Multiple phenotypes associated with Myc-induced transformation of chick embryo fibroblasts can be dissociated by a basic region mutation

D. H. Crouch*, R. Gallagher¹, C. R. Goding², J. C. Neil¹ and R. Fulton¹+¹

Beatson Institute for Cancer Research, CRC Beatson Laboratories, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD, UK, ¹Molecular Oncology Laboratory, Department of Veterinary Pathology, University of Glasgow, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1QH, UK and ²Marie Curie Research Institute, The Chart, Oxted, Surrey RH8 0TL, UK

Received April 25, 1996; Revised and Accepted July 8, 1996

ABSTRACT
Chimaeric alleles were constructed to assay the biological functions of an N-terminal deletion and C-terminal mutations which were found in a naturally occurring mutant of feline vMyc, T17. The mutant alleles were assayed for their ability to transform chick embryo fibroblasts in vitro by a number of criteria, namely the ability to induce morphological transformation, an accelerated growth rate and growth in soft agar. Feline cMyc could transform the avian cells, whilst T17 vMyc could not, and the N-terminal deletion was responsible for conferring the primary transformation defect on the mutant protein. The C-terminal mutations which consist of a point mutation adjacent to the nuclear localisation signal and a point mutation/amino acid insertion within the basic region (BR) could, however, dissociate the Myc-induced parameters of transformation. This effect was a specific function of the BR mutation alone, and the mutation could be transferred into avian cMyc with comparable biological consequences. The BR mutation did not disrupt the sequence specific DNA binding activity of the protein in vivo, despite exerting a biological effect. These data suggest a novel phenotype where the mutation may affect a subset of Myc-regulated genes through altered DNA binding specificity or protein–protein interactions.

INTRODUCTION
Deregulated expression of c-myc is a common feature of the neoplastic phenotype, and can occur through a number of mechanisms including retroviral insertion, retroviral transduction and chromosomal amplification (1). Myc is believed to function as a sequence specific transcription factor in association with another protein, Max (2), regulating genes which are crucial to the control of cell growth, differentiation and cell death (3–6). Both Myc and Max belong to the basic-helix–loop–helix-leucine zipper (B-HLH-LZ) class of transcription factors (7). Dimerisation with Max mediated through the HLH-LZ domain is a prerequisite for sequence specific DNA binding, which is itself mediated by the basic region (BR) (8,9). After binding to specific target sequences, transactivation can occur through multiple domains which have been mapped to the N-terminus of Myc (10). Recent data are consistent with a model in which the Myc–Max heterodimer is the active transforming complex (3,5).

The product of the c-myc protooncogene, cMyc, is transforming, although some virally transduced forms (vMyc) contain point mutations which potentiate their activity in vitro (11,12). More recently, point mutations have been detected in naturally occurring tumours in Burkitt’s lymphoma (13–15), suggesting that the point mutations may contribute to deregulating Myc function in vivo. Point mutations may alter the gene regulatory properties of the cMyc oncprotein by, for example, altering sequence specific DNA binding activity (16) or interfering with the transactivation properties of the protein (13,17). It is worthwhile noting that the point mutations identified to date in Burkitt’s lymphoma are clustered within the transactivation domain (15).

A naturally occurring mutant of cMyc, T17 vMyc, was originally isolated from a feline T-cell lymphosarcoma as a component of a virus mixture, which also contained FeLV helper virus and a retrovirally transduced copy of the T-cell receptor (18,19). T17 vMyc was shown to be the major oncogenic component of this virus mixture, since secondary tumours induced in neonatal cats using this virus mixture were shown to contain clonally integrated T17 vMyc DNA, and only trace amounts of T-cell receptor (18). We have shown that whilst T17 vMyc may be leukaemogenic in vivo, it is non-transforming in vitro, suggesting that it represents a novel and potential tissue specific form of the Myc protein (20).

T17 vMyc contains, in addition to a large N-terminal deletion (amino acids 49–124) which spans the transactivation domain, two other mutations. A single point mutation (D328 → G) lies adjacent to the nuclear localisation signal (NLS), and a point

* To whom correspondence should be addressed at present address: University of Dundee, Biomedical Research Centre, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK
+ Present address: Department of Biological Sciences, Caledonian University, City Campus, Cowcaddens, Glasgow G4 OBA, UK
mutation/amino acid insertion occurs within the BR (L362→FR).
We set out to establish the role of the individual point mutations in T17 vMyc, by measuring their biological activity in an in vitro system based on the transformation of chick embryo fibroblasts (CEF). We show that whilst the N-terminus is essential for all measurable biological functions of Myc in this system, the BR mutation (L362→FR) can dissociate these functions by promoting anchorage independent growth and the morphological changes associated with Myc overexpression, without inducing an accelerated growth rate. The role of this BR mutation within the context of Myc function is discussed.

MATERIALS AND METHODS

Cell culture and transfections

Secondary chick embryo fibroblasts (CEF) were grown at 41°C in DMEM supplemented with 10% tryptose phosphate (TP), 5% newborn calf serum and 1% chick serum. CEF (2 × 10^5 cells) were transfected with 10 µg of each SFCV construct, essentially as described previously (22) together with 4 µg of the replication competent avian retroviral vector RCAN (44). Selection with G418 (1 mg/ml) was applied a few days after transfection, and transformation assays performed on the resulting G418-resistant cells. The ability to grow in soft agar was assayed after 2 × 10^5 cells were seeded in 0.35% agar for 2 weeks. An accelerated growth rate was measured by plating 2 × 10^5 cells in a 35 mm dish at 41°C and cumulative cell counts performed each day. The growth curves presented have been reproduced in a number of independent experiments.

Western blot analysis

Western blot analysis of G418-resistant CEF was performed essentially as described (22) using a monoclonal antibody to the C-terminus of cMyc (the gift of K. Moelling, Zurich) at a concentration of 1:1000. Proteins were visualised using enhanced chemiluminescence (Amersham).

Oligonucleotide directed mutagenesis

Site directed mutagenesis was performed using the Amersham in vitro mutagenesis system, essentially as recommended by the manufacturers, except that a primer concentration of 10 pmol/µg was used. The mutagenic oligonucleotide (5′-CGAAGCGACACAAGTCTTTCGGAGCCAGGCGAAAGGAT-3′) was designed to introduce the L362→FR mutation into the avian cMyc background, and all mutations were confirmed by sequencing.

Yeasts strains, transformations and β-galactosidase assays

The yeasts strains, transformations and β-galactosidase assays were performed essentially as described (9,31). Briefly, the C-termini of feline cMyc and T17 vMyc containing the B- HLH-LZ domains were fused to the Pho4 transactivation domain in the vector, pMA132, while Max and Max9 were cloned into the centromeric plasmid, pRS314. Dimerisation and DNA binding in vivo were measured by co-transfection of the plasmids into the yeast strain Y700 essentially as described (31). The resulting β-galactosidase activity is representative of dimerisation and DNA binding mediated by the C-terminus of Myc and Max.

RESULTS

Cloning and expression of feline T17 vMyc and chimaeras

A diagrammatic representation of feline cMyc, T17 vMyc and the chimaeras is outlined in Figure 1A. When compared with cMyc, T17 vMyc contains, in addition to the large N-terminal deletion (amino acids 49–124), a point mutation adjacent to the NLS (G→D) and point mutation/amino acid insertion (L→FR) found in T17 vMyc are indicated. BR, basic region; HLH, helix-loop-helix; LZ, leucine zipper. (B) Expression of the feline myc genes in CEF detected by western blot analysis with a monoclonal Myc antibody. Numbers on the left indicate molecular masses in kilodaltons.
**Dissociation of Myc-mediated functions by a mutation in the basic region**

Having confirmed their efficient expression in CEF, the transforming potential of the chimaeras was determined. A number of parameters [an accelerated growth rate (Fig. 2A), morphological transformation (Fig. 2B) and anchorage independent growth (Fig. 2C)] were assayed. In agreement with others (23, 24), the N-terminus was shown to be essential for all Myc functions tested (Fig. 2A), since only cMyc and c/vMyc produced any detectable biological activity in vitro. c/vMyc, however, could dissociate the Myc-mediated functions by inducing both a morphological change and growth in agar (Fig. 2B and C) without inducing an accelerated growth rate (Fig. 2A). The number and size of the colonies in agar were, however, slightly reduced with c/vMyc compared with cMyc, presumably reflecting the slower growth rate of cells carrying this gene. c/vMyc and cMyc undergo similar rates of apoptosis (data not shown), suggesting that the reduced growth rate of c/vMyc compared with cMyc is not due to an increased rate of apoptosis which is associated with Myc overexpression under appropriate culture conditions (25).

The growth curves within each independent experiment show a reproducible trend where all the mutants (c/vMyc, c/cMyc and T17 vMyc) display similar growth rates which are not statistically different from the vector control, but are significantly reduced compared with cMyc (Fig. 2A).

These data demonstrate that whilst the N-terminal deletion is the major defect in T17 vMyc, the mutations within the C-terminus also have functional consequences.

**The basic region mutation is sufficient to dissociate Myc-induced parameters of transformation**

Immunocytochemical analysis showed that each of the mutant proteins localised to the nucleus, suggesting that the mutation (D328→G) adjacent to the NLS did not interfere with this intrinsic property of the Myc protein (26; D. Crouch, data not shown).

Comparisons of the sequences of the BR from a number of related proteins show an extremely high degree of conservation within this region (Table 1). We reasoned that if the BR mutation (L362→FR) alone was sufficient to dissociate Myc function, the mutation should be transferable into an avian cMyc background with similar consequences. This construct would also test the possibility that the feline Myc background contributes to the dissociated phenotype in avian cells. Site directed mutagenesis was used to introduce this mutation into avian cMyc, which was subsequently cloned into SFCV sat (27). The resulting construct, avian c/vMyc, was assayed for transforming potential in CEFs. The results, summarised in Table 2, show that the BR mutation alone in an avian cMyc background is sufficient to dissociate the Myc-induced parameters of transformation, since the growth promoting functions of Myc can be dissociated from the morphological changes and anchorage independent growth. These data confirm that this effect is not limited to the feline background, and that the specific BR mutation has general and reproducible biological effects.
Growth in – Accelerated

...participate cMyc and Arg-10 in T17 vMyc with the equivalent Leu residue through an E-box motif (8,9), and it has been proposed that the BR is essential for mediating sequence specific DNA binding (20). Given the stringent requirement for conserved amino acids equivalent contacts in their respective protein–DNA complexes, homologous amino acids of Myc and Max basic regions make due to the extra amino acid insertion in the T17 vMyc basic (Fig. 3A). To take into account the displacement which occurs within the BR, we have projected the amino acids onto a helical wheel to compare the basic regions of cMyc, T17 vMyc and Max (Fig. 3A). The conserved amino acids (Lys-3, Arg-4, His-7, Asn-8, Arg-12, Arg-14 and Arg-15) are boxed and, in Max, have been shown to make contact with either DNA bases or the phosphate backbone (29).

Table 1. Amino acid sequence homology between the basic regions of myc family members (20,32) and Max (29)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>cMyc</th>
<th>human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residues</td>
<td>Value</td>
<td>Value</td>
</tr>
<tr>
<td>Lys-3</td>
<td>&lt;100%</td>
<td>&lt;100%</td>
</tr>
<tr>
<td>Arg-4</td>
<td>&lt;100%</td>
<td>&lt;100%</td>
</tr>
<tr>
<td>His-7</td>
<td>80-90%</td>
<td>80-90%</td>
</tr>
<tr>
<td>Asn-8</td>
<td>&gt;70%</td>
<td>&gt;70%</td>
</tr>
<tr>
<td>Arg-12</td>
<td>&gt;50%</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>Arg-14</td>
<td>&gt;50%</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>Arg-15</td>
<td>&gt;50%</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>Max</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Amino acids in one-letter code are numbered according to the full-size feline cMyc. Numbering 1–15 identifies the amino acid when plotted on a helical wheel (see Fig. 3A). The conserved amino acids (Lys-3, Arg-4, His-7, Asn-8, Glu-11, Arg-12, Arg-14 and Arg-15) are boxed and, in Max, have been shown to make contact with either DNA bases or the phosphate backbone (29).

E-box mediated DNA binding is unaffected by the basic region mutation in vivo

The BR is essential for mediating sequence specific DNA binding through an E-box motif (8,9), and it has been proposed that homologous amino acids of Myc and Max basic regions make equivalent contacts in their respective protein–DNA complexes (28). Given the stringent requirement for conserved amino acids within the BR, we have projected the amino acids onto a helical wheel to compare the basic regions of cMyc, T17 vMyc and Max (Fig. 3A). To take into account the displacement which occurs due to the extra amino acid insertion in the T17 vMyc basic region, the helical wheels have been plotted to align Leu-10 of cMyc and Arg-10 in T17 vMyc with the equivalent Leu residue in the Max basic region. Conserved amino acids which participate in DNA binding (boxed residues in Table 1; circled residues in Fig. 3A) lie on one face of the α-helix, and in the Max–DNA crystal structure make contact with each other with DNA bases (His-7, Arg-15 and two contact point for Glu-11) or the phosphate backbone (Lys-3, Arg-14, Arg-4, Asn-8 and Arg-12). Within Max and Myc (Fig. 3A) and other CACGTG-binding proteins (9), six residues are most highly conserved (Lys-3, Arg-14, His-7, Glu-11, Arg-4 and Arg-15). We had initially assumed that the amino acid insertion in the T17 vMyc BR would result in disruption of the α-helix structure. It was, therefore, a surprise when the helical wheel analysis of the T17 vMyc BR showed that of the key residues along the face of the α-helix, only three amino acid changes resulted from the amino acid insertion: Lys-3, His-7 and Asn-8 become Arg-3, Asn-7 and Val-8, respectively. In the Max–DNA crystal structure (29), Lys-3 and Asn-8 are proposed to make contacts with the phosphate backbone, whilst in both Myc and Max, His-7 has been shown to contact a DNA base (28,29). It should be noted that Glu-11 which is crucial for DNA binding (30), is retained in the equivalent position in the T17 vMyc BR, and the hydrophobic Phe-9 in T17 vMyc is located at an equivalent position to the hydrophobic Val-9 in cMyc.

Since the helical wheel analysis demonstrated no major disruptions in the structure, we used a yeast based system to assess Myc-mediated DNA binding (31) to see whether the T17 BR mutation affected DNA binding in vivo. The C-terminus of Myc, fused to the transactivation domain of the yeast transcription factor, Pho4, (P4-Myc), is transfected into yeast with a plasmid encoding Max or Max9, together with a reporter plasmid containing an E box-containing element (PHO5-UAS) upstream of β-galactosidase. DNA binding mediated by the Myc/Max BR is therefore measured in terms of β-galactosidase activity resulting from the Pho4 transactivation domain. The results are summarised in Figure 3B and show that, in this assay, there is no discernible difference between the Myc proteins containing the wild type and mutant BR with respect to dimerisation with Max or Max9, and the ability to bind to DNA in vivo. The amino acid changes on the DNA-binding face of the α-helix which result from the amino acid insertion/mutation, whilst having a clear biological effect, do not affect the ability to bind to a consensus CACGTG motif. Consistent with the requirement for Max, no activation was observed with the Pho4-T17 vMyc and Pho4-cMyc chimaeras alone (data not shown).

DISCUSSION

Analysis of mutations of cMyc, either naturally occurring or experimentally designed, has yielded useful information about this important oncoprotein, providing strong links between both the biological function of the protein and its molecular mechanism of action as a transcription factor (5,32). In this study, we have investigated the role of the N- and C-terminal mutations contained within a novel mutant of vMyc, T17, to assess the relative contribution of each to its biological activity in vitro, and to relate this to its function as a transcription factor.

Compared with cMyc, the T17 vMyc mutant has certain defects: firstly, at the N-terminus, it contains a large deletion spanning the transactivation domain, and secondly, at the C-terminus, it contains a point mutation adjacent to the NLS and a point mutation/amino acid insertion within the basic region (BR), the domain responsible for mediating sequence specific DNA binding (8,9). We have previously shown that whilst this mutant is oncogenic in vivo, it is non-transforming in an in vitro transformation system (20). We now show that the individual T17 vMyc mutations have a differential effect on the biological activity of the protein. In agreement with other workers (23,24), we show that an intact N-terminus of Myc is essential for all the transformation parameters assayed in this system, and the mutation adjacent to the NLS does not affect nuclear localisation (D. Crouch, data not shown). However, by inducing the
Figure 3. (A) Helical wheel analysis of the basic regions of Max (29), Myc (20), and T17 vMyc (20), with amino acid numbering as depicted in Table 1. Amino acids have been aligned to take into account the amino acid insertion, with Leu-10 in Max and Myc aligned with Arg-10 of T17 vMyc. The highly conserved amino acids which participate in DNA binding (9) lie on one face of the α-helix and are circled. These amino acids are boxed in Table 1. On the helical wheel, the hydrophobic residues are boxed, and it should be noted that the inserted amino acid, Phe-9, in T17 vMyc BR is found at the same relative position as the hydrophobic Val-9 in Myc. (B) The cMyc and T17 vMyc BR bind to an E-box motif in vivo. E-box-specific DNA binding, mediated by the BR of cMyc and T17 vMyc, is measured in terms of β-galactosidase activity after cotransformation of yeast with vectors expressing Pho4-Myc, Pho4-T17 vMyc or the vector control, together with Max or Max9 (31).
isolation, the T17 vMyc BR mutation had a profound effect on the in vitro transformation properties of the protein, whilst, at the molecular level, not appearing to affect dimerisation and DNA binding in vitro or in vivo. This biological effect presumably reflects subtle alterations in DNA binding specificity or affinity, and three key residues proposed to be involved in DNA binding, and most notably His-7 (28,29) are altered in T17 vMyc (Fig. 3A). A recent report has identified the position and number of E-box elements within a bona fide target sequence (α-prothymosin) as being critical determinants of specificity for Myc in transcriptional activation (37). Assessing the transcriptional activity of T17 vMyc on such a target sequence may help to define how the BR mutation specifically affects its gene regulatory properties. A point mutation at an equivalent position to the T17 vMyc BR mutation in N-Myc can broaden the specificity of DNA binding (16), whilst methylation-sensitive sequence-specific DNA binding has been demonstrated for the cMyc BR, but not two other members of the HLH family (39). Different members of the bHLH family have distinct preferences for DNA sequences (30,40), and specific sequences flanking the core E-box motif strongly affect binding by Myc–Max heterodimers, but not Max–Max homodimers (41,42). In addition, the BR mutation may affect the transactivation potential of a protein. For MyoD, BR mutants have been identified which affect the ability to transactivate cMyc by analysing the effects of the mutant gene BR mutation may have a modulatory effect on the oncogenic potential of the chimaeric protein. The C-terminal mutation may be silent in the presence of the severe transformation defect conferred by the N-terminal deletion, and it arose because the selective pressure to maintain gene regulatory activity was lost in the tumour. Alternatively, the BR mutation may have a modulatory effect on the oncogenic effects of the mutant gene in vivo. This hypothesis may be tested by analysing the in vivo oncogenic potential of the chimaeric genes. However, whatever its origin, the C-terminal BR mutation clearly has an independent effect on the activity of cMyc in vitro, and will be a valuable tool for analysing the pleiotropic functions of this critical effector of oncogenic transformation.

ACKNOWLEDGEMENTS

We wish to thank David Brighty for helpful discussions, Jen Blake and Wei Li for reading the manuscript. This work was supported by the Cancer Research Campaign of Great Britain.

REFERENCES