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Original Paper

The Amino-terminal Phosphorylation Sites of C-MYC are Frequently Mutated in Burkitt's Lymphoma Lines but not in Mouse Plasmacytomas and Rat Immunocytomas

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We sequenced the region encoding the amino-terminal phosphorylation sites of C-MYC in the Ig/*MYC* translocation-carrying Burkitt lymphomas (BL), mouse plasmacytomas (MPC) and rat immunocytomas (RIC). Mutations affecting the Thr-58 codon or the immediate flanking region were found in seven of the 10 *in vitro* propagated BL lines. No mutations were found in any of the eight BL biopsies analysed. Germ-line sequences were also found in six *in vivo* and five *in vitro* passaged MPCs and in four *in vivo* transplanted RICs. These findings indicate that mutations in this region do not represent a general phenomena in Ig/*MYC* translocation-carrying tumours, but may confer growth advantage on BL cells under continuous *in vitro* propagation.

Key words: c-myc, Burkitt's lymphoma, mouse plasmacytoma, mutations
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INTRODUCTION

THE *C-MYC* gene encodes nuclear phosphoproteins with short half-lives that are synthesised throughout the cell cycle. The expression of *C-MYC* is tightly controlled and its deregulation appears to play a role in the development and/or progression of various tumours.

Reciprocal chromosomal translocations that lead to the juxtaposition of *C-MYC* and Ig-encoding sequences are consistently found in Burkitt lymphomas (BL), mouse plasmacytomas (MPC) and rat immunocytomas (RIC). This regularity and the resulting constitutive expression of *C-MYC* has been interpreted to suggest that the translocation represents a rate-limiting step in the pathogenesis of the tumours (for review see [1]).

The C-terminal part of the protein harbours three domains characteristic for proteins involved in transcriptional regulation. Two of them, the helix-loop-helix (HLH) and the leucine zipper (Zip), function as dimerisation motifs, while the basic region, which precedes the HLH domain, confers sequence-specific DNA-binding capacity [2–5]. C-MYC dimerises specifically with the bHLHZip protein MAX [4, 6], and the resulting hetero-dimers bind efficiently to the E-box sequence, CACGTG [7, 8]. Reporter gene constructs containing the *MYC/MAX* DNA binding site are transcriptionally modulated by *MYC* and *MAX*. This transactivation is mediated by the N-terminal domain of C-MYC [9–11].

Many transcription factors, such as C-FOS, C-JUN and C-ERB-A are regulated by phosphorylation (for review see [12]. It may affect the transactivating or the DNA binding potential of the protein, providing a rapid and reversible regulation mechanism.

C-MYC is phosphorylated at multiple sites *in vivo*, two of which we have identified within the highly conserved amino-terminal domain [13]. These sites, Thr-58 and Ser-62, can be phosphorylated by glycogen synthase kinase 3 (GSK3) *in vitro* [13, 14]. In addition, Ser-62 has been reported to be phosphorylated *in vitro* by a mitogen activated kinase (MAP) [15]. Mutagenesis of Thr-58 to alanine potentiated focus formation in rat embryo fibroblasts (REF), whereas substitution of Ser-62 severely inhibited transformation [13–15]. Furthermore, viral *myc* genes from three of four v-*myc* isolates have mutations resulting in a substitution at Thr-61, corresponding to human Thr-58, to Ala or Met [16–19]. Analysis of fusion proteins between C-MYC and v-Myc has shown that this mutation increased the transforming potential [20, 21]. These data suggest that C-MYC function is modulated via phosphorylation of the conserved amino terminal domain.

Studies on Ig/*MYC* carrying tumours have mainly been focused on the characterisation of the *C-MYC* breakpoints. Mutations were found in the coding region of the translocated allele in some BLs [22, 23], but they were not consistent and could not be attributed any functional significance. Recent

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studies, however, show that mutations within the coding region of *C-MYC* is much more common than previously thought [24, 25].

The identification of regulatory phosphorylation sites in the N-terminal domain of C-MYC prompted us to carry out a sequence analysis specifically aimed to determine the frequency of mutations in the vicinity of Thr-58 in a collection of Ig/*MYC* carrying tumours from humans, mice and rats. We show that the N-terminal phosphorylation sites are frequently mutated in BLs propagated *in vitro*. In contrast, the primary BLs included and tumours lacking the Ig/C-*MYC* translocation were devoid of mutations. Furthermore, no mutations were found in any *in vivo* or *in vitro* passaged MPCs or in *in vivo* passaged RICs analysed.

MATERIALS AND METHODS

Cell lines and tumours

Human cell lines and tumours. cDNA from 12 *in vitro* established BL cell lines and four LCLs (lymphoblastoid cell lines) were analysed. The characteristics have been described elsewhere [24–28]. Genomic DNA prepared from the eight primary BLs from Kenya and Uganda was previously described by Minarovits and associates [29]. BJAB is a B lymphoma [30] and NPC CAO is a nude-mouse passaged nasopharyngeal carcinoma [27].

Rodent cell lines and tumours. cDNA from the following cell lines and *in vivo* passaged (or transplanted) tumours were analysed: ABPC20 [31], ABPC22 [32], TEPCHi6 [33], YACUT [34], 38-B9-tk- [35]. ABPC422-1, ABPC429-1, TEPC M34/367, C4;12CB20134, C4;12CB20135 and TEPCIL6-9 were established at the Department of Tumour Biology as described elsewhere [36]. TEPC1165 and ABPC103-9 were gifts from F. Mushinski, NIH, U.S.A. cDNA from four transplanted RIC tumours were analysed (see [37]).

RNA and DNA isolation

Total RNA was extracted from the material by the guanidinium thiocyanate extraction procedure [38]. Genomic DNA was prepared as described previously [39].

PCR and primers

First strand cDNA was synthesised from 1–2 μg of total RNA using Avian Myeloblastosis Virus (AMV) reverse transcriptase. The RNA was incubated with 20 μl reaction buffer containing 50 mM Tris–HCl pH 8.5, 145 mM KCl, 10 mM $MgCl_2$, 4 mM DTT, 1.25 μM dNTP, 1 μM 14-mer random primer, 0.5 units of AMV reverse transcriptase. The reaction was incubated for 60 min at 42°C followed by 5 min at 95°C. Three microlitres were used for each PCR reaction. A 727 bp fragment including most of C-*MYC* exon 2 was generated from the complementary DNA by PCR using the same set of primers for both human, mouse and rat C-*MYC*.

PCR assays (50 μl) contained 10 pmol of primers, 1.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris–HCl (pH 8.5), 0.001% gelatin, 2 mM $MgCl_2$, 0.25 mM deoxyribonucleoside triphosphate, 2 U of Taq polymerase (Perkin–Elmer Cetus, Connecticut, U.S.A.). For amplification, we used 35 cycles of 94°C denaturation (30 s, 66°C annealing (1 min) and 72°C extension (2 min). The 5′ primer was GGA ACT ATG ACC TCG ACT ACG and the 3′ primer was AGA GTC GCT GCT GGT GGT G with a biotinylated 5′ end.

Sequencing reactions

The PCR products were purified using magnetic beads with covalently coupled streptavidin Dynabeads M-280-Streptavidin from Dynal, Oslo, Norway. A neodymium–iron–boron permanent magnet (Dynal AS) was used to sediment the beads during the washing procedures. 300 μg of beads were mixed with 40 μl of the PCR mixture and incubated for 15 min in a solution containing 10 mM Tris–HCl pH 8.0, 1 mM EDTA and 1 M NaCl. The immobilised doubled stranded DNA was washed with 50 μl TE buffer and then incubated with 0.10 M NaOH for 5 min. The supernatant was removed and the beads containing the immobilised single stranded DNA were washed with 50 μl 0.1 M NaOH and then three times with TE buffer. After the last wash, the beads were dissolved in 10 μl of water. The sequencing reactions were performed with reagents from the Autoread T7 sequencing kit according to the protocol provided by Pharmacia using a labelling-mixture including fluorescent dATP (Pharmacia LKB Biotechnology).

The sequencing primers were: ACT TCT ACC AGC AGC AGC A (human *C-MYC*) or AGA GAA TTT CTA TCA CCA (mouse and rat *C-MYC*).

5 μl of the sequencing reactions were loaded on a 6% sequencing gel run on an automated laser fluorescent sequencer (A.L.F., Pharmacia LKB Biotechnology, Uppsala, Sweden).

Table 1. Characteristics of human cell types analysed

Tumour	Origin/DNA	Translocation	Breakpoint location	EBV
BL				
Cell lines				
Eli	end. BL	8;14	II	+
P3HR1	end. BL	8;14	III	+
Chep	end. BL	8;14	II	+
Raji	end. BL	8;14	II	+
Rael	end. BL	8;14	ND	+
Akata	Japanese BL	ND	ND	+
Mutu1	end. BL	8;14	I/II	+
Namalwa	end. BL	8;14	ND	+
Daudi	end. BL	8;14	III	+
DG75	spor. BL	8;14	ND	−
Biopsies				
1	end. BL	ND	ND	+
2	end. BL	ND	ND	+
3	end. BL	ND	ND	+
4	end. BL	ND	ND	+
5	end. BL	ND	ND	+
6	end. BL	ND	ND	+
7	end. BL	ND	ND	+
8	end. BL	ND	ND	+
LCL				
KR4	LCL	—	—	+
IARC-0	LCL	—	—	+
IB4	LCL	—	—	+
Cherry	LCL	—	—	+
Other				
Bjab	B lymphoma	—	—	−
NPC CAO	NPC	—	—	+

end., endemic; spor., sporadic. Breakpoint definition: I, within the gene, II, immediately 5′, III, distant (from [40])
ND, not defined. NPC CAO was propagated in nude mice.

RESULTS

In order to assess the prevalence of mutations around Thr-58 of C-MYC in BLs, the second exon of C-*MYC* was amplified by PCR and the 5′ region sequenced using a primer located 60 nt 5′ of the Thr-58 codon. cDNA and/or genomic DNA was prepared from eight BL biopsies and 12 established BL lines, including three sublines of the Mutu 1 tumour (Table 1). Since only the translocated allele is expressed in BLs while the normal allele in most cases is transcriptionally downregulated [40], we preferentially used cDNA for the sequence analysis. For comparison, both cDNA and genomic DNA was sequenced in three tumours. Mutations detected in cDNA were also detected in genomic DNA in the three mutation-carrying tumours compared with both methods. These results permitted us to extend the study to tumours where only genomic material was available. Mutations in the immediate vicinity of Thr-58 were found in seven of the 10 independent BLs established for *in vitro* growth. As seen in Table 2, the mutations were clustered within five amino acids around Thr-58 and frequently affected Thr-58 itself. The three sublines of Mutu1 all carried the same substitution of Thr-58 to Asn, but two of the lines also had silent mutations further downstream, which shows that the process of mutation continued to occur during the *in vitro* growth. A typical sequencing result is shown in Figure 1.

No mutations were found in any of the eight BL biopsies or in any of the four EBV transformed LCLs of non-neoplastic origin. The translocation negative BJAB lymphoma line and one nasopharyngeal carcinoma passaged in nude mice were free from mutations.

For comparison with the BL system, we analysed the Ig/*MYC* translocation-carrying rodent tumours, MPC and RIC. We compared five MPC derived *in vitro* cell lines, five *in vivo* passaged, one primary MPC and four *in vivo* passaged RICs by the same methods as used for the BLs. None of these carried any mutations within the approximately 150 bp region around Thr-58 that was sequenced (Table 3).

Table 2. C-MYC *mutations in human cell types*

					Additional mutations			
Tumour	DNA	Codon	Nucleotide change	Protein change	Codon	Nucleotide change	Protein change	Sequenced region
BL								
Cell lines								
Eli		55	GAG-GAC	Glu-Asp				148–442
	cDNA	58	ACC-AGC	Thr-Ser				
		59	CCG-TCG	Pro-Ser				
		64	AGC-AAG	Ser-Lys				
P3HR1	cDNA	57	CCC-TCC	Pro-Ser	99	G-C	Gln-His	145–442
Chep	cDNA	60	CCC-TCC	Pro-Ser				160–282
Raji	cDNA/gen	58	ACC-ATT	Thr-Ile				155–274
		59	CCG-TCG	Pro-Ser				
Rael	cDNA/gen	58	ACC-AAC	Thr-Asn				156–442
Akata	cDNA	60	CCC-TTC	Pro-Phe				152–317
Mutu159	cDNA/gen	58	ACC-AAC	Thr-Asn				160–319
Mutu 1148	cDNA	58	ACC-AAC	Thr-Asn	71	G-C	—	160–335
					99	G-A	—	
Mutu1216	cDNA	58	ACC-AAC	Thr-Asn	99	G-A	—	
Namalwa	cDNA	—			106	G-A	—	145–348
Daudi	cDNA	—			112	C-A	—	145–442
DG75	cDNA	—			—		—	160–354
Biopsies								
1	gen.	—			—			145–420
2	gen.	—			—			145–393
3	gen.	—			—			145–396
4	gen.	—			—			164–324
5	gen.	—			—			160–330
6	gen.	—			—			152–353
7	gen.	—			—			149–280
8	gen.	—			—			147–312
LCL								
KR4	cDNA	—			—			152–342
IARC-0	cDNA	—			—			155–421
IB4	cDNA	—			—			149–383
Cherry	cDNA	—			—			160–331
Other								
Bjab	cDNA	—			—			152–317
NPC CAO	cDNA	—			—			145–270

gen, genomic DNA. The nucleotides are numbered from the first base in the ATG codon (amino acid 1).

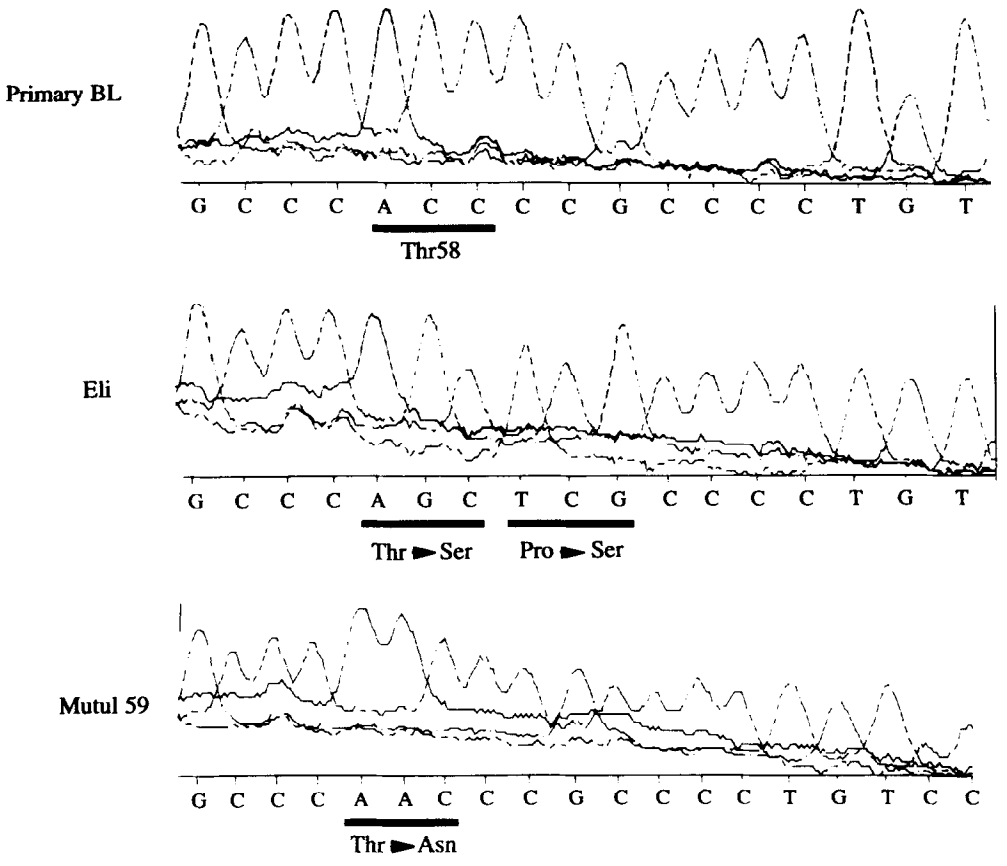

Figure 1. Examples of raw data output of *C-MYC* sequence analysis. The Thr-58 codon is indicated below the germline sequence of the primary BL and the amino acid changes caused by mutations are indicated below the Eli and Mutul 59 sequences.

Table 3. Rodent tumours analysed

Tumour	Type	Translocation	Sequenced region	Mutations
Cell lines				
ABPC22	MPC	6;15	142–220	–
TEPC1165	MPC	12;15	140–211	–
ABPC20	MPC	ND	135–221	–
ABPC422-1	MPC	ND	131–201	–
ABPC429-1	MPC	ND	135–241	–
YACUT	T-lymph	–	131–197	–
38-B9-tk$^-$	pre-B lymph	–	138–240	–
In vivo transplanted				
TEPCM34/367	MPC	ND	132–236	–
TEPCHi6	MPC	12;15	140–222	–
ABPC103-9	MPC	ND	131–224	–
C4;12CB20134	MPC	ND	142–241	–
C4;12CB20135	MPC	ND	131–226	–
IR50	RIC	6;7	134–242	–
IR209	RIC	6;7	128–222	–
IR223	RIC	6;7	133–243	–
IR304	RIC	6;7	135–220	–
Primary				
TEPCIL6-9	MPC	ND	122–245	–
Spleen		–	133–240	–

ND, translocation not defined. More than 90% of MPC carry either 12;15 or 6;15 translocations [1]. TEPC: induced by pristane alone. ABPC: induced by pristane + Abelson virus. The RIC arise spontaneously in the Lou strain of rats.

DISCUSSION

Our study shows that mutations in the region encoding Thr-58 were frequent in *in vitro* established BLs. The mutations within the sequenced region were targeted specifically to the region around the Thr-58 codon, since only a very limited number of additional mutations were detected within the 150–300 bp region sequenced (Table 2).

In a recent study by Bhatia and associates, 57 BLs were analysed using single stranded conformational polymorphism (SSCP) [25]. Mutations within the coding region of C-*MYC* in BLs were found in 65% of the tumours. Sequence analysis showed that 15 of the 30 tumours, including both biopsies and *in vitro* established samples from sporadic and endemic BLs, carried C-*MYC* mutations within the region that was analysed in our study. Yano and associates [24] sequenced C-*MYC* in 14 biopsies from sporadic BLs, and found mutations in the vicinity of Thr-58 in 5 of them. Furthermore, a study of BLs showed that mutations clustered between amino acids 57 and 62 in 13 of the 18 cell lines tested [41]. Together, these studies show that C-*MYC* mutations may occur in the BL tumour *in vivo*, even though the frequency of the mutations are higher in the established BL lines *in vitro*. The absence of mutations in our panel of BL biopsies could either be explained by the limited number of tumours studied, or alternatively reflect differences in frequencies in mutations depending on the origin of the endemic tumours.

Some of the mutations described by Bhatia and associates [25] were found to be homozygous, prompting the authors to suggest that the mutations occurred before the translocation event. Our approach was not designed to study this question specifically. In the three cases when both cDNA and genomic DNA was analysed, we were not able to determine unequivocally whether the mutations were homo- or heterozygous in the genomic material.

In accordance with previous studies, we were not able to find mutations in human cell lines or tumours without the Ig/*MYC* translocation, such as the B-lymphoma BJAB or LCLs, indicating that the juxtaposition of C-*MYC* to an Ig locus in BL makes the gene more vulnerable to mutation.

This tendency for mutation is restricted to BLs among the Ig/*MYC* carrying tumours as shown by the presence of mutations in seven of the 10 BL lines, but their absence from all 5 MPC lines tested. This may or may not be related to the phenotypic difference between BL cells and plasma cells, and the prerequisites for their propagation *in vitro*. Mutations may, however, occur in the coding region of *C-MYC* in MPC as found by Bhatia and associates [25] in four of 10 MPCs analysed. Only one of the tumours had a mutation that led to amino acid substitution in the vicinity of Thr-58, which, together with our analysis of 11 MPCs, show that mutations in this region do not carry a major selective advantage in MPC-derived lines.

In addition, several studies show that mutations are frequent in the 5′ region of C-*MYC* in both *in vitro* established and primary BLs [42]. These mutations were suggested to affect a block of elongation, thereby increasing the C-*MYC* expression [42–44]. No such mutations have been found in MPCs or RICs [39, 45].

The N-terminal conserved region of C-MYC harbours many important functions of the protein such as transactivation, transformation and autoregulation, and is possibly regulated by the phosphorylation status of Thr-58 and Ser-62. The apparent selection for mutation around Thr-58 in BLs and in viral myc indicate that these changes provide growth advantage to the tumour cells.

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