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Use of Histones to Increase the Frequency of Recombinant Plasmid Formation during Molecular Cloning

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ABSTRACT

By compacting DNA in eukaryotes, histones bring closer two distant regions on DNA. This is likely to facilitate the work of transcriptional enhancers at a long distance in gene regulation (Amouyal 1991). Since the classical strategy for plasmid engineering involves a step where the recombinant fragment is circularized, this has prompted the author to test this hypothesis by plasmid engineering. In fact, the unfractionated mixture of calf thymus histones facilitates the *in vitro* ligation of two fragments, resulting in an increased number of transformed *E. coli* cells under the appropriate conditions for about 7 and 12 kb large recombinant plasmids. This property can be exploited for plasmid engineering, when inserting a DNA fragment into a plasmid vector becomes increasingly difficult as the recombinant gets larger. The same effect was obtained with calf thymus histone H2B by itself, but was less marked with the linker histone H1.

Keywords: circularization, chromatin, compaction, DNA looping, *E. coli*, enhancer, ligation
Abbreviations: bp, base pair; **DNA**, deoxy-ribonucleic acid; **LB**, Luria-Bertani; **PEG**, polyethylene glycol; **X-gal**, 5-bromo-4-chloro-3-indolyl-β-D-galactoside

INTRODUCTION

Since 1984-1985, with my group, I have been working on the way in which transcriptional enhancers work and on DNA looping involved in this process (see for example, Krämer *et al*. 1987; Amouyal 2007). Thus a previous study was focused on the comparison between prokaryotic and eukaryotic enhancers (Amouyal 1991). It emerged from this work that a eukaryotic enhancer is not truly acting at a distance indicated by the number of base pairs separating the enhancer sequence from the gene, but actually closer when dealing with chromatin, since DNA is wrapped around histones in eukaryotes (Kornberg 1974, Luger *et al.* 1997, Schalch *et al.* 2005; for overviews, recent reviews or recent aspects related to chromatin structure, see for example van Holde 1989 and Wolffe 1998; Luger 2003; Nemeth *et al*. 2004; van Holde *et al*. 2006). This distance must take into account the compaction introduced by histones in DNA. Thus, the large, apparent discrepancy observed between the distances at which a eukaryotic enhancer and its prokaryotic counterpart operate, could be, at least partly explained and reduced. It also indicated that chromatin could play a passive role of activation in gene regulation by bringing the enhancer closer to the promoter region, thus facilitating DNA looping.

These remarks had an unexpected follow-up: the classical engineering of a recombinant plasmid implies the *in vitro* ligation of the insert with the linearized vector and the reclosure of the resulting recombinant fragment, before transformation of *E. coli* and subsequent selection of the transformed cells (for standard cloning procedures, see for example Sambrook *et al*. 1989). Therefore the ability of histones to bring closer two distant DNA regions can be tested by the ligation of a linearized plasmid with a *lacZ* gene from *E. coli*.

MATERIALS AND METHODS

DNA fragments and plasmids

Plasmids were prepared by standard protocols and purified either by the cesium chloride method (Birnboim *et al*. 1979) or by Qiagen Miniprep kits.

1- The pR4-LZ plasmid, which is 12034 bp long, results from the ligation of two fragments, fR4 and fLZ, as shown in **Fig. 1**.

- fR4 fragment (8159 bp)

This fragment results from the entire enzymatic digestion of the pR4 plasmid (10183 bp, Invitrogen) by *Sal*I (positions 7 and 1091) and *Spe*I (unique site at 9250). This plasmid was a kind gift from Frédérique and Irène Joab.

This fragment contains the colE1 replication origin for *E. coli* and the gene of resistance to ampicillin. It was separated from the two other fragments (1084 bp and 940 bp) by electrophoretic migration on a low melting point agarose gel (Seaplaque LMP agarose FMC Bioproducts), cutting out of the corresponding band and recovery of DNA from the band.

- fLZ fragment (3875 pb)

This fragment originates from a 6206 bp derivative of the pUT79 plasmid (Cayla, France), pLZ, kindly given by André

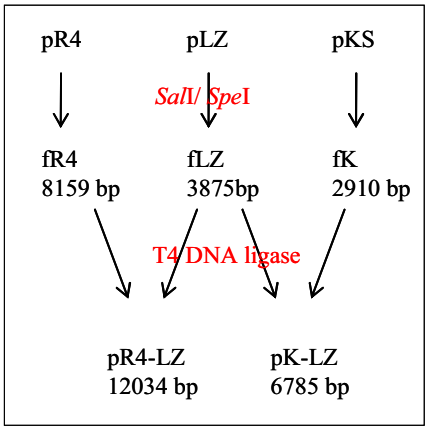

Fig. 1 Diagram of the plasmids/ fragments and how they relate to each other.

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Research Note

Choulika (Chevalier-Mariette *et al*. 2003). It contains the *E. coli lacZ* gene. It was separated from the 2331 bp fragment resulting from the enzymatic digestion of the plasmid at the unique sites *Spe*I (position 29) and *Sal*I (position 3904), after an electrophoretic separation of the fragments.

2- The pK-LZ recombinant plasmid, which is 6785 bp long, results from the ligation of two fragments, fLZ, already described, and fK (see **Fig. 1**).

- fK fragment (2910 bp)

This fragment stems from the digestion of the pBlueScript KS plasmid (Stratagene) by *Spe*I and *Sal*I. It contains a replication origin for *E. coli* and the β-lactamase gene conferring ampicillin resistance to the cell.

Protein

Histone preparations are those commercialized by Sigma.

Sigma H 9250 is the unfractionated mixture of histones from calf thymus containing the five histones H2A, H2B, H3, H4 and H1 (referred to as MixH in the text).

Sigma H 4524 is histone H1 from calf thymus.

Sigma H 4255 is histone H2B from calf thymus.

The protein, which is marketed in lyophilized form, was put in solution in the ligation buffer just before use to the desired concentration.

Ligations

The two fragments fR4 and fLZ were mixed in the proportions specified hereafter in the text, in 10 µl of ligation buffer (50 mM Tris-HCl, 10 mM magnesium chloride, 10 mM dithiothreitol, 1 mM adenosine triphosphate, 25 µg/ml bovine serum albumin) for the T4 DNA ligase (New Englands Biolabs). When necessary, the protein was added to the mixture of fragments at the desired concentration. T4 DNA ligase (200 units) was added after incubation for 3 to 5 minutes, when not otherwise specified. The ligation was carried out at 22°C for 24 hours.

Histone concentration

The amount of histone was determined by calculating the quantity of the natural mixture of histones (Mw = 130268 Da) assumed to be necessary for the formation of one nucleosome every 220 bp, and choosing deliberately to only take a part of the amount of protein thereby determined (one fourth in the given experiments) so as to avoid saturation of the fragment in protein. The amount of protein, m(protein), in nanograms for example, to add to the overall amount of fragments, m(DNA), in the same units (nanograms), that is derived from this calculation is the following:

$$m(protein) = m(DNA)\ Mw / 145640\ X \qquad [E]$$

where Mw is the molecular weight of the protein, X = 4 for the given experiments. 145640 is the product of the length of DNA occupied by a nucleosome (taken as 220 bp) by the mean molecular weight of one base pair of DNA (662 Da).

For the mixture of histones, MixH (Mw = 130268 Da), this equation becomes:

$$m(MixH) = O.22\ m(DNA) \qquad [E(mix)]$$

Each assay was in fact a triple assay performed in the presence of this calculated amount and two other flanking quantities at 50% and 200%, to compensate for pipetting and weighing discrepancies. In the first experiments, the amount of histones was calculated on the basis of complexation of the linearized vector alone. The procedure was later improved by considering complexation of the two fragments involved in the ligation process, the linearized vector and the *lacZ* insert.

When individual histones, H1 or H2B, were used, the amount of histone was determined on the basis of a quantity equivalent in moles to the quantity present in the natural mixture, when not otherwise specified.

Under these conditions, for H1(Mw = 21500 Da), equation [E] becomes:

$$m(H1) = 0.037\ m(DNA) \qquad [E(H1)]$$

and for H2B (Mw = 13774 Da), equation [E] becomes:

$$m(H2B) = 0.024\ m(DNA) \qquad [E(H2B)]$$

A gel retardation assay was consistently performed on the fR4 fragment prior to each assay with histones and under the same conditions, in order to monitor the complexation of the DNA by the protein, on the basis of this calculation. This gel shift assay was carried out with standard 0.4% agarose.

E. coli transformation by the ligation medium

The *E. coli* DH5α strain was transformed by 10 µl of the ligation mixture according to Mandel *et al*. (1970). The same batch of competent cells was used for all experiments. Selection of the cells containing the recombinant was performed on LB agar plates with 400 µg/ml ampicillin, covered with a 20 mg/ml solution of X-gal in dimethyl formamide, as an enzymatic test for the expression of β-galactosidase (Ullmann *et al*. 1967).

Tests for recombinant production

The colonies that are resistant to ampicillin and express β-galactosidase (blue coloration in the presence of X-gal) were selected and grown in LB medium containing ampicillin (100 µg/ml).

The plasmids extracted from the bacterial cultures were then submitted to an enzymatic digestion by *Eco*RI.

Cleavage by this enzyme of a correctly bound and reclosed R4-LZ recombinant (pR4-LZ plasmid, 12034 bp) should result in six fragments of the respective sizes: 4172 bp, 3076 bp, 2875 bp, 2 021 bp, 393 bp and 283 bp. In the same manner, cleavage by *Eco*RI of the K-LZ recombinant (pK-LZ plasmid, 6875 bp) should result in three fragments of the respective sizes: 3426 bp, 3,076 bp and 283 bp. These digestion profiles were electrophoretically visualized on a standard 1% agarose gel, stained with ethidium bromide (0.5 µg/ml).

RESULTS AND DISCUSSION

Cloning a 12034 bp recombinant with the unfractionated mixture of histones

The mixture of fR4 and fLZ fragments with the concentrations indicated in **Table 1** was incubated with the indicated amount of histones for less than 5 min or a longer period.

Fig. 2A shows the complexation of the fR4 fragment by the unfractionated mixture of histones (MixH) on the basis of the calculation detailed in Material and Methods for a typical assay. This preliminary test checks that the calculated amount of protein actually defines a range of concentrations for the formation of low molecular weight complexes in the reaction medium.

Following transformation of DH5α competent cells by the ligation mixture in the presence or absence of the histone mixture, blue and white transformants grew on the plates. The resistance to ampicillin conferred on the cell by the plasmid is a first indication for the presence of the R4 segment in the recombinant plasmid. The presence of the *lacZ* insert was indicated by the capacity of the selected colony to cleave X-gal (blue coloration). The obtention of the R4-LZ recombinant was further checked by the production of a correct profile of enzymatic cleavage (*Eco*RI test) as specified in Material and Methods and in **Fig. 3**.

The number of colonies expressing β-galactosidase is reported in **Table 1**. This analysis results from the data obtained with the various triple assays. For example, one triple assay gave 1, 1 and 0 blue colonies respectively when the MixH amount was respectively 33%(32nM), 67% (64nM) and 134% (128nM) of the value determined with equation E(mix) for 370ng of fR4 fragment and 190ng of fLZ fragment.

The most successful experimental conditions (those that enabled production with certitude of at least one recombi-

Table 1 Mean number of pR4-LZ or pK-LZ transformants resulting from ligation of the indicated fragments in the presence or absence of the unfractionated mixture of histones, MixH.

	Incubation time				
	<5 min	≥15 min	<5 min	<5 min	<5 min
[MixH] nM	**[fR4]=[fLZ]=7 nM**		**[fR4]=[fLZ]=2.8 nM**	**[fR4]=2.8nM[fLZ]=7 nM**	**[fK]=[fLZ]=9.5 nM**
0	0 (0; 16)		0 (0; 3)	0 (-; 1)	5 (3; 2)
32[1]	0.7 (1.1; 11)	0.3 (0.5; 3)	0 (-; 1)	-	4 (-; 1)
48[2]	9 (-; 1)	-	0 (-; 1)	-	-
64[1]	1.2 (1.0; 11)	0.7 (0.9; 3)	0 (-; 1)	-	6 (1; 2)
95[2]	2 (-; 1)	-	0.5 (0.5; 1)	-	-
128[1]	0.5 (0.7; 11)	0.3 (0.5; 3)	0 (-; 1)	-	10.5 (6.5; 2)
190[2]	2 (-; 1)	-	0 (0; 2)	-	-
320[3]	-	-	-	0 (-; 1)	-
380[2]	-	-	0 (-; 1)	-	-
640[3]	-	-	-	0 (-; 1)	-
1280[3]	-	-	-	0 (-; 1)	-

The first number in the brackets is the standard deviation from the mean value, the second one is the number of assays leading to this result. The superscript numbers relate the triple (or quadruple) assays described in Materials and Methods. The amount of MixH calculated from equation E(mix) corresponds to a molar concentration of 95 nM for [fR4]=[fLZ]=7 nM and to a molar concentration of 72 nM for [fK]=[fLZ]= 9.5 nM.

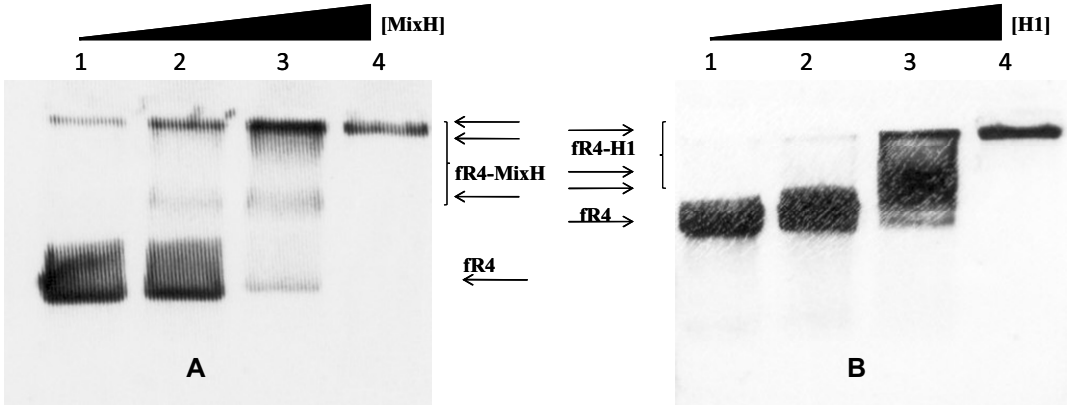

Fig. 2 Complexation of the fR4 fragment by histones. Panel A corresponds to a typical assay performed with the unfractionated mixture of histones (MixH). Panel B corresponds to another assay performed with histone H1. 370 ng of fR4 were incubated with the specified amount of histones for 5 mn, at room temperature and then loaded on a standard 0.4% agarose gel, stained with ethidium bromide. (**A**) Lane 1: 0 ng MixH, Lane 2: 41.5 ng MixH, Lane 3: 83 ng MixH, Lane 4: 166 ng MixH. (**B**) Lane 1: 0 ng H1, Lane 2: 0.68 ng H1, Lane 3: 1.37 ng H1, Lane 4: 2.74 ng H1. Migration of free DNA, as well as that of DNA-histone complexes, is indicated by arrows.

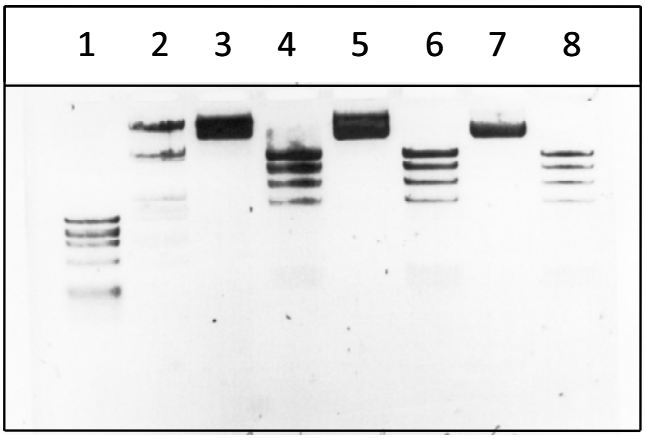

Fig. 3 Cleavage by *Eco*RI of 3 presumed recombinants pR4-LZ (expected size: 12,034-bp) issued from 3 ampicillin-resistant, blue transformants. Standard 1% agarose. Lane 1: ϕx174RF/*Hae*III ladder (Gibco BRL). Lane 2: λ/*Hind*III DNA ladder (NEB). Lanes 3,5,7: uncleaved plasmids. Lanes 4,6,8: respective *Eco*RI digestion with the 6 expected fragments (4172 bp, 3076 bp, 2875 bp, 2021 bp, 393 bp and 283 bp).

nant by triplet, even if the other conditions were modified, or those conditions that introduced an improvement in the cloning efficacy) were combined when the amount of histone was determined by the E(mix) law defined in Materials and Methods, the incubation time short (less than 5 minutes) and the highest DNA concentration (370 ng of fragment fR4 and 190 ng of fragment fLZ in 10 µl of ligation medium).

Note that the primary goal of a cloning procedure is not to yield the greatest number of transformants. A small number, even only one, is sufficient, provided that it reproducibly leads to this result.

Fig. 4 shows that the increased efficacy of cloning can be assigned to an increased amount of ligation products, and more specifically of circular R4-LZ recombinants in the ligation mixture. Note that in the ligation mixture, the pR4-LZ plasmid is mainly under the circular relaxed form. When purified from bacteria, the plasmid adopts the supercoiled form.

Cloning a 6785 bp recombinant

When fLZ is cloned into the pKS plasmid digested by *Spe*I and *Sal*I, a smaller plasmid of 6785 bp is obtained.

In this case, cleavage by *Eco*RI of the recombinant K-LZ results in three fragments of 3426 bp, 3076 bp and 283 bp (**Fig. 5**).

Transformants are now obtained even in the absence of histones, as reported in **Table 1**. However, the presence of histones during the ligation is also able to improve the cloning efficacy.

Cloning the 12034 bp recombinant in the presence of separate histones

Two histones have been employed separately: calf thymus histone H1 and calf thymus histone H2B. These two histones were chosen merely because they were commercially available at the time of the experiments.

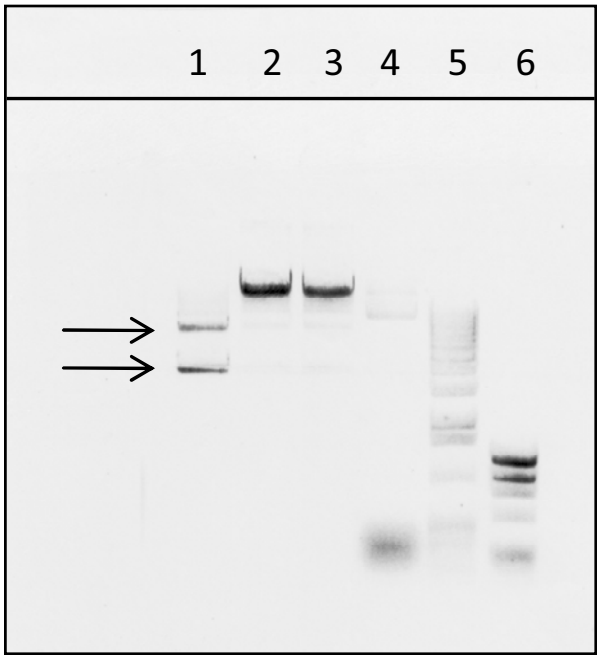

Fig. 4 Ligation products in the presence and absence of MixH. 370 ng of fR4 (8159 bp) and 180 ng of fLZ (3875 bp) were incubated with 0 ng MixH (lane 1), 62 ng MixH (lane 2) or 123 ng MixH (lane 3) and T4 DNA ligase in 10 µl of ligase buffer overnight. 5 µl of the ligation mixture were loaded on a standard 1% agarose gel. Lane 4: 250 ng of pR4-LZ recombinant purified from bacteria. Lane 5: 1Kb DNA ladder (Gibco BRL). Lane 6: ϕx174RF/HaeIII DNA ladder (Gibco BRL). The two fragments are indicated by arrows.

Ligation in the presence of histone H2B

The amount of histone H2B was calculated according to equation E(H2B) defined in Materials and Methods. It corresponds to a quantity equivalent in moles to the quantity present in the natural mixture.

The data are summarized in **Table 2**. Histone H2B by itself strongly increases the cloning efficacy.

Table 2 Mean number of pR4-LZ transformants resulting from ligation in the presence or absence of histone H2B.

[H2B] (nM)	[fR4]=[fLZ]= 7nM
0	0 (0 ; 5)
32[1]	1 (1 ; 2)
48[2]	10.7 (4.8; 3)
64[1]	0 (0; 2)
95[2]	3 (2.5; 3)
128[1]	0 (0; 2)
190[2]	1.7 (1.25; 3)

The amount of H2B calculated from equation E(H2B) corresponds to a molar concentration of 95 nM for the indicated molar concentration of fragments.

Table 3 Mean number of pR4-LZ transformants resulting from ligation in the presence of histone H1.

[H1] nM	[fR4]=[fLZ]=7nM	[fR4]=[fLZ]=1.4nM
0	0 (0 ; 4)	0 (- ; 1)
32[1]	0 (0 ; 2)	-
48[2]	0 (- ; 1)	-
64[1]	0.5 (0.5 ; 2)	-
85[3]	-	0 (- ; 1)
95[2]	0 (- ; 1)	-
128[1]	0 (0 ; 2)	-
170[3]	-	0 (- ; 1)
190[2]	0 (- ; 1)	-
340[3]	-	0 (- ; 1)
432[4]	0 (- ; 1)	-
855[4]	0 (- ; 1)	-
1152[4]	0 (- ; 1)	-

The amount of H1 calculated from equation E(H1) corresponds to a molar concentration of 95 nM for [fR4]=[fLZ]=7 nM.

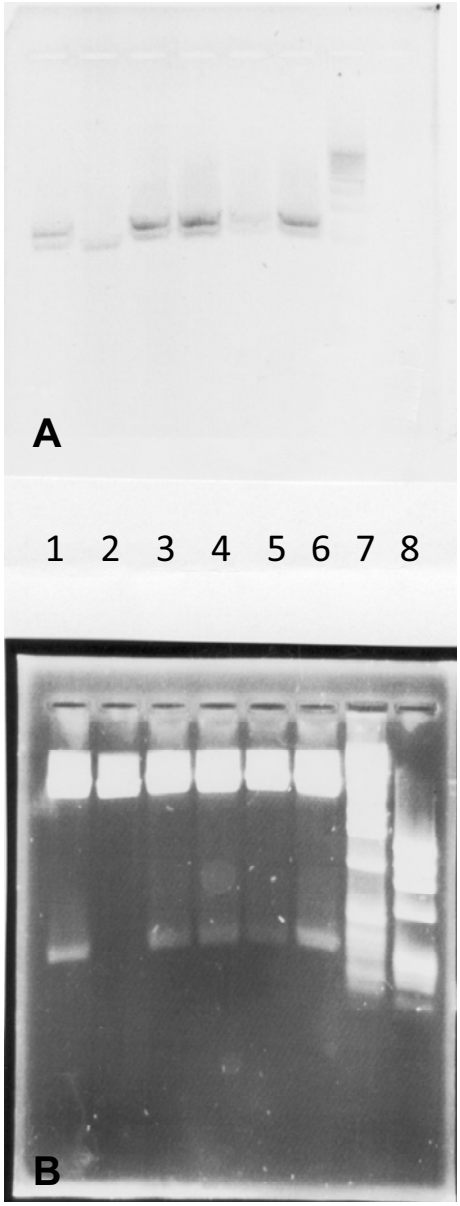

Fig. 5 Cleavage by *Eco*RI of the presumed pK-LZ recombinants (expected size: 6785 bp) prepared from six blue transformants: 3 fragments (3426 bp, 3076 bp and 283 bp) are expected. Panel A: Lanes 1-6: *Eco*RI digestions. Lane 7: 1 Kb DNA ladder. Lane 8: PhiX174RF/*Hae*III DNA ladder. Panel B: same gel, with a shorter electrophoretic run for best observation of the 283 bp band. Recombinants 1, 3, 4, 5 and 6 display both the 3426 bp, 3076 bp doublet in panel A, and the 28-bp band in panel B. Recombinant 2 does not show this digestion profile.

Ligation in the presence of H1

The amount of H1 determined on the basis of a quantity equivalent in moles to the quantity present in the natural mixture, is given by equation E(H1) in Materials and Methods. The amount of protein determined by this calculation was tested by a gel shift assay (**Fig. 2B**).

The data are presented in **Table 3**.

Histone H1 is not as efficient as the natural mixture or H2B under the same conditions, but provides a protein control. It is well-known that the activity of enzymes can be unspecifically stabilized by other proteins. This is why carrier proteins, such as BSA, are included in reaction buffers: they minimize unspecific interactions and adsorption of the enzyme on the tube walls. If the histone effect was not specific, H1 would have the same effect as MixH and H2B at the same concentration. This is not the case, especially given the fact that the ligase mixture already includes a carrier protein, 25 ng/µl BSA, and a high

amount of ligase (20 units/µl).

Histones are among the most abundant proteins in the nucleus; they are of small size (11 to 25 kDa) and of very basic nature. They are present in virtually all eukaryotes in association with most of the nuclear DNA. There are five types of histones which are referred to as H1, H2A, H2B, H3 and H4, respectively in higher eukaryotes. These five types are found with variants in all of the eukaryotes (with the exception of H1 which is replaced by histone H5 in certain cells and certain organisms). H2A, H2B, H3 and H4 are found *in vivo* in octamer form: a $(H3\text{-}H4)_2$ tetramer is associated with a $(H2A\text{-}H2B)_2$ dimer. The DNA (147 bp) coils twice around this octameric core so as to form a nucleosomic structure, as determined by X-ray crystallography (Luger *et al*. 1997; Schalch *et al*. 2005). An approximate 7-fold DNA compaction results from this structure. Histone H1 does not participate in this core structure but serves to seal the DNA around the octamer. The binding of histone H1 to linker DNA between nucleosomes leads to further chromatin compaction. Whereas the length of DNA protected by core histones is constant (147 bp), the spacing between the nucleosomes varies greatly between species and cell-types, and during development, with salts, H1 or non-histone chromatin components *in vitro* (see for example Tremethick *et al.* 1993, Blank *et al*. 1995) and H1 *in vivo* (Fan *et al*. 2005). In *Saccharomyces cervisiae*, this spacing is 165 bp; in most mammalian cells, it is about 200 bp; it can reach 260 bp in sea urchin sperm (van Holde 1989).

According to the first data reported in the present note, the insertion of a fragment into a plasmid is facilitated by the unfractionated mixture of histones, as determined by the number of transformants. The DH5αstrain was chosen in the first place because it is routinely used in cloning experiments. This strain is deficient in RecA1 protein, which does not promote recombinations and rearrangements of the DNA within the cell. Notably, the plasmids remain in the monomer form while deletions are avoided (Bedbrook *et al*. 1976; Yanish-Perron *et al*. 1985). An extensive study will include other strains, as well as other DNA lengths and other proteins. However, since the data presented in **Fig. 4** show that the unfractionated mixture of histones increases the amount of circular ligation products before the transformation, it can reasonably be expected that another strain will have a similar effect.

The ligation process includes both the intermolecular ligation of the insert to the linearized vector, and the intramolecular closure of the recombinant fragment. It is known that above around 500 bp, ring closure probability of a fragment decreases with its size (Shimada *et al*. 1984). This step might specifically benefit from the presence of histones.

Histone H2B has individually the same effect as the unfractionated mixture of histones. Most studies related to interaction of DNA with this histone are in fact related to the H2A/H2B couple, as this mixture is closer to the native chromatin than H2B alone (Li 1975). The data presented in this paper indicate that this protein might bridge distant DNA segments by itself and/or enhance cell entry. On the contrary, histone H1 does not appear to yield results as good as the other histones. In fact, histone H1 and the core histones play different roles in chromatin structure, since H1 is not a histone forming the octamer but rather a sealing protein for the nucleosome, which would restrict nucleosome movement (Pennings *et al*. 1994; Catez *et al*. 2006; van Holde *et al*. 2006). It has also been noted that this histone preferentially binds highly supercoiled DNA (Ivanchenko *et al*. 1997) and that it inhibits T4 and *E. coli* DNA ligases (Ray *et al*. 1996) as well as the eukaryotic DNA ligase IV / XRCC4 complex of ligation (Kysela *et al*. 2005). On the contrary, core histones bind linear DNA to reconstitute nucleosomes (see for example, Germond *et al*. 1976).

In any case, the present study provides a way to improve the cloning efficiency of DNA fragments in plasmid vectors by the classical strategies with the DH5α strain. It is known from the day-to-day laboratory practise (see also Sambrook *et al*. 1989, section 1.63) that the largest the plasmid, the more difficult is the cloning. Thus the 6785 bp long recombinants are easily obtained in the absence of histones, whereas none are obtained under the same conditions for a nearly double size of 12034 bp. *E. coli* transformation is made more difficult by the size of the plasmid (Hanahan 1983). Since DNA in the presented experiments is not purified at this stage, cell entry (whether by the calcium chloride procedure or by another mode, such as electroporation) might benefit from the use of histones. However, the present work also indicates that the *in vitro* ligation step is also limiting when the size of the recombinant is increased and that this drawback could be circumvented by the use of histones.

Other additives, mainly PEG (Pheiffer *et al*. 1983; Zimmerman *et al*. 1985), are known to speed-up ligation. This additive allows to lower the DNA and ligase concentrations. Furthermore, as it increases the amount of intermolecular products with respect to circularization, it is mainly used to flank the insert or the linearized vector with short oligonucleotides and new restriction enzymes recognition sequences. Thus, PEG does not specifically address the question of the vector size and would not have the same use as histones. It would be interesting in further studies to know if PEG and histones are antagonistic or synergistic.

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