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Regulatory elements in the immunoglobulin kappa locus induce c-*myc* activation and the promoter shift in Burkitt's lymphoma cells

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In Burkitt's lymphoma cells the proto-oncogene c-*myc* is constantly juxtaposed through chromosomal translocation to one of the immunoglobulin loci on chromosomes 14, 2 or 22. In the majority of cases the chromosomal breakpoint is localized 3′ or 5′ of the gene leaving the physiological c-*myc* transcription unit intact. As a consequence of the translocation the c-*myc* gene on the translocation chromosome becomes transcriptionally activated in such a manner that the c-*myc* promoter P1 is more active than promoter P2. In order to define elements involved in c-*myc* activation through t(2;8) translocation we have studied the expression of constructs consisting of c-*myc* and different parts of the immunoglobulin kappa locus after stable transfection into Burkitt's lymphoma cells. The c-*myc* gene under the control of the complete Ig kappa locus containing matrix attachment region, intron enhancer, constant kappa gene and 3′ enhancer was strongly activated with predominant usage of promoter P1. Deletion analysis revealed that the intron or 3′ enhancers alone activated c-*myc* to a much lesser extent and with normal promoter usage (P1 < P2). The cooperation of the same regulatory elements is required not only for transcriptional activation and induction of the promoter shift but also for down-regulation of promoter P1 of the translocated c-*myc* allele by sodium butyrate, another characteristic feature of Burkitt's lymphoma cells. This supports the notion that all elements involved in transcriptional activation and dysregulation of c-*myc* are contained within the *myc*-Ig specific minichromosome.

Key words: Burkitt's lymphoma/c-*myc*/oncogene deregulation/promoter shift/sodium butyrate

Introduction

Burkitt's lymphoma (BL) cells exhibit characteristic chromosomal translocations involving chromosome 8q24 carrying the proto-oncogene c-*myc* and the heavy or light chain loci of immunoglobulin (Ig). In some of the cases with t(8;14) and all cases with t(2;8) and t(8;22) translocations, the breakpoints are dispersed over a distance of at least 300 kb 5′ or 3′ of the gene (Henglein *et al.*, 1989; Joos *et al.*, 1992a,b). In all cases in which the translocation has left the c-*myc* transcription unit intact, the c-*myc* gene displays a number of characteristic features: (i) c-*myc* is predominantly expressed from the translocation chromosome, whereas the normal allele is silent or expressed at a very low level, (ii) c-*myc* is invariably characterized by structural alterations at the first exon/intron boundary, (iii) the block to RNA elongation is functionally missing and (iv) c-*myc* transcription initiates preferentially at promoter P1 in contrast to normal cells which show a ratio of P1/P2 promoter usage of ~1:3 to 1:5 (Taub *et al.*, 1984a,b; Yang *et al.*, 1985; Bornkamm *et al.*, 1988; Nishikura and Murray, 1988; Spencer and Groudine, 1991). The BL specific c-*myc* activation is apparently not only a consequence of the structural alterations within c-*myc*. Thus a mutated human c-*myc* gene exhibits a normal promoter usage upon transfer into mouse fibroblasts (Richman and Hayday, 1989a,b). Abelson virus transformed murine pre-B cells (Spencer *et al.*, 1990) and baby hamster kidney cells (Polack *et al.*, 1991). Moreover, a mutated c-*myc* allele cloned from a BL cell line with prominent promoter shift and reintroduced stably into BL cells with prominent promoter shift is only very weakly expressed with normal usage of promoters P1 and P2 (Polack *et al.*, 1991). Additional elements are therefore required for c-*myc* activation and induction of the promoter shift.

The functional changes of c-*myc* expression in BL cells are likely to be caused by juxtaposition of c-*myc* with regulatory elements of the immunoglobulin loci. In the case of t(8;14) in which c-*myc* and the Ig heavy (IgH) chain intron enhancer are colocalized, it was shown by transfection experiments that this enhancer was sufficient to activate c-*myc* (Hayday *et al.*, 1984). In cases with t(8;14) translocation in which the IgH intron enhancer is not colocalized with c-*myc*, the situation is more complicated. It is at present still unclear to what extent other elements located within the switch, constant or 3′ regions contribute to c-*myc* activation (Petterson *et al.*, 1990; Apel *et al.*, 1992).

To study the interplay between c-*myc* and regulatory elements in the Ig loci in the context of the chromosomal translocations, we have chosen the t(2;8) translocation as a model. This type of translocation offers several distinct advantages: (i) the kappa locus is structurally organized in a less complex fashion than the heavy chain and lambda light chain loci, so that our knowledge of the regulatory elements is more likely to be complete; (ii) the human kappa intron enhancer is among the best studied transcription control elements; (iii) the translocation breakpoints fall into a relatively small region on chromosome 2 within or in the vicinity of the kappa joining region even though the breakpoints are dispersed over a large distance 3′ of c-*myc* on chromosome 8 (Figure 2A) (Hartl and Lipp, 1987; Mengle-Gaw and Rabbitts, 1987; Henglein *et al.*, 1989). Therefore the kappa matrix attachment region (MAR), intron enhancer, constant region and 3′ enhancer element are colocalized with c-*myc* on the 8q + chromosome (Cockerill and Garrard, 1986; Meyer and Neuberger, 1989; Pongubala

and Atchison, 1991). The existence of a human kappa 3′ enhancer was postulated on the basis of a conserved sequence between mouse and man 12 kb 3′ of the human constant kappa gene (Müller *et al.*, 1990) and has been functionally identified by Judde and Max (1992). The enhancing activity of the 3′ enhancer was described to be much stronger than that of the intron enhancer in transient and stable transfection experiments (Blasquez *et al.*, 1989; Meyer and Neuberger, 1989). The presence of the 3′ enhancer provides an explanation for the fact that Ig kappa gene expression can take place in the absence of NF-κB, a transcription factor interacting with and absolutely required for the activity of the intron enhancer (Atchison and Perry, 1987, 1988). The intron enhancer alone can activate a juxtaposed c-*myc* gene only slightly and without inducing the BL specific promoter shift (Polack *et al.*, 1991). This suggested the study of the interaction of the c-*myc* promoter with the 3′ enhancer, either alone or in conjunction with other elements of the kappa locus including the intron enhancer.

We have previously proposed that drug induced c-*myc* down-regulation in BL cells might be useful for the identification of elements involved in c-*myc* deregulation (Polack *et al.*, 1987; Bornkamm *et al.*, 1988). This was based on the observation that sodium butyrate (BA) treatment of BL cells causes down-regulation of c-*myc* expression at a transcriptional level. Several arguments support the notion that BA does not primarily interact with the c-*myc* gene but rather disturbs the interplay between c-*myc* and the juxtaposed Ig locus: (i) BA reduces the transcription rate of the heavy chain locus (Polack *et al.*, 1987); (ii) BA exerts its effect not only on RNA initiated at the physiological promoters of c-*myc* but also on hybrid Ig−c-*myc* RNAs initiated at an antisense promoter located within the μ switch region (Eick *et al.*, 1985; Polack *et al.*, 1987; Apel *et al.*, 1992); (iii) BA slightly induced expression of the normal allele in BL cells where the normal c-*myc* allele is not completely silent (see below). The susceptibility of c-*myc* in BL cells to BA may provide a tool for the identification of target sites which on one hand mediate down-regulation in response to BA and on the other hand might be identical with the B cell specific long range enhancer postulated by Croce (1987).

It was our aim to set up a system in which all features of c-*myc* activation and perturbed regulation can be reconstructed. Such a system should be able to reveal (i) transcriptional c-*myc* activation, (ii) the Burkitt specific promoter shift with preferential usage of promoter P1, and (iii) a reduction of c-*myc* expression in response to BA.

Results

Sequence and functional identification of the human kappa 3′ enhancer

We have shown previously that part of the Ig kappa locus extending from J5 to 2.7 kb 3′ of the constant gene and carrying the canonical intron enhancer is not sufficient to mediate BL specific c-*myc* activation (Polack *et al.*, 1991). This may be due to the fact that the construct did not contain the enhancer element described in the 3′ region of the kappa locus (Meyer and Neuberger, 1989). The existence of a human kappa 3′ enhancer was suggested by sequence homology between mouse and man 12 kb 3′ of the kappa constant gene (Müller *et al.*, 1990). To localize the human enhancer element more precisely we have sequenced the 4.1 kb *Bam*HI fragment encompassing the described region of homology. Comparison of the human sequence with the murine sequence revealed a region of homology of ~700 bp (Figure 1). Judde and Max (1992) reported that the human kappa 3′ enhancer was entirely contained within a 218 bp fragment ending 5′ at the *Sca*I site. This 218 bp fragment,

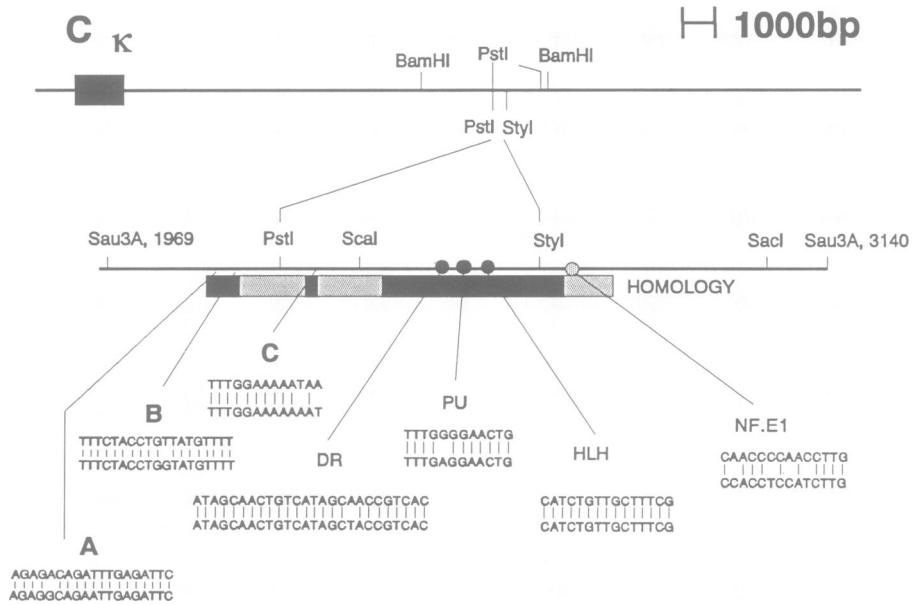

Fig. 1. Sequence homology between the murine and human kappa 3′ enhancer. The upper part shows the 3′ region of the human immunoglobulin kappa locus. The filled box indicates the kappa constant gene (Cκ). The part below is a magnification of the central portion of the 4.1 kb *Bam*HI fragment. The core elements which are highly conserved between mouse and man are indicated as filled circles (DR, PU and HLH) (Meyer and Neuberger, 1989; Müller *et al.*, 1990; Pongubala and Atchison, 1991; Judde and Max, 1992; Pongubala *et al.*, 1992). A weaker conserved element is shown as an open circle (NF.E1). Dark shading indicates the regions with the highest degree of homology between mouse and man. Three additional highly conserved motifs (A, B and C) were found upstream of the core of the human 3′ enhancer as has been described by Judde and Max (1992).

however, does not contain the entire region of homology. There are in addition three more highly conserved sequence motifs (designated A, B and C in Figure 1) extending 200 bp 5′ of the *Sca*I site (Figure 1). Computer analysis with transcription factor binding sites revealed no known binding site within the three conserved elements of this region. In order to delineate the extension of the human kappa 3′ enhancer, transient transfection experiments were performed with the P1 promoter of c-*myc* under the control of the 4.1 kb *Bam*HI fragment encompassing the 3′ enhancer. Deletion of the region of homology 5′ of the *Sca*I site decreased the activity of the 3′ enhancer by ~30% (data not shown). Therefore the entire 4.1 kb *Bam*HI fragment was used in all constructs described below (Figure 2B).

Induction of the BL specific promoter shift

To test the effect of different parts of the kappa locus on the expression of the c-*myc* gene a series of plasmids was constructed consisting of c-*myc* and various parts of the Ig kappa locus. The Epstein–Barr virus (EBV) derived eukaryotic vector pHEBO (Sugden *et al.*, 1985) was chosen since it replicates as an episome and therefore permits the study of gene regulation without interfering position effects. The structure of the different constructs is shown in Figure 2B. All constructs were transfected into the BL cell line Raji. After selection with hygromycin several stable transfectants were obtained for each construct. The integrity and copy number of the constructs in the transfectants were tested by Southern blot analysis (data not shown). The copy number of the transfected plasmids was in the range of 20–50. Raji cells transfected with pRF26-20, pRF128-12 and pRF245-10 contained about the same number of plasmids. The copy number in cell lines containing pRF261-4 and -6 was ~2-fold higher.

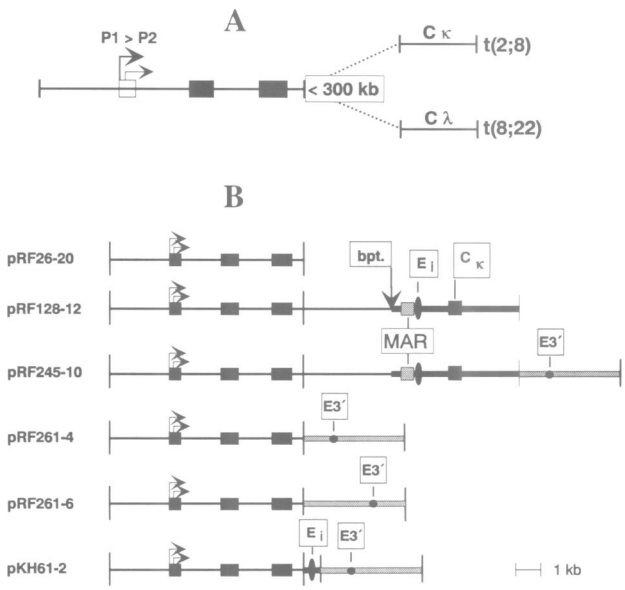

Fig. 2. (**A**) Schematic representation of chromosomal breakpoints of t(2;8) and t(8;22) chromosomal translocations. (**B**) Constructs used for stable transfection. Coding exons are shown as filled boxes, the first exon of c-*myc* is shaded. The two promoters of c-*myc*, P1 and P2, are indicated as horizontal arrows. The breakpoint region derived for LY91 is marked by a vertical arrow (bpt). The kappa intron and 3′ enhancers are shown as a solid filled ellipses and circles, respectively. All constructs are cloned into the polylinker site of the vector pHEBOpl.

The BL cell line Raji was chosen as a target for transfection for several reasons. First, the translocated c-*myc* allele in Raji displays a prominent promoter shift indicating that all factors '*in trans*' necessary for the dominant usage of promoter P1 are present in these cells. Secondly, the translocated c-*myc* allele in Raji carries a deletion at the end of exon 1, which allows differentiation by S1 protection analysis between transcripts derived from the endogenous translocated allele and transcripts from the transfected constructs (Rabbitts *et al.*, 1983). Figure 3 shows an S1 analysis of the different transfectants with an exon 1 specific probe, which allows detection of P1 and P2 derived transcripts. As described previously, mutations in the first exon and intron of the c-*myc* gene (pRF26-20 in Figure 2) were incapable of inducing c-*myc* expression, whereas a construct consisting of c-*myc*, a t(2;8) breakpoint and kappa sequences extending from the joining region J5 to 2.7 kb 3′ of the constant kappa gene (pRF128-12 in Figure 2) displayed only moderate activation of P2 (Polack *et al.*, 1991). This indicated that elements in addition to the kappa intron enhancer and/or the adjacent MAR are involved in the BL specific c-*myc* activation. The *Bam*HI fragment, which encompasses the human kappa 3′ enhancer was therefore added to the construct (pRF245-10, Figure 2B). S1 analysis revealed a dramatic increase in P1 and P2 specific transcripts from the transfected construct compared with the endogenous translocated c-*myc* allele (Figure 3). Visual inspection as well as densitometric analysis of the autoradiogram revealed a ratio of P1 to P2 derived transcripts >1 indicating the formation of a promoter shift as compared with a ratio of ~0.3 when the 3′ enhancer was missing from the construct. Five different transfectants were tested which showed essentially the same result (data not shown). The histogram in Figure 3C shows the densitometric data of two different exposures. Due to the addition of the kappa 3′ enhancer, the P1 promoter on construct pRF245-10 became ~30 times more active than on construct pRF128-12, whereas the P2 promoter was activated by a factor of eight. Northern blot analysis of cells stably transfected with this construct showed a strong increase in c-*myc* specific transcripts compared with untransfected cells (data not shown).

The kappa 3′ enhancer alone is not sufficient to activate c-myc

The enhancing activity of the human kappa 3′ enhancer was described to be 5- to 7-fold higher than that of the kappa intron enhancer (Judde and Max, 1992). Moreover, the fact that a kappa gene can be expressed in a cell which lacks NFκB, a major determinant of the activity of the kappa intron enhancer, indicates that the kappa 3′ enhancer is sufficient for the activation of the V promoter (Atchison and Perry, 1987, 1988; Blasquez *et al.*, 1989). Is this element therefore sufficient to activate c-*myc* and to induce the BL specific promoter shift? To address this question, the 4.1 kb *Bam*HI enhancer fragment was inserted in both orientations downstream of c-*myc* (pRF261-4 and pRF261-6, Figure 2). After establishment of stably transfected Raji cells the expression of the c-*myc* promoters was analysed. Transcripts derived from the P1 promoter on the construct were hardly visible and the P2 promoter became activated only slightly and independently of the orientation of the kappa 3′ enhancer. The result of the densitometric evaluation of the

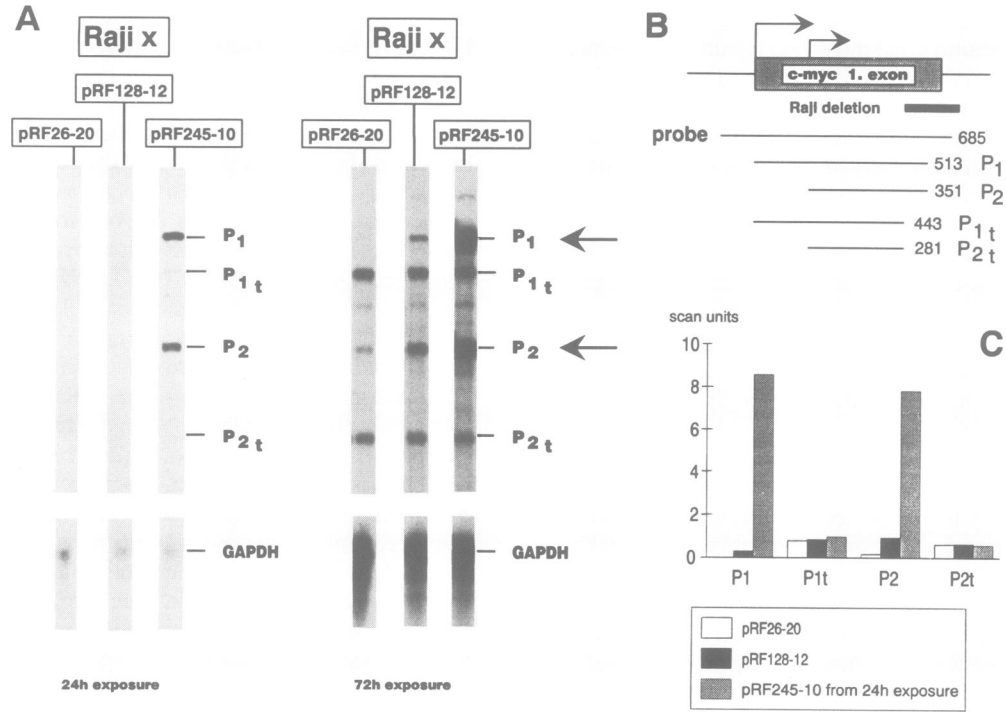

Fig. 3. Induction of the c-*myc* promoter shift through the Ig kappa locus. S1 analysis of c-*myc* promoter usage of Raji cells stably transfected with the constructs pRF26-20, pRF128-12 and pRF245-10. Total cellular RNA of the respective transfectants was analysed with the single-stranded probe shown in (**B**). Signals corresponding to the endogenous translocated allele are marked with P1t and P2t whereas signals derived from the transfected c-*myc* constructs are marked with horizontal arrows (P1 and P2). (**A**) Shows two different exposures of the same autoradiogram. As internal control a GAPDH probe was added to the hybridization mixture as described previously (Zimber-Strobl *et al.*, 1991). The results of densitometric scanning of the autoradiographs are plotted as a histogram in (**C**). The data for the transfectant containing the construct pRF245-10 were taken from a short exposure and adjusted according to the signal of P1t in the longer exposure.

autoradiogram is shown in Figure 4. The value of the endogenous P1 transcripts (P1t) served as an internal standard. The P1 promoters on the constructs containing only the kappa 3′ enhancers were ~35 times less active than on construct pRF245-10. The moderate activation of P2 did not exceed the one mediated by the kappa intron enhancer alone (pRF128-12 in Figure 3).

The intron and 3′ enhancers together are not sufficient for the BL specific c-myc activation

The next question was whether the intron and 3′ enhancer alone would be capable and sufficient to activate c-*myc* and to induce the BL specific promoter shift. To address this question, the 702 bp *Eco*RI fragment encompassing the kappa intron enhancer (Queen and Baltimore, 1983; Picard and Schaffner, 1984; Queen and Stafford, 1984; Sen and Baltimore, 1986) was inserted into the pRF261-4 construct between c-*myc* and the kappa 3′ enhancer (Figure 2). Analysis of the expression of this construct in stably transfected Raji cells revealed similar activation of P2 but weaker activation of P1 compared with pRF245-10 (Figure 4B). Densitometric analysis revealed that P1 on the construct pKH61-2 was about five times less active than P1 on pRF245-10. One or more additional elements present on pRF245-10 must therefore be involved in the BL specific c-*myc* activation.

Regulation of P1 activity by sodium butyrate

We have described previously that the expression of the translocated c-*myc* gene in BL cells can be transcriptionally down-regulated through the action of BA (Polack *et al.*, 1987). The effect of BA on the c-*myc* promoters P1 and P2 is shown in Figure 5 for Raji and LY91, a BL cell line with t(2;8) translocation. In Raji cells P1 and P2 transcripts from the translocation chromosome became almost undetectable in response to BA. In LY91 cells BA caused down-regulation of transcripts initiated at the P1 promoter by a factor of four whereas P2 transcripts remained more or less unchanged. Treatment of Raji cells transfected with pRF245-10 with BA strongly decreased P1 transcription derived from the construct with only little effect on P2 transcription (Figure 5). The effect of BA on c-*myc* expression from the construct thus closely resembled the effect of BA on the translocated c-*myc* gene of LY91 cells. This supports the notion that all elements contributing to c-*myc* deregulation in BL cells with t(2;8) translocation are indeed present on the construct.

In Raji cells, the P2 promoter of the normal c-*myc* allele was slightly active in the absence of BA and was moderately induced upon BA treatment (Figure 5). BA exerted a stimulatory, although more pronounced effect on the transfected c-*myc* gene without enhancers, particularly on promoter P2 (construct pRF26-20, Figure 5). The ratio of P1 and P2 derived transcripts was not affected by BA treatment. Induction of both c-*myc* promoters was also observed for c-*myc* under the control of the kappa intron or 3′ enhancer (Figure 5, Raji × pRF261-4 and Raji × pRF128-12) and was especially high in the cells carrying c-*myc* juxtaposed to the 3′ enhancer (pRF261-4). BA did not cause a prominent down-regulation of P1 transcripts in cells carrying the constructs of c-*myc* fused to both kappa intron and 3′ enhancers (data not shown) indicating that down-regulation by BA probably requires interaction with one or more elements not present on pKH61-2.

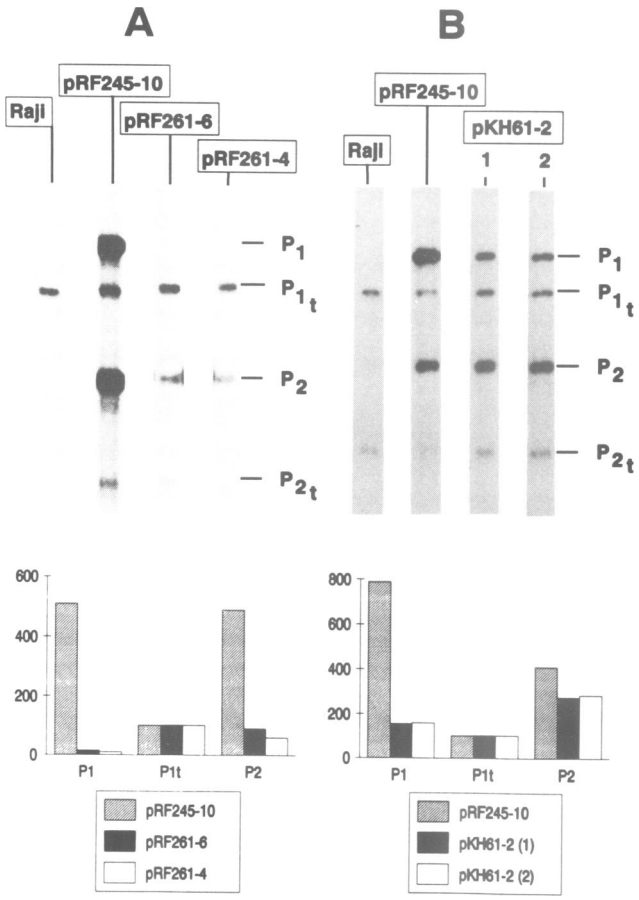

Fig. 4. Neither the kappa 3′ enhancer nor the canonical kappa intron enhancer together with the 3′ enhancer are sufficient to induce the promoter shift. S1 analysis of total cellular RNA from Raji cells stably transfected with the constructs indicated above each lane. Transfected cells containing c-*myc* either under the control of the kappa 3′ enhancer alone (pRF261-4 and -6, **A**) or the two kappa enhancers (pKH61-2, **B**) are shown in comparison with Raji cells transfected with the complete construct (pRF245-10) and untransfected Raji cells. For pKH61-2 two different transfected cell lines are shown (1 and 2). The S1 analysis of pRF245-10 transfected Raji cells shown in (A) and (B) was made from different RNA preparations. The probe used in these experiments is shown in Figure 3B. Data of the densitometric analysis are shown as histograms underneath.

Discussion

The concept that chromosomal translocations activate the c-*myc* gene and perturb its regulation in BL cells is well accepted, the precise molecular mechanism of c-*myc* activation is, however, not well understood. We have studied the interaction of regulatory elements in the Ig kappa locus with the c-*myc* gene to elucidate the mechanism of c-*myc* activation in BL cells with t(2;8) translocations. To this end, we have established a system which allows analysis of the contribution of different regulatory elements for c-*myc* activation and deregulation. The analysis of constructs which integrate into the host genome is hampered by the fact that the chromosomal surrounding of the integrated DNA may impose a negative or positive effect on the expression of the construct (position effect) (Brinster *et al.*, 1981; Palmiter and Brinster, 1985; Stief *et al.*, 1989). Blasquez *et al.* (1989, 1992) clearly demonstrated that expression of Ig kappa gene constructs stably integrated into the host genome varied by at least one order of magnitude. To overcome the problems associated with integration of expression vectors we have chosen an episomal vector system for our analysis. We and others have shown the high degree of reproducibility and the virtual lack of variability, when different cell clones transfected with identical constructs have been analysed (Grignani *et al.*, 1990; Polack *et al.*, 1991). Since it was our aim to reconstruct on episomal vectors c-*myc* activation and induction of the promoter shift, it was essential to have represented on the construct the elements of the c-*myc* gene which are required *in cis* to mediate the promoter shift. Studying the location of breakpoints in cell lines exhibiting a promoter shift, it was possible to narrow down the region to 340 bp upstream of c-*myc* promoter P1 (breakpoint of Ramos) (Hollis *et al.*, 1984) and 5739 bp downstream of P1 (breakpoint of BL37) (Wiman *et al.*, 1984). We have therefore chosen the 8 kb c-*myc* carrying the *Hin*dIII−*Eco*RI fragment spanning both of these breakpoints. The distance between c-*myc* and the Ig locus is highly variable on chromosome 8 ranging from <1 kb to >300 kb, whereas the translocation breakpoints on chromosome 2 are always localized within or very close to the joining region of the Ig kappa locus (Graham and Adams, 1986; Klobeck *et al.*, 1987; Hartl and Lipp, 1987; Henglein *et al.*, 1989). To reconstruct a t(2;8) translocation as closely as possible, a fragment spanning the breakpoint of LY91 cells was cloned (Henglein *et al.*, 1989) and inserted downstream of the 8 kb *Hin*dIII−*Eco*RI fragment carrying a mutated c-*myc* gene. This fragment carries 4.5 kb sequences of chromosome 8, the breakpoint in J5 and encompasses the MAR element, the intron enhancer and the kappa constant region. This construct exhibited only a moderate activation of c-*myc* without promoter shift after transfer into BL cells (Polack *et al.*, 1991). Inclusion of a 4.1 kb fragment carrying the 3′ enhancer into the constuct, however, strongly activated c-*myc* expression and induced the Burkitt specific promoter shift. To delineate the human kappa 3′ enhancer, we looked for regions of homology between the murine 3′ enhancer and human sequences 12 kb 3′ of the constant region (Müller *et al.*, 1990). Sequencing the entire 4.1 kb *Bam*HI fragment revealed a region of homology extending at the 5′ side the region recently defined by Judde and Max (1992) to represent the human 3′ enhancer. This additional stretch of homology, even though it does not contain a binding site for any known transcription factor, was found to contribute to the activity of the 3′ enhancer in transient transfection experiments (data not shown). To make sure not to miss any elements required for enhancer function, the complete 4.1 kb fragment has been included into the constructions.

The strong increase in c-*myc* transcription and promoter P1 usage left us with the possibility that the 3′ enhancer alone might account for the dramatic effect on c-*myc* transcription. However, fusion of the fragment carrying the 3′ enhancer directly to c-*myc* in both orientations had only a marginal effect on c-*myc* transcription and induced predominantly the promoter P2. The degree of activation thereby resembled the activation observed with pRF128-12 (the construct with the reconstructed breakpoint containing the intron enhancer and constant region without the 3′ enhancer). The effect of the 3′ enhancer on the c-*myc* promoter thus clearly differed from that on the β-globin, tk, or immunoglobulin gene promoters which were strongly induced by the 3′ enhancer (Blasquez *et al.*, 1989; Meyer and Neuberger, 1989; Pongubala and Atchison, 1991; Judde and Max, 1992).

Apparently, c-*myc* activation required the interaction of the intron and 3′ enhancer, but this does not imply that these

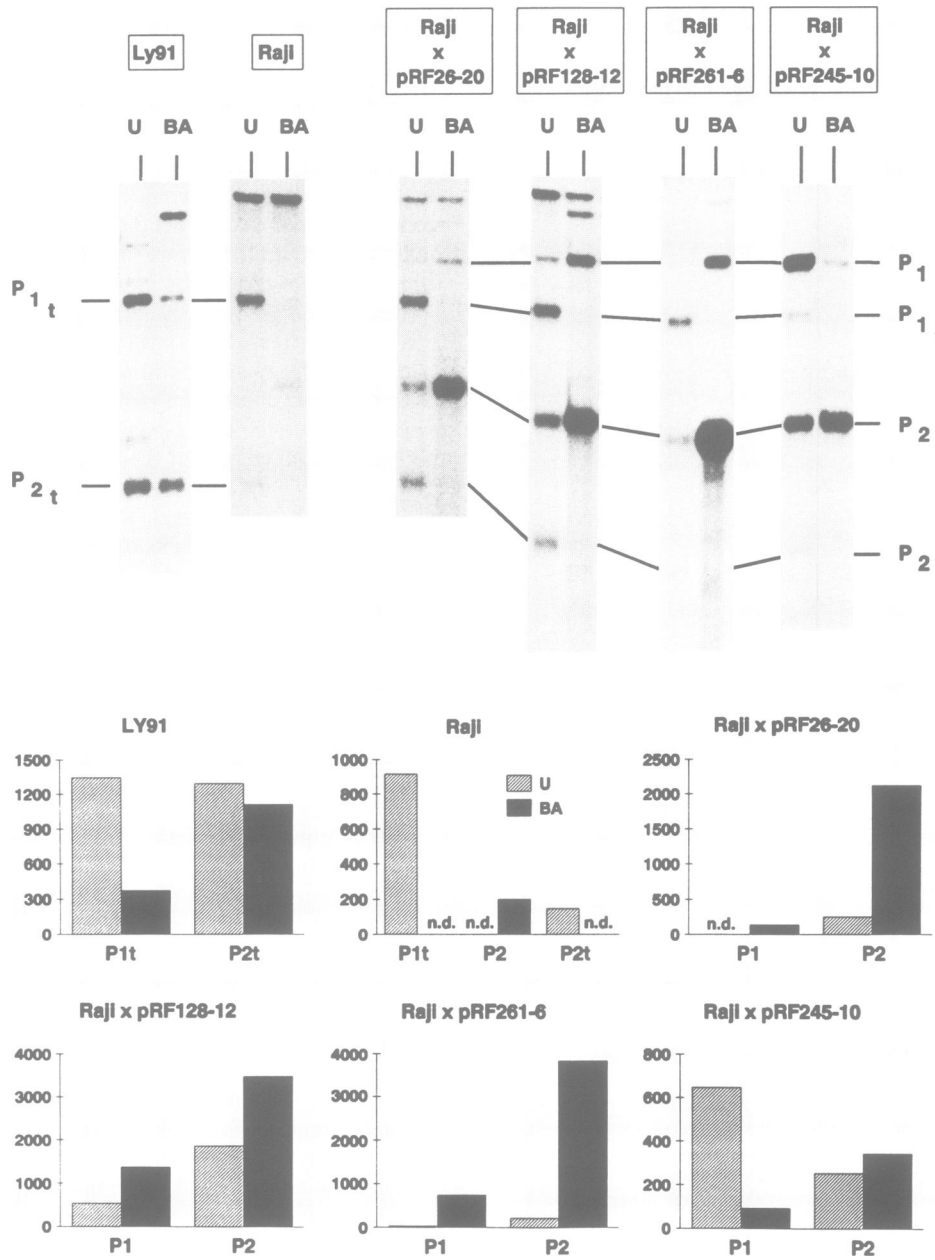

Fig. 5. Effect of BA treatment on c-*myc* expression in Raji and LY91 cells and Raji transfected with the constructs indicated. LY91 cells and pRF245-10 transfected Raji cells were treated for 4 h with BA whereas in all other experiments cells were incubated for 16 h with BA. The densitometric evaluation of each single experiment is shown as histograms underneath. n.d. indicates signal not detectable on the respective autoradiogram.

elements are also sufficient. To answer this question, the intron and 3′ enhancers alone were joined to the c-*myc* gene and the construct stably introduced into Raji cells. Analysis of the construct clearly revealed that both enhancers in conjunction are neither sufficient for full activation nor for induction of the promoter shift. This indicates that at least one additional element must be present in the kappa locus which interacts with the enhancers for full activation and deregulation of c-*myc*. The MAR element described by Cockerill and Garrard (1986) and reported by Blasquez *et al.* (1989) to have a stimulating effect on V-gene transcription in stable transfection experiments with integrating vectors is the prime candidate to represent this third interacting regulatory element. Experiments are under way to study the interaction of MAR with the kappa intron and 3′ enhancers either alone or in conjunction.

The interaction between the enhancer elements and the c-*myc* promoter requires the interaction of proteins binding to specific sites in the enhancer as well as in the promoter. It is generally assumed that proteins binding to remote sites on the DNA may interact specifically with each other and may thus bridge regulatory elements over considerable distance ('looping model'). By detailed mutation analysis of protein binding sites in both enhancers as well as in the c-*myc* promoter we will now address the question of which factors are involved in the interplay between the enhancers and the c-*myc* promoter over large distance.

Expression of the c-*myc* gene is not only regulated at the

level of transcription initiation but also at the level of transcript elongation (Bentley and Groudine, 1986; Eick and Bornkamm, 1986; Wright *et al.*, 1991; Krumm *et al.*, 1992; Meulia *et al.*, 1992; Strobl and Eick, 1992). In BL cells, regulation at the level of transcript elongation appears to be abrogated (Cesarman *et al.*, 1987; Eick *et al.*, 1988; Spencer *et al.*, 1990). Elongational blockage is, however, still operating on a construct carrying the c-*myc* gene fused to the 3′ enhancer which is stably introduced into Raji cells (Strobl *et al.*, 1993). The activation of the P1 and P2 promoters through the interaction with the complete kappa locus must therefore overcome this block to elongation. Whether the activation of c-*myc* through the complete kappa locus involves an increase in transcription initiation at P1 and P2 or exclusively the abrogation of transcriptional elongation, which consecutively may lead to prominent P1 promoter usage, remains to be determined.

The notion that we have identified all the elements in the kappa locus which are required for c-*myc* activation and perturbation of its regulation is strongly supported by a second line of evidence. We have shown earlier that treatment of BL cells with BA leads to a decrease of c-*myc* transcription from the translocated allele (Polack *et al.*, 1987). In BL cells with variant translocations this is mainly due to suppression of the P1 promoter. The negative effect of BA on c-*myc* transcription is specific for the translocated c-*myc* gene since the P2 promoter of the normal allele is slightly induced by BA in Raji cells (Figure 5). Construct pRF245-10 carrying c-*myc* fused to the kappa locus including the 3′ enhancer and stably introduced into Raji cells responded similarly to the translocated allele of LY91 cells to BA with P1 down-regulation. Remarkably, BA exerted its negative effect on P1 transcription only if the entire kappa locus including the 3′ enhancer was present on the construct. Constructs carrying the c-*myc* gene either alone or fused to the intron or 3′ enhancer responded differently to BA, i.e. BA induced transcription starting primarily at promoter P2. The effect of BA on these constructs thus resembled the action of BA on the normal allele of Raji cells except that the response of the constructs was much stronger than that of the endogenous normal allele, a difference presumably reflecting the differences in copy number. Stimulation of P2 transcription by BA was particularly strong with the construct carrying c-*myc* fused to the 3′ enhancer. BA appeared to unmask the strong activity of the 3′ enhancer by relieving negative control at the level of transcript elongation (Strobl *et al.*, 1993). The opposing effects of BA on the endogenous normal and translocated alleles as well as on constructs carrying the entire kappa locus or only parts of it strongly support the notion that we have functionally reconstructed a t(2;8) translocation.

Only little is known about the mechanism of action of BA in the cell. A well known effect is the inhibition of histone deacetylase leading to an accumulation of hyperacetylated histones (Riggs *et al.*, 1977). A link between the level of histone acetylation and gene regulation was recently presented by Lee *et al.* (1993). Studying transcription control of the 5S RNA gene, the authors could show that acetylation of histone facilitates access of transcription factors to DNA. The fact that the normal allele and constructs carrying the c-*myc* gene either alone or in conjunction with only the intron or 3′ enhancer are induced by BA, is compatible with the view that BA increases promoter accessibility and relieves negative constraints on transcription (Klehr *et al.*, 1992). Loosening the chromatin structure by hyperacetylation may at the same time interfere with protein−protein interactions over distance which supposedly require an organized chromatin configuration. It will be interesting to see whether the down-regulation of the translocated c-*myc* allele requires the presence of matrix attachment sites and whether another inhibitor of histone deacetylase exerts a similar effect to BA on c-*myc* transcription. For the identification of the transcription factors which are involved in c-*myc* activation by immunoglobulin regulatory elements on the one hand and in mediating the effect of BA on the other, a detailed mutational analysis of the c-*myc* promoter and Ig enhancer elements is now required.

Materials and methods

DNA cloning and sequence analysis

Constructs pRF26-20, pRF128-12, and the vector pHEBOPL are described in Polack *et al.* (1991). The 4.1 kb *Bam*HI fragment encompassing the kappa 3′ enhancer was described by Müller *et al.* (1990). This *Bam*HI fragment was inserted in inverted orientation compared with the germline configuration into the *Bam*HI site 3′ of the constant kappa gene of pRF128-12 giving rise to pRF245-10. For construction of pRF261-4 and -6, the enhancer carrying fragment was inserted into pRF115-3 at the *Bam*HI site 3′ of c-*myc* in construct. pRF115-3 carries the mutated BL60 c-*myc* as a *Hin*dIII−*Sal*I fragment inserted into pHEBOPL with a 5 bp tag inserted at the *Bst*EII site within the second exon of c-*myc*. To obtain pKH61-2, the kappa intron enhancer was inserted as a 702 bp *Eco*RI−*Eco*RI fragment after changing the *Eco*RI−*Sal*I sites into the *Sal*I site between c-*myc* and the kappa 3′ enhancer in pRF261-4. The 4.1 kb *Bam*HI fragment and the 702 bp *Eco*RI fragment encompassing the intron enhancer were sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977).

The search for transcription factor binding sites was done with the program FACTOR (1978 factor binding sites) which is part of the HUSAR program package available on the CONVEX computer at the German Cancer Center (Heidelberg).

Cells, tissue culture, stable transfection and BA treatment

Raji and LY91 are BL cell lines with t(8;14) and t(2;8) translocations, respectively (Bernheim *et al.*, 1981; Pulvertaft, 1965). Cells were grown to 3−6 × 10^5 cells/ml in 10% fetal calf serum, RPMI-1640 medium, supplemented with penicillin, streptomycin and L-glutamine. Transfection was performed by electroporation essentially as described by Toneguzzo *et al.* (1986). In brief, cells were resuspended in 250 μl of cold RPMI-1640 and 10 μg of plasmid DNA was added. The cell−DNA suspension was placed in an electroporation chamber (Bio-Rad: gene pulser with capacity extender), and a high voltage (220 V) pulse was applied. After 5 min on ice the cells were transferred to 10 ml of prewarmed growth medium. Cells were then grown for 48−72 h before selection with 300 μg/ml hygromycin B (Calbiochem). BA treatment was carried out with 3 mM sodium butyrate added to the cell culture from a 500 mM stock solution (Sigma).

RNA analysis

Total cellular RNA samples were prepared by extraction with guanidinium thiocyanate followed by centrifugation in caesium chloride (Sambrook *et al.*, 1989). RNA samples were quantitated by spectrophotometric analysis and ethidium bromide staining of formaldehyde−agarose gel. S1 analysis with a probe specific for the first exon of c-*myc* and Northern blotting was carried out as described previously (Eick and Bornkamm, 1989).

Expression levels were quantitated by densitometry using a video densitometer (Cybertech). Densitometric scanning data for Raji cells transfected with pRF26-20 and pRF128-12 were obtained from a longer exposure (Figure 3). Since the film does not respond to strong signals in a linear fashion, the data for the Raji cells transfected with pRF245-10 were taken from a shorter exposure and subsequently adjusted to the signal of the P1t promoter on the longer exposure.

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