A novel putative transcription factor protein MYT2 that preferentially binds supercoiled DNA and induces DNA synthesis in quiescent cells

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Abstract Myelin transcription factor 2 (MYT2), a putative transcription factor found in the human central nervous system, was cloned from an expression cDNA library from human T-cells. MYT2 shares weak similarity to bacterial type I topoisomerases and shares 63% sequence identity to a replicase from *Leuconostoc mesenteroides*. MYT2 preferentially binds supercoiled DNA (scDNA). Incubation of MYT2 and scDNA at or above equal molar ratios generated topoisomer-like patterns that were abolished by deproteination. Thus, MYT2 appears to relax scDNA via a non-enzymatic mechanism. The banding pattern of MYT2–scDNA complexes was shown to be quantisized, saturable and sequence-independent. Microinjection of MYT2 mRNA induced G_0 growth-arrested NIH 3T3 cells to enter the S phase of the cell cycle.

Key words: Myelin transcription factor 2; DNA-binding protein; Topoisomer-like pattern; DNA replicase; DNA synthesis; Microinjection

1. Introduction

DNA topology plays an important role in many key cellular processes such as DNA replication, recombination, repair, and transcription [1–3]. Proteins that bind and induce topological changes in DNA have profound effects on gene transcription [4]. For example, structural factors YY1 [5] and HMG1 [6] function as transcription factors by affecting DNA structure rather than by directly contacting the transcription machinery. The topology of DNA can be altered either enzymatically by topoisomerases or non-enzymatically by DNA-binding proteins, such as *Escherichia coli* single-stranded DNA-binding protein [7], prokaryotic DNA ligase [8], HIV-1 nucleocapsid protein [9], HMG [10], and p53 [11].

We recently cloned from a human T-cell cDNA expression library a novel DNA-binding protein that is almost identical to the newly described myelin transcription factor 2 (MYT2) [12]. MYT2 is a DNA-binding protein that is expressed in the central nervous system and was suggested to belong to a class of proteins involved in cellular growth control and survival in the nervous system. Intriguingly, MYT2 is secreted even though it binds to DNA, and it is a putative transcription factor function [12]. Here we present evidence that MYT2 preferentially binds and relaxes supercoiled DNA (scDNA), and is able to stimulate DNA synthesis in quiescent mammalian cells. This protein is homologous to the bacterial topoisomerase I family, and is 63% identical to a replicase from *Leuconostoc mesenteroides*. Interestingly, no identifiable homologs exist in the completed genomes of yeast and *Caenorhabditis elegans*.

2. Materials and methods

2.1. Cloning

A 5′-stretch human Hut-78 T-cell λgt-11 cDNA expression library was purchased from Clontech. This library was screened using a 43 bp oligonucleotide probe derived from the −81 to −39 region of the HIV-1 LTR and contained three methylated Sp1 transcription factor binding sites. The sequence of the probe was 5′AGGGAGGMGTGGCCTGGGMGGGACTGGGGAGTGGMGAGCCCTC3′ where M represents 5-methylcytosine. The probe was labeled with [γ-^{32}P]ATP at the 5′ end of the plus strand and annealed with 50% excess of unlabeled minus strand as previously described [13] and the labeled probe was further catenated [14]. The Hut-78 cDNA expression library was screened with this probe according to the procedure described by Singh and coworkers [15]. Purified positive phage plaques were used to infect *E. coli* Y1090r$^-$ according to the manufacturer's instructions. The supernatant containing the phage isolate was stored in 50% sterile glycerol at −80°C.

The insert was amplified from the phage isolate using nested PCR with the following primers: 5′-GACTCCTGGAGCCCG-3′ (5′ outer primer), 5′-GGTAGCGACCGGCGC-3′ (3′ outer primer), 5′-GGAATTCATATGGATAGTTCATTAAGTATTATAACATACTC-3′ (5′ inner primer with *Nde*I restriction site), and 5′-CCGCTCGAGACTATTAGTATTAGTTCTGTGCGTTAAAC-3′ (3′ inner primer with *Xho*I restriction site). PCR reactions were performed in 50 μl volumes containing 0.5 μM primers, 0.2 mM of each dNTP, 10 μl of 10x *Pfu* PCR reaction buffer and 2.5 units *Pfu* polymerase (Stratagene). PCR reactions were conducted as follows: 1 cycle of 94°C for 5 min, followed by 30 cycles of 94°C for 45 s, 51°C for 45 s, 72°C for 3 min, and finally 1 cycle of 72°C for 5 min. After gel purification, the PCR fragment was cloned into pCR-blunt (Invitrogen) and was digested with *Nde*I and *Xho*I. The insert was subcloned into pET24 (Novagen) with 6×His-tag at the 3′ end of the gene. This construct was named pMYT2.

2.2. Expression and purification

MYT2 was expressed in *E. coli* Bl21 (DE3). The cells were induced by 1 mM IPTG at $OD_{600} = 0.8$ and grown for 8 h at 20°C after

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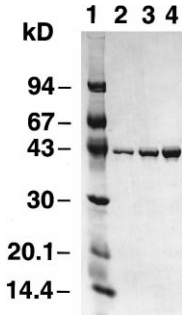

Fig. 1. SDS–PAGE analysis of purified MYT2 protein. Lane 1, molecular weight markers; lane 2, 1 μg of total protein; lane 3, 2.5 μg of total protein; lane 4, 5 μg of total protein.

induction. Cell disruption was performed by subsequent brief sonication in ice cold Dulbecco's PBS containing 0.5 M NaCl, 1 μM leupeptin, and 0.5 mM PMSF. Cell debris were removed by centrifugation (19 000 rpm, 20 min, Sorvall-34) at 4°C; the supernatant was applied to a POROS MC 20 resin Ni-IMAC column (4.6×100 mm) using a BioCAD 700E system (PerSeptives Biosystems). The protein was eluted with a linear gradient from 0 to 500 mM imidazole. The protein-containing fractions were combined and dialyzed against Buffer A (50 mM Tris–HCl, pH 7.5, 10% glycerol) at 4°C and were applied to a phosphocellulose column (20 ml Whatman P11) equilibrated in the same buffer, containing 1 mM EDTA. MYT2 was eluted with a linear gradient of 0–1 M NaCl. Fractions containing protein were combined, dialyzed overnight at 4°C against 1 l of dialysis buffer containing 30 mM HEPES, pH 7.5, 0.25 mM EDTA, and 10% glycerol, and stored at −80°C. The purity of MYT2 was examined with Novex 10–20% Tris–Glycin gel according to the manufacturer's instruction.

2.3. Gel shift assay

The plus strand of the 43 bp cDNA screening oligonucleotide was biotinylated at the 3′ end. It was either used as a single-strand probe or annealed with an equal amount of the minus strand to form a double-stranded probe. The methylated probe was designated as A and the non-methylated probe as B. The reaction was conducted as described [13], except for using a 50 fold more excess of poly(dIdC) as non-specific competitor. The reaction was incubated on ice for 20 min, loaded on a pre-run 6% DNA Retardation Gel (Novex), and run at 85 V in a Novex Xcell II electrophoresis apparatus for about 1 h. The gel was transferred to a Pall Biodyne A membrane (Gibco BRL Life Technologies) in a Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell at 50 mA for 30 min. The Southern-light Chemiluminescent Detection system (Tropix) was used to detect the free probe and binding complex according to the manufacturer's instructions.

2.4. Electron microscopy

Carbon-coated support-filmed grids were glow-discharged for 2 min and attached at the edges to a double-sided adhesive tape. The DNA-protein mixture (5 μl) was placed on the grids allowing the complex to adsorb for 2 min, then the grids were washed three times with glass-distilled water for 1 min each. The grids were dehydrated in 80% alcohol solution, stained with 80% alcohol containing 0.002% uranyl acetate, and washed in an 80% alcohol solution. The grids were air-dried on filter paper and rotary shadowed with platinum–palladium at an angle of 6° in a Denton vacuum evaporator. The grids were examined and photographed with a Hitachi H-7000 electron microscope operated at 50 kV.

2.5. Topoisomerase I activity assay

A topoisomerase I assay kit from TopoGen was used to test the possible topoisomerase activity of MYT2. Typically, the assay was done in 20 μl using the supplied assay buffer according to the manufacturer's instructions. The concentrations of MYT2 and DNA are indicated in the text. The reaction was carried out at 37°C for half an hour and terminated by adding stop and loading buffer. The mixture was then loaded on a 1% agarose gel and electrophoresed until the dye front reached the bottom of the gel. The gel was stained with 0.5 μg/ml ethidium bromide. As a positive control, human topoisomerase I (Gibco Life Technologies) was assayed under the same conditions as MYT2.

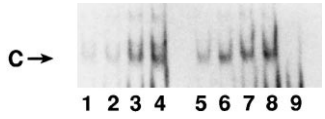

Fig. 2. The comparison of MYT2 binding to the methylated and non-methylated 43 bp oligonucleotide probes derived from the Sp1 binding site region of HIV-1 LTR. Lanes 1–4, methylated oligonucleotide; lanes 5–8, non-methylated oligonucleotides; lanes 1 and 5, 25 ng of oligonucleotides; lanes 2 and 6, 50 ng of oligonucleotides; lanes 3 and 7, 100 ng of oligonucleotides; lanes 4 and 8, 200 ng oligonucleotides. Protein MYT2 in lanes 1–8: 370 ng; lane 9, 200 ng non-methylated oligonucleotide, without protein MYT2. C: Represents MYT2:DNA probe complex.

2.6. Sequence analysis

Wisconsin Sequence Analysis Package (GCG) and MacVector 6.0 (Oxford Molecular Group) was used for sequence analysis.

2.7. Microinjection

Plasmid pMYT2 was linearized with *Xho*I and used as the template for in vitro transcription of the MYT2 gene with In Vitro Transcription kit MEGAscript (Ambion). The MYT2 mRNA was microinjected into approximately 50 quiescent NIH 3T3 cells and the subsequent incorporation of tritiated thymidine into their nuclei was detected according to the method previously described [16].

A

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1
DSSLSIITYSMSVHSLQKNGEPMKQEKQQFIKTDWTIDMINAVGNLRNMPLIFLT
56
ATKDIHRGGWVERIDNKAWQYVRVYENGDIEVLITLQIIRFHKYFDTPVWLQFNP
111
NHLLPADYVQLDHVMKYVDHSHLTRADLANDIYNINLQRYDFGLFGVTRDIYRS
165
LSGDLETRYWGRRKSERQIRLYDKMREMKKHGKADDIPDGITDWWRLEFQFRG
218
GKVESWQEEVMDKMQSFHVLAVDDNDDLSEIDKAILARVNADKFDFKRVGKR
270
YAAKIRKMVRENVGFDTTVAELSLKTFNEQKDELQRQLDSMLAKYNIGAQTEE
323
MTAYFEEELKQTGNLDFSVVESESALERNVIRNIAKSWREENSLTHRTNTNS*
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B

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RepC   6 HANHSNHLENHDLDNFSKTGYSNSRLDAHTVCISDPKLSFDAMTIVGNLN
         ... |    . .:.:: |.|   ..:....  : ...:.:| :. ||||
MYT2   2 DSSLSIITYSMSVHSLQKNG...EPMKQEKQQFIKTDWTIDMINAVGNL.
                .         .         .         .         .
        56 RDNAQALSKFMSVEPQIRLWDILQTKFKAKALQ.........EKVYIEYD
                ..:. :: ....   :: : .::..||:|          .|.|..:
        48 ....RNMPLIFLTATKDIHRGGWVERIDNKAWQYVRVYENGDIEVLITLQ
                .         .         .         .         .
        97 KVKADSWDRRNMRIEFNPNKLTRDEMIWLKQNIISYMEDDGFTRLDLAFD
            :: ..:    ::::||||.| ..: : | ::::.|::.. :|| ||| |
        94 IIRFHKYFDTPVWLQFNPNHLLPADYVQL.DHVMKYVDHSHLTRADLAND
                .         .         .         .  #      .
       147 FEEDLSDYYAMSDKAVKKTIFYGRNGKPETKYFGVRDSNRFIRIYNKKQE
           : :   : |.::  :|.:.|: : .|. ||:|:| |.|:| ||:|:|..|
       143 IYNINLQRYDFGLFGVTRDIYRSLSGDLETRYWGRRKSERQIRLYDKMRE
                .         .         .         .         .
       197 RKDNADAEVMSE...HLWRVEIELKRDMVDYWNDCFSD....LHILQPDW
           .|.::.|: :.:   .:||:|:::: :.|: |.: . |    :|:|..|
       193 MKKHGKADDIPDGITDWWRLEFQFRGGKVESWQEEVMDKMQSFHVLAVDD
                .         .         .         .         .
       240 KTIQRTADRAIVFMLLSDEEEWGKLHRNSRTKYKNLIKEISPVDLT..DL
           ..  .. |:||: .: .|. :: :: :.  .|.:.:::|  ..| |  :|
       243 NDDLSEIDKAILARVNADKFDFKRVGKRYAAKIRKMVRENVGFDTTVAEL
                .         .         .         .
       288 MKSTLKANEKQLQKQID.................FWQHEFK 311
             .|:.....:||:|:|                 :::.|:|
       293 SLKTFNEQKDELQRQLDSMLAKYNIGAQTEEMTAYFEEELK 333
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Fig. 3. The amino acid sequence of MYT2 and the sequence alignment of MYT2 and RepC. A: Amino acid sequence of MYT2. B: Sequence alignment of p44 and RepC. Highly conserved fragments are underlined. # represents the tyrosine residue essential for the topoisomerase I activity of RepC [21]. RepC contains 314 amino acids.

3. Results

3.1. Isolation and initial characterization of MYT2

We previously identified a HIV-1 methylated DNA-binding protein (HMBP) present in a human T-cell line, that preferentially binds to methylated Sp1 binding sites in the HIV-1 LTR [13]. In an attempt to isolate HMBP, a human Hut-78 cDNA expression library was screened with a methylated and catenated oligonucleotide probe derived from the −81 to −39 region of the HIV-1 LTR. One of the phage clones that yielded a positive probe-binding signal contained an insert of 1343 bp which encodes a polypeptide of 375 amino acid residues with a calculated molecular weight of 44.2 kDa (MYT2). BLAST analysis of the GenBank database revealed that MYT2 was almost identical to the newly described MYT2 [12] except that there is a valine substituted for a leucine at position 158 in MYT2. MYT2 was subcloned with a His6-tag at its C-terminus, expressed in *E. coli* BL21(DE3), and purified to homogeneity using metal chelate and phosphocellulose chromatography (Fig. 1).

We then characterized MYT2 binding to oligonucleotides derived from the Sp1 binding region of HIV-1 LTR (Fig. 2). In contrast to what we expected to find for HMBP, our data showed that MYT2 bound equally well to methylated (Fig. 2, lanes 1–4) and non-methylated oligonucleotide (Fig. 2, lanes 5–8) under the assay conditions. One explanation for this result is that MYT2 may be one component of HMBP and may not preferentially bind methylated DNA in the absence of other components. Alternatively, MYT2 may be unrelated to HMBP.

Sequence analysis indicates that MYT2 represents a novel human protein composed of a basic N-terminal region (residues 1–199 have a p*I* of 9.2) followed by an acidic C-terminal region (residues 200–375 have a p*I* of 4.8). In fact, a 43 residue segment (329–365) contains 11 glutamic or aspartic acid residues (Fig. 3A). Anionic regions with a high percentage of glutamic and aspartic acid residues have been implicated in protein–protein interactions [17], and also are characteristic of many proteins that interact with chromatin, such as, centromere protein CENP-B, chromatin assembly proteins N1/N2, topoisomerase I, and HMG [18]. The N-terminal region is typical of nucleic acid binding proteins which are rich in basic residues.

3.2. MYT2 generates a topoisomer-like pattern in scDNA

MYT2 shares sequence similarity with a family of plasmid-encoded *trans*-acting replication initiator (Rep) proteins from *Staphylococcus aureus*, all of which have type-I topoisomerase

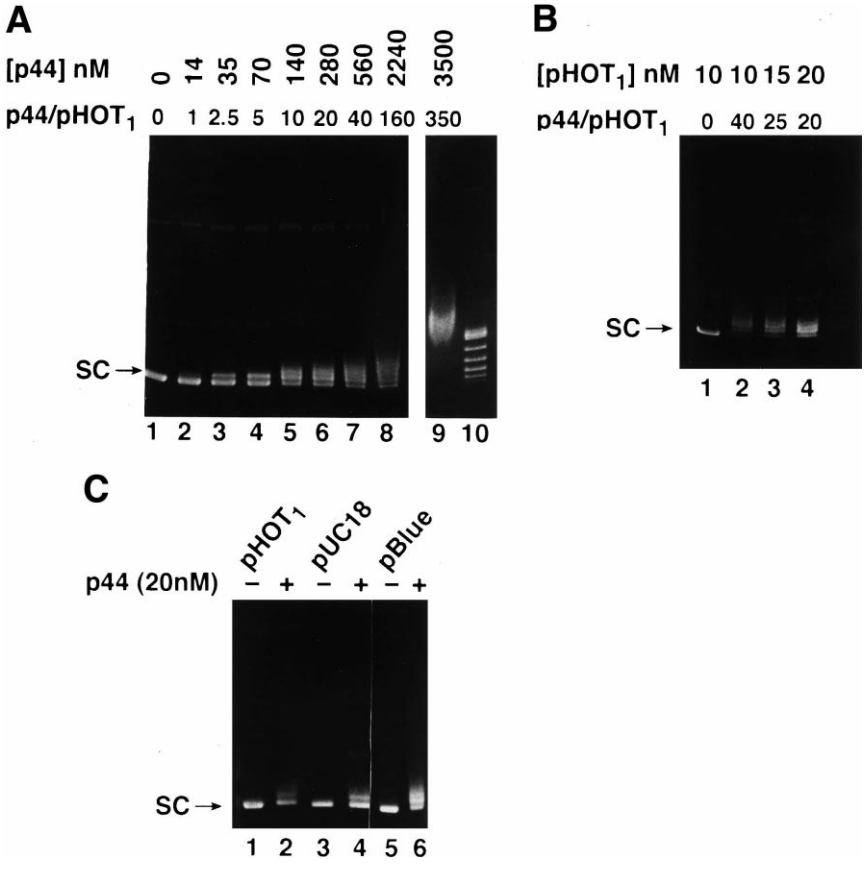

Fig. 4. MYT2 generates topoisomer-like pattern on scDNA. A: Topoisomer-like patterns of MYT2:scDNA complexes. The effect varying MYT2 concentrations on ladder pattern formation using 14 nM of pHOT1: lane 1, no protein; lane 2, 14 nM protein (protein:DNA ratio: 1:1); lane 3, 35 nM protein (2.5:1); lane 4, 70 nM protein (5:1); lane 5, 140 nM protein (10:1); lane 6, 280 nM protein (20:1); lane 7, 560 nM (40:1); lane 8, 2240 nM protein (160:1); lane 9, 3500 nM protein (350:1); lane 10, partially relaxed pHOT1 by human topoisomerase I. SC: scDNA. B: The effect of varying scDNA concentration on ladder pattern formation using 400 nM of MYT2: lane 1, 10 nM supercoiled pHOT1 without protein; lane 2, 10 nM supercoiled pHOT1 (protein:DNA ratio: 40:1); lane 3, 15 nM supercoiled pHOT1 (25:1); lane 4, 20 nM supercoiled pHOT1 (20:1). SC: scDNA. C: Ladder patterns using other plasmids at 10 nM. lanes 1, 3, and 5, no protein; lanes 2, 4, and 6, 200 nM protein; lanes 1 and 2: pHOT1; lanes 3 and 4; pUC18; lanes 5 and 6; pBluescript II SK(-). SC: scDNA.

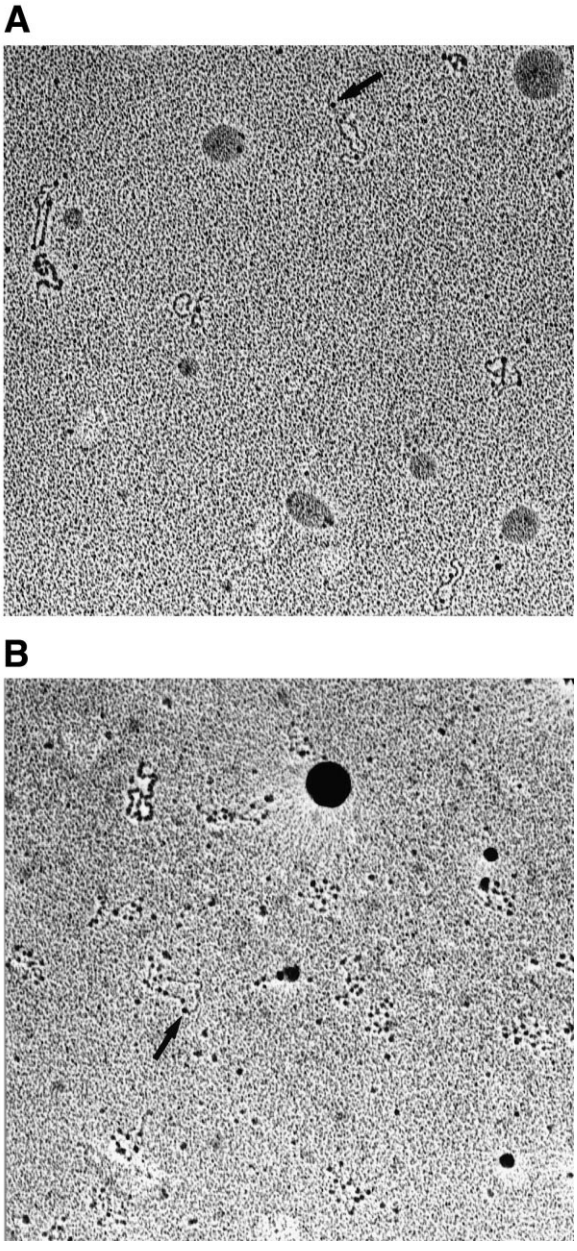

Fig. 5. Electron microscopic image of MYT2–DNA complexes formed from the incubation of 12 nM pHOT1 and (A) 0.5 μM MYT2; and (B) 2 μM MYT2. Arrows point to MYT2 bound to DNA.

activity [19]. Fig. 3B shows the alignment of MYT2 and RepC encoded by pT181 [20]. Although the overall sequence identity is only 23%, the alignment of MYT2 and RepC reveals several highly conserved segments, including FNPNH(K)L and IR-L(I)YD(N)K (Fig. 3B). The conserved tyrosine residue at position 191 in the second segment is the active site tyrosine of the RepC topoisomerase I [21].

To test for potential topoisomerase activity, MYT2 was incubated with supercoiled pHOT1 DNA at 37°C and analyzed on an agarose gel (Fig. 4A). Interestingly, this reaction yielded a pattern similar to the partial relaxation pattern generated by human topoisomerase I (Fig. 4A, lane 10). The number of the topoisomer-like bands increased as the molar ratio of MYT2 to scDNA increased from 1:1 to 160:1. However, the bands became less distinct as more protein was added, and at a ratio of MYT2:scDNA of 350:1, only a wide, diffuse band was observed (Fig. 4A, lane 9).

Similar results were observed when the DNA concentration was varied at a fixed protein concentration (Fig. 4B). At a MYT2:scDNA molar ratio of 40:1, several distinct bands were observed (Fig. 4B, lane 2). Reducing the MYT2:scDNA ratio by increasing the DNA concentration caused an increase in the intensity of the bands, but did not increase the number of bands observed (Fig. 4B, lanes 3 and 4). These results suggest that there is a fixed number of binding sites on the scDNA.

To test the sequence specificity of scDNA binding, MYT2 was incubated with two other plasmids, pUC18 and pBluescript II SK (-) (Fig. 4C). Similar banding patterns were formed with both plasmids, implying that scDNA relaxation by MYT2 is not sequence-dependent. Based on the concentration dependence of the banding patterns (Fig. 4A,B), we estimate the affinity between MYT2 and scDNA to be below 35 nM.

3.3. *MYT2 binds to scDNA in a manner similar to tumor suppressor p53*

The binding of MYT2 to scDNA (pHOT1) was visualized using electron microscopy. Fig. 5A shows that one or more MYT2 molecules bind to the pHOT1 plasmid when they are mixed at a stoichiometry of 42:1 (12 nM pHOT1 and 0.5 μM MYT2). However, after the same amount of pHOT1 was incubated with 2 μM MYT2, each pHOT1 plasmid had many bound MYT2 molecules, and some plasmids appeared to be covered with bound MYT2 (Fig. 5B). Interestingly, the pHOT1 plasmids displayed a relaxed conformation after MYT2 binding. A similar result was observed by electron microscopy with the tumor suppressor p53 which also induces relaxation of scDNA [11].

The MYT2 scDNA complexes were treated with proteinase K to better resolve the banding pattern. Surprisingly, the topoisomer-like patterns disappeared completely, and the DNA was restored to its original supercoiled conformation (Fig. 6). In contrast, proteinase K treatment did not affect the DNA

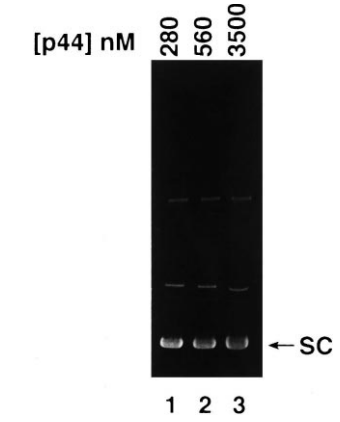

Fig. 6. Effects of proteinase K digestion on ladder patterns of MYT2:scDNA complexes. Proteinase K of a final concentration of 50 μg/ml was added to MYT2:scDNA complexes prepared using 14 nM pHOT1 DNA and MYT2 at the indicated concentrations. Digestion was performed at 37°C for 30 min. Lane 1, 280 nM MYT2; lane 2, 560 nM MYT2; lane 3, 3500 nM, MYT2. SC: scDNA.

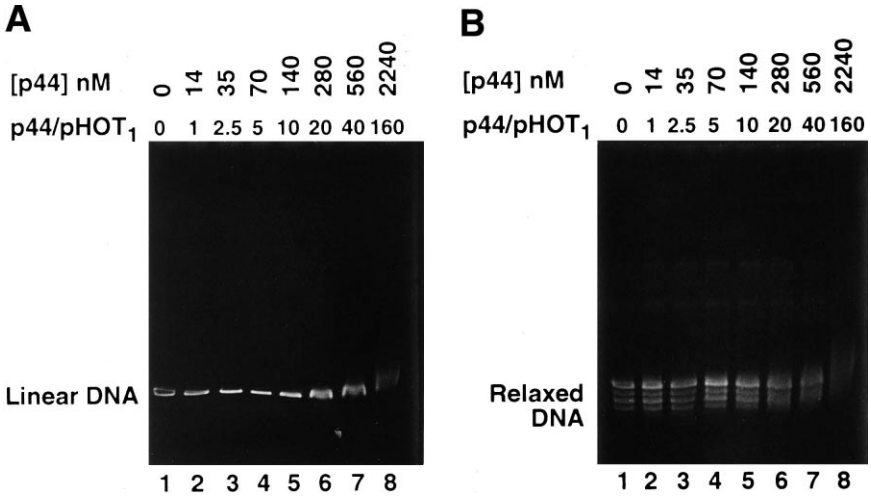

Fig. 7. Effect of MYT2 banding patterns of linear or partially relaxed DNA. A: Binding of MYT2 to linear pHOT1. Linear pHOT1 used in all lanes was 14 nM. Lane 1, without protein; lane 2, 14 nM protein (protein:DNA ratio: 1:1); lane 3, 35 nM protein (2.5:1); lane 4, 70 nM protein (5:1); lane 5, 140 nM protein (10:1); lane 6, 280 nM protein (20:1); lane 7, 560 nM (40:1); lane 8, 2240 nM protein (160:1). B: Binding of p44 to partially relaxed pHOT1. Partially relaxed pHOT1 used in all lanes was 14 nM. lane 1, without protein; lane 2, 14 nM protein (protein:DNA ratio: 1:1); lane 3, 35 nM protein (2.5:1); lane 4, 70 nM protein (5:1); lane 5, 140 nM protein (10:1); lane 6, 280 nM protein (20:1); lane 7, 560 nM (40:1); lane 8, 2240 nM protein (160:1).

topoisomers generated with human topoisomerase I (data not shown). Since the DNA linking number is evidently unaltered by the presence of MYT2, these results suggest that MYT2 does not possess topoisomerase I enzymatic activity under the assay conditions used here. Instead, MYT2 seems to behave more like tumor suppressor p53 in the sense that its effect on DNA conformation is reversed by proteolysis.

3.4. Topoisomer-like pattern formed only on scDNA

Since MYT2 must be present to maintain the topoisomer-like banding pattern, and since the number of bands depends on the protein:DNA ratio, it is possible that the pattern is generated by different numbers of MYT2 molecules binding to the DNA in a more or less quantized fashion. To test this possibility, MYT2 was added to partially relaxed sc and linear DNA forms generated using human topoisomerase I and *Eco*RI cleavage, respectively. In contrast to the case with scDNA, no topoisomer-like bands were generated with linear DNA at molar ratios from 1:1 to 160:1 (protein:DNA) (Fig. 7A, lanes 2–5), although band shifts were evident at the higher protein:DNA ratios (lanes 6–8). Similarly, MYT2 had only a slight effect on the banding pattern of partially relaxed DNA (Fig. 7B). Taken together, these results indicate that MYT2 preferentially binds to scDNA.

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MYT2   24 MKQEKQQFIKTDWTIDMINAVGNLRNMPLIFLTATKDIHRGGWVERIDNK
pFR18rep1 MEQEKAQLIKNHWSIDMVNVVGNLRRMPVAFI.....LNNGEWICRRENY
       74 AWQYVRVYENGDIEVLITLQIIRFHKYFDTPVWLQFNPNHLLPADYVQLD
       46 MWQYIRKFENGDTEVLITVQRLKFHKHFDDPVWVQYNPNHLENDDLKQLE
      124 HVMKYVDHSHLTRADLANDIYNINLQRYDFGLFGVTRDIYRSLSGDLETR
       96 RIMYSIDHTHLTRVDLACDIYNVDLSLYDFGLFNVTRDIYRTLSGKLETR
      174 YWGRRKSERQIRLYDKMREMKKHGKADDIPDGITDWWRLEFQFRGGKVES
      146 YWGRRKSERQIRLYDKANERKKHGKKDEIPDWAEEWWRLEFQFRQGKVDS
      224 WQEEVMDKMQSFHVLAVDDNDDLSEIDKAILARVNADKFDFKRVGKRYAA
      196 WQEEIIEKMSSFHVLAVDDNDNLTETEKAILERVNADKYNFKKVGKEKAA
      274 KIRKMVRENVGFDTTVAELSLKTFNEQKDELQRQLDSMLAKYNIGAQTEE
      246 KIRKMVRENVGFDTTVSDLAILDFNEQQEILQKQLNSLMTKYNIKPQTEE
      324 MTAYFEEELKQTGNLDFSVVESESALERNVIRNIAKS 360
      296 LTRYFEDAINSEQGITVE.VDTDDIVFKNAMRNVAKS 331
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Fig. 8. The comparison between MYT2 and pFR18 rep protein. The underlined fragment is the one corresponding to the topoisomerase I active site area of RepC. pFR18 rep protein contains 337 amino acids.

3.5. MYT2 induces DNA synthesis in resting cells

During the course of this study, we discovered that the sequence MYT2 is highly similar to a 337 residue replicase encoded by the plasmid pFR18 from *L. mesenteroides* [22]. The two proteins exhibit 63% amino acid sequence identity over their entire length with two long identical segments; the largest one is 20 residues in length and includes the topoisomerase I-like segment, IRLYDK (Fig. 8). The pFR18 replicase has been implicated in DNA synthesis in *L. mesenteroides* [22] and may provide clues about the function of MYT2 in mammalian cells. To test whether MYT2 plays a role in DNA replication, MYT2 mRNA was prepared and injected into quiescent NIH 3T3 cells grown in medium containing reduced serum to decrease the cell division rate. Tritiated thymidine was added to the medium 24 h after injection, and the cells were subsequently fixed and autoradiographed. Injection of 25 μg/ml MYT2 mRNA into NIH 3T3 cells led to an increased incorporation of [^{3}H]thymidine in 60% of cell nuclei (Fig. 9A). In contrast, no thymidine incorporation could be seen in cells after injection of 25 μg/ml mRNA of β-galactosidase (Fig. 9B). These results demonstrated that the injection of MYT2 mRNA induces quiescent NIH 3T3 cells to enter the S phase of the cell cycle and to synthesize DNA.

4. Discussion

MYT2 represents a novel human DNA-binding protein. The existence of a highly acidic C-terminal segment suggests that MYT2 might be involved in protein–protein interactions,

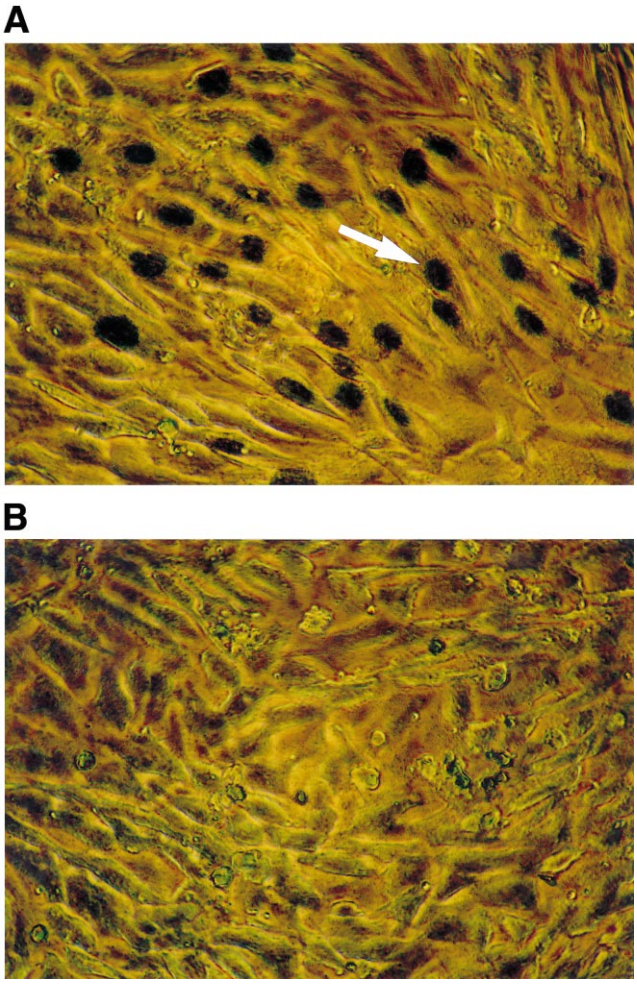

Fig. 9. MYT2 mRNA induces thymidine incorporation into nuclei (arrow pointed) of NIH 3T3 cells. NIH 3T3 cells injected with 25 μg/ml MYT2 mRNA (A), with 25 μg/ml β-galactosidase mRNA (B).

and possibly in chromosomal function [17,18]. However, we can not exclude the possibility that MYT2 has a specific binding target on chromosomal DNA yet to be found. Specific DNA-binding proteins often bind to non-specific DNA sequences with lower affinity as a mechanism to locate the specific sites as well as to regulate gene expression [23]. MYT2 is expressed in the cells of human central nervous system and reportedly binds to the proteolipid protein promoter [12].

We found that MYT2 binds preferentially to scDNA and generates a topoisomer-like pattern. However, that pattern is not the result of typical topoisomerase activity since it only occurs with stoichiometric amounts of protein and is abolished by proteolytic digestion of MYT2. One explanation for the banding pattern is that each band represents a fully scDNA molecule complexed with different numbers of MYT2 molecules. The separation of the bands would then be based on molecular weight differences of the different complexes. No effects on the banding pattern were seen with either partially relaxed circular or linear DNA, with MYT2:DNA ratios up to 10:1, indicating that these patterns were not simply generated by molecular weight shifts. However, gel shifts for MYT2 complexed under similar conditions with a small double-stranded oligonucleotide probe could readily be observed (data not shown). These results suggest that the lack of the observed gel shifts with the linear 1755 kDa DNA plasmid is due to an inability to detect small incremental molecular weight changes induced by binding with limited numbers of the MYT2 proteins. Another explanation for the ladder-like pattern is that there may be a limited number of MYT2 binding sites in the scDNA that are critical for maintaining a certain writhe in the scDNA structure. MYT2 is able to change the writhe of the DNA, without altering linking number, upon binding to these regions. This hypothesis is consistent with the saturable and quantized nature of the banding pattern of scDNA when MYT2 is bound. The preference of MYT2 for scDNA may be due to some special structural features, such as single strand or cruciform structures induced by DNA supercoiling [24,25]. Recent studies illustrate that tumor suppressor protein, p53, binds to scDNA and generates a protease sensitive ladder-like pattern similar to that obtained using MYT2 [11]. It was suggested that p53 binding to scDNA leads to local absorption of writhe and results in DNA relaxation, an interpretation supported by electron microscopy [11].

Other DNA-binding proteins have been reported to bind to scDNA and alter DNA topology. The most noticeable examples in eukaryotic cells probably are the non-histone chromatin-associated proteins (HMG). Like MYT2, HMG1 and 2 preferentially bind to scDNA [26]. However, unlike MYT2, detection of topological changes in the DNA required the addition of exogenous topoisomerase I [10].

The high sequence identity of MYT2 with the pFR18 replicase suggests that MYT2 might be involved in DNA replication. Indeed, microinjection experiments support this hypothesis. Injection of MYT2 mRNA into NIH 3T3 cells stimulated the G_0 cells to enter S phase and synthesize DNA in a dose-dependent manner. Microinjection is an established method to introduce macromolecules into living mammalian cells to study their mitogenic activity [27]. Several oncoproteins, proteins involved in signal transduction, and a number of cell cycle proteins have been shown to stimulate DNA synthesis and to induce quiescent cells to enter S phase using microinjection techniques. For example microinjection of *ras* gene product p21 showed that this oncoprotein was able to override the G_0 block of growth-arrested mouse fibroblast cells, inducing the cells to enter S phase, and inducing a temporary morphological transformation [16]. The microinjection of inositol phospholipid-specific phospholipase into NIH 3T3 cells produces similar effects as do c-myc [28], src [29], MAP kinase 1 [30], cyclin E [31], cyclin D1 [32], and G_1 cyclin-dependent kinase C [33]. Since DNA topology plays an important role in replication [1–3], it makes sense that MYT2 is able to alter DNA topology and stimulate DNA synthesis in NIH 3T3 cells. It is interesting that p53, which also binds to and relaxes scDNA, has an inhibitory effect on the cell cycle [34]. In summary, we suggest that MYT2 represents a novel DNA binding protein that can stimulate DNA synthesis in quiescent cells and may play a role in cell growth.

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