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RESEARCH ARTICLES

Identification of the Chimeric Protein Product of the *CBFB-MYH11* Fusion Gene in Inv(16) Leukemia Cells

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An expressed gene formed by fusion between the *CBFB* transcription factor gene and the smooth muscle myosin heavy chain gene *MYH11* is consistently detected by reverse transcription polymerase chain reaction (RT-PCR) in patients who have acute myeloid leukemia (AML) subtype M4Eo with an inversion of chromosome 16. We have previously shown that a *CBFB-MYH11* cDNA construct can produce a chimeric protein and transform NIH 3T3 cells. However, the presence of the chimeric protein in patient cells has not been demonstrated previously. Here, we show that such chimeric proteins can be identified in vivo, primarily in the nuclei of the leukemic cells, by use of antibodies against the C-terminus of the smooth muscle myosin heavy chain and the fusion junction peptide. A very high molecular weight protein/DNA complex is generated when nuclear extracts from patient cells are used in electrophoretic mobility shift assays, as seen in NIH 3T3 cells transfected with the *CBFB-MYH11* cDNA. Immunofluorescence staining shows that the proteins are organized in vivo into novel structures within cell nuclei. One isoform of the transcript of the *CBFB-MYH11* fusion gene, containing the MHC_{204} C-terminus, was the predominant form in all five cases studied. *Genes Chromosom Cancer 16:77–87 (1996).*

INTRODUCTION

A characteristic pericentric chromosome 16 inversion is a consistent finding in patients with acute myeloid leukemia (AML), subtype M4Eo (Le Beau et al., 1983; Testa et al., 1984; Larson et al., 1986). We recently cloned DNA sequences from both breakpoints of this inversion (Liu et al., 1993a). The *CBFB* gene at the q arm breakpoint and the *MYH11* gene at the p arm breakpoint were found to be broken and joined together by the inversion (Liu et al., 1993b). Fusion transcripts between the 5′ end of *CBFB* and the 3′ end of *MYH11* were detected in RNA of leukemia patients.

Since this initial publication, several groups have studied more than 100 patients and have reported the detection of *CBFB-MYH11* fusion transcripts in the vast majority of patients with a cytogenetically detected chromosome 16 inversion (Claxton et al., 1994; Hébert et al., 1994; Poirel et al., 1995; Schurloff et al., 1995; van der Reijden et al., 1995). Except in a few patients, the breakpoints in the *CBFB* gene are located in the last intron; in the *MYH11* gene, breakpoints in several introns have been identified, one of them being the most prevalent. All of the fusions identified so far, however, are in frame; therefore, a chimeric protein is predicted to be produced from the fusion transcripts (Liu et al., 1995).

We have prepared a full-length *CBFB-MYH11* cDNA construct and have used it in a variety of studies (Liu et al., 1994; Hajra et al., 1995a,b). NIH 3T3 cell lines have been generated that overexpress the *CBFB-MYH11* fusion cDNA. A fusion protein of the predicted size [designated CBFβ-smooth muscle myosin heavy chain (SMMHC)] can be detected in such cells. Electrophoretic mobility shift assay (EMSA) reveals a novel high molecular weight DNA-protein complex binding to the CBF cognate sequence in these cells. These CBFβ-SMMHC-expressing NIH 3T3 cells acquire a transformed phenotype: they form foci in cell culture, grow in soft agarose, and form tumors when injected into nude mice (Hajra et al., 1995a). This finding demonstrates that *CBFB-MYH11* is a transforming gene.

Despite the in vitro studies mentioned above, it has not yet been demonstrated that the CBFβ-SMMHC fusion protein is produced in vivo in patient leukemia cells. In this study, we report the identification of proteins of the predicted size for

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CBFβ-SMMHC by use of specific antibodies in each of five patients who had the chromosome 16 inversion. The CBFβ-SMMHC fusion protein was detected primarily in the nuclei by Western blot as well as by immunofluorescence staining (IF). We also show the presence of a higher molecular weight protein-DNA complex formed between nuclear extracts from inversion 16 patient cells and an oligonucleotide containing a CBF binding site. This complex is very similar to that observed in NIH 3T3 cells transformed with *CBFB-MYH11* (Hajra et al., 1995a), which correlates with the presence of the CBFβ-SMMHC fusion protein.

The *MYH11* gene transcript is known to be alternatively spliced at the 3′ end, resulting in the production of two isoforms of the SMMHC, MHC_{200} and MHC_{204}, with different molecular weights (200 and 204 kD, respectively) and carboxyl termini (Rovner et al., 1986a; Kawamoto and Adelstein, 1987; Eddinger and Murphy, 1988; Babij and Periasamy, 1989; Nagai et al., 1989). Alternative splicing in the *MYH11* part of the *CBFB-MYH11* fusion gene has been analyzed, and MHC_{204} was found to be the predominant isoform detected in the patient cells. A cDNA construct expressing this isoform can transform NIH 3T3 cells as readily as that expressing the alternative MHC_{200} isoform of the *CBFB-MYH11* gene product.

MATERIALS AND METHODS

Patient Description

Patient 1 was a 28-year-old man with a clinical diagnosis of AML-M4Eo and a chromosome 16 inversion, which was detected cytogenetically. Patient 2 was a 65-year-old man with a clinical diagnosis of AML-M1Eo. Patient 3 had AML-M4Eo, whereas patient 4 had CML in blast crisis associated with eosinophilia (Yanagisawa et al., 1991; Evers et al., 1992). Patient 6 was a 34-year-old woman with a clinical diagnosis of AML-M4 without elevated eosinophil count. The leukemia cells from all of these patients had a chromosome 16 inversion. Patient 7 had a leukemia-associated chromosome 16 inversion that generated a type E fusion gene between *CBFB* and *MYH11*, as described before (Liu et al., 1993b, 1995). Patient 5 was a 51-year-old man diagnosed with AML-M4, and his leukemia cells had a normal karyotype. Bone marrow samples were collected from all patients except patient 3. A cell line has been developed from patient 3 cells (Yanagisawa et al., 1991), and cultured cells were harvested for analysis.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA isolation, reverse transcription, and PCR reactions were all performed as described (Liu et al., 1992, 1993b). Primers used for the detection of fusion were C1 (nt 272–293 of *CBFB*) and M1 (nt 2,114-2,095 of *MYH11*; Liu et al., 1993b; Matsuoka et al., 1993). Primers used for nested PCR were CM5 (5′-GGACACGCGAATTTGAAGAT; nt 444–463 of *CBFB*) and SM3 (5′-TGGTTTTTGGTTTTCTTGCC; nt 3,345-3,325 of *MYH11*) for the first-round PCR and SM5 (5′-GCTGAAGGAAATCTTGCTGC; nt 2,892–2,911 of *MYH11*) and SM3 for the second-round PCR. Cycling conditions for the first-round PCR were 94°C for 1 minute, 60°C for 1 minute, and 72°C for 3 minutes for 30 cycles. The second-round PCR was done under the same conditions for 20 cycles. One microgram of total RNA was used for the reverse transcription, and one-fifth of the cDNA product was used for the first-round PCR; one-fiftieth of the first-round PCR product was then used for the second-round PCR. Digestions of PCR products were carried out with restriction enzymes from Boehringer Mannheim (Indianapolis, IN).

Hybridization of RT-PCR Products With Oligonucleotide

The oligonucleotide used as probe was SM1 (5′-CAGAGAAAGGCAATGCCA; nt 2,957–2,974 of *MYH11*). The oligonucleotide was labeled by polynucleotide kinase in the presence of γ-^{32}P-ATP. Hybridization of the labeled probe to the DNA blots was done in 40 mM $NaPO_4$, pH 7, 1 mM EDTA, and 7% sodium dodecyl sulfate (SDS) at 42°C for 18 hours. Blots were then washed at room temperature in 2 × SSC and 0.1% SDS for 15 minutes, in 1 × SSC and 0.1% SDS at 42°C for 15 minutes, and in 0.1 SSC and 0.1% SDS at 42°C for 30 minutes.

Sequencing

Sequencing of the RT-PCR products was performed as described previously (Liu et al., 1993b).

Construction of a cDNA Clone for the CBFβ-SMMHC MHC_{204} Isoform

The nested PCR product with RNA from patient 4 was used in the construction of the cDNA clone. The nested PCR was performed as described above, except for the addition of a *Not*I

recognition site at the 5′ end of primer SM3 (5′-ATAAGCGGCCGCTGGTTTTTGGTTTTCT-TGCC). The PCR product was digested with *Nco*I (nt 3,089–3,094 of *MYH11*) and *Not*I and was ligated to *Nco*I/*Not*I-digested pKL1, which is a full-length cDNA clone coding for the CBFβ-SMMHC MHC_{200} isoform in the pBluescript KS^+ vector (Liu et al., 1994). The ligation product was transformed into DH5α cells, and the selected clones were sequenced to confirm the construction.

Antibodies

Affinity-purified rabbit polyclonal antibodies specific for MHC_{204} (designated DAD) and MHC_{200} (designated GPPP) isoforms of SMMHC were generated as described previously (Kelley et al., 1992; Hajra et al., 1995a). An antibody against the inv(16) type A CBFβ-SMMHC chimeric junction (anti-inv16A) was produced by immunization of rabbits (Loftstrand Inc., Gaithersburg, MD) with synthetic peptide J42723 (residues 157–174 of the full-length CBFβ-SMMHC: DRSHREEME-VHELEKSKR) and was affinity purified with the same peptide (Wijmenga et al., 1996).

Indirect Immunofluorescence

Bone marrow cells were resuspended in 200 μl of tissue culture medium (1×10^4 cells/ml) containing 10% fetal calf serum. Cells were attached onto coverslips with a cytocentrifuge and then fixed with absolute methanol at −20°C for 7 minutes. Indirect IF was performed as described (Wijmenga et al., 1996).

Western Blotting

Cells were washed twice in PBS and then resuspended in 50 ml PBS with Triton (0.1%) and protease inhibitors (2.5 mM Leupeptin, 2.5 mM Aprotinin, and 0.1 mM PMSF) and incubated on ice for 20 minutes. The mixture was centrifuged for 10 minutes at 12,000 rpm in an Eppendorf microcentrifuge at 4°C. The supernatant containing the cytoplasmic fraction was removed and saved. The nuclear pellet was resuspended in 50 μl of phosphate-buffered saline (PBS) with protease inhibitors. Nuclear and cytoplasmic fractions from equal numbers of cells were loaded on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels. However, the amount of proteins loaded varied from patient to patient. Immunoblotting was performed according to standard procedures with an Amersham ELC kit (Amersham, Arlington Heights, IL).

EMSA

Nuclear extract preparation and EMSA were performed as described previously (Gumucio et al., 1988; Liu et al., 1994). The probe used was HA, which contains the high-affinity core site GGATATTTGCGGTTAGCA. The competitors for EMSA were the unlabeled double-stranded oligonucleotides HA and MUT. MUT is HA with two nucleotide changes in the core site: GGAT-ATCTGCCGTAAGCA (Wang and Speck, 1992).

NIH 3T3 Cell Transformation

Transfection of plasmid DNA into NIH 3T3 cells, selection of stable cell lines, focus formation assay, soft-agarose growth, and nude mouse tumor induction were all performed as described (Hajra et al., 1995a).

RESULTS

RT-PCR Detection of the *CBFB-MYH11* Fusion Transcript

Total RNA was isolated from leukemia patient bone marrow cells, and RT-PCR was conducted for detection of the *CBFB-MYH11* fusion transcripts in the RNA samples by use of the primers C1 and M1. RT-PCR with RNA from patients 1–3 and 6 showed amplified bands of a size consistent with *CBFB-MYH11* fusion type A (Liu et al., 1995). This was confirmed by restriction enzyme digestion with *Ava*II (an *Ava*II recognition site is created by the A-type fusion; for sequence information, see Liu et al., 1993b). RT-PCR with patient 4 RNA generated a fragment about 200 bp larger than the type A fragment. Sequencing of this RT-PCR product revealed a *CBFB-MYH11* fusion with the *CBFB* breakpoint at nt 495 and the *MYH11* breakpoint at nt 1708 (type B fusion; see Liu et al., 1995). No amplifiable products were detected with RNA from patient 5, who did not have inversion 16, as determined cytogenetically.

Generation of an inv(16)-Specific Antibody

In addition to existing antibodies recognizing either CBFβ or the carboxy terminus of the SMMHC part (DAD) of the CBFβ-SMMHC fusion protein, specific antibodies were raised against the junction region between CBFβ and SMMHC. Although multiple breakpoints within the *MYH11* gene give rise to in-frame fusion proteins, one major breakpoint (*MYH11* breakpoint at nt 1921) was found in about 84% of the patients (type A; see Liu et al., 1995). Therefore, a peptide encompassing

the type A junction was chosen for generation of antibodies (see Materials and Methods). The polyclonal rabbit antibody anti-inv16A was affinity purified with the same peptide and was characterized by Western blot analysis.

Detection of the CBFβ-SMMHC Fusion Protein in Patient Cells by Western Blotting

Whole cell lysates or cytoplasmic and nuclear fractions of cell lysates from the patient leukemia cells were electrophoresed on SDS-PAGE gels. The total cell lysate isolated from the NIH 3T3-derived cell line CM, which expresses CBFβ-SMMHC MHC_{200} (Hajra et al., 1995a). was used as a control.

A polyclonal antibody recognizing the SMMHC portion of the fusion protein was used, because the endogenous *MYH11* gene is not expressed in leukemia cells. This antibody, DAD, which was raised against the C-terminus of the MHC_{204} isoform of SMMHC, detected proteins of the expected size in all of the patients with inversion 16, but not in patient 5, who lacked the inversion, and not in cell line CM, which contained the MHC_{200} isoform. The protein was present predominantly in the nucleus, because most of the signal is in the nuclear fraction (Fig. 1A).

With anti-inv16A, the same protein was detected in patient 6 and in the cell line CM, but it was not detected in patient 5 or in patients with other types of fusion (type B for patient 4, type E for patient 7; Fig. 1B). In addition, the anti-inv16A antibody did not detect endogenous CBFβ or SMMHC (data not shown). These results indicate that anti-inv16A is an antibody that specifically detects the type A junction of the CBFβ-SMMHC fusion protein. In summary, the fusion gene generated by the chromosome 16 inversion does produce a CBFβ-SMMHC chimeric protein in vivo, as predicted based on the sequence of the RNA.

Indirect IF of Leukemic Cells

Leukemic cells from patients 4, 5, and 6 were used for IF. Cells from patients 4 and 6 demonstrated a speckled staining pattern throughout the nuclei with antibody DAD (Fig. 2A,B). A similar staining pattern was detected in patient 6 cells with anti-inv16A (Fig. 2D). Also evident was the cytoplasmic staining of these cells, consistent with the presence of the fusion protein in the cytoplasmic fraction by Western blot (see above). On the other hand, leukemic cells from patient 5 did not show any detectable staining in the nucleus and showed only faint background staining in the cytoplasm with DAD and anti-inv16A (Fig. 2C,E).

EMSA

EMSA was performed with nuclear extracts from three patients with inv(16) and the inv(16)-negative patient 5. A very slowly migrating complex was observed at the gel origin; this was specific for inversion 16, because 1) it was not detected in the control patient who did not have inv(16), and 2) this complex can be competed away by the addition of an excess amount of unlabeled probes (Fig. 3). Besides this slowly migrating complex, however, a broad complex similar to that in control patient 5 was seen in these inv(16) samples. This broad complex is likely to be the normal CBF-DNA complex.

The MHC_{204} Isoform Predominates in the CBFβ-SMMHC Fusion Protein

SMMHCs are known to exist as two isoforms with molecular masses of 204 and 200 kD (MHC_{204} and MHC_{200}, respectively) because of alternative 3′ splicing of mRNA from a single gene (Fig. 4A; Rovner et al., 1986a; Kawamoto and Adelstein, 1987; Eddinger and Murphy, 1988; Babij and Periasamy, 1989; Nagai et al., 1989). To investigate the isoform composition of SMMHC in the CBFβ-SMMHC fusion protein, we also used an antibody specific for MHC_{200} (GPPP; see Hajra et al., 1995a) in Western blot analyses. This antibody did not react with cell extracts from patients 1 and 2, and it reacted weakly with a protein of the expected size from patient 4 (data not shown).

Investigating the possibility of preferential alternative splicing of the *MYH11* transcript, we performed nested RT-PCR to amplify the 3′ region of *MYH11* from the leukemic fusion transcripts. For the first-round of PCR, we used primers CM5, which is 5′ to the breakpoint, and SM3, which is in the 3′ UTR region of the *MYH11* gene. For the second-round PCR, we used primers SM5 and SM3, which flank the alternative splicing site (Fig. 4A). PCR products were separated on agarose gels, transferred to nylon membrane, and hybridized with oligonucleotide SM1, a sequence 5′ to the alternative splicing site (Fig. 4A,B). To increase the specificity, we performed two restriction digests of the PCR products. An *Apa*I site is present in the MHC_{200} isoform but not in the MHC_{204} form. In addition, the *Xba*I fragments detected by SM1 will be larger for MHC_{200} (Fig. 4A) than for MHC_{204} because of the inserted exon in MHC_{200}.

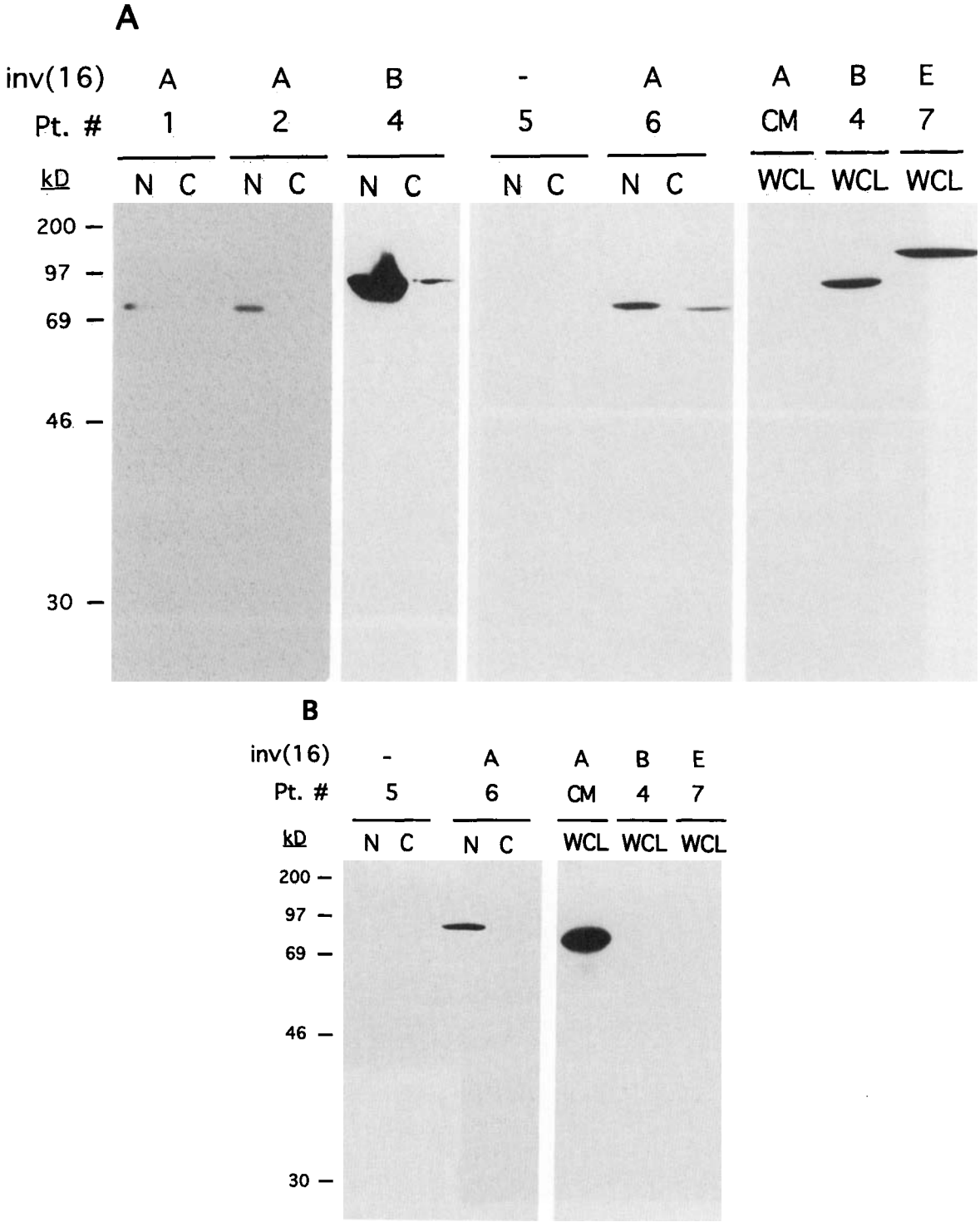

Figure 1. Detection of the CBFβ-smooth muscle myosin heavy chain (SMMHC) fusion protein in vivo in patient leukemia cells by Western blotting. Affinity-purified rabbit polyclonal antibodies specific for MHC_{204} (designated DAD) was used in **A**, and anti-inv16A was used in **B**. Inv(16), the presence and the fusion type of inversion 16 gene in each sample; N, nuclear fraction; C, cytoplasmic fraction; WCL, whole cell lysate; CM, the NIH 3T3 cell line with CBFβ-SMMHC MHC_{200}.

In Figure 4C, it can be seen that the *MYH11* portion of the inv(16) fusion gene is preferentially spliced to give the MHC_{204} isoform (the 416 bp *Apa*I fragment and the 266 bp *Xba*I fragment). The MHC_{200} isoform was detectable in patient 4 only after prolonged exposure (not shown).

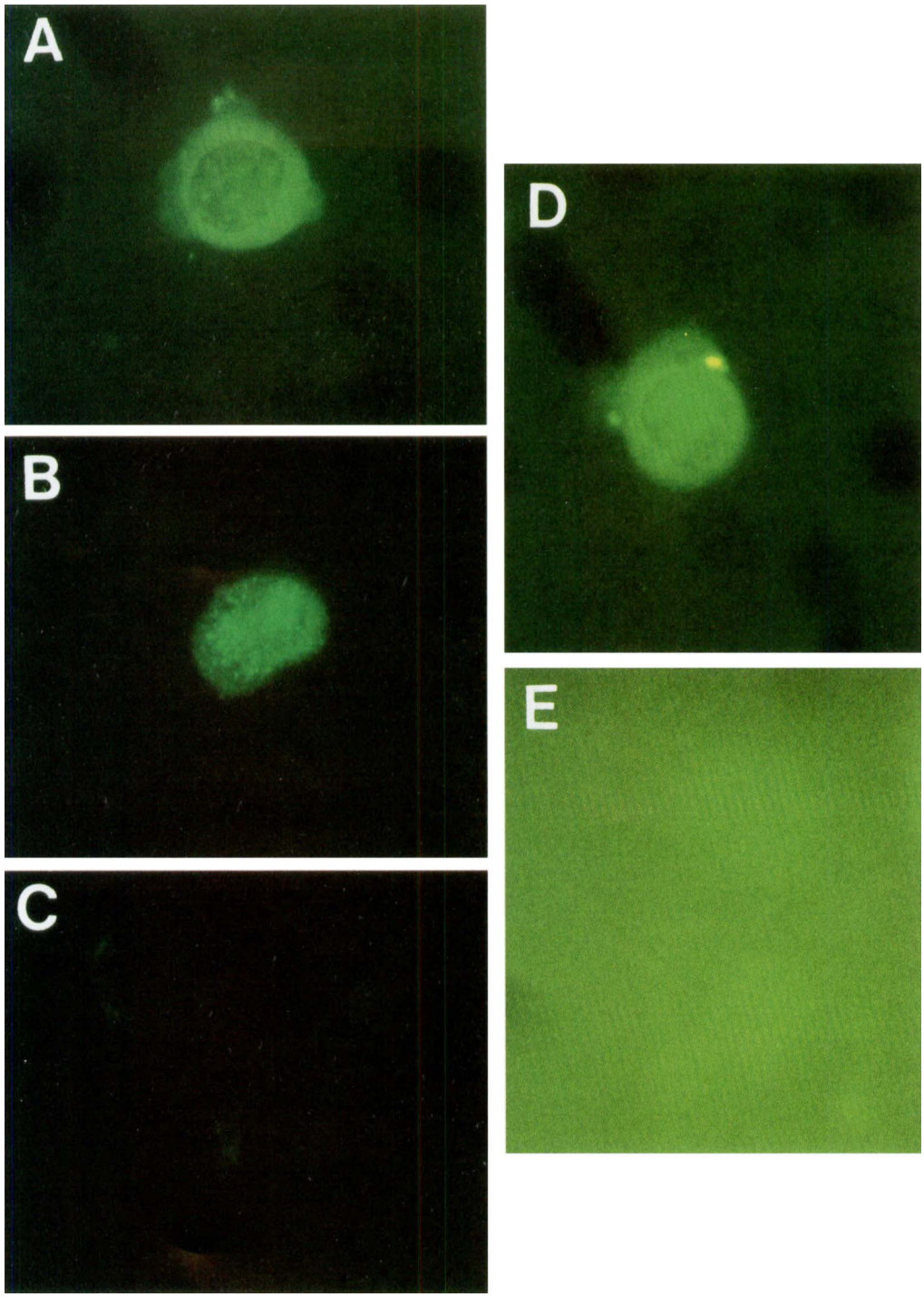

Figure 2. Indirect immunofluorescence staining of leukemic cells with antibodies DAD and anti-inv16A. **A:** A leukemia cell from patient 6 with DAD. **B:** A leukemia cell from patient 4 with DAD. **C:** Leukemia cells from patient 5 [inv(16$^-$)] with DAD. **D:** A leukemia cell from patient 6 with anti-inv16A. **E:** Leukemia cells from patient 5 with anti-inv16A.

Transformation of NIH 3T3 Cells by the MHC_{204} Isoform of the CBFβ-SMMHC Fusion Protein

In our previous NIH 3T3 cell transformation experiments (Hajra et al., 1995a), the inv(16) fusion cDNA construct used contained the MHC_{200} isoform. Given the finding that the fusion protein detected in vivo involves primarily the MHC_{204} isoform, it was important to determine whether

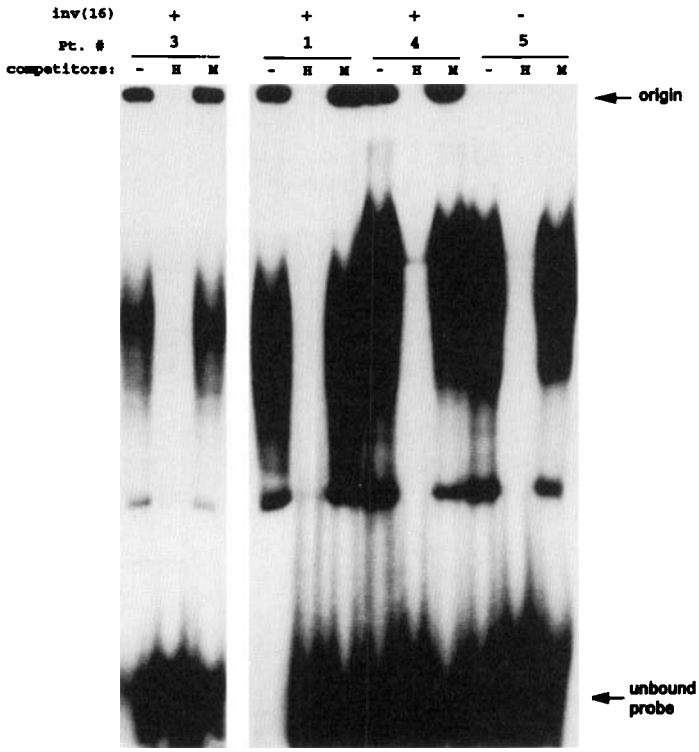

Figure 3. Electrophoretic mobility shift assay (EMSA). EMSA was performed with nuclear extracts from patients 1, 3, 4, and 5. Competitors used are H (high-affinity CBF site HA) and M (low-affinity mutant site MUT; see Materials and Methods for sequences). Origin, origin of the polyacrylamide gel.

this isoform can also transform NIH 3T3 cells. An inv(16) fusion cDNA construct containing the MHC_{204} isoform was transfected into NIH 3T3 cells, and several permanent cell lines with stably integrated cDNA constructs were isolated. These cell lines were shown to have acquired a transformed phenotype, as measured by their ability to form foci, grow in soft agar, and form tumors when injected into nude mice (Fig. 5, Table 1). The efficiency of focus formation and colony growth in soft agar was essentially indistinguishable from that of the MHC_{200} inv(16) cDNA construct (Table 1).

DISCUSSION

In this study, CBFβ-SMMHC fusion proteins were detected for the first time in vivo in leukemia cells from patients with chromosome 16 inversion. This protein probably plays a very important role in the pathogenesis of leukemias, especially AML-M4Eo. In NIH 3T3 cells overexpressing a transfected *CBFB-MYH11* construct driven by a cytomegalovirus (CMV) promoter, we have shown that the protein is localized mainly in the nucleus, consistent with its role in transcriptional regulation (Hajra et al., 1995a,b). In these same cells, the fusion protein is observed to form large rod-like structures in the nucleus (Wijmenga et al., 1996). In the present in vivo study, the fusion protein was visualized as speckled nuclear staining structures in the leukemia cells. The differences in these patterns may reflect differences in the levels of multimerization of the protein caused by differences in the levels of expression of the fusion protein in the nucleus: The expression level of the CMV-driven fusion gene in the NIH 3T3 cells was much higher than any of those in the patient cells (data not shown).

We have shown previously that the inv(16) protein forms a very slowly migrating complex with CBFα and a labeled DNA probe containing the CBF binding site in EMSA analyses (Liu et al., 1994; Hajra et al., 1995a). This was the case for both in vitro synthesized CBFβ-SMMHC plus CBFα and for nuclear extracts isolated from NIH 3T3 cells overexpressing the inv(16) fusion protein. Two complexes were formed with the synthetic protein: One was slightly larger than the wild-type CBFα/β complex, and the other did not migrate out of the gel origin (Liu et al., 1994; Hajra et al., 1995a). The smaller form was assumed to

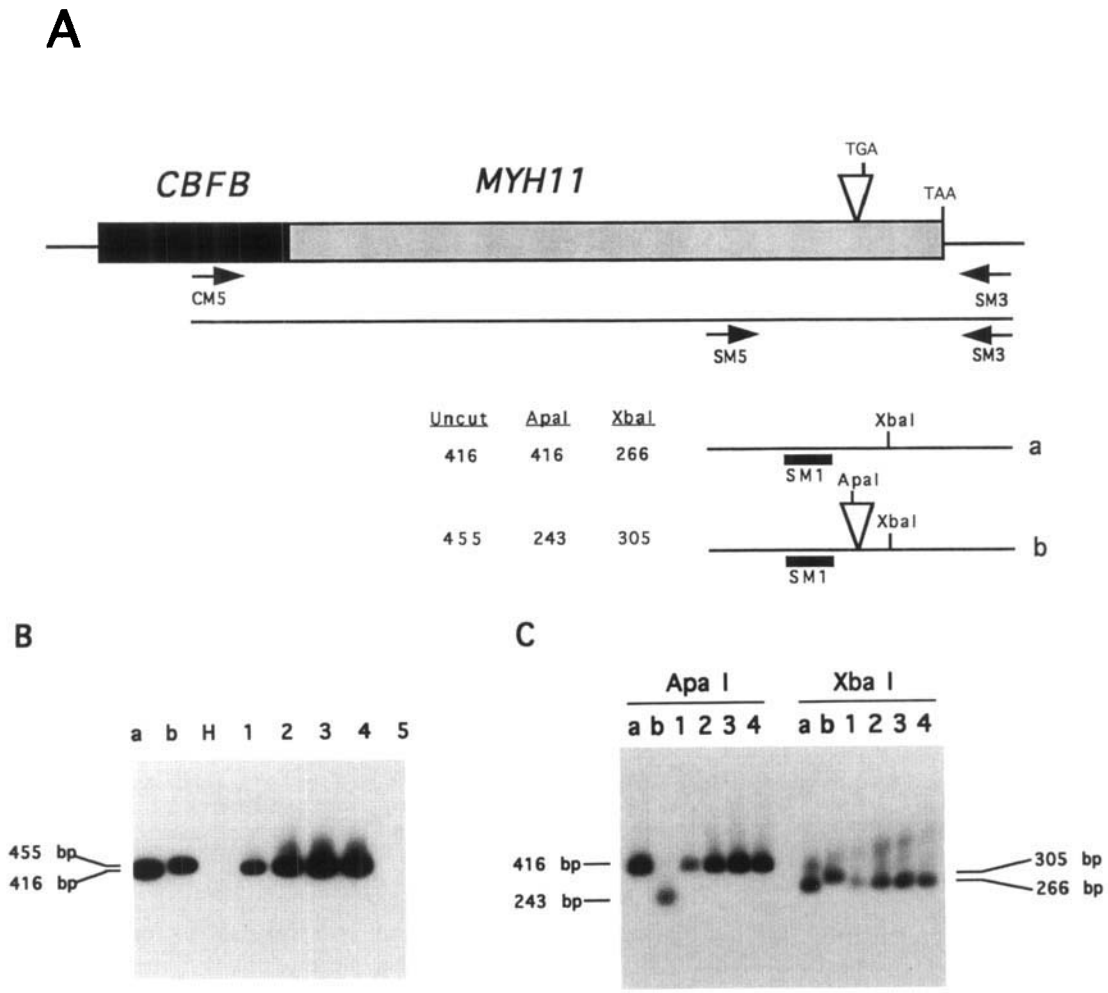

Figure 4. Alternative splicing of the 3′ end of the *CBFB-MYH11* fusion transcript. **A:** Diagrammatic representation of the *CBFB-MYH11* fusion transcript, including the locations of the polymerase chain reaction (PCR) primers (arrows), the locations of the alternatively spliced exon (triangles), stop codons (TGA for MHC_{200}, TAA for MHC_{204}), reverse transcription polymerase chain reaction (RT-PCR) products (horizontal lines), locations of restriction endonuclease recognition sites and predicted sizes of restriction fragments for MHC_{204} (**a**) and MHC_{200} (**b**) isoforms, and the location of the oligonucleotide probe SM1 used for hybridization (solid horizontal bar). **B,C:** Southern blots of RT-PCR products with primer pairs CM5-SM3 followed by SM5-SM3. The blots were hybridized with probe SM1. B: Undigested PCR products. C: *Apa*I- and *Xba*I-digested PCR products. **a:** PCR with a cDNA construct containing the MHC_{204} isoform. **b:** PCR with a cDNA construct with the MHC_{200} isoform. H, water; 1–4, patients 1–4 with inv(16); 5, patient 5 without inv(16).

represent a monomer or a dimer of the CBFβ-SMMHC fusion protein, and the larger form probably represented large multimers of the protein because of the capacity of the myosin heavy chain to multimerize (for a review, see Liu et al., 1995). When nuclear extracts from transfected NIH 3T3 cells were used, only the larger form of the protein-DNA complex was observed. The normal CBFα/β complex disappeared completely, even though wild-type CBFβ should also be present in these cells (Hajra et al., 1995a). This is likely to be a consequence of the fusion protein overexpression that may be required for transformation of NIH 3T3 cells. In fact, it has been shown that overexpression of CBFα can revert the transformed phenotype (Hajra et al., 1995b). It is shown here that both the high molecular weight multimers and the normal αβ complex are present in vivo in patient leukemia cells. This may indicate a lower level of the CBFβ-SMMHC fusion protein required for transformation of myeloid cells or a true dominant mechanism of transformation. Alternatively, the patient samples may have been a mixture of leukemic and nonleukemic cells, resulting in the appearance of both high molecular weight and normal DNA-protein complexes. However, patient 3 cells were harvested from an established cell line that contains only inv(16)$^+$ cells (Yanagisawa et al.,

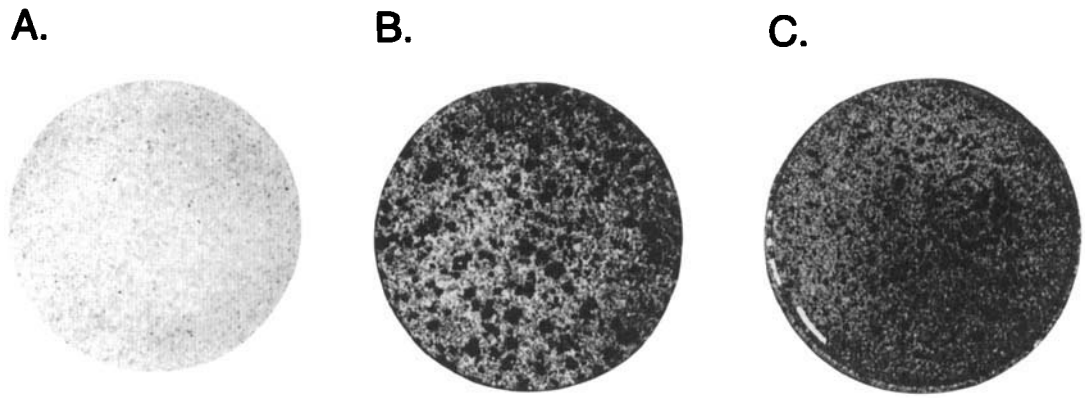

Figure 5. Focus formation by representative 3T3 cells stably expressing normal CBFβ (**A**), CBFβ-SMMHC of the MHC_{204} isoform (**B**), and CBFβ-SMMHC of the MHC_{200} isoform (**C**).

TABLE 1. Characteristics of NIH 3T3 Cells Stably Expressing the Two CBFβ-SMMHC Isoforms

Protein expressed[a]	No. of foci[b]	No. of soft agar colonies[b]	No. of tumors/injections[c]
CBFβ	23.6 ± 8.8	0.0 ± 0.0	0/6
C/M_{200}	576.4 ± 36.8	52.4 ± 8.8	6/6
C/M_{204}	612.0 ± 42.6	50.2 ± 6.6	6/6

[a]The table shows representative cell lines stably expressing the indicated proteins. C/M_{200} and C/M_{204} refer to the MHC_{200} and MHC_{204} isoforms of the CBFβ-SMMHC fusion protein, respectively.
[b]Per 1,000 cells. Values represent means ± standard errors from five separate experiments.
[c]Values are totals from all experiments. Two injection sites were used for each nude mouse.

1991). The fact that EMSA with nuclear extracts from cultured leukemia cells of patient 3 also showed both the high-molecular-weight and the normal complexes (Fig. 3) argues against this explanation.

The normal CBF-DNA complex observed in these patient samples was quite broad (Fig. 3). Similarly broad complexes have been observed when nuclear extracts from monkey COS cells and human myeloid leukemia cells Kasumi-1 and HL-60 were used (Sakakura et al., 1994; Tanaka et al., 1995). Such broad complexes may result from the presence of three different *CBFA* genes, alternative splicing of the *CBFA* gene transcripts, and proteolytic cleavage of their protein products (Bae et al., 1994; Levanon et al., 1994).

The almost exclusive use of the MHC_{204} isoform in the *CBFB-MYH11* gene transcripts was not completely unexpected. In smooth muscle tissue, the MHC_{204} isoform appears to be the result of constitutive splicing, because it occurs at all stages of embryonic development (Kuro-o et al., 1991), in adult smooth muscle tissue (Mohammad and Sparrow, 1989), as well as in smooth muscle cells in culture (Rovner et al., 1986b; Kawamoto and Adelstein, 1987). In contrast, alternative splicing to generate the MHC_{200} isoform occurs only late in embryonic development and in adult smooth muscle tissues. When differentiated smooth muscle cells are placed into tissue culture to proliferate, expression of the MHC_{200} isoform is quickly terminated. It appears, therefore, that this alternative splicing pathway is associated with a differentiated smooth muscle phenotype. Based on these observations, the predominant use of the MHC_{204} alternative splicing pathway in leukemia cells is not surprising.

The functional significance of an MHC_{204} carboxy-terminus as opposed to an MHC_{200} carboxy-terminus in the CBFβ-SMMHC protein is not clear. Several earlier reports indicate a potential functional difference between these two isoforms of SMMHC. First, these two isoforms apparently form only homodimers (Kelley et al., 1992), although there is one additional report that they can associate as heterodimers as well (Tsao and Eddinger, 1993). Second, it has been shown that MHC_{204}, but not MHC_{200}, is phosphorylated by casein kinase II in intact cells (Kelley and Adelstein, 1990). Third, it was shown that a short nonhelical sequence at the COOH-terminus of vertebrate nonmuscle myosin heavy chain (NMMHC) can enhance myosin filament assembly about 50-fold (Hodge et al., 1992). NMMHC is highly homologous to SMMHC, and the COOH-terminal sequence mentioned above is present only in the MHC_{204} isoform. In leukemia cells, the almost exclusive use of the MHC_{204} isoform of the CBFβ-SMMHC protein is of uncertain significance. We have shown before that the two isoforms of CBFβ-

SMMHC behave very similarly in assays for their solubility and EMSA (Hajra, 1995; Hajra et al., unpublished data). We demonstrate here that both isoforms can transform NIH 3T3 cells with similar efficiency and potency. However, we cannot rule out more subtle effects undetected by the analyses performed.

The demonstration of the presence of the CBFβ-SMMHC fusion protein in leukemia cells in vivo is another step in the path toward unraveling the molecular pathogenesis of this type of adult leukemia. The antibody described here should be a very useful tool for the detection of the fusion protein for clinical diagnosis and disease monitoring as well as for research purposes. Future studies will focus on determining the precise mechanism by which this oncoprotein induces malignant transformation, with the ultimate goal of designing specific therapy.

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