REVIEW — Current Perspective —

Gene Transcription Through Myc Family Members in Eukaryotic Cells

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ABSTRACT — c-Myc family proteins, encoded by c-myc family proto-oncogenes, play critical roles in mechanisms associated with proliferation, differentiation and apoptotic death in eukaryotic cells. These functions are mediated by transcriptional activity of these proteins through binding to the E-box core sequence CACGTG referred to as a Myc core element located at a promoter or enhancer region of the individual target genes in the nucleus. Recent studies have demonstrated the presence of novel nuclear proteins that specifically recognize a Myc core element, in addition to c-Myc, Max, Mad and Mxi1. On the other hand, a Myc core element has alternating purine/pyrimidine repeats which could undergo a conformational transition from right-handed (B-DNA) to left-handed (Z-DNA) forms in the presence of a high concentration of salts such as Mg$^{2+}$ and polyamines. Similarly, a Myc element has a homopurine-homopyrimidine site that may take a triplex configuration in particular situations. We have searched for nuclear proteins that can specifically recognize a Myc core element in different topological variations in murine brain.

Keywords: Transcription factor, c-Myc family protein, DNA binding, E-box element, DNA topology

I. Introduction
In eukaryotic cells, de novo synthesis of proteins is mainly controlled at the level of transcription of genomic genes by RNA polymerase II, which is responsible for the formation of mRNA from DNA in the nucleus. Gene transcription involves molecular mechanisms associated with protein-DNA and protein-protein interactions, in addition to topological alterations of DNA. Transcription factors are nuclear proteins that specifically recognize particular core nucleotide sequences at promoter and/or enhancer regions on target genes and thereby elicit quantitative control of mRNA formed from DNA as a result of modulation of the activity of RNA polymerase II in the nucleus. Therefore, both specific recognition of a particular core nucleotide sequence and transcriptional modulation are major functions of transcription factors in the nucleus.

c-Myc family proteins are shown to be involved in mechanisms underlying cell proliferation, differentiation and apoptosis. In model systems using various tumor cells, for example, overexpression of c-Myc protein is highly associated with induction of oncogenic transformation (1), blockade of cell differentiation (2) and induction of apoptosis (3). These functions are mediated by transcriptional activity of c-Myc family proteins through binding to the core sequence CACGTG, named as an E-box element, at the promoter or enhancer region of target genes. Cell growth cycles are at least in part associated with expression of different c-Myc family proteins such as Max and Mad, while alternative splicing variants of these proteins have also a possible correlation to the cell growth cycle including proliferation and differentiation (4).

In contrast to these trans-elements, nucleotide sequences are crucial for processes linked to modulation of the activity of gene transcription as a cis-element. For instance, the transcriptional activity could be regulated by different DNA topologies in a manner dependent on nucleotide sequences such as left-handed and triple-stranded DNA (5, 6). In fact, several nuclear proteins are shown to specifically recognize such DNA topological changes in eukaryotic cells (7–10). These nuclear proteins could play a role in mechanisms underlying long-term consolidation of the input of transient extracellular signals in the cell nucleus. In this review, we will mainly

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outline nuclear proteins related to c-Myc family members that have affinity for an E-box element and refer to their functions, interactions and cellular network. Possible presence of novel proteins with an ability to recognize a Myc core element under different topological changes is also discussed.

II. Functional domains of transcription factors

Transcription factors usually contain 3 functional domains as shown in Fig. 1. These include a transcription regulatory domain that can activate or repress the transcriptional activity of a target gene, a protein interaction domain that interacts with other family proteins or other regulatory proteins and a DNA binding domain. To date, the DNA binding domain is categorized into several different major classes according to their unique protein motifs such as helix-turn-helix, zinc finger and basic/helix-loop-helix/leucine zipper (bHLH-LZ). For example, a protein containing a bHLH-LZ motif could at first dimerize with another member protein, because both helix-loop-helix (HLH) and leucine zipper (LZ) motifs promote the formation of a dimeric complex for DNA binding through their basic regions. Afterwards, this dimer binds to DNA by using certain regions rich in basic amino acids. These functional domains and motifs are all required for regulation of the gene transcription. Dimerization could occur between bHLH-LZ family proteins without basic and transcription regulatory regions through their HLH and LZ motifs, whereas the dimer is unable to modulate gene transcription due to a lack of an ability to bind to DNA in the nucleus.

III. Transcriptional network

Functionally important regions of c-Myc protein exist at both the amino and carboxyl termini (Fig. 2). The amino-terminal region is a transactivation domain that contains two major phosphorylation sites on the threonine (T) residue at 58 and on the serine (S) residue at 62 amino acids away from the amino terminal, both of which are specifically phosphorylated by glycogen synthesis kinase-3 (GSK-3) and mitogen activated protein kinase (MAPK), respectively, but not by casein kinase II (CKII) (11). c-Myc/Ras co-transformation assay reveals that a point mutation of T58 to alanine markedly potentiates focus formation in primary cultures of embryonic fibroblasts, with a mutation of S62 leading to a significant reduction of the ability of transformation (11). In Burkitt's lymphoma cell lines, point mutations of T58 and S62 to aspartic acid, which is mimicry of constitutively phosphorylated forms of c-Myc protein, reduces the transactivation activity (12). Thus, post-translational modulation of c-Myc protein is associated with mechanisms underlying transactivation and transformation via phosphorylation at its amino-terminal region.

By contrast, the carboxyl-terminal of c-Myc protein has a bHLH-LZ motif that promotes dimerization with the partner protein Max. Max also has a bHLH-LZ motif as well as sequence-specific binding to the E-box core element CACGTG. Although c-Myc protein is unable to homodimerize under physiological conditions, Max
is able to bind to the identical core element by way of forming a homodimer in addition to a heterodimer with Mad. Another bHLH-LZ protein, Max, poorly forms a homodimer and does not heterodimerize with any members other than Max. Among these dimers including c-Myc/Max, Max/Max and Mad/Max, only a c-Myc/Max dimer has an ability to potentiate the transcriptional activity, because c-Myc protein only has a transactivation domain at the amino-terminal region. Both c-Myc/Max and Mad/Max heterodimers are more potent than a Max/Max homodimer in DNA binding activities as a result of formation of relatively stable complexes. c-Myc and Mad proteins have affinities not significantly different from each other for Max protein (4). It is thus suggested that relative levels of each protein are important for cell growth cycles. Elevated levels of c-Myc protein increase the ratio of a c-Myc/Max heterodimer to a Max/Max homodimer in proliferating cells, in fact, while overexpression of Max protein competes with the c-Myc/Max heterodimer for DNA binding (13). Overexpression of Mad protein antagonizes the transcriptional activity of c-Myc protein, however, while the endogenous level of Mad is increased in response to the differentiation inducer 12-O-tetradecanoylphorbol-13-acetate (TPA) with a concomitant increase in the endogenous level of c-Myc protein in human myeloid cell lines (14).

Although Max protein plays a central role in mechanisms underlying transcriptional activation and repres-
expression by c-Myc family members, expression of Mad protein leads to a drastic change in cell growth processes from proliferation to differentiation (Fig. 3). In addition, Mad protein is not a simple unified protein but has multiple forms such as Mad (Mad1), Mxi1 (Mad2), Mad3 and Mad4. As described above, Mad1 is primarily expressed late in cell differentiation and acts as an antagonist against c-Myc protein. Although Mad3 is detected in proliferating cells prior to differentiation, however, both Mxi1 and Mad4 are most abundant in cells under advanced differentiation cycles (15). Therefore, different members of such a Mad sub-family exert their own functions in distinct cellular processes during the growth cycle transition between proliferation and differentiation.

Several alternative proteins of c-Myc family could be expressed to modulate original transcriptional activities. For example, the c-myc gene is responsible for expression of two major proteins such as Myc1 (67 kDa) and Myc2 (64 kDa), which are both translated from the beginning sites at CUG and AUG, respectively. Myc1 is longer than Myc2 by 14 amino acids at the amino terminus in most tissues and cells, while Myc2 is the major isof orm with a role different from Myc1 in growth regulation. Overexpression of Myc1, but not Myc2, significantly inhibits cell growth (16). Moreover, a recent study has demonstrated the presence of a short c-Myc protein (c-Myc S) that lacks most of the amino terminal transactivation domain of c-Myc protein and is expected as a dominant-negative inhibitor of transactivation (17) (Fig. 4a). Accordingly, transcriptional activity seems to be dedicated to the Myc2 protein with a full-length. Similarly, the max gene has at least 2 alternative products identified as a 21-kDa (p21 Max) protein and a 22-kDa (p22 Max) protein with 9 more amino acids at the amino terminal. Both Max proteins can homodimerize with each other as well as heterodimerize with c-Myc to recognize the core DNA, while the association rate is faster with a dimer containing the shorter p21 Max than that containing the longer p22 Max. These result suggested that different Max complexes may be responsible for different kinetic properties (18). An alternative form of Max, dMax (delta Max), is also found during amplification of max-specific sequences from human erythrooleukemia cell cDNA by a polymerase chain reaction. dMax contains almost all bHLH-LZ domains of Max protein but lacks 62 amino acids including the nuclear localization signal and basic region at the carboxyl terminal. Therefore, dMax is a cytoplasmic protein with an ability to dimerize with c-Myc protein but unable to bind to a Myc core element (19) (Fig. 4b).

Furthermore, c-Myc protein can interact with other nuclear proteins. For example, Yin-Yang-1 (YY-1) (20), Miz-1 (Myc-interacting zinc finger protein) (21) and Nmi-1 (N-myc interactor-1) (22) all bind to the carboxyl terminal of c-Myc protein. As the carboxyl terminal has a HLH domain, c-Myc protein is unable to bind to an E-box core element together with Max protein when inter-

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**Fig. 4.** A transcriptional network among nuclear proteins with affinity for E-box. The dimer Max/Myc-S is unable to modulate gene transcription due to a lack of the transactivation domain (a), while a c-Myc/dMax dimer is unable to bind to an E-box element because it lacks a DNA binding domain (b). An interaction of c-Myc with other bHLH proteins may lead to binding to core elements other than an E-box at the upstream of target genes followed by different transcriptional activities (c), whereas co-regulator proteins could interact with c-Myc protein to potentiate or repress its transcriptional activity (d).
acted with these nuclear proteins. The complex between c-Myc and such other nuclear proteins could specifically bind to another core element on genomic DNA (Fig. 4c). c-Myc protein also interacts with other proteins including box-dependent myc-interacting protein-1 (BIN-1) (23) and AMY-1 (24) as a co-regulator protein at the amino terminal region (Fig. 4d). BIN-1 is related to the negative regulator of the yeast cell cycle and inhibits transformation by c-Myc protein in malignant cells (23), while AMY-1 is isolated as a 11 kDa-protein with an ability to stimulate the activation of E-box-dependent transcription by c-Myc protein (24).

IV. Other binding proteins

Several proteins with a bHLH/LZ motif and affinity for an E-box element have been identified to date, in addition to c-Myc, Max and Mad. These include USF, TFE3 and TFEB. Analysis of conformational changes reveals that no significant difference is seen in estimated bend angles induced by complexes of c-Myc/Max and Max/Max as compared with those of other bHLH-LZ proteins such as USF, TFE3 and TEF3 in major late promoters of the adenovirus and the immunoglobulin heavy chain. Accordingly, most bHLH-LZ proteins with affinity for a Myc core element may have common features in terms of interactions with DNA (25). However, these four (?) proteins have no identified effects on cell proliferation and/or apoptosis so far.

It is widely accepted that alternating purine/pyrimidine base repeats easily undergo a conformational transition from the usual right-handed (B-DNA) form to a unique left-handed (Z-DNA) form on double-stranded DNA in the presence of high concentrations of salts, Mg$^{2+}$ ions and polyamines. By using antibodies specific for DNA in a left-handed conformation, the presence of Z-DNA is demonstrated at transcriptional regulatory regions of a number of genomic genes including the c-myc gene (5) with a potential physiological role in mechanisms associated with regulation of gene transcription. Specific antibodies against Z-DNA strongly inhibit gene transcription activated by wheat germ RNA polymerase II due to formation of Z-DNA through a template of poly-[dG-C] (26). Some other proteins with affinity for the Z-conformation are also identified to date. Estrogen receptor (ER) normally binds to the estrogen response elements (ERE), GGTCANNNTGACC, and plays an important role in gene expression by estradiol. However, ER is shown to bind to a Z-conformation of poly(dG-m5dC)/poly(dG-m5dC) induced by polyamines, with maximal binding in the vicinity of the midpoint of B-DNA to Z-DNA transition of the polynucleotide (7), suggesting ER may have an ability to recognize some nucleotide sequences with a Z-form. Moreover, Z alpha protein is a Z-DNA binding protein confirmed by gel mobility shift analysis from chicken blood nuclei. Double-stranded RNA adenosine deaminase, named as human editing enzyme having a Z alpha region, can bind to the Z-conformation, suggesting a role of Z-DNA to edit nascent RNA (8). The E-box core element seems to be capable of easily taking a Z-DNA conformation, due to its alternating purine/pyrimidine sequence in the presence of Mg$^{2+}$ ions and polyamines. The possible presence of nuclear proteins with an ability to recognize an E-box element in a Z-form is conceivable.

In our hands, binding to a Myc core element (Myc binding) is markedly inhibited by the addition of MgCl$_2$ at concentrations above 5 mM. Gel retardation electrophoresis is performed by using a radiolabeled double

![Fig. 5. Binding to an E-box element. An aliquot of brain nuclear extracts was incubated in either the presence or absence of 10 mM MgCl$_2$ with two Myc probes that were separated from the original probe on preparative SDS-PAGE (upper and lower). These two radiolabeled probes showed different mobilities on the 12% gel. DNA binding was more potent for a Myc probe with a slow mobility than that with a fast mobility, while MgCl$_2$ was more potent in inhibiting binding of an upper probe than that of a lower probe.](image-url)
stranded oligonucleotide containing the core element CACGTG. Myc binding is specific for the core sequence judging from the competition experiments done in the presence and absence of MgCl₂. One possible interpretation is that MgCl₂ markedly reduces affinity of the dimer for an E-box element, whereas an alternative is that MgCl₂ may disclose DNA binding activity different from that found in the absence of MgCl₂ (27). In fact, regional variations are quite different from one another between Myc binding in the presence and absence of MgCl₂ in murine brain. Considering the requirement for Mg²⁺ ions of the transition of an E-box element from a B- to Z-form, the data cited above means that particular nuclear proteins may recognize an E-box element under a Z-DNA configuration in the presence of MgCl₂. Indeed, Z alpha protein could bind to (dG-dC)₁₂ or (dG-dC)₁₅ oligomers, but not to an E-box element, in the presence of 10 mM Mg²⁺ (28).

On the other hand, another topological variation is a triple stranded DNA that could appear at a homopurine-homopyrimidine site. A triplex is shown to be present at a promotor or enhancer region to modulate transcription of several genes including c-myc (6). The probe used to evaluate Myc binding in our study is designed as a double-stranded oligonucleotide by annealing two single strands of 5'-CTAGTGACACGTCGCCGGATCA-3' and 5'-TGATCCGGGCCACGTCACTAG-3'. Because these two single strands have a short homopurine-homopyrimidine site, a triplex oligonucleotide might be formed during procedures for preparation of a radiolabeled probe. To evaluate this possibility, gel retardation electrophoresis is done on a gel containing polyacrylamide at a high concentration (12%) for clear separation of possible radiolabeled probes with different strands. Two radioactive bands are detected on the gel only after the annealing of those two single strands. The data suggest that radioactive bands with different mobilities may correspond to triplex and duplex oligonucleotides, respectively. These two radioactive probes are isolated on preparative SDS-PAGE, followed by incubation with nuclear extracts of murine brain for determination of DNA binding (Fig. 5). Nuclear proteins could bind to both probes with different mobilities in a manner sensitive to inhibition by Mg²⁺ ions compared to each control lane. Interestingly, DNA binding is more potent with the upper probe than the lower one irrespective of the addition of MgCl₂. As Myc binding is specific for the E-box sequence CACGTG, a number of nuclear proteins may bind to an E-box element with a triplex conformation. Several proteins with affinity for triplex DNA are indeed demonstrated in vivo. For instance, a 55-kDa protein that does not have any affinity for single- and double-stranded oligonucleotides but for triplex DNA exclusively has been identified from human cells (9). Proteins of 100, 60 and 15 kDa seem to selectively recognize a GT-rich purine-motif triplex in Hela cells, while a 55 kDa-protein purified from Hela cells could bind to a TAT triplex in a sequence-specific manner (10). Our results are likely to be favorable for the possible presence of several nuclear proteins that can bind to an E-box element under a triplex transition in murine brain. Our goal is to search for and isolate novel nuclear proteins with an ability to recognize DNA topologies in brain to homogeneity and thereby to clarify molecular mechanisms underlying a variety of neurological disorders associated with abnormal gene transcription.

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