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A translocated human c-*myc* oncogene is altered in a conserved coding sequence

(translocation/DNA/Burkitt lymphoma)

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ABSTRACT **We have cloned and characterized a c-*myc* (now designated MYC) oncogene that had been translocated into the μ switch region of the immunoglobulin heavy chain locus in a Burkitt lymphoma cell line. The breakpoint of the translocation occurs within the first intron of the c-*myc* gene, thereby separating the untranslocated first exon from the two coding exons. Transcription from the translocated gene arises from a cryptic promoter within the first intron, which produces a 438-nucleotide untranslated 5′ region. The amino acid sequence of the protein encoded by the c-*myc* gene has been substantially altered. In particular, a compensating set of frameshift mutations alters a string of 24 amino acids in a region of the protein tightly conserved in human, mouse, and chicken c-*myc* genes as well as in the human N-*myc* and L-*myc* oncogenes. Despite this, the mutated gene retains a reduced transforming ability in a rat embryo fibroblast focus-formation assay.**

Burkitt lymphoma is a human B-cell malignancy characterized by consistent reciprocal chromosomal translocations involving chromosome segment 8q24, which joins the protooncogene c-*myc* (now designated MYC) and one of the chromosome segments bearing the immunoglobulin genes (1–6). These chromosomal translocations have been presumed to activate the c-*myc* gene by disturbing its regulated expression and several models have been proposed that suggest mechanisms by which this deregulation may be produced (7–12). None of these exclude the possibility that coding alterations may affect the transforming activity of the translocated c-*myc* gene, although there are several examples of translocated c-*myc* genes of unaltered coding capacity (10, 13).

One of the major impediments to our understanding of how this deregulation occurs and the role that c-*myc* plays in the process of malignant transformation is that the normal function of the gene product in the untransformed cell remains obscure. The protein encoded by the c-*myc* gene is known to be a DNA-binding protein that is localized within the nucleus (14–17), to be regulated with respect to cell growth (18), and to act as an intracellular mediator of the growth response of cells to growth factors (19). Because of its DNA-binding properties and its sequence homology with the adenovirus E1A protein (20), it has been proposed that the c-*myc* gene product may be a regulatory protein and it has recently been shown that the transcription of the *Drosophila* heat shock protein is markedly increased when DNA encoding the heat shock protein is cotransfected with a normal c-*myc* gene (21).

Despite these insights, however, the mechanism by which the c-*myc* gene product acts to cause transformation and the relation of the structure of the protein to its various DNA-binding and putative regulatory functions remains unknown. One approach to define the structural regions of the c-*myc* gene product important to its transforming potential is to identify naturally occurring mutants of the protein that alter its amino acid structure but allow it to retain its transforming capacity. We report here the cloning and characterization of a translocated c-*myc* oncogene from a Burkitt lymphoma cell line (Ly65) in which the breakpoint occurs within the first intron of the gene, thus removing the first untranslated exon from the coding region of the gene. The coding sequence of this translocated gene has been altered by both single amino acid substitutions and a frameshift mutation in the second exon. These extensive alterations occur in one of two regions conserved among other species and other members of the c-*myc* gene family. Despite these changes in the protein sequence the translocated gene retains its transforming ability, though at reduced efficiency.

MATERIALS AND METHODS

Cell Lines. The Ly65 cell line was obtained from the International Agency for Research on Cancer (Lyon, France). This cell line has been shown to be Epstein–Barr virus positive and to produce cytoplasmic μ heavy chains but no detectable light chains (3). The lymphoblastoid cell line IARC 100 was obtained by immortalization of normal peripheral lymphocytes by the B95/8 strain of Epstein–Barr virus. The cell lines were grown in RPMI 1640 medium supplemented with 20% fetal calf serum.

Genomic Cloning. DNA preparation, *Eco*RI restriction fragment enrichment, ligation into phage Charon 4A arms, and screening of plaques were carried out as described (13).

S1 Nuclease Protection and Primer-Extension Analysis of Ly65 mRNA. Single-stranded uniformly labeled DNA probes were prepared as described (13). S1 nuclease analysis was done as described by Taub *et al.* (11).

Hybridization mixtures intended for reverse transcription were ethanol precipitated. Then, the dry pellets were dissolved in 19 μl of buffer (50 mM Tris·HCl, pH 8.3/6 mM $MgCl_2$/40 mM KCl/0.2 mM dATP/dCTP/dGTP/dTTP), 24 units of avian myeloblastosis virus reverse transcriptase in 1 μl of buffer was added, and the mixture was incubated at 37°C for 1 hr. It was then ethanol precipitated, and the precipitate was washed and dried. The pellet was dissolved in 20 μl of S1 buffer and treated with S1 nuclease as above.

DNA Sequencing. DNA sequences were determined using the phage M13 dideoxy chain-termination method of Sanger *et al.* (22).

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Abbreviation: bp, base pair(s).

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Construction of Various Plasmids Used for Cotransfection. Plasmid pSV7neo was a gift from H. Potter. This plasmid is derived from pBR327 by replacement of the tetracycline-resistance region by a DNA fragment containing the simian virus 40 (SV40) enhancer/early region promoter, which controls the expression of the neomycin-resistance gene (derived from Tn*5*), followed by a polyadenylylation region of SV40. This plasmid was further modified by removing the DNA fragment containing the neomycin-resistance gene and the polyadenylylation region and replacing it with an M13 polylinker (derived from the replicative form of mp10). The plasmids containing the various c-*myc* genes used in the cotransfection experiment were constructed by insertion of the *Sma* I/*Eco*RI DNA fragment containing the second and third c-*myc* exons into the polylinker region. Construction of plasmid pSV7-Humyc Δpst was accomplished by removal of the *Pst* I/*Pst* I 400-base-pair (bp) region within the second exon of the c-*myc* gene. Religation of the plasmid after removal of the *Pst* I fragment restores the original reading frame of the c-*myc* gene. Plasmid pEJ6.6 containing the EJ-Ha-*ras* oncogene was a gift from R. Weinberg.

Transformation of Rat Embryo Fibroblasts and Focus-Formation Assay. Primary cultures of rat embryo fibroblasts were prepared as described (23). Cotransfection of secondary rat embryo fibroblast cultures, scoring of foci, and tumor formation in nude mice were performed according to the procedure of Land *et al.* (23) except that the serum concentration in the culture medium was reduced to 3% when the cells became confluent and the nude mice were not irradiated prior to injection of the transfected cells.

RESULTS

Characterization of the Translocated c-*myc* Gene from the Burkitt Lymphoma Cell Line Ly65. Cytogenetic analysis of the Ly65 cell line shows a characteristic 8;14 chromosomal translocation that brings one of the two c-*myc* alleles into the immunoglobulin heavy chain locus. Using probes isolated from germ-line c-*myc* and immunoglobulin μ genes, it has been shown by digestion of Ly65 genomic DNA that the c-*myc* coding region and the μ switch and μ constant regions comigrate on a single DNA fragment whereas the J_H and 5′ *myc* regions comigrate on another fragment (data not shown). The translocation breakpoint is located in the first intron of the c-*myc* gene and brings the c-*myc* coding region into the μ switch region with the two genes in a head-to-head fashion. This head-to-head orientation of the two loci has been defined based on the known chromosomal orientation of the two genes and restriction analysis of the translocated c-*myc* gene and its reciprocal product.

Transcriptional Analysis of the Translocated c-*myc* Gene from Cell Line Ly65. To define the site of initiation of the translocated Ly65 c-*myc* mRNA and to determine whether the translocation had altered the splicing and/or polyadenylylation sites of the mRNA, the experiments shown in Fig. 1 were performed. When DNA fragment C from the Ly65 translocated gene (Fig. 1*B*) was used in an S1 nuclease-protection experiment, the RNA initiation site, corresponding to a protected fragment length of 210 nucleotides, mapped to a position 438 nucleotides 5′ of the ATG translation initiation codon located in exon 2. Approximately 90% of the total radioactivity in the fragment C probe was present in the 210-nucleotide fragment; however, approximately 10% of the label was located in a band corresponding to full-length protection of the 365-nucleotide original fragment (Fig. 1*A*). This suggested the possible minor use of a second start site 5′ of the major promoter. Since the use of other DNA fragments immediately 5′ of fragment C failed to show any measurable S1 nuclease protection, a second approach to define the RNA initiation site for the translocated allele was followed. Using the probe A DNA fragment, we carried out a primer extension experiment with the Ly65 mRNA as template and found (Ly65 RNA, lane A′) that the DNA fragment generated in this manner is exactly the size of the protected DNA fragment B, confirming the position of the RNA start site in Ly65. No band corresponding to a larger fragment, which would be generated if a more 5′ site were also used, was found even with greatly increased exposures.

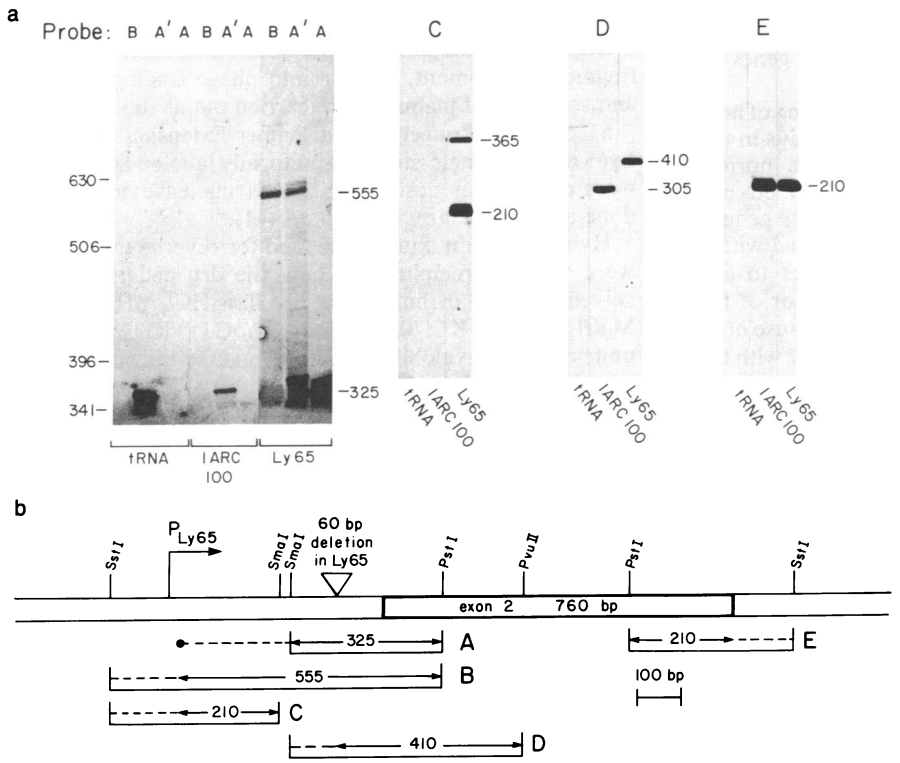

FIG. 1. (*a*) S1 nuclease protection and primer extension analyses of the Ly65 mRNA transcript. The following probes were used in the S1 nuclease analysis: A, the 325-bp *Sma* I/*Pst* I fragment from Ly65 DNA (probe A′, used in the primer extension analysis, was the same as probe A except that the probe A′ single-stranded fragment was formed by *Sma* I digestion instead of *Eco*RI digestion of the mp10 subclone); B, the 710-bp *Sst* I/*Pst* I fragment from Ly65 DNA; C, the 365-bp *Sst* I/*Sma* I fragment from Ly65 DNA; D, the 560-bp *Sma* I/*Pvu* II fragment from germ-line DNA; E, the 345-bp *Pst* I/*Sst* I fragment from Ly65 DNA. Ten micrograms of tRNA, IARC 100 RNA, or Ly65 RNA was hybridized to the probe at 56°C and S1 nuclease analysis was performed as described in *Materials and Methods*. (*b*) Schematic diagram of c-*myc* exon 2. P_{Ly65}, start site of Ly65 c-myc mRNA; ▽, site of a 60-bp deletion in Ly65 DNA. The locations of probes A–E are shown below the diagram and the sizes of the protected fragments from Ly65 c-myc mRNA are indicated. ●---, Region that is primer extended in Ly65 mRNA from probe A′.

The S1 protection pattern of the other DNA segments (A–D) depicted in Fig. 1 shows that the DNA that was part of the first intron, normally spliced out during processing of the germ-line mRNA, is entirely conserved during processing of the Ly65 transcript and becomes part of a new 5′ untranslated region of Ly65 c-*myc* mRNA. This Ly65 5′ untranslated region contains no AUG initiation codons. The protection pattern of fragment E shows that the usual splice donor site used in the germ-line joining of exons 2 and 3 is also used in Ly65 and that the single nucleotide change that occurs five nucleotides 3′ of the donor site in Ly65 DNA (see below) does not alter the normal splicing of the coding exons. The use of other DNA fragments covering the region 3′ of fragment E (data not shown) shows that in Ly65 mRNA the poly(A) addition site is identical to that used in the processing of the germ-line c-*myc* gene (13). Therefore, the size of the

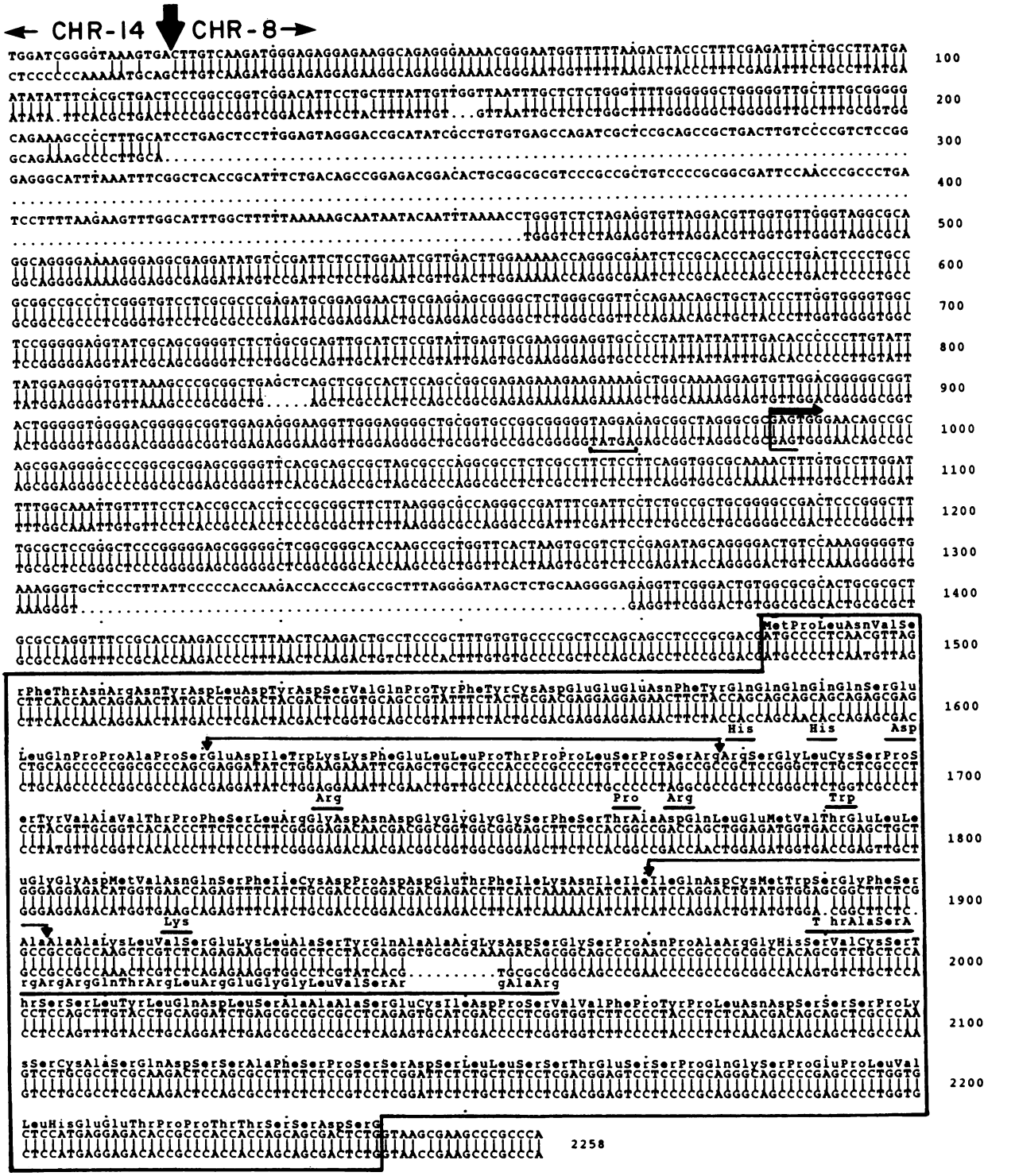

FIG. 2. Comparison of the nucleotide and amino acid sequences of the human c-*myc* germ-line gene and the Ly65 translocated *myc* gene. The sequence region presented represents the region of the Ly65 translocated c-*myc* gene from the breakpoint to the end of the first coding exon (bottom line of the nucleotide sequence). The sequence of the human germ-line c-*myc* gene is shown above the corresponding region of the Ly65 sequence. The normal amino acid sequence of the c-*myc*-encoded protein is shown above the sequence of the germ-line gene, and the amino acid changes in the translocated Ly65 c-*myc* gene product are shown below the Ly65 gene sequence.

cytoplasmic mRNA from the translocated c-*myc* allele is virtually identical to that of the c-*myc* mRNA of the germ-line transcript when the P2 promoter site is used.

Sequence of the Translocated c-*myc* Gene in the Ly65 Cell Line. The entire nucleotide sequence of the region of the c-*myc* gene from the breakpoint of the translocation to the end of the second exon is shown in Fig. 2. The breakpoint of the translocated c-*myc* gene occurs in the middle of the first intron [position 18 (vertical arrow) in Fig. 2] and is located 1155 nucleotides 5′ of the ATG translation start site in the second exon of the Ly65 gene. Comparison of the nucleotide sequence of the translocated gene with that of the germ-line gene shows the presence of many structural changes extending from the breakpoint into the coding region of the second exon. In the region 5′ of the second coding exon, there have occurred multiple point mutations and there are two major deletions (240 nucleotides and 60 nucleotides, respectively) located just 5′ of the start of the second coding exon. The RNA initiation site is shown at position 985 and, although there is no canonical "TATA" sequence immediately 5′ of the RNA start site, there has occurred a single point mutation 18 nucleotides 5′ of the start site, which changes the germ-line sequence TAGGA to TATGA beginning 20 nucleotides 5′ of the initiation site.

Comparison of the nucleotide sequences of the translocated and germ-line genes and of the amino acid sequences encoded by those genes shows that all of the amino acid changes in the c-myc protein have occurred within the region bounded by the two *Pst* I sites in the middle of exon 2. Single nucleotide mutations have resulted in a group of eight single amino acid substitutions between positions 32 and 112 in the protein molecule. There has also occurred a frameshift mutation beginning at nucleotide 1890 that extends for 24 amino acid codons (positions 136 to 159) before a compensatory deletion of 10 bases returns the sequence to its original reading frame.

Also noteworthy is a single base change at position 2245 located five nucleotides 3′ of the splice donor site at the end of the second exon. However, the results in Fig. 1 show that the mutation has not changed the use of the correct splice site. The nucleotide sequence of the third exon of the translocated gene is identical to that of its germ-line counterpart and consequently no change in the c-myc protein structure in the carboxyl-terminal half of the molecule has occurred.

***In Vitro* Transforming Ability of the Translocated c-*myc* Gene from Ly65 Cells.** Since the coding sequence of the translocated c-*myc* gene of Ly65 cells has been altered so substantially, it was important to confirm that the translocated allele retained its transforming potential. Land *et al.* (23) have shown that the c-*myc* oncogene, when complemented with the *RAS* oncogene in a cotransfection experiment, is capable of producing a change in growth properties (focus formation) of rat embryo fibroblasts and that, when these foci are injected into nude mice, they form tumors. We have used this assay system to compare the transforming ability of the germ-line c-*myc* gene with that of the translocated Ly65 gene. When each of the plasmid constructions is cotransfected with a plasmid containing the *EJ-RAS* gene into rat embryo fibroblasts the results shown in Table 1 were obtained. The construction made from the human germ-line c-*myc* gene was able to produce foci in each transfection and, in turn, produce tumors in all of the nude mice that were injected with cells from the transfection. The construction containing the translocated gene sequence from Ly65 cells also showed consistent transforming ability, although, as judged by numbers of foci per 10^6 cells transfected or by numbers of tumors in nude mice, the efficiency of transformation was reduced. Controls consisting of each of the oncogenes transfected together with either pBR322 carrier DNA or plasmid pSV7-Neo did not produce foci or tumors. In addition, a construction made by deleting the *Pst* I/*Pst* I fragment from the germ-line gene (within the region in which all the amino acid substitutions found in Ly65 cells are located) completely lost its transforming ability and did not produce foci or tumors.

DISCUSSION

The human cell line Ly65 carries an 8;14 reciprocal chromosomal translocation that brings the protooncogene c-*myc* into the μ switch region of the immunoglobulin heavy chain locus in a head-to-head fashion. The breakpoint of the translocation occurs within the first intron of the gene, thereby separating the coding region of the c-*myc* gene from the untranslated first exon. This pattern of translocation is characteristic of c-*myc* translocations in mouse plasmacytomas (6, 24, 25) and has been shown to occur in several human Burkitt lymphoma cell lines (8, 10, 12, 26, 27). In those few human translocations characterized to date, the breakpoint occurs within a 20-bp-

Table 1. Transformation of rat embryo fibroblasts by cotransfection of various gene combinations

DNA transfected	Experiment 1		Experiment 2		Experiment 3		Experiment 4		Tumorigenicity in nude mice	
	Plates with foci	Foci per 10^6 cells	Plates with foci	Foci per 10^6 cells	Plates with foci	Foci per 10^6 cells	Plates with foci	Foci per 10^6 cells	Tumors/ total injections	Tumors/ plates with foci injected
pSV7PL-Humyc + pEJ6.6	6/6	ND	6/6	23	6/6	15	6/6	33	15/15	15/15
pSV7PL-LY65 + pEJ6.6	3/6	ND	2/6	2	6/6	9	2/6	2	7/15	7/9
pSV7PL-Humyc Δpst + pEJ6.6	*	*	0/6	0	0/6	0	0/6	0	0/12	0 plates with foci
pSV7PL-Humyc + pBR327	*	*	0/6	0	0/6	0	0/6	0	0/12	0 plates with foci
pSV7neo + pEJ6.6	0/6	0	0/6	0	0/6	0	0/6	0	0/15	0 plates with foci
pSV7-LY65 + pBR327	*	*	*	*	*	*	0/6	0	*	*

ND, not determined; *, experiment not done.

long region of c-*myc* DNA and it has been suggested that this cluster may define a discrete region in which a breakpoint can occur and still result in a functional c-*myc* gene (28). The breakpoint in the Ly65 cell line occurs 100 nucleotides 5′ of this cluster but in the same general locale at the beginning of the first intron. This area is rich in switch-like pentanucleotide repeats (13) and in the tetranucleotide GAGG, which has been found by Piccoli *et al.* (29) in the breakpoint region in mouse plasmacytomas and by Showe *et al.* (27) in at least one human Burkitt cell line.

It has been reported that the quantity of c-myc mRNA present in the Ly65 cell line is 2–3 times the amount present in a lymphoblastoid control cell line and that ≈95% of the c-myc mRNA in Ly65 cells is transcribed from the translocated allele (11). We report here that transcription of the translocated allele makes use of a new cryptic promoter in the remaining region of the first intron. The portion of the intron between this promoter and the beginning of the second exon remains intact during mRNA processing and becomes part of a new 438-nucleotide untranslated 5′ sequence in Ly65 mRNA. The Ly65 mRNA uses the same splice sites in joining exons 2 and 3 and the same poly(A) addition site as the untranslocated germ-line mRNA. The size of the mRNA transcribed from the translocated allele is, therefore, virtually identical to that of the mRNA generated by use of the *P2* promoter in the germ-line gene (13).

It has been suggested that the translocation of the c-*myc* gene into the immunoglobulin heavy chain locus may allow the mechanisms responsible for the generation of variability in the immunoglobulin locus to act on the *myc* gene to produce the mutations frequently seen (9, 10, 30). The translocated allele in Ly65 cells has undergone extensive alteration in structure, including multiple point mutations and deletions in the 5′ untranslated region and extending into the coding region of the second exon. These alterations give rise to eight single amino acid changes and a large frameshift mutation that changes a string of 24 consecutive amino acids in the amino-terminal half of the myc protein. The above cluster of amino acid changes occurs in a region of the gene that is highly conserved in mouse, chicken, and human sequences (25) at both the nucleotide and the amino acid level. Also, it has been reported by Schwab *et al.* (31), based on a partial sequence determination of the N-*myc* gene, that there exist two regions of strong homology within the second exon between the N-*myc* and the c-*myc* genes. This and other regions of homology have been identified by Kohl *et al.* (32). In addition, similar "*myc* boxes" occur in the c-*myc*-like gene L-*myc*, which is amplified in certain small cell carcinomas of the lung (33). The two regions of homology shared by these genes (Fig. 2) substantially overlap the altered regions of the translocated allele in Ly65 cells, especially the second homology region. These later alterations suggest a paradox in that an evolutionarily conserved region appears to be indispensable for the biologic function of the c-myc protein and yet these mutations allow transformation, albeit at reduced efficiency. One of several ways to account for this is to assume that these conserved domains serve a biological function independent of that required for transformation—for example, a regulatory function mediating the activity of the c-myc protein. Interestingly, Ly65 is a Burkitt cell line in which the normal untranslocated c-*myc* allele retains a minimal level of expression (11). This observation fits well with the hypothesis advanced by Rabbitts *et al.* (34) that certain structural alterations of the c-*myc* gene may alter its postulated autoregulatory action (1, 18).

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