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Mapping Chromosomal Breakpoints of Burkitt's t(8;14) Translocations Far Upstream of c-*myc*[1]

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ABSTRACT

To analyze the region upstream of c-*myc*, a number of novel probes were established. These were generated by chromosomal walking starting from the breakpoint of the chromosomal translocation of the B-cell line 380 and by cloning the breakpoint of the translocation of the Burkitt lymphoma cell line IARC/BL72. Using the newly isolated probes a detailed physical map of 500 kilobases of the region upstream of c-*myc* was established applying pulsed-field gel electrophoresis. The chromosomal breakpoint of IARC/BL72 cells was mapped to a site 55 kilobases 5′ of c-*myc*. A region 20 kilobases in length and containing the breakpoints of 380, EW36, P3HR-1, and Daudi cells was identified 170–190 kilobases upstream of c-*myc*. In addition the HPV18 integration site in HeLa cells was located between 340 and 500 kilobases 5′ of c-*myc*. The probes were used to define the c-*myc* amplification units in Colo320-HSR and HL60 cells as well as in four cases of small cell lung cancer. Evidence is provided that the amplicon of HL60 cells is discontinuously organized.

INTRODUCTION

In many spontaneously occurring as well as experimentally induced tumors structural DNA alterations affect the locus of the protooncogene c-*myc*. These alterations include gene amplification, insertion of RNA or DNA viruses, and chromosomal translocations. Amplification of the c-*myc* gene has been observed in a number of human tumors including small cell lung carcinoma (1) and breast cancer (2). Virus insertion at the c-*myc* locus is a hallmark of B- and T-cell lymphomas induced by nondefective, slowly transforming retroviruses in avians (3), mice, and rats (4). Integration of DNA viruses at the c-*myc* locus is found in hepatocellular carcinomas induced by woodchuck hepatitis virus (5) as well as in some HPV[3]-associated cervical carcinomas (6). Chromosomal translocations affecting the c-*myc* locus and one of the immunoglobulin heavy or light chain loci are a consistent feature of Burkitt's lymphoma in humans, immunocytomas in rats, and pristane-induced plasmacytomas in mice (for a review see Ref. 7). Similar translocations of c-*myc* with T-cell receptor α or β genes are found in human T-cell leukemias and lymphomas (8).

Structural alterations can affect the c-*myc* gene itself or are located at a considerable distance from c-*myc*, leaving the transcription unit intact. A detailed map of the region downstream of c-*myc* including the positions of breakpoints of six Burkitt lymphoma cell lines with t(2;8) translocations has been established (9). This analysis showed that the breakpoints of chromosomal t(2;8) translocations are scattered over a distance of 320 kilobases downstream of c-*myc*, with three breakpoints clustered at a distance of 160 kilobases. This coincides with a region termed pvt-1 containing a cluster of breakpoints of mouse plasmacytoma variant translocations (10, 11) and which is a hotspot of retrovirus integration in T-cell lymphomas of mice (12) and rats (13). In four tumor cell lines the 3′ termini of the c-*myc* carrying amplification units was defined. While in HL60 cells about 40 kilobases downstream of c-*myc* are amplified (14, 15), the amplification unit of COLO320-HSR and some small cell lung cancer lines terminates between 160 and 260 kilobases (9, 14).

In contrast, little is known about the region upstream of c-*myc*. Haluska *et al.* (16, 17) have cloned the breakpoints of t(8;14) translocations from the cell lines 380, EW36, P3HR-1, and Daudi and found them to be located within 20 kilobases at an unknown distance upstream of c-*myc*. The breakpoints of three other Burkitt lymphomas were found to be outside of this region (18, 19). The integration of human papilloma viruses has been observed upstream of c-*myc* in two cervical carcinoma cell lines (6). In HeLa cells the integration site on chromosome 8q24, represented by a probe termed H4.1, has been assigned to a region located within the c-*myc* amplicon of HL60 and COLO320 cells. The HL60 amplification unit was shown to extend at least 40 kilobases downstream of c-*myc* (15) and to have an overall sequence complexity of about 80 kilobases (20). It was therefore concluded that H4.1 and the translocation breakpoints of the four cell lines mentioned above are located within approximately 40 kilobases upstream of c-*myc*.

Mapping breakpoints or viral insertion sites upstream of c-*myc* requires the establishment of a PFGE map of this region. An initial map was constructed by Gemmill *et al.* (21) using a few rare cutting restriction enzymes. However, to pinpoint breakpoints upstream of c-*myc* a map of higher resolution is needed. Here we describe a number of new probes generated from the region upstream of c-*myc* which were used to establish a detailed PFGE map of a region of 500 kilobases. Based on this map we could localize the chromosomal breakpoints of the cell lines IARC/BL72, EW36, P3HR-1, Daudi, and 380. Furthermore, the extension of amplification units within this region was determined in four small cell lung carcinomas and the cell lines Colo320-HSR and HL60.

MATERIALS AND METHODS

Tumor Cell Lines

The following cell lines were used: North African Burkitt lymphoma cell line IARC/BL72 and a lymphoblastoid cell line, IARC307, derived from the same patient, as well as LAZ385 were kindly provided by Gilbert Lenoir (22). LAZ385 is an Epstein-Barr virus-immortalized,

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[3] The abbreviations used are: HPV, human papilloma virus; IgH, immunoglobulin heavy chain; PFGE, pulsed-field gel electrophoresis.

lymphoblastoid cell line that was derived from a member of a family with a constitutional t(3;8) translocation predisposing to a highly enhanced risk for the development of renal cell carcinoma (23, 24). The chromosomal breakpoint of this translocation is more than 1000 kilobases upstream of c-*myc* (21, 25). Colo320-HSR and HL60 were provided by the American Type Culture Collection. Cell lines NCI-H82, NCI-H60, NCI-H446, and NCI-N417 were derived from patients with small cell lung cancer (1). DNA from these cell lines was kindly provided by J. D. Minna and coworkers.

All cell lines were grown as stationary suspension cultures at 37°C in an atmosphere of 5% CO_2 and 95% air in RPMI 1640 supplemented with 10% fetal calf serum (Gibco), 300 μg/ml glutamine (Gibco), 50 units/ml penicillin, 50 μg/ml streptomycin, and 1 μg/ml amphotericin B (Gibco).

Cloned Probes

Chromosome 8. As a c-*myc* probe a genomic *Hind*III/*Cla*I fragment (5′*myc*) was used, located immediatedly 5′ of the promoters P1/P2. This probe maps upstream of the CpG island found within c-*myc*, *i.e.*, 5′ of the restriction sites for *Not*I and *Nar*I. Probe p380j9 0.8Ss (p380j9) is close to the t(8;14) translocation breakpoints of the cell lines 380, EW36, Daudi, and P3HR-1 (Fig. 1) (16, 17). 380 was derived from a patient with pre-B acute lymphoblastic leukemia, EW36 from an undifferentiated lymphoma, and Daudi and P3HR-1 from Burkitt lymphomas. Additional probes were isolated by chromosomal walking starting with p380j9 as described in "Results." A further chromosome 8 probe, plasmid H4.1, represents a HPV18 integration site in HeLa cells (6).

Chromosome 14. Probe Cμ is a 1.2-kilobase *Eco*RI fragment containing the first and second Cμ exons (26). The enhancer probes were obtained by subcloning a genomic 3.1-kilobase *Eco*RI/*Hind*III fragment (27) which overlaps the IgH intron enhancer and part of the joining gene segment (see Fig. 2*a*), resulting in a 2.2-kilobase *Eco*RI/*Bgl*II fragment (5′E_i) and a 0.9-kilobase *Bgl*II/*Hind*III fragment (3′E_i).

Large-Scale Mapping Using PFGE

For PFGE analysis the protocol described by Smith *et al.* (28) was followed using the Chef-II system (Biorad). Nylon filters with blotted DNA were hybridized according to the method of Maniatis *et al.* (29). Probes were radiolabeled by the random priming method (30).

Construction of Genomic Libraries

Genomic DNA of the cell line IARC/BL72 was digested partially with Sau3A, dephosphorylated with calf intestine phosphatase, and size-fractionated in a sucrose gradient. The DNA was then ligated with *Bam*HI/*Sst*I and *Bam*HI/*Bst*EII-digested arms of the cosmid vector Lorist B (31, 32). Ligated DNA was packaged *in vitro* with Gigapack gold (Stratagene). Cosmids were propagated in ED8767 bacterial cells. The construction of the phage library containing the genomic DNA of EW36 cells has been described previously (17).

In Situ Hybridization

Metaphases were prepared according to standard techniques from human lymphocyte cultures. The cultures were labeled with bromodeoxyuridine to allow for fluorochrome-photolysis-Giemsa banding (RBG) analysis (33). The procedure for *in situ* hybridization with ^3H-labeled DNA probes was exactly as described previously (34).

RESULTS

Chromosomal Walking Starting from a t(8;14) Translocation Breakpoint Far Upstream of c-*myc*. In order to generate probes for the construction of a long-range map upstream of c-*myc*, we first investigated the region surrounding the breakpoint of the B-cell line 380 represented by the probe p380j9. This probe also detects the chromosomal breakpoints of three more cell lines with t(8;14) translocations (Daudi, P3HR-1, and EW36) (16, 17) and is located at an unknown distance upstream of c-*myc*. p380j9 was used as starting point for chromosomal walking. First a genomic library of cell line EW36 was screened and a phage clone containing about 18 kilobases of genomic DNA, termed E25C, surrounding p380j9 was isolated (Fig. 1). Single copy probes were obtained from the terminal parts of this insert and used to isolate additional phage clones with overlapping inserts. Accordingly, additional walking steps were carried out, and finally the 0.6-kilobase *Bam*HI/*Sst*I subclone pEW36-7D;SsB0.6 ($p380_c$) and the 2-kilobase *Hind*III subclone pEW36-9;H2,0 ($p380_t$) were isolated. As is indicated in Fig. 1, clone $p380_c$ is located approximately 20 kilobases in the centromeric direction and $p380_t$ approximately 26 kilobases in the telomeric direction of p380j9 on chromosome 8q24. The orientation of these probes can be concluded from the position of p380j9 relative to the IgH locus at the t(8;14) breakpoint site in 380 cells as has been described by Haluska *et al.* (16).

Cloning the Breakpoint of the Burkitt's Lymphoma Cell Line IARC/BL72. An additional probe of the region upstream of c-*myc* was obtained by cloning the breakpoint of the Burkitt lymphoma cell line IARC/BL72. This cell line is derived from a North African Burkitt lymphoma and has the translocation breakpoint more than 14 kilobases upstream of c-*myc*, as indicated by the germline configuration of the c-*myc*-carrying *Bam*HI fragment (data not shown). In addition, the breakpoint is not close to the t(8;14) breakpoints in the four cell lines described by Haluska *et al.* (16, 17). Screening of a cosmid library of IARC/BL72 by using the 1.2-kilobase IgH-Cμ probe revealed eight cosmids. These clones showed an overlapping restriction pattern after digestion with *Eco*RI and *Hind*III, concerning the IgH carrying part of the cosmids. The overlap

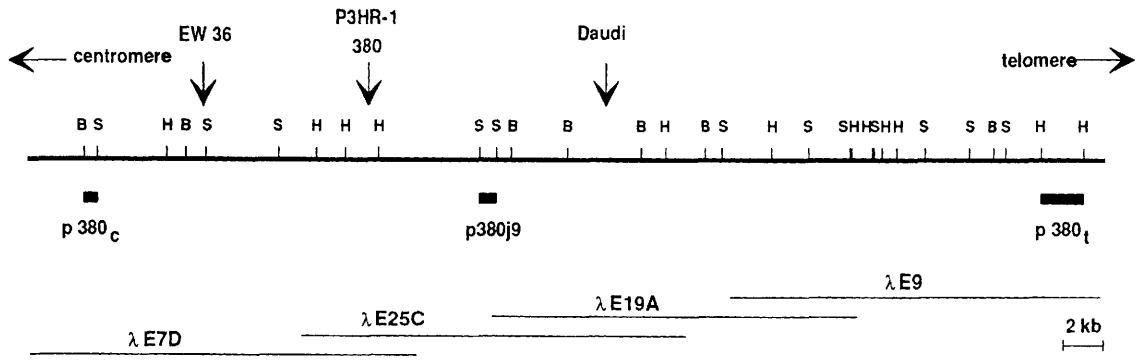


Fig. 1. Map of the breakpoint cluster region upstream of c-*myc* derived from Haluska *et al.* (16, 17) and including the relative position of overlapping phage clones obtained by chromosomal walking as well as the flanking $p380_t$ and $p380_c$ probes. *B*, *Bam*HI; *S*, *Sst*I; *H*, *Hind*III.

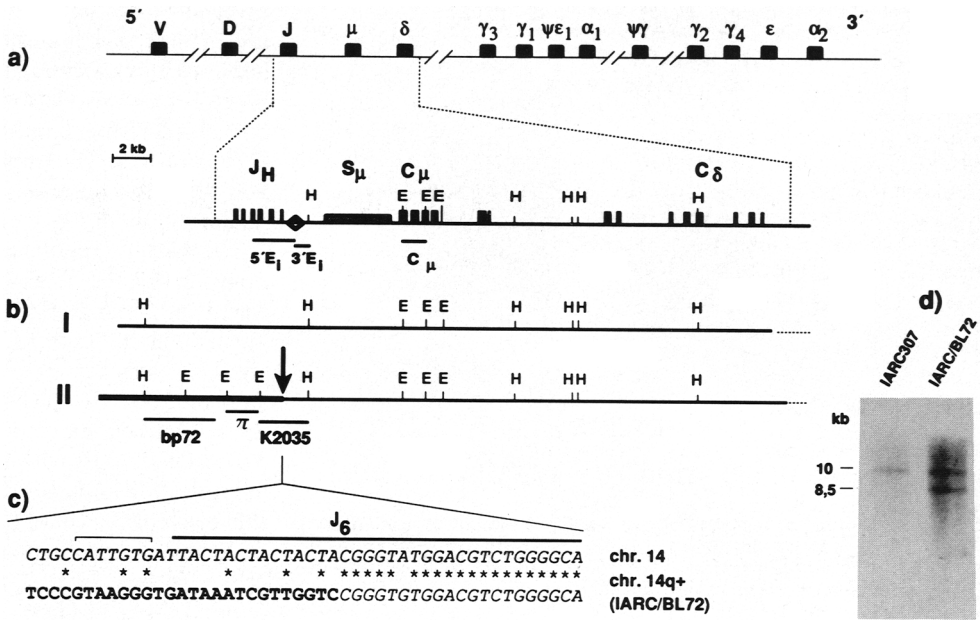


Fig. 2. *a*, physical map of the human IgH locus. *Bars*, probes; ♦, IgH intron enhancer. *b*, map of two cosmid types that were isolated from the IARC/BL72 library, showing a different restriction pattern 5′ of the IgH intron enhancer. Subclones π, K2035, and bp72 are described in the text. *c*, sequence of the chromosomal breakpoint in IARC/BL72 cells. In the *upper line* the germline configuration of the J6 gene segment (*overlined*) is shown (38). A heptamer recombinase signal is *bracketed*. The *lower line* indicates the corresponding sequence of the IARC/BL72 type II cosmid. *Boldface letters*, sequences without homology to chromosome 14, due either to chromosome 8-specific sequences or to additional nucleotides, inserted by the terminal transferase enzyme during the recombination of the IgH locus ("N-segment"). *d*, genomic Southern blot of IARC/BL72 and germline control DNA from the same patient (IARC307) digested with *Hind*III. An additional band is visualized by probe bp72 only in DNA of the tumor cells, indicating a rearrangement due to the chromosomal translocation. IgH gene segments: *V*, variable-; *D*, diversity-; *J*, joining region-; *Cμ*, IgM-; *Cδ*, IgD-; γ, IgG-; ε, IgE-; α, IgA-constant gene segments. *H*, *Hind*III; *E*, *Eco*RI.

extended to the *Hind*III site located downstream of the IgH intron enhancer between the IgH joining and switch region Sμ (Fig. 2, *a* and *b*). Two kinds of cosmids (types I and II) were found that differ in their restriction pattern upstream of the above-mentioned *Hind*III site (Fig. 2*b*). In order to distinguish which cosmid carries chromosome 14 sequences exclusively and which spans the breakpoint of IARC/BL72, subclones of the divergent regions were generated and hybridized to Southern filters containing placenta, HL60, and Colo320 DNA. A probe (π) derived from cosmid type II, although not free of repetitive sequences, visualized a smear and, in addition, distinct fragments of identical size in HL60 and Colo320 but not in placenta DNA (data not shown). This indicated that this probe maps within the c-*myc* amplification unit of HL60 and Colo320 cells. To demonstrate unequivocally that this part of the type II cosmid clones contains chromosome 8 sequences, additional subclones, free of repetetive sequences, were prepared. One clone, bp72, was subsequently used for *in situ* hybridization to metaphase chromosomes. Evaluation of 50 well-banded metaphases with a total of 208 grains resulted in a relatively broad distributed but highly specific signal within 8q23-q24, with the main accumulation within chromosome 8q24.1 (Fig. 3).

Southern analysis with bp72 revealed a distinct band of 10 kilobases in IARC 307 DNA and a second band of 8.5 kilobases in the cell line IARC/BL72 after digestion with *Hind*III (Fig. 2*d*). The 8.5-kilobase fragment was also detected by the $3'E_i$ and $5'E_i$ enhancer probes, indicating that these probes (*i.e.*, the IgH intron enhancer) are located downstream of the breakpoint. Although $5'E_i$ overlaps the chromosomal breakpoint (see below), only one fragment was detected by Southern analysis with this probe. Most probably the sequences representing the upstream part of $5'E_i$ were deleted in IARC/BL72 cells in the course of the VDJ rearrangement within the IgH locus.

To delineate the position of the chromosomal breakpoint more precisely sequence analysis of a further subclone (K2035) was performed, revealing that the breakpoint is located within the J6 segment of the IgH joining region (Fig. 2*c*). This is indicated by the abrupt ending of the sequence homology when comparing with the chromosome 14 germ line sequence. To

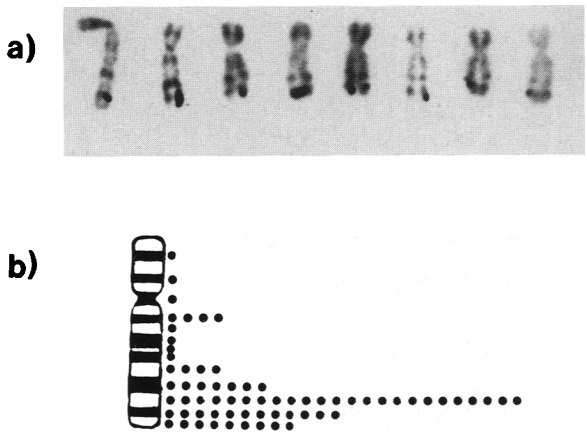


Fig. 3. *In situ* hybridization on metaphase chromosomes using probe bp72. *a*, several examples of RBG-banded chromosomes 8 with a specific signal of bp72. *b*, the distribution of silver grains as observed on chromosome 8.

define the breakpoint to the level of a single nucleotide, analysis of the reciprocal fragment of the translocation would be needed.

PFGE Analysis Maps the Chromosomal Breakpoints Up to 190 Kilobases Upstream of c-*myc*. PFGE mapping was started using *Sfi*I because this restriction enzyme generates relatively small fragments and is not sensitive to methylation. Hybridization of the various probes to *Sfi*I fragments revealed that the probes 5′*myc* and $p380_t$ visualized fragments of 85 kilobases, p380j9 and bp72 fragments of 25 kilobases, $p380_c$, a fragment of 160 kilobases, and H4.1, a fragment of 320 kilobases. 5′*myc* and $p380_t$ are located on different *Sfi*I fragments since double digestion with *Sfi*I and *Not*I revealed a *Not*I site only within the c-*myc* carrying fragment (Fig. 4). In addition detailed restriction analysis of the region around probe p380j9 (Fig. 1) and comparison to the breakpoint of IARC/BL72 cells showed that bp72 and p380j9 are located on different *Sfi*I fragments.

The relative order of the probes could be determined by digestion of DNA with *Nar*I, *Nar*I plus *Sal*I, and *Sal*I plus *Sfi*I (Fig. 5). Digestion with *Nar*I alone generated two fragments of 500 and 55 kilobases detected with the 5′*myc* probe. This could be due either to a polymorphism or to methylation of a *Nar*I site

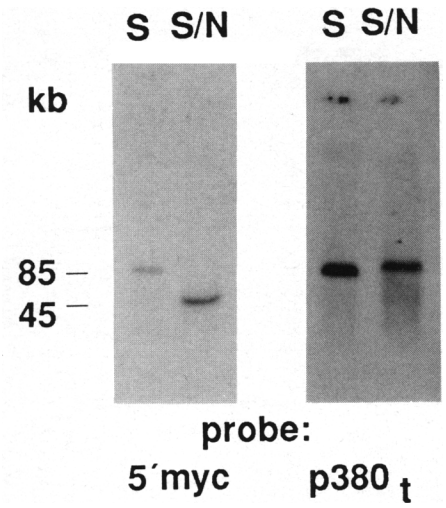


Fig. 4. PFGE blot of LAZ385 DNA after digestion with *Sfi*I (*S*) and *Sfi*I/*Not*I (*S/N*) and hybridization with probe 5′*myc* and p380$_t$ showing that both probes are located on different *Sfi*I fragments. LAZ385 represents the germline configuration within the analyzed region upstream of c-*myc*.

55 kilobases upstream of c-*myc* on one allele only. The same fragments of 500 and 55 kilobases were also detected with probe bp72. In contrast, the probes p380j9, p380$_t$, and H4.1 visualized fragments of 500 and about 445 kilobases. Double digestion with *Nar*I and *Sal*I generated fragments of 230 and 55 kilobases after hybridization with c-*myc* and bp72 probes, and fragments of 230 and 175 kilobases after hybridization with p380$_t$, p380j9, and p380$_c$. Therefore the IARC/BL72 breakpoint is located distally, and the chromosomal breakpoints in the cell lines described by Haluska *et al.* (16, 17) are located proximally to the *Nar*I site 55 kilobases upstream of c-*myc*. The breakpoints map within 230 kilobases 5′ of c-*myc*, as indicated by double digestion with *Nar*I/*Sal*I. Double digestion with *Sal*I plus *Sfi*I further indicated that the *Sal*I site is located on the p380$_c$-carrying *Sfi*I fragment of 160 kilobases (data not shown). Since H4.1 is also located on the 500-kilobase *Nar*I fragment, but on a *Sfi*I fragment different in size from the other probes (320 kilobases), H4.1 was assigned to a region between 340 and 500 kilobases upstream of c-*myc*. The data are summarized schematically in Fig. 5.

The HL60 Amplification Unit Is Discontinuous. The map presented in Fig. 5 was in contradiction to previous reports. Based on the size estimate of the HL60 amplification unit of about 80 kilobases by Kinzler *et al.* (20), the HPV18 integration site H4.1 was reported to be located within 40 kilobases upstream of c-*myc* (6). The size of the various *Sfi*I fragments and the established map indicated, however, a minimal distance of 340 kilobases. Provided that the size estimate of the HL60 amplification unit given by Kinzler *et al.* is approximately right, this discrepancy could only be explained by assuming that the region upstream of c-*myc* is discontinuously amplified in HL60 cells. To test this hypothesis, Southern blots containing *Hind*III-digested DNA of HL6O, COLO320, and human placenta were analyzed with the various probes. As shown in Fig. 6, all probes from chromosome 8 were amplified in COLO320, whereas only H4.1, bp72, and 5′*myc* were found to be amplified in HL60. Since H4.1 is located more distantly from c-*myc* than the probes p380j9, p380$_c$, and p380$_t$, the amplification unit of HL60 cells must in fact be organized in a discontinuous fashion.

Extension of Amplification Units Upstream of c-*myc* in Small Cell Lung Carcinomas. Using Southern blot hybridization as described above we also determined the extension of c-*myc* amplicons in DNA of four small cell lung carcinomas. In 3 cases (NCI-H82, NCI-H60, and NCI-H446) all chromosome

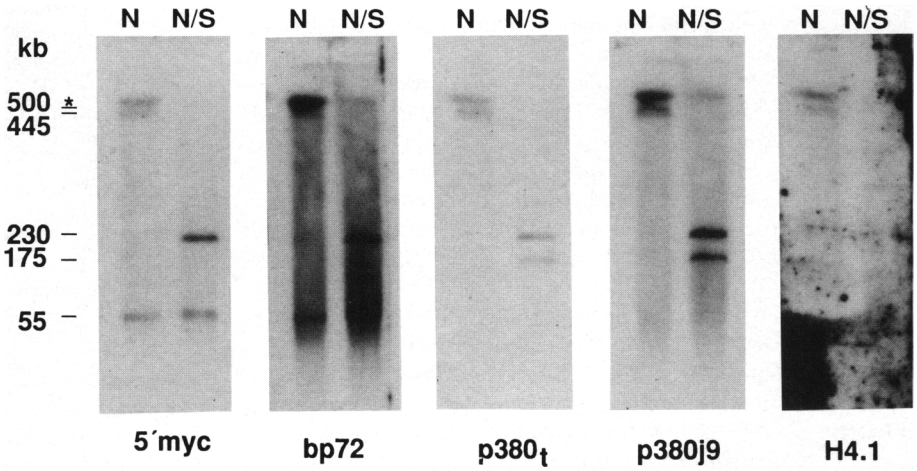
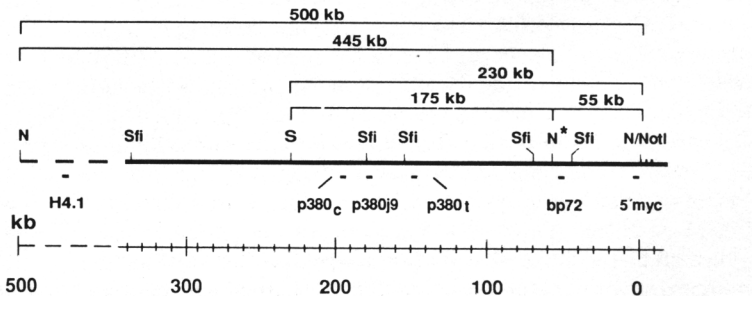


Fig. 5. PFGE analysis of the region upstream of c-*myc*. LAZ385 DNA was digested with *Nar*I (*N*) and *Nar*I plus *Sal*I (*N/S*) and hybridized with the probes indicated. *, due to unspecific hybridization to the "compression region" (39) caused by DNA of large size not resolved under the PFGE conditions applied. *Bottom*, the resulting map of the region upstream of c-*myc*, including the *Not*I and *Sfi*I (*S*) sites. *N**, partially cleaved *Nar*I site.



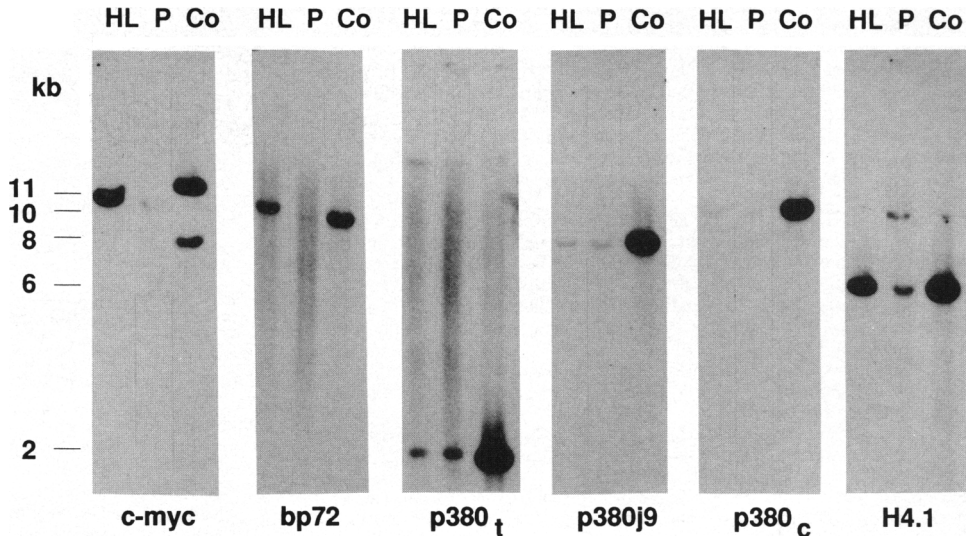


Fig. 6. Genomic Southern blot of HL60 (*HL*), placenta (*P*), and Colo320-HSR (*Co*) DNA digested with *Hind*III and hybridized with the indicated probes, revealing discontinuity of the HL60 c-*myc* amplicon. In Colo320-HSR cells c-*myc* gives rise to an additional fragment of approximately 8 kilobases due to a rearrangement in c-*myc* as described by Alitalo *et al.* (40).

8-specific probes used in this study were amplified, indicating that the amplicons carry sequences of at least 340 kilobases upstream of c-*myc*. In NCI-N417 the amplicon terminates between about 60 and 160 kilobases 5′ of the c-*myc* gene, since only the 5′*myc* and bp72 probes but not p380$_t$, p380j9, p380$_t$, and H4.1 were found to be amplified. The amplicon of this tumor terminates between 160 and 260 kilobases downstream of c-*myc* (14).

DISCUSSION

We have generated a number of new single copy probes of the region upstream of c-*myc* and have used them to establish a restriction map of about 500 kilobases. The probes were isolated by chromosomal walking starting from the breakpoint of the cell line 380 and by cloning the breakpoint of the chromosomal translocation of the cell line IARC/BL72. This allowed us to map the chromosomal breakpoints of 5 cell lines with t(8;14) translocation. The breakpoints in Daudi, P3HR-1, 380, and EW 36 cells are located between 170 and 190 kilobases upstream of c-*myc*. The IARC/BL72 breakpoint was mapped at a distance of 55 kilobases upstream of c-*myc*.

The finding that the HPV18 integration site H4.1 is located at least 340 kilobases upstream of c-*myc* was unexpected, since it was presumed to lie within 35 to 40 kilobases 5′ of c-*myc*. This estimate was based on the fact that H4.1 is located within the amplification unit of HL60 cells (6). The size of the amplification unit (80 kilobases) and its extension to at least 40 kilobases 3′ of c-*myc* (15) thus provided an upper limit of about 40 kilobases for the distance between H4.1 and c-*myc*.

The discrepancy can be readily explained by the fact that Kinzler *et al.* (20) estimated the sequence complexity of the HL60 amplification unit analyzing the reassociated fraction of amplified sequences. However, the sequence complexity is reflecting the size of the amplification unit only in the case in which a continuous piece of DNA is amplified. In contrast we show that the amplification unit in HL60 cells is organized in a discontinuous fashion compared to the germline situation. Similar sequence rearrangements have been described within the c-*myc* amplicon in Colo320-DM cells (35) as well as in the amplified N-*myc* region observed in neuroblastoma cells (36). Therefore mapping data which are based exclusively on the analysis of amplification units should be regarded with caution.

The region up to 200 kilobases upstream of c-*myc* is peculiar in several respects: (*a*) it is frequently the target of chromosomal translocations in Burkitt's lymphoma cells; (*b*) it is deleted in the HL60 amplicon; and (*c*) it carries the terminal end of the amplicons of NCI-N417 tumor cells. Thus it resembles the pvt locus which is a preferred site of recombination events in the region downstream of c-*myc* (14, 35, 37).

Structural alterations affecting the regulation of the c-*myc* gene can apparently occur at a distance of several hundred kilobases at either site of the gene. The availibility of probes from far upstream and far downstream of c-*myc* may help to improve the diagnosis and mapping of chromosomal translocations involving the c-*myc* locus in B- and T-cell lymphomas and leukemias and to identify structural alterations within or close to the c-*myc* locus in other malignancies.

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