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Sequences Involved in Accurate and Efficient Transcription of Human c-*myc* Genes Microinjected into Frog Oocytes

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By microinjecting a series of deletion mutant constructs into *Xenopus laevis* oocytes, transcriptional control regions, two promoters, of the human c-*myc* gene were defined. In the case of the first promoter, sequences between −60 and −37 relative to the transcription start site contained an element essential for promoter activity. In the case of the second promoter, sequences between −66 and −56 relative to the initiation site appeared to be involved in accurate and efficient transcription. In both cases, the region identified as the essential promoter element contained GGGCGG or GGCGGG,GC box-like sequences, suggesting that c-*myc* gene promoter activity may be controlled by transcription factor Sp1 binding in the microinjected oocytes.

Regulation of the c-*myc* oncogene, known to be involved in the genesis of many tumors, has been extensively studied in the past several years. A variety of activation mechanisms of this gene have been found in various tumors (24), in sensitive response to mitogen treatment (7, 19, 23, 31), and following in vitro-induced cell differentiation (7, 37). Several possible control mechanisms of the normal c-*myc* gene involving a transcriptional repressor (26) or emphasizing the importance of RNA stability or turnover (4, 9, 10) have been proposed. However, the molecular basis of the mechanisms, in particular the DNA sequences and interacting factors involved, is not yet known.

Transcriptional activation of many genes has been shown to involve the interaction of regulatory molecules with specific DNA sequences, usually located in the 5′-flanking region (5). The human c-*myc* gene contains a duplicate set of typical TATA sequences separated by about 165 base pairs (bp) near the 5′ end of the first noncoding exon (1). An obvious CAT box homology is absent in the human and mouse c-*myc* genes (1). In addition to two TATA sequences, the 5′-flanking region of the human c-*myc* gene contains some unique features, including a repeated 9-base sequence and four potential hairpin structures (1). However, the involvement of these unique sequences in a specific regulatory mechanism or in the basal promoter activities of human c-*myc* gene has not yet been determined.

Recently, it has been shown that *Xenopus laevis* oocytes can transcribe cloned human c-*myc* genes from their own two promoters very efficiently after microinjection (33). Previous studies have also indicated that the efficient and faithful transcription of cloned c-*myc* genes in oocytes is unaffected by the presence of the known enhancing elements in the construct in *cis* (33). In other words, the intact human c-*myc* gene subcloned as pLcMycHE (33) carrying 2.4 kilobases (kb) of 5′-flanking sequences in addition to three exons, two introns, and about 1 kb of 3′-flanking sequences is self-sufficient with respect to all control sequences necessary for active transcription from the correct initiation sites. As the first step of defining the transcriptional control region of the c-*myc* gene, I prepared in vitro-modified c-*myc* genes and studied the consequent alteration of their expression in oocytes after microinjection.

Construction of 5′ serial deletion mutants of human c-*myc* gene. First, I constructed a set of mutants of the human genomic c-*myc* gene recombinant pLcMycHE (33) with various internal deletions within the 5′-flanking sequence region of c-*myc* gene by using available restriction sites (Fig. 1). The mutants (0.5 ng per oocyte) were individually microinjected into oocytes, as described by Gurdon (20). The total polyadenylated [poly(A)$^+$] RNA was prepared 24 h after microinjection as described previously (32) and analyzed by an S1 mapping procedure (2). I found that the deletion between −2328 (*Hin*dIII) and −350 (*Pvu*II) did not affect upstream (P1) or downstream (P2) promoter activity (data not shown). In other words, the essential sequences of P1 and P2 are in a region on the 3′ side of the *Pvu*II restriction site, which is located about 350 bp upstream from the cap 1 site (Fig. 1).

To define the essential elements involved in accurate and efficient transcription of c-*myc* gene precisely, I then prepared a series of mutants having sequential deletions within the 5′-flanking region. This region has been shown to contain repeated nonanucleotide sequences and stretches of sequences for possible hairpin secondary structures, in addition to two TATA boxes (1). The 5′ deletion mutant constructs, carrying sequential deletions from the *Pvu*II site (−350) toward the two TATA sequences within the 5′-flanking region of the c-*myc* gene, were prepared as follows. A minigene recombinant, pMyc, which contains the 5′-flanking sequence plus the first noncoding exon as an *Hin*dIII-*Eco*RI (converted from *Pvu*II by linker ligation) 2.85-kb DNA fragment (Fig. 1) was used as a starting plasmid. Sequential 5′ deletions starting from the *Pvu*II site (−350) were prepared with Bal 31 exonuclease, and synthetic *Xba*I linkers were ligated to the remaining DNA. The precise endpoints of the 5′ deletion mutants of pMyc chosen for study were determined by DNA sequencing (41). The serial 5′ deletion mutants with intact second and third exons were reconstructed from pLcMycHE and sequential 5′ deletion constructs of pMyc. The resulting mutants differ from pLcMycHE by having sequential deletions within the 5′-flanking region from the *Pvu*II site (−350) and also by containing an extra *Xba*I site inroduced at the endpoint of the deletion.

Mapping of two promoters in human c-*myc* gene. Various 5′ deletion mutants were microinjected into oocytes, and c-*myc* transcripts were analyzed by the S1 mapping procedure. As has been previously shown (33), human c-*myc*-specific transcripts initiating from the cap 1 site (detected as 515 nucleotides of S1-resistant DNA) and cap 2 site (detected as 350

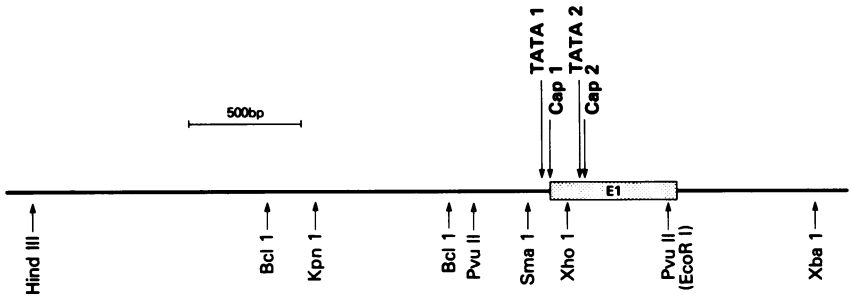

FIG. 1. Physical map of the region surrounding the first exon of the human c-*myc* gene. The physical map of a portion of human c-*myc* gene (1) is shown. Stippled box, First noncoding exon (E1). Horizontal line, Intron or nontranscribed sequences. The position of two TATA sequences and two cap sites are indicated.

nucleotides of S1-resistant DNA) were synthesized in oocytes microinjected with the parental pLcMycHE clone (Fig. 2, lane 2). As a control, I used RNAs prepared from Burkitt's lymphoma line BL2 (Fig. 2, lane 1). Each oocyte microinjected synthesized 2.5×10^8 copies of the c-*myc* RNAs, equivalent to the amount of c-*myc* mRNAs synthesized by 5×10^6 BL2 cells (33).

As shown in Fig. 2, transcription initiation from the cap 1 site was almost unaffected by the deletion of DNA sequences betwen −350 and −60 relative to the cap 1 site. The deletion to −37, which left 9 nucleotides at the 5′ side of the TATA box 1, decreased transcription initiation from the cap 1 site to a level of 5% of that in the parental clone. The deletion to −35, leaving 7 nucleotides on the 5′ side of TATA box 1, further decreased the level of transcription to 2% of that of the parental clone. The deletion to −26 into TATA box 1 completely abolished the activity of the P1 promoter. The deletion to −66 from the cap 2 site had no effect on transcription initiation from the cap 2 site, while the deletion of 10 more nucleotides (to −56) almost abolished P2 activity. The decrease of P2 activity by deletion to −56 was more drastic than the decrease of P1 activity by deletion to −37 or −35.

To see more precisely whether the deletion of various sequences within the 5′-flanking region affected the accuracy of initiation sites, I also carried out additional S1 mapping analysis with two different probes. To determine the precise transcription initiation site, I used shorter S1 probes, which will detect shorter S1 nuclease-resistant DNA products than those shown in Fig. 2. For example, the transcripts initiated by the P1 of various 5′ deletion mutants were now detected as a major product of 61 nucleotides together with additional minor products (Fig. 3), indicating that transcription by P1 in fact initiated at the multiple sites shown by the arrows in Fig. 5A. The use of multiple initiation sites is not due to inaccuracy of the *X. laevis* transcription machinery recognition of the human c-*myc* promoters, since the c-*myc* mRNAs from the control, BL2 cells, generated an identical set of S1 nuclease-resistant DNA products (Fig. 3, lane 2). The result suggests that deletion of the DNA sequences to −60 affects neither the frequency of the transcription initiations from the cap 1 site(s) nor the accuracy of initiation. It is noteworthy that the deletion to −37 and −35 decreased the frequency of transcription by 20- to 50-fold, but the exact sites of transcription initiation were not affected (Fig. 3, lanes 9 and 10).

Similarly, the effect of the deletion in the 5′-flanking sequences on accuracy of transcription initiation from the cap 2 site was analyzed by using a uniformly labeled, shorter S1 probe (Fig. 4). The c-*myc* transcripts initiated from the P2 promoter were now detected as shorter S1 nuclease-resistant products of 51 and 52 nucleotides, together with several minor products, indicating that once again transcription initiation from the P2 promoter occurred at multiple sites. These sites are indicated by arrows in Fig. 5B. I believe that the multiple S1 nuclease-resistant DNA products seen in Fig. 3 and also in Fig. 4 were not due to an artifact caused by

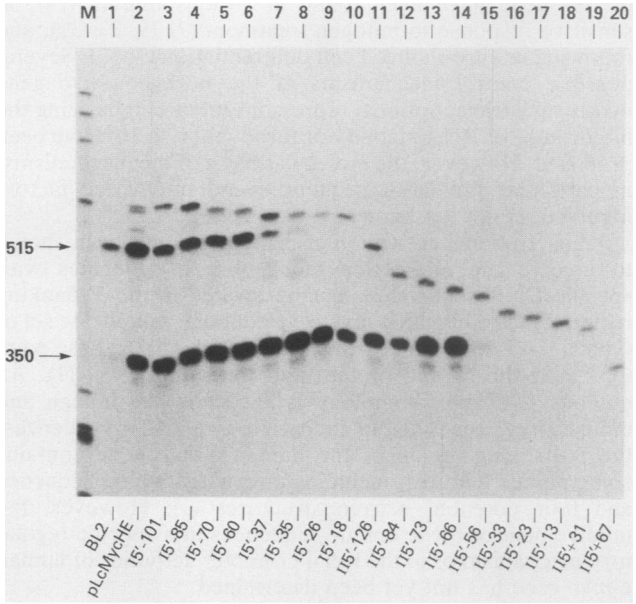

FIG. 2. S1 nuclease mapping analysis of c-*myc* RNAs synthesized in *X. laevis* oocytes injected with 5′ serial deletion mutants. Detection of two 5′ ends of the human c-*myc* RNAs from oocytes microinjected with various 5′ serial deletion mutants and the parental clone pLcMycHE (33) is shown. For example, the mutant designated I5′-85 has a deletion between −350 (*Pvu*II site) and −85 relative to the cap 1 site, whereas mutant II5′-23 lacks the sequence between the *Pvu*II site and −23 relative to the cap 2 site. Poly(A)$^+$ RNAs prepared from a single injected oocyte were subjected to S1 nuclease mapping analysis (2). The uniformly ^{32}P-labeled DNA probe (27), covering the region from the *Sma*I site to the *Pvu*II site located very near the 3′ end of exon E1 (see Fig. 1), was used as an S1 probe (33). The probe was heat denatured, hybridized in 80% formamide to the various RNA samples at 56.5°C for 10 h, digested with S1 nuclease, and analyzed by electrophoresis on a 7 M urea–4% polyacrylamide gel. Cytoplasmic RNAs (20 μg) from Burkitt's lymphoma cell line BL2 (lane 1) was also analyzed as a control. Lane M, size markers: ϕX174 digested with *Hae*III and ^{32}P-labeled at the 5′ end. The autoradiogram was composed from several separate gels, which resulted in the wavy line of 515- and 350-nucleotide bands.

"breathing" of DNA-RNA duplexes and subsequent nicking with S1 nuclease for the following reasons. First, lowering the concentration of S1 nuclease from 200 to 40 U/ml or decreasing the digestion temperature from 37 to 25°C essentially did not alter the S1 nuclease mapping results shown in Fig. 3 and 4 (data not shown). Second, replacing S1 nuclease with mung bean nuclease for trimming of the DNA-RNA duplex also did not significantly change the results (data not shown). I confirmed that the deletion to −66 relative to the cap 2 site(s) did not affect the frequency or accuracy of transcription initiation from the P2 promoter.

From these experiments with serial 5′ deletion mutants, I have concluded the following: (i) the essential sequences necessary for efficient transcription initiation by the P1 promoter reside on the 3′ side of −60; (ii) the essential sequences necessary for efficient transcription initiation by the P2 promoter are on the 3′ side of −66; and (iii) the remaining level of infrequent but accurate transcription is eliminated by the deletion of sequences to −26 in the case of P1 and to −33 in the case of P2.

Deletion of DNase I-hypersensitive sites does not alter promoter activity. The chromatin structure around the 5′-flanking region of human c-*myc* genes was previously investigated by DNase I hypersensitivity, and four hypersensitive sites, I, II, III_1, and III_2, were identified (14, 42). Sites I (−1900) and II (−750) have been suggested to be binding sites for the putative repressor and nuclear factor I, respectively, whereas sites III_1 (−128 relative to the cap 1 site) and III_2 (−72 relative to the cap 2 site) were considered to correspond to the P1 and P2 promoters, respectively (14, 42). However, my results with two sets of mutant constructs clearly show that deletion of all the DNase I-hypersensitive sites, including sites III_1 and III_2, essentially did not affect the efficiency or accuracy of transcription initiation of microinjected c-*myc* genes in frog oocytes.

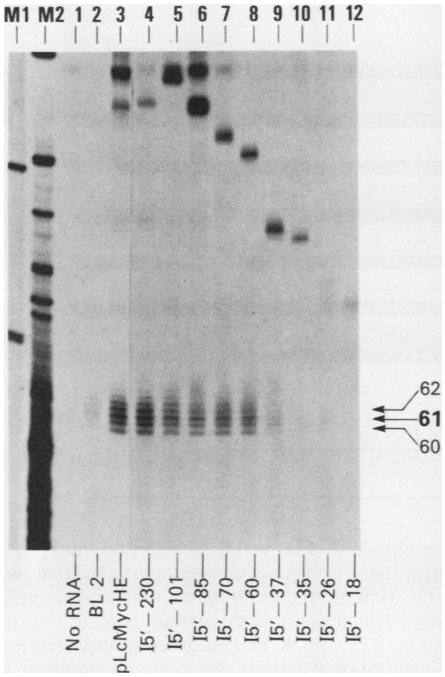

FIG. 3. Detection of 5′ end of c-*myc* transcripts initiated at the cap 1 sites in oocytes microinjected with various deletion mutants. The uniformly ^{32}P-labeled DNA probe, encompassing the region from the *Sma*I site (see Fig. 1) to +61 relative to the major cap 1 site (see Fig. 5A), was used as an S1 probe. Conditions for S1 nuclease analysis were identical to those described in the legend to Fig. 2. S1 nuclease-resistant DNA products were fractionated on a 7 M urea–10% polyacrylamide gel. Lane M1 and lane M2, 5′-end ^{32}P-labeled size markers: ϕX174 digested with *Hae*III and pBR322 digested with *Hae*III, respectively. Yeast soluble RNAs (20 μg) (lane 1) and 5 μg of cytoplasmic RNAs from BL2 cells (lane 2) were analyzed as controls. The sizes of protected fragments (in nucleotides) are indicated with arrows.

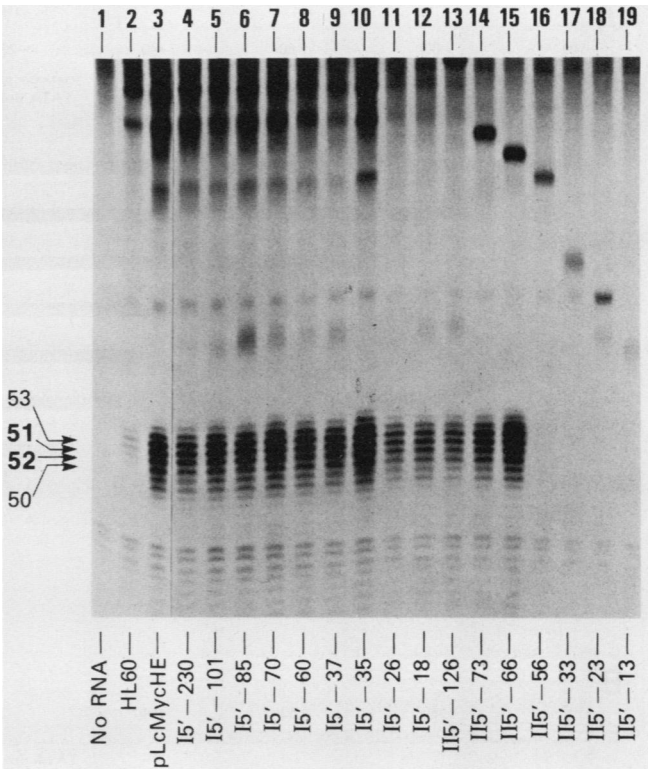

FIG. 4. Detection of 5′ end of c-*myc* transcripts initiated at the cap 2 sites in oocytes microinjected with various deletion mutants. The uniformly ^{32}P-labeled DNA probe, encompassing the region from the *Sma*I site (see Fig. 1) to +51 relative to the major cap 2 site (see Fig. 5B), was used as an S1 probe. Conditions for S1 nuclease analysis were identical to those described in the legend to Fig. 2 except the hybridization temperature used was 51°C instead of 56.5°C. S1 nuclease-resistant DNA products were fractionated on a 7 M urea–12% polyacrylamide gel. Yeast soluble RNAs (20 μg) (lane 1) and 5 μg of cytoplasmic RNA prepared from pro-myelocytic leukemia cell line HL-60 (lane 2) were analyzed as controls.

In addition, the results suggest that one of the unique features within the 5′-flanking region, the four repeats of the nonanucleotide $\frac{C}{T}C\frac{C}{T}TCCCCA$ located between −152 and −116 relative to the cap 1 site (1), is also not required for promoter activity.

GC-rich motifs or GC boxes are essential elements of human c-*myc* gene promoters. Neither region of sequences essential for the two promoter activities had obvious special features (Fig. 5), and the sequences around P1 and P2 were also quite different from each other except for TATA homology (Fig. 5). However, a possible element involved in transcription initiation of c-*myc* genes is the GC-rich hexanucleotides located between −39 and −44 in P1 and between −61 and −66 in P2 (indicated by open boxes in Fig. 5). These two GC-rich hexanucleotide sequences were identical near TATA box 1 and very similar near TATA box 2 to the sequence called the GC box, which was originally identified

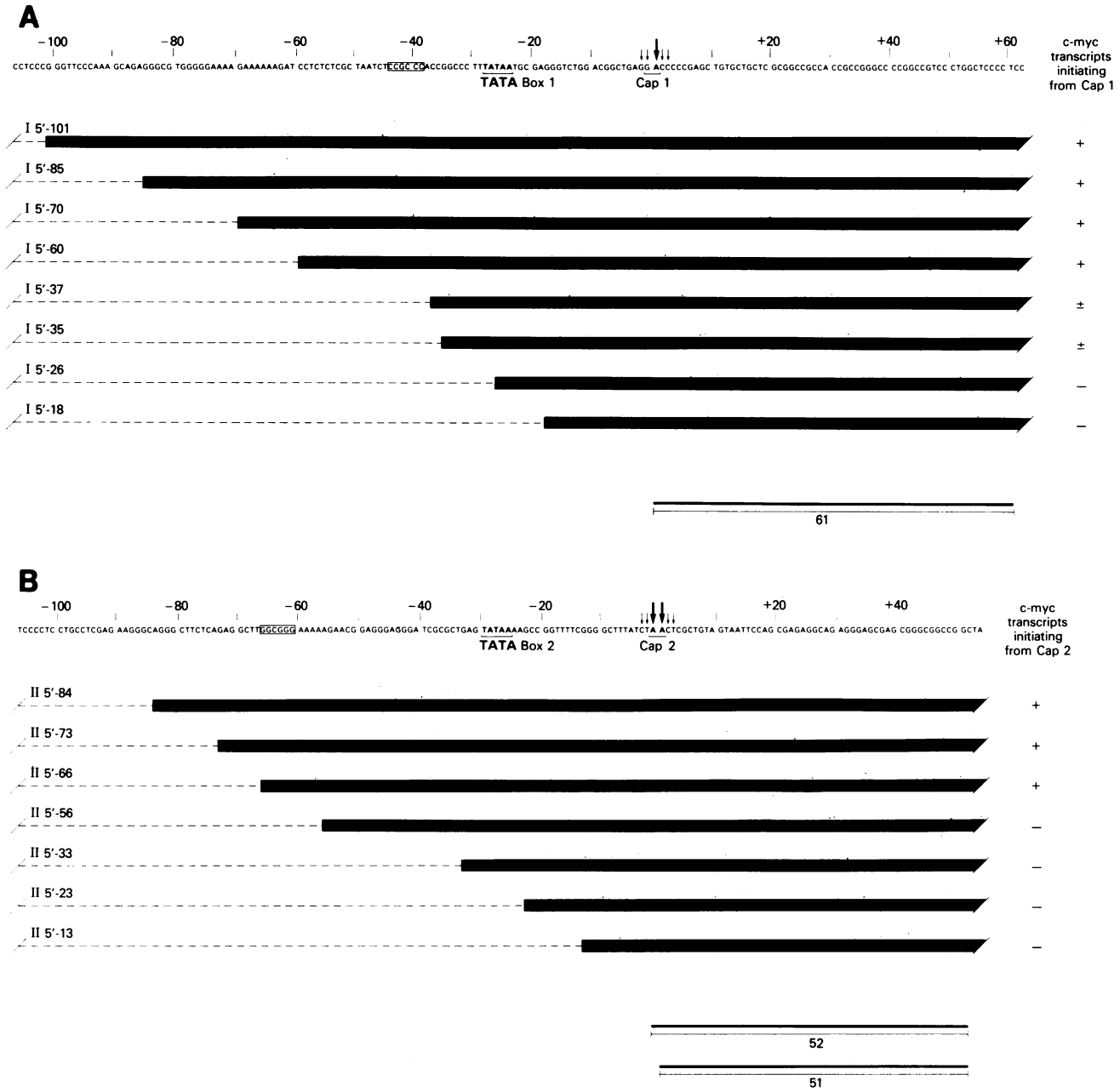

FIG. 5. Schematic diagrams showing the 5′ boundaries of two c-*myc* gene promoters. The serial 5′ deletion mutants are shown diagramatically. Solid lines, Intact c-*myc* DNA sequences; dashed lines, deleted sequences. TATA sequences are underlined, and cap sites are indicated by large arrows (major initiation sites) or small arrows (minor sites). GC-rich motifs identified as essential elements of c-*myc* gene promoters are shown in boxes. Accurate transcriptional activity of the individual 5′ deletion mutants is indicated at the right. Results are derived from quantitative analysis of S1 mapping results such as those shown in Fig. 2, 3, and 4. The autoradiograms were scanned with a densitometer for quantitation. Although there was some quantitative variation of transcriptional activity (for example, compare lanes 10 and 11 in Fig. 4), such differences often disappeared after averaging separate experiments (three independent microinjections into oocytes from different frogs). (A) 5′ boundary of the P1 promoter; (B) 5′ boundary of the P2 promoter.

as a part of the simian virus 40 gene promoter elements (13, 17, 18) and considered to be the binding site for the transcription factor Sp1. A number of GC box-containing promoters of cellular genes have been also reported (13). In all of those cases the promoters contain one or more perfect copies of the hexanucleotide GGGCGG, which may be present in either orientation with respect to transcription. In the human c-*myc* gene, the first GC box sequence located upstream of TATA box 1 (GC box 1) was indeed a perfect copy of the 5′-GGGCGG-3′ sequence but on the sense strand, whereas the second GC box sequence located upstream of TATA box 2 (GC box 2) was 5′-GGCGGG-3′ on the antisense strand. Although the second sequence was not a perfect match for the common GC box, my analysis of the deletion mutations around this region suggests that sequences between nucleotides −66 and −56 (GGCG

GGAAAA) contain an element essential for P2 activity. I believe that this GC box-like sequence, GGCGGG, may act like a GC box in this case. The presently accepted consensus sequence for the GC box and immediate surrounding sequences deduced from 19 Sp1-binding sites is 5'-$^{G}_{T}$GGGCGG$^{GGC}_{AAT}$-3' (12), whereas the sequence around GC box 1 and 2 is TGGGCGGAGA and TGGCGGGAAA, respectively. It is worthwhile to point out that two of these GC box or GC box-like sequences are perfectly conserved between human and mouse c-*myc* genes (3). In addition to these two GC boxes, one more GC-rich motif, located more proximal to TATA box 1 than GC box 1, may also contribute to P1 activity. The deletion of DNA sequences to −37 or −35, which completely removed the GC box 1 sequence, still left 1/20 to 1/50 the level of accurate transcription initiation from the cap 1 site(s) (Fig. 2 and 3), but deletion to −26 completely abolished residual promoter activity. The nucleotides located between −37 and −35 are part of the second GC-rich motif located between −30 and −37 (5'-GGGCCGG-3') on the sense strand. Once again the sequence did not perfectly match the typical GC box, but these sequences may be also a part of the P1 promoter. To demonstrate that the GC-rich motifs found upstream of the two TATA sequences of the human c-*myc* gene are indeed involved in Sp1 factor binding, foot-printing experiments with purified Sp1 factors have to be carried out. Such experiments are under way in my laboratory.

Control mechanism of c-*myc* gene expression. In this study, it was found that GC-rich motifs located near the two TATA sequences are essential elements of the human c-*myc* gene promoters recognized by the transcription machinery of frog oocytes, which are cells arrested in the G2 phase of the meiotic cell cycle (16). However, it is not clear at this stage whether the sequences identified also function as the promoter elements in somatic cells. I also do not know whether any other regions of the c-*myc* genes—for example, further upstream sequences such as DNase I-hypersensitive sites I and II (14, 42), which can be deleted without affecting the promoter activity of c-*myc* gene in the *X. laevis* oocyte assay system—are involved in specific regulatory mechanisms operating in a cell cycle-dependent or cell differentiation-dependent fashion controlled by putative repressors, as proposed previously (7, 23, 26). Alternatively, control of c-*myc* gene transcription may be simpler or even constitutive. Indeed, it has recently been shown that the c-*myc* transcriptional level stays constitutively high throughout the cell cycle of Chinese hamster fibroblast cells (4) and throughout in vitro-induced cell differentiation of F9 teratocarcinoma cells (10), suggesting that the posttranscriptional level of control, probably through the RNA turnover rate, is a major part of the control of c-*myc* gene expression.

X. laevis oocytes are known to carry out accurate and efficient transcription initiation of microinjected 5S rRNA (6) and tRNA (8, 25) genes by RNA polymerase III and also of rRNA genes by RNA polymerase I (30, 36). However, protein-coding genes, transcribed by RNA polymerase II, have been reported to be poorly expressed in oocytes, except for virus genes such as herpes virus thymidine kinase genes (28) and simian virus 40 genes (44), or housekeeping genes such as histone genes (15, 34). It is also of interest that most of the promoters so far reported to contain the GC box are found in either virus genes such as simian virus 40 (18), herpes simplex virus type 1 (21, 22), and AIDS retrovirus genes (35, 40), and eucaryotic cellular housekeeping genes such as dihydrofolate reductase (12), methallothionein-Ia (39), hydromethylglutaryl coenzyme A reductase (38), hypoxanthine phosphoribosyltransferase (29), adenine phosphoribosyltransferase (11), and adenosine deaminase (43). All of these are genes designed to function in various cells and tissues. In this study I have shown that in frog oocytes, the microinjected human c-*myc* gene is very efficiently transcribed from its own promoters located within a short stretch of the sequence containing GC-rich motifs. c-*myc* may thus be one of the housekeeping genes carrying strong promoters which allow constant or constitutive transcription controlled by rather universal transcription factors such as Sp1 in a variety of host cells and tissues. The varying level of c-*myc* mRNAs in the various stages of cell growth or cell differentiation may be the result of control at the posttranscriptional level as recently suggested (4, 10).

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