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Allele-specific gene probing supports the DQ molecule as a determinant of inherited susceptibility to Type 1 (insulin-dependent) diabetes mellitus

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Summary. Trans-racial analysis of disease associations has improved mapping of MHC-linked susceptibility to Type 1 (insulin-dependent) diabetes mellitus. In this study the contributions of the MHC class II DQA1 and DQB1 genes were investigated. Sequence-specific oligonucleotide gene probing in Type 1 diabetic and control subjects of North Indian origin supported the DQw1.18 allele of the DQB1 gene as a determinant of inherited protection against Type 1 diabetes (RR = 0.12, $p_c < 0.05$). The A3 allele of the DQA1 gene was positively associated with the disease, (RR = 3.6, $p_c < 0.05$), as was the DQw2 allele of the DQB1 gene (RR = 4.6, $p_c < 0.01$). Trans-racial comparison of these disease associations indicates that DQ alleles may directly determine an element of inherited susceptibility to Type 1 diabetes.

Key words: Trans-racial studies, North Indians, gene probing, HLA-DQ, Type 1 (insulin-dependent) diabetes.

Investigation of inherited susceptibility to Type 1 (insulin-dependent) diabetes has identified the existence of at least two MHC-associated genetic factors which predispose to the disease [1]. In white Caucasians, one factor is associated with DR3, the other with DR4. DR3 and DR4 are determined by polymorphisms of the DRB1 gene [2]. The frequencies of DR3 and DR4 in healthy white Caucasian populations are too great for these alleles themselves to explain the observed incidence of Type 1 diabetes, regardless of the penetrance of the predisposing genes [3]. It is likely that at least one of these DRB1 alleles is in linkage disequilibrium with an allele at another locus which directly predisposes to the disease. Many authors have focussed on the DQ-subregion of the MHC, particularly the DQB1 gene, as a possible determinant of Type 1 diabetes susceptibility [4–7]. Although strong associations between the disease and the DQB1 allele DQw8 (previously termed DQw3.2) have been found in Caucasians [8], it is unclear how the encoded DQβ chain might predispose to autoimmune destruction of the pancreatic Beta cell. The DQw8 allele may also be in linkage disequilibrium with an allele at another locus which directly predisposes to Type 1 diabetes. One method of distinguishing primary genetic associations from those secondary to linkage disequilibrium is to study disease associations in a variety of distinct ethnic groups [5, 9]. Although linkage disequilibrium within the DR/DQ subregion of the MHC is strong, recombination events have affected this part of the genome during evolution. These have produced different haplotypes which are preserved with varying frequencies in different races. Linkage disequilibrium between MHC alleles therefore shows racial variation. Assuming that Type 1 diabetes is the same disease in all populations studied, disease susceptibility alleles will show consistent associations with the disease in each group, in spite of variation in linkage disequilibrium. On the other hand, disease associations which are secondary to linkage disequilibrium are unlikely to be consistent in all ethnic groups. The DQA1 genes and DQB1 genes are closely linked to the DRB1 gene [10] and are, therefore, potential sites of Type 1 diabetes susceptibility.

Previous investigation of MHC associations with Type 1 diabetes in North Indian Asians has shown a marked positive association with DR3. In contrast to white Caucasian populations, the significant association between diabetes and DR4 was weaker than that with DR3, and DR2 was not significantly negatively associated with disease [11]. DQ restriction fragment length polymorphism (RFLP) analysis suggested the DQ subregion as a determinant of inherited susceptibility to Type 1 diabetes [11]. We have investigated this further by examining the frequencies of DQA1 and DQB1 alleles identified by sequence-specific oligonucleotide (SSO) gene probing in these individuals.

Subjects and methods

Type 1 diabetic and racially-matched control subjects of North Indian (Punjabi) origin were recruited for study. All subjects were resident in the United Kingdom, most of them since birth. Most subjects

Table 1. Priming sequences

Primer	Sequence
26A	GGTGTAAACTTGTACCAG
27A	GGTAGCAGCGGTAGAGTTG
27B	GTAGAGTTGGAGCGTTTA
GLPDQ1	GATTTCGTGTACCAGTTTAAGGGC
DQXB2	CCACCTCGTAGTTGTGTCTGCA
GLPDR1	TTCTTCAATGGGACGGAGCG

Primer sequences were obtained from refs. 4 and 19

were of Sikh denomination. Diabetic subjects were diagnosed at less than 30 years of age, ketosis-prone and continuously dependent on insulin from the time of diagnosis. Control subjects had neither personal nor family history of diabetes. All subjects had been serologically DR-typed and studied by HLA class II RFLP analysis in a previous study [11].

DNA amplification

Amplification of the second exon of the DQA1 and DQB1 genes was achieved by the polymerase chain reaction (PCR), [12]. The intensity and specificity of the amplified signal were improved by amplifying each gene in two stages. The primary stage of amplification used 2µg of genomic DNA in a total volume of 100 µl containing Tris hydrochloride (10 mmol l^{-1}), potassium chloride (50 mmol l^{-1}), magnesium chloride (1.5 mmol l^{-1}), gelatin (0.01%), deoxyadenosine triphosphate (0.2 mmol l^{-1}), deoxythymidine triphosphate (0.2 mmol l^{-1}), deoxyguanidine triphosphate (0.2 mmol l^{-1}), deoxycytosine triphosphate (0.2 mmol l^{-1}) and the priming DNA sequences, each at a concentration of 15 µg ml^{-1}. Taq Polymerase (4 units, Perkin Elmer Cetus, Beaconsfield, UK) was added to the reaction mixture which was incubated in a DNA thermocycler (Perkin Elmer Cetus). The temperature used were 92°C for denaturation, 55°C for primer annealing and 72°C for DNA extension. The times of each stage of the first cycle were: denaturation 5 min; annealing 2 min; extension 3 min. In the subsequent cycles the times were denaturation 1 min; annealing 2 min; extension 2 min. The DQA1 gene was amplified using the priming DNA sequences 26A and 27A (Table 1) for 25 cycles. Two µl of the mix were reamplified for the DQA1 gene using the priming sequences 26A and 27B (Table 1) for a further 25 cycles. The DQB1 gene was amplified using the primers GLPDQ1 and DQXB2 (Table 1) for 35 cycles. Two µl of the amplified mix were reamplified for the DQB1 gene using the primers DQXB2 and GLPDR1 (Table 1) for a further 35 cycles.

Sequence-specific oligonucleotide (SSO) dot blot analysis

Ten µl of the amplified mixture were added to 90 µl Tris/EDTA (10/0.1 pH 7.5), 6 µl EDTA (0.5 mol l^{-1} pH 8) and 8 µl NaOH (6 mol l^{-1}). The mix was incubated on ice for 10 min, neutralised with 116 µl ammonium acetate (2 mol l^{-1}) and dotted onto a nylon filter (Hybond, Amersham International, Amersham, UK) using a Hybridot apparatus (BRL, Paisley, UK). DNA was fixed to the filter using UV light.

Filters were prehybridised in 5.2 ml prehybridisation solution containing 6 × standard sodium citrate (SSC: 1 × SSC = 0.15 mol l^{-1} NaCl, 0.015 mol l^{-1} Na citrate), 2 mg denatured salmon sperm, 5 × Denhardt's solution and 0.5% sodium dodecyl sulphate (SDS), for 15 min at 34°C, with shaking.

Hybridisation of the filter was carried out in 2.5 ml hybridisation fluid containing 6 × SSC, 0.1% SDS and 0.2 µg of the radiolabelled SSO. SSOs were end-labelled using T4 kinase (Pharmacia, Milton Keynes, UK) and γ - ^{32}P ATP (Amersham International). The filter was shaken at 34°C overnight.

Filters were washed twice non-stringently in 6 × SSC at 20°C for 5 min, and autoradiographed to assess the degree of amplification. The filter was then washed twice stringently at the DNA duplex dissociation temperature (Td) [13] for 15 min in 6 × SSC, 0.1% SDS. The filter was autoradiographed for 2–4 h at −70°C. Adequate washing of the filter was ensured by checking signals from DNA amplified from a range of control cell lines dotted onto the same filter.

Allele detection

Twenty-three SSOs were synthesized. Eight were complementary to DQA1 alleles and 15 were complementary to DQB1 alleles. The nucleotide sequences of the SSOs are given in 5′ to 3′ order in Table 2. Some probes were allele-specific but others recognised two or more alleles. Conversely, some alleles were recognised by two or more probes. Table 3 shows how the SSOs recognised the various DQA1 and DQB1 alleles. The probes were designed so that an individual

Table 2. Sequence-specific oligonucleotides

Name	Sequence
A1.1	5′-ATGAGGAGTTCTACGTG-3′
A1.2a	5′-AGATGAGCAGTTCTACG-3′
A1.2b	5′-CCTGGAGAGGAAGGAGA-3′
A1.3	5′-CCTGGAGAAGAAGGAGA-3′
A2	5′-TCTAAGTCTGTGGAACA-3′
A3	5′-TTCCGCAGATTTAGAAGATTT-3′
A4.1	5′-GTTTGCCTGTTCTCAGA-3′
A4.2	5′-TGGAGACGAGCAGTTCT-3′
B2	5′-GCTGGGGCTGCCTGCCG-3′
B4	5′-TGGAGGAGGACCGGGCG-3′
B5a	5′-GGCGGCCTGTTGCCGAG-3′
B5b	5′-CGTGCGGGGTGTGACCA-3′
B6	5′-GGCGGCCTGATGCCGAG-3′
B7a	5′-CGTGCGTTATGTGACCA-3′
B7b	5′-GGCCGCCTGACGCCGAG-3′
B8	5′-GGCCGCCTGCCGCCGAG-3′
B9	5′-GCGTGCGTCTTGTGACC-3′
B1.9a	5′-AGGGGCGGCCTGACGCC-3′
B1.9b	5′-AGGAGTACGTGCGCTTC-3′
B1.12	5′-GAGAGGAGGACGTGCGC-3′
B1.18	5′-CGTCTTGTAACCAGACA-3′
B1.19b	5′-CTTGTAACCAGATACATC-3′
B1.AZH	5′-GCGGCCTAGCGCCGAGT-3′

Table 3. Allele identification

Allele		Probes for which allele is positive
A1.1	(DQA1*0101)	A1.1
A1.2	(DQA1*0102)	A1.2a, A1.2b
A1.3	(DQA1*0103)	A1.2a, A1.3
A2	(DQA1*0201)	A2, A1.2b
A3	(DQA1*0301)	A3, A1.2b
A4.1	(DQA1*0501)	A1.2a, A4.1
DQw2	(DQB1*0201)	B2
DQw5	(DQB1*0501)	B5a, B5b, B1.9b
DQw6	(DQB1*0602)	B6, B9
DQw7	(DQB1*0301)	B7a, B7b
DQw8	(DQB1*0302)	B8, B9
DQw9	(DQB1*0303)	B7b, B9
DQw1.AZH	(DQB1*0502)	B1.AZH, B5b, B1.9b
DQw1.9	(DQB1*0503)	B1.9a, B1.9b, B5b
DQw1.12	(DQB1*0601)	B1.12, B7a, B1.9a
DQw1.18	(DQB1*0603)	B6, B1.18
DQw1.19a	(DQB1*0604)	B5a, B1.18
DQw1.19b	(" ")	B5a, B1.19

Allele names and sequences are taken from Todd et al. [4] and Gyllensten and Erlich [15]. The recently designed nomenclature of the WHO nomenclature committee [2] is shown in brackets after each allele

heterozygous for any pair of DQA1 or DQB1 alleles could be typed unambiguously.

Statistical analysis

Comparison of allele frequencies between the diabetic and control groups was made by the X^2 test or Fisher's exact test as appropriate. p-values were corrected for the number of comparisons made, and considered significant if less than 0.05. Relative risks were calculated by the method of Woolf with the Haldane modification for small numbers [14].

Rank order analysis by the method of Thomson [3] was used to rank the DQA1 and DQB1 alleles after removal of the strongly predisposing alleles at each locus. Alleles were ranked in order of frequency in the disease and control groups. The rank order in the control group was subtracted from the rank order in the disease group to produce the net rank. Alleles which are positively associated with disease produce a negative net rank, and those negatively associated with disease produce a positive net rank.

Results

The SSOs used identified 6 distinct DQA1 and 12 DQB1 alleles (Table 3). The nomenclature of Gyllensten and Erlich [15] has been used for the DQA1 alleles, while the nomenclature of Todd et al. [4] has been used for the DQB1 alleles. The new nomenclature of the WHO nomenclature committee [2] is shown in brackets after each allele. The common DQ alleles were strongly associated with certain serological DR types (Table 4). A1.1 was associated with both DR1 and DRw10. A4.1 was associated with both DR3 and DR5. DQw2 was associated with DR3 and DR7. DQw5 was associated with both DR1 and DRw10.

The frequencies of the DQA1 and DQB1 alleles in the diabetic and control subjects are shown in Tables 5 and 6. The A1.3 allele was significantly decreased in the diabetic group compared with the controls, RR = 0.11 (0.032–0.4), $p_c < 0.003$. The A3 allele was significantly increased amongst the diabetic subjects, RR = 3.6 (1.5–8.8), $p_c < 0.05$. The A4.1 allele was significantly increased amongst diabetic subjects, RR = 6.3 (2.3–17.5), $p_c < 0.005$.

The DQw2 allele was significantly increased in the diabetic subjects, RR = 4.6 (2.0–10.4), $p_c < 0.01$. DQw8 was also significantly associated with diabetes, RR = 4.8 (2.0–11.7), $p_c < 0.01$. DQw1.18 was significantly reduced among diabetic subjects, RR = 0.12 (0.026–0.52), $p_c < 0.05$.

Rank order analysis of the DQA1 allele frequencies after removal of the predisposing alleles A3 and A4.1 showed the A1.3 allele to have the highest rank (+3). Rank order analysis of the DQB1 alleles after removal of the predisposing alleles DQw2 and DQw8 showed the DQw1.18 allele to have the highest rank (+4).

Discussion

Indian Asians have a low prevalence of Type 1 diabetes compared with white Caucasians [16]. It is unclear whether this is due to genetic and/or environmental factors. This study has determined the frequencies of the DQA1 and DQB1 alleles in North Indian subjects to allow more precise definition of MHC-associated susceptibility to Type 1 diabetes.

Table 4. Correlations between DQA1 and DQB1 alleles and serological DR types in North Indian control subjects

Allele	DR type	+ +	+ −	− +	− −	r
A1.1	DR1	6	0	24	60	0.4
A1.1	DRw10	12	0	18	60	0.55
A1.2	DR2	9	5	16	59	0.35
A1.3	DRw6	19	12	12	47	0.4
A2	DR7	26	1	1	62	0.95
A3	DR4	8	2	0	80	0.89
A4.1	DR3	29	24	1	36	0.54
A4.1	DR5	16	37	0	37	0.4
DQw2	DR3	29	24	1	36	0.63
DQw2	DR7	16	10	14	53	0.4
DQw5	DR1	8	13	0	71	0.56
DQw5	DRw10	14	5	0	73	0.83
DQw7	DR5	17	1	4	70	0.84
DQw8	DR4	7	2	1	82	0.8
DQw1.12	DR2	20	5	1	66	0.83
DQw1.18	DRw6	21	12	0	59	0.7

r is the correlation coefficient. All the above associations are statistically significant ($p < 0.001$)

Table 5. DQA1 allele frequencies in North Indian Type 1 (insulin-dependent) diabetic and control subjects

Allele	Diabetic subjects (n = 44)	Control subjects (n = 90)	RR	p_c
A1.1	7 (15.9)	30 (33.3)		NS
A1.2	5 (11.4)	14 (15.5)		NS
A1.3	2 (4.5)	31 (34.4)	0.11 (0.032–0.4)	< 0.003
A2	3 (6.8)	27 (30)		NS
A3	14 (31.8)	10 (11.1)	3.6 (1.5– 8.8)	< 0.05
A4.1	40 (90.9)	53 (58.8)	6.3 (2.3–17.5)	< 0.005

Figures in parentheses are percentages. RR is the relative risk with 95% confidence limits. p_c is the p-value corrected for the number of comparisons made between the diabetic and control groups

Table 6. DQB1 allele frequencies in North Indian Type 1 (insulin-dependent) diabetic and control subjects

Allele	Diabetic subjects n = 43	Control subjects n = 92	RR	p_c
DQw1.AZH	0 (0)	2 (2.2)		NS
DQw1.9	4 (9.3)	11 (12)		NS
DQw1.12	3 (7)	19 (20.6)		NS
DQw1.18	1 (2.3)	21 (22.8)	0.12 (0.026–0.52)	< 0.05
DQw1.19a	3 (7)	4 (4.3)		NS
DQw1.19b	0 (0)	2 (2.2)		NS
DQw2	35 (81.4)	44 (47.8)	4.6 (2.0–10.4)	< 0.01
DQw5	2 (4.7)	21 (22.8)		NS
DQw6	0 (0)	4 (4.3)		NS
DQw7	5 (11.6)	22 (23.9)		NS
DQw8	15 (34.9)	9 (9.8)	4.8 (2.0–11.7)	< 0.01
DQw9	1 (2.3)	13 (14.1)		NS

Figures in parentheses are percentages. RR is the relative risk with 95% confidence limits. p_c is the p-value corrected for the number of comparisons made between the diabetic and control groups

Mapping of MHC-associated susceptibility to disease has relied, until recently, on serological and cellular methods of MHC typing. These techniques are limited by the available antisera and T-cell clones. DNA sequence analysis allows exact definition of MHC class II gene polymorphism. The second exon of a class II gene encodes the

first domain of the class II α or β chain. The first domains interact directly with T-cell receptors [17], and form an antigen-binding cleft [18]. The second exon is, therefore, crucial to class II function, and polymorphism in this region affects T-cell activation [19]. SSO analysis of amplified DNA sequences allows rapid definition of polymorphism in the second exon. Polymorphism of some alleles is defined by a single allele-specific SSO. Other alleles are recognised by their reaction with a unique combination of SSOs. The SSOs presented in Table 2 allow unambiguous differentiation between the possible combinations of DQA1 and DQB1 alleles.

SSO analysis demonstrated strong associations between certain DQ alleles and Type 1 diabetes in this population. The DQA1 alleles A3 and A4.1 were positively associated with Type 1 diabetes, and A1.3 was negatively associated with the disease. The DQB1 alleles DQw2 and DQw8 were positively associated, and DQw1.18 was negatively associated with the disease. Rank order analysis demonstrated that the observed negative associations persisted after removal of the positively associated markers. This implies that the negative associations were real, and not secondary to an absence of the positively associated alleles.

A3 was strongly associated with DR4. It was also positively associated with Type 1 diabetes in Negroid [20] and Japanese populations [21]. It also occurred on virtually all DR4-positive haplotypes in white Caucasians [8]. This allele, therefore, has been positively associated with disease in all races studied. Although this satisfies one criterion for a disease susceptibility allele [9], A3 occurs on virtually all DR4-positive haplotypes. It does not, therefore, distinguish between the DR4-DQw7 and DR4-DQw8 haplotypes which show different associations with Type 1 diabetes in white Caucasians [4]. It is unlikely, therefore, that A3 determines DR4-associated disease susceptibility directly, unless an additional factor on non-disease-predisposing DR4 haplotypes modifies its effect.

DR4 was associated with the DQB1 allele DQw8, not with DQw7, in contrast to white Caucasians. The frequency of DQw8 in this North Indian population was similar to that in white Caucasians [22]. The low frequency of the DR4-DQw7 haplotype may explain the low frequency of DR4 in this group [11]. As in Caucasians, DQw8 was positively associated with Type 1 diabetes. DQw8 has been proposed as a primary determinant of DR4-associated disease susceptibility [8]. DQw8 was not significantly associated with Type 1 diabetes in Japanese subjects [7, 21, 23], however, suggesting that its association in other races is due to linkage disequilibrium with a distinct DR4-associated susceptibility allele. It appears, therefore, that factors distinct from the coding regions of the DQA1 and DQB1 genes are implicated in DR4-associated predisposition to Type 1 diabetes.

The DR3-associated DQA1 allele A4.1 was also positively associated with Type 1 diabetes in a white Caucasian population [24], but not in a Japanese population [21] in whom DR3 is rare [7]. The association between A4.1 and the disease, therefore, reflects linkage disequilibrium between A4.1 and DR3.

The DR3-associated DQB1 allele DQw2 was common among the diabetic subjects, consistent with a DR3-associated factor being the major MHC-associated disease susceptibility determinant in this population [11]. DQw2 also was positively associated with Type 1 diabetes in a white Caucasian [24], and a Negroid population (unpublished data). In Japanese subjects DQw2 is too rare to assess its association with disease [7]. The positive association between Type 1 diabetes and DQw2 may be due to linkage disequilibrium with DR3, or to a primary effect of the DQw2-encoded β chain. DQw2, however, also occurs on DR7 haplotypes which were not associated with Type 1 diabetes in this population [11]. If DQw2 predisposes to disease directly, the DR7 haplotype must possess an allele at another locus which alters the effect of DQw2 on susceptibility to diabetes.

A1.3 was associated with DRw6. Although this allele was also negatively associated with Type 1 diabetes in a white Caucasian population [24], it was neutrally associated with Type 1 diabetes in Negroid subjects (unpublished data). The negative association between A1.3 and disease is likely, therefore, to be secondary to linkage disequilibrium with an allele which directly protects against Type 1 diabetes.

The negative association between Type 1 diabetes and DQw1.18 suggested a protective effect of this allele against the disease. DQw1.18 was rare in Negroid subjects (unpublished data) in whom it was not significantly negatively associated with Type 1 diabetes. In a white Caucasian population, DQw1.18 occurred significantly less frequently among Type 1 diabetic subjects [24]. DQw1.18 occurs on the DRw6 (Dw18) haplotype and marks a subset of DRw6 haplotypes which confer protection against Type 1 diabetes. DRw6 is not negatively associated with Type 1 diabetes in white Caucasian populations [1], possibly due to a lower frequency of DRw6 (Dw18), and a higher frequency of other DRw6 haplotypes such as DRw6 (Dw9) and DRw6 (Dw19).

DQw1.18 is very similar to the DQw6 allele, differing in the second exon by only a single codon at position 30 [4]. DQw6 was absent from the diabetic group in this study, but was rare in the control group, making its effect on disease susceptibility difficult to assess. DQw6 was more common in other populations, and was significantly negatively associated with Type 1 diabetes in Negroid subjects (unpublished data), white Caucasian subjects [22, 24] and

Table 7. DQ allelic associations with Type 1 (insulin-dependent) diabetes in various ethnic groups

Allele	Caucasian	North Indian	Negroid	Japanese
A1.3	–	–	N	?
A3	+	+	+	+
A4.1	+	+	N	N
DQw1.18	–	–	r	?
DQw2	+	+	+	r
DQw6	–	r	–	–
DQw8	+	+	+	N

+ represents a positive association between Type 1 diabetes and the allele. – represents a negative association. N represents a neutral association. r indicates that an allele is too rare to assess its association with the disease. Associations are cited from studies quoted in the text

Japanese subjects [23]. The amino acid encoded by codon 30 lies outside the putative antigen binding cleft of a model class II molecule [18] and may cause little difference between the functions of the β chains encoded by the DQw6 and DQw1.18 alleles. If the DQw6-encoded β chain directly protects against Type 1 diabetes by altering T-cell recruitment and T-cell-mediated islet cell destruction, it is likely that the DQw1.18-encoded β chain has a similar effect and also protects against the disease [9].

DQw6 and DQw1.18 encode Asp57-positive DQβ chains. It has been suggested that Asp57 protects directly against Type 1 diabetes [4]. The Asp57-positive DQB1 allele, DQw4, however, is positively associated with Type 1 diabetes in the Japanese race [7, 21, 23]. The apparent protective effect of Asp57 in Caucasian subjects may result from its occurrence on the protective DQw6-encoded DQβ chain, rather than from a direct protective action.

These data support the hypothesis that MHC-associated susceptibility to Type 1 diabetes is partly class II-encoded. We propose that protection against Type 1 diabetes may be conferred by the DQw1.18 allele of the DQB1 gene. DR4-associated susceptibility to Type 1 diabetes may be partly determined by the A3 allele of the DQA1 gene. Another allele on DR4 haplotypes is also implicated in disease susceptibility. Similarly, the DQB1 allele DQw2 may determine DR3-associated susceptibility to the disease if DR7 haplotypes possess an additional disease-modifying allele. Further characterisation of disease-associated MHC haplotypes in a variety of races will allow more precise identification of the disease susceptibility loci. Increased knowledge of the immune mechanisms involved in islet β-cell destruction will further our understanding of how MHC alleles predispose to Type 1 diabetes.

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