韓國家畜繁殖學會誌 25(3) : 237~242 (2001)
Korean J. Animal Reprod.

Effect of DNA Conformation on Genomic Integration of Transgenes and Its Implications on Integration Mechanism

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외래유전자의 게놈내 삽입에 있어서 DNA 형태가 미치는 영향

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ABSTRACT

We recently demonstrated that both linear- and supercoil-form B1/B2 SINE (short interspersed elements) sequences could increase an integration frequency of a reporter gene in preimplatation mouse embryos. In those reports, when either a control or SINE-flanked DNA was separately applied to microinjection, the proportions of β-gal positives were 16% and 63%, respectively, in linear-form DNA, and 6% and 25%, respectively, in circular-form DNA. Here, we examined the contribution of a circular-form DNA moiety to integration frequency by using a mixed-form (linear and circular-form) DNA in microinjection. When examined in the blastocyst stage, the proportion of β-gal-positive embryos was 17.3% and 46.6% in control and SINE-flanked DNA, respectively. These results suggest that there is little contribution of circular-form DNA moiety to the resultant integration frequency, and that the majority of the integration events are mediated through a linear conformation of vector DNA. In addition, some clues on integration process could be obtained from the analysis of microinjection results.

I. INTRODUCTION

Introduction of an exogenous DNA into mammalian cells has become an increasingly important procedure. In areas such as transgenesis and gene therapy where the amount of target cells used in gene-delivery experiments is limited due to their expensiveness and rareness, the integration of a delivered DNA into the host genome is the most critical step of many biological applications. However, little information is available about the actual events involved in the integration process. To raise the integration efficiency of transgene into mammalian genome, it is essential to understand the mechanism of transgene integration in the molecular level.

SINE (short interspersed element) sequences

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have been discovered in the genomes of all vertebrate species studied to date. These repetitive DNA familiy members, such as B1, B2 in mouse and Alu sequences in human, are present in extremely high copy number, hundreds of thousands per haploid genome (Berg and Howe, 1989; Weiner et al., 1986). The efficiency of human Alu repeats in promoting homologous recombination has been reported in cultured cells (Ayares et al., 1986; Pavan and Reeves, 1991; Shen and Deininger, 1992; Watson et al., 1995). However, the effects of Alu sequences are still controversial, some reported to be efficient and others indistinguishable from entirely random recombinations. We previously demonstrated that the murine SINE sequences, such as a B1 and B2, could increase both integration frequency and expression level of a reporter gene in mouse preimplantation embryos (Kang et al., 1999; Kang et al., 2000).

In this study, using the two kinds of vectors in microinjection, we tested the mechanism on transgene integration by comparing integration frequencies of a linear-, a circular-, or a linear plus circular-form DNA in mouse preimplantation embryos. With usual transgenes, it is hard to calculate and compare the integration frequencies between linear and supercoiled DNA because of the relatively lower integration values of supercoiled DNA. The SINE-flanked transgene, however, due to its higher integration rates (25% and 63% in supercoiled and linear conformation, respectively), could make it possible to calculate and deduce the integration process.

Ⅱ. MATERIALS AND METHODS

1. Collection, Manipulation and Culture of Mouse Embryos

Vector construction was described previously (Kang *et al.*, 1999; Kang *et al.*, 2000). The procedures for the manipulation of mouse embryos were described previously (Hogan, 1994). Four- to five-week-old BCF1 mice were superovulated by intraperitoneal injections (48 hr apart) of PMSG (Folligon, Intervet) and hCG (Chorulon, Intervet), and mated with eight-week-old males of the same strain. Microinjection was performed 24 hr after hCG injection. The plasmid DNA used in microinjection was linearized with ScaI enzyme, purified through Elutip-d (Schleicher & Schuell) and adjusted to 4ng per microliter. Microinjected eggs were allowed to develop *in vitro* to blastocysts in M16 media (Sigma).

2. β-Galactosidase Assay with Blastocysts

Embryos were rinsed 3 times with PBS, fixed in 2% paraformaldehyde and 0.02% glutaraldehyde in PBS for 30 min at 4℃. Staining was done for 3 h at 30℃ in a solution containing 0.1% 4-chloro-5-bromo-3-indolyl--D-galactopyranoside (X-gal), 2 mM $MgCl_2$, 5 mM EGTA, 0.2% Nonidet P-40, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6 \cdot 6H_2O$. The stained samples were stored at 4℃ in PBS. β-Gal activity was observed on a microscope under 100× magnification.

Ⅲ. RESULTS

We constructed a vector ($p(B1/2)_2geo$) carrying two copies of fused SINE B1 and B2 sequences (B1/2) that flank both boundaries of a reporter gene, PGKgeo (Friedrich and Soriano, 1991), which consists of the promoter of the phosphoglycerate kinase-1 (PGK-1) gene, a fusion gene (geo) of the bacterial LacZ and neomycin resistant gene, and a poly(A) of the bovine growth hormone gene (Fig. 1) as described elsewhere (Kang *et al.*, 1999; Kang *et al.*, 2000). A pgeo vector, which is structurally identical to the $p(B1/2)_2geo$ except the presence of SINE sequences, was used as a control. To linearize

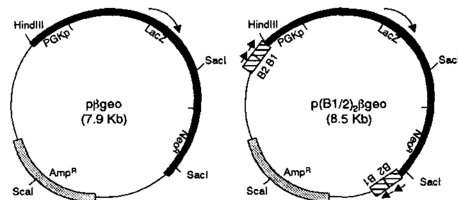

Fig. 1. Schematic representation of vector constructs used in microinjection. The reporter gene (black box) consists of a PGK-1 promoter (indicated as PGKp), the coding sequence of the geo (a fusion gene of bacterial LacZ and neomycin resistant gene (Friedrich and Soriano, 1991), and a poly (A) signal sequence of a bovine growth hormone (bGH) gene. The transcriptional direction of the reporter and fused B1/ B2 sequences (hatched box) is shown as arrows. The ScaI site in the Neo^R region was used to linearize the microinjection vectors. Except the two fused SINE sequences in $p(B1/2)_2geo$, both vectors are identical. pgeo, left panel; $p(B1/2)_2geo$, right panel.

both vectors, we used ScaI restriction enzyme that recognizes a unique sequence located in the ampicillin resistant gene region. Linearized vector was mixed in an equal volume with uncut, intact supercoil form vector and these were used in microinjection of mouse eggs.

As demonstrated in the previous studies (Kang *et al.*, 2000), when linearied vectors were applied in microinjection, only 16% (24/153) of the injected embryos revealed β-gal expression in the pgeo control, while the expression frequency increased to 63% (86/136) in the $p(B1/2)_2geo$. Also, when circular vectors were used, the pgeo control showed X-gal precipitates only in 5.9% (6/101) of the microinjected embryos whereas the SINE-carrying transgene expressed the β-galactosidase at a frequency of 25% (26/104). Since these microinjection vectors with different conformations represent a significant contrast in integration efficiency, it would be interesting to analyze the integration frequency of mixed (linear + supercoiled) DNA. This may allow us to determine to what extent the supercoiled molecules might contribute in the resultant integration. The rationale is that if the supercoiled SINE-flanked DNA could be independently integrated into the genome with its own conformation at a frequency of 25%, then when both linear and supercoiled forms are mixed and applied to microinjection, the resulting integration rate should be similar to the sum (63% + 25%) of the individual integration rates of both forms, or at least higher than that of the linear form (63%).

BCF1 mouse eggs were microinjected with a mixed (circular plus linearized) DNA and allowed to cleave to the blastocyst stage. Expanded or hatched blastocysts were fixed and then stained with X-gal. A significant difference in the frequency of β-gal expression was noticeable between the two vector groups (Table 1). The integration efficiency of mixed DNA was compared to that of the linear DNA due to its relatively high integration ability. When compared, the frequency was similar

Table 1. β-Gal expression in mouse blastocysts microinjected with a mixed (supercoiled+linear) form pgeo and $p(B1/2)_2geo$ vector

Conformation	Vector	1	2	3	Sum (%)
Supercoiled+linear	pβ geo	4/39	5/26	8/33	17/98 (17.3)
	$p(B1/2)_2geo$	11/29	10/19	14/27	35/75 (46.7)

Supercoiled and linear form DNA was mixed as 1:1 concentration to 4 ng/l.

in the pgeo (17.3%, 17/98 in mixed and 16 % in linear form), while in the p(B1/2)$_2$geo, it did not reach to the rate of the linear form (46.6%, 35/75 in mixed and 63% in linear form), indicating little contribution of the supercoiled DNA moiety to the resulted integration rate of the mixed DNA. By mixing both forms of DNA in an equal volume, the concentration of each form DNA is reduced to a half (2 ng) but it does not seem to affect the integration frequency according to a previous report (Brinster et al., 1985), which reported a similar integration efficiency with various DNA concentrations ranged from 0.1 to 45 ng per microlitter. Thus, this result proposes that the majority of the integration events are contributed by linear-form molecules irrespective of the initial conformation of microinjection DNA.

Ⅳ. DISCUSSION

Mouse eggs have abundant modifying enzyme activities that can make linear transgene copies ligated to long arrays or to supercoiled forms of various sizes and that also can make the supercoiled molecules relaxed to nicked or linear molecules (Bishop and Smith, 1989; Brinster *et al.*, 1985; Powell et al., 1992). This conformational convertibility of the microinjected DNA might influence the recombination mode and, in trun, the integration rates of a transgene. The conversion seems to be directional in some degree; if it occurs without any bias, then the integration efficiency should become nearly equal between both conformation. However, it seems not the case since a linear-form transgene is well known as much efficient in integration than a supercoiled one. In our recent microinjection results with liearized or supercoiled vector (Kang *et al.*, 2000), and also in a previous report (Brinster *et al.*, 1985), the integration frequencies were quite different between supercoiled and linear forms and revealed as consistently higher in linear form, whether the trangenes carried the SINE sequences or not. Brinster et al. reported (Brinster *et al.*, 1985) that when linear DNA molecules were injected, a large fraction of the molecules migrated in the position of supercoiled molecules on agarose gel after 24 h. Such results may prefer an assumption that the equilibrium of the conversion process goes highly tilted to the circularization process that entails relatively lower integration rates of supercoiled DNA. However, it remains to be examined in what degree the inter-conversion would occur in nuclei.

Also, It is unknown whether or not the results of microinjection using supercoiled DNA are originated solely from its own conformation. Although the equilibrium in the conformational conversion may favor circularization process than linearization, there also exist a few linear molecules derived from the circular-tolinear conversion process. Thus, these small amount of linear molecules might primarily contribute to the integration events due to their higher integration ability. However, an another possibility is that supercoiled form DNA has its own integration process, such as a simple insertion depending on a short homology (Allen et al., 1994; Hamada et al., 1993). We examined the possibilty of supercoiled molecules to integrate themselves into a genome (Table 1). The result does not support that supercoiled DNA has a unique integration pathway that is different from that of linear counterpart. Rather, the majority of the integration events are contributed by linear or linearized molecules.

In conclusion, we suggest that the equilibrium in the molecular conversion reaction between linear and supercoiled conformation is highly tilted to the circularization process, and that an actual integration efficiency of supercoiled DNA is much less than an apparent integration frequency, which may

be largely contributed by the linear molecules converted from supercoiled DNA.

V. 요 약

본 실험실에서는 최근에 SINE계 B_1과 B_2의 융합서열이 리포터유전자의 게놈내 삽입을 증가 시킴을 증명하는 결과를 보고하였다. Supercoil 형태일 때 대조구가 6%, SINE 벡터가 25%, 그리고 linear 형태일 때 각각 16%와 63%로 나타났다. 이번 실험에서는 이들 두가지 형태의 벡터를 같은 양으로 혼합한 DNA를 미세주입에 응용 함으로서, 전체 게놈내 삽입률 중에서 과연 supercoil 형태의 벡터가 어느 정도로 기여하는지를 조사하였다. 수정란의 전핵에 혼합형태의 DNA를 미세주입한 후 배반포기의 생쥐배아를 대상으로 베타-갈락토시데아제 발현 여부를 조사한 결과 대조구의 경우 17.3 %, SINE계 벡터의 경우 46.6%가 양성을 나타내었다. 이 결과는 아마도 supercoil 형태의 벡터는 독자적인 삽입 경로를 가지지 않음을 의미하는 것이며, 대부분의 경우 외래 유전자의 삽입은 linear 형태로 삽입이 이루어지는 듯 하다. 부가적으로 미세주입 결과로부터 일부 삽입 기작을 짐작할 수 있는 결과를 얻을 수 있었다.

VI. REFERENCES

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(접수일자 : 2001. 5. 30. / 채택일자 : 2001. 7. 12.)