Nucleic Acids Research, 1995, Vol. 23, No. 15 **3067–3068**

Random recombination of antibody single chain Fv sequences after fragmentation with DNaseI in the presence of Mn^{2+}

Ian A. J. Lorimer and Ira Pastan*

Laboratory of Molecular Biology, DCBDC, National Cancer Institute, National Institutes of Health, Building 37, Room 4E16, 37 Convent Drive MSC 4255, Bethesda, MD 20892-4255, USA

Received May 18, 1995; Accepted June 21, 1995

Protein engineering currently employs a mixture of rational design and random sequence generation followed by selection to isolate proteins with desirable properties. With the latter approach, a variety of procedures such as cassette mutagenesis (1) and error-prone PCR (2) can be used to introduce mutations in selected regions of DNA coding for the protein of interest. A variety of selection schemes can then be used to isolate mutants with the desired properties. Several studies have shown that different mutations in a protein (isolated with the same selection pressure) may be additive, or positively cooperative, in their effects on the selected function. This includes work on growth hormone (3) and β-lactamase (4). Stemmer has developed a procedure for randomly recombining sequences that exploits this property, called DNA shuffling (5) or sexual PCR (6), and shown that it can be used in the *in vitro* evolution of a β-lactamase enzyme with greatly improved activity.

The DNA shuffling technique consists of two main steps: fragmentation with DNaseI, and reassembly by thermocycling in the presence of a DNA polymerase. In the original DNA shuffling procedure (5), DNA was fragmented by DNaseI digestion in the presence of Mg^{2+}, followed by non-denaturing agarose gel purification of fragments of the desired size. This introduces single-strand nicks in the double-stranded PCR products used as substrates. Fragments of a particular size purified on a gel would thus consist of dsDNA with a large number of single-strand nicks, which would give rise to much smaller single-stranded fragments upon denaturation. If fragments <50 bp were isolated on a gel, much of this would be nicked so much that many of the single-stranded fragments generated with denaturation would be too short to function as primers for *Taq* polymerase at typical annealing temperatures. Here we describe the results of reassembly of fragments generated by DNaseI digestion in the presence of Mn^{2+}, which randomly introduces double-strand breaks into DNA (7). This procedure rapidly produced a set of small (<50 bp) fragments that could be efficiently reassembled without gel purification. We demonstrated the effectiveness of this procedure by using it to randomly recombine two antibody single chain Fv sequences. DNA shuffling between different scFv sequences has not been reported previously, although shuffling of synthetic oligonucleotides with a scFv sequence has been described (8).

Material for shuffling was generated by PCR of two scFv DNA sequences; one designated B3M that consists of Mab B3 VH and Mab B5 VL sequences (9,10), and one designated B1 that consists of Mab B1 VH and VL sequences (10). Each was in the VH–linker–VL orientation, with an identical linker sequence. The two sequences have 88% DNA sequence identity, coding for scFvs that differ by 49 amino acids. PCR was done with the primer pair B35′ and B5VLFX (for B3M) or the primer pair B15′ and B1VLFX (for B1). The primers contain *Sfi*I and *Not*I restriction sites for subcloning into phage display vectors, and a sequence coding for a factor X site to permit proteolytic cleavage of the Fv from its viral protein fusion partner (11). All PCR reactions were done on a Biometro Trio-thermoblock thermocycler (Biometro Inc., Tampa, FL). PCR products were purified with Wizard™ PCR Preps DNA Purification System (Promega) before DNaseI digestion.

Approximately 4.5 µg of PCR product was diluted to 45 µl in H_2O and 5 µl of 10× digestion buffer (500 mM Tris–HCl, 100 mM $MnCl_2$) were added. DNaseI (8.7 µg/ml, 21 500 U/mg protein, Sigma) was diluted 1000-fold in 1× digestion buffer and 1.5 µl of this was added to the PCR–DNA solution pre-equilibrated to 15°C. Digestions were done at 15°C, and terminated at specified times by heating at 90°C for 10 min. Figure 1 shows the type of cleavage obtained under the reaction conditions used. The digestion rapidly gave rise to fragments of <50 bp. Longer digestions (≤20 min) caused only minor decreases in the size and intensity of these fragments, suggesting that the enzyme is much less active on short fragments. This is a convenient property for fragment generation, as it makes timing of the reaction less critical. Also we found that this made it much easier to isolate sufficient amounts of fragments for efficient reassembly. As the gel analysis showed that little or no detectable fragments >~50 bp, we chose to omit size selection and purification of the fragments on a gel, and instead passed the digestion products over Centri-Sep Columns (Princeton Separations, Inc., Adelphia, NJ), small size exclusion spin columns that exclude dsDNA fragments >~16 bp. This removes Mn^{2+} ions that would interfere with *Taq* polymerase activity, and any very small DNA fragments which might be present.

* To whom correspondence should be addressed

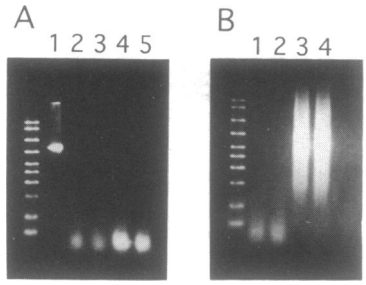

Figure 1. Fragmentation and reassembly of scFv DNA. Samples were analyzed on 1.8% agarose gels. (**A**) Lane 1, DNA from PCR of B3M scFv sequence before digestion; lane 2, scFv DNA after 1 min digestion with DNaseI in the presence of 10 mM Mn^{2+}; lane 3, after 2 min digestion; lane 4, fragments from lane 2 after purification on spin column; lane 5, fragments from lane 3 after purification on spin column. (**B**) Lanes 1 and 2 show fragments after 1 and 2 min digestion, as in (A). Lane 3 shows the reassembly of fragments from 1 min digest (after 40 thermocycles with *Taq* polymerase). Lane 4 shows the reassembly of fragments from 2 min digest. The leftmost lanes in A and B are AmpliSize™ DNA Size standards (Bio-Rad Laboratories, Hercules, CA). The sizes (from bottom to top) are 50, 100, 200, 300, 400, 500, 700, 1000, 1500 and 2000 bp.

To reassemble fragments, 20 µl of spin column-purified DNA fragments (10 µl from B3M digestion plus 10 µl from B1 digestion) were added to 20 µl of 2× PCR premix (5-fold diluted Perkin Elmer GeneAmp 10× PCR buffer, 0.4 mM each dNTP, 0.06 U/µl Elmer AmpliTaq® DNA polymerase). The reaction was overlaid with 50 µl mineral oil and thermocycling was done according to the following program: 96°C, 3 min; (94°C, 1 min; 55°C, 1 min; 72°C, 1 min + 5 s/cycle, 40 cycles), 72°C, 7 min, 4°C. Reassembly of the fragments with 40 temperature cycles in the presence of *Taq* polymerase gave a smear with an average length slightly less than the original PCR scFv sequence (Fig. 1). In this thermocycling reaction, the fragments function as both templates and primers to rebuild full-length genes. To isolate full-length reassembly products, 0.5 µl of this reaction was used as template in a 25 cycle PCR reaction (100 µl final volume) with an equimolar mixture of primers B35′, B5VLFX, B15′ and B1VLX described above. This PCR reaction gave a single sharp band that co-migrated with the original scFv DNA.

To analyse sequences generated using this modified DNA shuffling procedure, PCR products from the above step were subcloned using a TA cloning kit (Invitrogen Corp.). Plasmids containing inserts were purified and sequenced with T7 and SP6 primers on an Applied Biosystems 373A DNA Sequencing System using the Taq Dyedeoxy™ Cycle Sequencing kit. Sequences were analysed using the SeqEd™ 675 Sequence Editor software supplied with this system. Comparison of five randomly chosen sequences showed that five of five had a mixture of B1 and B3M sequences and that all were the result of different recombination events (Fig. 2). No insertions or deletions were found in the 3702 bases of sequence analyzed. The mutagenic rate was 0.2% (nine mutations in 3702 bases sequenced, with a range of 0–4 mutations per scFv sequence), which is not higher than the mutagenic rate expected from the known fidelity of *Taq* polymerase and the large number of cycles to which the DNA was subjected (12). The rate is significantly lower than the mutagenic rate of 0.7% reported for DNA shuffling after fragmentation with DNaseI in the presence of Mg^{2+} (5). It may be possible to further decrease this mutagenic rate by using higher-fidelity polymerases. Also the mutagenic rate could be increased by using error-prone PCR conditions (2), if desired.

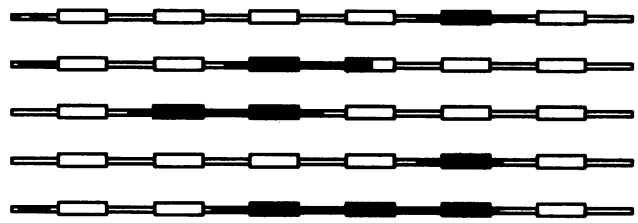

Figure 2. Recombination in five randomly-selected scFv sequences. Clones from reassembly of a mixture of B1 and B3M fragments were sequenced. DNA sequences are shown schematically, with the areas coding for the six complementarity-determining regions represented by boxes (from left to right H1, H2, H3, L1, L2 and L3). B1 sequences are shown in white, B3M in black. Crossovers are arbitrarily shown to be midway between characteristic bases of the two Fv sequences.

We have developed a modified procedure for DNA shuffling that is more rapid and convenient to perform than the previously published procedure, as gel purification of a size range of fragments is not necessary. Also our method is less mutagenic, which will be useful in applications where recombination alone is required. We have shown that the procedure can be used to randomly recombine scFv DNA sequences. This should be useful in the *in vitro* evolution of antibodies with enhanced binding or catalytic activities.

ACKNOWLEDGEMENT

Thanks to Dr Alfred Johnson for help with automated DNA sequencing.

REFERENCES

1 Wells, J.A., Vasser, M. and Powers, D.B. (1985) *Gene*, **34**, 315–323.
2 Leung, D.W., Chen, E. and Goeddel, D.V. (1989) *Technique*, **1**, 11–15.
3 Lowman, H.B. and Wells, J.A. (1993) *J. Mol. Biol.*, **234**, 564–578.
4 Stemmer, W.P.C. (1994) *Nature*, **370**, 389–391.
5 Stemmer, W.P.C. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 10747–10751.
6 Smith, G.P. (1994) *Nature*, **370**, 324–325.
7 Tabor, S. and Struhl, K. (1994) In Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (eds), *Current Protocols in Molecular Biology*. John Wiley & Sons, Inc., pp. 3.12.5–3.12.6.
8 Crameri, A. and Stemmer, W.P.C. (1995) *Biotechniques*, **18**, 194–196.
9 Brinkmann, U., Pai, L.H., FitzGerald, D.J., Willingham, M. and Pastan, I. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 8616–8620.
10 Benhar, I. and Pastan, I. (1994) *Protein Engng*, **7**, 1509–1515.
11 Soumillion, P., Jespers, L., Boucher, M., Marchand-Brynaert, J., Winter, G. and Fastrez, J. (1994) *J. Mol. Biol.*, **237**, 415–422.
12 Eckert, K.A. and Kunkel, T.A. (1991) In McPherson, M.J., Quirke, P. and Taylor, G.R. (eds), *PCR: A Practical Approach*. Oxford University Press, Oxford, pp. 225–244.