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Genetics

Endogenous retroviral long terminal repeats within the *HLA-DQ* locus

(major histocompatibility complex/polymorphism/retrovirus)

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ABSTRACT **Two endogenous retroviral long terminal repeats (LTRs) were found in the human major histocompatibility complex locus *HLA-DQ*. The solo LTRs, unlinked to retrovirus structural genes, are located ≈5 kilobases apart from each other and in the same transcriptional orientation, which is opposite to that for the *HLA-DQB1* gene. These elements exhibit >90% homology to the LTRs of the human endogenous retrovirus HERV-K10. The conservation of putative regulatory elements found within the LTRs and their position relative to the *HLA-DQB1* gene suggest that these elements may confer distinct regulatory properties on genes in the *HLA-DQ* region. Polymorphic variation between different *HLA* haplotypes for the presence of the LTRs at this location and of the molecular architecture within this subregion is supported by polymerase chain reaction and Southern blot analysis. Comparisons of chromosomes with and without the LTRs in this region will provide a unique opportunity in the human genome to analyze transposition or integration of retroviral sequences.**

Many of the genes located in the major histocompatibility complex (MHC) of man, the *HLA* region, are highly polymorphic, and products of different alleles of *HLA* encoding class I and II antigens are easily distinguished by serological analysis (1–3). Duplication or deletion of homologous genes within the MHCs of man and mouse has occurred to generate distinct haplotypes (4, 5). Recently, several genes, unrelated to class I and II genes or complement factors, have been found within the boundaries of the MHCs of man and mouse (6–8). Furthermore, endogenous retroviral genomes were found in multiple locations within the region containing the murine nonclassical class I genes (9).

At least three distinct class II A-B heterodimers, HLA-DP, HLA-DQ, and HLA-DR (henceforth referred to as DP, DQ, and DR), are encoded within a contiguous region within *HLA*. Each of the surface heterodimers has been implicated in the presentation of immunogenic peptide antigens to T cells and is expressed on relatively few tissues, primarily B lymphocytes (10). Transient expression of class II antigens is also associated with early stages of differentiation for hematopoietic cells or, after stimulation, with various soluble factors such as γ interferon and interleukin 4. We have directed our studies to understanding the organization and complexity of genes within the class II region and mechanisms that regulate their expression. In experiments designed to identify possible "new" genes in the *HLA* class II region, we searched for transcription of genomic sequences outside the class II genes in several cosmids containing *DP*, *DQ*, and *DR* genes. We report here the discovery of two endogenous retroviral long terminal repeats (LTRs) that appear to be present within the *DQ* region of several, but not all, distinct haplotypes.§

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MATERIALS AND METHODS

Cell Culture and Preparation of Extracts. All human B-cell lines were grown in RPMI 1640 medium (GIBCO-BRL) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (Whittaker M. A. Bioproducts), 2 mM glutamine (GIBCO), 100 units of penicillin and 100 μg of streptomycin per ml (both GIBCO), and 50 μM 2-mercaptoethanol (Sigma). Attached cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) containing the same supplements as above. All cell lines were grown at 37°C in 5% CO_2/95% air. Tera2 and DX-3 cell lines were obtained from A. Houghton of Memorial Sloan Kettering Cancer Center; both cell lines are from the Memorial Sloan Kettering Cancer Center Human Tumor Cell collection.

Sequence Analysis. *Bam*HI fragments of the cosmid LC14 were isolated by electrophoresis in 1% low-melting-temperature agarose gels (BRL) or adsorption of NA45 membranes (Schleicher & Schuell) and were subcloned into pUC12 or pGEM-1. A restriction map of the cosmid was generated by double digestion and Southern analysis. Selected fragments were subcloned into M13, and sequencing was performed by the chain-termination method (11) with Sequenase from United States Biochemical and with the universal primers and internal primers synthesized with a Codor 300 DNA synthesizer (DuPont).

Polymerase Chain Reaction (PCR) Amplification. Total genomic DNA (1 μg) from each *HLA* homozygous cell line was amplified by using a PCR amplification kit (Perkin–Elmer/Cetus) with flanking (external) primers (5′ primer, GTATCCAGGTTTTCTCATTGGTAT; 3′ primer, CTGATCAGGAGTACAGATCAGGTT) for *DQ* LTR3. During the first two cycles, the denaturation time was 1.5 min at 94°C, followed by an annealing cycle of 2 min at 45°C and synthesis for 3 min at 72°C. The 20 subsequent cycles were carried out with 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C (the last was extended 7 min to complete synthesis). One-tenth of each reaction mixture was then diluted into a fresh reaction mixture containing the corresponding junction (internal) primers (5′ primer, GGTGGAGCAACAGCCCACCCGAAGT; 3′ primer, CCCCTTGTGATTCTGTGGGAAAGC), and the same amplification steps were repeated. Only one

Abbreviations: LTR, long terminal repeat; HERV, human endogenous retrovirus; PCR, polymerase chain reaction; MHC, major histocompatibility complex.

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§The sequences reported in this paper have been deposited in the GenBank data base [accession nos. M33841 for *HLA-DQ* LTR3 and M33842 for *HLA-DQ* LTR5].

PCR amplification was performed with the control *HLA-DRA* primers (5′ within exon 2, GCCAGCTTTGAGGCTCAAGGTGCAT; 3′ within exon 4, CACCACGTTCTCTGTAGTCTCTGG) under the same conditions. The reaction mixtures were cooled to 4°C, and then 20 μl of each was subjected to electrophoresis on a 2% NuSieve (FMC)/2% regular agarose gel with ethidium bromide in 40 mM Tris-acetate/1 mM EDTA buffer for analysis.

Southern Analysis. High molecular weight human genomic DNAs were prepared by standard procedures and digested with restriction endonucleases under conditions recommended by the manufacturer, and 3 μg of each sample was subjected to electrophoresis on neutral 0.8% agarose gels. The DNAs were transferred to Zetabind membranes (AMF Cuno) in 0.4 M NaOH overnight, and the membrane was exposed to UV light in a Stratalinker (Stratagene). After prehybridization overnight, the blot was hybridized with a probe labeled uniformly with [^{32}P]CTP (12). The DNA fragment used to generate the probe was amplified from a 1.3-kilobase (kb) *Bam*HI fragment subcloned in pGEM-1 by using a primer corresponding to sequences at the 5′ junction of *DQ* LTR3 (ACAGAAGTCAACAAGGGGTAAGGG) and the T7 promoter primer from the pGEM-1 sequences next to the polylinker (Promega).

RESULTS

In an effort to identify transcribed sequences within the *HLA* class II region, Southern blots containing restriction digests of the cosmid DNAs were hybridized with labeled nuclear RNAs synthesized in isolated lymphoblastoid B-cell nuclei as described (13). Fragments that hybridized with nuclear RNA but not with nick-translated total human genomic DNA were selected for further analysis (unpublished data). One small (1.3 kb) *Bam*HI fragment of a cosmid from the *DQ* region was labeled and hybridized to RNAs from both lymphoid and/or nonlymphoid tissues in RNA (Northern) blotting experiments. Intriguingly, cytoplasmic RNA from a teratocarcinoma cell line, Tera2, and a melanoma cell line, DX-3, but not from B cells hybridized with the fragment. However, when the fragment was hybridized with restriction digests of human genomic DNAs, multiple diffuse bands were observed, indicating that the fragment was repetitive (unpublished data). Indeed, further experiments showed that the same cosmid contained another copy of the fragment. We concluded that the sequence was moderately repetitive, and at least closely related homologs of this sequence were efficiently transcribed in B cells but not processed into mature RNAs. Hybridization of the fragment to RNAs in Tera2 could have resulted from transcripts arising from elements homologous to, but distinct from, the sequences linked to *DQ*.

Nevertheless, we were curious about the identity of this unusual fragment and analyzed its nucleotide sequence. The sequences of both homologous repeats within the cosmid were obtained, and these are compared in Fig. 1. It was immediately apparent that each sequence was highly homologous (about 90%) to the LTRs of the human endogenous retrovirus HERV-K10 (14, 16). Cosmid LC14 in which these LTRs were found also contains a complete *DQB1* gene and a truncated *DQA1* gene (15). Mapping of the cosmid showed that the two LTRs were located upstream of the *DQB1* gene. The elements were called *DQ* LTR5 and *DQ* LTR3 (part of

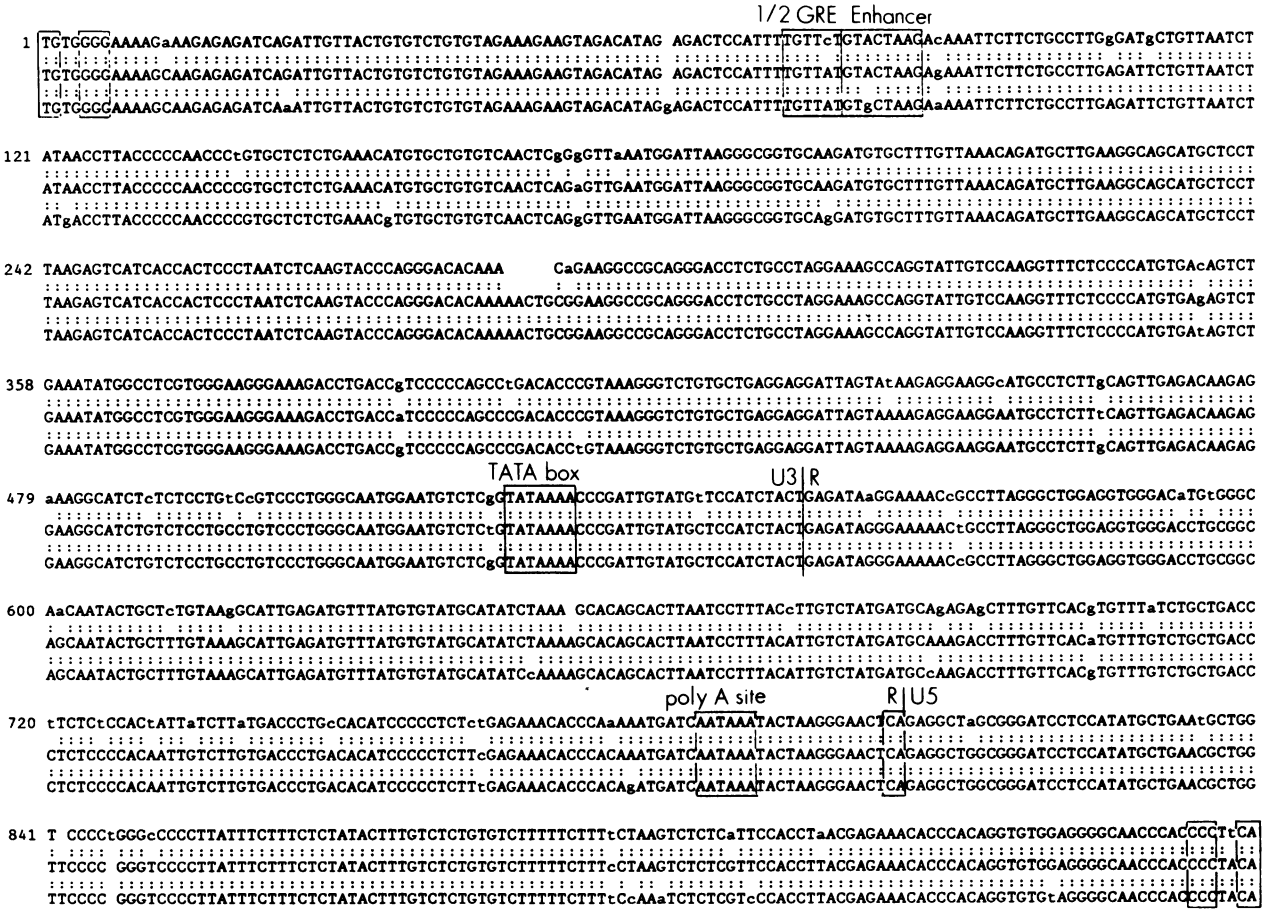

FIG. 1. Sequences of *DQ* LTR5 (top line), *DQ* LTR3 (middle line), and HERV-K10 5′ LTR (bottom line) (14). Sequences were derived from subcloned *Bam*HI fragments of cosmid LC14. Nucleotide differences are shown by small letters. Boxes indicate conserved elements. Most of the differences that are found in the *DQ* LTRs are also found in other copies of HERV-K family LTRs (15).

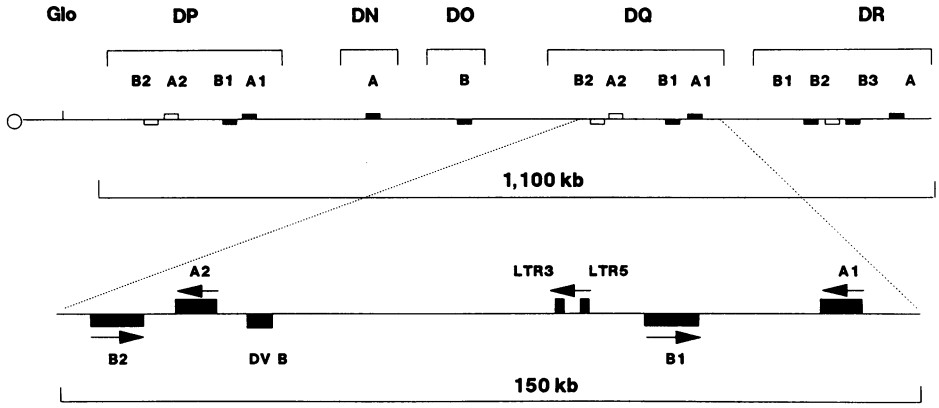

FIG. 2. Map of the *HLA* class II region with an expanded molecular map of the *DQ* region. The molecular organization of the *DQ* region was obtained by restriction analysis of cosmid LC14 (15). In the top line, black boxes indicate genes that are known to be transcribed, and open boxes indicate possible pseudogenes. In the expanded map of the *DQ* region, the black boxes are genes or pseudogenes [it is not clear whether *DQB2* and *DQA2* are functional or not (10)], with their transcriptional orientations indicated by arrows above or below the boxes.

the 1.3-kb *Bam*HI fragment) because of their orientations relative to each other, as illustrated in Fig. 2. More complete restriction maps for cosmids within this region from two distinct haplotypes are published (17, 18). *DQ* LTR5 located ≈11 kb upstream from the cap site for transcription of the *DQB1* gene. The *DQ* LTR3 element is ≈5 kb farther upstream of *DQB1*. Both *DQ* LTRs are opposite in transcriptional orientation with respect to the *DQB1* gene.

Examination of the sequences showed that certain features found in the 5′ LTR of HERV-K10 were for the most part preserved in the *DQ* LTRs. For example, the inverted repeats characteristic of LTRs are intact at the ends of both *DQ* LTRs (14). Approximately 70 nucleotides from the 5′ ends of each there are sequences [TGTT(C or A)T] corresponding to partial glucocorticoid response elements (1/2 GREs) followed directly by an element (GTACTAAG) that is similar to an enhancer core [GTGG(A or T)(A or T)(A or T)G] and identical to elements found in two other HERV-related LTRs that have been analyzed (14). The juxtaposition of these two elements creates a sequence that conceivably could act as a binding site for progesterone or related receptor complexes as suggested by Ono *et al.* (19). At position 563 are TATA-AAA sequences, presumably functional TATA boxes, and at position 825 are polyadenylylation signals. Thus, it is possible that either LTR could act as a promoter independently, although expression of *DQ* LTR3 could be suppressed by transcription of the *DQ* LTR5 (20).

Because the *HLA* class II region is highly polymorphic and the number of *HLA-DR* genes can vary according to haplotype (5), we wondered whether the *DQ* LTRs were present in all individuals or whether there might be variation for their presence in the population. First, in addition to the presence of the LTRs in cosmid LC14 (isolated from DNA of unknown *HLA* haplotypes), we detected the LTRs in *DQ* cosmids derived from *DR7*, *DQw2* homozygous cells (18) and from *DR4*, *DQw8* cells (17) by Southern blotting and PCR amplification (unpublished data). But a more formidable problem was to identify the *DQ* LTRs in total genomic DNA. Our sequence analysis of *DQ* LTR5 showed that it had presumably integrated within a highly repetitive *Kpn* I element (unpublished data). Thus, to derive a unique fragment that would detect only *DQ* LTR5 when used as a probe was extremely difficult. In addition, much of the DNA flanking the two *DQ* LTRs was also repetitive when used as a probe for Southern blotting. Therefore, we synthesized primers at the junctions of both *DQ* LTRs and flanking sequences to use with PCR amplification. As targets for amplification, we used genomic DNAs derived from *HLA* homozygous B-cell lines established or collected for the Tenth International HLA Workshop. In our initial experiments, several bands were observed after amplification by PCR (unpublished data). We presume that at least some of the bands are artifacts of the PCR amplification because sequences of the oligonucleotide primers are partially from repetitive elements. In addition, many of these bands were observed upon PCR amplification of the genomic DNA from the *HLA* class II deletion mutant 721.180 (21). For this reason, we also generated primers just 5′ to the LTRs (within a *Kpn* I repeat for *DQ* LTR5) and just downstream of the 3′ junctions. The outside or flanking primers were used for the first step of amplification and the inside or junction primers were used in the second step. Because the absence of a band in a sample could represent polymorphism in the primers, we performed the PCR under nonstringent conditions (two cycles at the beginning of each step used annealing at 45°C). Fig. 3 shows that only some of the DNA samples acted as templates for the PCR amplification of *DQ* LTR3, even though all except the deletion mutant 721.180 contained fragments from the *DRA* gene, which we were able to amplify. Moreover, both *DQ* LTR5 and LTR3 were found in those samples in which either was amplified (unpublished data). We conclude that the presence of the LTRs at this location within the *DQ* locus varies according to *HLA* haplotype.

The implied absence of both *DQ* LTRs in some of the DNA samples from homozygous cell lines suggested to us that the

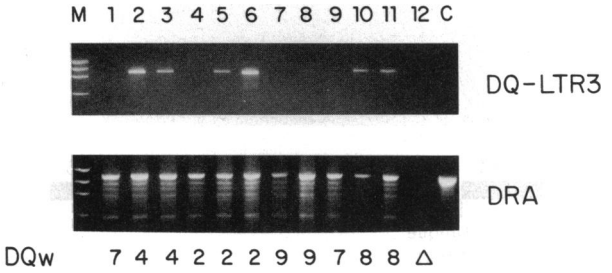

FIG. 3. PCR amplification of *DQ* LTRs. (*Upper*) Amplification from the *DQ* LTR primers. (*Lower*) One-step PCR amplification with control *DRA* primers. Cell lines in lanes: M, φX174/*Hae* III markers; 1, LUY; 2, MADURA; 3, RSH; 4, VAVY; 5, MOU; 6, PLH; 7, DBB; 8, DKB; 9, JHAF; 10, SAVC; 11, BOLETH; 12, 721.180; C, cosmid LC14 (*Upper*) or cosmid JG10-3 [containing DRA (15); *Lower*]. The positive control for *Upper* shows products amplified from 1 ng of cosmid LC14; only 1% of the product was loaded on the gel, in comparison with 20% of the products from genomic DNA samples. *DQw* allele designations are below the lower panel. The expected sizes of the PCR products were ≈960 nucleotides for *DQ* LTR3 and 1200 nucleotides for *DRA*. Smaller bands in the *DRA* PCR products may be single-stranded DNA or artifacts.

entire block of DNA between *DQ* LTR5 to *DQ* LTR3 might also be absent from the negative cells. We surmised that direct repeats of transcriptionally active elements, such as LTRs, might have led to excision of the LTRs and intervening DNA from the chromosome. This possibility was tested by generating a specific probe from unique sequences between *DQ* LTR5 and *DQ* LTR3 and hybridizing a Southern blot containing genomic DNA samples from several *HLA* homozygous B-cell lines. Fig. 4 shows that under stringent conditions of hybridization, single bands were observed, but only in some DNA samples. With one notable exception, those bands were found only in the samples in which the LTRs had been found (Fig. 3). The exception was DNA from cell line VAVY (*DR3*, *DQw2*; Fig. 4) from which the *DQ* LTRs could not be amplified, but a band hybridizing with the intervening fragment was found. This may indicate polymorphism in sequences at the *DQ* LTR junctions, although at least two of the PCR primers must be mismatched because neither *DQ* LTR could be amplified (Fig. 3). Alternatively, rearrangement of the sequences in this region without removal of the intervening fragment could explain these findings. In another sample, RSH (*DR18*, *DQw4*), the band was a different size from those in other samples, probably indicating restriction site polymorphism. In fact, in Southern blots with digests of the same genomic DNAs with another restriction endonuclease, the fragment in RSH DNA detected by the intervening unique fragment was not different in size from the other fragments (unpublished data).

Table 1 summarizes the data obtained so far regarding the associations of the LTRs with specific *DQ* alleles. In the few DNA samples analyzed (at least two for each *DQw* allele), the LTRs were found in *DQw2*-, *DQw4*-, and *DQw8*-bearing haplotypes but not in *DQw7*- and *DQw9*-bearing haplotypes. These results suggest that the *DQ* LTRs are associated with insertions within the *DQ* region and that they are relatively stable within the subset of haplotypes that possess them.

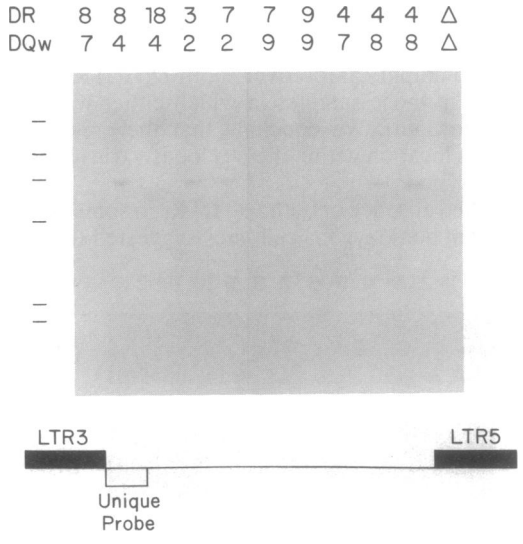

FIG. 4 Detection of fragment between *DQ* LTRs in genomic DNAs of *HLA* homozygous cell lines. Southern blots of genomic DNAs digested with *Kpn* I were hybridized with a probe of ≈300 nucleotides from the region between the LTRs. The probe was a PCR fragment generated from unique sequences adjacent to *DQ* LTR3 as described in *Materials and Methods*. The blot was washed at high stringency (65°C for 2 hr) to reduce background. Dashes at the left indicate the location of phage λ *Hin*dIII markers (23, 9.4, 6.6, 4.5, 2.3, and 2.0 kb, respectively). *DR* and *DQw* haplotypes are indicated above, and the location of the probe with respect to the *DQ* LTRs is indicated below. Cell lines in lanes were (from left to right): LUY, MADURA, RSH, VAVY, MOU, DBB, DKB, JHAF, SAVC, BOLETH, and 721.180.

Table 1. Association of LTRs with *HLA-DQ* and *-DR* alleles

Cell line*	Alleles		
	DR	*DQ*	*DQ* LTRs
9021 RSH	*w18*	*w4*	+
9023 VAVY	*3*	*w2*	−
9027 PF97387	*4*	*w7*	−
9030 JHAF	*4*	*w7*	−
9031 BOLETH	*4*	*w8*	+
9034 SAVC	*4*	*w8*	+
9038 BM16	*12*	*w7*	−
9043 BM21	*11*	*w7*	−
9047 PLH	*7*	*w2*	+
9050 MOU	*7*	*w2*	+
9052 DBB	*7*	*w9*	−
9069 MADURA	*8*	*w4*	+
9070 LUY	*8*	*w7*	−
9075 DKB	*9*	*w9*	−

*Tenth International HLA Workshop designation (22).

Haplotypes bearing *DQw2* might appear to be an exception, but sequence analysis has shown one amino acid substitution within the third exon (encoding the β_2 domain), and several nucleotide substitutions in the 3′ untranslated regions of cDNA clones indicate that the *DQw2*, *DQB1* genes associated with *DR7* are distinct alleles from those associated with *DR3* (J.S.L., unpublished observations). On the other hand, DNA from the *DR3*, *DQw2* cell line hybridized with the intervening fragment probe, suggesting that at least part of the *DQ* LTR segment might still be present.

DISCUSSION

We have shown that the *HLA* regions of some individuals contain two endogenous retroviral LTRs linked to the *DQB1* gene. Sequence analysis of the *DQ* LTRs revealed that each element was >90% homologous to the LTRs of the retrovirus HERV-K10 (Fig. 1) (14, 16). Furthermore, putative cis-acting regulatory sequences within the LTRs were largely conserved in comparison with other HERV-K retrovirus elements. Because of the remarkable polymorphism in protein products of *HLA* region genes and even variation in gene number per haplotype, we asked whether the *DQ* LTRs were ubiquitous in the *DQ* region or whether they might be peculiar to certain haplotypes. Accordingly, we found that these elements were associated with several, but not all, *DQ* alleles (Fig. 3). The uniform restriction pattern observed for a unique intervening fragment in different cell lines (Fig. 4), also when additional restriction endonucleases were used (unpublished data), suggests that transposition of the LTRs into this region was the result of original integration events that occurred before divergence of the *DQB1* sequences and provides evidence that these haplotypes might have evolved from a common ancestral haplotype.

In general, B cells express the *DR*, *DP*, and *DQ A* and *B* genes coordinately, suggesting that a common molecular mechanism regulates their expression (10). However, immature B cells, precursor cells, and many macrophages lack expression of *HLA-DQ* on their cell surfaces even though they express considerable amounts of *HLA-DR* and *HLA-DP*. Since both *DQA1* and *DQB1* promoters possess the conserved *X* and *Y* sequence elements just upstream from the initiation site for transcription, the mechanism for the disruption of coordinate regulation of the *DQ* genes with the other class II genes is not understood (10). However, other cis-acting regulatory regions also may modulate promoter activity. Some of these elements can act at significant distances from the promoter itself (23). The organization of the *DQ* LTRs with respect to the *DQB1* gene is reminiscent of the

patterns of integration for retroviruses that activate normally quiescent oncogenes to induce tumors (24, 25). A particularly salient example is the integration of mouse mammary tumor virus (MMTV) upstream of the *int-2* gene (26). When proviruses were inserted up to 40 kb upstream, they were able to activate transcription from the *int-2* promoter. Significantly, all of the proviruses had been integrated in the opposite transcriptional orientation. The HERV-K family of endogenous retroviruses can be stimulated by steroid hormones in cultured human tumor cells (19); thus, it is conceivable that activation of the *DQ* LTRs would also incite transcription of *DQB1*. Our data do not indicate whether *DQ* LTRs are transcriptionally active. Nevertheless, members of this LTR family are clearly transcribed in several cell types including B cells, although they are not always processed to stable, cytoplasmic RNAs (unpublished data). On the other hand, hormone receptor binding sequences may be involved not only in stimulating transcription of linked genes but also have been shown to mediate negative regulation (27). Thus, the endogenous retroviral LTRs upstream of *DQB1* may dissociate *DQ* expression, in some cases, from *DR* and *DP* expression.

A large body of evidence generated from family studies and population genetics indicates that recombination distance and physical distance between the various loci in the MHC do not always correspond (28). For example, recombinants between the *DP* and *DR* loci are relatively frequent, and little linkage disequilibrium is found between alleles of these loci, whereas between *DQ* and *DR* virtually no recombinants have been identified, and high linkage disequilibrium exists (29). Early analyses of polymorphisms for the two genes *DQA1* and *DQA2* in close proximity show relatively low linkage disequilibrium (15). Analysis of restriction fragment-linked polymorphisms associated with the *DQB2* and *DQA2* genes showed that the *DQ* LTRs could be found within each allele cluster (ref. 30 and Table 1). This suggests that one or more "hot spots" in recombination are situated between the *DP* and *DQ* loci within the class II region (31). A precedent for this is found in the murine class II region, *H-2*, in which a large number of crossovers has occurred within one intron of the *H-2I-E* β-chain gene (32). Another recombinational hotspot found in the murine class II region ($H\text{-}2I\text{-}A_{\beta 2}/A_{\beta 3}$) coincides with an element with high sequence similarity to LTR-IS (intervening sequence) elements (33). Similar LTRs have been shown *in vitro* to act as substrates for recombination (34).

Active transcription of genes in yeast has recently been shown to increase the frequency of recombination events between homologous sequences (35). If transcription of the LTRs does occur during germ-cell development, then a higher frequency of recombination between the *DQB1* and *DQA2* genes might be expected. Analysis of DNA from more homozygous cell lines should indicate whether the LTRs within this region might mediate increased levels of recombination between particular haplotypes. Alternatively, their absence in other haplotypes might explain the linkage disequilibrium that is observed between *DQ1* and *DQ2* genes. Understanding factors that influence recombination within this region is important in predicting suitability of transplant donors and also possibly to interpreting some associations of *HLA* alleles and autoimmune diseases. Furthermore, this model system should be useful in elucidating molecular events associated with transposition/integration of retroviral sequences in the human genome.

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