Nucleic Acids Research, 1996, Vol. 24, No. 10 **1979–1980**

Improved cloning of antibody variable regions from hybridomas by an antisense-directed RNase H digestion of the P3-X63-Ag8.653 derived pseudogene mRNA

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Received February 15, 1996; Accepted March 26, 1996

When antibody variable regions (V_L or V_H) are cloned from hybridoma cell lines using standard methods (1), often sequences of non-functional rearranged variable regions are obtained by PCR of the hybridoma cDNA (2,3). The origin of these sequences (GenBank accession nos X58634 for V_H and X05184, K00888 and M35669 for V_L) can be traced to the myeloma cell lines originally utilized for the fusion (4,5). Often, the non-secretor cell line P3-X63-Ag8.653 (as well as other cell lines derived from MOPC 21) is used for the establishment of hybridoma cell lines by the standard fusion technique described by G. Köhler and C. Milstein (6). In many cases these hybridoma cells not only transcribe the desired monoclonal antibody DNA, but also bear high levels of non-functionally rearranged mRNAs. These mRNAs represent pseudogenes and can greatly exceed the level of normal antibody mRNA. Due to a reading frame shift in the V–J recombination site, these pseudogene mRNAs cannot be translated into a functional protein (7).

Development of PCR primers which can avoid amplification of these pseudogenes was hampered by the nearly identical sequences of the N- and C-termini of the variable regions. Up to now, the discrimination between 'wrong' and 'right' clones was often achieved by DNA sequence analysis or by checking the binding properties of the expressed antibody fragments (2).

During the process of cloning a set of monoclonal antibodies directed against cytochrome c oxidase, we ran into the pseudogene problem many times. We developed two possible ways to solve the problem.

First, if the variable region of interest does not belong to the subtype γ1 for V_H or κ for V_L, it is possible to suppress transcription of the pseudogene mRNA (which is subtype γ1 and κ) into cDNA by using a specific primer for the first strand synthesis. This strategy worked well with a subtype IgG2a clone where the cDNA synthesis could be made subtype-specific with an IgG2a-specific primer (Fig. 1). Therefore, the V_H pseudogene (which belongs to subtype IgG1) could not be transcribed into cDNA. As a result, no pseudogene sequence could be detected using this procedure for cloning of the V_H domain of this clone.

Secondly, we developed another strategy which works independently from the antibody subtype. We used this strategy in the case of clone 11D3 (which is an IgG1/κ monoclonal antibody

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      122 121 120 119 118 117
5′-G ATA GAC CGA TGG GGC TGT T-3′
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Figure 1. Primer for IgG2a-specific first strand synthesis (numbering according to Kabbat).

Figure 2. Sequence of the CDR3 region of the V_L (κ) pseudogene mRNA with the annealed antisense DNA oligonucleotide. All three reading frames are shown. The CDR3 sequence is underlined to indicate the frame shift. The antisense oligonucleotide covers most of the CDR3 region, including the frame shift.

directed against subunit IV of the cytochrome c oxidase from *Paracoccus denitrificans*), where we only could clone the pseudogene for the V_L domain. We solved the problem by using a specific RNase H digestion of the V_L pseudogene mRNA. Specific digestion was directed by an antisense oligonucleotide which, under the experimental conditions used, can only bind to the pseudogene mRNA, but not to the (unknown) mRNA sequence of the V_L domain of clone 11D3. Then, RNase H can be used for the specific digestion of the RNA in the resulting double-stranded RNA–DNA hybrid (8). The antisense oligonucleotide used (Fig. 2) is directed against the pseudogene CDR3 region. In this region, four bases were lost during non-functional rearrangement leading to the κ pseudogene. The highly variable CDR3 region is the most suited gene region to discriminate between pseudogene and normal V_L sequences.

mRNA was prepared as described previously (3): mRNA prepared from 5×10^5 cells was dissolved in 20 µl 1 mM EDTA, pH 7.4 and incubated at 70°C for breakage of secondary structures for 10 min. After addition of 120 pmol (0.8 µl) antisense oligonucleotide, 5× RNase H buffer (8 µl), water (12 µl) and 1.6 U RNase H (0.8 µl, USB Nr. 70054) the mixture was

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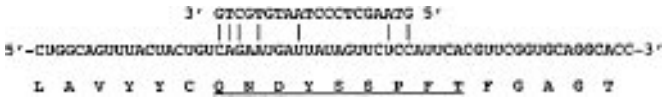

Figure 3. Sequence of the CDR3 region of the V_L (κ) 11D3 mRNA with the 'annealed' antisense DNA. The CDR3 region is underlined. The few paired bases cannot provide a substrate for RNase H.

incubated at 37°C. One sample was incubated for 30 min, another for 60 min. After phenol, phenol–chloroform and chloroform extractions the mRNA was precipitated. cDNA synthesis, PCR and cloning of the V_L gene was done as described (3). The correct sequence for the 11D3 V_L chain (Fig. 3) was obtained in 14% (seven clones were sequenced) upon the 30 min digestion and in 33% (12 clones were sequenced) upon the 60 min digestion. With untreated mRNA, it was impossible to get the right clone; in nine cases (from nine clones sequenced) the pseudogene sequences were obtained exclusively.

This result shows that it is possible to remove mRNA with high specificity by using an antisense directed RNase H digestion. This method may be of use not only for cloning variable regions from pseudogene containing hybridomas, but also in other more general cases.

ACKNOWLEDGEMENTS

We thank H. Müller for DNA synthesis, and Dr G. Kleymann, Dr Reiländer and Prof. J. Engels for discussion. This work was supported by the Fonds der Chemischen Industrie.

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