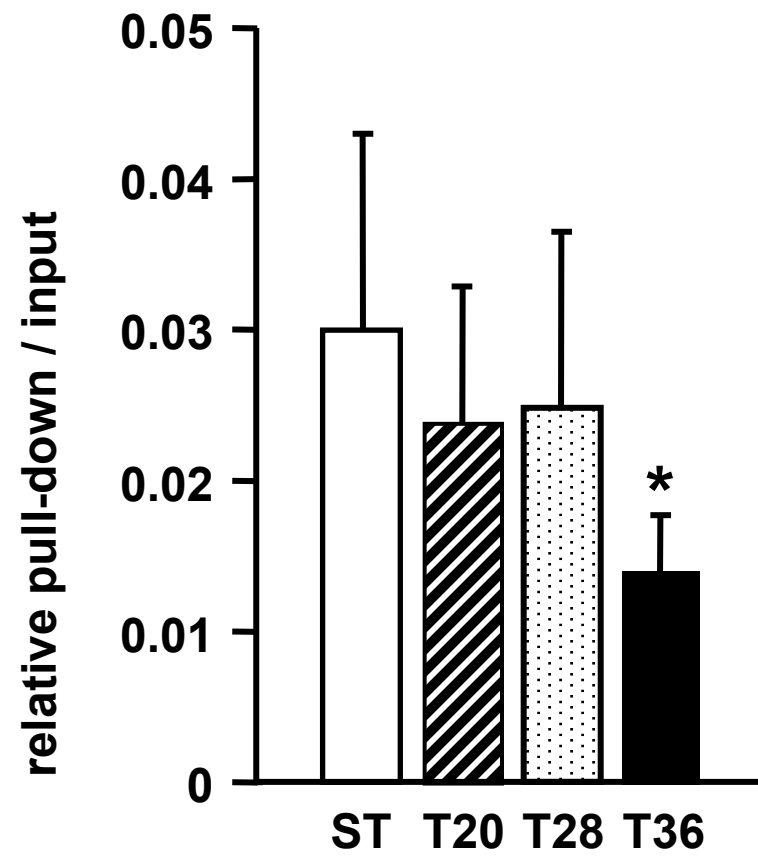
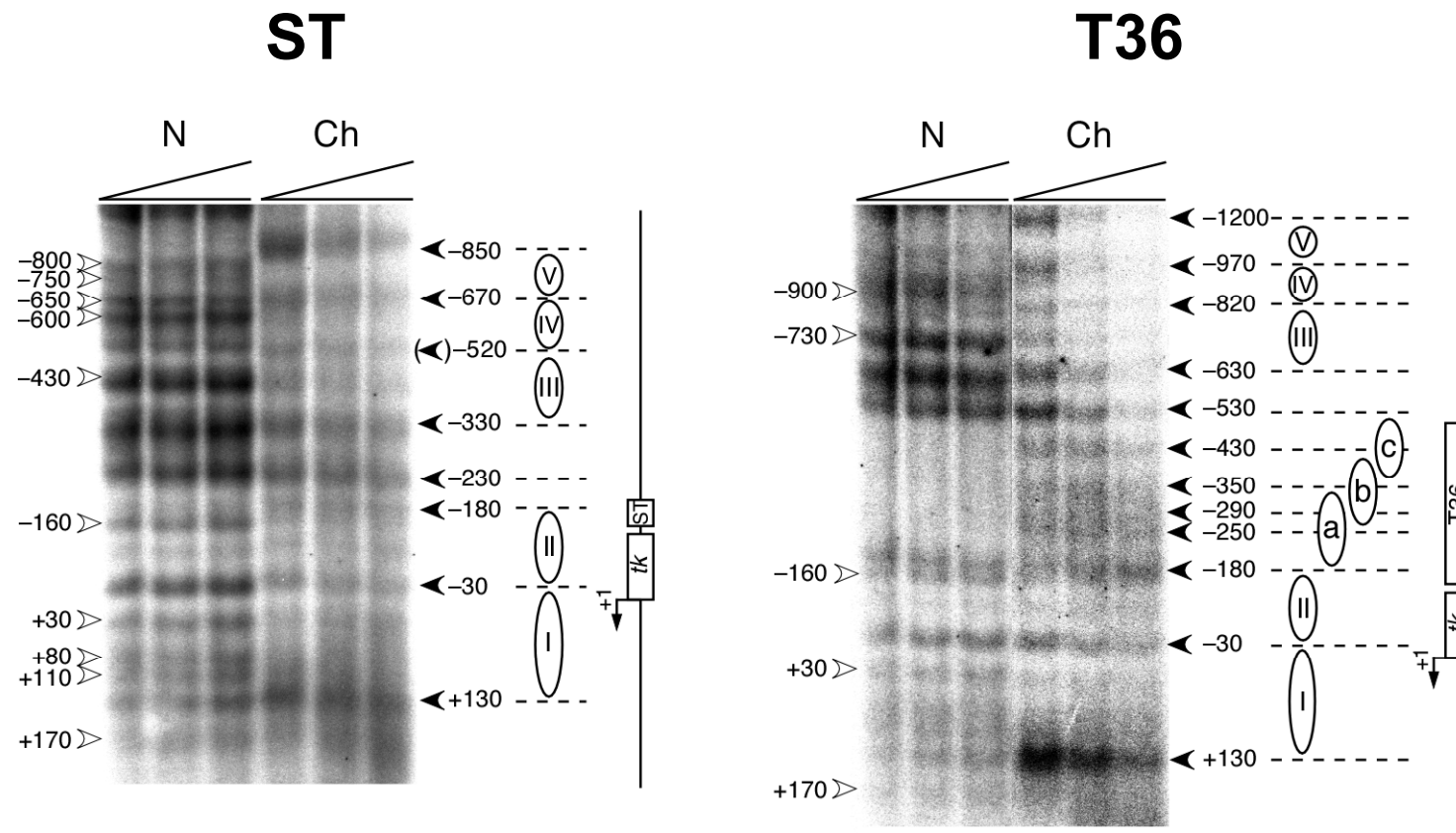
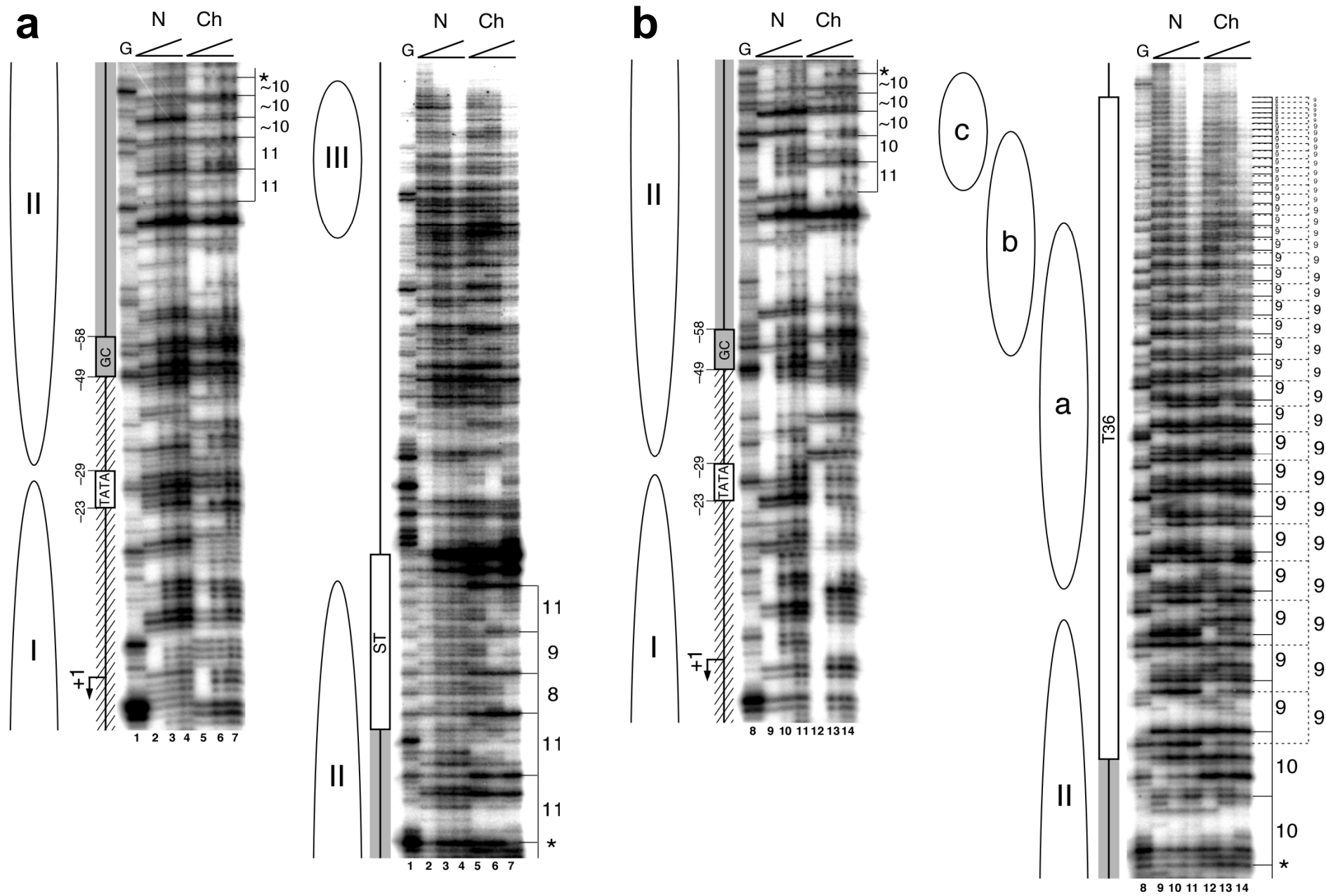


Fig. 5



**Fig. 6**



**Fig. 7**