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Involvement of HMGB1 and HMGB2 proteins in exogenous DNA integration reaction into the genome of HeLa S3 cells

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Abstract

High mobility group 1 and 2 proteins (HMGB1 and HMGB2) are abundant chromosomal proteins in eukaryotic cells. We examined the involvement of HMGB1 and HMGB2 in nonhomologous illegitimate recombination. The HMGB1 or HMGB2 expression plasmid, carrying the *neo*^{r} gene as a selection marker, was introduced into HeLa S3 cells to obtain stably-transfected cells. The number of G418-resistant colonies was about 10 times the number of colonies of control cells transfected with plasmids not carrying the HMGB genes. The copy number of the stably-integrated *neo*^{r} gene was higher in the cells transfected with the HMGB expression plasmids than in control cells. The exogenous DNA integration was suggested to have occurred by nonhomologous illegitimate recombination. On the contrary, the introduction of the HMGB antisense RNA expression plasmid with a reporter plasmid carrying the *neo*^{r} gene into HeLa S3 cells decreased the number of G418-resistant colonies. These results indicate that HMGB1 and HMGB2 each have a novel function as stimulators of stable integration of plasmid DNA into the host genome and that they may be important for the process of spontaneous DNA integration in living cells.

Keywords: HMGB1; HMGB2; Stable DNA integration; HeLa S3 cell; Protein overexpression; Nonhomologous illegitimate recombination

1. Introduction

High mobility group 1 and 2 proteins (HMGB1 and HMGB2) are abundant nonhistone chromosomal proteins in higher eukaryotic cells [1–3]. These proteins share a characteristic structure which contains two DNA binding regions, termed the HMGB1/2 boxes, and an acidic carboxyl-terminal [4]. They bind with DNA in a sequence-nonspecific manner and prefer cruciform DNA [5], B–Z junction [6] and kinked DNA [7] to linear B-formed DNA. These proteins are thought to be associated with active chromatin [8], and to have crucial roles in several important nuclear events including transcription, DNA replication and recombination [3,9,10]. HMGB1 and HMGB2 stimulate in vitro transcription [11–13], and function as general class II transcription factors [14]. HMGB1 stimulates transcription in cultured cells and in a cell line overexpressing HMGB1 by modulating the chromatin structure [15,16]. On the other hand, the expression level of endogenous HMGB2 is enhanced in exponentially growing cells and in cells transformed with various viral genes and oncogenes [17,18]. The progression of the cell cycle is repressed by expression of antisense RNA against HMGB2 mRNA [17]. These findings suggest that HMGB2 is involved in the regulation of cell proliferation activity. Recent studies suggest that HMGB1 and HMGB2 are involved in site-specific recombination such as V(D)J recombination in vitro [10,19]. Also, HMGB1 stimulates HIV1 integrase activity in vitro [20]. HMGB1 binds to the adeno-associated virus (AAV) Rep proteins and promotes Rep-mediated site-specific cleavage of DNA, ATPase activity and transcriptional repression [9]. HMGB1 and HMGB2 stimulate the ligation reaction of DNA double-strand break in vitro [21,22], suggesting that they are involved in DNA end joining reactions.

HMGB1 and HMGB2 were used as gene delivery agents. Plasmid DNA mixed with HMGB1 and HMGB2 could enter into the cells, at a similar level as to those of DNA transfected by traditional techniques [15,23–25]. Similar results were obtained by using recombinant HMGB1 expressed in yeast cells [26].

Exogenous DNA introduced into the mammalian cells can be integrated into the genome [27]. This phenomenon,

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called integration or illegitimate integration, arises out of illegitimate nonhomologous recombination process [28]. It requires terminus of DNA, joining into the genome with sequences of short homology and DNA strand-break repair process. Introduced DNA may be integrated into random genomic regions by preference for unstable chromosomal regions and in association with a chromosome rearrangement reaction. An illegitimate recombination is thought to be associated with chromosomal deletion, translocation and gene rearrangement. However, the molecular mechanism of illegitimate integration is not fully understood.

In this study, we examined whether HMGB1 and HMGB2 are involved in nonhomologous illegitimate recombination by assessing the effect of HMGB1 and HMGB2 on plasmid DNA integration into the genome of HeLa S3 cells. Overexpression of HMGB1 and HMGB2 stimulated the integration of plasmid DNA into the genome. In addition, the acidic C-terminal of HMGB was necessary for the full activation of integration. Expression of antisense RNA against HMGB1 and HMGB2 mRNA inhibited the plasmid DNA integration. These results are the first observation that HMGB1 and HMGB2 have a novel function in nonhomologous illegitimate recombination process in cultured cells.

2. Materials and methods

2.1. Construction of plasmids

An expression plasmid vector, pCI-neo (Promega), carrying the cytomegalovirus (CMV) immediate–early enhancer/promoter upstream from the multicloning site, the SV40 origin/early promoter and the neomycin phosphotransferase gene (*neo*^{r} gene), was used for the construction. Complementary DNA (cDNA) coding for the open reading frame (ORF) of HMGB1 or HMGB2 [15,29,30] was ligated into the multicloning site to obtain the plasmid pCI-HMGB1 or pCI-HMGB2.

In cotransfection experiments, an expression plasmid, pCMV, was constructed by removing the *neo*^{r} sequence from pCI-neo, by digestion with *Nae*I and *Cla*I and filling with Klenow fragment, followed by ligation. Plasmids expressing the full-length HMGB1 or HMGB2 (pCMV-HMGB1 or pCMV-HMGB2) were constructed by ligating the respective cDNA into the multicloning site. Antisense HMGB1 or HMGB2 RNA expression plasmids (pCMV-antiHMGB1 or pCMV-antiHMGB2) were constructed by ligating the HMGB1 or HMGB2 cDNA, obtained from pCI-HMGB1 or pCI-HMGB2 by digestion with *Xho*I and *Sal*I, into the multicloning site of pCMV digested with *Xho*I and *Sal*I, and then selecting the antisense direction construct. Acidic C-terminal deleted HMGB1 (1–178 amino acids) or HMGB2 (1–181 amino acids) expression plasmids (pCMV-1AlB or pCMV-2AlB) was constructed by ligating the respective cDNA derived from pMSC-1AlB or 2AlB [15] into the multicloning site. The plasmid pSV2neo, carrying the *neo*^{r} gene under the SV40 early enhancer/promoter, was used for the selection marker. All the plasmids were prepared by the alkaline-SDS method from *E. coli* DH5α [31] and purified by two cycles of cesium chloride ultracentrifugation [32].

2.2. Cell culture and DNA transfection

HeLa S3 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Nissui) supplemented with 10% fetal bovine serum (FBS, Invitrogen) under 5% CO_2 at 37 °C. Each plasmid was transfected into the cells by a calcium phosphate coprecipitation method or a cationic liposome method using the transfection reagent DOTAP (Roche Diagnostics). For calcium phosphate coprecipitation, 10 μg of the plasmid DNA was incubated in 1 ml of 21 mM Hepes–NaOH, pH 6.95, 138 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.5 mM glucose and 100 mM $CaCl_2$ for 30 min at room temperature. The solution was added to 5×10^6 HeLa S3 cells in a 10 cm dish. After 10 min at room temperature, 10 ml of DMEM with 10% FBS was added. Cells were incubated for 3 h in 5% CO_2 at 37 °C, and then the medium was replaced with 10 ml of fresh DMEM with 10% FBS. The cells were trypsinized 48 h after the transfection and an aliquot equivalent to one-tenth of the cells was plated into 10 cm dishes containing DMEM with 10% FBS in the presence of 500 μg/ml antibiotics G418 (GENETICIN, Invitrogen). After 12–14 days of culture during which the medium was replaced once every 2–3 days, the cells were fixed with methanol, stained with Giemsa's azure–eosin–methylene blue (Merck) and the number of G418-resistant colonies was counted. For the cationic liposome-mediated transfection, about 5×10^6 cells were transfected with 1 μg of each plasmid using DOTAP following the manufacturer's instructions. G418-resistant colony selection was performed by the same procedures as for the calcium phosphate coprecipitation method.

In the cotransfection experiments, 5 μg of the antisense HMGB1 or HMGB2 expression plasmid was cotransfected with 5 μg of the pSV2neo plasmid into HeLa cells by the calcium phosphate coprecipitation method and the G418-resistant colonies were selected by the procedure described above.

2.3. Analysis of the expression of HMGB1 and HMGB2 proteins

HeLa cells transfected with the expression plasmids were harvested and solubilized in SDS gel loading buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol and 10% glycerol). The whole-cell proteins were separated by 12.5% SDS polyacrylamide gel electrophoresis and then electrotransferred onto a nitrocellulose membrane filter (Advantec). The HMGB protein on the filter was reacted with anti-HMGB2 monoclonal antibody, which recognizes

both HMGB1 and HMGB2, and was detected with HRP-conjugated sheep anti-mouse Ig polyclonal antibody (Amersham) using an ECL assay system (Amersham). The resulting bands were scanned using a laser densitometer (Molecular Dynamics) for quantification.

2.4. Southern hybridization analysis

To analyze the transient transfection efficiency, we prepared whole-cell DNA or DNA from the nuclei of the transfected HeLa cells by the method of Hirt [33]. The DNA was digested with *Hin*dIII, separated by 1.0% (w/v) agarose gel electrophoresis, transferred to a Gene Screen Plus membrane (Du Pont) and hybridized with a ^{32}P-labeled DNA fragment (972 bp) excised from the *neo*^{r} gene in pCI-neo by digestion with *Hin*dIII and *Bam*HI. The radioactivity was measured using a BAS2000 imaging analyzer (Fuji Film).

To analyze the copy number of the stably-integrated *neo*^{r} gene, the genomic DNA of cloned G418-resistant cells was extracted by the method of Gross-Bellard [34]. The isolated DNA was digested with *Hin*dIII and *Bam*HI, separated by 0.7% (w/v) agarose gel electrophoresis, transferred to a Gene Screen Plus membrane, and hybridized with the *neo*^{r} DNA probe as described above. The radioactivity was measured using a BAS2000 imaging analyzer. The copy number of the integrated *neo*^{r} gene was estimated from radioactivity measurement relative to the radioactivity of a standard prepared on the same filter which contained a known copy number of *neo*^{r} gene fragment per cells.

2.5. Analysis of the product expressed by the stably-integrated reporter plasmid

We analyzed the product expressed by the stably-integrated reporter gene by using the reporter plasmid pActcat, carries the chloramphenicol acetyl transferase (CAT) gene under the chicken β-actin promoter. One microgram of the pActcat plasmid was cotransfected with 1 μg of either the pCI-neo, pCI-HMGB1 or pCI-HMGB2 plasmid into HeLa cells by a cationic liposome method using DOTAP and the G418-resistant colonies were selected by the method described above. Cell extracts were prepared from the cloned G418-resistant cells and the amount of CAT protein in the extract was quantified using CAT ELISA kit (Roche Diagnostics).

3. Results

3.1. The overexpression of HMGB1 and HMGB2 increased the number of G418-resistant colonies

To examine the effect of the HMGB1 and HMGB2 on the stable integration of plasmid DNA into the host genome, the HMGB1 or HMGB2 expression plasmid was introduced into HeLa cells. The ORF region of the HMGB1 or HMGB2 cDNA was inserted into the expression plasmid pCI-neo, which contains the *neo*^{r} gene as a selection marker. These plasmids (pCI-HMGB1 and pCI-HMGB2) were transfected into HeLa cells by a calcium phosphate coprecipitation method or a cationic liposome method using DOTAP. The transfected cells were selected with G418 for 12–14 days. This long selection period ensured that only cells with stably-integrated *neo*^{r} DNA were selected. The number of G418-resistant colonies was about 10 times the number of control cell colonies (Fig. 1A and B). Expression of green fluorescence protein (GFP) as well as CAT and β-galactosidase instead of HMGB1 and HMGB2 did not affect the number of G418-resistant colonies (Fig. 1B and data not shown). A similar increase in the number of G418-resistant colonies was observed when human 293 cells, human TIG-3 cells, monkey CV-1 cells, mouse C-127 cells or rat fibroblast 3Y1 cells were used instead of HeLa cells (data not shown), indicating that the increase induced by the overexpression of HMGB1 and HMGB2 is common to a wide variety of mammalian cell lines.

To confirm that HMGB1 and HMGB2 were overexpressed, the relative amounts of the proteins in the cells

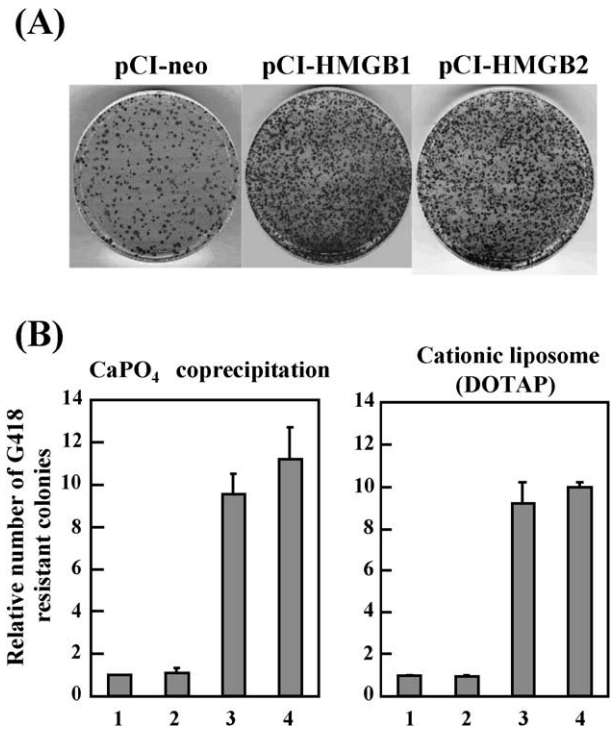

Fig. 1. The effect of HMGB1 and HMGB2 on plasmid DNA integration into HeLa cells. (A) A representation of the cultures stained with Giemsa after G418 selection of cells transfected with the HMGB expression plasmids pCI-HMGB1, pCI-HMGB2, or the control plasmids pCI-neo. (B) The relative number of the colonies after G418 selection of cells transfected with pCI-neo (column 1), pCI-GFP (the GFP expression plasmid, column 2), pCI-HMGB1 (column 3) or pCI-HMGB2 (column 4) by the calcium phosphate coprecipitation (left panel) and cationic liposome (right panel) methods. The values are means of six independent determinations, and bars represent standard deviations.

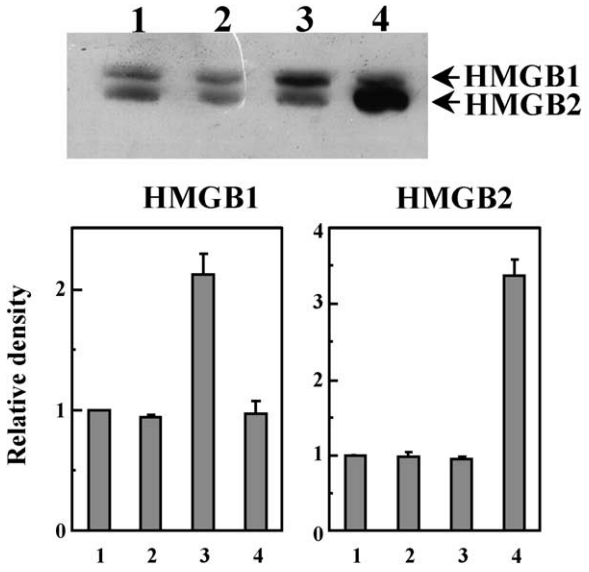

Fig. 2. Analysis of the expression level of HMGB protein in cells transfected with pCI-neo (lane 2), pCI-HMGB1 (lane 3), pCI-HMGB2 (lane 4) or in nontransfected HeLa cells (lane 1). The arrowheads at the top show the HMGB1 and HMGB2 bands, respectively. The amounts of HMGB1 and HMGB2 relative to that in mock-transfected HeLa cells are shown in the bottom panel. Bars represent standard deviations.

were estimated. The total protein extracted from cells harvested 48 h after transfection was subjected to SDS-polyacrylamide gel electrophoresis followed by Western blot analysis using anti-HMGB2 monoclonal antibody, which recognizes both HMGB1 and HMGB2 (Fig. 2). The amount of HMGB1 protein in cells transfected with pCI-HMGB1 was about 2-fold higher than the amount in HeLa cells transfected with pCI-neo and in mock-transfected HeLa cells. The amount of HMGB2 increased by about 3.5-fold in the cells transfected with pCI-HMGB2 in comparison with the controls (Fig. 2). Northern hybridization analysis showed that the mRNA expression levels were also enhanced (data not shown). The expression of HMGB1 and HMGB2 proteins was detected 24 h after transfection, reached a maximum 48 h after and decreased gradually up to 72 h (data not shown). These results show that the number of G418-resistant colonies increased as a result of the overexpression of HMGB1 and HMGB2.

3.2. Overexpression of HMGB1 and HMGB2 enhanced stable DNA integration, but did not affect the transient transfection of the plasmid DNA

The effect of the overexpression of HMGB1 and HMGB2 on the transient transfection efficiency and stable integration efficiency of the plasmids was examined. Total DNA prepared from cells transfected with each plasmid was analyzed by southern hybridization using the *neo*^{r} gene as the probe. The amounts of the plasmids present in cells transfected with pCI-HMGB1 or pCI-HMGB2 were similar to those of the control, from 12 to 72 h after transfection (Fig. 3A, left panel). In addition, the same results were obtained when the DNA was extracted from the purified nuclei of transfected HeLa cells (Fig. 3A, right panel). These results suggest that the overexpression of HMGB1 and HMGB2 did not affect the efficiency of the transient transfection, the stability of the transfected plasmids or the transfer of the plasmids into the nucleus.

To determine the copy number of the stably-integrated plasmid DNA in the host genome, genomic DNA was prepared from the G418-resistant clones. The genomic DNA was cleaved with *Hin*dIII and *Bam*HI, which excised a 972 bp DNA fragment containing the *neo*^{r} gene, and analyzed by Southern hybridization with the same fragment as the probe. Densitometric data of the resulting 972 bp DNA bands were used to calculate the copy numbers of the

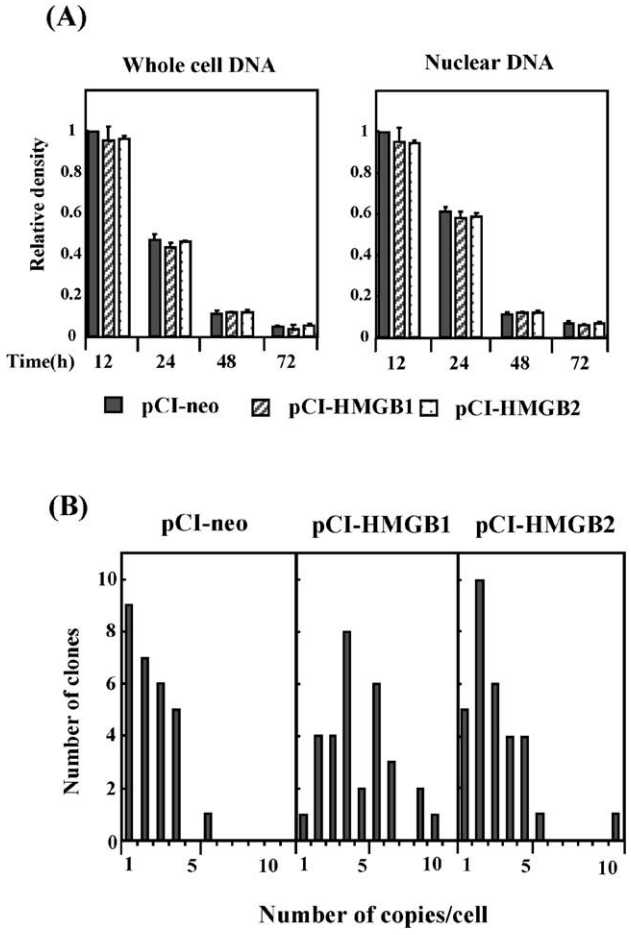

Fig. 3. The effect of the overexpression of HMGB1 and HMGB2 proteins on the transient transfection efficiency and on stable integration. (A) Determination of the amounts of transfected plasmid DNA in the cells and the nuclei. Total DNA prepared from whole cells or from purified nuclei 12, 24, 48 and 72 h after transfection with each plasmid was electrophoresed and subjected to Southern hybridization with the *neo*^{r} gene as the probe. Bars represent standard deviations. (B) Determination of the copy number of the stably integrated *neo*^{r} gene in the transfected HeLa cell genome. Genomic DNA prepared from cloned G418-resistant HeLa cells transfected with each plasmid was electrophoresed, subjected to Southern hybridization with the *neo*^{r} gene as the probe and quantified.

Table 1
The effect of overexpression of HMGB1 and HMGB2 proteins on the gene expression from the cotransfected reporter plasmid

Cotransfected plasmid with pActcat reporter	Expression frequency[a]	The number of clones to express CAT protein (pg CAT protein/μg cell protein)		
		<0.1	0.1–1.0	1.0<
pCI-neo	3/40	2	1	0
pCI-HMGB1	21/40	6	14	1
pCI-HMGB2	10/40	6	3	1

[a] Expression frequency shows the number of CAT expression clones per 40 clones of G418 resistance.

integrated plasmids, as shown in Fig. 3B. The transfection of pCI-HMGB1 resulted in the integration of four to six copies of the *neo*^{r} gene in each clone (average, 5.0), whereas transfection of pCI-neo resulted in the integration of only one or two copies (average, 2.2). Clones obtained by transfection with the pCI-HMGB2 had slightly lower copy number (3.1) than clones transfected with pCI-HMGB1. These results show that HMGB1 and HMGB2 promote the integration of exogenous DNA into chromosomal DNA.

The genomic DNAs prepared from the G418-resistant clones were fully digested with *Hin*dIII and the digests were separated by agarose gel electrophoresis. The southern hybridization profiles obtained using the *neo*^{r} gene as the probe showed a random distribution of the lengths of *Hin*dIII fragments that contained the *neo*^{r} sequence (data not shown). The various restriction fragments containing *neo*^{r} gene(s) were then analyzed to compare the sequences flanking the *neo*^{r} gene. The results showed that the flanking sequences originated from the various chromosomes and had no detectable homology with each other (data not shown).

To examine the expression of a stably-integrated reporter gene in the presence and absence of overexpression of HMGB1 and HMGB2, a reporter plasmid pActcat carrying the CAT gene under the control of the chicken β-actin promoter was cotransfected with pCI-neo, pCI-HMGB1 or pCI-HMGB2, and G418-resistant clones were selected. The amounts of CAT protein in whole-cell extracts prepared from the cloned G418-resistant cells are shown in Table 1. The results indicate that the relative number of clones expressing the CAT protein was increased by cotransfection with the HMGB expression plasmids in comparison with the control. In addition, the amount of CAT protein was richer in the cells cotransfected with the HMGB expression plasmid.

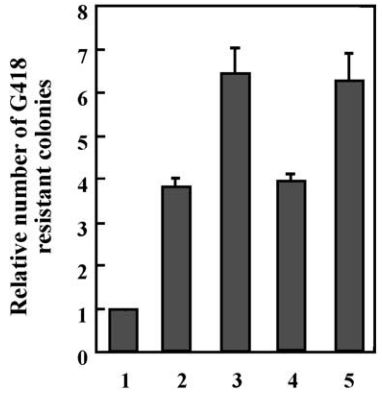

Fig. 4. The effect of expression of acidic C-terminal deleted of HMGB on the plasmid integration. The acidic C-terminal deleted HMGB expression plasmids, pCMV-1AlB (column 2) or pCMV-2AlB (column 4), or wild-type HMGB expression plasmids, pCMV-HMGB1 (column 3) or pCMV-HMGB2 (column 5) was cotransfected with the selection marker plasmid pSV2neo into the HeLa cells, and the number of G418-resistant colonies was counted. Bars represent standard deviations.

3.3. Acidic C-terminal of HMGB proteins was necessary for the full enhancement of integration reaction

Both HMGB1 and HMGB2 comprise unique C-terminals consisting of a tandem array of acidic amino acids. To examine the requirement of the acidic C-terminal in this stimulation, expression plasmid pCMV-1AlB or pCMV-2AlB encoding acidic C-terminal deleted HMGB1 or

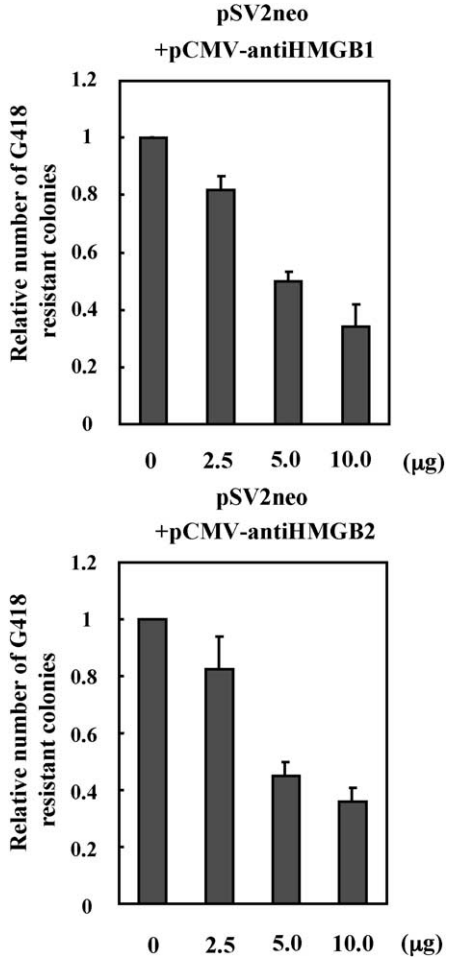

Fig. 5. The effect of expression of antisense RNA against HMGB1 or HMGB2 mRNA. The antisense HMGB1 or HMGB2 RNA expression plasmids, pCMV anti-HMGB1 or pCMV anti-HMGB2 was cotransfected with the selection marker plasmid pSV2neo into HeLa cells, and the number of G418-resistant colonies was counted. Bars represent standard deviations.

HMGB2 was cotransfected with pSV2neo into HeLa cells. Cotransfection of acidic C-terminal deletion mutant expression plasmids enhanced the number of G418-resistant colonies in weaker levels than those of full-length HMGB expression plasmids (Fig. 4). Western blot analysis showed that the expression level of these mutants were similar to those of full-length HMGB (data not shown). These results suggested that the acidic C-terminal of HMGB is necessary for the full enhancement of integration.

3.4. Antisense RNA against HMGB1 and HMGB2 mRNA reduced plasmid DNA integration into the genome

To examine whether endogenous HMGB1 and HMGB2 were involved in plasmid DNA integration into the host genome, the antisense RNA expression plasmids pCMV-antiHMGB1 and pCMV-antiHMGB2, containing HMGB1 and HMGB2 cDNA in the antisense direction, respectively, were cotransfected with pSV2neo into HeLa cells. The cotransfection of the antisense expression plasmids with pSV2neo decreased the number of the G418-resistant colonies dose-dependently (Fig. 5). This result indicates that DNA integration depends on endogenous HMGB1 and HMGB2, as did on the overexpressed exogenous HMGB1 and HMGB2, suggesting that HMGB1 and HMGB2 may be important to the process of DNA integration.

4. Discussion

In the present study, we have identified a novel function of HMGB1 and HMGB2. HMGB1 and HMGB2 were largely facilitated the integration reaction of plasmid DNA into the host genome (Fig. 1A and B). DNA molecules transfected into cells pass through the cell membrane, translocate into the nucleus and are integrated into the host cell genome. Endogenous HMGB1 and HMGB2 are present in the nucleus at the level of about 10^6 molecules per cell nuclei [35]. The amounts of HMGB1 and HMGB2 in our transfected HeLa cells were two to three times higher than the levels in mock-transfected cells (Fig. 2). The overexpression of HMGB1 and HMGB2 did not affect the uptake of the plasmid DNA into the cytoplasm or transfer of these molecules into the nucleus (Fig. 3A). Yet the copy number of the stably-integrated *neo*^{r} gene was higher in the cells transfected with the pCI-HMGB1 or pCI-HMGB2 compared with the control (Fig. 3B). The copy number of the integrated *neo*^{r} gene in cells transfected with the pCI-HMGB1 was larger than that in cells transfected with the pCI-HMGB2 (Fig. 3B), whereas the G418-resistant colonies in cells transfected with the pCI-HMGB1 were the same or slightly larger than those when transfected with the pCI-HMGB2 (Fig. 1B). These results suggested that foreign DNA integration more frequently occurred through the overexpression of HMGB1. The number of the integrated *neo*^{r} genes does not reflect the frequency of the foreign DNA integration in the present G418 selection condition, because one copy of the integrated *neo*^{r} gene into the cell gives the sufficient resistance to G418. Moreover, the expression of antisense RNAs for HMGB1 and HMGB2 mRNA repressed the plasmid DNA integration (Fig. 5). These results show that the facilitating integration of the plasmid DNA is related to HMGB protein per se and did not result from the secondary effect of the overexpression itself. Conclusively, HMGB1 and HMGB2 can stimulate exogenous DNA integration into the host genome.

HMGB1 and HMGB2 have two DNA binding motifs, HMG-boxes, within two-thirds from the amino-terminal and an acidic C-terminal [4]. The acidic C-terminal of HMGB is considered to be a functional region in the protein [15,36,37]. Indeed, the acidic C-terminal of HMGB1 is necessary for the transcriptional activation [15]. In the present experiment, the overexpression of the acidic C-terminal deletion mutant of HMGB induced the stimulation of plasmid DNA integration, but in weaker levels than that by overexpression of full-length HMGB (Fig. 4). This result suggested that the stimulation of plasmid DNA integration required both of DNA binding activity of two HMG boxes and acidic C-terminal. DNA binding activity of HMGB was enhanced by elimination of the acidic C-terminal [38,39]. On the other hand, HMGB bindings with unnatural structural DNAs, such as mini-circular DNA, four-way junction DNA and partial single-strand containing DNA, were modulated in the presence of the acidic C-terminal region [36,37]. Integration intermediate DNA structures may consist of bulge DNA containing single-strand, T-shaped and X-shaped DNAs [28]. The full-length HMGB is thought to bind tightly and stabilize the binding with such DNA structures than acidic C-terminal deleted HMGB. Thus, the decreased stimulatory effect may be obtained by the deletion of acidic C-terminal.

Some investigations showed that HMGB1 functions as a gene delivery agent [15,23–26]. These results were obtained under special conditions of mixing in excess molar ratio of HMGB to DNA (DNA: HMGB = 1:1000–10,000 in molar ratio). Under such a condition, DNA might aggregate with HMGB. These aggregates should protect DNA itself from nuclease attacks to render transportation of intact DNA into the nucleus by nuclear localization signal in HMGB1 protein bound with the DNA. The expression levels of HMGB1 and HMGB2 in HeLa cells transfected with the HMGB expression plasmid in the present study were only two to three times higher than that of mock-transfected HeLa cells (Fig. 2). HMGB protein molecule exists every 10 nucleosomes; about 2000 bp in DNA length [35]. Thus, the present experimental condition might be closed to one not to produce the DNA–HMGB aggregates. In addition, overexpression of HMGB neither effected on protection of the transfected plasmids from nuclease attack nor on transportation of them into the cell nucleus (Fig. 3A), whereas the plasmid DNA integration into HeLa cells was really enhanced (Fig. 3B). Thus, the present results are the first

observation that HMGB is concerned with the integration reaction step of the exogenous DNA.

Plasmid DNA integration is presently thought to arise from nonhomologous illegitimate recombination which involves chromosomal deletion, translocation and gene rearrangement [28,40]. The stably-integrated *neo*^{r} genes in the host DNA were distributed at random as shown by the irregular lengths in the *Hin*dIII DNA fragments containing the *neo*^{r} genes (data not shown). Furthermore, the sequences flanking the integrated *neo*^{r} genes originated from various chromosomes and showed no detectable homology each other. Taken together, HMGB1 and HMGB2 are involved in nonhomologous illegitimate recombination.

How are HMGB1 and HMGB2 involved in the illegitimate integration? The illegitimate integration reaction may need the presence of short homologous sequences at the ends in the exogenous and endogenous DNA strands and involvement of the DNA strand-break repair process [28]. HMGB1 and HMGB2, known to destabilize chromatin structure [16], may destabilize the transfected plasmid DNA to accept nuclease attacks, increasing the number of DNA ends. However, linearized-plasmid DNA obtained by restriction enzyme digestion prior to transfection was integrated to a similar extent with closed-circular DNA, based on the overexpression levels of HMGB1 and HMGB2 (data not shown). Thus, the stimulation of plasmid DNA integration may be independent of the formation of ends of the exogenous DNA strands. Second, HMGB preferentially binds with four-way junction DNA [5]. Thus, they may bind with junctions of plasmid and chromosomal DNAs, which form T-shaped or X-shaped DNA structures, to stabilize the junction structure resulting in the increase in the opportunity for DNA strand-break repair. Third, HMGB binds with active chromatin [1,3,8] and maintains the structures. That being the case, changing the HMGB expression level in the nucleus may vary the activity of chromatin structures [16]. In addition, the integration of retroviral cDNA into the host chromosome preferentially occurs at DNaseI hypersensitive sites [41,42] which linked to the active chromatin conformation. If plasmid DNA is integrated at a high frequency into the active chromatin region by the same mechanism as retroviral cDNA, HMGB may cause the structural activation of the chromatin necessary for integration. A recent study indicated that HMGB1 and HMGB2 proteins stimulate HIV1 integrase activity in vitro [20]. If both processes of plasmid DNA integration and retroviral integration into the host genome were same, then HMGB1 and HMGB2 proteins should be concerned with retroviral integration in vivo. Finally, recent studies indicated that HMGB1 and HMGB2 enhance the ligation reaction of DNA double-strand break in vitro [21,22]. Thus, HMGB may enhance the end joining reaction, which is an important step of DNA integration into the host genome.

The effect of HMGB on the DNA integration was independent of the sources of the cell line. In addition, the copy number of the stably-integrated gene was increased and the amount of protein expressed by the integrated gene was enhanced by overexpression of either HMGB protein (Table 1). Thus, overexpression of HMGB1 and HMGB2 could be a useful method for obtaining the stable gene integration and a higher level of gene expression in a variety of cell lines and species.

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