Binding of THZif-1, a MAZ-like Zinc Finger Protein to the Nuclease-hypersensitive Element in the Promoter Region of the c-MYC Protooncogene*

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A detailed analysis is reported of the binding of the zinc finger protein THZif-1 to the nuclease-hypersensitive element (NHE) in the promoter region of the c-MYC gene using the electrophoretic mobility shift assay and a series of mutants of a fusion protein composed of glutathione S-transferase and THZif-1. The THZif-1 protein bound specifically to the single-stranded (ss) pyrimidine-rich DNA of the NHE (ss c-myc NHE-C) with an apparent dissociation constant (Kd(app)) of 0.077 μM. By contrast, no binding to the single-stranded purine-rich DNA of the NHE (ss c-myc NHE-G) was detected. Moreover, the binding affinity of THZif-1 protein was 2-fold higher for the single-stranded 5-methyl-2′-deoxyctydine derivative of NHE (ss c-myc NHE-me5C) than for the unmethylated NHE. In the case of the binding of THZif-1 to methylated double-stranded (ds) NHE (ds c-myc NHE-me5CG), no significant binding to the DNA was observed. The decrease in binding to DNA of THZif-1 was significant in the case of mutated ds c-myc NHE, in which more than two sites of deoxycytidine residues were methylated. However, the binding affinity of THZif-1 protein for methylated and unmethylated triple-helical DNA of the NHE was almost identical. Moreover, the domain of the THZif-1 protein that made the major contribution to binding to ss c-myc NHE-C or ss c-myc NHE-me5C corresponded to the amino-terminal second zinc finger motif. Taken together, the results indicate that the THZif-1 protein exhibits preferential DNA-binding activity with ss c-myc NHE-C, ds c-myc NHE-CG, and ts c-myc NHE but not with ss c-myc NHE-G and ds c-myc NHE-me5CG in vitro.

Recent studies suggest that the c-Myc oncoprotein functions, in part, as a sequence-specific transcription factor. This protein is induced in a variety of cellular processes, which include regulation of progression of the cell cycle, proliferation, differentiation, and programmed cell death (apoptosis) (1–4). The c-myc gene is a member of a family of genes with basic, helix-loop-helix, and leucine zipper domains, all of which encode sequence-specific DNA-binding proteins (5). The c-Myc protein forms a heterodimer with the Max protein that is required for the oncogenic activity of c-myc. The Myc-Max complex recognizes the core sequence CACGTG (5).

Evidence for complementarity, but not fully redundant, activities among members of the Myc family (c-, N-, and L-myc) comes from studies of phenotypes of Myc-deficient mice. Although inactivation of c-myc and N-myc results in death of embryos by mid-gestation (6–9), a phenomenon that demonstrates that each is required for normal development, the survival of the mutant embryos to such a late stage of embryogenesis suggests that members of the c-myc family of genes might have overlapping functions at early, but not later, stages of development. At mid-gestation in the mouse, enhanced expression of c-myc is correlated with active proliferation, and down-regulation is associated with mitotic arrest and the onset of differentiation (10). Furthermore, in most experiments with cultured cells and transgenic mice, forced expression of c-myc prevents withdrawal from the cell cycle and inhibits differentiation (1–5), indicating that down-regulation of c-myc is required for mitotic arrest and terminal differentiation in the various cell lineages.

Transcription of the human c-MYC oncogene is subject to complex and, thus far, poorly understood regulatory mechanisms. Obvious difficulties arise from the fact that transcription of the gene is driven by at least three promoters, P0, P1, and P2 (11–13), which lack absolute polarity (12), and moreover, transcription is modulated at several different levels, such as initiation (14–16), elongation (14), premature termination (13), and attenuation (2, 3). Several cis- and trans-acting components that regulate the initiation of transcription of the c-MYC gene have been described (1–5).

It has also been suggested that transcription of c-MYC might be regulated at the level of template structure (17–20). A co-linear triplex formed between a site-specific oligodeoxynucleotide and c-MYC duplex DNA at −150 base pairs (bp1; counted from the P1 initiation site) can repress transcription of c-MYC from the P2 promoter in vitro (18–21). This result suggests that

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1 The abbreviations used are: bp, base pair(s); NHE, nuclease-hypersensitive element; EMSA, electrophoretic mobility shift assays; CAT, chloramphenicol acetyltransferase; nt, nucleotide(s); PBS, phosphate-buffered saline; PCR, polymerase chain reaction; tk, thymidine kinase; GST, glutathione S-transferase; ss, single-stranded; ds, double-stranded; ts, triple-stranded; TPA, 12-O-tetradecanoylphorbol-13-acetate; me5C, 5-methyldeoxycytosine; RNP, ribonucleoprotein; hn, heterogeneous nuclear.

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the region around -150 bp of the c-MYC promoter, which is rich in purine/pyrimidine sequences and is also hypersensitive to nuclease (we named it a nuclease-hypersensitive element III1 (NHE; 3, 12, 14, 17, 19–26), is important for the transcription of the c-MYC gene. This region is known as a putative intra- or intermolecular triple helix-forming element (18–20, 27) that might serve as a target of a specific transcription factor(s) needed for P2-directed expression. Previous studies on triplex formation by polypurine/polypyrimidine regions in gene promoters demonstrated the ability of a pyrimidine-rich third strand to form Hoogsteen hydrogen bonds with the purine-rich acceptor strand in the major groove of the target duplex (17). The third strand in pyrimidine-purine-pyrimidine triple helices adopts a parallel orientation relative to the polypurine strand of the duplex (28). The stability of a pyrimidine-purine-pyrimidine triple helix is dependent on nonphysiological acidic conditions required by the C-GC tripleplexes that are responsible for sequence specificity (29–31). In contrast, previous studies demonstrated triplex formation by a purine-rich or mixed purine/pyrimidine third strand that involved GGC and AAT or TAT triads at physiological pH (32–34). These triple helices arise through reverse Hoogsteen bonding, which results in the antiparallel orientation of the third strand (32, 35). Consistent with this view, it has been reported that this region of the c-MYC protooncogene exerts a modest stimulatory effect on P2-directed transcription and plays a much more important role in utilization of the P1 promoter (15, 36, 37). Moreover, in a recent report, Arcinas and Boxer (38) demonstrated that the nuclease-hypersensitive sites III1 and III2 in the 5′-end-flanking region of the c-MYC gene exhibit a decrease in sensitivity to DNase I during differentiation of HL60 cells. Thus, the changes in DNase I-hypersensitive sites might be correlated with structural changes in the NHE. Moreover, in vertebrate DNA, the cytosine residues in the dinucleotide sequence CpG and homopurine/homopyrimidine stretches in the flanking regions of genes are often methylated. Such CpG dinucleotides and homopurine/homopyrimidine stretches are often found clustered within so-called CpG islands or homopurine/homopyrimidine regions (29, 30, 39, 40). The presence of unmethylated cytidine residues is indicative of the presence of active genes. The 5′-ends of most housekeeping genes are, for example, located within unmethylated CpG islands or unmethylated homopurine/homopyrimidine regions (39–41). It was reported that the methylation of cytosine strongly enhanced the stability of triplex DNA (42) and that 5-methyl-2′-deoxycytidine in single-stranded DNA directed the methylation of CpG sites on the same strand (43). The NHE in the c-MYC promoter region can also be considered in these terms. It seems plausible that transcription factors that are involved in the control of the expression of c-MYC during differentiation might bind near these DNase I-hypersensitive sites or that some modification of the NHE DNA might occur during the differentiation of HL60 cells.

Davis et al. (20) reported the association of a ribonucleoprotein (RNP), which appeared to involve RNA-DNA hybridization, with the NHE sequence of c-myc. Postel and co-workers (21, 44) demonstrated the binding to c-MYC NHE of a protein of 27 kDa, which they later cloned as PaF/NM23/NDPK-3, a putative suppressor of tumor metastasis (44). The nuclease-sensitive element protein (NSEP-1) (45) and the heterogenous nuclear (hn) RNA protein K, which exhibit overlapping but distinct single-stranded DNA-binding, double-stranded DNA-binding, and RNA-binding specificities, have also been shown to interact with the NHE sequence in the human c-MYC promoter (46–48). Zinc finger proteins, such as SP1 (49), and cellular nucleic acid-binding protein (CNBP) (50), were also shown to bind to the NHE-III1 site of the human c-MYC gene. Distinct cis-acting elements, designated MEl2, E2F, and MEla1, are required for the optimal initiation of transcription from the P2 promoter (52). The DNase I-hypersensitive site III2 is situated almost exactly in the center of the sequence between the P1 and P2 promoters of the c-MYC gene (20–25). The zinc finger protein ZF87/Maz (or Pur-1) has been reported to bind to one of these sites, MEla1, in addition to binding to the NHE-III1 site (37, 52–55). Another zinc finger protein, CCCTC-binding factor or CTCF (51, 56), has also been reported to make contact with sequences in GC-rich core regions between the P1 and P2 promoters.

We showed previously that synthesis of the transcription factor THZif-1, a MAZ-like zinc finger protein, can be induced in the nuclei of HL60 cells that have been transformed with antisense c-MYC, and that it serves as a repressor of the endogenous transcription of the c-MYC protooncogene via binding to the NHE element (57–60). This THZif-1 factor is a zinc finger protein with strong binding affinity for the triple-helical conformation of the NHE of the c-MYC promoter, as well as for the single-stranded and double-stranded NHE (60). In this report, we compared the relative binding activities of THZif-1 protein to the methylated and unmethylated forms of c-myc NHE. Moreover, we identified the region of THZif-1 that binds to the NHEs using deletion and substitution mutants of the zinc finger motifs. The amino-terminal second zinc finger of the THZif-1 protein is required for binding to the methylated or unmethylated, single-stranded pyrimidine-rich NHE. Our results imply a significant role for THZif-1 in the regulated function of the NHE of the c-MYC gene during the growth and differentiation of HL60 cells.

**MATERIALS AND METHODS**

**Cell Culture, Transfection, and Assay of CAT Activity—**HL60 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and 60 μg/ml kanamycin monosulfate (Sigma). Transfection for long term expression was carried out as described previously (61, 62). Assays of chloramphenicol acetyltransferase (CAT) activity were performed as described elsewhere (61–63). Each thin-layer chromatography plate was exposed to RX film (Fuji, Tokyo, Japan). The extent of conversion of chloramphenicol to its acetylated forms was determined with a Bio-Imaging analyzer (model BAS 2000; Japan). The extent of conversion of chloramphenicol to its acetylated forms was determined with a Bio-Imaging analyzer (model BAS 2000; Japan). The extent of conversion of chloramphenicol to its acetylated forms was determined with a Bio-Imaging analyzer (model BAS 2000; Japan).
The exception that concentrations of Mg2+ in the promoter region of the c-MYC protooncogene is the target site of DNA-binding proteins that are induced in the antisense c-MYC transformed cell line AM93-4-12 (57–59). We purified a DNA-binding protein specific for this NHE from a nuclear extract of AM93-4-12 cells and isolated a recombinant cDNA clone (for THZif-1, triple helix-binding zinc-finger protein-1) of the corresponding gene (60). The deduced THZif-1 is a polypeptide of 253 amino acids with a molecular mass of 27,830 kDa. Nucleotide sequencing of the cDNA for THZif-1 demonstrated the extremely high degree of similarity to the Myc-associated zinc finger protein ZF87/Maz2 that consisted of 10 mM Tris (pH 7.8), 5 mM MgCl2, 1 mM spermidine, 10% sucrose, 25 μM ZnCl2, 5 mM dithiothreitol, and 1 μg poly(dI-dC) (Pharmacia). The THZif-1 fusion protein was added to the reaction buffer at the indicated amount, and the mixture was incubated for 60 min at 4°C. In competition experiments, indicated amounts of unlabeled oligomers were added, and incubation was continued for 15 min at 4°C. Samples mineralized with 1 N NaOH were analyzed by electrophoresis at 4°C at a constant current of 10 mA on polyacrylamide gels (4 or 12%) in 50 mM Tris-based buffer as described above. Gels were then dried and autoradiographed. Similarly, the rabbit polyclonal THZif-1-specific antiserum and preimmune serum were used for the supershifting experiment of EMSA as described elsewhere (65). Effect of protease digestion on DNA-protein complex with the THZif-1 protein and c-myc NHE was examined as described by Fang and Cech (66).

Preparation of Cell Extracts and DNA-binding Assay—After washing with phosphate-buffered saline (PBS), cells were harvested and lysed in 100 μl (per 0.1 g of cells, wet weight) of lysis buffer that contained 400 mM KCl, 10 mM Hepes (pH 8.0), 15% (w/v) glycerol, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (Sigma), and 2.5 μM each leupeptin, aprotinin, and pepstatin (all from Sigma). After centrifugation at 40,000 × g at 4°C for 15 min, each supernatant was divided into aliquots and stored at −70°C as described elsewhere (65). Cell extracts were incubated on ice with 2 μg of poly(dI-dC) (Pharmacia) in 20 μl of binding buffer (10 mM Hepes [pH 8.0], 0.5 mM MgCl2, 1 mM dithiothreitol, and 15% glycerol). After 10 min, the 32P-labeled probe (c-myc NHE-CG) and competitor DNAs were added, and the incubation was continued for 20 min. The reaction mixture was subjected to electrophoresis on a 5% polyacrylamide gel in 0.5 × TBE (44.5 mM Tris, 44.5 mM boric acid, and 1.25 mM EDTA). The gel was dried and exposed to x-ray film at −80°C. Monoclonal antibodies specific for SP-1 and for c-Jun (Santa Cruz Biotech, Santa Cruz, CA) and polyclonal antisera specific for ZF87/Maz2 were used for the “supershifting” experiment.

RESULTS

THZif-1 Is a Maz-like Zinc-Finger Protein—We reported previously that the NHE sequence in the promoter region of the c-MYC protooncogene is the target site of DNA-binding proteins that are induced in the antisense c-MYC transformed cell line AM93-4-12 (57–59). We purified a DNA-binding protein specific for this NHE from a nuclear extract of AM93-4-12 cells and isolated a recombinant cDNA clone (for THZif-1, triple helix-binding zinc-finger protein-1) of the corresponding gene (60). The deduced THZif-1 is a polypeptide of 253 amino acids with a molecular mass of 27,830 kDa. Nucleotide sequencing of the cDNA for THZif-1 demonstrated the extremely high degree of similarity to the Myc-associated zinc finger protein ZF87/Maz and to another zinc finger protein, Pur-1, that binds to purine-rich sequences (98.2 and 96.9% homology, respectively, at the amino acid level; 53–55). It appears that THZif-1 is a member of the ZF87/Maz (or Pur-1) family. However, the DNA

3 K. K. Yokoyama, unpublished observations.
THZif-1 Represses the NHE-mediated Expression of the c-MYC Gene—In an attempt to examine the intrinsic activity of c-myc NHE during the differentiation of HL60 cells, we compared the CAT activities of c-MYC promoter-CAT fusion constructs with and without NHE (pMycRNACAT versus pMycRNΔ55CAT; Fig. 1A). A significant reduction in the promoter activity of the c-MYC gene was observed 2 days after the short of treatment with 12-O-tetradecanoyl-phorbol-13-acetate (TPA; lanes 9–12). This reduction was similar to that in the case of pMycCAT2 (lanes 1–4). However, the deletion reporter construct of c-myc NHE did not show such a repression (lanes 5–8). Thus, the intrinsic activity of the c-myc NHE reflects the fact that the c-myc NHE is one of the major regulatory elements in the expression of the c-MYC gene during the differentiation of HL60 cells. Therefore, the characterization of factors that bind to c-myc NHE is important if we are to understand the mechanism of repression of the c-MYC promoter during the differentiation of HL60 cells (52–59).

To determine whether the THZif-1 protein was responsible for the negative regulation of transcription of the c-myc gene, we analyzed the effect of the THZif-1 protein on the promoter activity of c-MYC gene by introducing CAT reporter fusion constructs of c-MYC with or without NHE and an expression plasmid pTHZif-1 or its mutant plasmid pTHZif-1m of second zinc-finger motif, into HL60 cells. We also constructed a promoter for the gene for thymidine kinase (tk) from herpes simplex virus-1 with or without the NHE fused to the gene for CAT and examined CAT activities (61, 64). These plasmids were introduced into HL60 cells and isolated each stable clone. We assayed the reporter activities of these clones. As shown in Fig. 1B, CAT activity of the c-MYC promoter-CAT with NHE (pMycRNACAT) was repressed from 8- to 9-fold by THZif-1, whereas that of c-MYC promoter-CAT without NHE (pMycRNΔ55CAT) was not (lanes 1, 2, 4, and 5). Moreover this repression activity was not detected in the case of THZif-1 mutant construct of second zinc finger motif (lanes 3 and 6). These results indicate that at least the second zinc finger motif of THZif-1 protein is corresponded to the regulated expression of c-MYC-CAT reporter gene in HL60 cells. Similarly, we also observed a reduction in CAT activity in the case of the tk promoter-CAT with NHE (pTk-NHECAT) (lanes 7, 8, 10, and 11). We observed that the CAT activity of pMycRNACAT was reduced in the differentiated HL60 cells (7-fold reduction; lanes 13 and 14) and, however, the CAT activity of pMycRNΔ55CAT was not affected (lanes 16 and 17). We detected the similar reduction of pTk-NHECAT in the differentiated HL60 cells. It is clear that this repression was also detected at a significant level in U937 cells but not in F9 or P19 embryonic carcinoma cells (data not shown). These variations in CAT activity might reflect variations in endogenous levels of THZif-1 protein in the
respective cells (data not shown). These data suggest that at least THZif-1 protein, one of the transcription factors that bind to the NHE, is involved in the NHE-mediated negative regulation of the transcription of the c-MYC gene.

**THZif-1 Protein Is Included in the Protein-DNA Complex with c-myc NHE—**To assess the possibility that the THZif-1 protein binds to the c-myc NHE during the differentiation of HL60 cells, we performed a DNA-binding assay with oligodeoxynucleotides that corresponded to ds c-myc NHE-C as a probe (Fig. 1C). The binding of nuclear proteins to c-myc NHE-C was detected as a sequence-specific protein-DNA complex in extracts of differentiated HL60 cells (lane 2). The incubation of extracts with antisera specific for THZif-1 resulted in the supershifting of the DNA-protein complex (lane 3). The supershifted band disappeared in the presence of the GST-THZif-1 fusion protein (lane 4). Moreover, control preimmune serum did not cause any change in the complex (lane 5). Furthermore and to our surprise, the antisera specific for SP-1 also shifted the DNA-protein complex (lane 6). By contrast, antisera specific for c-JUN and ZF87/Max did not affect the binding to DNA (lanes 7 and 8). However, we did not detect any involvement of the THZif-1 protein in the formation of the DNA-protein complex with c-myc NHE when we used nuclear extracts of undifferentiated HL60 cells. This observation is consistent with previous data (58, 59), and these observations, taken together, indicate that the specific shifts observed in our DNA-binding assays were due to the THZif-1 protein or a closely related protein.

To address the question of whether the negative expression of c-MYC by the THZif-1 protein is primarily due to the binding of THZif-1 to the NHE sequence, we carried out a DNA-binding study using the single-stranded (ss), double-stranded (ds), and triple-stranded (ts) forms of the NHE of the c-MYC gene as DNA probes. The NHE of the c-MYC gene consists of pyrimidine-rich sequences in one strand that seem to be sensitive to de novo methylation and purine-rich sequences in the other strand which remain unmethylated. Therefore, it seems highly likely that the expression of the c-MYC gene is regulated by the methylation of the pyrimidine-rich strand of the NHE. Thus, we also attempted to study the effect of methylation of the cytosine residues in the NHE of the c-MYC promoter on the DNA-binding activity of the THZif-1 protein.

**Binding to Single Strand Unmethylated or Methylated NHE in Vitro—**To characterize the DNA-binding properties of THZif-1, we performed an EMSA study in the presence of Mg2+ ions (5 mM), using the recombinant GST-THZif-1 fusion protein and a single-stranded (ss) NHE oligodeoxynucleotide as a DNA probe. To circumvent the insolubility of recombinant intact THZif-1 protein in E. coli cells, we used the fusion construct with pGEX to produce a soluble protein for our assays. As shown in Fig. 2A, the THZif-1 protein had the capacity to bind to the pyrimidine-rich strand of NHE (ss c-myc NHE-C). The GST protein itself did not bind to any of the ss, ds, or ts c-myc NHE oligodeoxynucleotides used in this study (data not shown). It was also clear that a shifted DNA-protein complex appeared when the concentration of THZif-1 protein was increased (position B1, lane 2). When the dose of THZif-1 protein was increased further, a new shifted band of a DNA-protein complex which seemed to be the complex of DNA with a dimer of the THZif-1 protein, appeared (position B2, lanes 3 and 4). This result is consistent with the previous results that the dimer of THZif-1 protein had higher DNA-binding activity than that of the monomeric form of THZif-1 protein (data not shown). Moreover, we were surprised that the binding of THZif-1 to ss methylated NHE-C (ss c-myc NHE-me5C) resulted in 2-fold higher affinity than that to ss unmethylated NHE-C at all positions (compare lanes 2–4 with lanes 6–8; Table II). In contrast, the THZif-1 protein did not bind to the purine-rich strand of NHE (ss c-myc NHE-G). When the reaction products were treated with proteinase K, the shifted bands were lost in the case of both the methylated and the unmethylated DNA probe (data not shown). In addition, the antisera raised against THZif-1 protein caused supershifting of the DNA-protein complex (Fig. 2B). By contrast, the preimmune serum had no such activity (Fig. 2B). Thus, we concluded that these protease-sensitive bands really represented the complexes between the THZif-1 protein and its target DNA.

**Sequence Specificity of DNA Binding—**Under the same conditions as the EMSA for which results are shown in Fig. 2, we performed a competition experiment using various single-strand unmethylated oligodeoxynucleotides that corresponded to the known CT-elements reported by other investigators (45). As shown in Fig. 3A, the non-radiolabeled oligodeoxynucleotides that corresponded to the pyrimidine-rich strand of c-myc NHE (ss c-myc NHE-C) competed for binding to the THZif-1 protein (lane 3). In addition, the methylated oligodeoxynucleotide that corresponded to the pyrimidine-rich strand of c-myc NHE (methylated to yield the me5C derivative of the pyrimidine-rich strand of c-myc NHE, ss c-myc NHE-me5C) as a competitor gave a similar result (lane 4). We mutated the oligodeoxynucleotides by changing pyrimidine-rich sequences of the NHE to purine residues and analyzed their effects. Mutated oligodeoxynucleotides, such as ss c-myc NHE m1C and m2C, did not compete for binding to THZif-1 with radiola-
FIG. 3. Specificity of the binding to DNA of the THZif-1 protein. A, competition for binding of GST-THZif-1 protein to ss c-myc NHE-C. The GST-THZif-1 protein was incubated with a 100-fold molar excess of the indicated unw radiolabeled oligodeoxynucleotides as competitors for 10 min at 4°C and then mixed with the radiolabeled c-myc NHE-C oligodeoxynucleotides and incubated further for 50 min. The resultant DNA-protein complexes were resolved in a nondenaturing 4% polyacrylamide gel. Lane 1, without GST-THZif-1 protein; lanes 2–4, 0.2 μg of GST-THZif-1 protein; lane 5, ss c-myc NHE-C; lane 6, ss c-myc NHE-m5C; lane 7, ss K-ras NHE-C; lane 8, ss EGFR NHE-C. B, DNA-protein complex; F, free DNA probe. B, competition for binding of GST-THZif-1 protein to ss c-myc NHE-C. The GST-THZif-1 protein was incubated with the indicated unw radiolabeled oligodeoxynucleotides as competitors for 100 min at 4°C and then mixed with radiolabeled c-myc NHE-C oligodeoxynucleotides and incubated for another 50 min. The resultant DNA-protein complexes were resolved in a nondenaturing 4% polyacrylamide gel. Lane 1, without GST-THZif-1 protein; lanes 2–8, 0.2 μg of GST-THZif-1 protein; lane 3, ss c-myc NHE-C; lane 4, ss c-myc NHE-m5C; lane 5, ss K-ras NHE-C; lane 6, ss EGFR NHE-C. C, DNA-protein complex; F, free DNA probe. B, competition for binding of GST-THZif-1 protein to ss c-myc NHE-C. The GST-THZif-1 protein was incubated with the indicated unw radiolabeled oligodeoxynucleotides as competitors for 100 min at 4°C and then mixed with radiolabeled c-myc NHE-C oligodeoxynucleotides and incubated for another 50 min. The resultant DNA-protein complexes were resolved in a nondenaturing 4% polyacrylamide gel. Lane 1, without GST-THZif-1 protein; lanes 2–4, 0.2 μg of GST-THZif-1 protein; lane 5, ss c-myc NHE-C; lane 6, ss c-myc NHE-m5C; lane 7, ss K-ras NHE-C; lane 8, ss EGFR NHE-C. B, DNA-protein complex; F, free DNA probe.

FIG. 4. Binding of THZif-1 protein to ds c-myc NHE-CG. A, comparative binding of GST-THZif-1 protein to all 5-methyl-2′-deoxy- cytidine (me5C) or unmethylated ds c-myc NHE. The indicated amount of the recombinant fusion protein GST-THZif-1 was incubated with duplex c-myc NHE that consisted of radiolabeled c-myc NHE-C (lanes 1–4) or c-myc NHE-me5C (lanes 5–8) and unmethylated c-myc NHE-G for 60 min at 4°C. The resultant DNA-protein complex was resolved in nondenaturing 4% polyacrylamide gels. Lanes 1 and 5, without GST-THZif-1 protein; lanes 2 and 6, 1 μg of GST-THZif-1 protein; lanes 3 and 7, 4 μg of GST-THZif-1 protein, and lanes 4 and 8, 8 μg of GST-THZif-1 protein. B, DNA-protein complex; F, free DNA probe. The GST protein did not bind to the DNA probe of ds c-myc NHE-CG. B, competition for binding of GST-THZif-1 protein to ds c-myc NHE-CG. The indicated unlabeled duplex DNAs, as competitors, at a 100-fold molar excess were incubated with GST-THZif-1 protein (4 μg) for 10 min at 4°C and then the 32P-radiolabeled ds c-myc NHE-CG (radiolabeled DNA strand is c-myc NHE-C) was added as DNA probe, and the mixture was incubated for another 50 min at 4°C. The resultant complexes were resolved in nondenaturing 4% polyacrylamide gels. Lane 1, without GST-THZif-1 protein; lane 2, without competitor DNA; lane 3, ds c-myc NHE-CG; lane 4, ds c-myc NHE-m5C; lane 5, ds c-myc NHE-m2C; lane 6, ds K-ras NHE-CG and ds EGFR NHE-CG. B, DNA-protein complex; F, free DNA probe. C, effects of antiserum against the THZif-1 protein on the migration of the DNA-protein complex in the EMSA. Lane 1, without antiserum; lanes 2–4, addition of preimmune antiserum; lanes 5–7, addition of antiserum against THZif-1. Lanes 2 and 5, 10−4 dilution of antiserum; lanes 3 and 6, 10−3 dilution of antiserum; lanes 4 and 7, 10−2 dilution of antiserum. The upper and lower arrowheads indicate the supershifted bands of the DNA-protein complex and the DNA-protein complex between ds c-myc NHE-CG and the THZif-1 protein, respectively. DNA probe was prepared as in A.

c-MYC gene. These results imply that the sequence specificity of the binding to DNA of THZif-1 protein is restricted to single-stranded pyrimidine-rich sequences of c-myc NHE, irrespective of the presence or absence of methylation. The neu-1, neu-2, and Y-box NHEs, as well as poly(dC)12–18 and poly(dT)12–18, did not compete in the binding of GST-THZif-1 protein to ss c-myc NHE-C (45; data not shown). Thus, it appears that the THZif-1 protein binds preferentially to the pyrimidine-rich ss DNA of the c-myc NHE (ss c-myc NHE-C) in a sequence-specific manner.

Binding to Double-stranded Unmethylated or Methylated c-myc NHE—We prepared unmethylated DNA probes from the double-stranded (ds) NHE of the c-MYC gene, whose pyrimidine-rich strand or the purine-rich strand had been radiolabeled. Each probe was isolated from a nondenaturing gel as described under "Materials and Methods." The DNA-binding activity of THZif-1 protein was analyzed by the EMSA. As shown in Fig. 4A, a shifted band (lane 3) appeared, and the formation of the DNA-protein complex was dose-dependent in the case of ds c-myc NHE-CG, of which pyrimidine-rich strand was radiolabeled (lanes 1–4). However no shifted bands were
detected with the tested range of concentrations of THZif-1 protein in the case of ds c-myc NHE-me⁵CG, of which all cytosine residues were methylated (lanes 5–8). When the ds c-myc NHE-CG, of which the purine-rich strand had been radiolabeled, was used, the similar shifted band (band B) and the formation of the DNA-THZif-1 protein complex appeared (Table I). Thus we conclude that the THZif-1 protein has an ability to bind the ds c-myc NHE-CG.

We next examined the sequence specificity of the binding of THZif-1 protein to ds c-myc NHE-CG. As shown in Fig. 4B, oligodeoxynucleotides with the same sequence as ds c-myc NHE-CG competed for binding of THZif-1 protein (lanes 4 and 5) while the mutant oligodeoxynucleotides of ds c-myc NHE-CG did not have such inhibitory activity. It is noteworthy that ds c-myc NHE-CG of K-ras competed for the binding of THZif-1 protein with ds c-myc NHE-CG, but this was not the case for ds c-myc NHE-CG of EGFR (lanes 6 and 7). The differential binding of THZif-1 to the ds NHEs might have been due to the higher degree of similarity between the sequence of the NHE-CG of the K-ras gene and that of the c-MYC gene than is the case for EGFR NHE-CG. Antiserum against THZif-1 caused supershifting of the DNA-protein complex, an indication that the THZif-1 protein is included in this DNA-protein complex (Fig. 4C). Therefore, the sequence specificity of the binding to DNA of the THZif-1 protein was more restricted in the case of ds c-myc NHE-CG of the c-MYC gene, even though the binding affinity for the ds NHE was lower than for the ss c-myc NHE-C (Table I). The specificity of the binding of the THZif-1 protein to the ds NHE-CG might be slightly different from that of ss c-myc NHE-C.

We then examined the DNA-binding activity of the THZif-1 protein to various forms of double-stranded c-myc NHE-CG, in which the deoxycytidine residues were methylated at different positions (Fig. 5, Table I). To our surprise, the ds c-myc NHE probes, in which deoxycytidine residues at positions 3 or 33 were methylated, bound THZif-1 (Fig. 5). However, methylation at additional positions of NHE resulted in the absence of specific DNA-binding activity of THZif-1 (Fig. 5). These results are summarized in Table I. These data indicate that the effect of methylation of deoxycytidine residues at least at two positions, namely 3 and 6 (or 29 and 33), in c-myc NHE resulted in a decrease in the DNA-binding activity of the THZif-1 protein (Table I and Fig. 5).

We compared the K\textsubscript{d} values of the THZif-1 protein for its binding to various ss, ds, or ts forms of the c-myc NHE (Table II). It was clear that the THZif-1 protein had lower affinity for ds c-myc NHE-CG. The binding avidity of THZif-1 protein for ds c-myc NHE-CG was about 50-fold lower than that for ss c-myc NHE-me⁵C (all positions were methylated). Therefore, the methylation of the ds NHE of the c-MYC gene might be critical to the regulation of the affinity of the DNA for the THZif-1 protein, at least under our assay conditions.

**Potential Triple-helical Structure of the NHE of the c-MYC Gene**—We examined the structural differences associated with the formation of the triple-helical structure CCG with respect to the effect of methylation of the pyrimidine-rich strand of c-myc NHE (CGG versus me⁵CCG). We radiolabeled the oligodeoxynucleotides that corresponded to the pyrimidine-rich strand and the methylated me⁵C-derivatives of NHE-C and tested the capacity for triplex formation at a given concentration of the pyrimidine-rich strand, after addition of increasing amounts of the oligodeoxynucleotides that corresponded to the purine-rich strand of the NHE-G in the presence of 5 mM Mg\textsuperscript{2+} ions (Fig. 6). In both cases (Fig. 6, A and B) slowly migrating bands corresponding to double-stranded and triple-stranded NHES appeared, and that formation was dependent on the concentration of the unlabeled purine-rich oligodeoxynucleotides that corresponded to NHE-G. However, in the absence of Mg\textsuperscript{2+} ions, we did not detect the triple-helical NHE on gels, and we only found the shifted band corresponded to the ds NHE (Fig. 6, C and D). Then we radiolabeled the purine-rich NHE-G of the c-MYC gene, and the pyrimidine-rich strand of the NHE-C or the methylated pyrimidine-rich NHE-me⁵C was then added to the reaction mixture at a given concentration of the radiolabeled purine-rich NHE-G, in an attempt to produce a triplex such as CGG\textsuperscript{m} or CGG\textsuperscript{me} at both acidic and neutral pH. We detected only the ds DNA-protein complex and no triple-helical DNA-protein complex, no matter what the acidic or neutral pH (data not shown). Thus, we concluded that the
NHE of the c-myc gene was able to form a Mg$^{2+}$-dependent triplex, such as CGG and me$^5$CGG, irrespective of the methylation of NHE-C. There was no difference in the extent of the migration of the triple complex that consisted of unmethylated NHE or methylated NHE of the c-myc gene on a gel (Fig. 6). In view of observations that the amino terminus of the THZif-1 protein. Loss of the second amino-terminal zinc finger resulted in a significant reduction in the binding to ss c-myc NHE-C (Fig. 7, lines 9 and 10). In the case of amino-terminal deletions, we did not detect any contribution of the fifth finger motif of the THZif-1 protein to DNA-binding activity. Thus, the second amino-terminal zinc finger motif seemed to play a crucial role in the binding to ss c-myc NHE-C. The methylated NHE-me$^5$C was used as the DNA probe in the same assays, and slightly different specificities of binding to DNA were obtained with various mutant proteins. The carboxyl-terminal deletion of three zinc fingers caused a 50% reduction in binding to DNA (Fig. 7, lines 4 and 5), and the deletion of four carboxyl-terminal zinc fingers resulted in a 4-fold reduction in DNA-binding activity as compared with that of clone 5 (Fig. 7, lines 5 and 6). We next examined a series of constructs with deletions from the amino terminus of the THZif-1 protein. Loss of the second amino-terminal zinc finger resulted in a significant reduction in the binding to ss c-myc NHE-C (Fig. 7, lines 9 and 10). In the case of amino-terminal deletions, we did not detect any contribution of the fifth finger motif of the THZif-1 protein to DNA-binding activity. Thus, the second amino-terminal zinc finger motif seemed to play a crucial role in the binding to ss c-myc NHE-C. The methylated NHE-me$^5$C was used as the DNA probe in the same assays, and slightly different specificities of binding to DNA were obtained with various mutant proteins. The carboxyl-terminal deletion of three zinc fingers caused a 50% reduction in binding to DNA (Fig. 7, lines 4 and 5), and the deletion of four carboxyl-terminal zinc fingers resulted in a 4-fold reduction in DNA-binding activity as compared with that of clone 5 (Fig. 7, lines 5 and 6). The results with the amino-terminally deleted GST-THZif-1 protein yielded a similar conclusion (lines 9–11). The second and third zinc fingers of the THZif-1 protein, counted from the amino terminus, and in particular the second finger motif, were important for binding of THZif-1 protein to ss c-myc NHE-me$^5$C. Therefore, it appears that the second amino-terminal zinc finger is very important for binding of the THZif-1 protein to the methylated and unmethylated ss c-myc NHE-C of the c-MYC gene in vitro. Moreover, the contributions of other zinc finger motifs, such as the fifth zinc finger, to the binding to ss c-myc NHE-C and the third zinc finger to the binding to ss c-myc NHE-me$^5$C were smaller. The deletion mutant without the second zinc finger and the mutation of the second zinc finger motif confirmed the significant reduction in

**Fig. 5.** Binding of the THZif-1 protein to various 5-methyl-2′-deoxycytidine derivatives of ds c-myc NHE-CG. Eight μg of the recombinant fusion protein GST-THZif-1 were incubated with the indicated 5-methyl-2′-deoxycytidine derivatives with methylation at various positions in c-myc NHE. The reaction mixtures were incubated for 60 min at 4 °C. The resultant DNA-protein complexes were resolved in non-denaturing 5% polyacrylamide gels. In some cases, the unlabeled double-stranded oligodeoxynucleotides, as competitors, were incubated at 100-fold molar excess with GST-THZif-1 protein (4 μg) after 10 min at 4 °C, and then the $^{32}$P-radiolabeled 5-methyl-2′-deoxycytidine derivatives of ds c-myc NHE-CG were added as DNA probes, and mixtures were incubated for another 50 min at 4 °C. Lanes 1 and 2, without GST-THZif-1 protein; lanes 3 and 4, 4 and 8 μg of GST-THZif-1 protein, respectively; lane 5, ds c-myc NHE-CG, and lane 6, ds c-myc NHE-m1CG. Lane 1, the DNA probe was radiolabeled ss c-myc NHE-G; lane 2, the DNA probe was a 5-methyl-2′-deoxycytidine derivative of ds c-myc NHE-CG. B(ds)$_A$, DNA-protein complex. F(ss)$_A$, the free DNA probe, namely ss c-myc NHE-G; F(ds)$_A$, the free DNA probe, namely a 5-methyl-2′-deoxycytidine derivative with methylation at appropriate positions in ds c-myc NHE-CG. As defined in Table I, A, mA; B, mB; C, mC; D, mD; E, mE; F, mF; G, mG; H, mH, mP.

**Domains of the THZif-1 Protein That Bind to Unmethylated and Methylated ss-NHEs**—In view of observations that the binding to DNA of the THZif-1 protein is specific for ss c-myc NHE-C and for the methylated derivative of the NHE of the c-myc gene (ss c-myc NHE-me$^5$C), we next attempted to identify the domain(s) of the THZif-1 protein required for the specific binding to the DNA. We constructed a set of serially deleted mutant GST-fusion proteins with removal of each zinc finger motif of THZif-1 from either the amino terminus or the carboxyl terminus, and we examined their binding to the ss pyrimidine-rich NHE-C in both its methylated and its unmethylated form (Fig. 7). We compared the radioactivity of the shifted bands of DNA-protein complexes relative to the total radioactivity of the input DNA probe and calculated the relative DNA-binding efficiency based on the binding avidity of clone 1 as a control, which encoded the intact recombinant GST-fusion protein with five zinc fingers and for which the results are indicated as 100%. With ss c-myc NHE-C as probe, the mutant construct without the fifth finger motif in the carboxyl-terminal region had about half the DNA-binding activity of the intact GST-THZif-1 fusion protein (see Fig. 7, lines 1 and 2). The mutant without the carboxyl-terminal four fingers exhibited an approximately 5-fold reduction in DNA-binding activity, as compared with that of clone 5 (Fig. 7, lines 5 and 6). We next examined a series of constructs with deletions from the amino terminus of the THZif-1 protein. Loss of the second amino-terminal zinc finger resulted in a significant reduction in the binding to ss c-myc NHE-C (Fig. 7, lines 9 and 10). In the case of amino-terminal deletions, we did not detect any contribution of the fifth finger motif of the THZif-1 protein to DNA-binding activity. Thus, the second amino-terminal zinc finger motif seemed to play a crucial role in the binding to ss c-myc NHE-C. The methylated NHE-me$^5$C was used as the DNA probe in the same assays, and slightly different specificities of binding to DNA were obtained with various mutant proteins. The carboxyl-terminal deletion of three zinc fingers caused a 50% reduction in binding to DNA (Fig. 7, lines 4 and 5), and the deletion of four carboxyl-terminal zinc fingers resulted in a 4-fold reduction in DNA-binding activity as compared with that of clone 5 (lines 5 and 6). The results with the amino-terminally deleted GST-THZif-1 protein yielded a similar conclusion (lines 9–11). The second and third zinc fingers of the THZif-1 protein, counted from the amino terminus, and in particular the second finger motif, were important for binding of THZif-1 protein to ss c-myc NHE-me$^5$C. Therefore, it appears that the second amino-terminal zinc finger is very important for binding of the THZif-1 protein to the methylated and unmethylated ss c-myc NHE-C of the c-MYC gene in vitro. Moreover, the contributions of other zinc finger motifs, such as the fifth zinc finger, to the binding to ss c-myc NHE-C and the third zinc finger to the binding to ss c-myc NHE-me$^5$C were smaller. The deletion mutant without the second zinc finger and the mutation of the second zinc finger motif confirmed the significant reduction in

**Fig. 6.** EMSA of CGG and me$^5$CGG triplex formation with human c-myc NHE. The indicated amounts of unlabeled ss c-myc NHE-G oligodeoxynucleotide were added to a reaction mixture that consisted of $^{32}$P-radiolabeled ss c-myc NHE-C (A and C) or c-myc NHE-me$^5$C (B and D) oligodeoxynucleotides in the presence (A and B) or absence (C and D) of 2 mM Mg$^{2+}$ ions. After heating of the reaction mixture at 70 °C for 15 min, the mixture was gradually cooled to room temperature. The resultant mixtures were analyzed on nondenaturing 12% polyacrylamide gels. A and B, lane 1, without unlabeled c-myc NHE-G; lanes 2–5, the amount of unradiolabeled c-myc NHE-G was 1, 2, 4, 8, 16, 36, and 80 pmol, respectively. C and D, lane 1, without unlabeled c-myc NHE-G; lane 2, 1 pmol of c-myc NHE-G; lane 3, 8 pmol of c-myc NHE-G; lane 4, 16 pmol of c-myc NHE-G. The positions of ss, ds, and ts DNA are indicated by S, D, and T, respectively.
that the THZif-1 protein might be a key regulator of the differentiation of the HL60 cells to normal macrophages.

The gel shift assay using nuclear extracts of differentiated HL60 cells demonstrated that the THZif-1 protein is included in protein-DNA complexes with c-myc NHE-GC. As shown in Fig. 1C, the antisera specific for THZif-1 protein shifted the band of the protein-DNA complex, whereas the antisera specific for c-JUN and ZF87/Maz did not shift the complex of protein and DNA. However, the THZif-1 protein bound weakly to the c-myc NHE when we used extracts of undifferentiated HL60 cells (58, 59, data not shown). Thus, it is clear that the THZif-1 protein can bind to the NHE of the human c-MYC gene in differentiated HL60 cells. We next examined the binding of the recombinant THZif-1 protein to the NHE sequence to determine the DNA-binding specificity and to characterize the effect of the methylation of the cytidine residues in c-myc NHE on the binding to DNA of the THZif-1 protein.

**Effect of Methylation of NHE on Binding of GST-THZif-1**—We found that the recombinant GST-THZif-1 protein bound to the single-stranded pyrimidine-rich NHE (ss c-myc NHE-C) of the c-MYC gene in a dose-dependent manner (lanes 1–4 in Fig 2A). By contrast, binding of the THZif-1 protein to the single-stranded purine-rich c-myc NHE-G was not detected (lanes 9–12 in Fig 2A). Thus, the DNA-binding specificity of the THZif-1 protein was restricted to the pyrimidine-rich single-stranded NHE of c-MYC gene. Moreover the binding avidity of GST-THZif-1 protein for ss c-myc NHE-me5C was about twice that for unmethylated ss NHE-C (lanes 5–8 in Fig 2A). According to the migration of DNA-protein complexes, the slowest migrating bands seemed to correspond to dimeric proteins in a complex with DNA (see Band B2; lanes 3, 4, 7, and 8). The binding specificity of THZif-1 protein for both methylated and unmethylated ss NHE-C was almost identical. The competition experiment yielded the same results (Fig. 3, A and B).

We next tested the differential requirements for zinc finger motifs in the THZif-1 protein for binding to methylated or unmethylated c-myc NHE-C. According to the results of the DNA-binding study with methylated and unmethylated NHE-C and the deletion mutants of the GST-THZif-1 fusion protein, the second zinc finger motif of the THZif-1 protein plays a central role in binding to the ss c-myc NHE-C (see Fig. 7). It is consistent with the results of transcriptional repression of THZif-1 on the expression of human c-MYC gene.

The DNA-binding activity of THZif-1 protein with methylated and unmethylated ds NHE of the c-MYC gene was examined. To our surprise, the THZif-1 protein did not bind to the methylated deoxycytidine derivatives at all positions of NHE-me5CG (see Fig. 4A). However, the exact position of 5-methyl-2'-deoxycytidine residues of c-myc NHE in *vivo* has not been known. Thus, we introduced methyl residues into every cluster of deoxycytidine residues in c-myc NHE and examined the binding of THZif-1 to these methylated sequences. We also radiolabeled the purine-rich c-myc NHE and allowed it to hybridize with the pyrimidine-rich strand to form ds c-myc NHE as a DNA probe. We detected DNA-binding activity of THZif-1 similar to that to ds c-myc NHE-CG (Fig. 5), in which the pyrimidine-rich strand of NHE-CG had been radiolabeled (Fig. 4). Thus, we concluded that the THZif-1 protein binds the ds c-myc NHE-CG DNA. The THZif-1 protein did not bind to c-myc NHE-CG, when at least two deoxycytidine residues in the c-myc NHE had been methylated (see Table 1). Therefore, our studies *in vitro* indicate that the THZif-1 protein has differential DNA-binding activities that depend on the extent of methylation of c-myc NHE-CG.

We present here two tentative models that might explain how the THZif-1 protein recognizes the single-stranded NHE-C.
antiparallel orientation of the third strand (32, 35). We examined the effects of the methylation of the pyrimidine-rich strand of c-myc NHE on the formation of intermolecular triplexes of the CGG and meCGG types. Under our experimental conditions, no differences in the formation of a triplex were observed between methylated and unmethylated pyrimidine-rich strands of NHE (see Fig. 6). Thus, we can conclude there is no major difference in the capacity for intermolecular formation of a triplex of c-myc NHE in the presence of Mg$^{2+}$ ions between the methylated and the unmethylated pyrimidine-rich strand of NHE (Fig. 6). In fact, the THZif-1 protein bound to the triplex NHE in a similar manner irrespective of whether NHE was in the methylated or unmethylated form (see Table II). The apparent dissociation constant of THZif-1 protein was almost the same for c-myc NHE-CGG and c-myc NHE-meCGG ($K_d = 0.89$ versus 0.80 nM). It has been known that the presence of 5-methylcytosine in place of cytosine in the third strand of C-GC triple helices increases the apparent pK for triplex formation and permits formation of detectable triplex up to neutral pH (67). Firulli et al. (19, 20, 27) reported that the DNA sequences of NHEs that form intramolecular triple helices seem to be positive indicators of promoter strength. Transcriptionally active, stable triplexes included C-GC triplets in an intramolecular complex that induced formation of a single-stranded portion and the parallel orientation of polypyrimidine triplex-forming strands. Firulli et al. (21, 32–34) suggested that one or more of the proteins known to bind to the NHE might function to stabilize such a structure at neutral pH. However, we did not detect the intramolecular formation of a triplex, such as C-GC, under our experimental conditions in vitro, a result that is consistent with those in other reports.

Possible Role for Binding of THZif-1 to c-myc NHE—The capacity of the c-myc NHE to form a triplex to inhibit transcription of the c-MYC gene was first demonstrated by the addition of an oligodeoxynucleotide targeted to the NHE (18). Subsequently, several studies have shown that the transcription of c-MYC is repressed in human cervical carcinoma and ovarian carcinoma cells (21, 68), indicating an inhibitory effect on cell proliferation. Previously, our studies indicated that the c-myc NHE might involve the negative regulation of c-MYC gene by introduction of antisense c-MYC gene in HL60 cells. We also detected the similar negative regulation of c-myc NHE during the cell differentiation of HL60 cells (Fig. 1A). Although the mechanism for inhibition of transcription has not been elucidated, it has been suggested that triplex formation prevents the binding of essential regulatory factors (69). Similarly, triplex-induced inhibition of the binding of nuclear factors to an enhancer-promoter element had been demonstrated in other systems, for example in the case of the NFkB that binds to the interleukin-2a promoter (70), a factor that binds to the HER1 promoter (71), and SP-1 that binds to the human Ha-RAS promoter (72, 73, 74). These studies demonstrated in vitro and in vivo the gene-specific repression of transcription that accompanied formation of triplex targeted to the nuclear protein-binding sites. However, we found in the present study that the THZif-1 protein has the ability to bind to the triple helix of c-myeNHE. Moreover, the binding avidity of the THZif-1 protein to ss, ds, and ts c-myc NHEs was well correlated with the negative regulation of transcription of the c-MYC gene (60, but not shown) since the THZif-1 protein with a mutant second zinc finger did not have the DNA-binding activity and did not repress transcription of the c-MYC gene (Figs. 1B and 7) (60).

Previous deletion studies of the c-MYC promoter clearly showed that the c-myc NHE is a positive element (3, 15, 20) that is required for transcription from the c-MYC P1 promoter.
THZif-1 protein has twice the affinity for ss methylated NHE sites. In such a case, the THZif-1 protein might play a role in myc ME1a1. However, repression of transcription of the c-myc gene. Arcinas and Boxer (38) reported that the nuclear hypersensitive site III1 in the NHE of the c-myc gene exhibits decreased sensitivity to DNase I during differentiation of HL60 cells. It is possible that the THZif-1 protein might bind to the c-myc NHE and prevent digestion by DNase I.

Other relevant cis-acting regions are the GC-rich sequences at the ME1a1 site in the promoter of the c-myc gene. This site has been shown to enhance the initiation of transcription of the c-myc gene in vitro (68) and to be essential for initiation at P2 in vivo (26). Moreover, the E2F and the ZF87/Maz (Pur-1) proteins both bind to two of the three distinct elements within the mouse c-MYC promoter that are required for transcription (52, 53). The novel triplex-forming sites encompass half of the E2F site and all of the sites of the canonical cis elements of ME1a1. However, repression of transcription of the c-myc gene might additionally require the melting of the target c-myc NHE or the formation of a triplex among P1 and P2 and the NHE sites. In such a case, the THZif-1 protein might play a role in changing the conformation of the DNA.

It is clear from our DNA-binding study in vitro that the THZif-1 protein has twice the affinity for ss methylated NHE-meC as it does for NHE-C and that it has the capacity to bind to the triple-helical conformation of c-myc NHE, irrespective of methylation of the NHE-C or the absence of methylation. By contrast, the THZif-1 protein does not bind to the methylated ds c-myc NHE-meC. Such differential binding of the THZif-1 protein suggests that the protein might be one of the key regulators of the altered conformation and topology of the DNA in the chromatin, with resultant changes in the rate of transcription of the c-MYC gene in vivo. Further studies are clearly required if we are to understand the molecular basis of the conformation and the extent of the methylation of c-myc NHE and the combinatorial interactions between the THZif-1 protein, other DNA-binding proteins and the NHE sequence during the differentiation of HL60 cells.

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