Analysis of the 3' flanking region of the human c-myc gene in lymphomas with the t(8;22) and t(2;8) chromosomal translocations

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Received 27 February 1986; Accepted 25 April 1986

ABSTRACT

We have cloned and mapped the sequences extending 38 kb 3' of the c-myc gene. This region is found to be highly repetitive in nature and hybridizes extensively with a BLUR 8 Alu probe. Unique sequence probes derived from this region were used to map the chromosomal breakpoints of a number of lymphoma cell lines with t(2;8) or t(8;22) translocations. In five of the cell lines (PA682, LY67, LY47, LY66 and LY91), the immunoglobulin light chain locus translocates into a region which is greater than 47 kb downstream of c-myc. For one of the cell lines, JI, the location of the breakpoint on the 8q+ chromosome was found to be 25-32 kb 3' of c-myc. The breakpoint for the BL2 cell line had been previously mapped at 10 kb 3' of the c-myc oncogene. Analyses of steady-state levels of c-myc mRNA in cell lines with chromosomal breakpoints ranging from 10 kb to greater than 47 kb 3' of c-myc range from 0.5 to 10X the levels in lymphoblast controls. The different levels of c-myc transcripts is not a direct function of the distance between the c-myc gene and the translocated immunoglobulin light chain locus.

INTRODUCTION

The chromosomal translocations seen in Burkitt lymphomas have been shown to involve the c-myc gene on chromosome 8 and one of the chromosomes on which the immunoglobulin genes are located. In 75% of these translocations, the c-myc gene translocates from its normal position on chromosome 8, to the heavy chain locus on chromosome 14 (1,2). The breakpoints on chromosome 8 are located either within the first intron of c-myc or in the 5' flanking region (3,4). The variant translocations, which comprise the remaining 25%, carry either a t(2;8) or t(8;22) translocation. In these cases, the c-myc gene always remains on chromosome 8, and the kappa or the lambda light chain locus translocates into the 3' flanking region of c-myc (5-7). Hollis *et al.* (7) have described a t(8;22) translocation in the cell line, BL37, in which the C λ locus was translocated to within 400 bp of the 3' end of c-myc. In all other cases, no DNA rearrangement could be detected within 20 kb 3' of c-myc on Southern analysis (8,9). This observation raises the question of how the immunoglobulin locus might influence the expression of the c-myc gene involved

in these translocations over such distances and whether the effect of this influence might be reflected in c-myc RNA levels for translocations which are more or less proximal to c-myc. We report here the cloning and characterization of 38 kb of the 3' flanking region of c-myc. Using probes generated from this region, we have examined six lymphoma cell lines carrying variant translocations in an attempt to map the positions of these breakpoints. In addition, we have compared steady-state levels of c-myc mRNA for five of these six cell lines to determine whether c-myc expression might be a function of the relative distances between the c-myc gene and the translocated immunoglobulin locus in these cell lines.

MATERIALS AND METHODS

Cell Lines

The PA682 cell lines were derived from the peripheral blood (PB), pleural effusion (PE), and bone marrow (BM) of a patient with a B-cell lymphoma and stigmata of the AIDS syndrome (10). The three cell lines, PA682 (PB), PA682 (PE) and PA682 (BM) are clonally related based on immunoglobulin gene rearrangements (10). LY47, LY67 (11), LY66, LY91 (12), BL2 (13) and JI (14) are Burkitt lymphoma cell lines with t(2;8) or t(8;22) translocations and have been previously described. Molt 4 is a T-cell line (15). GM1056 and GM1500 are human lymphoblastoid cell lines which do not contain a c-myc translocation and were obtained from the Human Genetic Mutant Cell Depository (Camden, NJ). PA682, B12, LY67, LY47 are t(8;22) translocations. JI, LY91 and LY66 are t(2;8) translocations.

Genomic Library Construction and Screening

Genomic libraries are constructed in the phage vector EMBL3A. High molecular weight DNA from the PA682 (PB) cell line was partially digested with the restriction enzyme Sau3A and size-fractionated on a 10-40% sucrose gradient. DNA fragments of 18-23 kb were ligated with EMBL3A phage arms and packaged according to Hohn and Murray (16). Packaging efficiency was 4×10^5 recombinant phage per μg of insert DNA. The genomic library was screened at a density of 15,000 recombinant plaques per 150-mm diameter petri dish. Hybridizations were carried out in 5X SSC at 65°C for 18 h. Final washes were in 1.0X or 0.5X SSC at 65°C.

DNA Analysis

Genomic DNA was digested with restriction endonucleases, fractionated by electrophoresis through a 0.7% agarose gel, and blotted onto nitrocellulose (17). Hybridizations were in 4X SSC and 50% formamide at 37°C for 48 h unless

otherwise noted. Final washes were in 0.5-0.2X SSC at 65°C. High stringency hybridization conditions for probes containing repetitive sequences were in 4X SSC and 50% formamide at 55°C for 24 h (18). Final washes were in 0.2X SSC at 65°C for 10 min.

RNA Isolation and Analysis

Cytoplasmic RNA was extracted from logarithmic cell cultures according to Scott and Frankel (19). RNA samples were denatured at 60°C with 50% formamide and 2.2 M formaldehyde and electrophoresed on 1% agarose gels containing 2.2 M formaldehyde. RNA was transferred to nitrocellulose and hybridized essentially as described by Thomas (20). Removal of hybridized probe was accomplished by washing the blot in 1 mM EDTA at 68°C for 1-2 h. The efficiency of the removal was checked by exposure on X-ray film before the blot was hybridized with another probe. Autoradiograms were analyzed on a Corning 750 scanning densitometer. At least two exposures were analyzed to assure that the film response was in the linear range and several tracings were cut and weighed.

Subcloning

Restriction fragments of bacteriophage clones were ligated into PUC18 or PUC19 and used to transform *E. coli* HB101 (21).

Probes

The c-myc probe is a 1092-bp fragment of a cDNA clone pRyc7.4 which includes all of exon 3 and 200 bp of exon 2 (22). The Alu repeat sequence probe is subclone BLUR 8 which contains 300-bp human repeated DNA sequence of the Alu family (23). The phosphoglycerate kinase (PGK) probe is a human cDNA clone pHPGK-7e containing the complete PGK coding sequences (24). The actin probe is a human genomic subclone pHA4.1 containing β -actin coding sequences (25). 32 P-labelled probes (specific activity, 5×10^8 cpm/ μ g) were prepared by using calf thymus primers (26). Free nucleotides were removed by centrifugation through a Sephadex G-50 mini-column.

RESULTS

We have used unique sequence probes derived from the 3' flanking region of c-myc to screen a genomic DNA library derived from the lymphoma cell line, PA682 (PB), which has a t(8;22) translocation (10). Figure 1 shows representative bacteriophage λ clones isolated with probes pCA1.7S and pPA1.3SB which are indicated below the restriction map. The 38 kb of DNA covered by these clones is indicated in the composite restriction map. The far 3' of the map is not included in the genomic clones but is drawn from Southern analyses

Figure 1. Restriction map of the *c-myc* locus. The restriction map was generated from overlapping bacteriophage λ clones derived from the genomic DNA library of Burkitt's lymphoma cells PA682 (PB). Two probes used in the library screening were pCA1.7S containing a 1.7-kb *Sst*I fragment, and pPA1.3SB containing a 1.3-kb *Sst*I/*Bam*HI fragment. The three exons of *c-myc* are indicated by numbered boxes 1, 2 and 3. Distances in kb from the 3' end of *c-myc* are marked on the bottom line. Restriction sites beyond 38 kb from the 3' end of *c-myc*, as shown on a discontinued line, were obtained by Southern blot analysis with probe pPA0.2S. The two-way arrow below the map indicates the region within which the breakpoint is located in the Burkitt's lymphoma cell line JI. Restriction sites are abbreviated as follows: K, *KPN*I; E, *Eco*R I; H, *Hind*III; B, *Bam*HI; S, *Sst*I; Bg, *Bgl*II. *Sst*I *KPN*I and *Bgl*II sites are not complete. Triangles identify published breakpoint positions in T-cell neoplasias and the circles the breakpoint positions in B-cell neoplasias; the numbers are references.

using the most 3' probe pPA0.2S. This map represents the normal configuration for this region, as identical restriction fragments were detected on Southern analysis of DNA from PA682 cells and control cell lines. Hybridization with 32 P-labelled Alu repeat sequence probe BLUR 8 (23) or total human DNA of either single restriction enzyme digests or double digests with *Bam*HI and *Xba*I, *Bam*HI and *Hind*III, or *Hind*III and *Eco*R I resulted in cross-hybridization with fragments derived from the entire region. Therefore, there is no fragment greater than 3 kb within this locus that does not contain a sequence that cross-hybridizes with the BLUR 8 probe and as a result unique sequence probes were difficult to identify. The unique sequence probes derived from this region, as indicated in Fig. 1, were used on Southern blot analysis to examine

Figure 2. Southern blot hybridization with probes derived from the 3' flanking region of *c-myc*. Ten μ g of DNA samples of Burkitt's lymphoma cells were digested with restriction enzyme *EcoRI*, *XbaI*, *BglII* or *HindIII* as indicated, fractionated on a 0.7% agarose gel, and blotted onto nitrocellulose. The filter was hybridized with probe pCA1.7S (A), pPA1.3SB (B, C) or pPA0.2S (D). High stringency conditions (4X SSC and 50% formamide at 50°C) were used with probe pPA0.2S in (D) lane 1, Molt 4, a T cell line used as germline control; lane 2, PA682 (PB), a Burkitt's lymphoma cell line with t(8;22) translocation; lane 3, DNA shown is derived from the LY67 cell line, however, all of the following four Burkitt's lymphoma cell lines gave identical hybridization bands: LY67, LY47 [both with t(8;22) translocations], LY66 and LY91 [both with t(2;8) translocations]; lane 4, JI, a Burkitt's lymphoma cell line with t(2;8) translocation.

the PA682 (PB) cell line as well as five Burkitt lymphoma cell lines with variant translocations: LY67, LY47 (2), LY66, LY91 (28) and JI (5).

Figure 2a shows the results from Southern analysis using probe pCA1.7S. Germline patterns are found in all DNA samples of these various cell lines. Therefore none of the cell lines contain a rearrangement within the 20 kb detectable with this probe. We have previously shown that the t(8;22) Burkitt cell line BL2 is rearranged with the pCA1.7S probe (data not shown) and that this rearrangement has been mapped 10 kb 3' of *c-myc* (43). Probe pPA1.3SB, which is derived from a region 20 kb 3' of *c-myc*, detects altered restriction fragments in addition to the germline bands in *XbaI* and *BglII* digests of DNA from the JI cell line. The 17-kb *XbaI* and 24-kb *BglII* rearranged bands are shown in Fig. 2b and 2c, respectively. Since pPA1.3SB does not detect a DNA rearrangement in a *HindIII* digest of JI DNA (data not shown), the breakpoint in JI cells must occur 3' to the *HindIII* site and 5' to the *XbaI* site which are located between 25 kb and 32 kb from the 3' end of *c-myc*. This position corresponds to the region underlined by the two-way arrow in Fig. 1. Only the germline patterns were detected with probe pPA1.3SB for the remaining cell lines (Fig. 2b, c). As we were unable to identify a unique sequence probe at the 3' end of the cloned sequence, we have subcloned a 200-bp *SstI* fragment, pPA0.2S, which contains some repetitive sequences. Figure 2d shows the

Figure 3. Level of c-myc transcripts in Burkitt lymphoma cells with variant translocations. Twenty μ g of total cytoplasmic RNA from the indicated cell lines were electrophoresed through 1% agarose gel containing 2.2 M formaldehyde, blotted onto nitrocellulose, and hybridized with the c-myc probe. In the lower panel, the hybridized probe was removed and the same blot rehybridized with the human phosphoglycerate kinase probe. Lymphoblastoid cell lines, GM1056 and GM607, are included as controls. In lanes containing RNA from PA682 (PB) and GM607, the filter hybridized with the c-myc probe was exposed approximately 7 times longer than the remaining lanes. The upper band in the prolonged exposure is due to cross-hybridization between the c-myc probe and 28S rRNA.

Southern blot analysis with this probe using high stringency hybridization conditions described by Fisher *et al.* (18). This probe allows us to extend the map for 9 kb beyond the cloned sequences. Germline patterns were obtained for DNA samples of all these cell lines digested with several restriction enzymes. The rearrangements in the lymphoma cell lines PA682, LY67, LY47, LY66 and LY91 are more than 47 kb beyond the 3' end of the c-myc gene.

Analysis of c-myc transcription in somatic cell hybrids has indicated that the c-myc gene involved with the translocation is differentially regulated as compared with the nontranslocated gene (29). It has been further

Table 1. Level of c-myc transcripts in Burkitt's lymphoma cell lines with variant translocations.

Cell line	Chromosomal translocation	Distance between c-myc and breakpoint	Level of c-myc transcripts	
			(A) ^a	(B) ^b
GM607	None	None	1.0	N.D. ^c
GM1056	None	None	1.5	N.D.
BL2	8;22 ^d	10 kb ^e	4.0	4.0
JI	2;8 ^f	25-32 kb	4.0	4.8
LY91	2;8 ^g	> 47 kb	4.4	3.6
LY47	8;22 ^h	> 47 kb	3.7	N.D.
LY67	8;22 ^h	> 47 kb	10.4	9.8
PA682 (PB,BM,PE)	8;22 ⁱ	> 47 kb	0.5	0.3

(a) The amount of c-myc mRNA was normalized relative to that of phosphoglycerate kinase (pGK) mRNA. The level in lymphoblastoid cell line GM607 is taken to be 1.0. (b) The amount of c-myc mRNA was normalized relative to that of action mRNA. The level of c-myc transcripts in Burkitt's lymphoma cell line BL2 is taken to be 4.0 for comparison with column (A). (c) N.D. = not determined. (d) Ref. 6. (e) Ref. 42. (f) Ref. 5. (g) Ref. 28. (h) Ref. 27. (i) Ref. 10.

suggested that this deregulation may be due in part to the influence of the associated immunoglobulin gene locus (30-32). As the majority of the breakpoints in the variant translocations we have analyzed are greater than 47 kb away from the 3' end of c-myc and greater than 55 kb away from the 5' control regions, this raises the question as to how the influence of the immunoglobulin locus is exerted over these long distances. In order to determine whether a correlation may be made between the levels of c-myc transcription and the relative distances between c-myc and the immunoglobulin locus in these cell lines, we have determined the steady state levels of c-myc mRNA in a number of cell lines in which the chromosomal breakpoints range from 10 kb to greater than 47 kb 3' of c-myc. Total cytoplasmic RNAs from several lymphoma cell lines with variant translocations were analyzed by RNA blot hybridization using the c-myc probe containing the second and third coding exons (22). The amount of RNA in each lane was quantitated by using phosphoglycerate kinase (PGK) (24) transcripts as an internal standard. The results are shown in Fig. 3. The amounts of steady-state mRNA transcripts were determined by den-

Figure 4. Levels of c-myc transcripts in Burkitt's lymphoma cells with variant translocations. Twenty μ g of total cytoplasmic RNAs from the indicated cell lines were electrophoresed and blotted as described in legend of Fig. 3. The nitrocellulose filter was hybridized with the c-myc probe. In the lower panel, the hybridized probe was removed and the same blot rehybridized with the actin probe. In lanes containing RNA from PA682 (PB) and PA682 (PE), the filter hybridized with the c-myc probe was exposed approximately 6 times longer than the remaining lanes.

sitometer scanning of the autoradiograms. The level of c-myc mRNA was normalized relative to that of PGK mRNA in each cell line, and the results are summarized in column A of Table I. Analysis using a human actin probe (25) as an internal standard gave similar relative values, as shown in Fig. 4 and column B of Table I. The steady-state levels of c-myc transcripts for BL2, JI, LY47 and LY91 cells appear to be equivalent and are approximately three- to four-fold of that in the control lymphoblastoid cell lines, GM607 and GM1056. The distances between c-myc and breakpoints for these cell lines vary from 10

kb in BL2 (38), 25-32 kb in JI, to greater than 47 kb for LY47 and LY91. In addition, although both LY67 and PA682 cells both also have breakpoints greater than 47 kb 3' of c-myc, the level of c-myc mRNA in LY67 is 10-fold increased and the level of c-myc in PA682 is twofold decreased relative to that in the control lymphoblastoid cells. Although the c-myc deregulation appears to be a function of the association with the Ig loci (29-31), the relative steady state levels of c-myc transcripts in these cell lines is not a function of the distance between the c-myc gene and the associated immunoglobulin gene loci.

DISCUSSION

The consistent translocations between the c-myc genes and the heavy and light chain immunoglobulin genes seen in Burkitt's lymphoma and mouse plasmacytomas produce a compelling model for the deregulation of the translocated c-myc gene under the influence of these B cell-specific genes (3,4). Evidence for the influence of the Ig loci on c-myc expression comes primarily from studies of somatic cell hybrids (5,6,29). These studies suggest that the normal and the Ig-associated c-myc genes are differentially regulated in B cells at different stages of differentiation. The translocated c-myc gene is efficiently expressed in the plasma cell background while normal transcripts are not detected (29,30). In a lymphoblastoid background transcription of the translocated gene associated with a heavy chain switch region is repressed while the normal endogenous c-myc gene is expressed (30,31). Using transfection studies, Prehn *et al.* (32) and Feo *et al.* (33) have shown that in rearrangements in which the first noncoding exon is separated from the second and third coding exons, the elimination of the normal regulatory sequences is not sufficient in itself to activate the c-myc gene. These interpretations are based on the inability to express the transfected, decapitated c-myc genes in mouse plasmacytoma cell lines. They further suggest that it is the associated Ig locus which may be influencing c-myc transcription (32-34) and it has been suggested that this effect may be due to the action of enhancer sequences capable of acting over long distances (30,31). Enhancer influences on gene expression have been primarily described as being effective over distances of several hundred base pairs to a few kilobases (34,35). These limits of influence are primarily derived from the analysis of transcription of cloned gene constructions (34,35). Recently Wang and Calame (36) have demonstrated that when in the normal chromosomal context of the IgH locus, the immunoglobulin heavy chain enhancer is able to activate a heavy chain V gene promoter over a

distance of 17 kb. The cis-acting effect of this enhancer on transcription of the upstream V_H gene is only slightly diminished in comparison to the closely associated rearranged V_H gene (36). Clearly the upper limit for this type of activation has not yet been determined and may be well within the ranges for the activation of the most distal translocations.

We have shown here that the putative effect of the Ig locus on c-myc expression is not distance-dependent, in that translocations which place lambda light chain sequences closer to the c-myc gene do not result in significantly higher levels of c-myc transcription. Therefore, if the cis activation of the c-myc gene is potentiated by the association with the various Ig loci, then the differences in the steady-state levels of c-myc mRNA for the different cell lines must be determined by additional factors. These could include rate of transcription, message stability, or the availability of transacting factors in different B-cell stages. Mutations within the first exon of translocated c-myc gene which could result in changes in either transcription rates or message stability have been previously described (9). LY67, which has the highest level of c-myc transcripts (Table I), has been shown to have multiple base substitutions in the noncoding first exon (9) and a minor mRNA species of 3.3 kb not present in the other cell lines can be seen on Northern blot analysis (Fig. 3). Recent studies by several groups have demonstrated that either truncated c-myc m-RNAs which are initiated within the first intron (38,39) or c-myc transcripts which initiate within flanking immunoglobulin sequences (40) are more stable than the normal c-myc m-RNA (33-35). Except for LY67, the c-myc transcripts in the other cell lines appear normal on northern blots and several also appear normal by S_1 nuclease protection studies (29-31) which eliminates the possibility of any extensive mutations within the 5' exon although single base pair changes might not be detected.

The translocated c-myc gene in the PA682 cell lines has also been shown to be differentially regulated as compared to the normal c-myc gene in somatic cell hybrids with mouse plasmacytoma cells (unpublished results). The level of c-myc transcripts in the PA682 cell lines is striking as it is even lower than in the lymphoblastoid controls. PA682 has been described as being more lymphoblastoid in character than most Burkitt cell lines based on cell surface markers and growth characteristics (10). In addition the PA682 cell lines are non-tumorigenic in nude mice while Burkitt lymphoma cell lines are (Croce, unpublished observations). The low level of c-myc expression in PA682 would then be consistent with observations that the translocated c-myc genes may not be expressed in the lymphoblastoid background but are expressed in the plasma-

cytoma background (29-31). The low level of c-myc transcripts may then reflects different requirements for transformation of cells at various stages of B-cell differentiation. The PA682 cell lines were derived from a patient with stigmata of the AIDS syndrome and have been shown to be Epstein-Barr virus-positive (10). EBV-positive lymphomas are uncommon in the United States but have been observed in patients with AIDS (10). It is not clear what role these factors have played, if any, in the generation of the malignant phenotypes. It seems possible that the PA682 cell lines may represent prelymphoma cells in which the c-myc oncogene involved in the chromosomal translocation has not yet been fully activated. This possibility is suggested by the non-tumorigenicity of PA682 cells in nude mice. We are presently analyzing somatic cell hybrids between the PA682 cell line and a mouse plasmacytoma to assess the effect of the cellular milieu on the c-myc transcription levels. Thus, although the presence of the Ig locus may be a requirement for the c-myc deregulation additional events may be required to fully activate this gene.

Recently translocations have been described in T-cell neoplasias which involve the locus for the α -chain of the T cell antigen receptor and the 3' region of c-myc (41-43). The mapped position of several of these breakpoints are shown in Figure 1 (1, 41-44). The breakpoint positions appear in limited stretches of repeat free sequences in that short probes (600-800 bp) which span the breakpoint regions on chromosome 8 detect single copy sequences on Southern blots. The B and T cell breakpoints mapped to date, appear to cluster within a few limited regions. We have recently identified heptamer-nonamer joining signals in association with one sequenced breakpoint which occurs >400 bp 3' of c-myc suggesting that the translocations into the 3' flanking region of c-myc may occur by an immunoglobulin joining mechanism (45) as had been previously shown for the t(14;18) and the t(11;14) translocations (46,47). This would suggest that the translocations involving the α TCR locus and c-myc may occur by a similar mechanism, and more importantly that there may also be a common mechanism for the deregulation of c-myc in both the B and T cell neoplasias.

The highly repetitive nature of the 3' flanking region of c-myc which extends through the entire 38 kb of DNA is rather unusual. The 47-kb region which is detected by our probes is included in the HL60 and Colo 320 c-myc amplification unit, the Colo 320 amplification also extends greater than 20 kb 5' of c-myc (unpublished results). Alu repeats have been frequently described in relationship to deletions and rearrangements and the presence of such an extensive repetitive unit in the c'-myc flanking region might play some role

in the amplification process. Genomic regions which are enriched in Alu repeat sequences have been demonstrated to be unstable showing frequent restriction length polymorphisms and variation in copy number (48) and have also been implicated in regulation of the transcription of a number of eukaryotic genes including the ϵ -globin gene (49). The association of the Ig locus with this region as a result of the translocation could possibly interfere with the normal regulatory functions of this region by perturbing the normal structure of this chromosomal domain.

ACKNOWLEDGEMENTS

We thank The Wistar Institute editorial staff for preparing the manuscript. We are grateful to Dr. S. H. Orkin for the human PGK probe, to Dr. R. Weinmann for the actin probe and to Dr. I. Magrath and Dr. G. M. Lenoir for providing cell lines. This work was supported by the National Institutes of Health grants CA-16685, CA-36521, and GM-20700 to C.M.C. and GM-32583 to L.C.S.

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