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Immunoglobulin gene organisation and expression in haemopoietic stem cell leukaemia

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We have analysed the organisation and expression of μ genes in the granulocytic phase and in the lymphoid and myeloid blast crises of Philadelphia chromosome (Ph^1) chronic granulocytic leukaemia (CGL), a leukaemia which is known to arise in mutipotential stem cells. We find that μ chain gene rearrangement occurs exclusively in lymphoid blast crisis leading in some, but not all, cases to the synthesis of small amounts of cytoplasmic μ chains characteristic of early pre-B lymphocytes. In Southern blots, only one or two rearranged μ chain genes are seen, suggesting that a clonal event leading to blast crisis can occur in a committed B cell precursor rather than in the multipotential stem cell precursor, in which the Ph^1 chromosome originated. The pattern of μ gene rearrangement observed in Ph^1 CGL blast crisis is compared with that in normal B cells, other B lineage malignancies, myeloid leukaemias and T cell leukaemias.

Key words: B-lymphocyte differentiation/diagnosis of leukaemia/gene organisation/immunoglobulin genes/Ph^1 CGL

Introduction

Chronic granulocytic leukaemia (CGL) with the distinctive marker, the Philadelphia chromosome (Ph^1;22q$^-$) is of great interest both because its multi-step pathogenesis allows separate stages of the malignancy to be analysed within the same clone and because of the insights it provides into haemopoiesis (reviewed in Greaves, 1982). The initial events of this leukaemia occur in multipotential stem cells and usually result in an overt selective advantage for the granulocytes whose population levels are raised. Karyotype and glucose-6-phosphate dehydrogenase isoenzyme studies (Fialkow *et al.*, 1977) at the chronic stage of CGL suggest, however, that all the members of the myeloid lineage, and possibly lymphoid cells, are part of a dominant clone derived from the transformed (Ph^1 positive) stem cell (Fialkow, 1982; Greaves, 1982). The chronic phase inevitably progresses to an acute phase, or blast crisis, involving immature cell types having the phenotype of myeloblasts, immature lymphoblasts or, more rarely, erythroblasts, megakaryoblasts or mixtures of these (Janossy *et al.*, 1978; Greaves, 1982; Griffin *et al.*, 1983).

The evidence for immature lymphoid phenotype in blast crisis of CGL is based on cell surface and enzyme markers (Greaves, 1982). Also, about a third of these lymphoid blast crises produce low amounts of cytoplasmic μ chains (Greaves *et al.*, 1979a) in common with normal 'pre-B' cells (Cooper *et al.*, 1980). The occurrence of cells with an immature lymphoid phenotype in the blast crisis of CGL raises a number of questions. Are the cells involved really members of the lymphoid lineage? Does this type of blast crisis arise in the transformed pluripotent stem cell population or in progeny committed to the lymphocyte lineage?

Functional μ chain genes are the result of at least two translocation events in the DNA of the B cell in which the gene coding for the variable region (V_H) is assembled from a V_H, a D and a J_H segment within an array of six J_H segments located some 8 kb 5′ to the Cμ gene (Takahashi *et al.*, 1980; Rabbitts *et al.*, 1981; Matthyssens and Rabbitts, 1980; Ravetch *et al.*, 1981; Siebenlist *et al.*, 1981). A similar translocation event, but not involving D segments, leads to a functional light chain gene (Hieter *et al.*, 1980, 1981). Immunoglobulin gene rearrangement is not known to occur in any other cell type, with the possible exception of T lymphocytes (Korsmeyer *et al.*, 1981a, 1983).

The early stages of B cell differentiation are difficult to analyse because the Ig gene products are the only known markers exclusive to B cells, and homogeneous populations of the earliest recognisable progenitors are difficult to obtain. However, both Abelson virus (A-MuLV)-induced leukaemias in mice and the common variant of acute lymphoblastic leukaemia (ALL) in humans probably originate in pre-B cells (Baltimore *et al.*, 1979; Vogler *et al.*, 1978; Greaves *et al.*, 1981a; Korsmeyer *et al.*, 1983). Analysis of these cells has confirmed that μ genes rearrange at a very early stage of B cell differentiation and before the rearrangement of light chain genes (Alt *et al.*, 1981; Korsmeyer *et al.*, 1983). The results also suggest a hierarchy of light chain gene rearrangement in which λ light chain genes remain in the germ line configuration unless the κ chain gene rearrangement is unproductive (Korsmeyer *et al.*, 1981b, 1983). Later steps in B cell differentiation include the 'heavy chain switch' in which the heavy chain class may change from μ to γ, α or ϵ, an event which usually leads to the deletion of the constant region of the μ chain gene (reviewed in Marcu, 1982), and somatic mutation in the V regions of the expressed genes (reviewed in Baltimore, 1981).

The occurrence of immunoglobulin gene rearrangement is unambiguous evidence of the lymphocyte phenotype and the stage of rearrangement can help to define the time in lymphocyte differentiation at which a particular B cell clone acquired selective growth advantage or enhanced malignancy. We have used specific gene probes for the J_H and Cμ regions to analyse the state of the μ genes in the lymphoid and myeloid blast crisis and in the chronic phase of Ph^1 CGL. The Ig gene rearrangements are exclusive to the lymphoid clones and appear to be monoclonal suggesting that the clonal expansion leading directly to lymphoid blast crisis occurs after the initial events in Ig gene rearrangement in a B cell progenitor.

Results and Discussion

Cells from nine individuals with Ph^1 CGL in blast crisis were analysed for the rearrangement and expression of μ genes. Four expressed the gene but all nine were discovered to have at least one, and at most two, rearranged genes (Table I), as judged by restriction endonuclease digestion of the

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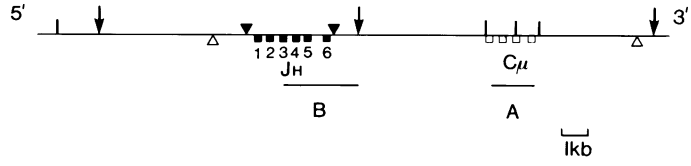

Fig. 1. Restriction enzyme map of human μ gene. The restriction map is based on previously published sites and sequence data (Takahashi *et al.*, 1980; Rabbitts *et al.*, 1981; Ravetch *et al.*, 1981) except for the extreme 5' *Eco*RI and *Hind*III sites. The position of these sites has been determined from fragment sizes found in this work in digests of DNA from myeloid cells and do not agree with the previously published map (Ravetch *et al.*, 1981) which places both sites 4 kb closer to the J_H region. The other DNA fragment sizes have been confirmed in this work. **Probe A** is a 1.6 kb long μ cDNA clone isolated from the lymphoblastoid cell line RPMI 1788 and contains only constant region sequences and 3'-untranslated sequences. **Probe B** was isolated from C76R51 a subclone of λC76 (Rabbitts *et al.*, 1981) (a kind gift of T.H. Rabbitts) and contains sequences from the J_H3 sequence to the *Hind*III site in the J_H-Cμ intron. △ *Bam*HI; ⬇ *Hind*III; ▮ *Eco*RI; ▼ *Bgl*II.

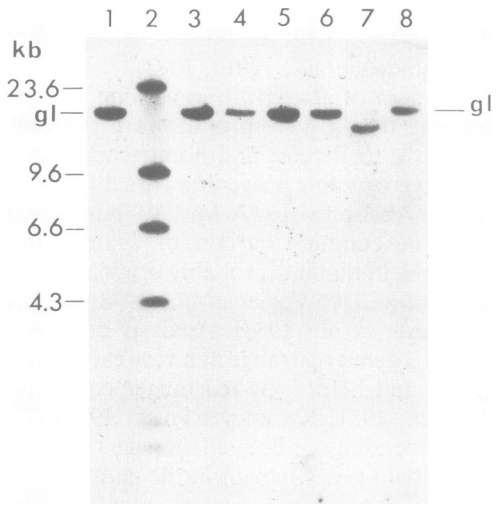

Fig. 2. Southern transfer of *Bam*HI digest of AML and CGL DNA. 10 μg of DNA from each cell line was digested with *Bam*HI, electrophoresed in 0.7% agarose, transferred to nitrocellulose membrane and hybridised with the μ cDNA probe as described above in Materials and methods; DNA from (1) a myeloid CGL blast crisis, (2) *Hind*III digest of λ DNA and labelled with [^{32}P]dCTP using T4 polymerase, (3) chronic phase of CGL, (4) a T-ALL, (5) AML, (6) HL60 (AML), (7) lymphoid blast crisis of a CGL (R.B.), (8) myeloid chronic phase of same patient (R.B.) as in (7). gl indicates the position of the germ line band.

DNA and Southern blotting, using probes A or B (Figure 1). In contrast, all of the 11 myeloid blast crisis DNA samples retained the μ gene in the germ line configuration (Table I; Figure 2, lane 1). In two cases (R.B. and D.P.), cells from the same patient in the chronic granulocytic phase of the disease and in the lymphoid blast crisis were compared, as shown in the example (R.B.) in Figure 2 (lanes 7 and 8). As with the results from cells taken from different individuals, only those in the lymphoid blast crisis display μ gene rearrangements and confirm that the observed rearrangement was not due to a polymorphism in the DNA sequence. The combined data also indicate that polymorphism in the 16.5-kb *Bam*HI μ gene fragment is rare, since we observed a single fragment of this size in 18 different myeloid leukaemias and in a sperm DNA sample (examples shown in lanes 1, 3–6 and 8, Figure 2). These results provide convincing confirmation of the assignment of some blast crises to the lymphoid lineage.

Table I. μ gene rearrangement in human leukaemic cells

Cell type	μ expression[c]	Number of patients or cell lines	μ gene state[a]
1. Chronic myeloid leukaemia			
(a) Chronic phase	–	3 (M.K., R.B., D.P.)	1°
(b) Acute phase/blast crisis:			
(i) 'Lymphoid'[b]	+	1 (T.H.)	1+1°
	+	3[d] (D.P., C.P., D.F.), NALM 1	2+
	–	1 (W.D.)	2+
	–	3[d] (T.R., R.B., L.P.)	1+
(ii) 'Myeloid'[e]	–	10, K562	1°
2. Common acute lymphoblastic leukaemia[b]	+	NALM 6, Ru-3	2+
	–	Reh,Mapp, NALM 16[h], KM3	2+
3. Acute myeloblastic leukaemia	–	3, HL60	1°
4. T cell leukaemias			
(a) T-ALL	–	6, GH1, CCRF-CEM, MOLT3	1°
	–	JM, CCRF HSB2, T-ALL-1, HPBALL	1+1°
(b) T-CLL	–	1	1°
5. B-lymphoblastoid cell lines	+	RPMI 1788 (μ,λ)	2+
	n.d.	Bristol 7 (γ,κ)	1+[f]
6. Myeloma	–	266BL (ε,λ)	0[g]

[a]Determined by *Bam*HI digests with probe A and by one or more of *Bam*HI + *Hind*III, *Hind*III, *Eco*RI and *Bgl*II digests, with probe B. In cases where a band was apparently germ line in one enzyme combination but rearranged with another, it has been described as rearranged. 1+ denotes one rearranged band, 2+ two rearranged bands and 1° a germ line band.

[b]Lymphoid phenotype includes expression of the common acute lymphoblastic leukaemia antigen (gp100) and terminal deoxynucleotidyl transferase (see Greaves *et al.*, 1982).

[c]Synthesised μ chains as revealed by biosynthetic labelling or staining cell cytoplasm by affinity purified antibody against human μ chains (Greaves *et al.*, 1979).

[d]One sample had 15–20% contaminant cells. A band of corresponding intensity at the germ line position has been discounted.

[e]'Myeloid' phenotype reflects lack of expression of lymphoid markers (see above) plus usual but not invariable expression of antigens detected by anti-myeloid monoclonal antibodies and myeloid cytochemistry (sudan black or peroxidase staining).

[f]Two rearranged bands are seen when probe B(J_H) is used.

[g]One rearranged band is seen when probe B(J_H) is used.

[h]In this case, NALM 16, our result differs from that reported by Korsmeyer *et al.* (1983) who observe rearrangement only with *Eco*RI and a J_H probe and then only of one allele.

A large number of rearrangements are possible in a normal B cell population and discrete rearranged bands can only be detected in a population of cells containing one or a few clones. We detect at the most two μ hybridising bands in the lymphoid blast crisis. This result implies that blast crisis is the

consequence of clonal expansion of a B cell progenitor that had already commenced μ gene rearrangement. It is unlikely that μ gene rearrangement would have been restricted if clonal expansion had occurred before μ gene rearrangement had begun, e.g., in the Ph¹-positive stem cell population itself. The essentially monoclonal pattern of μ gene rearrangement also implies that rearrangement does not occur during clonal expansion in the blast crisis. The observed pattern of immunoglobulin gene rearrangement probably reflects the state of differentiation of the B cell at the time of the event which gave rise to blast crisis.

A striking feature of the pattern of μ gene rearrangement in the lymphoid blast crisis is the occurrence of three cases (T.R., R.B. and L.P.) where one μ gene allele has been deleted. The deletion includes the J_H region and its 3′-flanking sequences as well as the Cμ region, since neither the J_H nor the Cμ probes (probes B and A in Figure 1) detect a second allele (an example of a result with probe A is shown in Figure 2, lane 7 and one with probe B, Figure 3, lane 9). The deletion is unlikely to be the result of a normal heavy chain switch in which part of the J_H region and its 3′-flanking sequence would have been retained. The rearranged J_H region which is now part of a γ gene is easily identified as one of the two bands in DNA from the lymphoblastic cell line Bristol 7 (lane 7, Figure 3), in this DNA only one allele is seen with the Cμ probe (lane 6, Figure 4). We cannot formally exclude that the second allele is heterogeneously rearranged but we believe this to be very unlikely. Korsmeyer *et al.* (1983) also report deletion of one heavy chain allele in ALL (five out of 25 cases analysed) which in some cases included J_H. In this study, all six cell lines of Ph¹-negative common ALL had both μ alleles rearranged. The deletions we detect have all occurred in blast crises in which cytoplasmic μ has not been detected. This suggests that the remaining allele has undergone an incomplete or unproductive rearrangement. The deletion could be an example of the chromosomal abnormalities that Ph¹ CGL patients commonly acquire before or during blast crisis, although the gross translocations or abnormalities that could be detected in the course of karyotyping rarely involve chromosome 14 (Whang-Peng and Knutsen, 1982). Furthermore, none of the patients reported here with μ gene deletions had abnormalities or loss of either chromosome 14. The two other lymphoid blast crises (D.F. and W.D.) shown in Figure 3 (lanes 4 and 8) have two rearranged alleles which in this case co-migrate. Two bands are resolved when these blast crises are analysed with other restriction enzymes.

In cases where no germ line allele is observed we usually observe a faint band at the germ line position. We believe this band is due to the presence of contaminant cells in the sample (see for example, Figure 2, lane 7; Figure 3, lanes 4, 8 and 9). We also frequently observe other faint bands when using a J_H region probe. We do not know if these are due to minor populations of cells or if they represent regions sharing some sequence homology with the J_H region probe. They are not usually observed when the Cμ probe is used (see for example, Figure 2). The faint bands common to all tracks (Figures 3 and 4) are probably trace plasmid contaminants present in the DNA being analysed and in the probes.

Only one of the blast crises retains an apparently germ line μ gene and none of the cell lines we have analysed, which were derived from either pre-B like common cALLs (Ph¹ negative) or from myelomas or normal B cells, retain a germ line allele

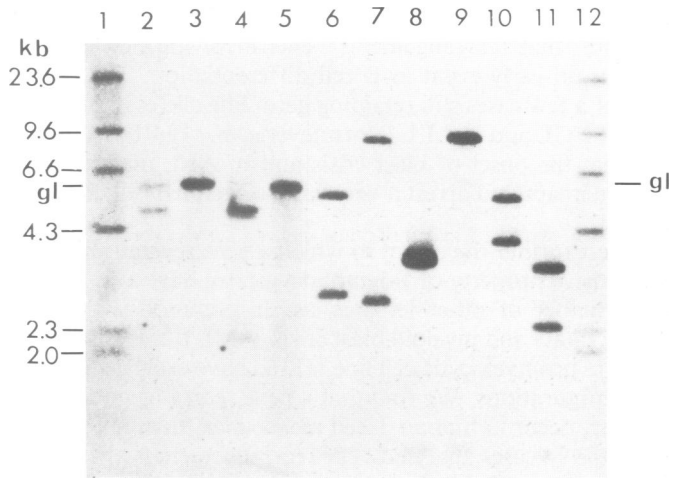

Fig. 3. Southern transfer of DNA analysed by *Bam*HI and *Hind*III double digests. About 10 μg of DNA from each sample was analysed as described in Figure 2 except that the DNA was digested by both *Bam*HI and *Hind*III and the transfer was hybridised with a J_H region probe (probe B, Figure 1). **Lane** (1) *Hind*III-digested λ size markers as in Figure 2 above. DNA from (2) HPBALL a T-ALL line, (3) HL60, (4) a lymphoid blast crisis of CGL (D.F.), (5) a myeloid blast crisis of CGL, (6) RPMI 1788, (7) Bristol 7, (8) (W.D.) and (9) (L.P.) lymphoid blast crisis of CGL, (10) REH a cALL line, (11) NALM 6 a cALL line, (12) *Hind*III digest of λ. gl indicates the position of the germ line band.

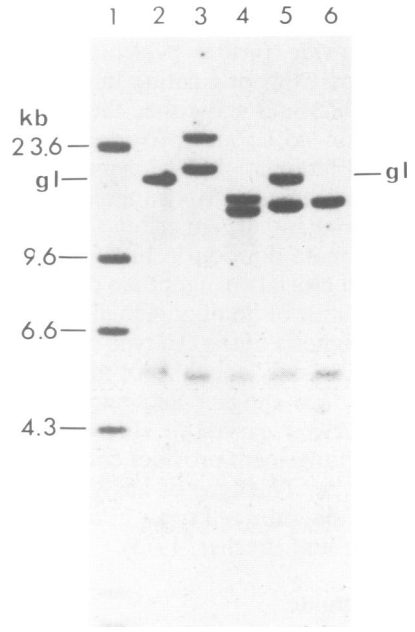

Fig. 4. Southern transfer of DNA analysed by *Bam*HI. 20 μg of DNA from each sample analysed as described in Figure 2. **Lane** (1) *Hind*III digest of λ, (2) HL60, (3) REH, (4) NALM 6, (5) RPMI 1788, (6) Bristol 7. gl indicates the position of the germ line band.

(Table I; Figure 3, lanes 6 and 7, 10 and 11; Figure 4, lanes 2–6). Korsmeyer *et al.* (1983), however, report that seven out of 25 cases of ALL retain a germ line gene, as analysed by *Bam*HI and *Eco*RI restriction endonuclease digestion. Nothing is known about the onset of μ gene rearrangement in normal human B cells. In mouse, >90% of the μ genes have rearranged in B cells (Nottenberg and Weissman, 1981) and essentially no germ line μ genes were found in A-MuLV-

induced pre-B cell lines (Alt *et al.*, 1981). It seems likely, therefore, that rearrangement events involving both μ gene alleles is an early event in B cell differentiation. The occurrence of a few cases still retaining germ line alleles in the blast crisis of CGL and in ALL (Korsmeyer *et al.*, 1981b, 1983) implies that the onset of blast crisis and of ALL may in some cases interrupt and arrest a very early stage of B cell differentiation.

To test further the extent to which μ gene rearrangement is an exclusive property of B lymphocytes, we have also analysed a number of other leukaemias. In common with CGL chronic phase and myeloid blast crisis, AML (three cases) and the acute promyelocytic cell line HL-60 have exclusively germ line configurations. We find that μ gene rearrangement does, however, occur in human T cell luekaemias (thymic), in four out of the 14 cases analysed. The rearrangement is always only on one allele (Table I, and see an example in Figure 3, lane 2). We have only observed the rearrangement in T-ALL cell lines (in four out of the eight analysed). No rearrangements were found in the fresh (i.e., uncultured) samples from seven patients. Korsmeyer *et al.* (1981a, 1983) have reported only one example of rearrangement out of 12 samples of cell lines of T-ALL. In contrast, μ gene rearrangement frequently occurs in mouse T cells (Zuniga *et al.*, 1982) and in the cases analysed seem to involve only D to J translocations (Kurosawa *et al.*, 1981). It seems unlikely that these gene rearrangements are essential to T cell function (Kronenberg *et al.*, 1980) and their significance is obscure.

Our results provide further evidence for a multi-step, clonal evolution of CGL originating in multipotential stem cells (Fialkow, 1982) and show that the blast crisis of Ph^1 CGL and common ALL both provide access to the early stages of B cell differentiation. The demonstrated restriction of Ig gene rearrangements to lymphoid subclones of the transformed multipotential stem cell in CGL formally establishes the approximate position in haemopoietic differentiation at which such crucial changes take place.

Analysis of the state of immunoglobulin genes is also clearly useful in the diagnosis of the cell type of immature cells. In Ph^1 CGL blast crisis, only one third of the cells typed as lymphoid (by cell surface antigens and nuclear terminal transferase; Greaves, 1982) express μ. For the remaining two thirds, μ gene rearrangement provides convincing evidence of lymphoid phenotype. This type of analysis should therefore be of value in defining the cell types in other 'unclassifiable' leukaemias (Bessis and Brecher, 1975).

Materials and methods

Isolation and characterisation of cells

Fresh leukaemia cells from newly diagnosed (untreated) patients were received as part of an immunodiagnostic service linked to the MRC chemotherapy trials in the UK (Greaves *et al.*, 1981b). Blood or bone marrow samples were separated on Ficoll-isopaque. Leukaemic blasts were characterised with a panel of monoclonal antibodies plus affinity purified rabbit antibodies specific for nuclear terminal deoxynucleotidyl transferase (Greaves *et al.*, 1982). Cytoplasmic immunoglobulins were detected in methanol-fixed cells by immunofluorescence using affinity purified antibodies as previously described (Greaves *et al.*, 1979b). Aliquots of cells were stored in liquid nitrogen after controlled rate (1°C/min) freezing. All samples, with three exceptions (see Table I), had high proportions (>90%) of leukaemic cells.

Leukaemic cell lines were established from patients, maintained and characterised as previously described (Minowada *et al.*, 1978).

Isolation and analysis of DNA

DNA was isolated from the cells essentially as described (Gross-Bellard *et al.*, 1973) except that in most cases the dialysis steps were replaced by ethanol precipitations. The DNA was digested with restriction enzymes supplied by Boehringer or BRL and used as recommended by the suppliers.

The restricted DNA was electrophoresed in 0.7% agarose in 0.036 M Tris, 0.03 M NaH_2PO_4, 1 mM EDTA at 1.0 or 0.2 volts/cm for 18 or 48 h. The DNA was transferred to nitrocellulose membranes as described by Southern (1975). DNA probes were nick-translated (Rigby *et al.*, 1977) essentially as described (Amersham International, 1980). The nitrocellulose-bound DNA was prehybridised in 3 x SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate) containing 0.2% w/v of each of bovine serum albumin, Ficoll and polyvinylpyrolidone (Denhardt, 1966), 50 μg/ml denatured salmon sperm DNA, 10 mM EDTA, and 0.2% SDS for 30–60 min and in the above solution containing, in addition, 9% w/v dextran sulphate also for ~60 min. The nick-translated probe was added to the dextran sulphate containing prehybridisation buffer at a concentration of ~10 ng/ml and at a specific activity of ~10^8 c.p.m./μg. Hybridisation was carried out at 65°C overnight. After hybridisation, the membranes were washed at 65°C twice in the hybridisation buffer without dextran sulphate and then 3–4 times for ~15 min each in 0.1 x SSC, 0.05% SDS. The hybridised membranes were exposed against prefogged Kodak Xomat RP5 film with intensifying screens for 1–7 days.

Identification of μ gene rearrangement

The principal restriction enzyme sites in the region containing the J_H gene segments and in the Cμ gene are shown in Figure 1. D to J_H or V to D to J_H translocation events will alter the restriction enzyme sites upstream of the J_H region and will generate Cμ- and J_H-containing fragments which will usually differ from the germ line 16.5-kb DNA fragment generated by *Bam*HI. The J_H-containing fragments generated by double digests of *Bam*HI and by *Hind*III (6 kb in germ line DNA), *Eco*RI (17 kb in germ line DNA), *Bgl*II (3.3 kb in germ line DNA) will usually also be altered by D or V and D to J_H translocation events. These DNA fragments which were used to recognise μ gene rearrangements were identified with the probes A and B shown in Figure 1.

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