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Activation of a translocated c-*myc* gene: Role of structural alterations in the upstream region

(translocation/oncogenes/lymphomagenesis)

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ABSTRACT **The translocated c-*myc* gene in AW-Ramos, a Burkitt lymphoma cell line carrying the 8;14 translocation, is expressed at 2- to 5-fold higher levels than c-*myc* in lymphoblastoid cell lines. The translocation event has joined c-*myc* to the *IgM* switch region. As a consequence, a recently identified immunoglobulin transcriptional enhancer element is not linked to the translocated c-*myc* gene. Chromosomal recombination occurs ≈340 nucleotides upstream of the c-*myc* 5′ cap site, leaving all three c-*myc* exons intact. The nucleotide sequences of the two coding exons in the translocated c-*myc* gene are identical to those of the normal c-*myc* gene. Nucleotide sequence analyses of the first, noncoding c-*myc* exon and of the region between this exon and the chromosomal recombination point reveal two single-base differences from normal c-*myc*. Our data indicate that altered expression rather than an altered gene product is responsible for c-*myc* activation in AW-Ramos cells and that this is a result of either loss of regulatory sequences located >340 nucleotides upstream of c-*myc* or disruption of normal c-*myc* regulation by one or both base substitutions. Alternatively, unidentified enhancer-like sequences in the *Ig* locus may alter the expression of c-*myc*.**

The genomes of vertebrate cells contain a class of genes termed cellular oncogenes, or proto-oncogenes, which have the potential to induce neoplastic transformation (1). A variety of mutational events can convert a proto-oncogene into an "activated" oncogene. Analyses of chicken B-cell lymphomas induced by avian leukosis virus (ALV) have provided strong evidence that proviral integration adjacent to the c-*myc* gene causes enhanced transcription of this gene and neoplastic growth (2–4). In addition, amplification of the c-*myc* gene in cell lines derived from a promyelocytic leukemia (5, 6) and a colon carcinoma (7) has been associated with augmented levels of c-*myc* mRNA. Thus, altered expression of the c-*myc* gene appears to be the crucial factor in activation of the oncogenic potential of this gene.

Many types of tumors carry specific chromosomal defects (8–10). This information and the demonstration of c-*myc* activation by proviral integration led to the suggestion that chromosomal translocation might activate a proto-oncogene by joining it with positive control elements belonging to another gene (2, 8). In Burkitt lymphomas and some non-Burkitt lymphomas the c-*myc* gene, which is normally located on chromosome 8 (11–13), is recombined with one of the *Ig* loci on chromosomes 2, 14, and 22 (14–17). It seems likely that these translocations result in altered expression of c-*myc* and that this is causally related to the development of neoplasia.

Analyses of c-*myc* mRNA levels and molecular cloning of this gene from a number of Burkitt lymphomas have brought to light several notable features: (*i*) The translocated c-*myc* gene is expressed at significant but variable levels in Burkitt lymphomas (18–23). (*ii*) In most Burkitt lymphomas carrying the 8;14 translocation, the c-*myc* gene is recombined with the *IgM* switch region, although recombination outside this region has also been observed (21, 22, 24–26). (*iii*) The breakpoint in the c-*myc* gene varies considerably in different Burkitt lymphomas and has been mapped upstream of c-*myc* exon 1 as well as in the intron between exon 1 and exon 2 (20–22, 26, 27).

The variability in levels of c-*myc* expression and in the chromosomal recombination points on chromosomes 8 and 14 makes it difficult to draw conclusions as to a general mechanism for c-*myc* activation in Burkitt lymphomas. Explanations for specific cases have been put forward, including transcriptional activation by an *Ig* enhancer element (21) and structural alterations in the c-*myc* gene product resulting from mutations in coding sequences (28). In addition, it has been proposed that sequences within the 5′ noncoding c-*myc* exon are involved in control of c-*myc* expression (29–31) and that removal or extensive mutation of this exon may be responsible for transcriptional activation (23).

We describe here a translocated c-*myc* gene that does not fall into any of these categories. Because this c-*myc* gene has been joined to the *IgM* switch region the known *Ig* enhancer element is not retained on chromosome 14 and cannot influence the expression of the translocated c-*myc* gene. Nucleotide sequencing failed to reveal any differences between the coding region of this gene and that of the normal c-*myc* gene. Furthermore, no extensive mutations were found in the first, noncoding exon or in the region comprising the 340 nucleotides upstream of the first exon, although we do find two single-base substitutions in these regions. We discuss mechanisms that might be responsible for altered control of c-*myc* expression in AW-Ramos.

MATERIALS AND METHODS

Cell Lines. The AW-Ramos cell line (provided by J. Fogh, Memorial Sloan–Kettering Cancer Center) is derived from an American Burkitt lymphoma (32). Manca, a non-Hodgkin lymphoma cell line, has been described (33). Both cell lines carry the 8;14 translocation, express surface IgM, and form tumors in nude mice. The HL-60 cell line has been described (5, 6). Lymphoblastoid cell lines (LCs) derived from Epstein–Barr virus-infected peripheral blood lymphocytes were provided by T. Albino and L. Old (Memorial Sloan–Kettering Cancer Center).

Southern and RNA Blot Hybridization Analyses. Restriction-enzyme-digested size-fractionated DNA was transferred to nitrocellulose paper (34). Glyoxal agarose gel elec-

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Abbreviations: LC, lymphoblastoid cell line; kb, kilobase(s); bp, base pair(s).

trophoresis and RNA transfer were performed as described (35, 36). Hybridization was for 24 hr in 0.3 M NaCl/30 mM sodium citrate, pH 7/50% formamide/5% dextran sulfate using a probe labeled by nick-translation to a specific activity of $\geq 10^8$ dpm/μg (37). Hybridized filters were washed in 15 mM NaCl/1.5 mM sodium citrate, pH 7/0.1% $NaDodSO_4$ at 37°C.

Molecular Cloning. A cosmid library of Manca DNA was constructed in cosmid pTCF (38). One cosmid clone, Cosmyc, hybridized both to a v-*myc* probe and to an *IgM* probe. A 6.0-kilobase (kb) *Eco*RI/*Hin*dIII fragment containing c-*myc* as well as *IgM* sequences was isolated from Cosmyc and subcloned into pBR322. A library of AW-Ramos DNA was constructed in λgtWES, and the 7.5-kb *Eco*RI fragment from a recombinant phage that hybridized to a c-*myc* probe was subcloned into pBR322.

Nucleotide Sequence Analysis. Nucleotide sequencing was performed according to the method of Maxam and Gilbert (39).

RESULTS

Points of Chromosomal Recombination Suggest Different Modes of c-*myc* Activation. Fig. 1 shows restriction maps of the translocated c-*myc* genes in Manca and AW-Ramos cells. The recombination point in Manca cells is such that the c-*myc* gene is close to a recently identified *Ig* transcriptional enhancer element (21). The translocation event results in truncation of the c-*myc* gene, joining this gene to the *Ig* sequences at a point within the first intron. In contrast, the translocated c-*myc* gene in AW-Ramos cells has been recombined to the *IgM* switch region; all three c-*myc* exons remain intact. This gene arrangement does not permit the known *Ig* enhancer to act on the translocated c-*myc* gene.

Nucleotide Sequencing Across the Chromosomal Recombination Points Reveals Short Stretches of Homology. To localize more precisely the points of chromosomal recombination in Manca and AW-Ramos, we have determined the nucleotide sequence across the points of recombination in both cell lines (Fig. 2).

In Manca, chromosomal linkage has occurred between a point 291–293 nucleotides downstream of exon 1 of the c-*myc* gene and a point between the *IgM* switch region and the *J* segments, a few hundred nucleotides downstream of J_H6. (Because the sequence T-C occurs in both the germ line *IgM* and the normal c-*myc* in the region of the recombination point an unambiguous localization of this point is not possible.) The junction is at a point that is not used for normal immunoglobulin heavy chain gene rearrangement. An almost perfect direct repeat (seven out of eight nucleotides) occurs on both sides of the recombination point (Fig. 2).

Comparison of the sequence across the chromosomal junction in AW-Ramos cells to the normal c-*myc* sequence

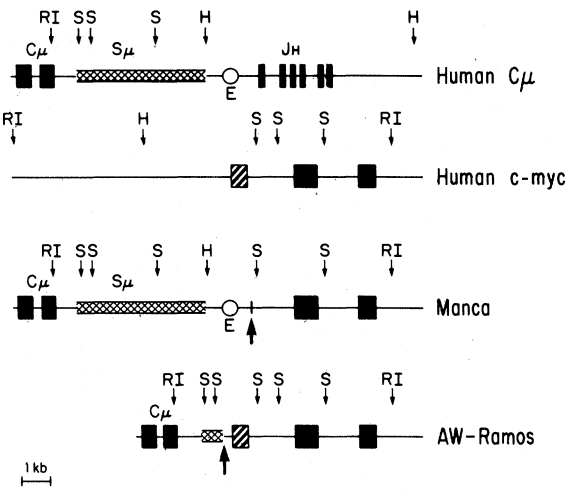

FIG. 1. Restriction maps of part of the human *IgM* locus (40), the normal human c-*myc* gene, and the translocated c-*myc* genes of Manca and AW-Ramos. The heavy chain constant domain-encoding exons (C_μ), the switch region (S_μ, cross-hatched regions), and the six functional human *J* segments (J_H) are indicated. E denotes the position of a recently identified transcriptional enhancer element (21). The first, noncoding exon in the human c-*myc* gene is represented by the hatched box; black boxes indicate coding exons. Large arrows indicate chromosomal junction points in Manca and AW-Ramos. Restriction enzyme cleavage sites for *Eco*RI (RI), *Hin*dIII (H), and *Sac* I (S) are indicated by small arrows.

reveals that the sequences diverge at a point 340 base pairs (bp) upstream of c-*myc* exon 1. Thus, chromosomal recombination has probably occurred within a few nucleotides of this point. As suggested by restriction mapping data, the c-*myc* gene has been recombined with a sequence containing multiple *IgM* switch pentamers: G-G-G-C-T, G-A-G-C-T, and G-A-A-C-T. In Fig. 2 the pentamers are shown as they occur on the c-*myc* sense strand: A-G-C-C-C, A-G-C-T-C, and A-G-T-T-C. No switch-like pentameric units are apparent in the c-*myc* sequence. It is noteworthy that a 4-bp inverted repeat is found immediately upstream and downstream of the chromosomal junction in AW-Ramos (Fig. 2).

Levels of c-*myc* mRNA in Manca and AW-Ramos Cells. Poly(A)$^+$ RNA from exponentially growing cells was characterized by RNA blot hybridization analysis (Fig. 3). Hybridization was carried out using a probe corresponding to c-*myc* exon 2. In a LC derived from Epstein–Barr virus-infected peripheral blood lymphocytes, the c-*myc* gene was expressed as one major mRNA species of ≈2.4 kb and possibly as a minor species of ≈2.6 kb (Fig. 3*A*). This observation is consistent with the presence of two transcription start sites in the c-*myc* gene (26, 31). The translocated c-*myc* gene in

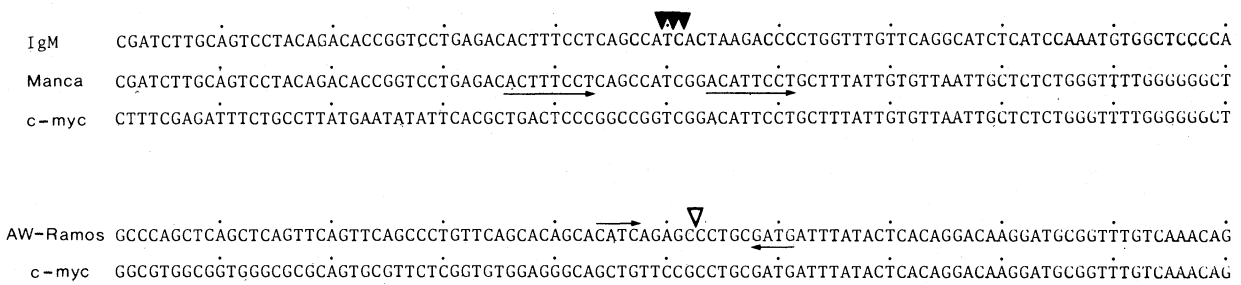

FIG. 2. Nucleotide sequence across the chromosomal junction points in Manca and AW-Ramos. The sequences are compared with the sequence of an unrearranged c-*myc* gene (ref. 31; unpublished data). For Manca, the sequence of the germ line *IgM* region is also included for comparison. Solid triangles indicate possible points of chromosomal junction (see text). In AW-Ramos, comparisons with the germ line *IgM* sequence cannot be made, because the nucleotide sequence of this region in the *IgM* locus has not been determined. The open triangle indicates the point at which the two sequences diverge. As in Manca, some ambiguity remains as to the exact nucleotide at which the junction occurs. Nucleotide sequence repeats near the chromosomal junction points are indicated by arrows.

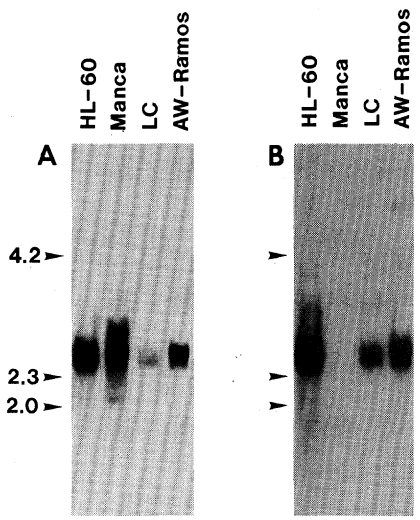

FIG. 3. RNA blot hybridization analysis of c-*myc* mRNA in Manca and AW-Ramos. Five micrograms of poly(A)$^+$ RNA, isolated from exponentially growing cells of the indicated type, were applied to each lane. DNA probes were labeled by nick-translation; autoradiograms of the hybridized filters are shown. Size markers are in kb. (*A*) Hybridization using a probe corresponding to the second exon (a 2.0-kb *Sac* I fragment derived from Cosmyc). (*B*) Rehybridization of the filter used in *A* with a probe corresponding to the first exon [a 1.3-kb *Sma* I/*Xba* I fragment derived from cAIDS.4 (31)].

Manca cells was transcribed into multiple mRNA species; however, two major transcripts of 2.5 kb and 2.8 kb, respectively, could be discerned (Fig. 3*A*). The level of c-*myc* mRNA in Manca cells was equal to or higher than the level of c-*myc* mRNA in the HL-60 cell line (Fig. 3*A*), in which the c-*myc* gene is amplified 8- to 32-fold (5, 6). We have previously shown that the high level of c-*myc* expression in Manca cells results from the placement of the *Ig* enhancer element adjacent to c-*myc* and that the initiation of transcription from cryptic promoters in the intron upstream of exon 2 gives rise to multiple transcripts (21). The translocated c-*myc* gene in AW-Ramos cells was expressed as mRNA species with apparent sizes corresponding to those of the mRNA species detected in the LC (Fig. 3*A*). The major mRNA species comigrated with the 2.6-kb mRNA transcribed from the unrearranged c-*myc* gene in the LC. The level of c-*myc* mRNA in AW-Ramos cells was at least 5-fold elevated compared to that in the LC, as judged by the intensity of the hybridized bands. Although the c-*myc* mRNA levels in different LCs were variable, the c-*myc* mRNA level in all LCs that we have analyzed was ≤20% of the level in AW-Ramos (data not shown).

The same filter was washed and rehybridized with a probe corresponding to the first c-*myc* exon. As expected, this probe detected the same mRNA species in the LC and in AW-Ramos cells as did the probe corresponding to exon 2, confirming that these mRNAs contained sequences derived from both exon 1 and exon 2 (Fig. 3*B*). The fact that no hybridizing bands were seen for Manca mRNA is consistent with restriction mapping of the translocated Manca c-*myc* gene and nuclease S1 analysis of Manca mRNA (21); no exon 1 sequences are present in the translocated c-*myc* gene in Manca cells or in the mRNA synthesized from the truncated gene. Moreover, because normal c-*myc* mRNA should hybridize to the exon 1 probe, the data in Fig. 3*B* indicate that the expression of the normal unrearranged c-*myc* allele in Manca cells was below the level of detection.

The Coding Sequence of the Translocated c-*myc* Gene in AW-Ramos Cells Is Identical to the Coding Sequence of the Normal c-*myc* Gene. Because the translocated c-*myc* in AW-Ramos cells is not linked to the known *Ig* transcriptional enhancer element, and because the level of expression of this gene is only moderately augmented, we wished to assess whether mutations affecting the primary structure of the c-myc protein could be responsible for activating the oncogenic potential of this gene. Therefore, we determined the nucleotide sequence of the two coding c-*myc* exons and some flanking sequences (data not shown). No nucleotide changes were found within the coding sequence of the translocated AW-Ramos c-*myc* gene when it was compared with the sequence of the normal c-*myc* gene (41, 42). We conclude that the protein encoded by this translocated gene is identical to the normal c-*myc* gene product.

The First c-*myc* Exon and the Region Immediately Upstream Are Not Extensively Mutated in AW-Ramos Cells. We also sequenced exon 1 of the AW-Ramos c-*myc* gene and the region between this exon and the chromosomal junction point to determine whether mutations had been introduced in this region (Fig. 4). For comparison, the nucleotide sequence of the corresponding region of a normal c-*myc* gene (42) is shown. [The sequence shown for this normal c-*myc* gene is identical to that of another unrearranged c-*myc* gene (ref. 31; unpublished data)]. As can be seen, the AW-Ramos c-*myc* sequence differs by only two single-base substitutions from the normal c-*myc* sequence. One substitution (adenine for guanine) is located at a position 158 bp upstream of the 5′ cap site. The other substitution (thymine for adenine) occurs at a position 33 bp upstream of the 3′ end of exon 1. Two other recently published normal c-*myc* sequences (23, 44) differ from the normal sequences discussed here and from each other by a few scattered substitutions, deletions, and insertions but they do not contain the two substitutions found in AW-Ramos c-*myc*.

DISCUSSION

We have characterized the translocated c-*myc* genes in two human B-cell lymphoma lines in an attempt to elucidate the mechanism(s) responsible for c-*myc* activation. In the case of Manca, c-*myc* is not joined to the *IgM* switch region. Accordingly, no switch-like pentamers are present in the sequence close to the chromosomal junction point. However, we do find an imperfect direct repeat immediately upstream and downstream of the breakpoint (Fig. 2). The nucleotide sequence across the chromosomal junction point in AW-Ramos contains typical switch-region pentamers in the sequence derived from chromosome 14 and a short inverted repeat on both sides of the junction point. It is possible that the repeats around the junction points in both cell lines are involved in mediating the joining of the two chromosomes.

Activation of the c-*myc* gene in various tumors has been associated with altered regulation (2–7). We have shown that a high level of expression from the translocated c-*myc* gene in Manca cells occurs as a result of a nearby *Ig* enhancer element (21). Juxtaposition of the c-*myc* gene and the immunoglobulin heavy chain enhancer has recently been observed in several other lymphomas (M. Diaz, personal communication; refs. 22 and 49). However, a majority of translocated c-*myc* genes in Burkitt lymphomas are recombined with the immunoglobulin heavy chain locus in a manner that does not allow the known *Ig* transcriptional enhancer element to exert an effect on the c-*myc* gene (20, 22, 26, 27). The mode of activation of the translocated c-*myc* genes that are not juxtaposed to an *Ig* enhancer element and that are expressed at only moderately elevated levels has remained obscure. We have analyzed a c-*myc* gene of this type from the Burkitt lymphoma cell line AW-Ramos. As in many other Burkitt lymphomas (20, 22, 26), the breakpoint on chromosome 8 in this cell line is located upstream of the c-*myc* 5′ cap site so that all three exons are intact. Thus, translational activation of the expression of the AW-Ramos c-*myc* gene by trunca-

FIG. 4. Comparison of the sequence of the first exon of a normal c-*myc* gene (ref. 42; unpublished data) and a region comprising 340 nucleotides immediately upstream with the corresponding sequence of the translocated c-*myc* gene in AW-Ramos. Dashes indicate positions in the AW-Ramos sequence that are identical with those in the normal sequence. The two initiation sites for c-*myc* transcription (▼; ref. 31) and the splice donor site at the 3′ end of exon 1 (▽) are indicated. "TATA" sequences (43) are within boxes.

tion of this gene (31) is ruled out. Neither an effect of the known *Ig* enhancer element nor major rearrangements within the c-*myc* gene can be invoked to explain c-*myc* activation in lymphomas such as AW-Ramos.

Recent reports have suggested that activation of c-*myc* might in some cases result from mutations within coding sequences. Rabbitts *et al.* (28) found multiple mutations in the coding sequence of the translocated c-*myc* in the Raji cell line. Likewise, at least one mutation that would lead to an amino acid change in the c-myc protein was found in the chicken c-*myc* gene from an avian leukosis virus-induced lymphoma (45). It is interesting to note, therefore, that our nucleotide sequence analysis of the AW-Ramos c-*myc* gene failed to detect any mutations within the coding region. Furthermore, the c-*myc* mRNA appears to be structurally normal by several criteria: (*i*) sequences corresponding to exon 1 are present, (*ii*) hybridization analysis reveals c-*myc* mRNAs of normal size, (*iii*) at least the most upstream of the two normal transcription start sites is used (data not shown), and (*iv*) nucleotide sequences of the normal splice sites are unchanged. These results and the absence of mutations in the coding region of the translocated c-*myc* gene in the Burkitt lymphoma cell line BL22 (26) provide strong evidence that changes in the structure of the c-*myc* gene product are not a requirement for c-*myc* activation in Burkitt lymphomas.

The hybridization analyses indicate substantial c-*myc* transcription in AW-Ramos cells. In addition, by analogy with Manca cells and with other Burkitt lymphomas (21, 46), it is likely that c-*myc* transcription in AW-Ramos cells derives almost exclusively from the translocated allele. Two distinct interpretations of this can be made. First, the absolute level of c-*myc* mRNA in AW-Ramos cells and other Burkitt lymphomas may be less important than its constitutive production. Recent studies (47, 48) suggest that c-*myc* expression is regulated in a cell-cycle-dependent manner, the perturbation of which may contribute to neoplasia. Second, the absence of transcription from the unrearranged allele suggests that Burkitt lymphomas are composed of cells whose normal counterparts do not express c-*myc*. In this case, the consequence of translocation may be to activate transcription from the previously silent gene, in which case c-*myc* expression itself is perhaps the crucial proliferative stimulus. The mechanism responsible for transcriptional enhancement or deregulation of those translocated c-*myc* genes on which the known *Ig* enhancer cannot act remains to be elucidated. Several groups (29–31) have proposed that the first, noncoding exon plays a role in regulation of the c-*myc* gene and that somatic mutations within this region might activate c-*myc* (23). The translocated c-*myc* gene in AW-Ramos, however, has not acquired extensive somatic mutations in this region. The nucleotide sequence of the first exon and the region upstream of it is in fact very similar to the normal c-*myc* sequence. Yet, two single-base substitutions are present in the AW-Ramos c-*myc* gene. The upstream substitution occurs in a region that might be involved in regulation of c-*myc* expression. An identical octameric sequence, C-G-G-C-T-G-A-G, is altered in both AW-Ramos and Manca. As a result of the substitution the sequence in AW-Ramos is C-G-A-C-T-G-A-G. In Manca, the same octamer [located downstream of exon 1 at one of the major new initiation sites (21) instead of upstream] is changed to C-G-G-C-T-G-A-A (data not shown). It remains to be seen whether these substitutions play a role in c-*myc* activation.

The common feature of all known c-*myc* translocations in Burkitt lymphomas is the juxtaposition of the c-*myc* and *Ig* loci. If a single mechanism for activation of the translocated c-*myc* gene exists, it may be related to this juxtaposition. Known properties of the immunoglobulin heavy chain locus in mature B cells are its high transcriptional activity and its

hypermutability. Either or both of these properties may be directly involved in activation of translocated c-*myc* genes in individual lymphomas. Our results show that neither introduction of mutations within the coding region, nor displacement of the first exon, nor introduction of extensive mutations within the first exon are prerequisites for c-*myc* activation. Instead, we suggest that activation of the translocated c-*myc* gene in AW-Ramos occurs as a result of changes in upstream regulatory sequences, either by limited base substitutions or by removal of regulatory sequences located more than 340 bp upstream of the c-*myc* gene. Alternatively, unidentified *cis*-acting positive transcriptional regulators within the immunoglobulin heavy chain locus, brought close to c-*myc* by chromosomal translocation, might override the normal regulation of c-*myc* expression.

Note Added in Proof. Siebenlist *et al.* (49) have recently identified several DNase I-hypersensitive sites upstream of the c-*myc* gene and have suggested that these may play important regulatory roles. Three of these sites have been removed from the rearranged c-*myc* gene in AW-Ramos as a result of the translocation.

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