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The leukemic core binding factor beta-smooth muscle myosin heavy chain (CBF beta-SMMHC) chimeric protein requires both CBF beta and myosin heavy chain domains for transformation of NIH 3T3 cells.

Amitav Hajra
University of Michigan-Ann Arbor

Pu Liu
National Institutes of Health

Qing Wang
Dartmouth College

Christine Kelley
National Institutes of Health

Medical Sciences. In the article "The leukemic core binding factor β-smooth muscle myosin heavy chain (CBFβ–SMMHC) chimeric protein requires both CBFβ and myosin heavy chain domains for transformation of NIH 3T3 cells" by Amitav Hajra, Pu P. Liu, Qing Wang, Christine A. Kelley, Terryl Stacy, Robert S. Adelstein, Nancy A. Speck, and Francis S. Collins, which appeared in number 6, March 14, 1995, of *Proc. Natl. Acad. Sci. USA* (**92,** 1926–1930), the authors request that the following be noted. "Recent information has come to light that experiments reported in this paper can in several instances not be verified as correct. The critical experiments are being repeated, but in the interim we regretfully wish to retract the paper."

Robert S. Adelstein
Francis S. Collins
Amitav Hajra
Christine A. Kelley
Pu P. Liu
Nancy A. Speck
Terryl Stacy
Qing Wang

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The leukemic core binding factor β–smooth muscle myosin heavy chain (CBFβ–SMMHC) chimeric protein requires both CBFβ and myosin heavy chain domains for transformation of NIH 3T3 cells

AMITAV HAJRA*†, PU P. LIU*, QING WANG‡, CHRISTINE A. KELLEY§, TERRYL STACY‡, ROBERT S. ADELSTEIN§, NANCY A. SPECK‡, AND FRANCIS S. COLLINS*¶

*Laboratory of Gene Transfer, National Center for Human Genome Research, Building 38A, Room 605, National Institutes of Health, Bethesda, MD 20892-6050; †Department of Human Genetics, University of Michigan Medical School, Ann Arbor, MI 48109; ‡Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755; and §Laboratory of Molecular Cardiology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **An inversion of chromosome 16 associated with the M4Eo subtype of acute myeloid leukemia produces a chimeric protein fusing the β subunit of the transcription factor core binding factor (CBFβ) to the tail region of smooth muscle myosin heavy chain (SMMHC). We investigated the oncogenic properties of this CBFβ–SMMHC chimeric protein using a 3T3 transformation assay. NIH 3T3 cells expressing CBFβ–SMMHC acquired a transformed phenotype, as indicated by their ability to form foci, grow in soft agarose, and form tumors in nude mice. Cells expressing normal CBFβ or the SMMHC tail domain did not become transformed. Electrophoretic mobility-shift assays showed that extracts from cells transformed by CBFβ–SMMHC no longer formed the normal CBF/DNA complex but instead formed a much larger complex that did not migrate into the gel. Analysis of CBFβ–SMMHC deletion mutants demonstrated that the chimeric protein was transforming only if two domains were both present: (*i*) CBFβ sequences necessary for association with the CBFα subunit, and (*ii*) SMMHC sequences important for the formation of multimeric filaments. These results are direct evidence that CBFβ–SMMHC can function as an oncoprotein.**

A characteristic pericentric inversion of chromosome 16 [inv(16)(p13q22)] is present in virtually all cases of the M4Eo subtype of acute myeloid leukemia (1, 2). We have recently shown (3) that this inversion produces a chimeric protein in which the tail region of smooth muscle myosin heavy chain (SMMHC) is fused in-frame to the end of the β subunit of core binding factor (CBF).

CBF (also known as PEBP2) was first identified as a murine transcription factor that binds to viral enhancers at the sequence RACCRCA (where R represents a purine) (4, 5). CBF has subsequently been shown to regulate the transcription of T-cell- (6) and myeloid-specific genes (7) and cooperate with the transcription factors Ets-1 and c-Myb in DNA binding and transcriptional activation (8, 9). CBF consists of two subunits: a DNA-binding α subunit and a β subunit that does not bind DNA directly but stabilizes DNA binding by CBFα. Three different CBFα subunits have been identified (10–12), and one of them (*AML1*) is disrupted by leukemia-associated t(8;21) and t(3;21) translocations (13–16, 32). CBFβ, in contrast, is encoded by a single, ubiquitously expressed gene (17, 18). The inv(16) rearrangement fuses all but the last 22 amino acids of CBFβ to SMMHC (3).

SMMHC (19) is a member of the myosin II class of proteins. It forms two of the subunits of hexameric smooth muscle myosin and consists of an amino-terminal globular domain that binds actin and has ATPase activity as well as a tail region that dimerizes to form a long rod of α-helical coiled–coil structure. The tail region becomes fused in-frame to CBFβ as a result of inv(16) (3).

We are interested in studying how CBFβ–SMMHC functions in oncogenesis. Transformation assays using 3T3 cells have been previously used to investigate the properties of leukemia-associated oncogenes (20, 21). In addition, 3T3 cells express functional CBF, and CBF/DNA complexes are altered in 3T3 cells transformed by Ha-*ras* (10, 11, 18, 22). Therefore, we reasoned that a 3T3 transformation assay would be useful to analyze the oncogenic properties of CBFβ–SMMHC.

MATERIALS AND METHODS

Generation of Antibodies. An affinity-purified polyclonal antibody (designated GPPP) specific for the 200-kDa isoform of SMMHC was generated against a synthetic peptide corresponding to the carboxyl-terminal nine amino acids (GPPPQETSQ) of human umbilical artery $SMMHC_{200}$ (19) by using described methods (23). Monoclonal antibodies β13 and β14 were raised against bacterially expressed CBFβ by using NS-1 myeloma cells according to standard procedures (24). The antibody isotypes were IgG1 (β13) and IgG2a (β14), and immunoblot analysis of CBFβ deletion proteins indicated that the antibodies recognized epitopes between amino acids 26 and 55 (β13) and 141 and 165 (β14) (data not shown).

Generation and Characterization of Stably Transfected Cell Lines. Full-length cDNAs encoding human CBFβ and CBFβ–SMMHC (25) were cloned into the mammalian expression vector pRc/CMV (Invitrogen). 3T3 cells were transfected with these plasmids by calcium phosphate coprecipitation and grown as described (26). Colonies resistant to G418 sulfate (Geneticin, GIBCO/BRL) were isolated and expanded to generate stably transfected cell lines.

Expression of recombinant proteins was determined by immunoblotting with standard procedures (24). For detection of CBFβ, cell extracts were first immunoprecipitated with antibody β13 and protein G (Pharmacia) before gel electrophoresis. Blotted proteins were detected with either GPPP (diluted 1:10,000) or β14 (diluted 1:200) antibodies using the Lumi-GLO chemiluminescent immunoblot kit (Kirkegaard & Perry Laboratories). Nuclear extractions and electrophoretic mobility-shift assays (EMSAs) were done as described (25, 33). The doubling times and saturation densities of the cells were measured as described (20). Assessment of foci formation, growth in 0.6% soft agarose, and *in vivo* tumor formation in nude mice were done as described (20, 21).

Construction and Characterization of Deletion Mutants. Truncated derivatives of murine CBFβ prepared by PCR

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Abbreviations: CBF, core binding factor; EMSA, electrophoretic mobility-shift assay; GST, glutathione-*S*-transferase; SMMHC, smooth muscle myosin heavy chain.

¶To whom reprint requests should be addressed.

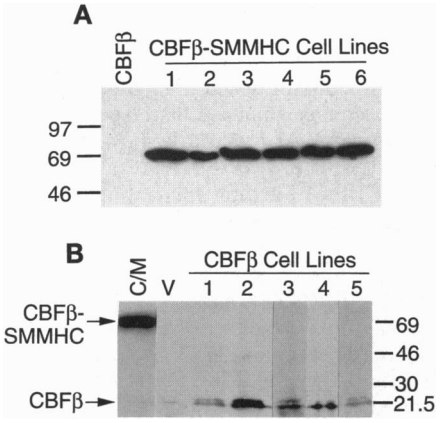

FIG. 1. Expression of CBFβ and CBFβ–SMMHC proteins in clonal, stably transfected cell lines. The predicted sizes of the CBFβ and CBFβ–SMMHC proteins are 21.5 and 67 kDa, respectively. (*A*) Immunoblot of cell extracts probed with antibody GPPP. The first lane contains an extract from cells expressing CBFβ, and six representative CBFβ–SMMHC cell lines are shown. The positions of molecular mass standards (in kDa) are shown at left. (*B*) Immunoblot of cell extracts immunoprecipitated and probed with anti-CBFβ monoclonal antibodies. Extracts from five different CBFβ cell lines are shown, as well as extracts from cell lines expressing CBFβ–SMMHC (C/M) or vector sequences alone (V). Arrows at left indicate positions of the CBFβ and CBFβ–SMMHC proteins, and the positions of molecular mass standards (in kDa) are indicated at right.

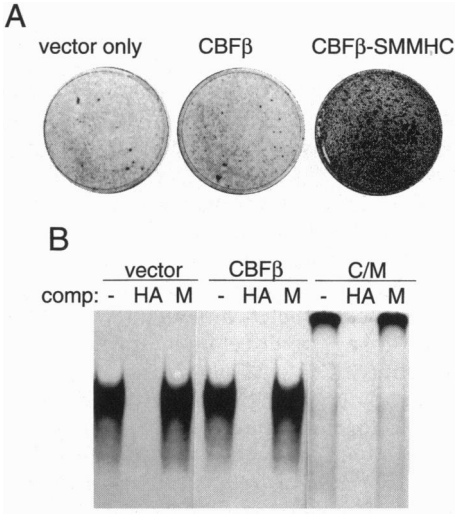

FIG. 2. (*A*) Foci formation by representative cells stably expressing insertless vector, normal CBFβ, or CBFβ–SMMHC. (*B*) EMSA of nuclear extracts from representative NIH 3T3 cells stably expressing vector alone, CBFβ, or CBFβ–SMMHC (C/M) incubated with a probe containing a high-affinity CBF-binding site. comp., Competitor probe added to binding reaction. Binding reactions were done either without additional unlabeled competitor (−) or in the presence of a 200-molar excess of unlabeled probes containing high-affinity (HA) or mutated (M) CBF-binding sites.

amplification were cloned into the glutathione-*S*-transferase (GST) fusion vector pGEX2T (Pharmacia) and expressed in bacteria and purified as described (17). EMSAs were done by mixing the CBFβ proteins with purified bovine CBFα and incubating with a probe containing a high-affinity CBF-binding site as described (17). One and one-half micrograms of CBFβ protein was used for the binding reactions, so that it was present in great excess over CBFα.

CBFβ–SMMHC deletion mutants were constructed by PCR amplification of the appropriate cDNA regions using *Pfu* polymerase (Stratagene) and primers slightly altered to introduce the appropriate truncation/fusion sequence. After the PCR product was cloned into the full-length cDNA, regions amplified by PCR were sequenced to confirm the presence of the correct sequence. Cells expressing the mutants were characterized as described above, except that *in vivo* tumorigenicity assays in nude mice were not done.

Determination of Myosin Solubility Curves. Protein solubility curves were determined by using a variation of a described procedure (27). Briefly, stably transfected cells were lysed in various concentrations of KCl, and lysates were treated with DNase (Sigma) and centrifuged at 500 × *g* for 10 min at 4°C. The amount of recombinant protein present in the supernatant and pellet at each KCl concentration was determined by ELISA with the appropriate antibody. Horseradish peroxidase-labeled goat anti-rabbit IgG (for antibody GPPP) or anti-mouse IgG (for β13/β14) antibodies (Kirkegaard & Perry Laboratories) were used as secondary antibodies, and immunoreactive products were detected as described (24).

RESULTS

Analysis of Transfected Cells. Stably transfected, clonal cell lines expressed the appropriate recombinant proteins at high levels. As shown in Fig. 1*A*, the GPPP antibody detected CBFβ–SMMHC with high specificity in extracts from stably transfected cells. The anti-CBFβ monoclonal antibodies could also detect CBFβ–SMMHC as well as recombinant human CBFβ in transfected cells and also the endogenous murine CBFβ (Fig. 1*B*). A CBFβ protein doublet was often seen in both transfected and untransfected cells.

CBFβ–SMMHC-expressing cells had a more rounded, refractile morphology than CBFβ-expressing cells, which had the same morphology as untransfected cells (data not shown). CBFβ–SMMHC cell lines grew more slowly than CBFβ cell lines but had a higher saturation density (Table 1). Phenotypic characterization of the cells demonstrated that CBFβ–SMMHC cell lines acquired a transformed phenotype, as indicated by their ability to form foci, grow in 0.6% soft agarose, and form tumors when injected into nude mice (Table 1 and Fig. 2*A*). Cells expressing normal CBFβ or insertless vector retained a normal phenotype (Table 1 and Fig. 2*A*). As shown in Fig. 2*B*, extracts from cells expressing CBFβ formed the CBF/DNA complex normally seen in 3T3 cells (5), but extracts from cells expressing CBFβ–SMMHC formed a much larger complex that barely migrated into the gel.

Table 1. Characteristics of clonal, stably transfected NIH 3T3 cell lines

Cell line expression	Doubling time, hr	Saturation density, cells × 10^{-6}	Foci, no. per 1000 cells	Soft agar colonies, no. per 1000 cells	Tumors in nude mice, no. per no. of injection sites
Vector alone	24.0 ± 0.4	3.2 ± 0.2	8.4 ± 1.1	0.0 ± 0.0	0/2
CBFβ	24.0 ± 0.6	3.3 ± 0.3	9.4 ± 1.0	0.0 ± 0.0	0/10
CBFβ–SMMHC	40.0 ± 0.8	6.3 ± 0.2	620.1 ± 27.3	61.3 ± 7.7	12/12

Representative cell lines expressing vector only (cell line RC-1), CBFβ (cell line C182-2), and CBFβ–SMMHC (C/M-5) are shown. Values for doubling times, saturation densities, and numbers of foci and soft agar colonies are means ± SEMs from five separate experiments. The number of tumors in nude mice are total numbers from all experiments (two injection sites per mouse).

Characteristics of Cell Lines Expressing CBFβ–SMMHC Deletion Mutants. Fig. 3 schematically shows the deletion mutants constructed to identify regions of CBFβ–SMMHC important for 3T3 transformation. First, we made constructs with only the CBFβ or SMMHC portions of CBFβ–SMMHC (C165, MHCR). We also constructed mutants with deletions in CBFβ sequences (CΔC24/M, CΔC32/M, CΔC78/M, CΔex3/M, CΔN11/M). Another group of mutants (C/MΔC68, C/MΔC158) had deletions of the SMMHC carboxyl terminus, which has been shown to be necessary for formation of multimeric myosin filaments (28, 29). In addition, a mutant (C/MΔI182) was constructed with an internal SMMHC deletion predicted to have no effect on multimerization (28). The first two columns of Fig. 3 summarize the ability of these mutants to associate with CBFα in DNA-binding assays and self-associate to form multimers (see below).

We established at least four stably transfected, clonal cell lines for each mutant construct. Proteins of the appropriate size, as predicted from the amino acid sequence, were detected in all the cell lines by immunoblotting (data not shown). The phenotypes of these cell lines are summarized in the last column of Fig. 3. Cells expressing CΔC24/M and C/MΔI182 had transformed phenotypes and formed approximately the same numbers of foci (≈600) and soft agarose colonies (≈65) as cells expressing CBFβ–SMMHC. Cells expressing the CΔC32/M construct appeared to have an attenuated transformed phenotype; while the cells formed the same number of foci and soft agarose colonies as other transformed cells, these foci and colonies were distinctly smaller (data not shown). Cells expressing all other deletion mutants retained a nontransformed phenotype and formed very few foci and no soft agarose colonies.

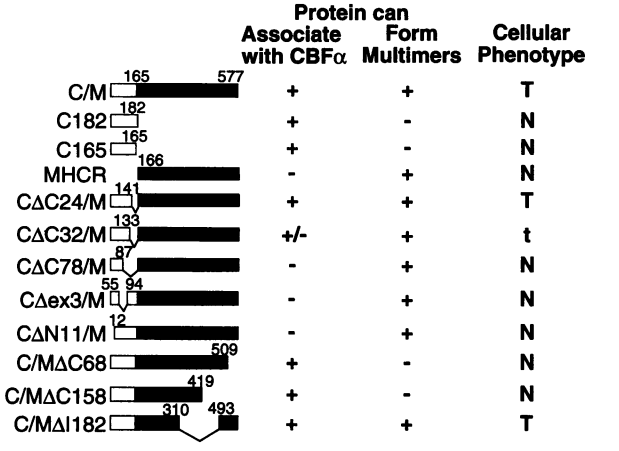

FIG. 3. Schematic drawing of mutants with deletions of various regions of CBFβ–SMMHC. Full-length CBFβ–SMMHC (C/M) and CBFβ (C182) proteins are also shown for comparison. The white boxes represent CBFβ sequences, whereas black boxes represent SMMHC sequences. Numbers above boxes are the amino acid locations of the indicated boundaries, and lines under the boxes show which regions become fused together in the deletion mutants. The CΔex3/M and CΔC32/M constructs represent alternatively spliced isoforms of *CBFB* lacking exons 3 and 5, respectively (17, 18). Columns to the right of the boxes summarize whether the deletion mutants can (+) or cannot (−) associate with CBFα (Figs. 4*A* and *B*) and form multimers (Fig. 4*C*). The +/− in the first column indicates that CΔC32/M associates weakly with CBFα. The last column summarizes whether cells expressing the constructs have a transformed (T, t) or nontransformed (N) phenotype. Transformed cells formed approximately the same number of foci and soft agarose colonies as CBFβ–SMMHC-expressing cells, whereas nontransformed cells formed very few foci and no soft agarose colonies, like CBFβ-expressing cells. t, CΔC32/M cells formed smaller foci and soft agarose colonies than other transformed cells (data not shown).

Ability of Deletion Mutants to Associate with CBFα. The ability of bacterially expressed, truncated CBFβ proteins to associate with purified CBFα is shown in Fig. 4*A*. Although CBFα could form a complex with DNA by itself (α arrow), it formed a larger complex when associated with the CBFβ/GST proteins (α + β arrow). As previously shown (18), the first 141 amino acids of CBFβ (1–141/GST) retained the ability to associate with CBFα. Further truncation of the carboxyl terminus to amino acid 133 (1–133/GST) greatly weakened association with CBFα, as indicated by the smearing between the α + β and α complexes, whereas deletion of the first 10 amino acids (11–141/GST) abolished all association with CBFα. The GST domain had a rather dramatic effect on the mobility of the α + β complex (Fig. 4*A*, second and last lanes). The CBFβ mutant corresponding to the CΔex3/M construct has been previously shown not to associate with CBFα (17).

These CBFα association properties were maintained in CBFβ–SMMHC deletion mutants. As shown in Fig. 4*B*, extracts from cells expressing mutants unable to associate with CBFα formed only the normal CBF/DNA EMSA complex (lanes 2–4). Extracts from cells expressing mutants able to associate with CBFα but unable to multimerize formed larger complexes (lanes 7, 8), due to the large size of the mutant proteins relative to the normal CBF subunits. Extracts from cells expressing mutants able to both associate with CBFα and form multimers formed the large complex in the gel wells seen with full-length CBFβ–SMMHC (lanes 6, 9). Extracts from cells expressing CΔC32/M formed a faint EMSA complex in the gel wells in addition to the normal complex (lane 5).

Multimer Formation by Proteins Expressed in NIH 3T3 Cells. We assessed whether the proteins expressed in NIH 3T3 cells were forming multimers by determining their solubilities over a range of salt concentrations, as has previously been done for various myosin heavy chains (27–29). As shown in Fig. 4*C*, CBFβ was completely soluble in 3T3 cells at all salt concentrations. CBFβ–SMMHC, however, was relatively insoluble, indicating that it is present in the cells predominantly as multimers. The β13 and β14 antibodies crossreacted with endogenous murine CBFβ, causing the solubility curve to be ≈15% higher than the curve obtained when antibody GPPP was used for protein detection (data not shown). The SMMHC tail region alone had the same solubility properties as CBFβ–SMMHC (data not shown). Mutants with truncated carboxyl termini were completely soluble (Fig. 4*C*, C/MΔC68), whereas mutants with deletions of other regions had the same solubility as full-length CBFβ–SMMHC (Fig. 4*C*, C/MΔI182).

DISCUSSION

This study demonstrates that the CBFβ–SMMHC chimeric protein associated with M4Eo acute myeloid leukemia is a potent transforming agent in 3T3 cells. This transforming ability depends on regions of the protein necessary for association with CBFα and formation of myosin filaments. These results provide functional evidence for the central role of this protein in inv(16) leukemia, which previously has only been inferred from the presence of the fusion transcripts in patients (3, 30, 31).

While immunoblots showed that human CBFβ was expressed in stably transfected cells, there was no increase in the intensity of CBF/DNA complexes formed by extracts from these cells. This result may indicate that the DNA-binding activity of CBF is limited by the amount of CBFα in the cell. Cells expressing CBFβ–SMMHC, however, showed a dramatically altered CBF/DNA complex. This complex can be formed by CBFα and CBFβ–SMMHC proteins alone (25) and is much different than the complex formed by extracts from NIH 3T3 cells transformed by Ha-*ras* (22). It therefore appears to be a specific characteristic of CBFβ–SMMHC expression. The large size of the complex is most likely due to multimer

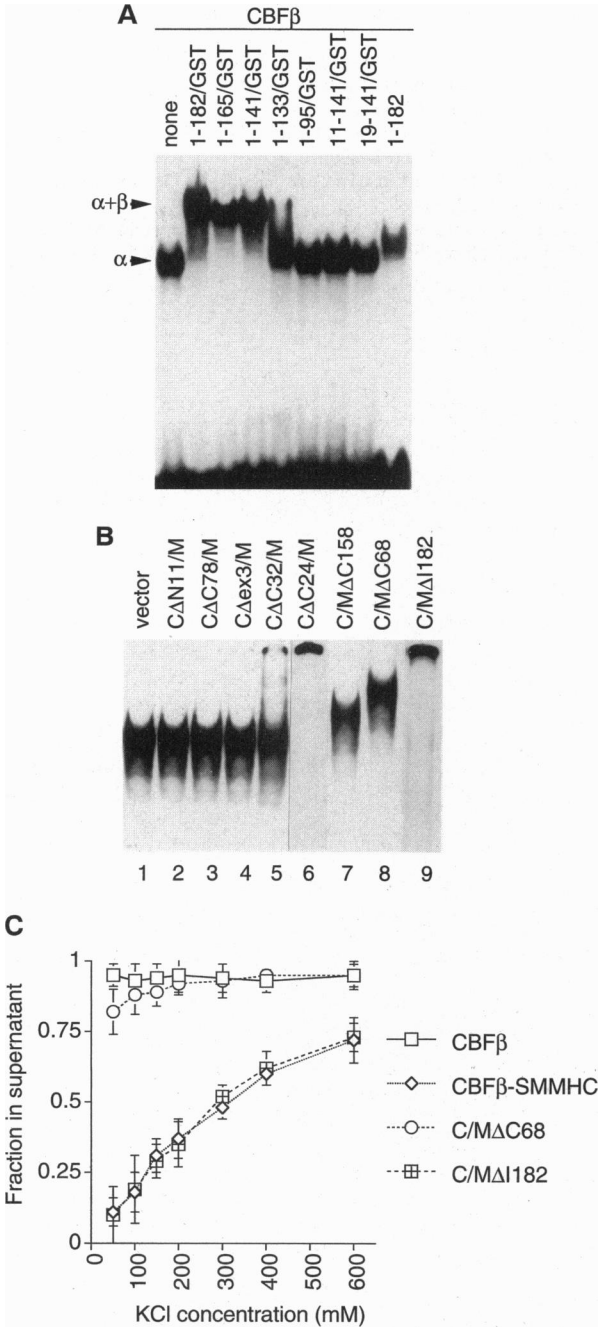

FIG. 4. (*A*) EMSA of purified bacterially expressed, truncated CBFβ proteins fused to GST. The CBFβ amino acids fused to GST are indicated above each lane. The truncated CBFβ/GST proteins were mixed with purified CBFα and incubated with a probe containing a high-affinity CBF-binding site. Arrows at left indicate the position of complexes resulting from DNA binding by CBFα alone (α arrow) or by CBFα and CBFβ together (α + β arrow). The first lane contains no CBFβ protein, and the last lane contains full-length CBFβ protein cleaved from GST. (*B*) EMSA of nuclear extracts from cells stably expressing the indicated mutants incubated with a probe containing a high-affinity CBF-binding site. The first lane has nuclear extracts from cells expressing insertless vector. Cells expressing C165 and MHCR constructs had an EMSA complex identical to that seen in cells transfected with vector alone (data not shown). All of the complexes detected by EMSA disappeared in the presence of a large excess of unlabeled probe but not in the presence of a large excess of probe with a mutated CBF-binding site (data not shown). (*C*) Solubility curves of recombinant proteins in NIH 3T3 cells. Proteins were detected by using antibodies β13 and β14. The bar for each data point represents the SEM from five different experiments. C/MΔC158 had the same solubility curve as C/MΔC68 (data not shown).

formation mediated by the SMMHC carboxyl terminus. Whether these multimers are typical bipolar myosin filaments or less ordered protein aggregates is not yet clear.

This study suggests that NIH 3T3 transformation is caused by multimeric CBFβ–SMMHC/CBFα complexes that retain the ability to bind CBF-binding sites. These complexes may interfere with the normal function of CBF in a dominant negative manner, either by causing the mislocalization of CBFα within the cell or by binding DNA at CBF sites and interfering with the adjacent binding of cooperating transcription factors (8, 9). This complex may also acquire novel properties and thereby transform cells through a dominant positive mechanism.

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