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Immunoaffinity Purification and Characterization of CACGTG Sequence-Binding Proteins from Cultured Mammalian Cells Using an Anti c-Myc Monoclonal Antibody Recognizing the DNA-Binding Domain

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c-Myc protein contains a basic helix-loop-helix and leucine zipper (bHLHLZ) structure in its carboxyl terminal region, forms heterodimers with Max, and binds to the CACGTG sequence in DNA. A number of bHLHLZ proteins are present in cells, and some of them bind to the same DNA sequence. Using an anti c-Myc monoclonal antibody, MYC5, raised against a bacterially synthesized c-Myc protein, we have carried out immunoaffinity purification of c-Myc proteins from cultured mammalian cells. The immunoaffinity-purified fraction was found to contain not only c-Myc but also other CACGTG sequence-binding proteins, such as Max, Mad, and USF, indicating a wide cross-reaction to CACGTG sequence-binding proteins. The MYC5 antibody recognized the common structural motif in their basic region required for sequence-specific DNA binding and was shown to inhibit their DNA-binding activities. The immunoaffinity-purified N-Myc, Max, Mad, and, presumably, c-Myc were highly phosphorylated, and phosphatase treatment increased the DNA-binding activity of Myc, suggesting that the DNA-binding activity of c-Myc was regulated by phosphorylation *in vivo*. From these results, we can conclude that the MYC5 antibody constitutes a powerful tool for the purification and characterization of c-Myc and other CACGTG sequence-binding proteins.

Key words: CACGTG sequence-binding protein, c-Myc, immunoaffinity purification, Max, monoclonal antibody.

c-Myc oncoprotein plays an important role in cell proliferation, differentiation and apoptosis (*1-3*). Recent studies have indicated that c-Myc protein is involved in the regulation of gene expression (*4*). Myc family proteins, including c-, N-, and L-Myc, are nuclear phosphoproteins with short half-lives which possess the ability to bind to DNA. The amino-terminal domain of c-Myc has been reported to be required for transformation and transcriptional activation (*5*). The carboxy-terminal region of c-Myc protein shares a common structural motif with other transcriptional regulatory proteins: a basic region, helix-loop-helix and leucine zipper (bHLHLZ) (*6, 7*). The helix-loop-helix and leucine zipper have been demonstrated to mediate protein oligomerization, while the basic region is essential for sequence-specific DNA binding, but not for protein oligomerization. For a number of basic helix-loop-helix proteins (bHLH), the commonly recognized DNA sequence has been defined as CANNTG and named the E-box (*8*). The transcriptional factor E3 (TFE3) (*9*) and the upstream stimulatory transcriptional factor (USF) (*10*) bind to the same CACGTG sequence. Comparison of the amino acid sequence of c-Myc protein with those of TFE3 and USF revealed a high degree of similarity in the basic region among these proteins. Blackwell *et al.* (*11*) demonstrated specific DNA binding of c-Myc protein to the CACGTG sequence using a bacterially synthesized fusion protein containing the carboxy-terminus of c-Myc. However, this binding activity was only observed at very high concentrations. Using protein-protein interaction cloning, Blackwood and Eisenman identified a c-Myc partner protein, Max (*12*), which contains a bHLHLZ motif similar to that of the Myc family, including N- and L-Myc. Max could be oligomerized with c-Myc protein and the resulting heterodimer also bound to the CACGTG sequence. The association with N- and L- as well as c-Myc protein (*13*) suggests that Max is a common partner for all Myc family proteins. In contrast to c-Myc, Max is expressed constitutively at a high level in cells and is very stable, exhibiting a long half-life. Recently, two proteins, called Mad (*14*) and Mxi1 (*15*), were identified as partners of Max. Both contain a bHLHLZ domain, heterodimerize with Max, and bind to the same CACGTG sequence as the Myc/Max heterodimer and Max homodimer. These recent findings have revealed a network of bHLHLZ proteins in which Max plays a central role in mediating interactions (*16*), and complicated regulation of the DNA binding of Myc. Furthermore, many other CACGTG sequence-binding proteins, such as USF and TFE3, are present *in vivo* and compete with Myc/Max heterodimers for binding to the same sequences.

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Myc, Max, and Mad are phosphorylated *in vivo*. c-Myc is phosphorylated *in vitro* by at least four kinases; glycogen syntheses kinase type 3 (GSK3), mitogen activated protein kinase (MAPK) (*17, 18*), DNA dependent protein kinase (DNAPK) (*19*), and casein kinase II (CKII) (*20*). Max and Mad are also phosphorylated by CKII (*14, 21*). It has been shown by electrophoretic mobility shift assay (EMSA) that *in vitro* CKII phosphorylation modulates DNA-binding activity by increasing both the on- and off-rates of Max homo- and Myc/Max heterodimers, which results in a decrease of DNA-protein complexes involving Max (*22*).

Many studies have been carried out to demonstrate the CACGTG sequence-specific DNA-binding activity of Myc proteins involving either translation products in reticulocyte lysates or expression products in *Escherichia coli* cells. In the case of *in vitro* translated Myc proteins, specific DNA-binding activity was demonstrated, but the possibility could not be excluded that a certain proportion of the Myc proteins had formed complexes with Max-like proteins in the reticulocyte lysate, and that such heterodimers might therefore have been responsible for the majority of the specific DNA-binding activity. With bacterially synthesized c-Myc proteins, no specific DNA-binding activity was demonstrated. In this case, however, a lack of modification of the c-Myc proteins, such as phosphorylation, may have affected the DNA-binding activity.

In the present study, we analyzed the DNA-binding properties of c-Myc proteins using a monoclonal antibody, designated as MYC5, raised against exon 3 of the c-Myc polypeptide (*23*). We showed that the antibody cross-reacts with other CACGTG sequence-binding proteins which could be immunoaffinity-purified along with Myc proteins from mammalian cells. To characterize their DNA-binding activity, the DNA immunoprecipitation assay, EMSA, DNA affinity column chromatography, and modified Southwestern blotting were applied. The immunoaffinity-purified c-Myc protein alone was found to have little DNA-binding activity, but in the presence of 23 and 24 kDa nuclear phosphoproteins (P23 and P24) co-purified on immunoaffinity and proven to be phosphorylated forms of P21 and P22 Max, respectively, high-affinity binding was apparent. After phosphatase treatment of the immunoaffinity-purified fraction, the DNA-binding activity of c-Myc protein increased about twofold for both the homodimeric and heterodimeric forms.

These results indicate that the anti c-Myc monoclonal antibody, MYC5, is useful for analysis of the network of CACGTG sequence-binding proteins.

MATERIALS AND METHODS

Cell Culture and Preparation of Nuclear Extracts—HL60 and IMR32 cells were grown in RPMI1640 medium supplemented with 10% fetal calf serum at 37°C. To prepare nuclear extracts, the following steps were performed at 0°C except where indicated. The cells were collected, washed three times with phosphate buffered saline (154 mM NaCl and 10 mM sodium phosphate buffer, pH 7.6) containing 2 mM $MgCl_2$ (PBS-Mg), frozen in liquid N_2, and then stored at −80°C. The frozen cells were thawed, washed with PBS-Mg, and then suspended in isolation buffer [20 mM HEPES, pH 7.6, 20 mM NaCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 0.05 mM spermine, 0.125 mM spermidine, and protease inhibitors (0.5 mM PMSF, 0.5 μg/ml pepstatin, and 2 μg/ml leupeptin)], at 8×10^7 cells/ml. Digitonin was added at 0.1% to the cell suspensions and the cells were homogenized with 10 strokes of a Dounce homogenizer. Nuclei were collected by centrifugation, washed twice with isolation buffer, and then suspended in extraction buffer (20 mM HEPES, pH 7.6, 0.5 mM EDTA, 10% glycerol, and the protease inhibitors described above) containing 20 mM NaCl, at 4×10^8 cells/ml. An equal volume of extraction buffer containing 0.68 or 0.98 M NaCl was added to the nuclear suspensions, and after 1 h gentle agitation, the preparations were centrifuged at 30,000 rpm for 1 h at 4°C in a Type 30 rotor (Beckman). The supernatant fraction was recovered as the nuclear extract (0.35 M or 0.5 M NaCl nuclear extract) and either frozen in liquid N_2 and stored at −80°C, or further purified with immunoaffinity columns.

Antigen and Antisera—P42 Myc is a truncated c-Myc polypeptide comprising the 342 amino acids from the 98th Gln to the C-terminus fused with the N-terminal 21 amino acids of Ha-ras (*24*). Anti-P42Myc rabbit antiserum was raised against the bacterially synthesized P42Myc and then the specific IgG was affinity purified using Sepharose beads to which P42Myc was coupled (anti P42Myc). Anti c-Myc rabbit antiserum (anti c-Myc) was obtained from Medac (USA). The anti-Max, anti-Mad, anti-Mxi1, and anti-USF antibodies were obtained from Santa Cruz Biotechnology (USA). Anti N-Myc rabbit antiserum (anti N-Myc) was provided by K. Kato and H. Kondo (*25*). The anti c-Myc monoclonal antibody, MYC5, prepared against P42Myc was described previously as MYC-5 (*24*).

Oligonucleotide Probes—The CM1 probe (*11*) was prepared by annealing the following complementary oligonucleotides, 5′-GATCCCCCCACCACGTGGTGCCTGA-3′ and 5′-GATCTCAGGCACCACGTGGTGGGGG-3′. The single-stranded ends of the CM1 probe were ^{32}P-labeled by means of repair reaction with the DNA polymerase I Klenow fragment. The CM1mut probe contained a CAGCTG sequence instead of the CACGTG sequence in CM1. The control probe was the same size as CM1 but composed of an upstream sequence of the c-Myc gene, 5′-GATCCTCTTATGCGGTTGAATAGTG-3′, reported to be the replication origin by Ariga *et al.* (*26*).

DNA Immunoprecipitation Assay—The binding activity of Myc protein to specific oligonucleotides was determined by immunoprecipitation of DNA as described by Masumoto *et al.* (*27*) with modifications. Nuclear extracts or affinity-purified protein fractions were incubated with 0.5 ng of ^{32}P-labeled oligonucleotide probes in 100 μl of DNA-binding buffer (20 mM HEPES, pH 7.6, 1 mM EDTA, 1 mM DTT, 150 mM NaCl, 10% glycerol, 0.5% NP40, 4 mg/ml BSA, and the protease inhibitors described above) containing the indicated amounts of sonicated salmon sperm DNA (ssDNA) for 1 h at 0°C. Then 1 μl or the indicated amounts of antisera or 0.5 μg of affinity-purified IgG were added to the mixture, and after incubation for 1 h at 0°C, 25 μl of protein A Sepharose, which was diluted twofold with the DNA-binding buffer, was added to the mixture, followed by 30 min incubation at 4°C with gentle agitation on a rotator. The mixture was centrifuged, and the precipitate was washed twice with 1 ml of the DNA-binding buffer at 4°C and its radioactivity was determined with a liquid scintillation counter.

Immunoaffinity Purification—For immunoaffinity purification of Myc protein, affinity resins were prepared as follows. Protein A-purified anti c-Myc monoclonal antibody, MYC5, was coupled to a 1 ml Hi-Trap NHS Sepharose column (5 mg IgG/ml gel) (MYC5 Sepharose). Hi-Trap NHS Sepharose without the coupling reaction was used as a control. The 0.35 M NaCl nuclear extract from HL60 cells was supplemented with 0.5% NP40 and applied to a control Sepharose column. The flow-through fraction was directly applied to a MYC5 Sepharose column. Then each Sepharose column was washed successively with low salt washing buffer (the extraction buffer plus 0.35 M NaCl and 0.5% NP40), high salt washing buffer (the extraction buffer plus 1 M NaCl and 0.5% NP40), and low salt washing buffer. Proteins bound to MYC5 Sepharose or control Sepharose were then eluted with 7.5 M guanidine hydrochloride (the immunoaffinity-purified Myc fraction or the control fraction, respectively).

DNA Sepharose Column Chromatography—DNA Sepharose was prepared with the CM1 oligonucleotide as described by Muro *et al.* (*28*). A 200 μl aliquot of the affinity-purified c-Myc fraction was diluted 100-fold with DNA-binding buffer containing 150 mM NaCl, mixed with 200 μl of CACGTG sequence-containing oligonucleotide Sepharose, and then incubated for 2 h on a rotator at 0°C. The mixture was loaded onto a column and washed with DNA-binding buffer containing 150 mM NaCl. The bound protein was then eluted stepwise with 2 ml aliquots of DNA-binding buffer containing 200 mM to 1 M NaCl.

SDS-PAGE and Transfer to Membranes—SDS-PAGE was performed by standard methods under reducing conditions (*29*). Proteins were visualized by CBB staining or transferred to PVDF membranes (Millipore, USA) according to the method of Towbin *et al.* (*30*) except for the use of blotting buffer without SDS.

Immunoblotting—All steps were performed at room temperature. The protein-blotted membranes were preincubated with 10% skim milk in TPBS (154 mM NaCl, 10 mM Na-phosphate buffer, pH 7.6, and 0.05% Tween 20) for 1 h and then incubated with monoclonal antibody MYC5 or affinity-purified rabbit c-Myc specific IgG (1 μg IgG/ml) in TPBS for 1 h. After washing with TPBS five times (1 min × 2, 10 min × 3), the membranes were incubated in TPBS with anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad, USA) (1:3,000 dilution), and washed with TPBS five times, and then signals were detected on Kodak RP film using an ECL system (Amersham, UK).

Electrophoretic Mobility Shift Assay (EMSA)—Protein fractions were incubated with ^{32}P-labeled oligonucleotides in EMSA buffer (20 mM HEPES, pH 7.6, 1 mM EDTA, 1 mM DTT, 150 mM NaCl, 4% Ficol 400, 0.5% NP40, 4 mg/ml BSA, and the protease inhibitors described above) containing the indicated amounts of ssDNA for 1 h at 0°C. Then the mixtures were electrophoresed on 5% polyacrylamide gels (acrylamide:bis acrylamide, 80:1) in 0.25 × TBE buffer (1 × TBE: 50 mM Tris borate and 1 mM EDTA) at 20 V/cm and 4°C. The gels were dried and exposed to imaging plates (Fuji Photo Film, Tokyo), and signals were visualized with a Bio Image Analyzer (Fuji Photo Film).

Modified Southwestern Blotting—P42Myc or immunoaffinity-purified fractions were electrophoresed on 12.5% SDS polyacrylamide gels and then transferred to PVDF membranes. The following steps were performed at 4°C. The proteins bound to membranes were denatured with 6 M guanidine hydrochloride in 20 mM HEPES, pH 7.6, 1 mM EDTA, and 1 mM DTT for 1 h, and then renatured in protein binding buffer (20 mM HEPES, pH 7.6, 1 mM EDTA, 1 mM DTT, 0.35 M NaCl, 10% glycerol, 0.5% NP40, and the protease inhibitors described above) for 1 h. The membranes were blocked with 10% skim milk and 1% BSA in the protein binding buffer for 1 h, and then incubated with P42 Myc (10 μg/ml) or the immunoaffinity-purified c-Myc fraction (1:20 dilution) in the protein binding buffer for 1 h. After washing twice with protein binding buffer and twice with the same buffer containing 1 M instead of 0.35 M NaCl, the membranes were blocked again with 10% skim milk and 1% BSA in DNA-binding buffer, and then incubated with ^{32}P-labeled oligonucleotides (10 ng/ml) in DNA-binding buffer containing 1 μg/ml ssDNA for 1 h. After being washed four times for 5 min each, the membranes were exposed to imaging plates at 4°C, and the signals were visualized with a Bio Image Analyzer.

Phosphatase Treatment—Phosphatase treatment was carried out as described by Kitagawa *et al.* (*31*) with modifications. Nuclear extracts were prepared from HL60 and IMR32 cells in the presence of phosphatase inhibitors (50 mM NaF and 10 mM sodium phosphate buffer, pH 7.6). Proteins coupled to MYC5 Sepharose were washed twice with dephosphorylation buffer (50 mM Tris HCl, pH 8.0, 5 mM $MgCl_2$, 100 mM NaCl, and protease inhibitors) in the presence or absence of the phosphatase inhibitors, suspended in the same buffer, and then incubated with or without calf intestine alkaline phosphatase (250 U/ml) (Boehringer Mannheim, Germany) for 30 min at 37°C. The reaction was stopped by washing twice in dephosphorylation buffer containing phosphatase inhibitors.

RESULTS

Detection of DNA-Binding Activity of Myc Proteins in Crude Nuclear Extracts by DNA Immunoprecipitation—We tried to detect and characterize the DNA-binding activity of Myc protein in crude cell extracts using a DNA immunoprecipitation assay involving rabbit anti c-Myc and anti N-Myc polyclonal antisera. Nuclear extracts were prepared from HL60 and IMR32 cells, overexpressing c-Myc and N-Myc, respectively, and DNA immunoprecipitation assays were performed as described under "MATERIALS AND METHODS." The rabbit anti c-Myc and anti N-Myc polycolonal antisera used did not cross-react with each other on immunoblotting (data not shown). The two anti c-Myc antisera (anti-P42Myc and anti c-Myc) specifically precipitated CM1 oligonucleotides containing the CACGTG sequence from the reaction mixture with the HL60 nuclear extract at almost the same efficiency (Fig. 1A). One microliter of antiserum was sufficient for this assay. A low level of non-specific precipitation was observed with control serum. To reduce the background further, affinity-purified IgG was used where indicated. From the HL60 nuclear extract, CM1 oligonucleotides were precipitated with the anti c-Myc antiserum, and from the IMR32 nuclear extract with the anti N-Myc antiserum. The CM1mut oligonucleotide, containing the CAGCTG sequence in place of the CACGTG sequence, was precipitated with neither the anti c-Myc nor the anti N-Myc antiserum

(Fig. 1B). These results indicated that the DNA immunoprecipitation assay was easy and useful for detecting and estimating the sequence-specific DNA-binding activity of Myc proteins.

It has been reported that c-Myc protein is tightly associated with the nuclear matrix and hardly extractable under mild conditions (*32, 33*). By increasing the concentration of sodium chloride up to 0.35 M without any detergent or denaturing agent, the amount of c-Myc protein extracted from HL60 nuclei increased, but a further increase in the salt concentration did not affect the efficiency of the extraction (Fig. 1C, lower panel). The DNA-binding activity of c-Myc protein in each extract increased essentially in parallel with the amount of c-Myc protein in the extract (Fig. 1C, upper panel). These results indicated that both the c-Myc and N-Myc proteins are extractable under mild conditions and that this approach is useful for the characterization of DNA-binding properties.

Immunoaffinity Purification of c-Myc Protein and Other CACGTG Sequence-Binding Proteins Using the MYC5 Monoclonal Antibody—NaCl nuclear extracts (0.35 M) from HL60 cells were immunoaffinity purified using columns on which the anti c-Myc monoclonal antibody, MYC5, was immobilized (*23*). High efficiency absorption of c-Myc and Max proteins was achieved with efficiencies, as estimated on immunoblotting, of about 90 and 98%, respectively (Fig. 2B). The recovery of c-Myc protein from the MYC5 column in the eluate was estimated on immunoblotting to be 27%, and that of DNA-binding activity by means of DNA immunoprecipitation assay to be about 50% (data not shown). For the immunoaffinity-purified fraction, a number of polypeptide bands were detected on CBB staining (Fig. 2A). However, when the MYC5 monoclonal antibody or anti-P42Myc polyclonal antibody was used for immunoblotting analysis, bands corresponding to the full length c-Myc protein and many possible degradation products were detected (Fig. 2C). P18 was detected strongly with MYC5 but very weakly with anti-P42Myc on immunoblotting. Some of the bands for the immunoaffinity-purified fraction in Fig. 2A were not detected with either antibody on immunoblotting, but recognized by anti-Max, anti-Mad, or anti-USF antibodies (Fig. 2C). These results suggested that MYC5 had reacted with the native forms of CACGTG sequence-binding proteins in solution, but that when the proteins were immobilized on membranes it was only able to react with Myc and P18. The amino acid sequences of c-Myc and other CACGTG sequence-binding proteins are highly conserved in their basic regions which are essential for their sequence-specific DNA-binding activities (Fig. 2D). To confirm the epitope of the MYC5 antibody, we synthesized two peptides, one containing the basic region of c-Myc protein (amino acid nos. 353-375) and other containing its helix region, which is most highly conserved between c-Myc and N-Myc (amino acid nos. 373-403), and then measured the affinity of the MYC5 antibody to these peptides by means of an enzyme-linked immunosorbent assay (ELISA). A specific interaction only occurred with the peptide containing the basic region of c-Myc protein (data not shown). This result is consistent with the fact that the immunoaffinity-purified fraction also contained other CACGTG sequence-binding proteins, such as Max, Mad, and USF.

c-Myc protein by itself cannot bind to the CACGTG sequence, requiring another bHLHLZ protein, such as Max protein, for the binding activity (*12*). Thus, in crude nuclear extracts or immunoaffinity-purified fractions, Myc proteins were demonstrated to exhibit specific DNA binding, while the human truncated c-Myc protein synthesized

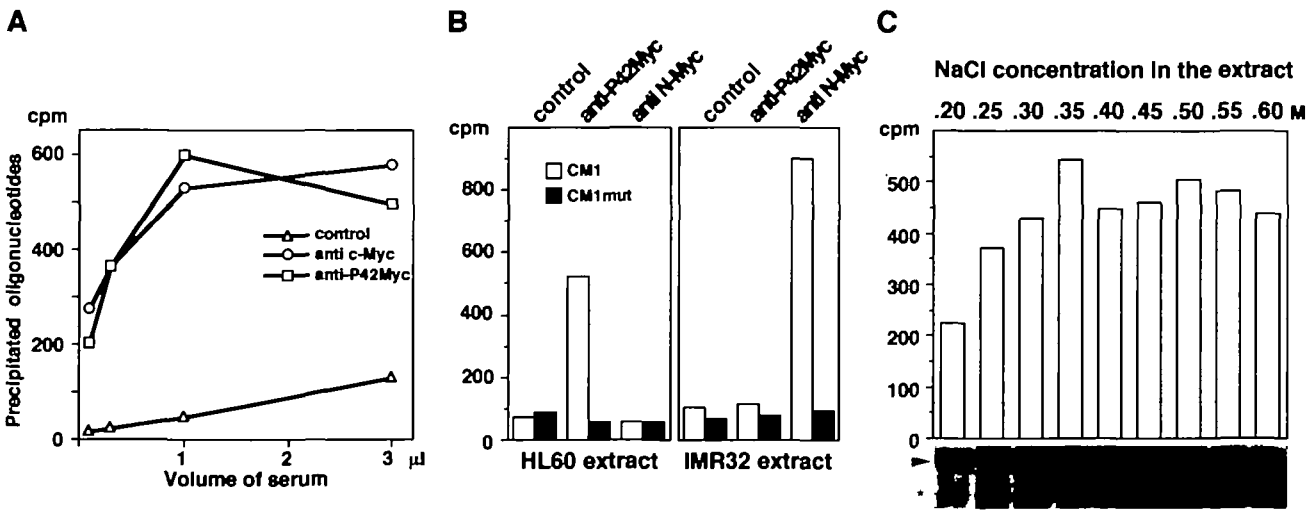

Fig. 1. **DNA immunoprecipitation assay of nuclear extracts.** The DNA-binding activities of Myc proteins in nuclear extracts were estimated by means of a DNA immunoprecipitation assay with anti Myc antibodies. Ten microliters of nuclear extract was incubated with ^{32}P-labeled CM1 or CM1mut oligonucleotides in DNA-binding buffer containing 5 μg of ssDNA. (A) A 0.35 M NaCl nuclear extract from HL60 cells was incubated with CM1 oligonucleotides, and protein-DNA complexes were precipitated with the indicated amounts of two distinct anti c-Myc antisera, anti-P42Myc (circles) and anti c-Myc (squares), or control serum (triangles). (B) 0.5 M NaCl nuclear extracts from HL60 and IMR32 cells were incubated with ^{32}P-labeled CM1 (clear bars) or CM1mut (shadow bars) oligonucleotides, and protein-DNA complexes were precipitated with 1 μl of anti-P42Myc, anti N-Myc, or control antiserum. (C) Nuclear extracts from HL60 cells treated with the indicated concentrations of sodium chloride were incubated with ^{32}P-labeled CM1 oligonucleotides, and protein-DNA complexes were precipitated with 0.5 μg of affinity-purified anti-P42Myc antibodies. c-Myc proteins in each extract were detected by immunoblotting (lower panel). The arrowhead indicates intact c-Myc proteins and the asterisk indicates c-Myc degradation products.

in bacteria, P42Myc, was found to exhibit only very weak and nonspecific DNA-binding activity (*24*). To detect the presence of the partner protein of c-Myc in the immunoaffinity-purified fractions, the changes in the DNA-binding activities of the fractions were determined after heterodimerization with externally added P42Myc. In parallel with the increase in P42Myc added to the fractions, the DNA-binding activity of c-Myc determined on the immunoprecipitation with the anti-P42Myc antibody increased, whereas that of P42Myc alone gave a background value (Fig. 3A). Furthermore, when P42Myc was added to the immunoaffinity-purified N-Myc fraction, the DNA-binding activity of c-Myc determined with the anti-P42Myc antibody increased, while that of N-Myc determined with the anti N-Myc antibody decreased (Fig. 3B). These results indicated that in the immunoaffinity-purified fractions, excess c-Myc partner proteins were present, and c-Myc proteins competed with N-Myc proteins for the partner proteins. The results also suggested that the MYC5 antibody directly reacted with the partner proteins.

The Major Proportion of Immunoaffinity-Purified c-Myc Does Not Show Affinity for the CACGTG Sequence—To determine what proportion of the cellular c-Myc protein binds to the specific sequence, the immunoaffinity-purified fraction was diluted and mixed with oligonucleotide Sepharose. The DNA-binding activity of c-Myc protein remaining in the supernatant fraction was reduced to about 20% of that of the starting material, as measured by the

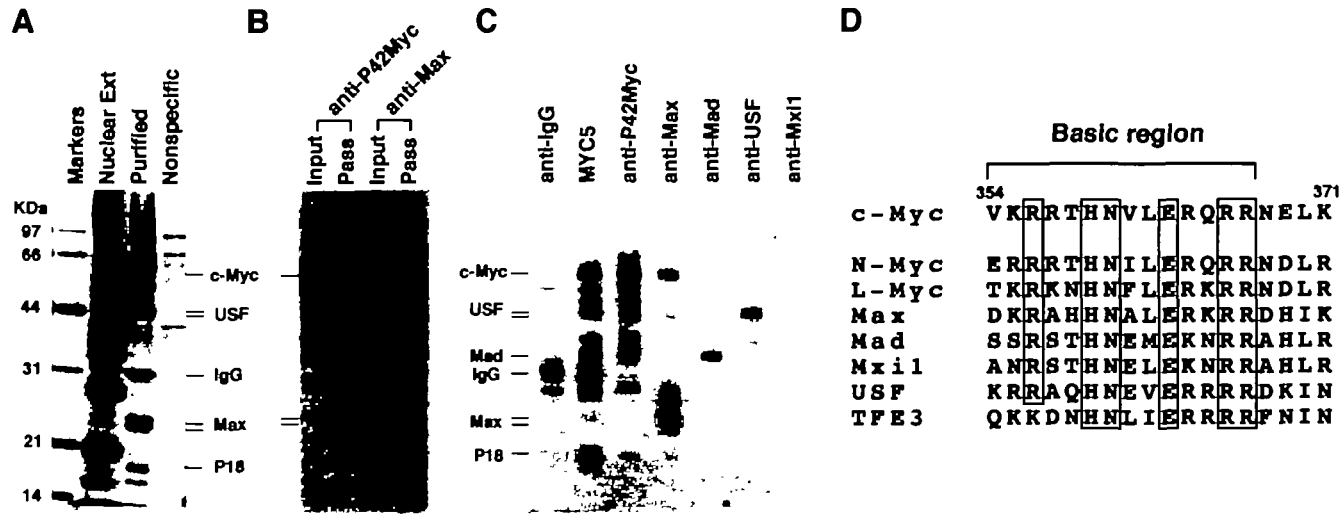

Fig. 2. **Immunoaffinity purification of c-Myc protein using the anti c-Myc monoclonal antibody, MYC5.** Proteins in various fractions from the MYC5 column were separated by 12.5% SDS-PAGE, and visualized by CBB staining (A) or immunoblotting with various antibodies (B, C). (A) Bio-Rad molecular weight markers, 0.25 μg/band (Markers), the 0.35 M NaCl nuclear extract of HL60 cells, equivalent to 2×10^8 cells (Nuclear Ext), the immunoaffinity-purified fraction, equivalent to 2×10^9 cells (Purified), and the fraction nonspecifically bound to Sepharose beads, equivalent to 2×10^9 cells (Nonspecific), were loaded. (B) The absorption efficiency of the immunoaffinity column was estimated by immunoblotting. The input fraction, equal to the nuclear extract of HL60 cells, and the flow-through fraction from the immunoaffinity column were separated by SDS-PAGE. To each lane, a sample equivalent to 2×10^6 cells was loaded. c-Myc and Max proteins were detected by immunoblotting with anti-P42Myc or anti-Max antibodies. (C) Detection of various CACGTG sequence-binding proteins in immunoaffinity-purified fractions by immunoblotting with various antibodies. A sample equivalent to 2×10^6 cells was loaded on each lane and proteins were detected with the antibody shown at the top of each lane. (D) Comparison of the amino acid sequence of the basic region of c-Myc protein (amino acid nos. 354-371) with those of other CACGTG sequence-binding proteins.

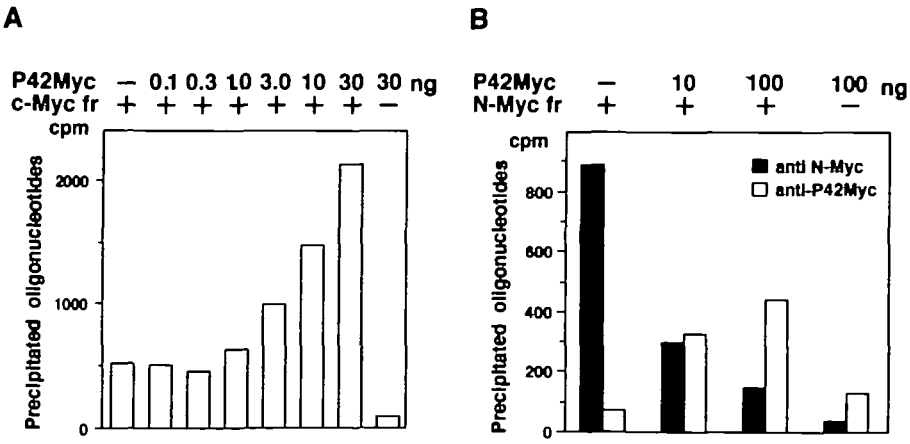

Fig. 3. **DNA immunoprecipitation assay of immunoaffinity-purified fractions in the presence or absence of P42Myc.** The DNA-binding activities of c-Myc and N-Myc proteins were determined by DNA immunoprecipitation assay. The indicated amounts of P42myc were added to DNA-binding buffer containing 100 ng of ssDNA in the presence (+) or absence (−) of 0.5 μl of the immunoaffinity-purified c-Myc (A) or N-Myc (B) fraction from HL60 or IMR32 cells, respectively, and then incubated with CM1 oligonucleotides. Protein-DNA complexes were precipitated with 1 μl of anti-P42Myc (clear bars) or anti N-Myc (shadow bars) antiserum.

DNA immunoprecipitation assay (Fig. 4B), while the amount of c-Myc protein detected on immunoblotting was almost the same as that of the starting material (Fig. 4A). In contrast, the c-Myc protein recovered from the DNA-affinity column by stepwise elution with NaCl buffer was estimated on immunoblotting to be less than 10%, but the recovery of DNA-binding activity was estimated on DNA immunoprecipitation to be 140%. The peak fraction was eluted with 0.6 M NaCl. The NaCl concentration used for preparation of nuclear extracts was 0.35 M NaCl. Thus, although the partner proteins of c-Myc required for DNA-binding activity (Max) were present in excess of c-Myc (Fig. 3A), in the immunoaffinity-purified fraction only a small proportion of c-Myc proteins possessed DNA-binding activity. This suggests that efficient DNA binding of c-Myc protein requires either other cellular factors or modification of c-Myc or partner proteins.

The MYC5 Monoclonal Antibody Widely Cross-Reacts to CACGTG Sequence-Binding Proteins and Inhibits Their DNA Binding—To examine the cross-reactivity of the MYC5 antibody to CACGTG sequence-binding proteins, EMSA was carried out using the input, flow-through, and purified fractions from the immunoaffinity column. Using the input fraction (crude extract), several DNA-protein complexes (complexes A to E) were observed with the CM1 oligonucleotide (Fig. 5A, lane 1), while with the flow-through fraction, only complexes C and D were detected (lane 2). Using the immunoaffinity-purified fraction, complexes A, B, B′, E, F, and G were detected (lane 3). On the other hand, with the CM1mut probe, the EMSA patterns did not differ between the input and flow-through fractions except for complex B, which was commonly observed with both the CM1 and CM1mut probes (lanes 4 and 5). Using the control probe, which was of the same size but composed of the upstream sequence of c-Myc, no difference was observed between the two fractions (lanes 7 and 8).

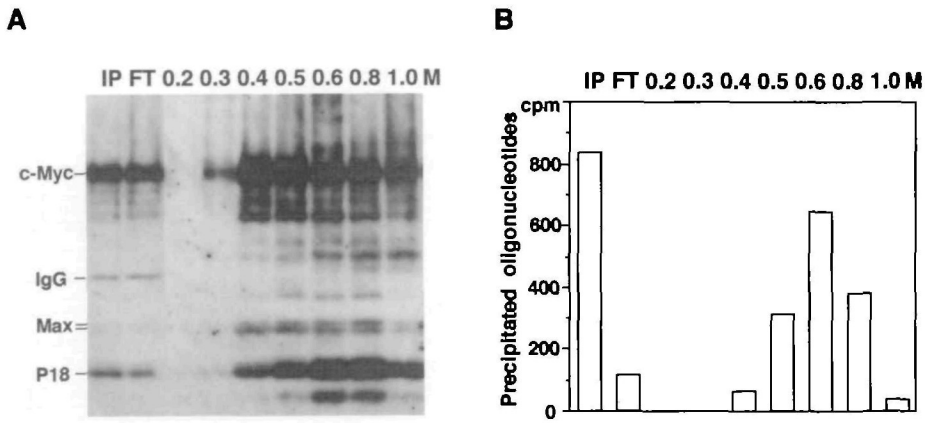

Fig. 4. **DNA affinity chromatography of the immunoaffinity-purified fraction.** The immunoaffinity-purified fraction was diluted and mixed with oligonucleotide-Sepharose. Bound proteins were eluted stepwise with buffer containing the indicated concentrations of NaCl. (A) The input fraction (IP), flow-through fraction (FT), and eluted fractions precipitated with acetone were dissolved in the sample buffer, subjected to SDS-PAGE, transferred to a membrane, and then probed with the MYC5 monoclonal antibody. IgG indicates the light chain of IgG. (B) The DNA-binding activity of each fraction was determined by DNA immunoprecipitation assay. One hundred microliter aliquots of the input and flow-through fractions were incubated with ^{32}P-labeled CM1 oligonucleotides, and protein-DNA complexes were precipitated with affinity-purified anti-P42Myc antibodies. Ten microliters of each fraction eluted from the column was diluted 10-fold with the DNA-binding buffer and adjusted to a final NaCl concentration of 150 mM, and then the DNA-binding activity was determined.

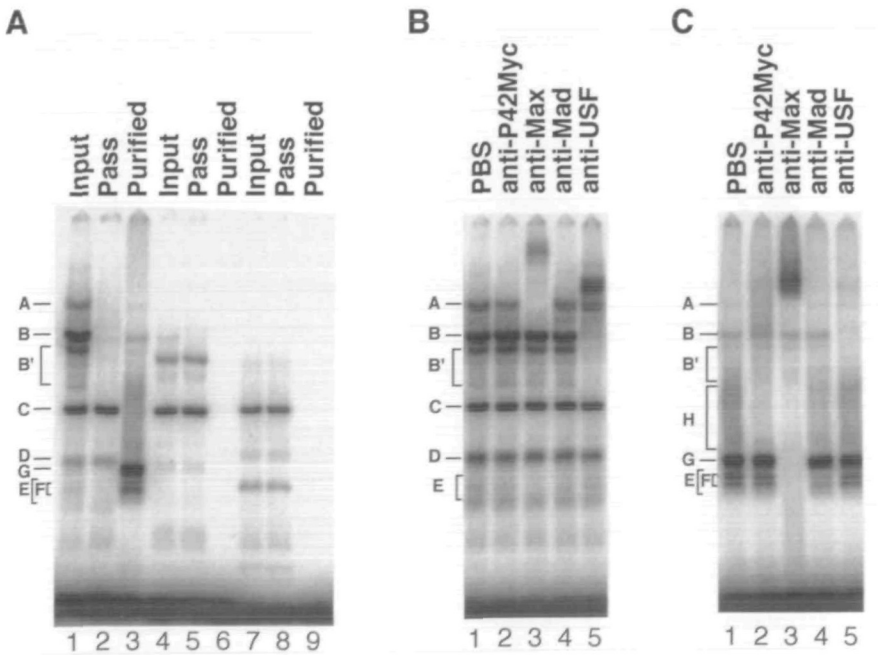

Fig. 5. **Detection of CACGTG sequence-binding protein in the immunoaffinity column fractions by EMSA.** EMSA was carried out using reaction mixtures containing the ^{32}P-labeled CM1, CM1mut or control oligonucleotide probe, and 1 μl of the input (Input) or flow-through (Pass) fraction, or 0.1 μl of the immunoaffinity-purified c-Myc fraction using the MYC5 antibody (Purified). 1 μg of ssDNA was added to the reaction mixture for the input and flow-through fractions, and 100 ng for the purified fraction. (A) EMSA was performed with the indicated samples, and the ^{32}P-labeled CM1 (1-3), CM1mut (4-6), or control (7-9) oligonucleotide probe. (B) The input fraction and (C) immunoaffinity-purified fraction were preincubated with the CM1 oligonucleotide probe to form DNA-protein complexes, and then 1 μl of the indicated antibody (1 mg/ml) or PBS was added and the reaction products were analyzed by EMSA.

Complex C was commonly observed with all three probes, indicating nonspecificity. The purified fraction gave no band with either the CM1mut or the control probe (lanes 6 and 9). These results indicated that the MYC5 antibody on the immunoaffinity column widely cross-reacted with proteins having CACGTG specific binding ability.

To identify the proteins in each EMSA complex with the input and immunoaffinity-purified fractions, supershift analysis were carried out using anti c-Myc, anti-Max, anti-Mad, and anti-USF antibodies (Fig. 5, B and C). Complexes A, E, and G disappeared on addition of anti-Max, complexes B and B′ with anti-USF, complex F with anti-Max or anti-Mad, and complex H with anti-Max or anti c-Myc. With the input fraction, complex A was the main complex which disappeared with the anti-Max antibody, while with the purified fraction, complex A was minor and the main complexes which disappeared were G and F. Complex G presumably comprises a Max homodimer because the same bands were detected on EMSA using the Max-containing gel-filtration fractions under denaturing conditions (data not shown). Complex A appeared to contain Max and a protein with a higher molecular weight. The Max protein formed different complexes in the input and purified fractions, and the c-Myc/Max heterocomplex could not be detected on EMSA using the nuclear extract in this experiment. With the purified fraction, the complexes in region H disappeared on the addition of either anti c-Myc or anti-Max, indicating that they are c-Myc/Max heterocomplexes. The results suggest that the MYC5 monoclonal antibody recognizes the basic regions of bHLH family proteins which are commonly required for sequence-specific DNA binding. We therefore examined by EMSA whether or not the antibody affects the specific DNA-binding activities of these proteins. The addition of the MYC5 antibody to the reaction mixture prior to the formation of DNA-protein complexes diminished complexes A, B, B′, and E in a short time (Fig. 6A), producing an identical pattern to that observed with the immunoaffinity column flow-through fraction (Fig. 5A, lane 2). Time course analysis revealed different patterns of diminution for each complex when the antibody was added to the reaction mixture after the formation of DNA-protein complexes. Complex A diminished very rapidly, and then complex E and complexes B and B′ more slowly (Fig. 6B). The pattern after 16-h incubation with the MYC5 antibody was nearly identical to that of the flow-through fraction from the immunoaffinity column. As the inhibitory effect of the MYC5 antibody on each complex was observed with the same efficiency when the antibody was added to the mixture prior to the formation of DNA-protein complexes, the affinity for each protein appeared essentially constant (Fig. 6A). Therefore, the different effect of MYC5 on each complex may not depend on the affinity of the antibody to each protein, but rather on the stability of each DNA-protein complex.

Analysis of c-Myc Partner Proteins as to DNA-Binding Reactions—Proteins other than Max have hitherto not been reported to be partners for c-Myc to effect on DNA binding. To explore this, modified Southwestern blotting analysis was carried out. This is a useful method for identifying sequence-specific DNA-binding proteins, if they bind as monomers or homodimers. However, it is difficult to identify the proteins which bind to DNA after hetero-oligomerization with other proteins. To detect DNA-binding activity, it is essential for c-Myc to form heterodimer complexes on the membrane prior to the probing reaction. The bacterially expressed P42Myc and proteins in the immunoaffinity-purified fraction were therefore separated

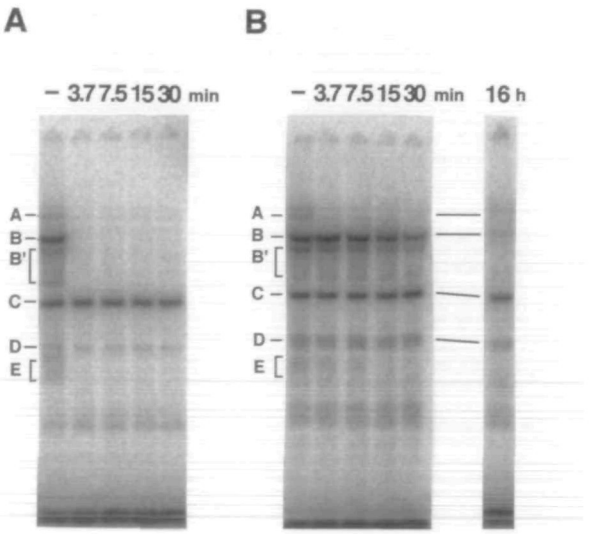

Fig. 6. **Inhibition of the DNA-binding activities of CACGTG sequence-binding proteins by the MYC5 monoclonal antibody.** The time course of inhibition of DNA-protein complex formation by the MYC5 monoclonal antibody was analyzed by EMSA. (A) Nuclear extracts were preincubated with the MYC5 monoclonal antibody for the indicated times prior to the addition of CM1 oligonucleotides, after which incubation was performed for 1 h. (B) Nuclear extracts were incubated with CM1 oligonucleotides for 1 h, and then 10 μg of the MYC5 monoclonal antibody was added and the incubation was continued for the indicated times.

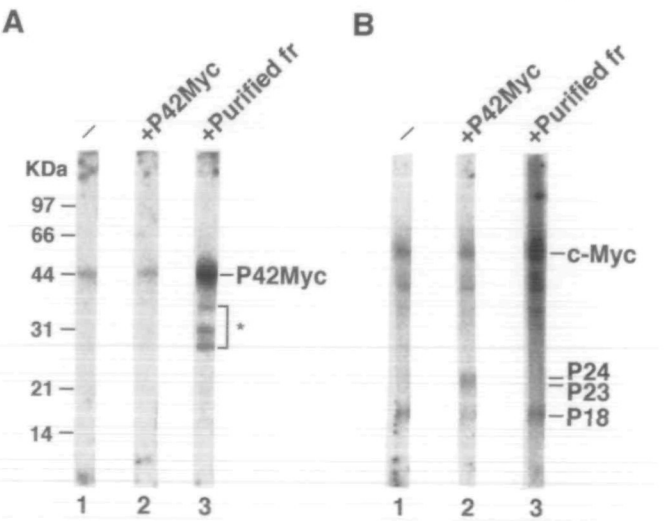

Fig. 7. **Detection of DNA-binding activity by modified Southwestern blotting.** Twenty nanograms of P42Myc (A) or 5 μl of the immunoaffinity-purified c-Myc fraction (B) were precipitated with acetone, subjected to 12.5% SDS-PAGE, and then transferred to membranes. The membranes were incubated with no protein (lane 1), or 10 μg/ml of P42Myc (lane 2) or the immunoaffinity-purified c-Myc fraction (1:20 dilution) (lane 3) in the protein binding buffer. After washing, the membranes were probed with ^{32}P-labeled CM1 oligonucleotides. The asterisk indicates P42Myc degradation products.

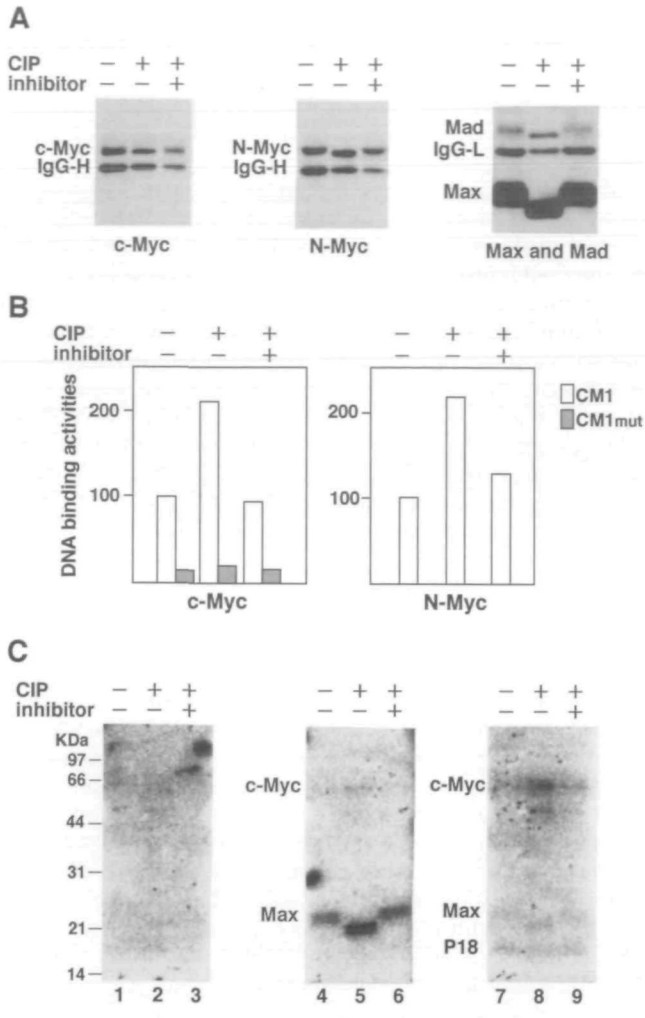

Fig. 8. **Phosphatase treatment of immunoaffinity-purified fractions.** NaCl nuclear extracts (0.35 M) were prepared from HL60 or IMR32 cells in the presence of phosphatase inhibitors and immunoaffinity purification was carried out with the MYC5-conjugated Sepharose. Then proteins bound to the Sepharose were treated with or without calf intestine alkaline phosphatase (CIP) in the presence or absence of the phosphatase inhibitor. Then aliquots of the MYC5-conjugated Sepharose were dissolved in Laemmli sample buffer for immunoblotting (A, left and middle panels). Bound proteins were eluted from the rest of the MYC5-conjugated Sepharose with 7.5 M guanidine hydrochloride, and then aliquots of the eluted proteins were used for the DNA immunoprecipitation assay (B), or immunoblotting (A, right panel) and modified Southwestern blotting (C) after precipitation with acetone. (A) Each sample was subjected to 12.5% SDS-PAGE, transferred to a membrane, and then probed with the MYC5 monoclonal antibody (left and middle panels) or the anti-Max and anti-Mad antibodies (right panel). IgG-H and IgG-L indicate the heavy and light chains of IgG. (B) Relative DNA-binding activities were determined by DNA immunoprecipitation assay. One microliter of the immunoaffinity-purified c-Myc or N-Myc fraction was incubated with ^{32}P-labeled CM1 (clear bars) or CM1mut (shadow bars) oligonucleotides in the presence of 100 ng ssDNA, and then protein-DNA complexes were precipitated with 1 μl of anti-P42Myc or anti N-Myc antiserum. (C) Modified Southwestern blotting was carried out as described in Fig. 7. The protein-blotted membrane was incubated with no protein (left panel), P42Myc (middle panel) or the immunoaffinity-purified c-Myc fraction (right panel), and then probed with ^{32}P-labeled CM1 oligonucleotides.

by SDS-PAGE and then transferred to a membrane, which was incubated with the P42Myc or immunoaffinity-purified fraction to form protein-protein complexes, before being probed with ^{32}P-labeled CM1 oligonucleotides (Fig. 7). Without any additional protein, P42Myc exhibited very weak DNA-binding activity, and after incubation with P42Myc, its activity did not increase. In contrast, after incubation with the immunoaffinity-purified fraction, the activity increased several fold. Only weak activities were detected with c-Myc and P18 without prior incubation with any proteins. After incubation with P42Myc, the activities of P23 and P24 became evident, but those of c-Myc and P18 did not change. P23 and P24 correspond to Max, as shown previously (Fig. 2, B and C). These results indicated that c-Myc protein forms complexes with P23 and P24, and that this increases DNA binding. After incubation with the immunoaffinity-purified fraction, the c-Myc and P18 activities increased several fold. Although the addition of the purified fraction did not stimulate the DNA-binding activities of P23 and P24 in this experiment (Fig. 7B, lane 3), we obtained stimulation in another experiment (Fig. 8C, lanes 7-9). These results suggest that the amounts of c-Myc protein in the purified fraction may be less than the Max protein level.

Phosphatase Treatment of the Immunoaffinity-Purified Fraction Increases the DNA-Binding Activity—To analyze the effect of phosphorylation on the DNA-binding activity of Myc, the immunoaffinity-purified c-Myc and N-Myc fractions were treated with phosphatase. Mobility shifts on SDS-PAGE with phosphatase treatment were observed with N-Myc protein but were only very slight, if any, with c-Myc. Furthermore, mobility shifts were observed with Max (P24, P23) and Mad (Fig. 8A). These results indicated that Max, Mad, and N-Myc are largely phosphorylated *in vivo*. DNA immunoprecipitation assay showed that DNA binding of c-Myc and N-Myc in the immunoaffinity-purified fraction was increased about twofold by phosphatase treatment (Fig. 8B). Further analyses were carried out with modified Southwestern blotting using the P42Myc and immunoaffinity-purified c-Myc fraction. After incubation with either, the binding activities of Max and c-Myc to CM1 oligonucleotides increased two- to threefold in samples treated with phosphatase (Fig. 8C). Neither the mobility nor the intensity of P18 changed (Fig. 8C). These results indicate that the *in vivo* phosphorylation of CACGTG sequence-binding proteins regulates their DNA-binding activities.

DISCUSSION

Many previous studies on Myc protein DNA-binding activity have been carried out, using recombinant proteins or *in vitro* translated proteins, by EMSA. However, the recent identification of Max (*12*), Mad (*14*), and Mxi1 (*15*) revealed the protein network regulating the sequence-specific DNA-binding activity of c-Myc (*16*). Many other CACGTG sequence-binding proteins, such as USF (*10*) and TFE3 (*9*), are now known to be present *in vivo*, and they compete with c-Myc for binding to the same sequences in chromosomal DNA. Their phosphorylation *in vivo* has been observed (*34*), and in many cases, it regulates their sequence-specific DNA-binding activities (*35*). Though c-Myc protein was reported to be tightly associated with

nuclei and hardly extractable with salt under mild conditions (*32*), we were able to extract an appreciable amount from HL60 or HeLa nuclei with 0.35 M NaCl without any detergent or denaturing agent (Fig. 1). Under the same conditions, N-Myc protein could be extracted from IMR32 nuclei. The yield of cellular c-Myc protein was in the range of 30 to 50%, and all of the CACGTG sequence-binding proteins extracted apparently retained their sequence-specific DNA-binding activities (Figs. 1, 4, and 5). Therefore, extraction under mild conditions is in fact suitable for analysis of the DNA-binding properties of c-Myc and other proteins.

The anti c-Myc monoclonal antibody, MYC5, originally raised against a protein synthesized in bacteria (*23, 24*), reacted with almost all proteins specifically binding to the CACGTG sequence, but not CAGCTG, except for one protein in complex B, which was formed with both sequences (Fig. 5A). In the immunoaffinity-purified fraction, some uncharacterized proteins were visualized on CBB staining, and one of them, P18, exhibited sequence-specific DNA binding, as revealed on DNA-Sepharose column chromatography (Fig. 4A) and modified Southwestern blotting (Fig. 7B). P18 was recognized strongly by the MYC5 antibody but only very weakly by anti-P42Myc or anti-Max (Fig. 2C). The fact that its DNA-binding activity was stimulated by the immunoaffinity-purified protein fraction but not by P42Myc (Fig. 7B) suggests that P18 is a novel CACGTG sequence-binding protein which has a homologous domain to Myc and Max, and can heterodimerize with a CACGTG sequence-binding protein other than c-Myc. Thus, unidentified CACGTG sequence-binding proteins may be present in the immunoaffinity-purified fraction, and direct cloning of the target proteins of the MYC5 antibody using expression libraries might allow their identification.

The observation of an inhibitory effect of the MYC5 antibody on DNA-binding activities of CACGTG sequence-binding proteins on EMSA (Fig. 6) is consistent with the results of epitope mapping analysis of the MYC5 antibody with a synthetic peptide containing the basic region, which is conserved among CACGTG sequence-binding proteins and essential for their specific binding (Fig. 2). In spite of the presence of an excess amount of the partner protein (Fig. 3), less than 10% of c-Myc protein in the fraction bound to DNA-Sepharose (Fig. 4), indicating that the remainder was in an inactive form, for example, monomers (*36*). The main DNA-protein complex in the immunoaffinity-purified fraction detected on EMSA using the CM1 probe contained Max but not c-Myc or Mad (Fig. 5). However, the Max-containing complex formed with the crude nuclear extract showed slower mobility than that formed with the purified fraction (complex A *vs.* complex G), and neither contained c-Myc or Mad (Fig. 5, B and C). The DNA-Max complex detected with the crude nuclear extract on EMSA (complex A) might therefore contain mSin3 and Mxi1 proteins in place of Mad (*37, 38*).

EMSA analysis detected c-Myc complexes only weakly with the purified fraction, and not at all with the crude nuclear extract. In contrast, c-Myc complexes were clearly detected on DNA immunoprecipitation assay or modified Southwestern blotting. EMSA takes about two hours to separate complexes in gels after their formation in the binding buffer, while the other approaches take only 15 to 20 min for washing after complex formation is completed. When the washing was prolonged in these assays, the DNA-binding activity of c-Myc decreased. Therefore, these methods, when conditions are carefully controlled, are superior to EMSA for detecting the DNA-binding activities of unstable complexes.

The present investigation of partner proteins of c-Myc by modified Southwestern blotting of the immunoaffinity-purified fraction containing almost all CACGTG sequence-binding proteins revealed 23 and 24 kDa proteins. These appear to be isoforms of previously reported Max proteins (P21 and P22) but with different mobilities on SDS-PAGE due to *in vivo* phosphorylation. In the immunoaffinity-purified fraction almost equal amounts of the two proteins were present (Fig. 2B), and their DNA-binding activities detected on modified Southwestern blotting were apparently the same (Figs. 7 and 8). The latter method can be concluded to be convenient for comparing the DNA-binding activities of many proteins simultaneously. In addition, it is applicable for analysis of the effect of phosphorylation on the DNA-binding activity if the mobility on SDS-PAGE differs between phosphorylated and dephosphorylated forms.

c-Myc and Max are known to be phosphorylated *in vitro* by CKII, and the DNA-binding activity of Max homodimers, but not that of Myc/Max heterodimers, has been reported to be thereby inhibited (*34*). The phosphorylation of amino-terminal sites of Max by CKII may, however, increase both the on- and off-rates of Max homo- as well as Myc/Max heterodimers, and thus result in an apparent decrease in binding (*22*). c-Myc is also phosphorylated *in vitro* by at least three kinases other than CKII (*17, 19*), and mitosis-specific phosphorylation has been observed and reported to reduce nonspecific-binding activity to DNA cellulose (*39*). In this study, the immunoaffinity-purified fraction contained the phosphorylated forms of Max, Mad, and N-Myc, as shown by the mobility shifts on SDS-PAGE, while with the c-Myc protein, presumably comprising the phosphorylated form, no mobility shift was observed (Fig. 8). The fact that phosphatase treatment of these fractions increased the DNA-binding activity of c-Myc or N-Myc about twofold (Fig. 8) suggests that the DNA-binding activity of c-Myc is regulated by phosphorylation *in vivo*.

In conclusion, this study showed that many CACGTG sequence-binding proteins are present in nuclear extracts, c-Myc being only a minor constituent. The MYC5 antibody is very useful not only for the purification but also the characterization of DNA-protein complexes. Furthermore, this antibody might be used to find other members of the Myc superfamily or CACGTG sequence-binding proteins.

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