Volume 236, number 1, 23-26 FEB 06184 August 1988

A simple strategy to amplify specifically the HLA-DQβ gene region with genomic DNA as template

Xue Fan Gu°+, Jacques Elion*, Malika Ouagued°, Eric Clauser, Roger Assan+ and Rajagopal Krishnamoorthy°

°*INSERM U15, 24 rue du Fg St Jacques, 75014 Paris,* +*Service et Laboratoire de Diabétologie, Hôpital Bichat, 46 rue Huchard, 75018 Paris,* **Laboratoire de Biochimie Génétique, Hôpital Robert Debré, 48 Bd Sérurier, 75019 Paris* and *INSERM U36, 17 rue du Fer à Moulin, 75005 Paris, France*

Received 21 June 1988; revised version received 24 June 1988

The nature of codon 57 in the HLA-DQβ gene was recently reported as a potential marker of genetic susceptibility to insulin-dependent diabetes mellitus. When exploring the relevance of this marker by using genomic DNA amplification, we encountered difficulties resulting from the coamplification of the homologous DXβ region. A simple strategy is proposed to amplify the DQβ region exclusively. It involves the preliminary digestion of genomic DNA with a restriction enzyme which cleaves DXβ specifically, leaving intact the DQβ sequence. The amplified material is suitable for dot blot analysis and restriction enzyme digestion. This strategy is of general interest when homologous sequences impair the specificity of enzymatic DNA amplification.

HLA-DQβ locus; HLA-DXβ locus; Polymerase chain reaction; Insulin-dependent diabetes mellitus

1. INTRODUCTION

In vitro enzymatic amplification of specific DNA sequences from a complex template, viz. human genomic DNA, by the polymerase chain reaction (PCR), has become a powerful tool for molecular diagnosis of genetic diseases, direct cloning and sequencing of fragments of specific interest [1-5]. This approach, however, is often hampered by sequence homologies between members of a given gene family. When the interfering sequence belongs to a pseudogene or to a gene whose expression is limited to a particular cell type, the general approach has been to use mRNA isolated from the appropriate tissue as the starting material. This mRNA is first copied into cDNA before one can proceed to the amplification step. Such a strategy weighs too heavily, in terms of time and material, to be applicable to a very large series.

Using this latter approach, Todd et al. [6] have explored the sequence polymorphism of the four major expressed HLA class II gene products, in relation with the genetic susceptibility to insulin-dependent diabetes mellitus (IDDM). They found that the structure of the DQ molecule, especially the nature of residue 57 in DQβ polypeptide chain, is critical in specifying both disease susceptibility and resistance to IDDM. Very early diagnosis of susceptibility to IDDM may be crucial for the success of newly introduced preventive and curative approaches involving immunosuppressive drugs [7].

In this context, we have initiated studies to characterize the structural variations in the region of the HLA-DQβ codon 57 on a large series of patients and controls of multiple ethnic origin to appreciate its value as a marker for IDDM susceptibility and resistance. In setting up a technique applicable to such a large series, using genomic DNA as template, we encountered difficulties due to the coamplification of the homologous HLA-DXβ region. We have overcome this problem by devising a simple strategy

Correspondence address: R. Krishnamoorthy, INSERM U15, 24 rue du Fg St Jacques, 75014 Paris, France

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

which should be of general use in any similar situation involving homologous sequences.

2. MATERIALS AND METHODS

Human genomic DNA was prepared by any of the three following procedures: phenol-chloroform extraction [8], guanidinium chloride method [9] or protein salting out [10]. Supernatant of 200 μl of boiled human whole blood was also satisfactorily used as a source of DNA template. HLA-DQβ exon 2 was amplified using the primers reported by Todd et al. [6]. Relevant synthetic oligonucleotides used as primers and probes [6] were prepared on a Pharmacia gene assembler by the β-cyanoethyl phosphoramidite method and purified by electrophoresis on 20% polyacrylamide gels. PCR was performed manually using *Taq*I polymerase from New England Biolabs or from Cetus Corporation according to the instructions of the first manufacturer. All restriction enzyme digestions were carried out according to the manufacturer's advice (New England Biolabs). Analytical minipolyacrylamide gels were run in a Protean II cell (Biorad).

3. RESULTS AND DISCUSSION

When 1μg of genomic DNA was submitted to 30 cycles of PCR and the product analyzed by electrophoresis on agarose gel, a single major amplification product of 241 bp size was observed (fig.1B), which hybridized with a HLA-DQβ cDNA probe (fig.1A). However, when this product was submitted to digestion with *Sma*I, a restriction enzyme which should cleave the amplified DQβ sequence, a smaller 199 bp fragment was clearly visible, but the 241 bp fragment, although diminished in intensity, remained consistently present despite extensive digestion (fig.2A, lane 1). Given the fact that no *Sma*I recognition site is present in the homologous region of DXβ [11], coamplification of DXβ together with DQβ is a likely explanation for the persistence of the 241 bp fragment upon digestion with this enzyme. This hypothesis was tested by digestion with the restriction enzyme *Taq*I which should cleave both sequences. As shown in fig.2A (lane 3), the 241 bp fragment then totally disappeared in favor of four fragments of smaller sizes, all corresponding to the known restriction map of the amplified DXβ and DQβ regions. Therefore, these two regions indeed coamplified.

We have taken advantage of these restriction recognition sequence differences between DXβ and DQβ regions to devise a strategy involving selective cleavage of DXβ prior to the amplification step. Disruption of the DNA covalent structure between the two primers should naturally abolish the exponential kinetics of the amplification reaction of DXβ. The enzyme *Bst*EII was chosen for its capacity to cleave within DXβ, leaving intact the target DQβ sequence. Digestion of the DNA template with *Bst*EII resulted in a consistent decrease (around 50%) in the intensity of the amplified fragment in agarose gel when compared to that of an undigested sample (fig.2B). However, doubling the amount of template restored the original intensity of the amplified material, suggesting that the target DQβ sequence was indeed the limiting factor and that the intensity reduction was due to the absence of amplification of DXβ and not to nuclease contamination. In order to verify if the amplified sequences were now strictly restricted to the DQβ target, they were digested with *Sma*I and *Taq*I restriction enzymes, respectively. As could be visualized by polyacrylamide gel electrophoresis, *Sma*I treatment now resulted in the complete digestion of the 241 bp fragment

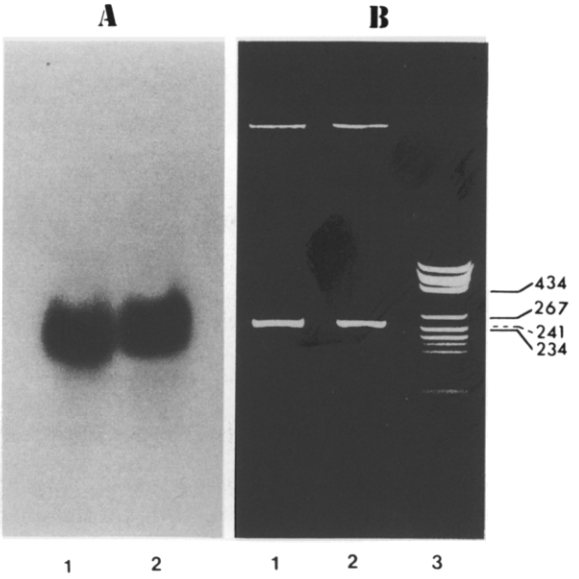

Fig.1. Amplification of the HLA-DQβ region. HLA-DQβ exon 2 was enzymatically amplified using as template 1 μg of native DNA from two independent individuals (lanes 1 and 2). (A) Hybridization of the amplified material with a ^{32}P-labelled HLA-DQβ cDNA probe (kindly provided by Dr P.A. Peterson [12]). DNA was capillary blotted from gel B to a nylon membrane (Gene Screen Plus, New England Nuclear). (B) Analysis of a 5% aliquot of the amplified material by electrophoresis on a 2.2% agarose gel (lane 3 = size markers). The gel was stained with ethidium bromide.

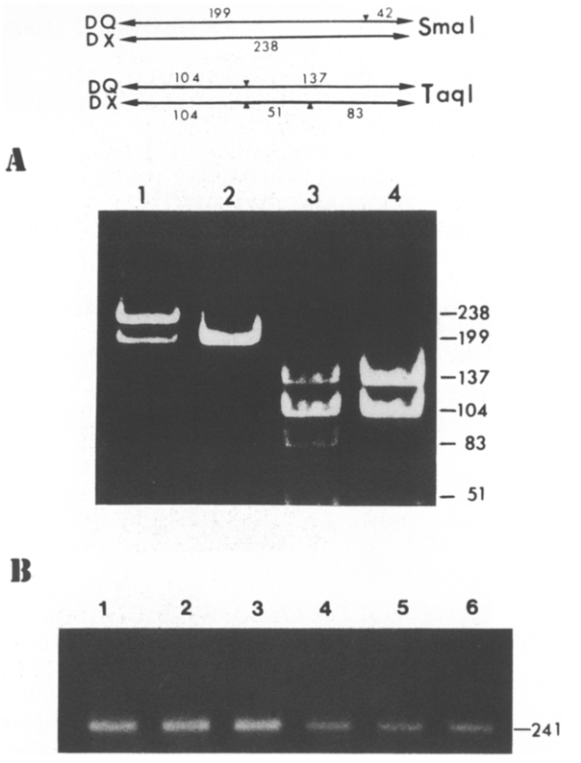

Fig.2. Effect of *Bst*EII digestion of the DNA template. (A) Restriction analysis of the amplified HLA regions. Genomic DNA used as the amplification template was either native (lanes 1 and 3) or predigested with *Bst*EII (lanes 2 and 4). A 10% aliquot of the amplified product was submitted to digestion with *Sma*I (lanes 1 and 2) or *Taq*I (lanes 3 and 4). Digests were size-fractionated by electrophoresis on a 8% polyacrylamide gel and stained with ethidium bromide. The upper part of the figure indicates *Sma*I and *Taq*I sites in the DQβ and DXβ homologous regions as well as the sizes of the expected fragments. (B) Analysis of the amplified material from three different individuals. The amplification template was either native DNA (lanes 1–3) or the same DNA samples predigested with *Bst*EII (lanes 4–6). The 2.0% agarose gel was stained with ethidium bromide.

(fig.2A, lane 2) and the four-band pattern previously observed with *Taq*I was reduced to two bands (fig.2A, lane 4). These data are consistent with the complete absence of amplification of the DXβ region and the selective amplification of DQβ.

From the sequence data, two other enzymes, *Hae*II and *Bal*I, should also cleave differentially the DQβ and DXβ regions and therefore could potentially be used for preamplification digestion. Actually, *Bal*I, an expensive enzyme, gave irreproducible digestion efficiency on the whole, whereas *Hae*II produced results with wide interindividual variations due, we believe, to sequence polymorphism. This was not the case with *Bst*EII predigestion as has been experienced by us with several samples of DNA from different ethnic groups.

Restriction enzymes have already been used to characterize PCR products [1,13]. However, such an analysis is obviously complicated if two, or more, homologous regions are coamplified, in particular when, as here, those sequences are naturally polymorphic. In any such situation, we propose to cut the DNA before amplification in order to specifically generate a unique sequence. Generalization of this strategy is exemplified by the fact that conversely, we were able to amplify selectively DXβ by predigesting the template DNA with *Sma*I. We have also tested this approach successfully on an other duplicated gene family, viz. the highly homologous human γ-globin genes.

Allele specific oligonucleotides have been used by Todd et al. [6] to detect the sequence variations at the position of DQβ codon 57 by dot blot analysis of the PCR product of genomic DNA. At present, because the relation between the nature of codon 57 and IDDM is not absolute and still controversial [14], such a targeted approach is very much restricted. It seems therefore important to look for other allelic variations in this polymorphic region through population studies. This is conceivable either by restriction enzyme analysis or by direct sequencing of the amplified product. Both approaches necessitate that the amplified material be unique for the DQβ locus. This is achieved by the strategy we propose, which by bypassing the mRNA and cDNA preparations has two further advantages, (i) it requires only small quantities of starting material, even obtainable by non-invasive methods [13], and (ii) potential reverse transcriptase-induced errors are avoided.

Acknowledgements: This work was supported by INSERM grants to the Unités 15 and 36 and by grants from the Universities Paris 5 and 7.

REFERENCES

[1] Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N. (1985) Science 230, 1350–1354.

[2] Saiki, R.K., Bugawan, T.L., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1986) Nature 324, 163–166.
[3] Scharf, S.J., Horn, G.T. and Erlich, H.A. (1986) Science 233, 1076–1078.
[4] Wong, C., Dowling, C.E., Saiki, R.K., Higuchi, R.G., Erlich, H.A. and Kazazian, H.H. (1987) Nature 330, 384–386.
[5] Engelke, D.R., Hoener, P.A. and Collins, F.S. (1988) Proc. Natl. Acad. Sci. USA 85, 544–548.
[6] Todd, J.A., Bell, J.I. and McDevitt, H.D. (1987) Nature 329, 599–604.
[7] Feutren, G., Assan, R., Karsenty, G., Du Rostu, H., Sirmai, J., Papoz, L., Vialettes, B., Vexiau, P., Rodier, M., Lallemand, A. and Bach, J.F. (1986) Lancet ii, 119–124.
[8] Gross-Bellard, M., Oudet, P. and Chambon, P. (1973) Eur. J. Biochem. 36, 32–38.
[9] Jeanpierre, M. (1987) Nucleic Acids Res. 15, 9611.
[10] Miller, S.A., Dykes, D.D. and Polesky, H.F. (1988) Nucleic Acids Res. 16, 1215.
[11] Larhammar, D., Hyldig-Nielseln, J.J., Servenius, B., Anderson, G., Rask, L. and Peterson, P.A. (1983) Proc. Natl. Acad. Sci. USA 80, 7313–7317.
[12] Larhammar, D., Schenning, L., Gustafsson, K., Wiman, F., Claesson, L., Rask, L. and Peterson, P.A. (1982) Proc. Natl. Acad. Sci. USA 79, 3687–3691.
[13] Lench, N., Stanier, P. and Williamson, R. (1988) Lancet i, 1356–1358.
[14] Klitz, W. (1988) Nature 333, 402–403.