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Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the *HLA-DQA* locus

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ABSTRACT **Single-copy sequences can be enzymatically amplified from genomic DNA by the polymerase chain reaction. By using unequal molar amounts of the two amplification primers, it is possible in a single step to amplify a single-copy gene and produce an excess of single-stranded DNA of a chosen strand for direct sequencing or for use as a hybridization probe. Further, individual alleles in a heterozygote can be sequenced directly by using allele-specific oligonucleotides either in the amplification reaction or as sequencing primers. By using these methods, we have studied the allelic diversity at the *HLA-DQA* locus and its association with the serologically defined *HLA-DR* and *-DQ* types. This analysis has revealed a total of eight alleles and three additional haplotypes. This procedure has wide applications in screening for mutations in human genes and facilitates the linking of enzymatic amplification of genes to automated sequencing.**

A number of human heritable diseases have been mapped to specific chromosomal regions by using recombinant DNA techniques (1). To determine the molecular basis for these diseases, it is often necessary to examine the same gene segment in a number of individuals. Several methods for rapid screening of mutations in a target sequence have been developed (for a review, see ref. 2). In particular, the polymerase chain reaction (PCR) (3, 4) has been used to identify mutations in the β-globin gene, responsible for the development of sickle cell anemia and β-thalassemia (4–6), and for analysis of the association between sequence variation in *HLA* genes and susceptibility to autoimmune diseases (7–9). The PCR procedure involves repeated cycles of denaturation of the DNA, annealing of oligonucleotides homologous to sequences flanking the segment of interest, and primer extension by a DNA polymerase, resulting in a doubling of the amount of the specific DNA fragment with each cycle (3). This procedure results in a double-stranded DNA (dsDNA) fragment whose sequence can be identified indirectly by hybridization to allele-specific oligonucleotide probes representing the various alleles studied or whose sequence can be determined. The sequence of DNA fragments generated by PCR has been determined either by cloning them into M13 (7, 9) or by direct sequencing of the double-stranded template by using a third "internal" primer (5, 10). However, the M13 cloning method is time consuming and requires that several sequences be determined to distinguish mutations occurring in the original sequence from (*i*) random point mutations introduced by lack of fidelity of the DNA polymerase and (*ii*) PCR artifacts, such as the formation of mosaic alleles by *in vitro* recombination (6). Direct sequencing of double-stranded templates on the other hand often presents difficulties due to the rapid reannealing of strands and the presence of sequences on both strands homologous to that of the sequencing primer, resulting in compound sequence ladders. These problems can be overcome by modifying the PCR reaction in such a way that an excess of full-length single-stranded DNA (ssDNA) of a chosen strand is produced that is suitable for sequence determination. This method has been applied here to the analysis of allelic variation at the *HLA-DQA* locus.

MATERIALS AND METHODS

Enzymatic Amplification of Genomic DNA. Genomic DNA (1 μg) was subjected to amplification with 2 units of the DNA polymerase from *Thermus aquaticus*, as described (6). The reaction mixture, including 50 pmol of primer GH26 (5′-GTGCTGCAGGTGTAAACTTGTACCAG-3′) and 0.5–1 pmol of primer GH27 (5′-CACGGATCCGGTAGCAGCGGTAGAGTTG-3′) (7), was subjected to repeated cycles of 30 sec at 94°C, 1 min at 55°C, and 2 min at 72°C, by using a Perkin–Elmer Cetus Thermocycler (model PCR1000).

Electrophoretic Analysis of Amplifications. Aliquots of the reaction mixture from successive cycles were dried, resuspended in 5 μl of TE (10 mM Tris·HCl, pH 7.4/1 mM EDTA) and electrophoresed through a 1% regular agarose/3% Nusive agarose gel in 1× TBE (1× TBE = 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) for 2 hr at 5 V/cm. Two micrograms of the replicative form of DNA from the phage φX174 digested with *Hae* III was used as the size standard. The agarose gel was then denatured for 45 min in 0.4 M NaOH/1.5 M NaCl and neutralized for 45 min in 0.5 M Tris·HCl, pH 7.5/1.5 M NaCl, and the DNA was transferred overnight in a solution of 4× SSC (1× SSC = 0.15 M NaCl/0.013 M sodium citrate, pH 7.0) to a nylon filter (Genatrans; Plasco, Woburn, MA). The filter was baked for 1 hr at 80°C in a vacuum and prehybridized in 5× SSPE/5× Denhardt's solution/0.5% sodium dodecyl sulfate (SDS) for 15 min at 55°C (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA and 1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin).

Oligonucleotides were labeled with [γ-^{32}P]dATP by using polynucleotide kinase and unincorporated nucleotides were removed with the Centricon-30 microconcentrator (Amicon). Blots were hybridized in the same solution as that for the prehybridization, with the addition of labeled oligonucleotide at a concentration of 0.1 pmol/ml for 5 hr and then washed in 1× SSC at 42°C for 30 min. The blots were exposed to Kodak X-Omat film, and various exposures were taken for densitometry scanning. The oligonucleotides were stripped off the blot by immersing it into distilled water at 60°C for 15 min, prehybridized, and rehybridized as above.

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Abbreviations: PCR, polymerase chain reaction; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; HTC, homozygous typing cell line; ASO, allele-specific oligonucleotides.

Sequencing of ssDNA. The amplification reaction mixture (100 μl) was mixed with 2 ml of distilled H_2O, applied to the microconcentrator Centricon-30 (Amicon), and spun at 5000 rpm in a JA-20 (Beckman) rotor to remove excess dNTPs and buffer components, and the retentate was collected in 40 μl. Approximately 10–20 μl of the retentate was dried and resuspended in 9 μl of 1× sequencing buffer (40 mM Tris·HCl, pH 7.5/20 mM $MgCl_2$/50 mM NaCl) and 1 μl of 10 μM sequencing primer. This resulted in an approximate primer/template molar ratio of 10:1. After annealing at 65°C for 5 min, the solution was brought to 10 mM dithiothreitol, 75 nM dGTP, 75 nM dCTP, 75 nM dTTP, and 5 μCi of adenosine 5-[γ-[^{35}S]thio]triphosphate (1000 Ci/mmol; 1 Ci = 37 GBq), and 2 units of modified T7 DNA polymerase (Sequenase) was added. The labeling reaction (16 μl) was continued for 5 min at room temperature and then divided among four tubes with 2.5 μl of termination mixture (with 80 μM of each deoxynucleoside triphosphate to 8 μM of the appropriate dideoxynucleoside triphosphate). After a 5-min incubation at 37°C, the reaction was stopped by adding 4 μl of 95% (vol/vol) formamide/20 mM EDTA, heated to 75°C for 2 min, and loaded on a 0.4-mm-thick 6% polyacrylamide/7 M urea gel. The gel was electrophoresed at 40 mA/1.8 kV for 2.5 hr, fixed in 10% (vol/vol) glacial acetic acid/10% (vol/vol) methanol for 10 min, dried, and exposed to Kodak X-Omat film overnight. In general, if this procedure resulted in faint sequencing ladders, indicating insufficient amounts of ssDNA template, one of four modifications of the protocol was used: (*i*) 5–10 more PCR cycles were done, (*ii*) dNTPs in the labeling reaction were diluted 1:2, (*iii*) more Taq polymerase was added during the last 5 cycles, or (*iv*) the reciprocal asymmetric primer ratio was used.

RESULTS

Generation of ssDNA by PCR. The principle for the ssDNA-producing PCR reaction (ssDNA PCR) is shown in Fig. 1. The two amplification primers are present in different molar amounts—e.g., GH26/GH27 ratio of 50 pmol to 0.5 pmol (referred to hereafter as 50:0.5). During the first 10–15 cycles, exclusively dsDNA will be produced. However, when the primer added in limiting amounts has been used up, an excess of ssDNA will be produced in each cycle. Theoretically, the amount of dsDNA should increase exponentially, whereas the production of ssDNA should only follow a linear growth.

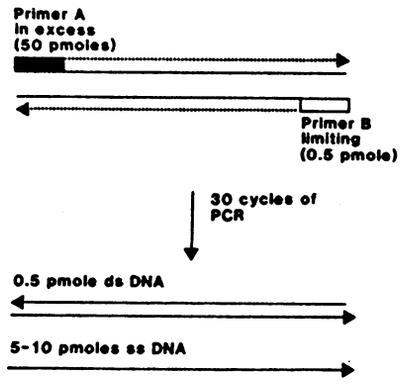

FIG. 1. Outline of the procedure for generating ssDNA by the PCR. The concentrations of the primers (primer A/primer B or GH26/GH27) used in the amplification reaction were initially set to 50 pmol and 0.5 pmol as indicated. After ≈0.5 pmol of dsDNA had been generated, ssDNA started to accumulate at a rate of 0.5 pmol per cycle of amplification. The resulting ssDNA can be sequenced either by adding more of the limited amplification primer or by using an internal primer.

It is, therefore, important that the production of dsDNA is allowed to reach a certain level before ssDNA production is initiated. The optimal point for initiating the generation of ssDNA would be a few cycles before the reaction has reached the level at which the amount of enzyme present in the reaction mixture, limits the growth in copy number (see below).

To test this procedure we amplified a 242-base-pair segment of the *HLA-DQA* gene by using primers flanking the polymorphic region of exon 2. First, by using a GH26/GH27 molar ratio of 50:0.5, the accumulation of product was followed for 43 cycles of amplification. A product of the expected size appeared on the ethidium-stained gel from about cycle 19 and its accumulation appeared to stop at cycle 25 (Fig. 2*a*). When a Southern blot of this gel was probed with an internal oligonucleotide complementary to the ssDNA strand produced, hybridization was found both to the dsDNA band and a component with a lower molecular weight, presumably representing the ssDNA (Fig. 2*b*). To study whether this represented ssDNA of only one strand, the blot was stripped and reprobed with an oligonucleotide made to the same strand as the ssDNA (Fig. 2*c*). Only the dsDNA PCR product hybridized to this oligonucleotide, indicating that the lower molecular component in Fig. 2*b* indeed contains ssDNA of only one strand. The accumulation of dsDNA (as determined by densitometry scanning of autoradiographs) appears to follow the pattern predicted above with

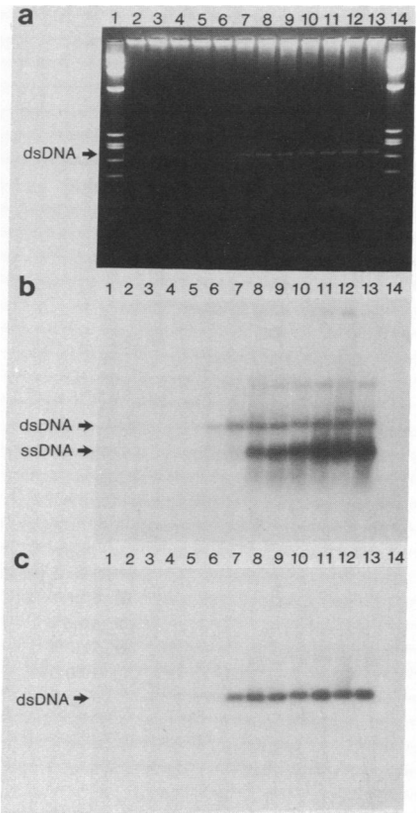

FIG. 2. Accumulation of dsDNA and ssDNA of a *HLA-DQA* fragment as a function of number of cycles of amplification. (*a*) Genomic DNA of a typed *DR3* homozygous individual was subjected to amplification with 50 pmol of primer GH26 and 0.5 pmol of primer GH27. Lanes 1 and 14 contain 2 μg of the replicative form DNA from the phage φX174 cut with *Hae* III used as size standard. Lanes 2–13 contain samples subjected to 5, 10, 13, 16, 19, 25, 28, 31, 34, 37, 40, and 43 cycles of amplification, respectively. (*b*) Southern blot of the agarose gel in *a* probed with an oligonucleotide (GH85) hybridizing to both the dsDNA and ssDNA. (*c*) Same blot reprobed with GH67, an oligonucleotide made to the same strand as the ssDNA generated.

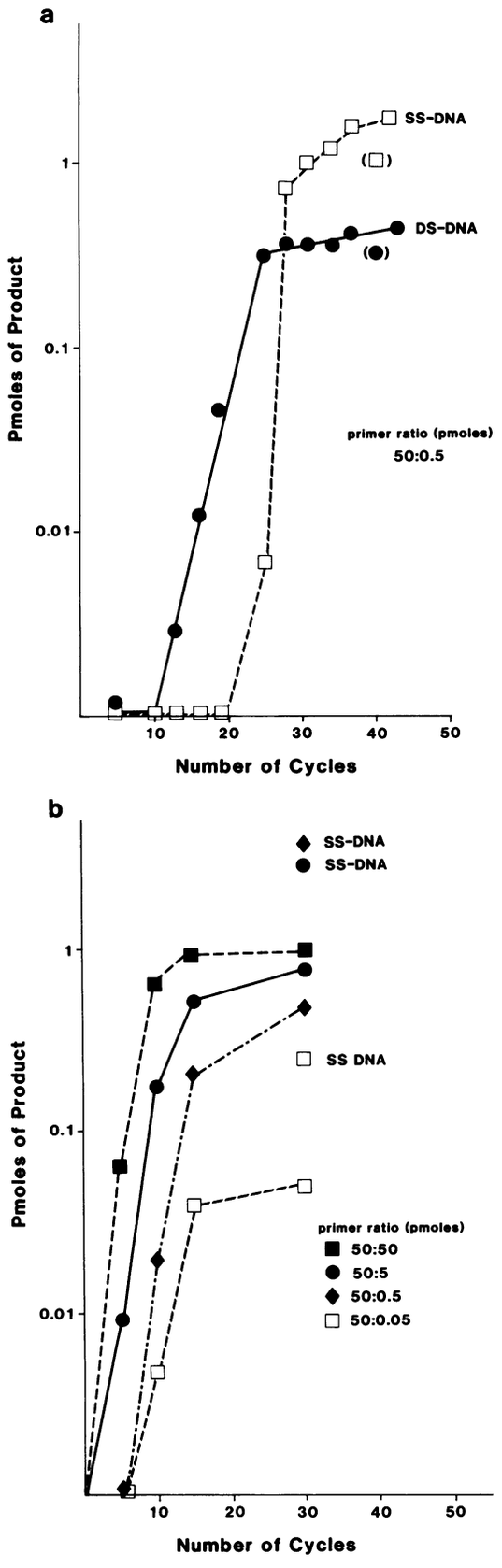

FIG. 3. Accumulation of dsDNA and ssDNA at various GH26/GH27 primer ratios. (*a*) Primer ratio of 50:0.5 up to 43 cycles of amplification. (*b*) Four primer ratios of 50:50, 50:5, 50:0.5, and 50:0.05. The curves are based on densitometry scanning of autoradiograms.

a short exponential phase of increase followed by a phase of linear growth (Fig. 3*a*). From the slope of the curve, we can calculate the efficiency of amplification at the various stages of the reaction. During the exponential growth phase the efficiency of accumulation of dsDNA was ≈70%, while the efficiency of accumulation of ssDNA during the linear phase was only 30%.

A similar experiment with four GH26/GH27 molar ratios of primers, 50:0.05, 50:0.5, 50:5, and 50:50, showed that all three unequal molar ratios result in accumulation of ssDNA amounts exceeding that of dsDNA after 30 cycles (Fig. 3*b*). Surprisingly, with a primer ratio of 50:5, ≈0.8 pmol of dsDNA and several pmol of ssDNA were produced after 30 cycles, indicating that ssDNA was produced even under conditions where the limiting primer was not exhausted (≈4 pmol remaining). This is probably due to the fact that the reaction had reached a level where the amount of enzyme available was insufficient to completely extend all the templates present during each cycle (6). Although the amount of ssDNA generated will vary between primer sets, a ratio of from 1:50 to 1:100 will, after 30 cycles of PCR, generally produce a sufficient excess of ssDNA (1–5 pmol) for several sequencing reactions.

Direct Sequencing of Homo- and Heterozygous Individuals. The method was initially employed to sequence the *HLA-DQA* locus of several homozygous typing cell lines (HTCs) (Table 1). The sequence ladder obtained shows that only a single template was generated in the PCR reaction (Fig. 4*a*). Given the extensive polymorphism of *HLA* genes, most individuals are heterozygous at these loci; since these alleles differ at multiple positions including small deletions, direct

Table 1. *DQA* alleles in homozygous typing cells and heterozygous individuals

Sample	HLA-DR	DQw	DQA genotype
LG2*	*1*	*1*	*A1.1/A1.1*
PGF*	*2*	*1*	*A1.2/A1.2*
AVL*	*3*	*2*	*A4.1/A4.1*
RSH	*3*	Blank	*A4.1/A4.1*
ER	*4*	*3*	*A3/A3*
KT3	*4*	Blank	*A3/A3*
FPF*	*5*	*1*	*A1.3/A1.3*
SWE1	*5*	*3*	*A3/A3*
HERLUF	*5*	*3*	*A4.1/A4.1*
BM15	*5*	*3*	*A4.1/A4.1*
SPO	*5*	*1*	*A1.2/A1.2*
TISI	*5*	*3*	*A4.1/A4.1*
TUBO	*5*	*3*	*A4.1/A4.1*
APD*	*w6 (Dw18)*	*1*	*A1.3/A1.3*
ABO	*w6 (Dw9)*	*1*	*A1.1/A1.1*
AMALIA	*w6 (Dw14)*	*3*	*A4.1/A4.1*
CB6B	*w6 (Dw18)*	*1*	*A1.3/A1.3*
EKOH	*w6 (Dw9)*	*1*	*A1.1/A1.1*
HHK	*w6 (Dw18)*	*1*	*A1.3/A1.3*
EMJ	*w6 (Dw19)*	*1*	*A1.2/A1.2*
LL107	*w6 (Dw14)*	*3*	*A4.1/A4.1*
ZUK	*w6 (Dw18)*	*1*	*A1.3/A1.3*
128	*5.6*	*1,3*	*A1.1/A4.1*
SCHOM	*5.6*	*1,3*	*A1.2/A4.1*
WITHER	*4.6*	*3*	*A3/A4.1*
LG10*	*7*	*2*	*A2/A2*
TAB*	*8*	*1*	*A1.3/A1.3*
LUY*	*8*	*3*	*A4.3/A4.3*
ARC*	*8*	Blank	*A4.2/A4.2*
OLG	*8*	Blank	*A4.1/A4.1*
8854	*8*	*3*	*A4.1/A4.1*

**DQA* sequences derived from the HTCs were determined by M13 cloning of the PCR-amplified products and were reported in Horn *et al.* (9).

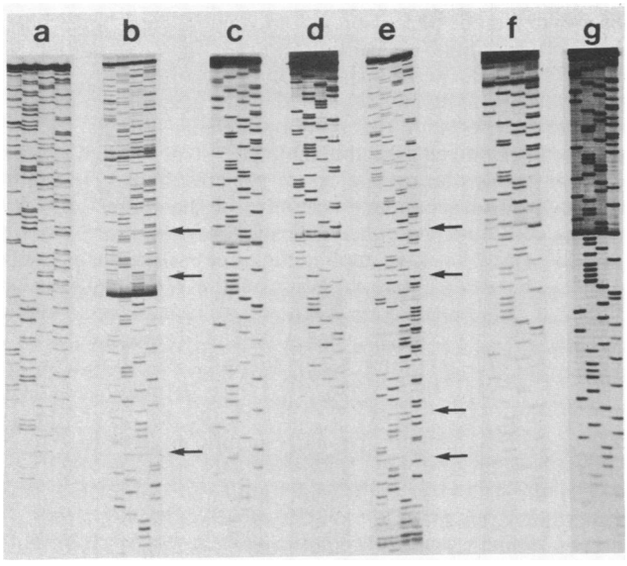

FIG. 4. Direct sequencing of ssDNA generated by the PCR. Templates are as follows. (*a*) HTC line KT3 amplified with primers GH26 and GH27 and sequenced with GH27. (*b*) *DR1/DR3* individual amplified with primers GH26 and GH27 and sequenced with GH27. (*c*) Reciprocal amplification of that shown in *b* sequenced with GH66. (*d*) Same amplification as in *b* sequenced with GH75. (*e*) *DR3/DR4* individual amplified with primers GH26 and GH27 and sequenced with GH27. (*f*) Reciprocal amplification of that in *e* sequenced with GH66. (*g*) *DR3/DR4* individual amplified with primers GH27 and GH66 and sequenced with GH66. The arrows indicate positions where the two allelic templates differ in their nucleotide sequence.

sequencing of *HLA* variants will be feasible only if individual alleles can be distinguished without cloning them apart. This is possible if allele-specific oligonucleotides (ASO) are used either for (*i*) sequencing of only one allele at a time in a mixture or (*ii*) amplification of only one of the two alleles. For example, when a *DR1/DR3* heterozygote individual was sequenced with one of the *DQA* PCR primers, a compound sequence was generated (Fig. 4*b*). The two alleles differ at several positions close to the primer and a three-base-pair deletion. The sequences obtained by using oligonucleotides specific for the *DQA1* and *DQA4.1* alleles, respectively, as sequencing primers are identical to that of the two alleles of the *DR1/DR3*; *DQA1/DQA4.1* heterozygote (Fig. 4 *c* and *d*). In general, only one of the two alleles needs to be sequenced separately, since by "subtracting" this from the heterozygote sequence the other allele can generally be identified. For example, the two alleles of a *DQA4.1/DQA3* individual differ by several nucleotide substitutions (Fig. 4*e*). By sequencing with a *DQA4* ASO primer, one of the alleles was identified (Fig. 4*f*), and the other could be subsequently reconstructed from the heterozygote sequence. It is also possible to use the ASOs to amplify selectively a specific allele in the heterozygote (Fig. 4*g*). These methods are suitable for sequencing alleles that are similar or identical to those previously described, but any different between the ASO and the allele will be hidden and lost in the amplification. To identify the true genomic sequence to which the ASO anneals, we used a second oligonucleotide (GH84 or GH85, Fig. 5) that is homologous to a region flanking the polymorphic central part of the *DQA* fragment as a sequencing primer (data not shown).

Sequence Variation at the *HLA-DQA* Locus. A total of eight allelic variants has been found thus far at the *HLA-DQA* locus by direct sequencing and other sequencing methods (ref. 9 and Fig. 5). In this study, the *DQA* sequences from 25 haplotypes were determined. No additional alleles were identified, suggesting that these eight sequences contain most, if not all, of the allelic variation at this locus. The *DQA1* (1.1, 1.2, and 1.3) alleles are invariably associated with the *DQw1* specificities (Table 1). This "supertypic" specificity is found on *DR1*, *DR2*, and most *DRw6* haplotypes, as well as rare *DR5* (e.g., FPF) and *DR8* (e.g., TAB) haplotypes, which were derived, presumably, from a crossover between the *DR* and *DQ* region. The *DQA1.1* allele is found on all *DR1* haplotypes and a rare subset of *DRw6* haplotypes with the mixed lymphocyte culture-defined *Dw9* type (see below). The *DQA1.2* allele is found on *DR2* and *DRw6,Dw19* haplotypes, and the *DQA1.3* is found on *DRw6,Dw18* haplotypes as well as on the unusual FPF (*DR5*) and TAB (*DR8*) haplotypes that probably arose from a recombination within the *DQ–DR* interval between a *DRw6,DW18* haplotype and a conventional *DR5* and *DR8* haplotype, respectively. The *DQA2* allele is found only on *DR7,DQw2* haplotypes, the *DQA3* allele is found only on *DR4*, *DQw3* and *DR9,DQw3* haplotypes, and the *DQA4* allele is found only on *DR3,DR5* and *DR8* haplotypes (Table 1).

However, several unusual combinations of *DQA* alleles and *DR* specificities (or haplotypes) were also revealed by this analysis (Table 1). In the *DRw6,Dw9* HTCs ABO and EH/OH, a *DQA1.1* allele, previously found only on *DR1* haplotypes was identified. This allele was also found on the *DRw6* haplotype of individual 128, a *Pemphigus vulgaris* patient, and by oligonucleotide hybridization analysis on the *DRw6* haplotypes of most (90%) patients but only 5% of control *DRw6* haplotypes (11).

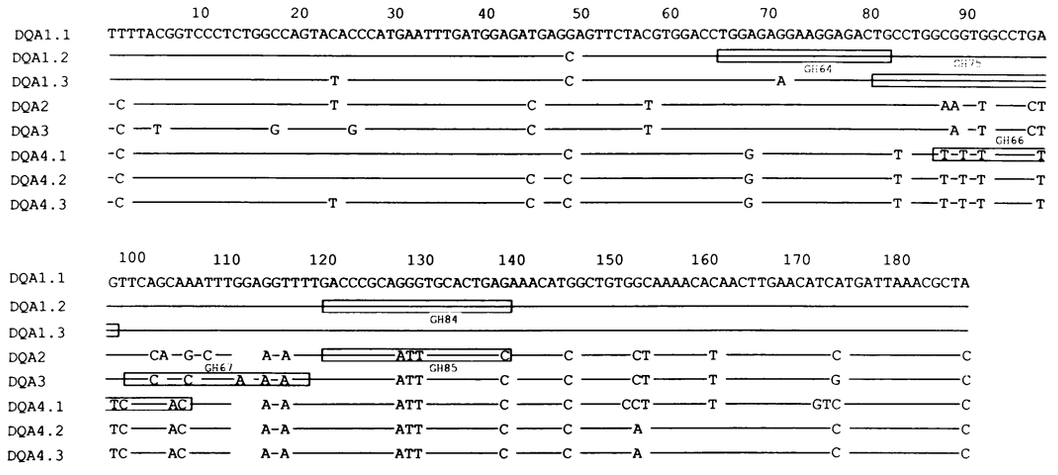

FIG. 5. Alignment of *DQA* alleles. The boxes indicate the oligonucleotides used for direct sequencing of the various alleles.

Another unusual *DRw6* haplotype was identified in the HTCs AMALIA and LL107 (*DQw3,Dw16*) and contained the *DQA4.1* alleles found on virtually all *DR3*, DR5, and *DR8* haplotypes. A rare *DR5* haplotype from the HTC SPO (*DQw1*) has the reciprocal combination; it contained the *DQA1.2* associated with *DR2* and *DRw6* (*Dw19*) haplotypes. Thus the *DR5,DQw1* haplotypes in SPO and FPF must have arisen from independent recombination events.

DISCUSSION

The development of a simple PCR protocol for generating specific single strands significantly facilitates the direct sequencing of the amplified product as well as the preparation of hybridization probes. The overall efficiency of amplification appears somewhat lower (70%) when an asymmetric primer ratio is used compared to when both are present in vast excess (80–90%, ref. 6). In practice, this can usually be compensated for by increasing the number of PCR cycles. The rapid identification of mutants or allelic variants can be accomplished by amplifying DNA segments by using locus-specific primers. Similarly, the analysis of unknown sequences can be carried out by amplifying a cloned insert with vector-specific primers that flank the insertion site (6). In both cases, a single-tube reaction mixture for producing single-stranded PCR products could ultimately be linked to an automated sequencing system for rapid sequence determination. An alternative approach to produce ssDNA templates of PCR products for direct sequencing has been described (12). This method involves attaching a phage promoter to one of the PCR primers, transcribing the PCR product to obtain an RNA copy, and sequencing this with reverse transcriptase. This procedure has more limited applicability since it involves additional enzymatic steps after the amplification reaction and is restricted to using reverse transcriptase as the sequencing enzyme.

Our direct sequence procedure is also capable of identifying both alleles in a heterozygous individual (Fig. 5). The resolution of two alleles that differ by more than two nucleotides requires oligonucleotides (ASOs) that are capable of either priming the sequencing reaction in an allele-specific fashion or of allele-specific PCR amplification (Fig. 5). Alternative approaches to the problem of sequencing heterozygotes are to use restriction enzymes to cleave one of the two alleles (11) or to use denaturing gradient gels (13) to revolve the allelic PCR products. One virtue of direct sequence analysis vs. cloning of PCR products in M13 is the simultaneous detection of both alleles. Consider, for example, an individual serologically typed as *DQw3*; this could either be homozygous *DQw3/DQw3* or *DQw3*/blank (there are some "blank" alleles that are not reactive with—i.e., typed by—existing serologic reagents). Direct sequence analysis could immediately reveal the "hidden" blank allele but one would have to sequence several M13 clones before being able to distinguish the two potential genotypes. Another advantage of the direct sequencing method is that "erroneous" PCR products generated by nucleotide misincorporation will not interfere with the sequence determination. Point mutations due to lack to fidelity of the DNA polymerase will, even if they arise in the first cycle of amplification from a single template, only represent 25% of the intensity of the nucleotides of the consensus sequence when the chain-termination reactions are analyzed by gel electrophoresis. For amplification reactions starting with more than a single DNA template, the frequency of PCR products containing a misincorporated nucleotide is sufficiently low that they will not be detected. Occasionally mosaic alleles have been observed, presumably resulting from partially extended PCR products that can act as primers on other allelic templates in later cycles (6). Such alleles are likely to accumulate primarily in later cycles of the reaction because of insufficient enzyme to extend all available templates. Unless such *in vitro* recombinant or mosaic alleles are very frequent—for example, because of secondary structure of the template—they will not confound the consensus sequence.

In this study, we have applied the direct sequence protocol to the analysis of allelic variation at the *HLA-DQA* locus. Although 25 haplotypes were examined, no additional allelic sequences were identified, suggesting that the eight described sequences (Fig. 5) contain essentially all the variation at this locus. In general, these *DQA* alleles are strongly associated with specific *DR* haplotypes (Table 1 and ref. 9) and in the case of the *DRw6* haplotype, with specific mixed lymphocyte culture-defined subtypes. In the analysis of a panel of *DRw6* HTCs, the *DQA1.1* allele was found only on *DRw6,Dw9* haplotypes, the *DQA1.2* allele only on *DRw6,Dw19* haplotypes, and the *DQA1.3* allele only on *DRw6,Dw18* haplotypes. Although no additional *DQA* sequences were revealed in this study, several additional combinations of *DQA* alleles and *DR* types were identified on *DR5* and *DRw6* haplotypes, illustrating the role of recombination in generating the diversity of the *HLA* haplotypes.

In summary, the direct PCR sequencing method reported here significantly facilitates the analysis of allelic variants at a known locus as well as the determination of unknown sequences. In conjunction with automated sequencing procedures, it provides a rapid and simple approach to the analysis of nucleotide sequences.

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