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Functional Analysis of Burkitt's Lymphoma Mutant c-Myc Proteins*

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The c-*myc* gene encodes a sequence-specific DNA binding protein that activates transcription of cellular genes. Transcription activation by Myc proteins is regulated by phosphorylation of serine and threonine residues within the transactivation domain and by complex formation with the retinoblastoma-related protein p107. In Burkitt's lymphoma, missense mutations within the c-Myc transactivation domain have been found with high frequency. It has been reported that mutant c-Myc proteins derived from Burkitt's lymphoma cell lines are resistant to inhibition by p107, thus providing a rationale for the increased oncogenic activity of these mutant c-Myc proteins. It has been suggested that these mutant c-Myc proteins resist down-modulation by p107 because they lack cyclin A-cdk2-dependent phosphorylation. Here, we have examined three different Burkitt's lymphoma mutant c-Myc proteins found in primary Burkitt's lymphomas and one mutant c-Myc protein detected in a Burkitt's lymphoma cell line. All four have an unaltered ability to activate transcription and are sensitive to inhibition of transactivation by p107. Furthermore, we provide evidence that down-modulation of c-Myc transactivation by p107 does not require phosphorylation of the c-Myc transactivation domain by cyclin A-cdk2. Our data indicate that escape from p107-induced suppression is not a general consequence of all Burkitt's lymphoma-associated c-Myc mutations, suggesting that other mechanisms exist to deregulate c-Myc function.

The proto-oncogene c-*myc* encodes a short-lived nuclear phosphoprotein with important roles in cellular proliferation, differentiation, neoplasia, and apoptotic cell death (for review, see Lüscher and Eisenman (1990) and Marcu *et al.* (1992)). A rapid increase in c-*myc* expression is observed when quiescent cells are stimulated to re-enter the cell cycle by growth factor stimulation (Kelly *et al.*, 1983). In continuously proliferating cells, however, c-*myc* mRNA and protein levels are invariant throughout the cell cycle (Hann *et al.*, 1985; Thompson *et al.*, 1986). Insight into the function of Myc proteins came from the identification of two motifs in the carboxyl terminus of Myc proteins that are present in several transcription factors: the basic-helix-loop-helix (bHLH) motif and the leucine zipper (Zip). c-Myc forms a heterodimer through its bHLH-Zip domain with the Max protein (Blackwood and Eisenman, 1991), and a specific DNA binding site (CACGTG) has been identified for the complex (Blackwell *et al.*, 1990; Blackwood and Eisenman, 1991; Blackwood *et al.*, 1992; Halazonetis and Kandil, 1991; Prendergast and Ziff, 1991). A strong and highly conserved transcriptional activation domain is located within the amino terminus of Myc proteins (Kato *et al.*, 1990). Importantly, both the transactivation domain and the DNA binding domain are required for transformation (Stone *et al.*, 1987). In addition to specific transcriptional activation, repression by c-Myc has been described for several genes including c-*myc* itself, the *neu* proto-oncogene, and the *cyclin D1* gene (Penn *et al.*, 1990a; Philipp *et al.*, 1994; Suen and Hung, 1991). Together, these data indicate that Myc proteins are transcription factors that affect cellular physiology by altering the expression of key cellular genes, the altered expression which is ultimately responsible for the *myc*-induced phenotype.

The activity of Myc proteins is tightly regulated by several independent mechanisms. During the process of cellular differentiation, Myc is replaced by Mad or Mxi in the Max complex, generating novel heterodimers that suppress transcription by interacting with the Sin3 repressor (reviewed by Bernards (1995)). In addition, the amino-terminal transactivation domain is regulated in at least two different ways. Phosphorylation of threonine 58 and serine 62 within the transactivation domain has been shown to occur in a cell cycle-regulated fashion (Lutterbach and Hann, 1994), and mutation of these phosphorylation sites affects transactivation and transformation by c-*myc* (Albert *et al.*, 1994; Gupta *et al.*, 1993; Henriksson *et al.*, 1993; Pulverer *et al.*, 1994; Seth *et al.*, 1991). In addition, the transactivation domain of c-Myc can form a specific complex with the retinoblastoma-related protein p107, resulting in inhibition of c-Myc transactivation (Beijersbergen *et al.*, 1994; Gu *et al.*, 1994).

Activation of the proto-oncogene c-*myc* by gene amplification, proviral insertions, as well as chromosomal translocation involving the c-*myc* locus has been described in a number of human tumors. The translocation of the c-*myc* gene on chromosome 8q24 to immunoglobulin gene loci on chromosome 14q32, 22q11, or 2p12 is considered to be a central molecular event in the pathogenesis of Burkitt's lymphoma (BL)[1] (Dalla-Favera *et al.*, 1982). Variability in the breakpoint positions distinguish between the subtypes endemic and sporadic (Pelicci *et al.*, 1986). The human c-*myc* gene is composed of three exons, of which the first is noncoding. Sporadic BLs typically possess breakpoints in the first exon or intron of the c-*myc* gene, whereas the endemic cases usually have the chromosome 8 breakpoints far 5′ to the c-*myc* gene. A putative repressor binding site has been identified at the 5′-end of the first exon of c-*myc* (Siebenlist *et al.*, 1984). Translocations that separate this exon from the rest of the gene as well as accumulated mutations within this region could result in a release of the c-*myc*

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[1] The abbreviations used are: BL, Burkitt's lymphoma; CHO, Chinese hamster ovary; CAT, chloramphenicol acetyltransferase.

gene from the action of the repressor. Exon 1 mutations have also been found to abrogate the transcriptional attenuation that occurs at the 3′-end of exon 1 (Bentley and Groudine, 1986a, 1986b; Cesarman *et al.*, 1987). Within the first intron of the c-*myc* gene, a 20-base pair region has been identified as the binding site of a nuclear protein named MIF. This interaction was abrogated by point mutations present in a BL cell line (Zajac-Kaye *et al.*, 1988). Mutations in the same region have also been described for other BL cell lines, supporting the idea that the MIF protein may be an important factor for control of c-*myc* expression.

Apart from mutations that affect c-*myc* expression, recently a number of endemic and sporadic BL cell lines and some primary tumors have been reported to have mutations within the protein-encoding exons 2 and 3 of c-*myc* (Albert *et al.*, 1994; Bhatia *et al.*, 1993; Yano *et al.*, 1993; Clark *et al.*, 1994; Murphy *et al.*, 1986; Rabbitts *et al.*, 1983, 1984; Showe *et al.*, 1985). In some cases, it has been shown that these mutations are present before the chromosomal translocation takes place, suggesting that mutations within the protein-encoding region of c-*myc* are an early event in lymphomagenesis (Bhatia *et al.*, 1993). This suggests that the presence of the mutation is not neutral with respect to Myc function but rather that it confers a growth advantage onto the BL tumor cells. This is supported by the finding of several groups that BL-derived mutant c-*myc* alleles have increased transforming activity *in vitro* (Frykberg *et al.*, 1987; Henriksson *et al.*, 1993; Hoang *et al.*, 1995).

It has recently been reported that BL-derived mutant c-Myc proteins are resistant to p107-mediated suppression of transactivation (Gu *et al.*, 1994; Hoang *et al.*, 1995). Surprisingly, these mutant c-Myc proteins had a wild type ability to bind p107 (Hoang *et al.*, 1995). It has been suggested that BL-derived mutant c-Myc alleles lack phosphorylation of threonine 58, the phosphorylation of which is dependent on prior phosphorylation of serine 62 by a p107-cyclin A-cdk2 complex. Thus, it has been proposed that in normal cells cyclin A-cdk2 is recruited to the c-Myc transactivation domain by p107, causing it to phosphorylate serine 62. After this has occurred, additional phosphorylation on threonine 58 is possible, leading to down-modulation of c-Myc transactivation (Hoang *et al.*, 1995).

In the present report, we have studied three different BL-derived c-*myc* point mutants, each described to be the only alteration in the c-*myc* coding region of the corresponding primary tumor, and one BL-derived c-*myc* point mutant detected in a cell line. The four mutant c-Myc proteins were analyzed for their ability to transactivate transcription both as GAL4 fusion protein as well as a full-length protein. In contrast to other reports, we find no significant effect of these mutations on c-Myc transactivation and sensitivity to p107 suppression.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—The human osteosarcoma cell line U2-OS and CHO cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfections were performed using the standard calcium phosphate precipitation technique (Van der Eb and Graham, 1980).

Plasmid Constructs—The coding sequence of the human c-*myc* gene was isolated from pSP64 c-*myc* (generously provided by J. Woodgett) and cloned into the pJ3Ω vector (Morgenstern and Land, 1990) and the pALTER-1 vector (Promega), creating pJ3Ω c-*myc* and pALTER-1-c-*myc*, respectively. The c-*myc* mutants Ser-57, Ser-86, and Leu-115 pALTER-1 constructs were made by using pALTER-1 c-*myc* and the altered sites *in vitro* mutagenesis system (Promega). The following three oligonucleotides were used: Ser-57, 5′-GCGGGGTGGACAGCAGCTC-3′; Ser-86, 5′-CCGCCGTCGCTGTCTCCC-3′; and Leu-115, 5′-CGCAGATGAGACTCTGGTT-3′. After verifying the presence of the mutations by sequencing, the c-*myc* coding sequences were isolated from the pALTER-1 vectors and cloned into the pJ3Ω vector creating pJ3Ω c-*myc* Ser-57, pJ3Ω c-*myc* Ser-86, and pJ3Ω c-*myc* Leu-115. The c-*myc* mutant Ala-58 was obtained from J. Woodgett in the pSP64 vector. This mutant c-*myc* cDNA was also recloned in pJ3Ω. The pSV-GAL4/c-*myc* 1–262 construct (GM(1–262)) was obtained from Dr. C. V. Dang and has been described previously (Kato *et al.*, 1990). The *GAL*4 fusion constructs of the c-*myc* mutants Ser-57, Thr-58, Ser-86, and Leu-115 were made by polymerase chain reaction using the different pJ3Φ c-*myc* mutant plasmids as templates. The polymerase chain reaction-amplified c-*myc* fragments encoding c-Myc amino acids 1–262 were cloned into a pJ3Ω vector containing the *GAL*4 1–147 coding sequence. The c-*myc* mutants generated by polymerase chain reaction were verified by DNA sequencing. The expression vectors pCMVp107AS (antisense) and pCMVp107 (sense) were described previously (Zhu *et al.*, 1993). The reporter plasmid with five GAL4 DNA binding sites upstream of a minimal promoter linked to the chloramphenicol acetyltransferase (CAT) gene has been described (Kato *et al.*, 1990). The reporter plasmid with four c-Myc/Max binding sites (CACGTG), upstream of a minimal promoter linked to the CAT gene, was kindly provided by Dr. R. N. Eisenman (Kretzner *et al.*, 1992).

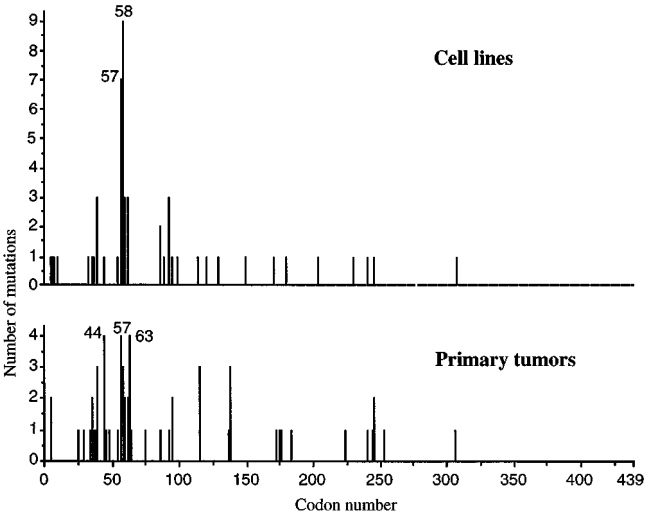

FIG. 1. **Frequency of c-Myc missense mutations in primary Burkitt's lymphoma and in Burkitt's lymphoma cell lines.** The *numbers* above the bars represent amino acid numbers.

CAT Assays—U2-OS cells or CHO cells on 100-mm dishes were transiently transfected with 5 μg of CAT reporter construct, 0.2 μg of pSV40 luciferase plasmid, 0.5 μg of c-Myc activator plasmid, and 3 μg of pCMVp107 or pCMVp107AS plasmid as indicated. Where indicated, 3 μg of cdk2-DN was added. The total amount of DNA was made up to 18 μg for each transfection by adding herring sperm DNA. 36–40 h after transfection, cells were collected, and CAT activity was determined using the phase extraction method (Seed and Sheen, 1988). In U2-OS cells, CAT counts were normalized to luciferase activity as determined using the luciferase assay system (Promega). In CHO cells, CAT counts were normalized to protein content using a modified Bradford assay. This procedure was used to precisely replicate the experimental procedure used by Hoang *et al.* (1995).

Western Blot Analysis—For Western blot analysis of transiently transfected cells, total cell lysates were collected from 100-mm dishes 36–40 h after transfection. Lysates were boiled in 200 μl of 2 × protein sample buffer. One-half of the lysates was resolved by 10% SDS-polyacrylamide gel electrophoresis and subjected to immunoblot analysis using the polyclonal c-Myc antibody pan Myc (1:10.000 dilution, obtained from Cambridge Research Biochemicals) and a peroxidase-coupled anti-rabbit second antibody (1:10.000 dilution) (Tago). The Western blots were developed using the enhanced chemiluminescence system (ECL, Amersham).

RESULTS

Generation and Expression of Mutant c-Myc Proteins—A summary of reported mutated c-*myc* codons detected in BL cell lines and primary tumors is given in Fig. 1. Although the numbers are small, the majority of the mutations result in amino acid substitutions within the amino-terminal transactivation domain of c-Myc. Many of the reported mutations in c-*myc* were identified in BL cell lines rather than in primary tumors. It has recently been reported that in a BL cell line,

mutations in the c-*myc* coding region continue to accumulate during cell culture (Albert *et al.*, 1994). It is therefore not clear whether all of the mutations found in BL cell lines were already present in the primary tumor. To circumvent this potential problem, we selected three mutant c-*myc* alleles from primary BL in which only a single missense mutation was present. The mutant c-*myc* alleles used were Ser-57, Ser-86, and Leu-115 (Bhatia *et al.*, 1993). We also used the mutant Ala-58 detected in a BL cell line (Albert *et al.*, 1994) and studied by others (Gupta *et al.*, 1993; Henriksson *et al.*, 1993; Hoang *et al.*, 1995; Pulverer *et al.*, 1994). To study the function of these mutant c-Myc proteins, we generated by site-directed mutagenesis three mutant human c-*myc* cDNAs, each containing a single amino acid substitution that corresponds to the mutation found in primary BL.

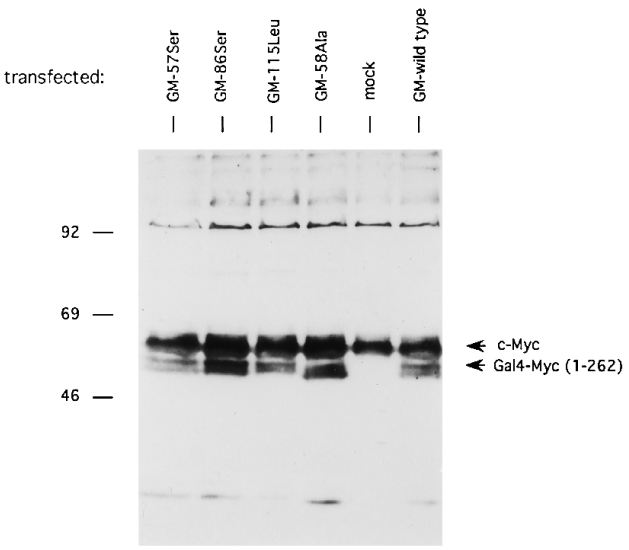

FIG. 2. **Expression of Gal4-Myc fusion proteins.** U2-OS osteosarcoma cells were transiently transfected with the various GAL4-myc (*GM*) chimeric expression vectors. After 2 days, protein lysates were prepared from transfected cells and separated on an SDS-polyacrylamide gel. After this, proteins were transferred to a nitrocellulose filter, and Myc proteins were detected with polyclonal rabbit anti pan Myc antiserum.

To study transactivation by the mutant c-*myc* alleles, we fused the amino-terminal transactivation domain (amino acids 1–262) of wild type and the four mutant c-*myc* alleles to the DNA binding domain of the yeast transcription factor GAL4. In a first experiment, we compared expression levels of the various GAL4-myc chimeric proteins. U2-OS osteosarcoma cells were transiently transfected with the wild type and mutant GAL4-myc expression vectors. After two days, cell lysates were prepared from transfected cells and subjected to polyacrylamide gel electrophoresis. The size-fractionated proteins were transferred to a nitrocellulose filter and detected with a polyclonal Myc antibody. As can be seen in Fig. 2, the Myc antibody detects both endogenous c-Myc protein (which provides an internal control for protein loading) and the slightly faster-migrating transfected GAL4-myc fusion protein. Fig. 2 also shows that the four mutant GAL4-myc fusion proteins are approximately equally well expressed as GAL4-myc wild type.

Transactivation by BL GAL4-Myc Fusion Proteins—It has recently been reported that several BL-derived mutant c-Myc proteins resist suppression of transactivation by p107 (Gu *et al.*, 1994; Hoang *et al.*, 1995). We therefore tested the chimeric GAL4-myc proteins for their ability to activate transcription both in the presence and absence of cotransfected p107. U2-OS osteosarcoma cells were transfected with GAL4-myc expression vectors and a CAT reporter gene that was linked to a core promoter and five upstream GAL4 sites in the presence and absence of p107. Fig. 3*A* shows that the wild type and mutant GAL4-myc expression vectors did not differ significantly in their ability to activate the GAL4-CAT reporter gene. Cotransfection of p107 expression vector caused a greater than 5-fold inhibition of transactivation of both the wild type and BL GAL4-myc expression vectors. We conclude from this experiment that these BL c-*myc* alleles do not differ significantly in their ability to activate transcription and sensitivity to suppression by p107.

These results are in apparent contrast with earlier studies by Hoang *et al.* (1995) and Gu *et al.* (1994) who found that ten

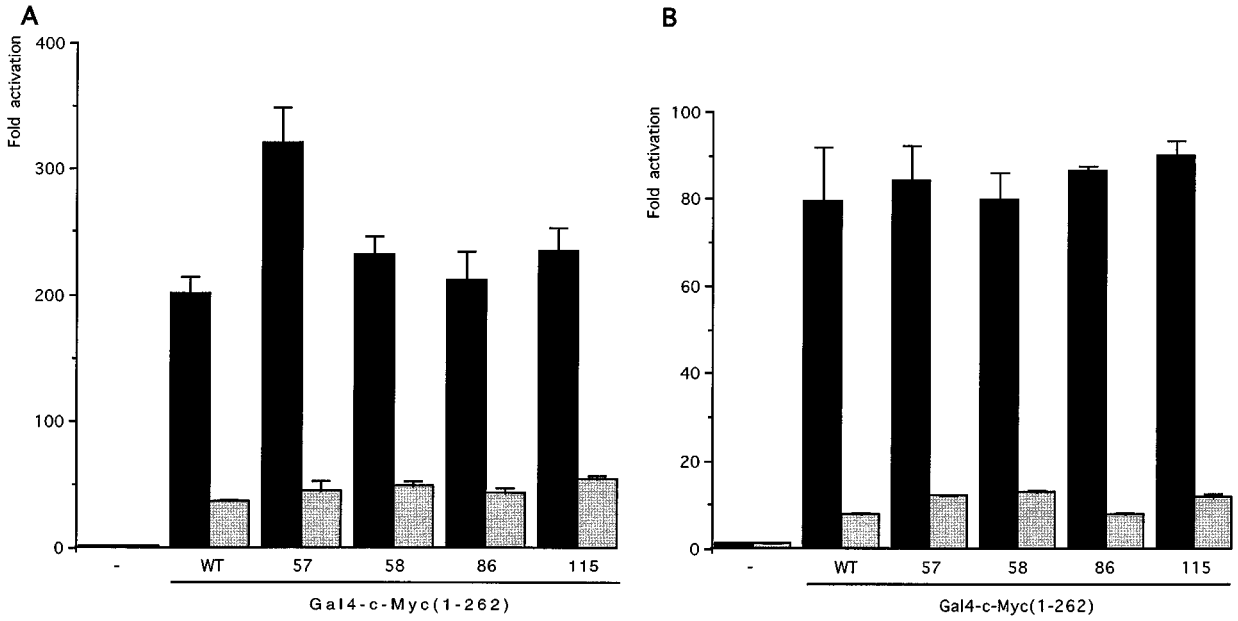

FIG. 3. **Transactivation by GAL4-Myc and repression by p107.** *A*, U2-OS cells were transfected with GAL4 reporter plasmid alone or cotransfected with reporter plasmid and pJ3-GAL4-myc expression vectors with specific missense mutations within the coding region (*numbers* below bars indicate the position of the mutations in c-Myc; *WT*, wild type), together with pCMV-p107 sense (*gray bars*) or p107 antisense (*black bars*). Data are representative of at least three independent experiments, each performed in duplicate. *B*, same experiment as in *A* performed in Chinese hamster ovary cells.

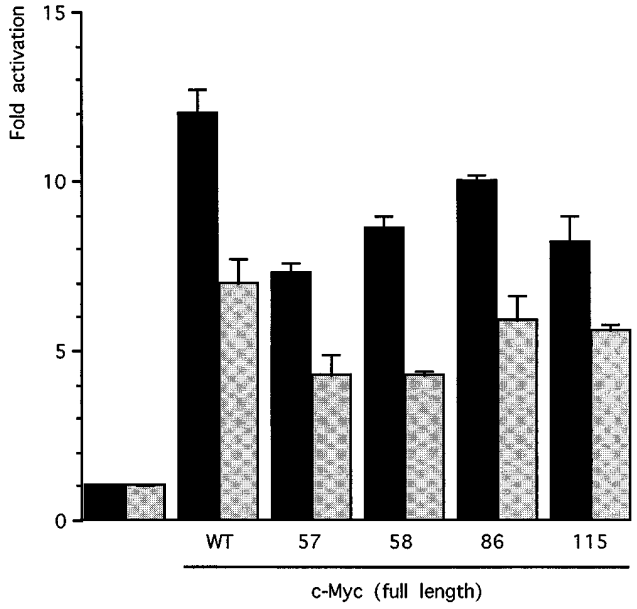

FIG. 4. **Transactivation by full-length mutant c-Myc proteins.** U2-OS cells were transfected with Myc/Max reporter plasmid alone or cotransfected with reporter plasmid and pJ3-myc expression vectors with specific missense mutations within the coding region (*numbers* below bars indicate the position of the mutations in c-Myc; *WT*, wild type), together with pCMV-p107 sense (*gray bars*) or antisense (*black bars*). Data are representative of at least three independent experiments, each performed in duplicate.

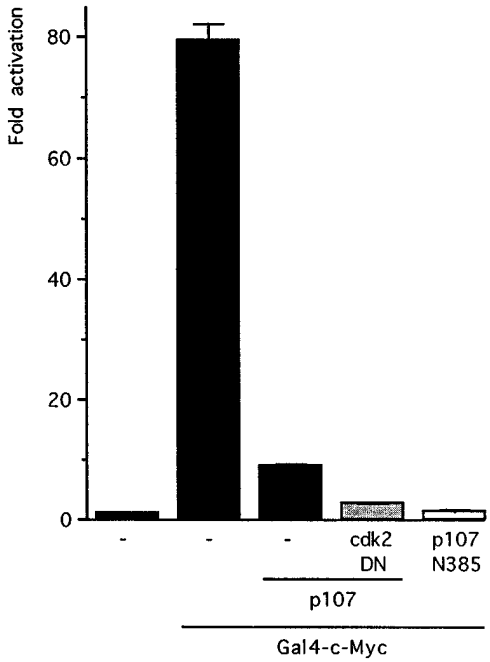

FIG. 5. **Effect of cyclin-cdks on c-Myc transactivation.** U2-OS cells were transfected with GAL4 reporter plasmid alone (*column 1*) or cotransfected with reporter plasmid and pJ3-GAL4-myc expression vector (*column 2*), together with pCMV-p107 (*columns 3* and *4*) and cdk2-dominant negative expression vector (cdk2-DN, *column 4*). in addition, GAL4-myc was cotransfected with a mutant p107 expression vector that directs the synthesis of a truncated p107 protein unable to bind cyclins (p107-N385, *column 5*). Data are representative of at least three independent experiments, each performed in duplicate.

different c-Myc alleles from BL cell lines did resist inhibition by p107. Since Hoang *et al.* (1995) used CHO cells in their studies, we repeated the experiment shown in Fig. 3*A* in CHO cells. Fig. 3*B* shows that all four mutant c-Myc chimeric proteins were equally sensitive to p107 suppression in CHO cells as compared to the human osteosarcoma cells. These data indicate that differences in cell type are not likely to be responsible for the observed difference in sensitivity to p107 suppression.

Transactivation by Full-length BL c-Myc Proteins—In addition to testing GAL4-myc fusion constructs, the corresponding full-length c-*myc* expression vectors were also tested for their ability to activate transcription and sensitivity to p107 suppression. The reporter plasmid used in these experiments contained four consensus c-Myc/Max DNA binding sites (CACGTG) upstream of a minimal promoter and a CAT reporter gene (Kretzner *et al.*, 1992). The results of this experiment, shown in Fig. 4, indicate that again both BL mutant and wild type c-Myc proteins activate transcription to the same extent. Consistent with their similar transactivation ability, we found that the wild type and mutant c-*myc* expression vectors were expressed equally in transiently transfected cells (data not shown). Again, no significant differences in susceptibility to p107-induced inhibition of transactivation could be observed for any of the mutant c-Myc proteins compared to the wild type (Fig. 4). Together, our data indicate that the three mutant c-Myc proteins derived from primary BL as well as the mutant derived from a BL cell line do not differ significantly from wild type c-Myc, both with respect to transactivation and sensitivity to suppression by p107.

Sensitivity to Cyclin/cdk Phosphorylation—It has been suggested that p107 inhibits c-Myc transactivation by recruitment of an active cyclin A-cdk2 kinase complex to the amino-terminal transactivation domain. Circumstantial evidence has been presented to indicate that this kinase complex phosphorylates serine 62 within the c-Myc transactivation domain, leading to down-modulation of transactivation. BL-derived mutant c-Myc proteins may therefore escape p107 inhibition of transactivation as a result of mutations that prevent efficient cyclin A-cdk2-dependent phosphorylation of c-Myc (Hoang *et al.*, 1995). We used two approaches to evaluate the possible role of the cyclin A-cdk2 complex in the down-modulation of c-Myc transactivation. First, we used a dominant-negative mutant of cdk2 (cdk2-DN) that effectively inhibits cdk2 kinase activity in transiently transfected cells (van den Heuvel and Harlow, 1993). We argued that if cyclin A-cdk2 would be responsible for down-modulation of c-Myc transactivation by p107, cotransfection of the dominant negative cdk2 expression vector should interfere with inhibition by p107 by preventing c-Myc phosphorylation. Fig. 5 shows that the opposite result was found: cotransfection of p107 and cdk2-DN caused a further inhibition of c-Myc transactivation. This suggests that cyclin A-cdk2 does not contribute to down-modulation of c-Myc transactivation by p107. As a control, we transfected a p107 expression vector in the presence or absence of a cdk2-DN expression vector. Fig. 6 shows that p107 is heterogeneously phosphorylated in transiently transfected U2-OS cells. Cotransfection of cdk4-DN or cdk2-DN strongly inhibited p107 phosphorylation, which results in a decrease in the mobility of p107 in SDS-polyacrylamide gels (Beijersbergen *et al.*, 1995). We conclude from this experiment that cdk2-DN is effective as an inhibitor of endogenous cyclin-cdk complexes in transfected cells.

In a second approach, we used an expression vector that directs the synthesis of an amino-terminally truncated p107 protein. p107-N385 is unable to bind cyclin A-cdk2 complexes (Zhu *et al.*, 1995) and may therefore be unable to inhibit c-Myc transactivation. Fig. 5 shows that p107-N385 was more effective than the wild type p107 protein in suppression of c-Myc transactivation. This result further supports the notion that recruitment of cyclin A-cdk2 to c-Myc by p107 is not required to mediate inhibition of transactivation.

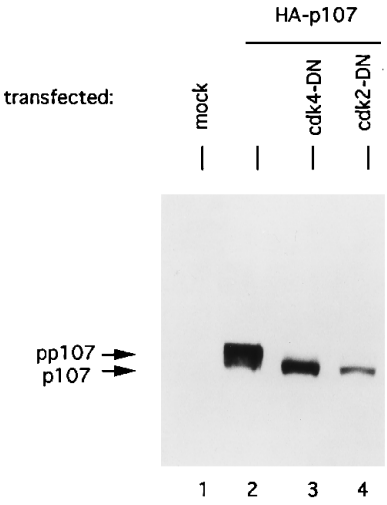

FIG. 6. **p107 phosphorylation is inhibited by dominant negative cdk2.** U2-OS cells were transfected with HA-epitope-tagged p107 expression vector (*lanes 2–4*) in the presence of either cdk4-DN expression vector (*lane 3*) or cdk2-DN (*lane 4*). After 2 days, protein lysates were prepared from transfected cells and separated on a SDS-polyacrylamide gel. After this, proteins were transferred to a nitrocellulose filter, and p107 protein was detected with monoclonal antibody 12CA5 directed against the HA epitope of transfected p107.

DISCUSSION

We describe here the functional characterization of three mutant c-*myc* alleles from primary BL in addition to one c-*myc* mutant derived from a BL cell line. All four mutant BL c-*myc* alleles were equally active in transactivation and did not differ from wild type c-*myc* in their sensitivity to suppression of transactivation by p107. These results differ significantly from those of two other studies in which it was shown that ten different mutant c-Myc proteins from BL cell lines were resistant to inhibition of transactivation by p107 (Gu *et al.*, 1994; Hoang *et al.*, 1995). One difference between our study and those of Gu *et al.* and Hoang *et al.* is that in our assays we used single missense mutations, three of which were identified in primary BL. In contrast, Hoang *et al.* (1995) and Gu *et al.* (1994) used mutant c-*myc* alleles found in BL cell lines, most of which carried multiple missense mutations scattered throughout the transactivation domain. We focused on c-*myc* mutations found in primary BL rather than BL cell lines because BL cell lines frequently acquire additional mutations during *in vitro* cultivation. Thus, BL cell lines derived from the same tumor have been reported to have different mutations (Albert *et al.*, 1994) and the BL cell line CA46 had additional mutations when compared to the primary tumor (Yano *et al.*, 1993). In addition, the translocated c-*myc* allele in the BL cell line Raji also continues to accumulate mutations in cell culture (Albert *et al.*, 1994). One possible explanation for the difference in results between this study and the two previous studies may be that resistance of c-Myc to p107 suppression is not frequent in primary BL but is a secondary phenomenon that BL tumor cells acquire when they accumulate additional c-*myc* gene mutations during *in vitro* cultivation. It remains unclear, however, why the Thr-58 to Ala mutant is resistant to p107 suppression in the study of Hoang *et al.* (1995), whereas in the same cell type, the same mutant was found to be sensitive to p107 suppression in the present study (Fig. 3*B*).

The clustering of mutations in exon 2 and especially in the region encoding amino acids 39–63 may suggest that the sequence alterations found are not neutral with respect to c-Myc function but provide a growth advantage to the cells. This region of the c-Myc protein is part of the transcriptional activation domain of c-Myc (Kato *et al.*, 1990). That transactivation by c-Myc is essential, for its transforming activity is supported by several lines of evidence. First, deletions within the amino-terminal transactivation domain severely affect c-Myc transformation (Kato *et al.*, 1990; Stone *et al.*, 1987). Furthermore, Barrett *et al.* (1992) have demonstrated a correlation between the transcriptional transactivation potency of Myc proteins and their ability to transform. The c-Myc protein is phosphorylated *in vivo,* and several studies have suggested that c-Myc transactivation and transformation is influenced by phosphorylation (Albert *et al.*, 1994; Gupta *et al.*, 1993; Henriksson *et al.*, 1993; Pulverer *et al.*, 1994; Seth *et al.*, 1991). Importantly, the two major phosphorylation sites in the transactivation domain, Thr-58 and Ser-62, and their flanking amino acids are most frequently affected in BL (Fig. 1). This may indicate that phosphorylation of at least some of these sites leads to down-regulation of c-Myc growth-promoting activity. Consistent with this is the finding that v-*myc* oncogenes frequently contain mutations in these phosphorylation sites (Frykberg *et al.*, 1987; Symonds *et al.*, 1989). In this study, we found no evidence that BL mutations affect c-Myc transactivation (Figs. 3 and 4), and similar results have been obtained by two other groups (Gu *et al.*, 1994; Lutterbach and Hann, 1994), whereas two other groups found that mutation of c-Myc phosphorylation sites leads to reduced transactivation ability (Albert *et al.*, 1994; Gupta *et al.*, 1993). Thus, although different constructs and different cell types have been used in different reports, all studies report that the transactivation ability of mutant BL c-Myc proteins is either comparable to that of wild type c-Myc or reduced. The lack of BL mutations with increased transactivation ability raises the possibility that mutation of amino-terminal residues may affect some other aspect of c-Myc physiology other than transactivation. One possibility is that some of the BL-derived mutant c-*myc* alleles contribute to tumorigenesis because of a reduced ability to induce apoptosis. That Myc-induced apoptosis depends on amino-terminal motifs within c-Myc has recently been demonstrated (Evan *et al.*, 1992). However, all BL mutant c-Myc protein mutants investigated thus far have unaltered ability to induce apoptosis (Hoang *et al.*, 1995). Another possibility is that transrepression by c-Myc is affected by the BL mutations. That transrepression requires the c-Myc amino terminus is supported by several lines of experimentation (Li *et al.*, 1994; Penn *et al.*, 1990a, 1990b; Philipp *et al.*, 1994). It will therefore be worthwhile to study the effects of BL-derived mutant c-Myc proteins in transcriptional repression assays.

The resistance of BL mutant c-Myc proteins to suppression by p107 is not due to a decreased affinity of the mutant c-Myc proteins for p107 (Hoang *et al.*, 1995). It has been suggested that transactivation by wild type c-Myc is down-modulated by an initial phosphorylation of Ser-62 by a cyclin A-cdk2-p107 complex. Subsequently, additional phosphorylation at Thr-58 is allowed by other kinases that inactivate Myc transactivation. BL-derived mutant c-Myc proteins frequently lack phosphorylation of Thr-58, which renders these mutant c-Myc proteins resistant to inactivation by the cyclin A-cdk2-p107 complex (Hoang *et al.*, 1995). Our data do not support this model for two reasons. First, a mutant of p107 that fails to form a stable complex with cyclin A-cdk2 has wild type ability to suppress c-Myc transactivation (Fig. 5). This indicates that cyclin A-cdk2 plays no role in the p107-mediated down-modulation of c-Myc transactivation. Furthermore, a dominant negative mutant of cdk2 that effectively blocks all cdk2 kinase activity in transiently transfected cells (Fig. 6 and van den Heuvel and Harlow (1993)) did not interfere with inhibition of c-Myc transactivation by p107. This further supports the notion that a cyclin A-cdk2 complex does not play a major role in down-modulation

of c-Myc transactivation. A prediction of the model proposed by Hoang *et al.* (1995) would be that Ser-62 and Thr-58 mutants of c-Myc would have an increased transactivation ability by escaping down-regulation by p107. As was discussed above, this is not found (Gu *et al.*, 1994; Lutterbach and Hann, 1994; Albert *et al.*, 1994; Gupta *et al.*, 1993). We favor the view that p107 binds to the c-Myc transactivation domain to prevent it from interaction with proteins that mediate transactivation. We and others have previously shown that c-Myc can interact with the TATA binding protein, and indeed the p107 binding site on c-Myc appears to overlap with that of the TATA binding protein (Hateboer *et al.*, 1993; Beijersbergen *et al.*, 1994; Maheswaran *et al.*, 1994). It should be noted, however, that this fails to explain why BL mutant Myc proteins bind p107 without being sensitive to its inhibitory effect (Gu *et al.*, 1994; Hoang *et al.*, 1995).

The results in the present paper indicate that escape from p107-induced suppression is not a general consequence for all BL-associated c-Myc mutations. It seems more likely that the heterogeneous BL mutations enhance the growth stimulatory ability of the c-Myc protein in different ways, and several mechanisms, operating either singly or in concert, lead to the abnormalities in cell growth found in BL.

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