Clustered somatic mutations in and around first exon of non-rearranged c-myc in Burkitt lymphoma with t(8;22) translocation

Marie-France Szajnert, Simon Saule¹, Georg W.Bornkamm², Henri Wajcman, Gilbert M.Lenoir³ and Jean-Claude Kaplan

Institut de Pathologie Moléculaire, INSERM U129, CHU Cochin, Paris 75014, ¹INSERM U 186, Institut Pasteur, Lille, France, ²Institut für Virologie, University of Freiburg, Freiburg i. Breisgau, FRG and ³IARC, Lyon, France

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ABSTRACT

We have examined the restriction map of the c-myc gene in 15 BL cell lines carrying the variant t(8;22) translocation in which c-myc is known to remain on chromosome 8. Using 3 restriction enzymes cutting outside the c-myc domain (EcoRI, BamHI, HindIII), we found no evidence for a c-myc/Ig lambda rearrangement in 14 BL cell lines. In the last one, BL 37, the 3' flanking region was rearranged corresponding to the already identified breakpoint located 400 pb downstream from the c-myc gene (9). Using 4 restriction enzymes cutting inside the c-myc gene (PvuII, PstI, SacI, HincII) we looked for discrete abnormalities within the gene limits, and we found in 9 BL cell lines several abolished and created sites, compatible with multiple independent somatic mutations. They are significantly clustered in the 5' non coding region, with a striking prevalence at the end of exon 1. The role of mutations in the non-coding first exon region for the deregulation of c-myc expression is discussed.

INTRODUCTION

Chromosomal translocations bringing together the c-myc oncogene and an immunoglobulin (Ig) domain are a characteristic and constant feature of Burkitt's lymphoma (BL). As a consequence c-myc gene expression is deregulated, and several mechanisms have been postulated to explain this loss of control (see review in ref 1-4). In the common BL translocation t(8;14), present in up to 75 % of BL cases, the chromosomal breakpoint on chromosome 8 occurs in the 5' region of c-myc, at several possible sites, suggesting that the gene disruption itself directly affects some regulatory elements. In all cases c-myc is translocated to chromosome 14. In the variant translocations, t(2;8) and t(8;22), in contrast, c-myc remains on chromosome 8, and a C region of a light chain Ig gene is fused downstream (3') of c-myc, usually at a considerable distance (10 to 100 kb) (5-6). This raises the challenging question of how c-myc can be deregulated by a remote event.

In the present study, we have analysed a large number of BL cell lines with the t(8;22) variant translocation, to look systematically for somatic mutations and see whether they cluster in a given region of the c-myc gene.

As an alternative to the cloning and sequencing of DNA from each case, we scanned the 8 kb domain comprising the entire c-myc gene by a number of restriction enzymes and probed the resulting fragments with a battery of overlapping probes. This enabled us to find many somatic mutations occurring significantly in and around the first exon of an otherwise non-disrupted c-myc gene.

MATERIALS AND METHODS

Cell lines

Fifteen Burkitt cell lines with fully documented (8;22) chromosome translocation (3) were investigated. They are listed in table I with their specifications. The cells were harvested from cultures of BL cell lines which had been frozen early after establishment. The following controls were also investigated :

- lymphoblastoid cell lines (LCL) established by EBV immortalization of peripheral lymphocytes of individuals from whom some Burkitt cell lines originated : IARC 304 (subject with BL2), IARC 261 and 277 (subject with BL 60) ; IARC 176 (subject with BL 37) ;
- peripheral leukocytes from 11 normal unrelated individuals ;
- a control LCL established by EBV from a subject without Burkitt (LL84) and having already undergone more than 100 cell divisions.

DNA analysis

Genomic DNA was phenol extracted from frozen cell pellets, digested by one of the following restriction endonucleases : EcoRI, BamHI, HindIII, PvuII, PstI, SacI, HincII, electrophoresed in 0.6 to 1.0 % agarose and blotted on nylon membrane (Zetabind, Biorad) as described by Southern (7). Hybridization was carried out for 20 h at 42°C in the presence of 50 % formamide and 10 % dextran sulfate with the ³²P nick translated probe (specific activity : 5-8 x 10⁸ cpm/μg DNA). Filters were washed in 0.1 M SSC, 55° C (final wash), and exposed to Kodak X Omat AR5.

Probes

Several genomic probes covering altogether the entire c-myc domain were used (see fig 1). The numbering of nucleotides given below (5' to 3') starts at the first promoter (P1) :

probe_1 : a 2395 bp HindIII-XhoI fragment, flanking exon 1 on its 5' side (spanning -2325 to + 70)

probe_2 : a 862 bp PvuII-PvuII fragment containing exon 1 (spanning -349 to + 513)

probe_3 : a 1533 bp SacI-SacI fragment covering the 3' half of intron 1 and exon 2 (spanning 1545 to 3078)

probe_4 : a 1410 bp ClaI-ClaI fragment encompassing exon 3 and the 3' adjacent sequence (spanning 4355 to 5765)

probe_5 : a 606 bp SacI-SacI fragment corresponding to the middle part of intron 1 (spanning 939 to 1545).

These probes explored all the sites existing for the 4 enzymes used. Sometimes a same site was explorable with two adjacent probes (fig 1).

Sequence of c-myc and normal restriction sites

The precise location of the restriction sites of the enzymes used in the study was computed from the 8 kb long Hind III-EcoRI fragment sequenced by Gazin et al (8). It extends from 2303 nucleotides upstream exon I (HindIII site) to 869 nucleotides downstream exon 3 (EcoRI site).

All "cryptic" sites, ie sites in which a single base substitution can generate a new 6 nucleotide sequence recognized by each of the restriction enzymes used, were also computed.

RESULTS

Restriction map with enzymes cutting outside the c-myc gene :

Restriction fragments obtained by EcoRI (12.5 kb), HindIII (11.5 kb) and BamHI, (ca 26 kb) digestion allow the exploration of the "whole" c-myc gene. In 13 out of 15 Burkitt cell lines with t(8;22) the c-myc restriction pattern was normal, indicating that no gross rearrangement of c-myc, as could be produced by the nearby transposition of a lambda Ig gene, had occurred.

In BL 37 an abnormal pattern of the 3' site was detected (data not shown), corresponding to the rearrangement of this region with a lambda constant Ig gene already described by Hollis et al (9).

In BL 104 the size of the HindIII and BamHI fragments were apparently unmodified. Using EcoRI we found, in addition to the normal 12.5 kb allele, an abnormal fragment of 7.5 kb with probe 2 (exon 1) or of 5 kb with probe 4 (exon 3). Both fragments were detected with probe 5 (intron 1), indicating the insertion of an additional EcoRI site within the first intron.

Abnormalities of the restriction map with enzymes cutting within the c-myc gene (fig 1) :

The absence of important rearrangement within the c-myc gene does not exclude the possibility of mutations, or micro-rearrangements in this region. Four enzymes possessing well separated sites within the c-myc gene, PvuII (5 sites), PstI (4 sites), SacI (3 sites) and HincII (5 sites), were selected

figure 1 :

Restriction map of the normal c-myc gene (8) and of c-myc in 8 BL with t(8;22). Restriction sites are mentioned as : H3 : HindIII ; RI : EcoRI ; Ps : PstI ; H : HincII ; Pv : PvuII ; S : SacI. Bold letters indicate the modified sites, either abolished (symbol⁻) above the line or created (symbol⁺) below the line. BL cell lines BL 37, BL 104 and L 660 are not shown.

to scan the 8 kb of the HindIII-EcoRI fragment. Their even distribution (fig 1) and the use of 5 subcloned probes corresponding to the 5' part of exon 1 (probe 1), exon 1 (probe 2), the 3' half of intron 1 + exon 2 (probe 3), exon 3 and the 3' adjacent region (probe 4), middle part of intron 1 (probe 5) (cf materials and methods and fig 1) enabled us to screen the whole c-myc region in the 15 t(8;22) Burkitt cell lines (table I). Altogether 17 different sites were explored in each cell line.

Table I BURKITT CELL LINES WITH t(8;22) INVESTIGATED IN THIS STUDY

Cell line	Ig light chain produced	Geographical origin	EBV
LY 47	none	AF	+
LY 67	lambda	AF	+
BL 2*	lambda	E	-
BL 33	lambda	E	-
BL 37*	kappa	E	+
BL 60*	lambda	NAF	+
BL 47	none	E	-
BL 50	lambda	E	-
BL 84	lambda	E	-
BL 89	lambda	NAF	+
BL 90	lambda	NAF	+
BL 99	lambda	E	+
BL 104	kappa	E	-
MWIK	lambda	AF	+
L660	none	E	+

* : LCL were established by EBV immortalization of peripheral lymphocytes of subjects BL 2 (IARC 304), BL 37 (IARC 176), BL 60 (IARC 261 and IARC 277) and BL 104 (IARC 629). All cell lines had been established by one of us (G.M.L.) at IARC, except for lines Mwika and L 660 kindly provided by A. Rickinson and V. Diehl respectively.

AF : black Africa ; NAF : North-Africa ; E : Europe.

Eight Burkitt cell lines (LY 47, LY 67, BL 2, BL 33, BL 60, BL 89, BL 90, Mwika) exhibited abnormal restriction fragments in addition to the normal allele. They are listed in table II and partially illustrated in fig 2. These results were systematically confirmed using several consecutive probes, and were compatible with the abolition of preexisting restriction sites or the creation of new ones. Altogether 26 sites were found modified : 6 in LY 47, 2 in LY 67, 2 in BL 2, 4 in BL 33, 7 in BL 60, 2 in BL 89, 1 in BL 90 and 1 in Mwika (fig 1). It was observed that a same site could be abnormal in more than one cell line : the PvuII site at 513 (at the 3' end of exon 1) was abolished in 7 out of 8 cell lines (fig 1) ; the SacI site (position 1545) was abolished in 3 different lines ; the SacI (position 1435), the PstI site (position 1313), were created in 2 different lines (table II).

In the BL 104 cell line, in which an additional EcoRI site was found in intron 1, all the other explored sites (PvuII, SacI, HincII, and PstI) in that region were normal. These results confirm that this EcoRI site is not due to a c-myc Ig rearrangement at a chromosomal breakpoint, but is a new created site in the 360 bp interval between unperturbed PvuII-XbaI sites.

Using probe 4 the 3' flanking region of c-myc was also explored by PvuII (0.7 kb 3' of EcoRI site) and Pst I (6.5 kb 3' of EcoRI site) (not shown in

TABLE II LOCATION OF ABNORMAL RESTRICTION SITES ALONG THE c-myc GENE IN 8 BURKITT CELL LINES WITH t(8;22)

Location	Event	Cell line
5' flanking sequence		
- 2120	Hinc II(cr)	BL 33
- 1156	Hinc II(ab)	BL 33
- 743	Pvu II(cr)	BL 60
- 349	Pvu II(ab)	BL 60
- 217	Hinc II(cr)	LY 47
- 195	Pvu II(cr)	LY 47
Exon I		
513	Pvu II(ab)	LY 47, LY 67, BL 33, BL 60, BL 89, BL 90, MWIKA.
Intron I		
991	Pvu II(cr)	BL 89
1313	Pst I(cr)	BL 33, BL 60
1393	Pvu II(ab)	BL 60
1435	Sac I(cr)	BL 2, BL 60
1545	Sac I(ab)	BL 2, BL 60, LY 67
2070	Pst I(cr)	LY 47
1182/1545	Eco RI(cr)	BL 104
Exon 2		
2313	Pst I(ab)	LY 47
2355	Pst I(cr)	LY 47

The location is referred to Promoter 1. (ab) : abolished restriction site ; (cr) : created restriction site.

fig 1). In the L 660 cell line, which retains a normal restriction pattern map within the 8 kb limits, a PvuII site 700 bp downstream from the 3' end of c-myc was abolished. Similarly BL 37 exhibited a created PstI site 600 bp 3' of c-myc corresponding to the c-myc Ig lambda joining region fully characterized by Hollis et al (9).

Analysis of the restriction map perturbations found in BL cell lines with t(8;22)

The new fragments observed could be interpreted either by the abolition of pre-existing sites or by the creation of new sites.

A precise map of the abolished and created sites, deduced from the known sequence of the region is shown in fig. 1 and 4, and table II. The location of the created sites, as deduced from the size of the fragments, correlates

figure 2 :

Hybridization pattern with c-myc exon 1 probe (PvuII - PvuII probe n° 2) of some PvuII digested t(8;22) BL DNA. The new fragments are due (a) to abolition, (b) to creation of PvuII sites. 0.86 kb is the normal allele.

perfectly with the position of cryptic sites (n-1 specific sequences) pre-existing in the sequence. In all cases the creation of a new site is compatible with a point mutation. In BL 104, however, no cryptic site for EcoRI was found in the PvuII/XbaI 360 bp sequence in which the new site was created. Therefore we cannot give a precise location of this event.

The mutated sites are not randomly distributed on the whole c-myc region explored. Altogether (fig. 1 and 4, table II) in the 9 BL cell lines we found 6 abnormalities upstream exon 1, 7 abnormalities in exon 1 (all consisting in the abolition of the same PvuII restriction site near the end of exon 1, at position 513), 11 abnormalities in intron 1, 2 abnormalities in exon 2 and none in exon 3 and in the 3' flanking sequence of the gene. Interestingly, whereas the amount of cryptic sites is higher in exon 2 + intron 2, the created sites occurred preferentially in the 5' non-coding half of the c-myc gene (fig 1 and 4).

Search for restriction map abnormalities of c-myc in non BL cells

To exclude the possibility that some restriction site modifications could be actually due to RFLPs, we analysed, with the same enzymes and probes, DNA from peripheral leukocytes of 11 normal unrelated individuals. We failed to detect any perturbation at the sites that had been found abnormal in the BL cell lines. In addition (i) the constitutional DNA from LCL established from

figure 3 :

Absence of mutated restriction sites in lymphoblastoid cell lines derived from peripheral blood of BL subjects compared to their BL cell line counterpart. IARC 304 is the LCL of the BL 2 subject, IARC 261 and 277 derive from the BL 60 subject. SacI digested DNA was hybridized with c-myc exon 2 probe (SacI - SacI probe n° 3).

peripheral blood of subjects BL 2, BL 37, BL 60 and BL 104 did not display any of the abnormal patterns found in their corresponding BL cell line DNA (fig 3) ; (ii) the restriction map of c-myc in the DNA from control LCL which had undergone more than 100 cell divisions was also normal.

DISCUSSION

To search for possible alterations in the non-translocated c-myc gene of variant Burkitt cells, we have investigated the restriction map of DNA from 15 BL lines with a documented t(8;22) translocation (table I).

With the exception of BL 37, there was non gross rearrangement of c-myc suggestive of a breakpoint lying within the gene or at a short distance. We then systematically scanned the c-myc region with 4 enzymes exploring altogether 17 "full" sites (pre-existing normal restriction sites) and 186 "cryptic" sites (6 nucleotides sites with a single nucleotide difference from

figure 4 :

The cumulated number of created sites for SacI, PvuII, PstI, HincII and EcoRI (white blocks) found in 8 BL with t(8;22) is clearly not related to the distribution of cryptic sites present in the normal c-myc gene (shaded blocks). The position of cumulated abolished sites (black blocks) is also given to date.

a full restriction site), all evenly distributed on a 8 kb DNA sequence (HindIII-EcoRI fragment) containing the c-myc gene (fig 1 and 4).

In 9 BL cell lines (LY 47, LY 67, BL 2, BL 33, BL 60, BL 89, BL 90, BL 104 and Mwika), we found an abnormal pattern coexisting with the normal c-myc allele, compatible with the abolition of existing sites and/or the creation of new ones. Because the general framework of adjacent normal restriction sites was always maintained (data not shown) we believe that these modifications correspond to point mutations, or to small insertions or deletions that are below the resolving power of standard agarose gel electrophoresis, rather than extensive DNA rearrangement. However, in some cases (LY 47, BL 2, BL 60) the distance between two adjacent perturbations is short enough to be compatible with microrearrangements. The size of the c-myc transcripts in LY 47 and BL 2 was normal (10). These variations of restriction sites correspond to somatic events and do not reflect constitutional polymorphisms because : (1) the constitutional DNA of 3 patients (BL 2, BL 60, BL 37) did not exhibit the abnormal restriction

pattern found in their Burkitt cells (fig 3) ; (ii) no RFLP was found with the same set of enzymes and probes in the normal population.

The abolished and created restriction sites that we observed are concentrated in and around the first non coding exon of c-myc, with 24 perturbed sites against only 2 in the rest of the gene (fig 1 and 4, table II). This non-random distribution is highly significant : $.005 > p > .001$ (χ^2 test). It could have resulted from an uneven distribution of the normal sites explored by the arbitrary selection of enzymes used. As seen in fig 1 and table II this is not the case for the abolished sites. The number of restriction sites explored in the 5' non coding half of the gene (10 sites on 4520 nucleotides spanning from the 5' HindIII site to the beginning of the second exon), is equivalent to that corresponding to the 3' half (7 sites on 3561 nucleotides spanning from the second exon to the 3' EcoRI site) ; however all the abolished sites are in the 5' half (table II). As to the created sites, assuming that they originated by a single base change, we searched for all existing "cryptic" sites exhibiting only one difference with the 6 nucleotide sequence specific for each restriction enzymes. As seen in fig 4 186 such sites pre-exist. Since they are almost evenly distributed all through there was no bias introduced by our choice of enzyme and the observed clustering of the created sites is not circumstantial.

Also if a bias against mutations occurring in the exons is conceivable, the tolerance of mutations should be the same in the two introns and their distribution in both introns should be equal. This is not the case : no abnormal site was found in intron 2, whereas altogether 11 abnormal sites were found in intron 1.

Because the few selected enzymes explore only few existing site (17 altogether), the actual number of abolished sites, -and created ones as well-, is necessarily underestimated. Accordingly the 4 lines exhibiting no abnormality with 4 enzymes (BL 47, BL 50, BL 84, BL 99), may indeed harbour other perturbed sites that would be displayed by other enzymes. Even if only extensive sequencing of the c-myc abnormal gene could bring a definitive inventory of all mutational events in each BL cell line the clustering of mutated sites we found in some BL cell lines is striking. Whether mutations in c-myc are obligatory for tumorigenesis is not known. This important question could not be answered until it is proven that, at least in one Burkitt cell lines, the c-myc sequence is entirely normal.

Rabbitts et al (11,12) first emphasized the existence and significance

of somatic mutation in c-myc by partial sequencing of two BL with t(8;14), Raji and Daudi, and one BL with t(8;22), LY 67. Concerning the BL with t(8;14) Rabbitts et al (12) provided the tempting hypothesis that in its new location, i.e. upstream and close to a C_H Ig gene, the translocated c-myc occupies the place of a V_H gene and undergoes the same propensity to somatic mutations as would a normal rearranged V gene. This might also hold true in the case of the (8;22) translocation and more generally in all Burkitt translocations, since whether it is transposed (8;14) or not (2;8 and 8;22) the c-myc gene ends invariably in a position upstream from an Ig gene. However it is not known whether the generation of V-like somatic mutations can occur when a large distance still separates the two genes, as observed in most of the t(8;22) (5).

The most important question concerns the relevance of the clustered somatic mutations in c-myc. Since they were first reported in BL with t(8;14) (11-14), other groups also found mutations of c-myc in other BL cell lines with (8;14) (15-17). Concerning the BL variants with t(8;22) two reports already indicated the existence of somatic mutations found on a sequenced c-myc gene : in LY 67 (12) and KK 124 (18). Recently Pelicci et al (19), investigating the restriction map of a series of BL with t(8;14), found like us evidence for numerous mutations in the same non-coding region of c-myc. Particularly the same PvuII site at the 3' end of exon 1 was found abolished in 7 out of our 15 cases with t(8;22) and in 10 out of the 13 BL with t(8;14) investigated by Pelicci et al (19). Moreover, sequencing data obtained in Ramos with t(8;14) (14), in LY 67 with t(8;22) (12) and in KK 124 with t(8;22) (18) showed a cluster of multiple microdeletions or mutations in the immediate vicinity of this "hot spot" site, respectively 6 bp, 70 bp and 60 bp 5' of it. In addition amongst the 26 mutational events that we found in 8 BL lines, 18 are located in a 600 bp region comprising the 3' half of exon 1 and the 5' half of intron 1 (fig 1 and table II). Significantly this is precisely the domain which is disrupted in the t(8;14) BL belonging to the class I type as categorized by Cory (4). Our present report reinforces the importance of somatic alteration in a critical region of the c-myc gene. Many models have been proposed to explain how c-myc can be deregulated when not directly disrupted by the translocation (2,12,20-28). A possible transcriptional down regulation mechanism has been recently suggested (29,30). The mutation we found are precisely clustered around the boundary of exon 1 and intron 1, a region which is believed to be essential for transcriptional arrest (30). In particular the PvuII site at the end of exon

I is part of a proposed stem and loop structure which might act as a transcription termination signal (30). A partial or complete constitutive release of this block might be produced by point mutations, leading to the deregulation of c-myc. The fact that in our series of BL with t(8;22) the PvuII mutation was strikingly prevalent in African subjects (6 out of 6), as compared with European cases (1 out of 9), is compatible with the existence of more than one mechanism of deregulation of the c-myc gene even when it is not translocated.

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