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A new method for constructing linker scanning mutants

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ABSTRACT

A new procedure for the construction of linker scanning mutants is described. A plasmid containing the target DNA is randomly linearized and slightly shortened by a novel combination of established methods. After partial apurination with formic acid a specific nick or small gap is introduced at the apurinic site by exonuclease III, followed by nuclease S1 cleavage of the strand opposite the nick/gap. Synthetic linkers are ligated to the ends and plasmids having the linker inserted in the target DNA are enriched. Putative linker scanning mutants are identified by their topoisomer patterns after relaxation with topoisomerase I. This technique allows the distinction of plasmids differing in length by a single basepair. We have used this rapid and efficient strategy to generate a set of 32 linker scanning mutants covering the chicken lysozyme promoter from −208 to +15.

INTRODUCTION

The development of powerful new in vitro techniques during the last few years has made possible the performance of site-directed mutagenesis of DNA in vitro to reveal the relationship between structure and function of the genetic material. These new techniques are well suited for studying regulatory regions of genes. As a first step in the analysis of gene function, deletion mutants are generated. They allow the dissection of long stretches of DNA; however, for the analysis of a complex eukaryotic promoter containing multiple control sequences, deletions are not ideal. Analyzing deletions only permits definition of the borders of important regions. Furthermore, the spatial relationship of sequences is changed and new DNA sequences at the fusion junctions are generated. There are examples which show that changing the spacing of sequences affects gene expression (1,2,3,4). Once a functionally important region has been delimited using deletion mutants, this site can be characterized further by introducing point mutations. Such an analysis is only feasible for a short stretch of DNA. So far only the promoter of the mouse β-major-globin gene has been characterized in this way, using more than 100 single base substitutions (5). Alternatively, linker scanning (abbreviated LS) mutants, which contain clustered sets of point mutations at desired locations, can be used for the fine structure genetic analysis of regulatory regions (6).

The number of mutants needed to scan a region of interest is reduced and the original spacing between sequences is conserved. However the construction of LS mutants has proven to be very laborious and time consuming. The original procedure by McKnight (6) as well as the modified version by Haltiner et al. (7) require the construction and sequence analysis of a large number of 5' and 3' deletion mutants to obtain a limited number of LS mutants. As a consequence, this method has seen rather little usage despite its recognized advantages (3,6,8,9,10).

Here we describe a new procedure that facilitates the construction of LS mutants. First, a randomly placed nick is generated within the plasmid containing the target DNA by partial apurination with formic acid and exonuclease III, which introduces a single strand breakage at the apurinic site. The second strand is then cleaved opposite the nick or small gap by nuclease S1, thus assuring a random linearization and a slight shortening of the starting plasmid. A synthetic linker is ligated to the newly created ends and those plasmids which have a linker inserted in the target DNA are enriched. Finally, the mutants are screened by comparing their topoisomer patterns with that of the starting plasmid after relaxation with topoisomerase I. Differences in length as small as one basepair can be resolved by this technique (11). Plasmids showing the wildtype topoisomer pattern and which are therefore supposed to be of wildtype length, are sequenced using a rapid dideoxy sequencing method for supercoiled plasmid DNA. We have used this rapid mutagenesis protocol to generate a set of 32 linker scanning mutants spanning the promoter region of the chicken lysozyme gene from – 208 to + 15. This segment was chosen because previous gene transfer experiments have indicated that this region is required for oviduct specific expression as well as for inducibility by progesterone and dexamethasone (12).

MATERIALS AND METHODS

Enzymes and oligonucleotides

Restriction enzymes, T4 DNA polymerase and Klenow fragment were purchased from Bethesda Research Laboratories, exonuclease III from Boehringer Mannheim, nuclease S1 from Sigma, T4 DNA ligase from New England Biolabs and BglII linkers (5' CAGATCTG 3') from Pharmacia. Topoisomerase I prepared from calf thymus was a kind gift of Dr. H. – P. Vosberg, Heidelberg.

Plasmids

The plasmid pUClysΔ– 208A is a variant of pUClysΔ– 208 (12). The lysozyme promoter sequences were isolated as a *BamHI/EcoRI* fragment and cloned into the HincII site of pUC12 in the orientation shown in the map.

The plasmid pBL2 was constructed in the following way: The coding region of the aminoglycoside 3' – phosphotransferase gene from transposon Tn903 (13) was isolated as a 1.4kb HaeII fragment from the plasmid pHSG262 (14). After T4 DNA polymerase

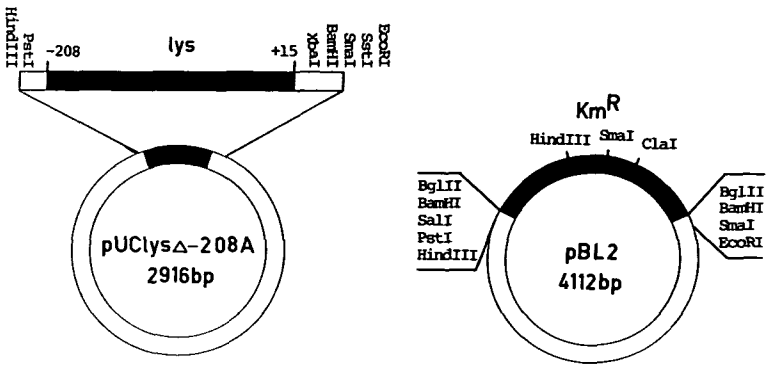

Figure 1. Restriction maps of the plasmids used for the construction of linker scanning mutants of the chicken lysozyme promoter.
Both plasmids are described in MATERIALS AND METHODS.

treatment BglII linkers were ligated to the ends and the fragment was cloned into the BglII site of a pUC8 variant, in which a BglII linker had been inserted at the BamHI site. The maps of both plasmids are shown in figure 1.

Apurination of plasmid DNA using formic acid

For apurination 200µg of supercoiled pUClysΔ- 208A DNA were dissolved in 400µl double - distilled water and preincubated at 15°C. Then 40µl of 2% formic acid (pH = 2, also preincubated at 15°C) were added and the reaction was stopped after 4min at 15°C by the addition of 1600µl 100mM Tris/HCl pH 7.5. The apurinated DNA was ethanol precipitated and washed once.

Specific nicking at apurinic sites using exonuclease III

Apurinated pUClysΔ- 208A DNA (200µg) was dissolved in 800µl 66mM Tris/HCl pH 8.0, 125mM NaCl, 5mM $CaCl_2$, 10mM DTT and preincubated at 37°C. The reaction was started by adding 800u exonuclease III. After 1min, 3min and 9min at 37°C 270µl samples were taken and the reaction was stopped by adding EDTA followed by a phenol/chloroform extraction. After further extractions with chloroform/isoamylalcohol and ether, the DNA was ethanol precipitated, washed and resuspended in TE buffer. The mixture of nicked and gapped circles was separated from the unreacted material by a CsCl/EtdBr gradient.

Linearization of nicked/gapped circles using nuclease S1

For linearization 100µg pUClysΔ- 208A DNA (nicked and gapped circles) were dissolved in 500µl 50mM sodium acetate pH 5.7, 200mM NaCl, 1mM $ZnSO_4$, 0.5% glycerol and preincubated at 37°C. The reaction was started by adding 5000u nuclease S1. Samples were taken after 4min, 15min and 60min at 37°C and stopped as described above for exonuclease III. Finally the DNA was precipitated with ethanol, washed twice and resuspended in TE buffer.

Ligation of linkers to the ends of linear plasmids

In order to increase the portion of blunt ended plasmids after the S1 reaction, a treatment with T4 DNA polymerase was performed using conditions recommended by the supplier. For the linker ligation 10µg pUClysΔ-208A form III (about 10pmoles 5' ends) and 1000pmoles BglII linkers kinased in the presence of [γ-^{32}P]ATP were dissolved in 200µl 50mM Tris/HCl pH 7.8, 10mM $MgCl_2$, 20mM DTT and 0.5mM ATP containing T4 DNA ligase at a concentration of 40u/µl. The ligation was done for at least 15 hours at 15°C. The linker multimers were subsequently digested to completion with an excess of BglII (500u, 6 hours 37°C). Selection for plasmids carrying linkers at both ends was enabeled by insertion of the Km^R fragment from plasmid pBL2. A DNA concentration of 20 - 30µg/ml and a T4 DNA ligase concentration of 400u/ml were used. Both DNAs were present in a 1:1 molar ratio.

Transformation of competent bacteria

Competent bacteria from the E.coli strains HB101LM1035 or JM109 were prepared according to published procedures (15). Transformation was done essentially as described (15). Transformed bacteria were plated on LB plates containing kanamycin and/or ampicillin each at 100µg/ml.

Intramolecular ligation (circularization) of plasmid DNA

The reaction was carried out at 15°C in the ligase buffer described above at a DNA concentration of 1µg/ml and a ligase concentration of 40u/ml. It was performed on a 50ml scale. Before the ethanol precipitation the DNA was concentrated using 2 - butanol.

Isotachophoresis

This method was used to recover DNA fragments almost quantitatively from agarose and polyacrylamide gels. It was performed as described (16), substituting Econo - columns (Bio - Rad) for the specific apparatus mentioned. The outlet was closed during electrophoresis with a dialysis membrane and a female luer fitting. As the leading electrolyte 40mM Tris/HCl pH 7.5 was used. When the DNA had been displaced, the dialysis membrane was removed and fractions were collected. The fractions containing the DNA were identified either by measuring the radioactivity if the sample was labeled or by a simple spot test as described (17).

Size fractionation of lysozyme inserts carrying a linker

The size fractionation was done on a 40 x 20 x 0.1cm 5% polyacrylamide gel. Vector DNA had been removed in a preceeding step in order to avoid overloading of the gel. Lysozyme fragments of 263bp, 254bp and 248bp were used as internal size standards in order to excise a band of about 254 ± 5bp containing mutated lysozyme inserts of wild-type length.

Small scale preparation of plasmid DNA ("miniprep")

This was done according to Holmes and Quigley (18) with the following modifications:

Routinely 1.5ml of a liquid culture grown over night were used. The bacteria were lysed in 400µl STET buffer using 32µl lysozyme (10mg/ml) and an incubation for 50 seconds in a boiling water bath. The debris was removed by centrifugation and the nucleic acids were precipitated with isopropanol. The pellet was washed once and then resuspended in 50µl TE buffer. Neither RNase treatment nor any further purifications were necessary. The plasmid preparations could be used immediately for the topoisomerase I reaction as well as for the supercoil sequencing procedure. Large scale preparation of plasmid DNA was performed as described (18). The DNA was purified twice on CsCl/EtdBr gradients.

Relaxation of plasmid DNA using topoisomerase I

Miniprep DNA (2µl, about 300ng) was relaxed with 1µl topoisomerase I in a total volume of 10µl. As a topoisomerase I preparation of unknown activity was used, the amount of enzyme needed had to be determined empirically. The reaction was usually carried out over night at 37°C in a warm room. The buffer described by Wang (11) containing 10mM Tris/HCl pH 8.0, 200mM NaCl and 0.1mM EDTA was used. The reaction was stopped by adding 10µl TE buffer containing 0.1% SDS and loaded directly on an agarose gel for analysis.

Analysis of DNA topoisomer patterns using agarose gels

DNA topoisomers were resolved on horizontal 1.5% agarose gels of the following dimensions: 235 x 195 x 6mm. The gels contained 25 lanes and 5 of them were always loaded with wildtype DNA serving as standard. The running buffer contained 40mM Tris/HCl pH 7.9, 5mM sodium acetate and 1mM EDTA. The buffer had to be circulated with a pump between the 2 buffer chambers and was used only once. The gels were run at room temperature with 100V until the xylene cyanol has migrated 20cm (about 24 hours). Gels were stained for 30 min to 1 hour in water containing 1µg/ml ethidium bromide and subsequently photographed.

DNA sequencing of supercoiled plasmids

Sequencing was performed as described by Chen and Seeburg (19) with the following modifications: "Miniprep" DNA was used without additional purification and was denatured with alkali. Just before primer annealing the solution was briefly centrifuged and the supernatant was transferred to a new Eppendorf tube. Only 8µCi of [α-^{35}S]dATP were used for the reaction and the samples were not dried in vacuo after the chase but 5µl formamide loading buffer were added directly to the solution. Usually 3µl aliquots were applied to standard 0.35mm thick sequencing gels.

RESULTS

Because of the inherent difficulties of published procedures for constructing linker scanning mutants, we have developed a new approach which accelerates the construction of those mutants. In order to demonstrate its feasibility, we have constructed a series of

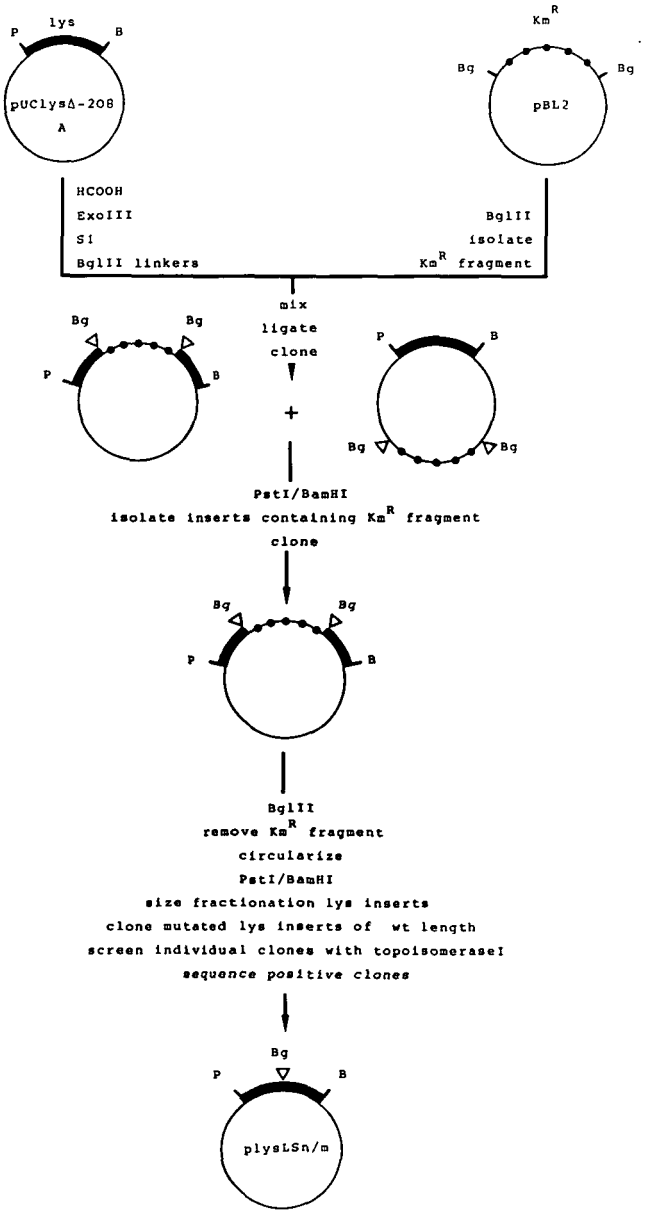

Figure 2. Schematic outline of the construction of linker scanning mutants of the chicken lysozyme promoter.
Abbreviations: P = PstI, B = BamHI, Bg = BglII, lys = chicken lysozyme promoter from −208 to +15, Km^R = aminoglycoside 3' – phosphotransferase gene from Tn903. The open triangle symbolizes a BglII linker.

LS mutants spanning the chicken lysozyme promoter from −208 to +15. The construction is schematically outlined in figure 2.

The first step entailed a random linearization of the plasmid containing the target DNA, achieved by a combination of formic acid, exonuclease III and nuclease S1. The DNA was apurinated with formic acid in a way that at most one third to one half of the plasmids contained a single apurinic site. A subsequent exonuclease III treatment introduced nicks specifically at the apurinic sites. The exonucleolytic activity of exonuclease III could be suppressed to a high extent by substituting Ca^{2+} ions for Mg^{2+} ions in the reaction buffer whereas the endonucleolytic activity was not affected (20). The endproducts of this reaction were therefore plasmids with specific nicks or small gaps of a few basepairs. Nuclease S1 was then used to cut the DNA strand opposite the nick or small gap. This enzyme also removes a few basepairs (0 − 30bp under our conditions) by nibbling at the ends of the linear plasmids. In preliminary experiments a strong S1 hypersensitive site was found in pUClysΔ−208A located in the lysozyme insert. To avoid a high background of plasmids opened specifically at that particular site, it was necessary to separate the randomly nicked circles after the exonuclease III reaction from the remaining supercoils before performing the S1 reaction. Three different time points were used for the exonuclease III as well as for the S1 reaction in order to compensate for differences in reactivity of various sequences. The pH of the S1 reaction buffer was adjusted to 5.7 in order to avoid any additional apurination of the nicked circles. Figure 3 shows pUClysΔ−208A DNA at various stages of the random linearization procedure.

The linearized plasmids were treated with T4 DNA polymerase to increase the number of blunt ended molecules. Octameric BglII linkers were then ligated to the ends of the slightly shortened plasmids. As linker ligations are usually not very efficient, those constructs having linkers attached at both ends were selected for after insertion of the Km^R fragment from plasmid pBL2 (figure 1). Double selection for ampicillin and kanamycin resistance was performed, thereby excluding plasmids containing the Km^R fragment within the β−lactamase gene of the vector. As expected, the portion of clones having a BglII linker inserted in the lysozyme part of pUClysΔ−208A (7.6% of the plasmid length) was increased up to 14.6% by this double selection. Approximately 250 000 independent double−resistant clones were plated. Plasmid DNA was extracted and digested with the restriction enzymes PstI and BamHI to excise the lysozyme inserts. Inserts containing the Km^R fragment were separated by gel electrophoresis and subcloned in pUC12. Approximately 50 000 clones were plated and used to isolate a pool of plasmid DNA having the Km^R fragment inserted in the lysozyme insert. This DNA was digested with BglII in order to excise the resistance fragment and the plasmids were re−circularized in vitro on a preparative scale. The lysozyme inserts carrying a BglII linker were excised with the restriction enzymes PstI and BamHI and sized on a polya−

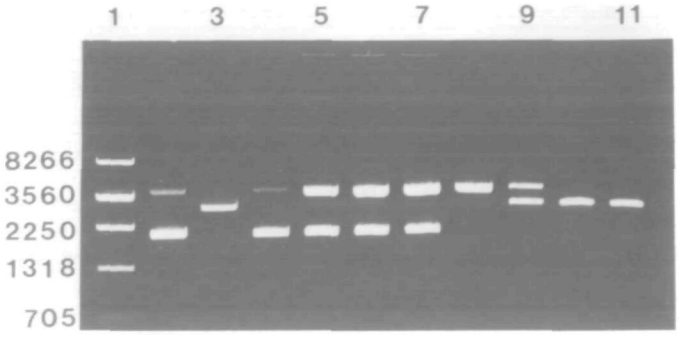

Figure 3. Random linearization of pUClysΔ- 208A DNA.
Lane 1: Size standards. Lane 2: pUClysΔ- 208A, form I. Lane 3: pUClysΔ- 208A, form III. Lane 4: pUClysΔ- 208A x HCOOH. Lanes 5 - 7: pUClysΔ- 208A x HCOOH x exonuclease III (1min, 3min, 9min). Lane 8: pUClysΔ- 208A x HCOOH x exonuclease III, form II. Lanes 9 - 11: pUClysΔ- 208A x HCOOH x exonuclease III, form II, x nuclease S1 (4min, 15min, 60min).

crylamide gel. The fraction of mutated lysozyme fragments differing in length from the wildtype insert by no more than ± 5bp was isolated and subcloned in pUC12, using blue/white color screening for inserts (21). Transformants obtained at this step were screened individually with topoisomerase I. Miniprep DNA from randomly picked white colonies was relaxed to completion with topoisomerase I and resolved on agarose gels. This technique allowed the discrimination of plasmids that differed in length by a single basepair (11), assuring that the majority of clones chosen for sequencing were correct LS mutants (figure 4A and 4B).

In order to establish a relatively complete series of linker scanning mutants of the chicken lysozyme promoter, 1000 individual clones were screened with topoisomerase I. The evaluation of this screen is given in table 1. Plasmids displaying the wildtype topoisomer pattern and which carried a linker at a desired position were sequenced. Altogether 54 clones were sequenced using a rapid sequencing protocol for supercoiled plasmid DNA. The outcome is summarized in table 2. The sequences of 32 mutants chosen to represent a scan of the lysozyme promoter from - 208 to + 15 are shown in figure 5.

DISCUSSION

In this paper we describe a new method of general applicability for the construction of linker scanning mutants. They are the mutants of choice for the analysis of complex regulatory sites such as eukaryotic promoters, but they have the disadvantage that their construction is very laborious and time consuming. Published methods (6,7) rely on the

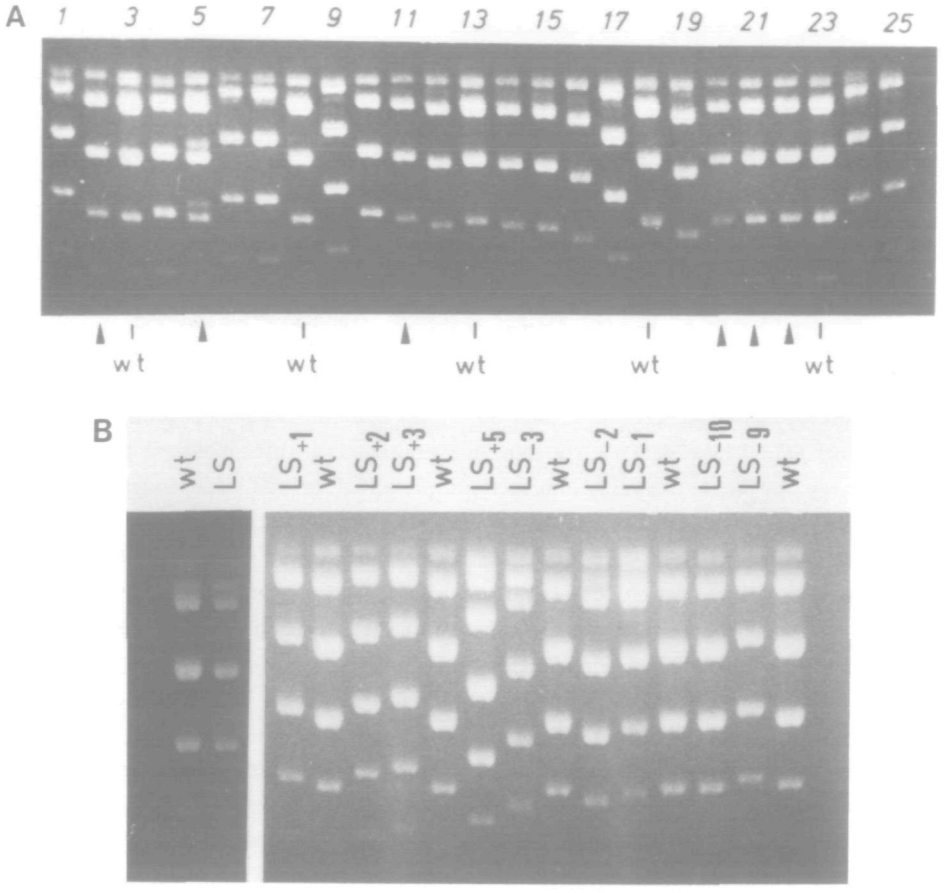

Figure 4A. Screening for linker scanning mutants with topoisomerase I.
Lanes 3, 8, 13, 18, 23: pUClysΔ- 208A DNA relaxed to completion with topoisomerase I (wildtype). Lanes 1, 2, 4 - 7, 9 - 12, 14 - 17, 19 - 22, 24, 25: Plasmid DNA of individual mutant clones after relaxation with topoisomerase I. Clones displaying the wild-type topoisomer pattern are marked by an arrowhead.
Figure 4B. Topoisomer patterns of plasmids of different length.
Supercoiled plasmids from selected mutants were relaxed to completion with topoisomerase I and resolved on agarose gels. For a description of the plasmid types see table 2. Every mutant was verified by sequencing. wt = pUClysΔ- 208A.

generation and sequencing of 2 sets of narrowly spaced 5' and 3' deletions of the target DNA, carrying the same synthetic linker sequence at the ends. Sequence analysis of a large number of deletions is required to find a few matching pairs which can subse - quently be used for the construction of a correct LS mutant. McKnight and Kingsbury sequenced 43 5' and 42 3' deletions to obtain 15 matching pairs (6). Haltiner and col -

Table 1. Summary topoisomerase I screen

total number of clones analyzed	1000
pUC12 vector without lysozyme insert	27
dimers	103
very promising clones (+ +)	85
promising clones (+)	84
less promising clones (+/−)	166
wrong clones (−)	535

The clones were classified according to their topoisomer patterns. (+ +)clones showed exactly the wildtype pattern, (+)clones showed a very similar, (+/−)clones a slightly shifted and (−)clones a totally shifted topoisomer pattern in comparison to the wildtype.

leagues analyzed 35 5' and 45 3' deletions and were able to construct 7 LS mutants (7). Buetti and Kühnel sequenced 60 5' and 49 3' deletions in order to produce 9 correct LS mutants (8). These data clearly show the limitations of the published procedures. The strategy for linker scanning mutagenesis presented in this paper overcomes the inherent difficulties of the established protocols. The number of mutants requiring sequencing to find a correct LS mutant has been reduced significantly. Of 26 mutants displaying topoisomer patterns identical to wildtype, 21 proved to be correct LS mutants. In order to complete the linker scan of the chicken lysozyme promoter, an additional 28 clones with topoisomer patterns similar or slightly different from wildtype were sequenced (see table 2). Although designed primarily for the construction of LS mutants, the method presented here is also applicable for the isolation of 5' and 3' deletion mutations as well as for the identification of mutants with small internal deletions or insertions.

Two steps appear to be important for a succesful application of this method. First, the initial linearization has to be as random as possible. Initially DNase I was used for this step, but the limited sequence specificity shown by this endonuclease makes it unsuitable for truly random linearization of DNA. This led to the development of an alternative procedure relying on formic acid, exonuclease III and nuclease S1. As every basepair

Table 2. Summary DNA sequence determination

mutant type	(+ +)clones	(+)clones	(+/−)clones
LS	21	7	0
$LS_{\pm 1}$	2	9	8
LS_{-2}	0	0	1
LS_{-9}	0	1	0
LS_{-10}	3	2	0

Classification of clones was performed as described in table 1. The different mutant types have the following meaning: LS, a mutant of exact wildtype length; $LS_{\pm 1}$, mutants with an insertion or deletion of 1bp; LS_{-2}, LS_{-9}, LS_{-10}, mutants with deletions of 2bp, 9bp and 10bp.

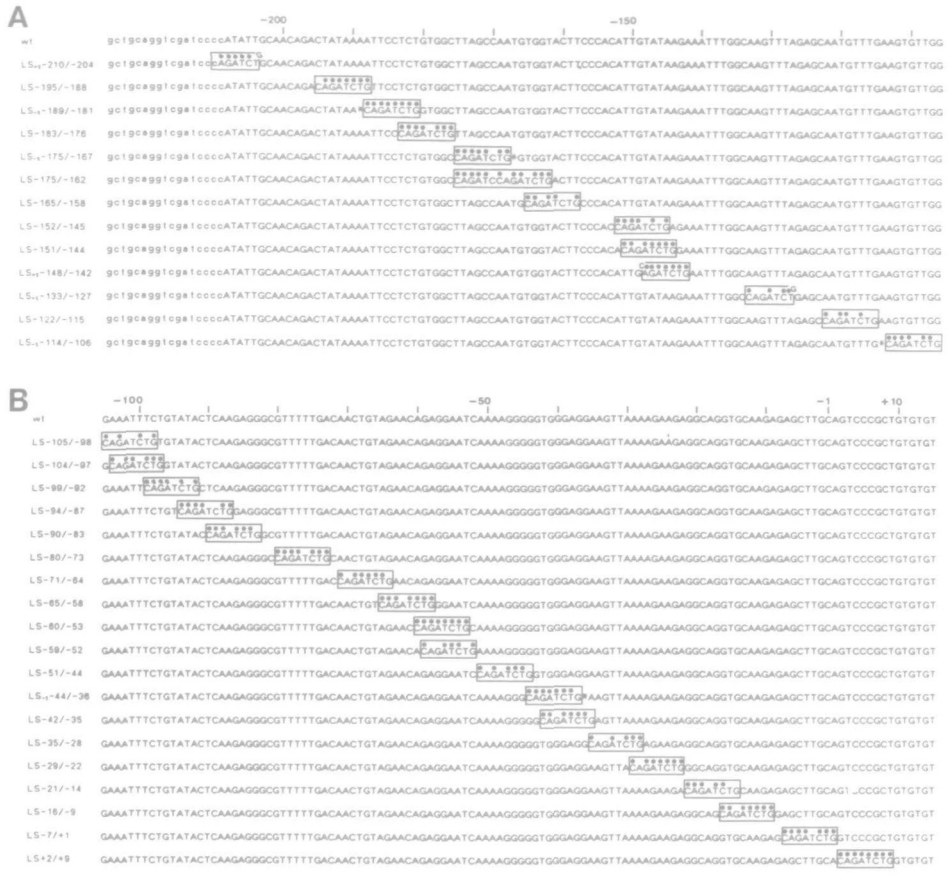

Figure 5. Nucleotide sequence of linker scanning mutations of the chicken lysozyme promoter.
The nucleotide sequence of the wildtype DNA from −208 to −106 and −105 to +15 is displayed in the top line of figure 5A and 5B, respectively. The synthetic linker sequence is boxed. The nucleotides that are changed by substitution of promoter sequences by the linker are indicated by dots. In the case of LS mutations that are not exact substitutions, the alignement has been adjusted to provide minimal sequence conservation within the mutated region. The nomenclature for the LS mutants is plysLSn/m, whereby n and m give the first and the last nucleotide of the wt sequence that has been substituted by the linker sequence. The mutant plysLS−175/−162 was constructed from plysLS$_{-1}$−175/−167 by digesting with BglII, filling in with T4 DNA polymerase, cutting with RsaI at −163 and ligating octameric BglII linkers.

contains a purine and a pyrimidine, each position represents a potential target for acid-catalyzed apurination thus insuring that cuts occur in all regions of the target DNA with about the same probability. The second crucial step is the screening of indi-

vidual clones with topoisomerase I. In order to minimize the number of clones that have to be screened as well as to avoid false - positive clones which have insertions or deletions of 10, 20,... bp and which cannot be distinguished from clones of wildtype length, a stringent size fractionation of the mutated inserts should be performed. As a set of LS mutants requires in any case the screening of a considerable number of clones, a rapid and simple screening procedure is required. For the procedure described here, screening is performed by a one - step enzymatic reaction followed by a facile electro - phoretic analysis for which "miniprep" DNA is suitable. "Miniprep" DNA from up to 100 clones per day can be prepared and the subsequent analysis with topoisomerase I requires an additional day.

What are the problems and limitations of the method presented here? First, large amounts of topoisomerase I are needed. Using impure "miniprep" DNA for the reac - tion requires the use of 30 times more enzyme than for CsCl purified DNA. Topoiso - merase I is commercially available but still rather expensive. Since highly purified enzyme is not essential for the screening procedure described, it is worth considering the use of partially purified enzyme. An alternative is the use of chloroquine to relax the plasmids (22). Although this is effective, the electrophoretic separation of different topoisomers in the presence of chloroquine is, in our hands, not as satisfactory as after relaxation with topoisomerase I. A second potential problem regards the stability of pooled populations of bacterial transformants. Our original strategy for constructing LS mutants required 5 transformations with pooled DNA. Although in the first pool the inserted linkers were randomly distributed, the distribution became more and more non - random with further amplification steps. Mutants containing the linker at some specific sites within the lysozyme promoter clearly seemed to have a poorly understood selective advantage and were enriched after each cloning step. This problem could be solved to a great extent by reducing the number of transformation and amplification steps. The remaining irregularities of representation are revealed by a closer look at the series of mutants obtained using this procedure. An almost perfect distribution of link - ers was observed in the lysozyme promoter from - 105 to + 15, but clones having the linker inserted between - 208 and - 106 were clearly underrepresented. It is important to stress that this underrepresentation of some regions of the target DNA is not due to less frequent cuts during the initial linearization but rather due to differences in the growth rates of individual mutants. It is possible that this phenomenon is restricted to certain DNA fragments or that it depends on the bacterial strain used for the transfor - mation.

In summary the method presented in this paper significantly simplifies the construction of linker scanning mutants thus allowing greater use of these mutants in molecular biol - ogy.

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