Alternative Translation of the Proto-oncogene c-myc by an Internal Ribosome Entry Site*

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The human proto-oncogene c-myc encodes two proteins, c-Myc1 and c-Myc2, from two initiation codons, CUG and AUG, respectively. It is also transcribed from four alternative promoters (P0, P1, P2, and P3), giving rise to different RNA 5'-leader sequences, the long sizes of which suggest that they must be inefficiently translated by the classical ribosome scanning mechanism. Here we have examined the influence of three c-myc mRNA 5'-leaders on the translation of chimeric myc-cAT mRNAs. We observed that in the reticulocyte rabbit lysate, these 5'-leaders lead to cap-independent translation initiation. To determine whether this kind of initiation resulted from the presence of an internal ribosome entry site (IRES), COS-7 cells were transfected with bicistronic vectors containing the different c-myc 5'-leaders in the intercistronic region. An IRES was identified, requiring elements located within the P2 leader, between nucleotides −363 and −94 upstream from the CUG start codon. This is the first demonstration of the existence of IRES-dependent translation for a proto-oncogene. This IRES could be a translation enhancer, allowing activation of c-myc expression under the control of trans-acting factors and in response to specific cell stimuli.

The c-myc proto-oncogene has a fundamental role in various cellular events, including proliferation, differentiation, and apoptosis (1). The human c-myc gene is transcribed from four alternative promoters. In normal cells, most of the transcripts start at the P1 and P2 promoters, the latter accounting for 75–90% of the c-myc mRNA. In Burkitt’s lymphoma cells that have a c-myc chromosomal translocation, the P1 promoter is preferentially used. In addition, two additional promoters, P0 and P3, are located 600 nucleotides upstream from the P1 promoter and in the first intron of the gene, respectively (2, 3).

In Burkitt’s lymphoma cell lines, P0 mRNA can reach levels of >10% of the total c-myc mRNA (4).

Interestingly, the coding capacity of the four c-myc mRNAs is different. P1 and P2 mRNAs encode two proteins of 64 and 67 kDa, designated as c-Myc1 and c-Myc2, respectively, by a process of alternative initiation of translation starting at two in-frame codons, CUG and AUG (5). c-Myc1 and c-Myc2 proteins, whose ratio is regulated in response to methionine starvation, are transcription factors with distinct DNA targets (6, 7). P0 mRNA is a natural polycistronic mRNA containing three open reading frames (ORFs). The 3'-ORF codes for c-Myc1 and c-Myc2 proteins, whereas the middle and 5'-ORFs, which have initiation codons upstream from the P1 promoter, code for proteins of 188 and 114 amino acids, respectively (2, 8). Only the 188-amino acid product has been characterized in HeLa cells. The function of this MycHEX1 protein remains unknown, however (9). In contrast to the coding capacity of P0, P1, and P2 mRNAs, that of P3 mRNA is restricted to the 64-kDa c-Myc2 protein.

The transcription starting points of the P0, P1, and P2 c-myc mRNAs are located 1172, 524, and 363 nt upstream from the CUG initiation codon, respectively. According to the classical cap-dependent scanning model, such long leader sequences are expected to impair translation initiation by preventing the ribosome scanning from the capped mRNA 5'-end (10). It has indeed been reported that the c-myc P1 leader has an inhibitory effect on translation of the c-myc mRNA in rabbit reticulocyte lysate (RRL) and in Xenopus oocytes (11, 12). However, in several mRNAs, mainly of viral origin, the structure of long leader sequences has been shown to constitute an internal ribosome entry site (IRES) (13, 14), allowing translation initiation to be cap-independent. The internal entry process, first shown for picornaviruses (15, 16), has also been reported for a few cellular mRNAs, but never for a proto-oncogene (17-21). In picornaviruses, the internal entry process has been shown to require cellular trans-acting factors. The best characterized of these factors is the polypyrimidine tract-binding protein, also known as a splicing factor (22-24). Due to their positive regulation by cellular factors, IRESs can be considered as translation enhancers, allowing activation of tightly controlled genes in response to specific stimuli (25). In this report, we demonstrate the presence of an IRES in P0, P1, and P2 c-myc mRNAs, implying an element located between nucleotides −363 and −95 upstream from the CUG start codon.

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MATERIALS AND METHODS

Plasmid Construction—The m-cyc P1 eDNA was obtained by reverse transcription and polymerase chain reaction (PCR) amplification of total mRNA from human tumorigenic cells (kindly provided by D. Morello) using Superscript reverse transcriptase (Life Technologies, Inc.) and Goldstar Tq DNA polymerase (Eurogentec). The 5'- and 3'-primers used for this amplification, 5'-TCGCTCTAGTAAGGACC- CCGTGGAG-3' to 5'-TTTGGCGCCGAG-3', respectively, gave a cDNA fragment extending from positions 1 to 712 from the P1 leader 5'-end (positions 1 and 12 are 1361 nt downstream from the P0 leader 5'-end and 143 nt downstream from the AUG codon, respectively). This fragment was inserted into the plasmid pFC1, derived from the pSCT vector, upstream from the CAT coding sequence and under the control of cytomegalovirus and T7 promoters (26). The plasmid pSCT-MyCAT-P1 was expected to encode two Myc-CAT proteins of 29 and 31 kDa, respectively.

The m-cyc P2 eDNA was obtained from the construct MyCMyAT-P1 by PCR amplification and cloning of the fragment extending from the P2 5'-end (610 nt downstream from the P0 5'-end) to 3' of CAT using oligonucleotides 5'-AGAAGCTTAAAACATGGCATGATATCCTTTATTCACAACAAGGCGGCGCCG-3' and 5'-AATACTCAGATTTACATGTGTCAGCTTACCACCGCCGCTGGCA-3' (CA-Trev) as 5'- and 3'-primers, respectively. The plasmid pSCT-MyCAT-P2 was constructed by insertion of the Myc-CAT-P2 fragment into the pSCT vector.

The m-cyc P0 cDNA was obtained by PCR amplification of the P0 eDNA from the plasmid pHLM-P1 containing the m-cyc genomic sequence (kindly provided by D. Morello) with oligonucleotides 5'-AGAAGCTTAAAACATGGCATGATATCCTTTATTCACAACAAGGCGGCGCCG-3' and 5'-CA-CAAGTCGTAGCTATTATTCACAACAAGGCGGCGCCG-3' (pSCT-MyCAT-P0) and 5'-CAAGTCGTAGCTATTATTCACAACAAGGCGGCGCCG-3' and 5'-CA-CAAGTCGTAGCTATTATTCACAACAAGGCGGCGCCG-3' (pSCT-MyCAT-P0*). The cDNA fragment extending from positions 1 to 712 of the P0 leader 5'-end was inserted into the pSCT vector, upstream from the CAT coding sequence and under the control of cytomegalovirus and T7 promoters (26). The cDNA fragment extending from positions 1 to 712 of the P0 leader 5'-end was inserted into the pSCT vector, upstream from the CAT coding sequence and under the control of cytomegalovirus and T7 promoters (26). The new plasmid was called pSCT-MyCAT-P0.

The construct pSCT-MyCAT-P0 DNA. The first fragment, obtained with oligonucleotides 5'-P0 5'-TCGCTCTAGTAAGGACC-CGGTGGAG-3' to 5'-TTTGGCGCCGAG-3', respectively, from the putative activity of these internal promoters by mutating the two mutated promoters, was designated MyCAT-P0* (Fig. 1D, lane 12). In contrast, the P0 and P2 TATA boxes. To distinguish between the transcription and translation capacities of this plasmid in transfected cells, we abolished the putative activity of these internal promoters by mutating the two P1 and P2 TATA boxes in the MyCMyAT-P0 construct (as described under “Materials and Methods”); the new construct was designated MyCAT-P0* (Fig. 1A).

Cell Transfections and Western Immunoblotting—COS-7 monkey cells were transfected with 1 μg/ml DNA by the DEAE-dextran method (20). Cell lysates were prepared 48 h later. Total proteins were prepared, quantified, and analyzed by Western immunoblotting (5 μg of proteins from each cell lysate) as described previously (20). CAT proteins were immunodetected using rabbit polyclonal anti-CAT antibodies prepared in our laboratory (1:20,000 dilution).

Cellular RNA Purification and Northern Blotting—Total cellular RNA was prepared with the Trizol method (Life Technologies, Inc.) from pellets containing 5 × 10^6 transfected scraped cells. Northern blotting was performed as described previously (20). DNA probes were labeled with [32P]dATP using a random priming kit (Promega). Total cellular RNA (1 μg/lane) was subjected to electrophoresis through 1.2% formaldehyde-agarose gels, electrotransferred to nylon membrane, and hybridized under the conditions described previously (20).

RESULTS

Cap-independent Translation Is Conferrred by m-cyc 5'-Leaders—To evaluate the translational regulation of c-Myc protein expression by the different leaders P1, P0, and P2, the three leader sequences, measuring 1172, 524, and 363 nt, respectively, were fused to the CAT coding sequence (Fig. 1A, pSCT-MyCAT-P0, pSCT-MyCAT-P1, and pSCT-MyCAT-P2). Two deletions were also created in the 5'-region of the MyCMyAT-P2 construct, resulting in small leaders of 94 and 14 nt for the constructs MyCMyAT-D2 and MyCMyAT-D1, respectively (Fig. 1A). All these constructs were expected to code for two Myc-CAT proteins translated from the c-myc CUG and AUG start codons, located at positions 1173 and 1218 from the P0 5'-end. In addition, the vector pSCT-MyCAT-P0 contains two 5'-open reading frames of 114 and 188 amino acids, starting at positions 53 and 625 from P0 5'-end, respectively (Fig. 1A) (2, 8).

The 188-amino acid ORF codes for the MycHEX1 protein. The vector pSCT-MyCAT-P1 should allow the synthesis of a slightly truncated form of the MycHEX1 protein from a CUG codon located 10 nt downstream from the P1 mRNA 5'-end (in fact, 24 nt from the T7 promoter used for in vitro transcription).

The MyCMyAT-P0 construct contains the P1 and P2 TATA boxes. To distinguish between the transcription and translation capacities of this plasmid in transfected cells, we abolished the putative activity of these internal promoters by mutating the two P1 and P2 TATA boxes in the MyCMyAT-P0 construct (as described under “Materials and Methods”); the new construct was designated MyCAT-P0* (Fig. 1A).

Capped and uncapped mRNAs were transcribed in vitro and translated in RRL in the presence of [35S]methionine. Their translational efficiencies were compared (Fig. 1, B and C). The translational levels of capped P0, P1, P2, and δ2 mRNAs were approximately 2, 10, 50, and 100% of that of the capped δ1 mRNA devoid of leader sequence, respectively (Fig. 1, B (lanes 2, 6, 8, 10, and 12) and C). P0* mRNA, with mutations of the P1 and P2 TATA boxes, was translated at the same level as P0 mRNA (Fig. 1B, lanes 2 and 4). The ratio of the two proteins MycCAT1 and MycCAT2 was not influenced by the presence of the 5'-leaders P1, P2, and δ2 (Fig. 1, B (lanes 6, 8, and 10) and C). The δ1 5'-leader was responsible for lower MycCAT1 expression, probably resulting from its shortness (Fig. 1B, lane 12). In contrast, the P0 and P0* 5'-leaders led to a relative increase in MycCAT1 expression (Fig. 1B, lanes 2 and 4).

The translation efficiencies of the capped and uncapped mRNAs were compared to determine the cap dependence of translation initiation. The expression of the Myc-CAT proteins using P0 and P0* mRNAs was cap-independent, whereas the expression of 5'-ORF1 was increased in the presence of the cap.
The results shown correspond to a multimer of MycHEX1 (9). This was checked by trans-

Fig. 1. Influence of the cap on translation of chimeric myc-CAT mRNAs in RRL. A, shown is schematic representation of the chimeric Myc-CAT constructs. The 5'-end of c-myc cDNA is fused to CAT 143 nt downstream from the ATG codon, with the two initiation codons CTG and ATG in frame with the CAT ORF (see "Materials and Methods"). The plasmid pSCT-MyCAT-P0 contains the complete 1172 nt P0 leader, whereas the other MyCAT plasmids start at positions 649, 810, 1078, and 1158 for MyCAT-P1, MyCAT-P2, MyCAT-P3, and MyCAT-D1, respectively, counting from the P0 mRNA 5'-end. The sizes of the corresponding leaders, as well as the positions of ORF1 and the MycHEX1 ORF, are indicated. P0* corresponds to the P0 leader with mutations of the P1 and P2 TATA boxes (see "Materials and Methods"). T7 Pr, T7 promoter. B, the RNAs described in A were synthesized in vitro with T7 RNA polymerase. 1 fmol of each capped (+) and uncapped (−) mRNA was translated in RRL (20 μl) in the presence of [35S]methionine (see "Materials and Methods"). Translation products were analyzed by electrophoresis on a 12.5% polyacrylamide gel, followed by autoradiography of the gel. The migrations of Myc-CAT1, Myc-CAT2, ORF1, and MycHEX1 are indicated by arrows. For P0 and P0*, two additional bands migrating just below and above Myc-CAT1 correspond to ORF1 multimers. For P1, the band at the top of the gel corresponds to a multimer of MycHEX1 (9). This was checked by translating shorter RNAs (not shown). The results shown correspond to representative experiments that were repeated three to eight times (depending on the constructs). The inset shows Myc-CAT1 and Myc-2

Fig. 2. Construction of bicistronic Myc-CAT DNAs. The chimeric Myc-CAT constructs described in the legend of Fig. 1A were subcloned downstream from the CAT coding sequence, again in the pSCT vector, under the control of the cytomegalovirus promoter (CMV Pr) (20). The resulting plasmids (BI series) encode bicistronic mRNAs having two tandem CAT ORFs with different sizes. The HP-MyCAT DNAs are derived from the BI DNAs with the addition of a 5'-hairpin (ΔG = −40 kcal/mol). The lengths of the different c-myC leaders present between ORF1 (CAT) and ORF2 (Myc-CAT) are indicated.

In conclusion, these experiments clearly show that cap-independent expression of CUG- and AUG-initiated Myc-CAT proteins is conferred by the three P0, P1, and P2 c-myC 5'-leaders. However, a weak cap-dependent translation initiation was also observed with the P1 and P2 5'-leaders, whereas synthesis of the 5'-ORFs was cap-dependent in P0 and P1 mRNAs.

An IRES Controls the Synthesis of c-Myc1 and c-Myc2 Proteins—To determine whether the cap-independent translation initiation, observed in the RRL system for the P0 leader and also for the P1 and P2 leaders, occurred by a process of ribosome entry, we looked for the presence of an IRES in the c-myC mRNA. The same bicistronic vector assay as that described for the identification of FGF-2 and murine leukemia virus IRESs in previous reports (20, 27) was used. This strategy is based on the principle that, according to the cap-dependent ribosome scanning model (10), the second ORF of a bicistronic mRNA will not be expressed unless it is preceded by an IRES. Two series of bicistronic vectors were constructed; the first one had as the first ORF the CAT coding sequence and as the second ORF the above-described Myc-CAT fusion sequence (Fig. 2, BI-MyCAT). The presence of two tandem CAT genes encoding proteins of different sizes allowed comparison of the expression of the two cistrons (20, 27). The different c-myC leaders described in Fig. 1A were inserted in the intercistronic region. The second series of bicistronic vectors was derived from the first by the introduction, upstream from the first ORFs, of a stable hairpin intended to inhibit the cap-dependent, but not the IRES-mediated, initiation of translation (Fig. 2, HP-My-

The different bicistronic DNAs, with or without a hairpin, were used to transiently transfect COS-7 cells. The natural with a shorter exposure of the gel for lanes 6–13 (3 versus 20 h). C, the bands were quantitated with a PhosphorImager. The values (corresponding to arbitrary units given by the PhosphorImager) are represented in a histogram under each lane of B. The inset shows an enlarged view of P0 and P0* quantitation (at the same scale). −R, −RNA.
We conclude from these data that an IRES is present in both the P0 and P2 5’-leaders of c-myc mRNA. This structure therefore involves elements located between nucleotides −363 and −94 upstream from the CUG initiation codon. Furthermore, the IRES allows a higher Myc-CAT translational level compared with the CAT first cistron, indicating that it is very efficient.

**Sequence Alignment of the c-myc IRES with the FGF-2 IRES and Secondary Structure Prediction** — Fig. 4A shows a sequence alignment of c-myc P2 leader sequence with nucleotides 189–487 of human FGF-2 mRNA containing the IRES. Although there is no striking sequence homology, several conserved GC-rich stretches are apparent. According to a recent report, several of these blocks are involved in stems of the E1 and E2 motifs predicted to form the FGF-2 IRES structure (28): in particular, the a/a’, b/b’, and d/d’ elements of the E2 motif (Fig. 4A). The c-myc P2 RNA structure predicted by the Zuker method (35), with aΔG of −122 kcal/mol, divides the leader into three major domains (Fig. 4B). The large D1 domain alone has a ΔG of −78 kcal/mol. The D1 and D2 domains, extending from nucleotides 1 to 205 and from nucleotides 206 to 293, respectively, could participate in the IRES as they are not present in the IRES-minus Δ2 construct (starting at nucleotide 269 from the P2 5’-end).

**DISCUSSION**

In this study, we demonstrate the presence of an IRES in the c-myc mRNA. So far, it is the first IRES to be characterized in the mRNA of a proto-oncogene. Most of the few cellular mRNAs described up to now as containing IRESs code for proteins involved in the control of cell proliferation and differentiation and require stringent regulation, as do c-myc mRNAs; these genes need to be expressed at very specific stages of cell life and/or in response to different stimuli (17–21). The IRES, being a recognition site for translational regulatory factors, allows translational activation of the expression of such messengers presenting a low or even non-existent level of cap-dependent translation; thus, internal ribosome entry constitutes a novel mechanism of gene expression regulation. This has been shown in the case of FGF-2, whose CUG-initiated isoforms are translationally activated in response to stress (25). Furthermore, the constitutive expression of these FGF-2 isoforms has been observed in transformed cells (25). Such observations suggest that, in the case of c-myc, a relationship may exist between IRES activation and cell transformation.

To corroborate this hypothesis, it has been recently reported that c-myc expression is activated at the translational level in cell lines derived from patients with the cancer-prone disorder Bloom’s syndrome (29). This activation does not appear to be mediated by the cap-binding eukaryotic initiation factor 4E, suggesting that it occurs through a cap-independent pathway. In the light of our results, one can postulate that in these cells, c-myc is activated by an IRES-dependent translation pathway. This activation may be triggered by the increased level of DNA strand breaks that occurs in Bloom’s syndrome cells, as previously proposed (29). This supports our hypothesis, already suggested by the stress activation of FGF-2 isoforms (25), that the internal ribosome entry process is a cellular regulation pathway allowing a rapid response to disorder-generating stimuli.

The c-myc mRNA is the third example of an mRNA with alternative initiation codons to contain an IRES (20, 27, 30). However, in contrast to Moloney murine leukemia virus retroviral mRNA, showing an IRES between its CUG and AUG initiation codons and allowing translation of the AUG-initiated protein exclusively, the c-myc mRNA has an IRES located 2 B. Galy, unpublished results.
upstream from the two initiation codons. Thus, this IRES controls the expression of both c-Myc1 and c-Myc2 proteins and is similar in this respect to that found in the FGF-2 mRNA. The translation efficiency of oncogene expression emerges more and more in the literature as an important regulatory target in malignant conversion. This translation efficiency can be regulated by at least two pathways. The first pathway is cap-dependent; oncogene messengers with highly structured untranslated regions can be up-regulated by overexpression of eukaryotic initiation factor 4E, characterized as a proto-oncogene (31, 32). We show here that a second possible pathway involving the IRESs should be considered. These specific structures are under the control of internal entry factors. Work is in progress to identify the factors involved in this regulation mechanism. Furthermore, both processes are related to one another in a regulatory loop: eukaryotic initiation factor 4E is indeed activated by growth factors (33), and its expression is induced by several oncogenes, including c-myc (34). Thus, the disregulation of the c-myc IRES will have a

Fig. 4. The c-myc IRES: sequence alignment with the FGF-2 IRES and Zuker secondary structure prediction. A, a sequence alignment of the human c-myc P2 leader (nt 1–366) and part of the FGF-2 leader (nt 189–487) containing c-myc and FGF-2 IRESs, respectively, was obtained with Geneworks software. Homologous nucleotides are boxed. D1, D2, and D3 correspond to the c-myc RNA domains of the secondary structure shown in B. E1 and E2 correspond to the two predicted IRES structural motifs of FGF-2 RNA proposed in the RNA structure studies of Le and Maizel (28). The elements a, a', b, b', c, c', d, and d' form stems in each E1 and E2 motif, as reported by these authors. The 5'-end of the Δ2 mutant is indicated with an arrow at position 269 of the c-myc sequence. B, the secondary structure of the c-myc P2 RNA leader (366 nt) was predicted with the Zuker folding program (35). ΔG = −122 kcal/mol. The P2 RNA 5'-end and the CUG initiation codon are indicated, as well as the position of the Δ2 deletion. The three structural domains are shown (D1, D2, and D3). D1 alone has a ΔG of −78 kcal/mol.
cascade effect that is likely to have drastic consequences on the control of cell growth. Finally, since this IRES is remarkably efficient, it is also particularly interesting in a biotechnological point of view: it can serve for the design of polycistronic vectors expressing several proteins from the same mRNA.

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