Functional Analysis of the AUG- and CUG-Initiated Forms of the c-Myc Protein

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Submitted February 22, 1994; Accepted April 1, 1994

Monitoring Editor: J. Michael Bishop

Activation of the *c-myc* proto-oncogene by chromosomal translocation or proviral insertion frequently results in the separation of the *c-myc* coding region from its normal regulatory elements. Such rearrangements are often accompanied by loss or mutation of *c-myc* exon 1 sequences. These genetic alterations do not affect synthesis of the major *c-myc* protein, p64, which is initiated from the first AUG codon in exon 2. However they can result in mutation or loss of the CUG codon located in exon 1 that normally serves as an alternative translational initiation codon for synthesis of an N-terminally extended form of c-Myc (p67). It has been hypothesized that p67 is a functionally distinct form of c-Myc whose specific loss during *c-myc* rearrangements confers a selective growth advantage. Here we describe experiments designed to test the functional properties of the two c-Myc protein forms. We introduced mutations within the translational initiation codons of a normal human *c-myc* cDNA that alter the pattern of Myc protein synthesis (p64 vs. p67). The functions of each of these proteins were experimentally addressed using co-transformation and transcriptional activation assays. Both the p64 and p67 c-Myc proteins were independently able to collaborate with *bcr-abl* in the transformation of Rat-1 fibroblasts. In addition, both the exon 1- and exon 2-initiated forms of the c-Myc protein stimulated transcription of a Myc/Max-responsive reporter construct to a similar level. Given the apparent absence of functional differences between p64 and p67, we conclude that the basis for c-Myc oncogenic activation lies primarily in the overall deregulation of its expression and not in alterations in the protein. The existence of the CUG translational initiator may reflect a mechanism for the continued synthesis of c-Myc protein under conditions where AUG initiation is inhibited.

INTRODUCTION

The generation of neoplastic disease is considered to be a multistep process in which the sequential mutagenic

activation of oncogenes and/or inactivation of tumor suppressor genes results in a selective proliferative advantage (for reviews see Bishop, 1991; Vogelstein and Kinzler, 1993). *c-myc* is one oncogene whose activation via chromosomal rearrangement, proviral insertion or genomic amplification leads to its deregulated expres-

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sion in a wide variety of neoplasias (for reviews, see Alitalo *et al.*, 1987; DePinho *et al.*, 1991; Marcu *et al.*, 1992; Meichle *et al.*, 1992). In experimental models of tumor progression, *myc* can collaborate with activated *ras* (Stone *et al.*, 1987; Mukherjee *et al.*, 1992) or *bcr-abl* tyrosine kinase (Lugo and Witte, 1989; Sawyers *et al.*, 1992) in the transformation of fibroblasts in vitro or hematopoietic tissues in vivo (Sandberg, 1980). Our understanding of Myc's role in promoting tumorigenesis is currently being extended by molecular studies into its normal regulation, function, and interplay with other cellular factors.

The c-Myc gene product is a member of the helix-loop-helix and leucine zipper families of transcriptional regulators (Murre *et al.*, 1989). Beyond the apparent structural homology, Myc has been shown to possess sequence-specific DNA binding properties when in complex with its heterodimeric partner, Max (Blackwell *et al.*, 1990; Blackwood and Eisenman 1991; Prendergast and Ziff, 1991; Prendergast *et al.*, 1991). In transient transfections, such a Myc/Max complex is able to stimulate the transcription of a reporter construct bearing a Myc/Max binding site, CACGTG (Kretzner *et al.*, 1992). As an important corollary, mutational analysis demonstrates that the N-terminal transcriptional activation domain and the C-terminal DNA binding/dimerization domain of c-Myc are essential for cell transformation (Stone *et al.*, 1987; Kato *et al.*, 1990; Penn *et al.*, 1990) and apoptosis mediated by Myc (Evan *et al.*, 1992).

The differential regulation of c-Myc and Max in vivo also appears to be a critical determinant of Myc function. Whereas the Max proteins are stable (Blackwood *et al.*, 1992b; Kato *et al.*, 1992) and constitutively expressed in many cell types (Berberich *et al.*, 1992; Blackwood *et al.*, 1992b), the c-Myc proteins are unstable (Lüscher and Eisenman, 1988), and their expression is regulated. For example *c-myc* expression is induced by mitogenic signals (Kelly *et al.*, 1983; Greenberg and Ziff, 1984; Dean *et al.*, 1986) and correlates with cell proliferation (Hann *et al.*, 1985; Thompson *et al.*, 1985; Waters *et al.*, 1991). Indeed, its transcriptional regulation is subject to tight control at nearly every level, from transcriptional initiation (Moberg *et al.*, 1992) and elongation (Bentley and Groudine, 1986; Spencer and Groudine, 1991) to mRNA stability (Nepveu *et al.*, 1987; Wisdom and Lee, 1991). Interference with proper Myc expression has been shown to preclude entry into the cell cycle or to block differentiation (Holt *et al.*, 1988; Karn *et al.*, 1989; Eilers *et al.*, 1991; Roussel *et al.*, 1991). Thus Myc appears to be the rate-limiting component of a nucleoprotein complex whose function is required for cell proliferation.

Although the primary consequence of genomic rearrangement or proviral insertion is to separate the Myc gene from elements that normally control its expression, these genetic lesions can also alter transcript structure and are often accompanied by point mutations (Alitalo

et al., 1987). Normally, the *c-myc* gene is comprised of three exons that direct the synthesis of two primary translation products, p64 and p67 (Hann *et al.*, 1983; Hann and Eisenman, 1984). These proteins differ not as the result of alternative splicing or post-translational modification, but by alternative translational initiation (Hann *et al.*, 1988). The predominant Myc polypeptide, p64, is the product of an AUG-initiated open reading frame contained within exons 2 and 3, while the p67 form arises from a CUG initiation within exon 1. Since the initiation from this upstream, in-frame and highly conserved CUG codon yields a Myc protein with a unique N-terminus, this 14-amino acid extension could potentially alter the properties and function of p67. The observation that exon 1 sequences are frequently lost or mutated in Burkitt's lymphoma (Magrath, 1990), murine plasmocytoma (Ohno *et al.*, 1984), and other lymphoid neoplasias (McCarthy *et al.*, 1984) has led to the hypothesis that the specific loss of p67 expression contributes to oncogenesis (Hann *et al.*, 1988). A more recent study has shown that the levels of the CUG-initiated form increase during density-dependent growth arrest as a result of methionine depletion, providing further support for the idea that the two c-Myc proteins have unique functions (Hann *et al.*, 1992). Furthermore, following the identification of the CUG initiation in *c-myc*, similar upstream alternative CUG initiations have been identified in a number of genes including *int-2* (Acland *et al.*, 1990), basic FGF (Florkiewicz and Sommer, 1989; Prats *et al.*, 1989; Bugler *et al.*, 1991), *hck* (Lock *et al.*, 1991), *pim-1* (Saris *et al.*, 1991), *lxl-1* (Mellentin *et al.*, 1989), and the glycosylated gag protein of murine leukemia virus (Prats *et al.*, 1989a). In some cases the CUG-initiated forms exhibit subcellular localization properties distinct from their AUG-initiated counterparts (Prats *et al.*, 1989a; Acland *et al.*, 1990; Bugler *et al.*, 1991; Lock *et al.*, 1991).

In this work, we have designed mutations that alter the translation pattern of the p64 and p67 c-Myc proteins, and experimentally address the possibility of their functional difference. We find that both p64 and p67 are independently able to transform Rat-1 fibroblasts in collaboration with *bcr-abl*, and moreover, are equally able to stimulate transcription from a reporter construct containing Myc/Max consensus sites. We conclude that the p64 and p67 Myc proteins are not functionally distinct as judged by the major available criteria for Myc function.

MATERIALS AND METHODS

Myc Mutations

An oligonucleotide-directed in vitro mutagenesis system (Amersham, version 2.1) was used to introduce mutations into the region spanning the exon 1/exon 2 boundary of the 0/1 human c-Myc cDNA (Hann *et al.*, 1988). Mutation 3 (e.g., Mut 3) inserted a stop codon at the exon 1/2 junction by a CAG → TAG transition. Mutation 13 altered the exon 1 start site from CUG to AUG, while mutation 15 additionally

substituted CUG for AUG at the exon 2 translational initiation site (i.e., conservative substitution of leucine for methionine). Mutation 15b modified only the p64 translational start site from AUG to CUG. When the exon 1 start site was altered, the adjacent upstream codon was also modified to prevent cryptic initiation (ACG → ACC). Each mutation was verified by dideoxy-sequencing. Structural mutations within the c-Myc gene have been described elsewhere (Blackwood and Eisenman, 1991).

Viruses

To generate virus producer lines, the Ψ 2 ecotropic packaging cell line was transiently transfected with the parental pLXSN retroviral vector or with a vector into which had been subcloned the mutated c-Myc cDNA's (pLMycSN) (EcoRI inserts: Mut 3, Mut15b, Δ CKII, BR21M, Δ BR, Δ N100; BamHI inserts: 0/1 [wild-type], Mut 13, Mut 15). Low titer viral supernatants were subsequently harvested and used to infect the PA317 amphotropic packaging cell line. Amphotropic producer lines were established by drug selection of cells plated at limiting dilution (G418 @ 500 μ g/ml). Characterization of several clones allowed the selection of a producer line, which met the following criteria: (1) a high Neo^R titer as measured on NIH 3T3 TK- cells (10⁶ cfu/ml), (2) expression of the exogenous protein as verified by radio-immunoprecipitation, and (3) correct proviral size and a single integration site as verified by Southern blotting of genomic DNA.

The BCR-ABL virus was generated by infection of the PA317 amphotropic packaging line with Moloney-rescued pJW-RX (previously described in Lugo and Witte 1989) and selection for a helper-free clone. The resulting p210 viral producer line gives a lower Neo^R titer on TK-cells (1.5 × 10⁵ cfu/ml) but appears to exhibit reduced toxicity (see Daley *et al.*, 1987 for description of the toxic effects of *bcr-abl* in fibroblasts). The amphotropic virus stocks contain no helper activity as judged by the inability to propagate Neomycin resistance from infected NIH 3T3 TK-cells.

Construction of an Alternative Initiation Site in Lamin C

The plasmid pUC9 cDNA-7, containing the human lamin C cDNA (McKeon *et al.*, 1986), was linearized by cutting 5' of the lamin C ATG translational initiation codon with *Sac*II. The plasmid was "blunt-ended" and the terminal G nucleotide removed by employing T4 DNA polymerase in the sole presence of dCTP. This allowed the insertion of the c-myc exon 1 sequences in the appropriate reading frame. The exon 1/2 region proximal to the c-myc CTG segment was released by *Fnu*DII digestion and ligated into this modified lamin cDNA. Fragments were inserted from both the wild-type c-myc and the Mut11 c-myc clones (see above). The structures of the clones were verified by sequencing. For in vitro transcription, the lamin C cDNA was subcloned into pSP64.

In Vitro Transcription/Translation

The c-Myc mutants were subcloned into the T3/T7 RNA polymerase transcription vector, pBlueScript (Promega, Madison, WI). In vitro transcription and translation were performed under the conditions recommended by the Promega Protocols and Applications guide. [³⁵S]methionine-labeled products from programed rabbit reticulocyte lysates were resolved under reducing conditions on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and analyzed by autoradiography.

Radio-Immunoprecipitations

Proliferating fibroblasts were pulse-labeled with [³⁵S]methionine (EXPRE³⁵S [³⁵S]protein labeling mix, NEN Research Products, Boston, MA) for 30 min. Standard high stringency immunoprecipitations were performed as described elsewhere (Lüscher *et al.*, 1989). Briefly, cells were lysed in the following Ab buffer (in mM): 20

tris(hydroxymethyl)aminomethane (Tris) pH 7.4, 50 NaCl, 1 EDTA, 0.5% NP-40, 0.5% deoxycholate, 0.5% SDS, and protease inhibitors, sonicated on ice, and the lysate clarified by centrifugation. The clarified supernatants were normalized to equivalent trichloroacetic acid (TCA) precipitable counts, and subjected to immunoprecipitation with affinity-purified antibodies specific for the C-terminal 12 amino acids of human or murine c-Myc (Hann and Eisenman, 1984). Immunocomplexes were captured on Protein A-Sepharose beads, washed twice with Ab buffer, once with high salt buffer (2 M NaCl, 10 mM Tris pH 7.4, 0.5% deoxycholate, 1% NP-40), and finally, twice with RIPA buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, 0.5% aprotinin). Reduced, heat denatured samples were resolved by SDS-PAGE (10% acrylamide).

In Vitro Autophosphorylation Assays

Cells (2 × 10⁶) were lysed in 1.5 ml low stringency buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 1% NP-40, protease inhibitors), sonicated on ice, and centrifuged. The clarified lysate was subjected to immunoprecipitation with a rabbit polyclonal antiserum directed against exons 2 and 3 of c-*abl* (Lugo and Witte, 1989). This antiserum does not interfere with kinase activity. The immune complexes captured on Protein-A Sepharose resin were washed four times with the low stringency buffer, followed by two washes with Tris-buffered saline (20 mM Tris pH 7.5, 150 mM NaCl). The beads were then resuspended in 25 μ l of kinase buffer (20 mM Tris pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 5 mM MnCl₂, 5 μ M ATP, 0.5% aprotinin, and 1 μ l/reaction [γ -³²P]ATP) and allowed to incubate at 30°C for 30 min. The kinased samples were electrophoresed on an 8% discontinuous SDS-PAGE gel and analyzed by autoradiography.

Myc/BCR-ABL Cotransformation Assay

Rat-1 cells were plated at 5 × 10⁴ cells/well in a 6-well plate [10% fetal calf serum (FCS)/Dulbecco's modified Eagle's medium (DMEM)/pen/strep]. On the following day, the cells were infected with control or Myc virus, using a Multiplicity of Infection (MOI) of one (assuming that cells have divided once, 1 × 10⁵ cfu = MOI of 1.0). To enhance infection, polybrene was routinely added at 8 μ g/ml. On the day following infection, cells were split 1:10 into media containing G418 (500 μ g/ml) and selected in drug for 5–7 d. These bulk populations of Neo^R, "Myc-expressing" cells were seeded at 5 × 10⁴ cells/well in a 6-well plate. Again on the following day, cells were infected with 1.0 ml of p210/bcr-abl virus. In parallel, the p210 virus was titered on Rat-1 cells for a direct comparison of Neo^R with colony formation. After the overnight infection, 1000–2000 cells/well were plated in soft agar (in duplicate) or as a monolayer culture. The soft agar culture was comprised of three layers: an underlayer and overlay (2.5 ml/well in a 6-well plate: DMEM, 5% FCS, 0.65% noble agar), and a cell layer (4.0 ml/well in a 6-well plate: DMEM, 12.5% FCS, 0.25% noble agar). After 14–18 d in soft agar, the number of colonies exceeding 250 μ m in diameter were scored by image analysis using Visage 2000 software (BioImages, Millipore, Ann Arbor, MI) and the average number of colonies arising per 1000 cells plated was tabulated.

Northern Analysis

Total RNA was prepared from G418-resistant bulk populations by the acid-guanidinium thiocyanate-phenol-chloroform extraction procedure as described (Chomczynski and Sacchi, 1987). Ten micrograms of total RNA was resolved in a formaldehyde gel, transferred to a nylon membrane, and hybridized with the 3' *Clal*-EcoRI fragment of the human c-Myc cDNA (0/1). To normalize for unequal loading, the blot was stripped and rehybridized with a rat GAPDH probe.

CAT Assay

Chloramphenicol acetyl transferase (CAT) activity of a reporter gene was measured essentially as described (Kretzner *et al.*, 1992). Briefly,

NIH 3T3 cells were co-transfected with reporter plasmid (3 μ g M4-minCAT), Myc expression vector (e.g., 10 μ g pSP271-Myc) and a β -galactosidase vector used to normalize for transfection efficiency (0.6 μ g CMV β -gal). Cell lysates were collected 36 h after CaPO₄ transfection, normalized to β -galactosidase activity, and assayed for CAT activity (Gorman *et al.*, 1982). The mono- and diacetylated products were resolved from unmodified substrate by thin layer chromatography. To calculate the fold activation, the percent conversion values were normalized to that of the control pSP271 expression vector.

RESULTS

Design of Mutations in the c-Myc cDNA that Alter the Translational Expression Pattern of p64 and p67

Site-directed mutagenesis was used to introduce specific point mutations into the wild-type human *c-myc* cDNA (denoted 0/1; Hann *et al.*, 1988) that would block or enhance translation from either the AUG initiation site within exon 2, or the CUG-initiation site within exon 1-containing RNAs. These mutations are depicted in Figure 1A (note that when referring directly to DNA sequences we use T, whereas, we employ U in reference to translational initiation codons in the context of RNA).

Mutation 3 (Mut 3) changed the CAG codon present at the exon 1-2 junction to a TAG stop codon; this mutation was expected to effectively terminate translation products originating from the upstream CUG and result in the sole production of p64. In Mutation 13 (Mut 13), the production of p67 was enhanced by replacement of the less efficient CUG initiator with an AUG. Mutation of the guanine base 5' the AUG (G $\rightarrow$ C), not only prevented potential cryptic initiation from the ACG codon (Peabody, 1989) but also introduced a convenient *Nco* I site to be used for other experimental purposes. Because both AUG start sites in Mut 13 support translational initiation, resulting in the translation of p64 as well as p67, we sought to eliminate the production of p64 by mutating its initiation codon. As a methionine to leucine conversion represented the most conservative amino acid substitution, we replaced the exon 2 AUG with a CUG (Mut 15). Last, the fact that the N-terminal fragment of p67 had never proven amenable to Edman degradation (Hann *et al.*, 1988) suggested the presence of an unusual N-terminal blocking group. We postulated that the naturally occurring CUG-encoded start site might contain additional information encrypted within its sequences that might promote such a co-translational modification of the protein. Therefore, we chose to retain the exon 1 CUG, yet downregulate the translation of p64 by substituting a leucine triplet (CUG) at its initiation codon (Mut 15b). Each of these mutations was confirmed by dideoxy-sequencing of the 5' cDNA sequences.

Having introduced these mutations into the *c-Myc* cDNA, we next verified that the transcripts were translated into the predicted protein products. In vitro transcription was used to synthesize mRNA, and equivalent amounts of uncapped transcript were used to program

rabbit reticulocyte lysates. The resulting [³⁵S]methionine labeled proteins, as analyzed by SDS polyacrylamide gel electrophoresis, are shown in Figure 1B. The wild-type *c-Myc* transcript (0/1) produces significant amounts of both p64 and p67 protein, although the difference in specific activity of the radiolabeled proteins should be considered (3 vs. 4 methionine residues, based on the presumption that the N-terminal methionine is removed following translation [Moerschell *et al.*, 1990]). As predicted, the insertion of a stop codon between the two translational start sites permitted only the expression of p64 (Mut 3), while the conservative substitution of CUG at the exon 2 AUG produced only p67 (Mut 15). Mut 13, which contains good context consensus AUGs (Kozak, 1989a) in both exons 1 and 2, expressed the inverse ratio of p64 to p67. Apparently increasing the efficiency of translation initiation at the exon 1 start site decreases the efficiency of initiation at subsequent sites. The CUG/CUG combination of translational initiation sites is much less efficient at the initiation of either protein (Mut 15b). Mutations that alter sequences surrounding the start site have shown that the efficiency of translational initiation at non-AUG codons is highly dependent on a good context sequence e.g., A at the -3 and G at the +4 positions surrounding the AUG (Street and King, unpublished data) (Kozak, 1989a, b). The panel of mutations shown in Figure 1A, which allowed the independent expression of p64 or p67, permitted us to ask specific questions about the biological significance of these two Myc variants.

We were also interested in determining whether the *c-myc* CUG codon could only function in translational initiation within the context of the *c-myc* coding region. A *Fnu*DII fragment, comprising the CUG codon in exon 1 and extending to five nucleotides upstream of the AUG in exon 2, was inserted upstream and in-frame of the AUG initiation codon of a human lamin C cDNA (Figure 1C, bottom). Initiation at the CUG codon should result in a translation product with a 23 residue N-terminal extension. As shown in Figure 1C, in vitro transcription/translation of the lamin C cDNA lacking the insert results in the synthesis of a single major band with an apparent molecular weight of 68 kd, as expected for lamin C. However translation of the lamin C cDNA containing the *c-myc* exon 1 CUG initiation region generates two polypeptides: a smaller protein which comigrates with the lamin C product and an additional protein that is 1-2 kd larger in apparent molecular weight. That this larger protein is initiated from the *c-myc*-derived CUG codon was indicated by the fact that it is not present when the CUG codon in the *Fnu*DII insert was mutated to a noninitiating UAG codon. These results show that the CUG-containing codon can function as an alternative translational initiation site in contexts other than the *c-myc* gene and is consistent with other reports of CUG initiations (see DISCUSSION).

Figure 1. Mutations in the c-Myc cDNA designed to alter the ratio of p64/p67 protein expression. (A) Oligonucleotide-directed mutagenesis was used to insert a stop codon (TAG) between the two translational initiation sites, or to modify the initiation codon(s) and its context. The amino acid sequence of the N-terminal extension of p67 is shown above the corresponding nucleotide triplet, while the exon 1-2 junction is denoted by the bent double arrow. The asterisk indicates an ambiguity in the 3' splice acceptor site, which can result in the inclusion of a second CAG triplet. Nucleotides, which define the consensus for efficient translational initiation, (Kozak, 1989a,b) are underlined. (B) In vitro translation products from the altered c-myc cDNAs. Equivalent amounts of synthetic transcripts were used to program a rabbit reticulocyte lysate, and the resulting [³⁵S]methionine-labeled proteins were analyzed by SDS-PAGE and autoradiography. (C) In vitro translation of lamin C cDNAs. The human lamin C cDNA was transcribed and translated to generate the protein product expected from its AUG-initiated ORF (left lane). Insertion of a *Fnu*DII fragment from human c-myc places the CUG containing initiator upstream of the lamin AUG and results in synthesis of the normal lamin C and a higher molecular weight protein (middle lane). Mutation of the CUG to UAG abolishes the synthesis of the higher molecular weight protein (right lane).

Generation of Retroviral Vectors Expressing the c-myc or bcr-abl Gene Products

To test the functional potential of p64 and p67 c-Myc proteins in vivo, we chose to exploit the advantages of retroviral vectors. Unlike plasmid transfections, where coding sequences may become rearranged, and prolonged culturing is required for the selection of stable integrants, retroviruses permit the rapid linear transduction of the transgene. In addition, the copy number of the introduced gene can be controlled from the titer of the viral stock. Because infection yields a bulk population of transgene-expressing cells, artifacts that arise as the result of insertional mutagenesis are also minimized. Given these advantages, the mutant c-myc cDNAs were subcloned into the pLXSN retroviral vector

(Miller and Rosman, 1989) and used to develop stocks of amphotropic virus. As a strong promoter, the retroviral long terminal repeat (LTR) was used to drive Myc expression. The Myc proteins are translated from the resulting full length viral genomic mRNA, while neomycin resistance is conferred by an SV-40 promoted subgenomic transcript. The bcr-abl virus was generated by the conversion of the previously described pJW-RX stock (Lugo and Witte, 1989) to a helper-free amphotropic packaging line. The elimination of the MoMLV helper virus not only allowed the controlled introduction of the p210 virus into the Rat-1 cell line but may have also reduced its toxicity in both the viral producer and recipient fibroblasts (Daley *et al.*, 1987; Lugo and Witte, 1989).

To test for the accurate transduction and expression of the exogenous proteins, Rat-1 cells were infected with the different Myc viruses at a multiplicity of one, and selected in G418 for the minimal amount of time required to establish neomycin resistance. The rodent and human Myc polypeptides were subsequently identified by immunoprecipitation from metabolically labeled cells using species-specific antisera prepared against the C-terminal 12 amino acids of the mouse and human Myc proteins (Hann and Eisenman, 1984). As seen in Figure 2A, the pattern of exogenous Myc polypeptide expression is similar, although not identical, to that seen *in vitro*. *In vivo*, the 0/1 *c-myc* cDNA produces primarily the 64-kd species of c-Myc (p67 is barely visible in this exposure; it can be better seen in Figure 7). As enhanced translation of the p67 form has been attributed to methionine deprivation at high-cell densities (Hann *et al.*, 1992), the rather small amount of p67 produced here may reflect the health and proliferative state of the culture as well as *in vivo* preferences for AUG over CUG initiation. With respect to the translational initiation mutants, the replacement of CUG with AUG in Mut13 effectively inverts the ratio of the two proteins. Mut 3 and Mut 15 produce only p64 or p67, respectively, as expected from the *in vitro* translation results (see Figure 1A). Interestingly, the 15b mutant appears unable to produce appreciable amounts of Myc protein, even though by Northern analysis its levels of transcript are comparable to those of the other *c-myc* genes tested (Figure 2B).

Because different antibodies are also likely to differ in antigen affinity, it is difficult to make a quantitative comparison between the levels of endogenous and exogenous c-Myc protein. However use of cross-reacting antisera permitted discrimination between rodent and human c-Myc proteins based on slight differences in electrophoretic mobility. Under these immunoprecipitation conditions, the virally introduced protein appeared to be expressed at only a 2- to 3-fold higher level than that seen in proliferating cells, though this small increase may partially reflect negative autoregulation of the endogenous allele (Penn *et al.*, 1990). This suggests that the viral LTR promotes moderate constitutive overexpression of the c-Myc protein. Characterization of the independently expressed p64 and p67 c-Myc proteins revealed no significant differences in their predominant nuclear localization nor in protein half-life (A.J. Street, unpublished observations).

Figure 2A also shows proteins expressed by several previously described *c-myc* mutants in which functional regions were deleted (Blackwood and Eisenman, 1991). The Δ N100 mutation initiates translation at residue #101 and removes a major portion of the previously defined transcriptional activation domain (residues 40–143) (Kato *et al.*, 1990) resulting in a 46-kd polypeptide (Figure 2A). Δ BR specifically deletes 15 amino acids within the DNA binding basic region of the Myc protein,

Figure 2. (A) Myc protein expression in infected Rat-1 cells. Myc polypeptides were immunoprecipitated from [35 S]methionine-labeled G418^R cell populations using antisera specific for either rodent (Endogenous) or human (Exogenous) c-Myc protein. Specific immunoprecipitation was blocked by the inclusion of the cognate peptide immunogen (Block). Samples were analyzed by SDS-PAGE and autoradiography. The migration of molecular weight markers is indicated. Myc virus designations are described in the text. (B) Expression of Myc-encoding viral transcripts. Total RNAs derived from G418-resistant bulk populations were analyzed by Northern blotting. In the top panel, hybridization with a *myc* probe detected the steady-state levels of Myc mRNA expressed from the viral LTR. The major 4.4-kb transcript corresponds to the predicted genomic mRNA (LTR-Myc); subgenomic species are also present. To normalize for potential loading disparities, the blot was rehybridized with a rat GAPDH probe (bottom panel). The positions of EtBr-stained ribosomal RNA bands (18S and 28S) are indicated.

while the Δ CKII mutation removes nine amino acids at the CKII phosphorylation sites adjacent to the basic region. Finally the BR21M replaces the Myc basic region with that of MyoD. The expected small differences in electrophoretic mobility are apparent when these proteins are expressed (Figure 2A).

The BCR-ABL virus was also evaluated for its ability to introduce the p210 tyrosine kinase into cultured cells. Immunoprecipitation with a c-Abl-specific antiserum revealed that the infected cells expressed a 210-kd protein with *in vitro* autophosphorylation activity. The comparison of *bcr-abl* kinase activities shown in Figure 3 demonstrates that a bulk population of Rat-1 cells transduced with the virus express as much p210 kinase

Figure 3. p210 protein kinase activity in infected Rat-1 cells. Immunoprecipitation of BCR-ABL protein was followed by in vitro autophosphorylation in the presence of [γ - 32 P]ATP. Samples were resolved by SDS-PAGE and visualized by autoradiography.

activity as a clonal cell line (RV688E) expressing *bcr-abl* (Lugo and Witte, 1989). Importantly, the levels of *bcr-abl* activity are comparable with that of the naturally occurring Philadelphia chromosomal translocation seen in the erythroleukemia cell line, K562 (Daley *et al.*, 1987). We also note that the levels of endogenous c-Myc are not significantly altered in cells expressing *bcr-abl* (Figure 2A).

p64^{c-myc} and p67^{c-myc} Transform Rat-1 Fibroblasts in Collaboration with P210^{bcr-abl}

We chose the Myc/BCR-ABL co-transformation assay for one comparison of p64 versus p67 c-Myc function for two reasons. First, co-transformation represents a biologically relevant assay, especially since *abl* and *c-myc* are known collaborators in the generation of murine

plasmacytomas (Rosenbaum *et al.*, 1990) and in the transformation of cell lines (Lugo and Witte, 1989; Street *et al.*, 1990; Sawyers *et al.*, 1992). Second, co-transformation appears to be a more sensitive assay for c-Myc biological function than the ability to block the induced differentiation of cell lines (E.M. Blackwood, unpublished observations).

In the assay schematized in Figure 4, Rat-1 cells are infected with the various Myc viruses at a titer ensuring that the majority of the cells obtain a single integrated copy of the transgene. Selection in drug for the minimal time required to establish the Neo resistant character of the bulk population (5–7 d) limits the acquisition of secondary mutational events that might contribute to transformation. These bulk populations are then infected with the *bcr-abl* virus and are cultured in monolayer or soft agar to score for the transformed phenotype. In parallel, the p210 virus is titrated on Rat-1 cells to allow for direct comparison of colony formation with conferral of Neo resistance.

As seen in Figure 5, the Rat-1 fibroblasts arrest as dense cultures, which continue to display a basically flat morphology. When infected with control (Lx) or Myc-expressing virus, the cultures retain this "normal" phenotype. While the Myc expressing cells appear to proliferate more rapidly and arrest at a somewhat higher density, neither of these phenomena score above a 20% increase. On the other hand, infection with the BCR-ABL virus results in regions of dysplasia within the monolayer (Figure 5). Prolonged culture, with repeated passaging, eventually selects for a population that is no longer subject to density dependent growth arrest. Thus the p210 kinase may contribute to a general genomic destabilization and thereby increase the frequency of secondary activation events. Given the elevated background when infected Rat-1/p210 bulk populations are subsequently infected with the Myc virus, we relied on the inverse order of transgene introduction. Transformation by these oncogenes has been shown to be in-

Figure 4. Myc/BCR-ABL co-transformation assay. Rat-1 cells were infected with control or Myc-expressing virus (MOI = 1.0) and selected in G418 for 5–7 d. The resulting bulk populations were infected with the BCR-ABL virus (MOI < 0.1) and directly assayed in monolayer or soft agar culture for transformed phenotype.

Figure 5. Monolayer cultures of infected and doubly infected Rat-1 cells. Phase-contrast photomicrographs demonstrate cell morphology of 10-d monolayer cultures. Parental and G418-resistant cell populations are shown in the left-hand column (Control), while the same cultures subjected to serial infection with the BCR-ABL virus (+p210) are shown in the right-hand column. Magnification, 100 $\times$.

dependent of the order in which they are introduced into cells (Lugo and Witte, 1989). Using the Myc/BCR-ABL co-transformation assay, we tested the ability of either or both the p64 and p67 c-Myc polypeptides to promote soft agar colony formation. As seen in Figure 6 and tabulated in Table 1, wild-type Myc as well as p67 or p64 alone were functionally comparable in the establishment of substratum-independent growth. Of the translational initiation mutants, only Mutation 15b (CUG/CUG) was unable to proliferate in soft agar. However as shown in Figure 2, A and B, the inability of Mut 15b to initiate the translation of appreciable amounts of either p64 or p67 protein presumably accounts for this functional failure.

Use of c-Myc mutants deficient in known functional regions of the protein confirmed the co-dependence of p210^{bc_r-abl} and Myc in transformation in this system. Thus removal of most of the Myc N-terminal transactivation domain (Δ N100) severely reduced the number of soft agar colonies, while mutations that inactivate

the DNA binding domain or modify its specificity (Δ BR and BR21M, respectively) were completely unable to cooperate with *bcr-abl* to transform the Rat-1 fibroblasts (Figure 6 and Table 1).

Comparison of the Neo^R titer with the transformation potency of the p210/*bcr-abl* virus demonstrates a one-to-one correspondence between the number of colonies that proliferate in soft agar and the presence of both oncogenes (Table 1). For example, of 10⁵ cells, 10⁴ are normally infected with Neo^R-conferring virus particles. Given this low titer, only $\sim$ 10% of the Rat-1/Myc bulk population receives the second oncogene, and a corresponding 10% of the 1000–2000 cells plated in soft agar proliferate into macroscopic colonies. Indeed, colonies which were subcultured from soft agar expressed both the human c-Myc protein as well as the p210^{bc_r-abl} kinase (E.M. Blackwood, unpublished observations).

The observation that either the p64 or p67 forms of the c-Myc protein can independently collaborate with the p210^{bc_r-abl} kinase to transform Rat-1 fibroblasts, suggests that the p64 and p67 proteins function equally well in the context of cellular transformation (see DISCUSSION).

p64 and p67 Independently Transactive Reporter Gene Expression in NIH 3T3 Cells

Recent studies have shown that Myc can function as a transcriptional activator of reporter genes containing promoter-proximal Myc:Max CACGTG binding sites (Amati *et al.*, 1992; Kretzner *et al.*, 1992; Amin *et al.*, 1993; Gu *et al.*, 1993). We therefore examined whether the two alternatively initiated forms of c-Myc might differ with respect to their ability to activate transcription of a reporter gene. In our experiments a minimal promoter, which contains a four-fold repeat of the Myc/Max recognition sequence controlled the expression of the chloramphenicol acetyltransferase gene (pM4-minCAT). Transient transfection of this reporter construct alone (Figure 7A, pSP) revealed that proliferating NIH 3T3 cells contain endogenous factor(s), including c-Myc itself, that are able to stimulate transcription from the CACGTG response elements (Kretzner *et al.*, 1992). However, as the levels of Myc protein were supplemented by co-transfection of the Myc expression vector (pSP271-0/1), reporter activity coordinately increased (Figure 7A). Max, as the essential partner for Myc function, is normally in molar excess in these cells (Blackwood *et al.*, 1992a,b) and thus its supplementation by expression vector was not required.

When the CUG/AUG translational initiation mutants were analyzed for their ability to stimulate transcription from the pM4-minCAT reporter construct, we found that all the mutants elevated the levels of CAT activity regardless of whether the c-Myc proteins were initiated from the exon 1 or exon 2 sites (Figure 7A). The finding that Mut15b also functioned in the transcription assay

was unexpected since this mutant failed to transform Rat-1 cells in conjunction with *bcr-abl*. To determine whether Mut15b produced c-Myc protein, we performed parallel immunoprecipitations from the transiently transfected cultures. As seen in Figure 7B, Mut 15b transcripts produce human c-Myc proteins, though at a reduced level which correlates with the lower levels of CAT activity. The apparent discrepancy between the translation efficiency of viral *myc* constructs used in the co-transformation assays and the plasmid constructs used in the transcription study may lie in the fact that the viral genomic RNA is full-length and contains the Ψ packaging sequences. This RNA secondary structure could potentially influence the ability of ribosomes to initiate translation at non-AUG sites. In the absence of these viral sequences, the pattern of Myc polypeptides that initiate from the Mut 15b transcript include not only p64 and p67 [including their phosphorylated counterparts (p65/p68)] but p46 as well. This smaller protein corresponds to initiation at methionine 100 (see Δ N100 in Figure 2A). Thus, a reduction in the efficiency of initiation at upstream sites may allow ribosomes to scan through to more internal AUG consensus sites. A similar effect has been observed for internal translational initiation products derived from the thyroid hormone receptor (Bigler *et al.*, 1992). Clearly, both the p64 and p67 c-Myc polypeptides are independently able to stimulate reporter gene activity, suggesting that they are functionally similar if not equivalent.

Table 1. Myc/BCR-ABL cotransformation assay

Cell line	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Rat 1	0	0	4	1
Lx	0	2	3	1
Myc 0/1	115	91	110	43
Mut 3	136	91	138	34
Mut 13	108	104	146	32
Mut 15	144	125	143	32
Mut 15b	c	3	2	2
Δ N100	1	10	6	5
Δ BR	0	1	1	2
BR21M	1	1	ND	1
Δ CKII	96	70	ND	38
p210 Titer Neo ^R cfu/ml	1×10^4	1×10^4	1×10^4	4×10^3

c, contaminated; ND, not done.

DISCUSSION

The consequences of chromosomal translocation and proviral insertion within the *c-myc* locus include the deregulation of Myc expression and alterations in the pattern of Myc protein synthesis. To understand the significance, if any, behind the specific loss of p67 expression, we sought to differentiate between the p64 and p67 c-Myc polypeptides on the basis of function. We found that both p64 and p67 could independently transform Rat-1 fibroblasts in conjunction with p210^{bcr-abl}

Figure 6. Soft agar assay for Myc/BCR-ABL co-transformation. G418-resistant bulk populations were superinfected with the BCR-ABL virus for 24 h, then plated in 0.25% agar. Photomicrographs of the soft agar plates were taken on day 18. Magnification, 1 $\times$. The number of colonies (per 1000 cells plated) scored by computer-aided analysis is shown in parentheses.

Soft Agar Colonies per 1000 cells plated

Rat 1/Myc + Bcr-Abl p210 Kinase

A **B**

Figure 7. Sequence-specific transcriptional activation by p64 and p67 c-Myc proteins. (A) Transient transfection was used to introduce various Myc expression vectors into NIH 3T3 cells, along with the M4-minCAT reporter plasmid. This reporter construct contains a 4-fold reiteration of the defined Myc/Max binding site, CACGTG, proximal to a minimal promoter. Chloramphenicol acetyl transferase activity was determined and the activation of the reporter construct was normalized to that of the vector control (pSP). Samples are shown in duplicate. (B) In a parallel experiment, transiently transfected cells were metabolically labeled with [35 S]methionine and Myc polypeptides were immunoprecipitated with either rodent- or human-specific c-Myc antisera. Plates of cells transfected with a control expression vector were used for the immunoprecipitations shown in the first 3 lanes (I and B), while transfections with the various Myc expression vectors were used for each of the last 5 lanes. Specific immunoprecipitation could be blocked by the cognate peptide immunogen (B).

and that both Myc proteins were equivalent in terms of their capacity to transcriptionally activate a reporter gene through a promoter proximal Myc:Max binding site.

The Myc/BCR-ABL co-transformation assay is exceptionally relevant as a model for tumor progression. In animal models, plasmacytomas induced by injection of the Abelson virus into pristane primed mice, often contain a chromosomal translocation that places the *c-myc* gene under the influence of the immunoglobulin α promoter (Ohno *et al.*, 1984). Mice carrying *v-abl* transgene under the control of the immunoglobulin μ -chain enhancer ($E\mu$) develop monoclonal plasmacytomas with similar *c-myc* rearrangements. Hybrid mice carrying both $E\mu$ -*abl* and $E\mu$ -*myc* transgenes develop polyclonal tumors with a significantly shorter latency period com-

pared with either transgene alone (Rosenbaum *et al.*, 1990).

Human chronic myelogenous leukemia (CML), which is characterized by the fusion of the *bcr* and *c-abl* loci into a single transcription unit (for review see Clark *et al.*, 1989), may also involve activation of *c-myc* during the acute phase transition. Leukemic cells in blast crisis accumulate nonrandom secondary karyotypic abnormalities, including the frequent appearance of trisomy of chromosome 8 (the location of the human *c-myc* gene) (Sandberg, 1980). Occasionally, amplification of *c-myc* sequences has also been observed in these cells (Nowell *et al.*, 1983; McCarthy *et al.*, 1984). Several reports have documented generally elevated steady state levels of *c-myc* mRNA in blast crisis cells when directly compared with chronic phase cells from the same patient or with normal white blood cells. The upregulation of the *c-myc* gene product has also been confirmed in acute phase CML cells by antibody staining (Preisler *et al.*, 1988; Ferrari *et al.*, 1990). While these observations implicate the *c-myc* gene product as participating in the malignant progression of CML, the mechanism by which this is accomplished is yet unclear. One potential scenario is that a Myc:Max nucleoprotein complex is responsible for the transcriptional regulation of genes that are essential for DNA replication and cell division. Deregulation of Myc expression could perturb the normal hierarchy of cell-cycle controls, resulting in constitutive proliferation, loss of commitment and a corresponding block to differentiation (Blackwood *et al.*, 1992a).

The importance of *c-myc* function in direct transformation of hematopoietic cells by *bcr-abl* was also underscored by experiments demonstrating that dominant negative c-Myc protein could prevent transformation (Sawyers *et al.*, 1992). In the assay employed here the parental Rat-1 fibroblasts are already immortalized, yet the progression to the malignant phenotype clearly relies on the introduction of two activated oncogenes: *bcr-abl* and *c-myc*. While previous work has indicated that *v-myc* can cooperate in transformation with *bcr-abl* (Lugo and Witte, 1989), the data shown here demonstrate that an unmutated wild-type *c-myc* allele is also capable of co-transformation. This suggests that it is the constitutive, and therefore inappropriate, expression of c-Myc that accounts for its transforming properties.

Our results underscore the link between c-Myc transcriptional and transforming activities (Kato *et al.*, 1990). The presence of the DNA-binding region within Myc has been demonstrated previously to be essential for both activities (Penn *et al.*, 1990b; Kretzner *et al.*, 1992; Amati *et al.*, 1993; see Figure 6). However we also show here that the BR21M mutation, which specifically replaces the basic region of c-Myc with that of MyoD, fails to collaborate with the p210 tyrosine kinase. This substitution has no negative impact on heterodimerization with Max (Blackwood and Eisenman, 1991), yet

it presumably alters the DNA binding characteristics of the complex (e.g., lower affinity, altered, or broadened specificity, etc.) (Davis *et al.*, 1990). This transformation incompetent mutant suggests that sequence-specific recognition may be requisite for Myc function and lends further support to the notion that it is the transcriptional modulation of authentic Myc-responsive elements *in vivo* that is required for the transformed phenotype (Barrett *et al.*, 1992).

Our inability to detect functional differences between the two alternative c-Myc initiation products was surprising in light of their differential expression in lymphomas. Additionally, in at least four other proteins found to use alternative CUG initiation, the N-terminally extended forms localize to a different cellular compartment from the AUG forms (Prats *et al.*, 1989a; Acland *et al.*, 1990; Bugler *et al.*, 1991; Lock *et al.*, 1991). However both the CUG- and AUG-initiated forms of c-Myc localize to the cell nucleus and have the same short half-life (Street and Blackwood, unpublished data; Lüscher and Eisenman, 1988). Furthermore, in lymphomas induced in *Eμ-myc* mice, analysis of the *myc* transgene failed to detect rearrangements or exon 1 mutations (Webb *et al.*, 1989), demonstrating, in accord with the results in this paper, that loss of the CUG initiation site is not a requirement for transformation.

Although we have found no functional difference between the two forms of Myc protein using either the co-transformation or transient transactivation assays, future research may expand the repertoire of Myc's capabilities. However, it should be noted that while the use of a non-AUG codon to generate a protein doublet is a hallmark of the c-Myc gene that is conserved in many species (human, rodent, avian, and frog) (Hann *et al.*, 1988), the amino acid sequence of each N-terminal extension is not conserved. Perhaps the N-terminal extension may exist solely to restrict a subset of Myc functions, such that the coding sequences between species could evolve without structural constraint. Alternatively, it is possible that the sequence within the N-terminal extension is irrelevant and that only the cellular mechanism that produces the p67 Myc protein in response to physiological stress (such as methionine deprivation) may have been preserved (Hann *et al.*, 1992). Why conserve CUG-initiation as a translational option? Perhaps the cell requires a mechanism to maintain the production of a minimal level of Myc protein to prevent differentiation, senescence, or inopportune exit from the cell cycle under conditions of metabolic stress that disrupt normal AUG initiation.

In summary, the p64 and p67 c-Myc proteins appear functionally indistinguishable in either co-transformation or transactivation assays. This suggests that specific loss of the exon 1-initiated p67 protein, *per se*, is unrelated to the activation of this proto-oncogene. Rather, the promoter insertions that accompany chromosomal translocation and proviral integration most likely select

for breakpoints, insertion sites, and mutations that allow optimal expression. For example, within the Myc promoter or its exon 1/intron 1 sequences, negative regulatory elements or dominant control elements may be preferentially lost. Other mutations within the coding region may contribute to its oncogenic potency (Bhatia *et al.*, 1993; Yano *et al.*, 1993) but many tumors lack such consistent mutations, and wild-type c-myc is clearly able to act as a transforming gene. Thus we conclude that it is primarily the deregulation of Myc expression that underlies its oncogenic activation.

ACKNOWLEDGMENTS

We are grateful to Jaclynn Mac for expert technical assistance, Peter Whyte for reagents, Paul Goodwin for image analysis, and Päivi Koskinen, and Adam Geballe for critical readings of the manuscript. We also thank Frank McKeon for his kind gift of the lamin C cDNA. This work was supported by the Molecular Training Program in Cancer Research (National Cancer Institute grant T32 CA-09437) to E.M.B.; R01 CA-20525 to R.N.E., and R35CA-53867 to O.N.W., who is an Investigator with the Howard Hughes Medical Institute; a Medical Genetics Intercampus Training Grant (1T32GM08243) (T.G.L.); and fellowships from the American Cancer Society (L.K. and M.W.K.).

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