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Tetrahymena Nuclear Proteins that Bind to a Micronucleus-Specific Sequence during Vegetative Growth

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ABSTRACT—*Tetrahymena thermophila* has two nuclei: a micronucleus is transcriptionally silent during vegetative growth and a macronucleus is active. Extensive programmed DNA rearrangement is known to occur during the development of the somatic macronucleus from the germ-line micronucleus. We previously found a 1.4 kb micronucleus-specific sequence, C-element, which was located upstream of the micronuclear calmodulin gene and was eliminated from the macronuclear genome during macronuclear development. Here, using gel mobility shift assays, we show that C-element binding factors, CBFs, are present in the nuclear extract prepared from vegetative cells. Competition experiments demonstrate that CBFs bind to two regions within the C-element. A sequence motif common to these regions is 5'-ATAGATTT-3'.

INTRODUCTION

The ciliated protozoan *Tetrahymena thermophila* has two structurally and functionally distinct nuclei: a micronucleus and a macronucleus. The micronucleus is transcriptionally silent during vegetative growth, but is active only at early stages of conjugation (Sugai and Hiwatashi, 1974; Martindale *et al.*, 1985). The macronucleus is transcriptionally active and sustains vegetative growth (Gorovsky, 1973). Two nuclei replicate their DNA and divide at different times in the cell cycle (McDonald, 1962; Charret, 1969; Woodard *et al.*, 1972). In addition, the way of nuclear division is also different: the micronucleus divides by typical mitosis and the macronucleus divides by an amitotic process that does not involve spindle formation or chromosome condensation. The micronucleus carries out meiosis and serves as germ-line nucleus, but the macronucleus is degraded every sexual generation. A new macronucleus develops from a mitotic product of the zygotic nucleus, accompanying with its transcriptional activation and extensive changes in genome organization (Karrer, 1986; Yao, 1989). In the resulting macronucleus, nearly 15% of the micronuclear sequence complexity are lost (Yao and Gorovsky, 1974; Karrer, 1983). Such micronucleus-specific (mic-specific) sequence elements are regularly eliminated during the development of the macronucleus from the zygotic nucleus, suggesting that these elements confer the functional difference between micro- and macronuclei (Blackburn and Karrer, 1986; Karrer, 1986).

Some mic-specific sequence elements have been cloned and sequenced completely (Austerberry and Yao, 1987, 1988; Katoh *et al.*, 1993) or partially (Heinonen and Pearlman, 1994; Wells *et al.*, 1994). Very little sequence identity is shared among them so far. A 1.4 kb mic-specific sequence (named C-element) that we previously found (Katoh *et al.*, 1993) is located about 3.5 kb upstream of the micronuclear calmodulin gene and is eliminated during macronuclear development. It contains no prominent structures of transposable elements, such as long ORF and long terminal repeats or inverted repeats. The C-element is closely related to the DNA rearrangement, since it has a large family which is also eliminated during macronuclear development (Katoh *et al.*, 1993). It is considered that the C-element and its family may have roles for the maintenance of micronuclear characteristics or micronuclear genome organization including higher-order structure.

Although mic-specific sequence elements are being analyzed in detail (Godiska *et al.*, 1993; Saveliev and Cox, 1994, 1995; Yao and Yao, 1994), nuclear proteins that directly bind to these elements remain unknown. In this report, we describe the first evidence for the direct interactions between C-element and proteins in the nuclear extract prepared from vegetative cells. We also demonstrate that 5'-ATAGATTT-3' included in the C-element is responsible for the interactions.

MATERIALS AND METHODS

Cell culture

Cultivation of *Tetrahymena thermophila*, an inbred strain B1868, was performed as previously described (Watanabe *et al.*, 1994).

Nuclear extract

The mixture of micronuclei and macronuclei was isolated by the method of Niles and Jain (1981) with the following modifications. Cells were lysed at 0°C in 9 volumes of sucrose buffer [0.25 M sucrose, 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 3 mM $CaCl_2$, 25 mM KCl] containing 0.18% (v/v) Nonidet P-40. To this lysate, 0.815 g sucrose

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was added per milliliter of lysate, and the lysate was centrifuged at 27,000 ×g for 30 min at 2°C. The nucleus pellet was stored at -80°C. The nuclear sample was suspended in an equal volume of extraction buffer [40 mM Tris-HCl (pH 7.9), 3 mM $MgCl_2$, 0.4 mM EDTA, 0.8 M NaCl, 50% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 0.02 mM TLCK, 5 μg/ml Leupeptin] for 30 min at 0°C and centrifuged at 300,000 ×g for 30 min. The resulting supernatant was dialyzed against dialysis buffer [20 mM Tris-HCl (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, 0.2 mM PMSF, 0.01 mM TLCK, 5 μg/ml Leupeptin], and was stored at -80°C.

For partial purification of C-element binding factors (CBFs), the nuclear extract was diluted to a final concentration of 40 mM NaCl and was applied to a Mono Q column (Pharmacia, 5 × 50 mm) pre-equilibrated with buffer Q [20 mM Tris-HCl (pH 7.9), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 40 mM NaCl, 10% glycerol, 0.5 mM DTT, 0.5 mM PMSF], and eluted with a linear gradient of 0-1 M NaCl in buffer Q. Each fraction was dialyzed to the dialysis buffer. The active fraction for C-element binding was determined by gel mobility shift assays.

Fragments, oligonucleotides, and gel shift probes

The C-element was divided into seven subfragments by the treatment of restriction enzymes: *Dde* I, *Xba* I, *Sau*3A I, *Hinf* I, and *Pac* I. The most 5' end of short terminal repeat (ATTA) which is located at the distal junction from the calmodulin gene is tentatively defined as +1 in the present study. The digested-fragments 1 (+19 to +232), 2 (+230 to +340), 3 (+337 to +566), 4 (+563 to +754), 5 (+752 to +989), 6 (+987 to +1174), and 7 (+1173 to +1377) were labeled by filling-in with dNTPs containing [α-^{32}P]dATP using Klenow enzyme. All oligonucleotides used in gel mobility shift assays were synthesized, annealed, and labeled as described above.

Gel mobility shift assays

The standard gel mobility shift assay (Singh *et al.*, 1986) was performed in a volume of 21-25 μl consisting ^{32}P-labeled DNA (1 × 10^4 cpm), poly(dI-dC)-poly(dI-dC) (4 μg), nuclear extract (2-6 μl); and 25 mM Tris-HCl (pH 7.9), 0.5 mM EDTA, 0.4 mM DTT, 0.4 mM PMSF, 35 mM KCl, 12% glycerol. All operations were done on ice. The mixture was preincubated for 5-10 min prior to the addition of labeled probe. The complete mixture was incubated for 15-30 min and then loaded onto a 4% polyacrylamide gel (acrylamide : bisacrylamide ratio of 39 : 1) in TGE buffer (50 mM Tris-HCl, 38 mM glycine, 2 mM EDTA, pH 8.4) at a cold room. The gel was electrophoresed, dried, and autoradiographed. In competition experiments, each unlabeled fragment or double-stranded oligonucleotide was added to the nuclear extract before the binding reactions.

RESULTS

Presence of CBFs in vegetative cells

To investigate the interactions between C-element and nuclear proteins, gel mobility shift assays were performed. A crude nuclear extract was prepared from vegetative cells, and was incubated with each labeled subfragment of C-element, which was illustrated in Fig. 1A. In this assay, specific complex formation failed to be detected with all subfragments except for fragment 2 (data not shown). However, when fragment 2 was employed as a probe, three complexes were detected (Fig. 1B, lane 2). The lowest mobility complex probably resulted from non-specific binding, since it was abolished by the addition of excess of unrelated subfragments (Fig. 1B, lanes 4 and 5). The formation of the other two complexes (c-I and c-II) on fragment 2 was competed with an excess of unlabeled fragment 2 (Fig. 1B, lane 3), but not with unrelated fragment 3 or 5 (Fig. 1B, lanes 4 and 5). These complexes were sensitive to 0.1% SDS or 100 μg/ml proteinase K treatment (data not shown). These results indicate that certain nuclear proteins in vegetative cells are capable of binding specifically to the C-element.

Partial purification of CBFs

To characterize CBFs binding in detail, the crude nuclear extract from vegetative cells was applied to a Mono Q anion-exchange column, and eluted fractions were assayed for the specific binding to fragment 2 in gel mobility shift assay (Fig. 2). Two major complexes (c-I and c-II) were detected in fractions No. 8-No. 13 (about 300-500 mM NaCl elution). The

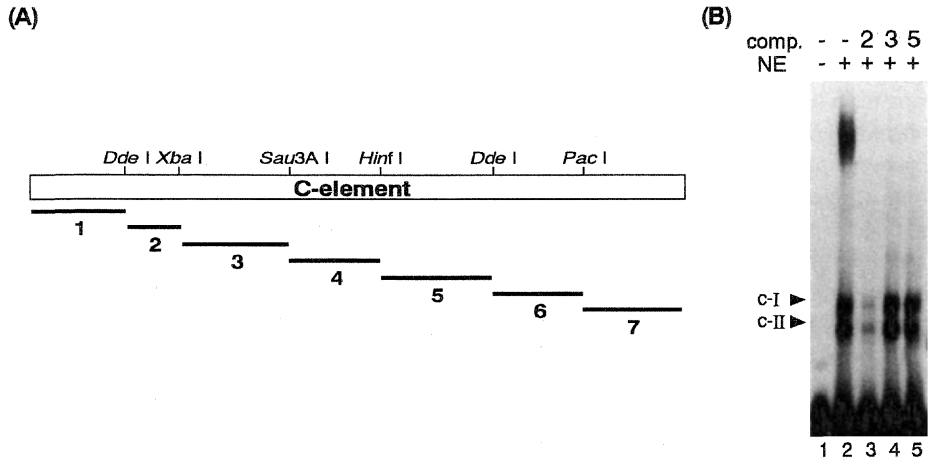

Fig. 1. Detection of CBFs by gel mobility shift assay. (A) Diagrammatic representation of the C-element (open box) and its subfragments (solid bars) used as DNA probes in the gel shift assay. (B) Fragment 2 shown in (A) was assayed with the crude nuclear extract (2 μg) prepared from vegetative cells in the gel shift assay. The minus signs above the lanes indicate the absence of the nuclear extract (NE) or unlabeled competitor (comp.). The presence of the nuclear extract (NE) and that of the competitor (comp.) are indicated by the plus sign and the number of fragment used as a competitor, respectively. Arrowheads indicate sequence-specific complexes (c-I and c-II).

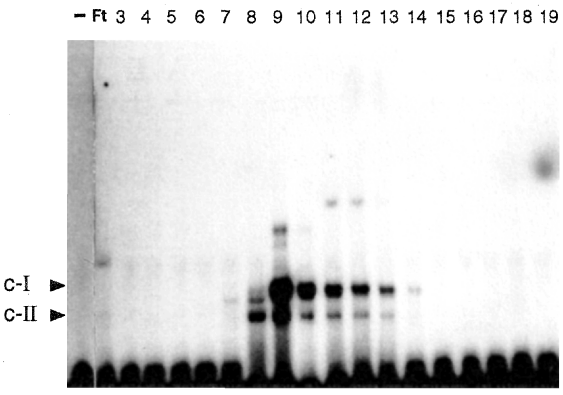

Fig. 2. DNA-binding activity in fractions from Mono Q column. The nuclear extract was fractionated by Mono Q column chromatography. The indicated fractions were assayed for the ability to form protein-DNA complexes on fragment 2. Lane -, ^{32}P-labeled fragment 2 without proteins; Ft, flow-through fraction.

active fraction (No. 9) was then subjected to the following assays.

Sequence specificity of CBFs binding

The sequence specificity of CBFs binding was tested. With increasing amounts of Mono Q active fraction, there was an increase of bound ^{32}P-labeled fragment 2 (Fig. 3, lanes 1-4). Competition experiments were then carried out using unlabeled fragment 2 as a specific competitor or pBR322 fragment as a non-specific competitor. The formation of complexes c-I and c-II competed with increasing amounts of

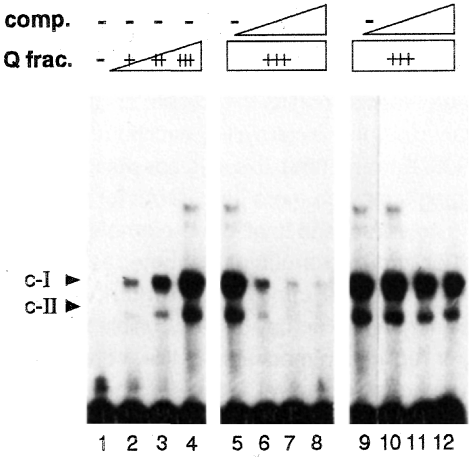

Fig. 3. Specificity of CBFs binding to fragment 2. Gel mobility shift assay was performed using fragment 2 as a probe. From left to right the lanes are as follows: lanes 1-4, pattern of binding to fragment 2 in response to increasing amounts (0, 2, 4, 6 μl) of Mono Q active fraction (Q frac.); lanes 5-8, effect of increasing amounts (0, 50, 100, 200-fold molar excess) of unlabeled fragment 2 as a specific competitor; lanes 9-12, effect of increasing amounts (0, 50, 100, 200-fold molar excess) of the 154-bp *Hinf* I fragment isolated from pBR 322 as a non-specific competitor. The amount of the Mono Q active fraction used in lanes 5-12 is the same as in lane 4.

unlabeled competitor fragment 2 (Fig. 3, lanes 5-8), and was completely inhibited by a 100-fold molar excess (Fig. 3, lane 7). The pBR322 fragment had virtually no effect on the formation of these complexes (Fig. 3, lanes 9-12). Furthermore, these complexes could not be abolished by 0.5-4 μg of unlabeled poly(dA-dT)-poly(dA-dT), although the C-element has high A+T content (data not shown). These results strongly suggest that CBFs never bind non-specifically to only an A+T rich sequence, but effectively distinguish a certain sequence motif on fragment 2 of the C-element.

CBFs bind to two regions within fragment 2

In order to determine the regions involved in these protein-DNA complexes, oligonucleotides corresponding to four regions (A-D) that spanned fragment 2 were synthesized, annealed, and used as competitors (Fig. 4A). Oligonucleotides A and D, as well as unlabeled fragment 2, competed for the formation of both complexes c-I and c-II (Fig. 4B, lanes 3, 4, and 7). Neither oligonucleotide B nor C was an effective competitor (Fig. 4B, lanes 5 and 6). In addition, gel mobility shift assays using oligonucleotides A and D as probes showed that CBFs bound directly to both oligonucleotides, and reciprocal competition experiments demonstrated that one major complex on each oligonucleotide was abolished by both oligonucleotides (data not shown). These results indicate that the sequence motif recognized by CBFs is located at two regions (A and D) within fragment 2.

The sequence motif responsible for the formation of the protein-DNA complexes

Competition experiment using altered versions of oligonucleotide A (Fig. 4A, oligo E and F) demonstrated that both oligonucleotides E and F were effective competitors for the formation of both complexes c-I and c-II (Fig. 4C, lanes 4 and 5). This result indicates that oligonucleotide F, the shortest sequence, contains sufficient sequence to form both complexes. Together with the data shown in Fig. 4B, the sequence conserved among the four regions (A, D, E, and F) is presumably the recognition site of CBFs. The sequence shared among them is 5'-ATAGATTT-3' (Fig. 4D).

DISCUSSION

In this study, using gel mobility shift assays, we demonstrated for the first time that nuclear proteins which directly bound to a mic-specific sequence (C-element) exist in the nuclear extract from vegetative cells. These proteins (CBFs) were shown to bind to two regions within the C-element, because each oligonucleotide corresponding to these regions could compete for all binding by CBFs. The simplest interpretation of these results is that the same protein binds to each region. It appears reasonable to assume that the motif shared between these regions, 5'-ATAGATTT-3', is minimum unit recognized by CBFs, although other sequence structures besides this octanucleotides must help to determine the specificity of CBFs binding *in vivo*. The result that CBFs appear

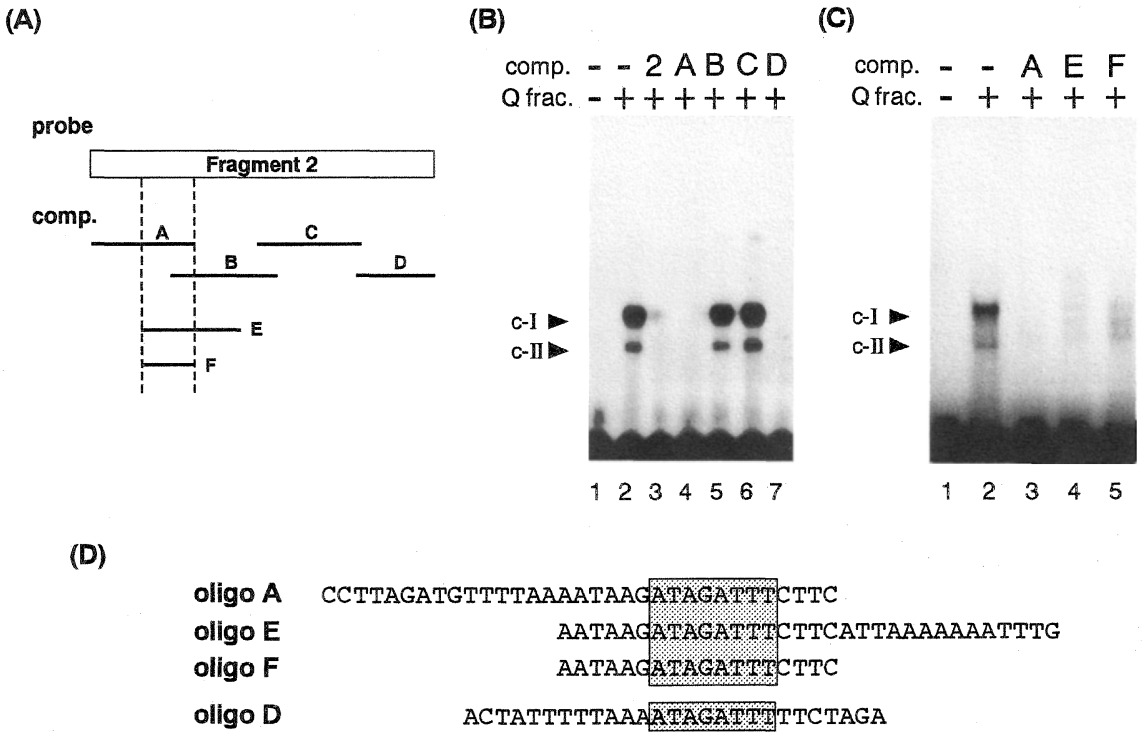

Fig. 4. Competition experiment to determine the DNA motif responsible for the protein-DNA complex formation. (A) Schematic showing of fragment 2 and the relative positions of oligonucleotides used as competitors. (B) Gel mobility shift assay using oligonucleotides A-D of spanning fragment 2 as competitors. ^{32}P-labeled fragment 2 was incubated in the absence (–) or presence (+) of the active fraction (Q frac.) from Mono Q column. At the top of each lane the presence (the source) of the competitor used at 500-fold molar excess is indicated. (C) Competition experiment using oligonucleotides A, E, and F as competitors. (D) Comparison of synthetic oligonucleotides acted as effective competitors. The nucleotide sequences of oligonucleotides acted as effective competitors in Fig. 4B and C are indicated. Shaded boxes correspond to the common sequence among all oligonucleotides.

to have higher affinity to oligonucleotide A than oligonucleotide F (Fig. 4C) may also suggest the necessity of other structures. The important question whether CBFs are derived from the micronucleus or macronucleus remains, owing to using of the extract prepared from the mixture of the micro- and macronucleus. Our preliminary data shows that C-element binding activity is not virtually present in macronucleus rich extract. CBFs may be derived from micronucleus, although we cannot affirm this since we failed to obtain the enough amount of a micronuclear extract to use for gel mobility shift assay so far.

It has been discussed about the biological significance of DNA rearrangement in ciliates, especially the elimination of mic-specific sequence elements, for a long time (Gorovsky, 1980; Blackburn and Karrer, 1986; Brunk, 1986; Karrer, 1986; Prescott, 1994; Madireddi *et al.*, 1995; Yao, 1996). One possibility is that the elimination of these elements may lead to large-scale transcriptional activation in the macronuclear genome (Blackburn and Karrer, 1986; Brunk, 1986; Karrer, 1986; Prescott, 1994; Yao, 1996). These elimination would not always create the coding regions or the promoter regions of every micronuclear genes, since DNA elimination does not occur within such region of several genes (Bannon *et al.*, 1984; Allen *et al.*, 1984; Pederson *et al.*, 1984; Katoh *et al.*, 1993). Even if these elements do not exist in flank of every micronuclear genes, it may be possible that these elements could provide for a global organization of micronuclear chromatin which maintains genetic repression (Prescott, 1994; Yao, 1996). Another is that these elements may act as the micronuclear specific origins to replicate at different times from the macronucleus in the cell cycle (Blackburn and Karrer, 1986; Brunk, 1986; Karrer, 1986). It is also possible, in extreme case, most elements may be mere junk DNAs for the cell.

CBFs may also bind to all the C-element family which are scattered on the micronuclear genome; accordingly, these interactions may be capable of regulating globally the micronuclear genome or may be responsible for the postulated silencing or replication mechanism. We, here, reported the *in vitro* interactions between C-element and nuclear proteins (CBFs) during vegetative growth, but further work is needed to confirm their *in vivo* interactions. If CBFs specifically bind to C-element or C-element family in the micronucleus *in vivo*, CBFs appear to be important as the molecular tools for analysis of the biological function of C-element family.

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