Cell Research(1995),5,25–34

Affinity chromatography-dependent selection (ACDS) of genomic DNA fragments bound specifically to bacterial synthesized Myc / Myn proteins[1,2]

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ABSTRACT

This paper describes an approach to seek for mouse c-Myc/Myn proteins-bound specific sequences among genomic DNA. cDNA fragment of myn gene was obtained through RT-PCR technique from RNA of NIH3T3 cells. DNA fragments encoding BR/HLH/LZ structure of Myc and Myn proteins were cloned in frame into pGEX-2T vector respectively. Fusion GST-Myc and GST-Myn synthesized in E.coli hosts showed affinity to CACGTG E-box DNA and subsequently interacted with genomic fragments prepared through whole-genome-PCR. A PCR-assisted procedure which combines protein-DNA interaction and affinity chromatography was designed to enrich Myc/Myn bound DNA. At least two genomic DNA fragments obtained exhibit specifical binding capacity to Myc/Myn complex but not to GST alone. Significance of the work and of the technique itself as well as identification of the DNAs are discussed.

Key words: *Myc / Myn proteins, ACDS, genomic DNA binding.*

1. The work is specifically dedicated to Prof. Zhen YAO for his 80-years birthday.
2. Supported by grant No. 39270742 of the National Natural Science Foundation.
3. Both made equal contribution to the work.
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INTRODUCTION

The protooncogene c-myc has been shown tightly related to cell growth, differentiation, cell cycle progression and tumorigenesis, even apoptosis (see review[1,2]). c-Myc protein seems playing important roles in these processes. Although the anthentic functions of c-Myc protein have still been obscure, achievements have been made during recent years since the elucidation of "basic region-helix-loop-helix-leucine-zipper" (BR /HLH /LZ) structure in Myq and discoveries of partner proteins of Myc including Max[3] / Myn[4], Mad[5] and Mxi1[6]. Myc protein, similiar to other BR /HLH /LZ proteins, possesses specific DNA binding activity to CACGTG (E-box) sequence[7,8]. Myc is therefore supposed to function as transcription regulator while cooperates with its partner(s).

It has been proposed that heterodimer formed bv Mvc and Max /Myn or so on can bind CACGTG or similiar consensus of downstream target gene(s) thereby affecting expression of these gene(s) (see review[2]), arid subsequently determine destiny of the cell. Several Myc-regulated genes have been proposed, including p53[9], ornithine decarboxylase gene (ODC)[10], or-prothymosin(α -PT)[11]and ECA39[12]. Recent finding, however, that Myc may participate TFII-1 mediated transcription initiation[13], raised an interesting question that if Myc may involve in general transcription machinery.

It is therefore still necessary to make further efforts for the identification of Myc-regulated target genes or DNA elements. Through SAAB technique, some authors have obtained important information of Myc-bound sequences aiming at characterization of the genes. However, data from Myc bound genomic DNA which may reflect more confident cases *in vivo* is limited.

In this paper we describe our approach to investigate Myc-bound DNA targets. Mouse fragments of c-myc / myn genes were cloned into expressing vector, the Myc and Myn proteins fragments with their BR / HLH/LZ structures were expressed from bacteria. An affinity chromatography-dependent Myc / Myn-DNA binding system was designed and used successively to screen PCR-amplified genomic DNA flagments. At 1east two interesting fragments were obtained and put in further analysis.

MAT'ERIALS AND METHODS

DNA, enzymes, bacteria and chemicals

Plasmid pGEX-2T (Pharmacia) is a gift of Prof. U. Schwarz and Prof. W. Hennig of Marx-Planck Institution. pSVc-myc-1 is supplied by Dr. M. Habernig, University of Vienna. pUC119 is product of Promega. Oligonucleotide primers for synthesis of c-myn gene fragment were synthesized by Eurogenetic. A 20mers/ 16mers adaptor-primer (adaptor 20/ 16) used for whole genome PCR was synthesized in CeU Co.. Restriction enzymes BamH I, Xho I, Sca I, EcoR I, S ma I, Pst I, Ava II, Polynucleotide Kinase, T4 DNA ligase, MMLV reverse transcriptase, Klenow flagment, dNTPs were purchased from Promega, BRL, Pharmacia respectively. Glutathione-agarose beads, glutathione, poly (dI-dC)·poly(dI-dC), Hae III digested pBR322 DNA markers, acrylamide are products of Sigma. E.coli strains JMl01, JMl09 were purchased from Promega. Conventional

chemicals were basically obtained from Shanghai Chemicals Co..

c-Myn cDNA fragment synthesis

c-Myn gene fragment from nucleotide No. 174 to No. 499[4] was amplified from total RNA of NIH3T3 cells through RT-PCR directed by 5' primer 5' ATGACATCGAGGTGGAGAGCGAC3' and 3' primer 5' GGTCTGCAGTTGGGCACTTGATCT 3'[4]. One microgram of total RNA was mixd with the following: 4 μl 5 x reverse transcriptase buffer (BRL), 0.1 μl 3'primer (100 pmol/ μl), 2μl of each dNTP (5 mM /μ1), 0.25μl RNase inhibitor (37 u/μ1), 2μ1 MMLV reverse transcriptase (BRL, 200 u /μl), in 20μl total volume. The mixture was incubated at 56℃ for 60 min. The sample was then heated to 95℃, 5 min, cool in ice-cold water. For PCR amplification, 1μl above RT product was mixed with 10 pmo13' primer and 10 pmol 5' primer in total volume of 25 μl containing 1 x Taq buffer (10 m*M* Tnis·HCl, pH 8.3, 50 m *M* KCl, 3 m *M* $MgCl_2$, 0.01%(W/V) gelatin) and 1u Ampli Taq DNA polymerase (Perkin Cetus). The mixture was amplified for 30 cycles each consists of 95℃, 30 sec, 60℃, 60 sec, 72℃, 120 sec, with final extension 72℃ 10 min. A single approximate 300 bp cDNA band was identified in 3% Nusieve agarose + 1% conventional agarose gel. The band was then recovered and purified for ligation.

DNA and RNA manipulation

Mini-and medi- preps of plasmid DNA were done as described[14]. The method was also used to prepare DNA for sequencing employing Taq-Track DNA Sequencing System (Promega) with a little modification. Total RNA was purified from NIH3T3 cell according to methods of Strohman[15].

Cloning of c-myc and c-myn DNA fragments to expressing vector

A ~290 bp mouse c-myc fragment of exon 3 was excised by BamH I and EcoR I from pUC119-myc which contains Ava II / Xho I portion of c-myc exon 3 originally derived (unpublished data, Wang et a1.) from pSVc-myc-1[16] and ligated to BamH I /EcoR I sites of pGEx-2T (Phamacia). Correction of reading frame of selected colony named as pGEX-Myc was confirmed by sequencing of Sca I-EcoR I fragment of this pGEX-Myc recloned in pBluscript II KS(+) (Stratagene). c-Myn cDNA was blunted by T4 DNA polymerase, and ligated to pUCl19. After transformation, 5 positive colonies containing insert were identified through dot-blot hybridization with [α ^{32}P]d CTP labelled myn DNA same as protocol described. Two positive orientated colonies were selected by Sma I digestion. Adjustment of BamH I site in 5'end of the insert was achieved by excising the myn DNA again from the vector with BamH I and ligated to pBluescript II KS(+) (Stratagene) and sequenced. DNA with expected 5' end BamH I site was then ligated to pGEX-2T thereafter called pGEX-myn. Similiar to pGEX-myc, correction of reading frame was confirmed by sequencing Sca I-EcoR I fragment of pGEX-myn recloned in pUCl19. pGEX-myc and PGEX-myn were then transformed to JM109 or JM101 in standard procedure described elsewhere.

Expression, isolation and affinity purification of GST-Myc and GST-Myn Proteins

Procedure for selection of minipreps, isolation and purification of both GST-Myc and GST-Myn proteins were basically as described[17] with small modification. 12% polyacrylamide gel was used to check quality and quantity of the proteins. Proteins were visulized through Coomassie blue or silver stain in methods described elsewhere.

Preparation of 1abelled E box-containing DNA

15 mers oligonucleotides NNNNNNGACCACGTG was annealed in 25℃, 30 min, then filled by Klenow fragment of DNA polymerase 1 with [α ^{32}P]dCTP as tracer becoming 24 bp dsDNA and end labelled through phosphorylation with [γ ^{32}P]ATP(Amersham) by T4 DNA polynucleotide kinase as recommended by manufacturer.

Gel retardation assay

The procedure is basically as described[18]. Briefly, 1-5μl raw preparation of GST, GST-Myc and GST-Myn or 5-10 μl affinity purified GST, GST-Myc and GST-Myn were mixed respectively with 24 bp labelled E-box DNA or genomic DNA fragments in buffer similiar to described[4], put in ice for 30 min, run in 4-6 % polyacrylamide gel. The gel was then dried for autoradiography.

Affinity chromatography-dependent selection (ACDS) for genomic DNA possessing specific binding acitivity to Myc/ Myn complex

Genomie DNA of NIH3T3 cells digested by Hae III was dephosphorylated by alkaline phosphatase and then ligated to the phosphorylated blunt end of adaptor 20 /16. The ligates were PCR amplified directly using 20 mer as primer with [α-^{32}P]dCTP incorporated as tracers and ready for selection. For ACDS, 10μl each of unpurified GST-Myc and GST-Myn were mixed together with 10 #g above PCR amplified DNA, kept in ice for 3 h. The protein-DNA complexes were then incubated with glutathione-agarose beads 5 rain at room temperature. E. coli proteins were washed out with 1 ml 1 X binding buffer for 4 times. The DNA fragments captured by Mye/ Myn complex were reamplified for 60 cycles and suffered same selection step as above. Second selected DNA fragments were then amplified again by 60 cycles PCR. These possible Myc/ Myn-binding DNA fragments were separated via 8% PAGE. The gel was stained with ethidium bromide. Single band was cut from the gel, eluted with 0.5 M NH4oAc and recovered through ethanol precipitation. The DNA was end labelled directly using [γ ^{32}P]ATP 5' end phosphorylation for further gel retardation assay or amplified again by PCR.

RESULTS

RT-PCR amplification of mouse myn gene fragment

Using synthetic oligonucleotides both 5' end sense 5'ATGACATCGAGGTGGAGAGCGAC3' and 3' end antisense 5'GGTCTGCAGTTGGGCACTTGATCT3 which correspond to nucleotides sequence No. 174-196 and No. 476-499 respectively [4], RT-PCR was carried out to amplify myn cDNA fragments encoding basic-region/helix-loop-helix/leucine zipper (BR/HLH/LZ) structure of Myn protein. Upon the PCR procedure, the products run in 4% agarose gel showed a single ~300 bp band (data not shown). Identity of the amplified myn DNA was confirmed by sequencing after the fragment was inserted into pUC119 vector. Sequence of the fragment showed a deletion of 27 nucleotides which contains potential casein kinase II region as desribed[4]. Although it is still unclear, the deletion seems to be not essential for Myc / Myn association[4]. The myn DNA obtained was therefore utilized for further cloning.

Cloning e-myc and myn DNA fragments into expressing vector

Murine c-myc exon 3 AvaII - XhoI fragment excised originally from pSVc-myc -1[16] was cloned into bacterial expressing vector pGEX-2T (Pharmacia). The fragment encodes entire BR/HLH/LZ structure of Myc protein. In practice, reading frame was corrected by pUCl19 -myc cloning and recloning of pGEX-2T-myc to compensate unexpected shift of the frame raised during cloning. Linkage region between vector and 5' end of insert was always checked by cloning of this region

to pUC119 and sequencing. Finally obtained pGEX-myc possessed exact in frame integration confirmed by sequencing (Fig 1, a). Cloning of Myn was similiar to that of Myc. Myn cDNA was first cloned into pUC119 and inserts of myn were identified through bacterial colony hybridization. One of 5 strongly hybridized colonies was sequenced and cloned again into pBluescript II KS(+) thereafter into pGEX-2T to get expected restriction enzyme sites. Correction of reading frame of final pGEX-Myn was testified through sequencing (Fig 1, b). Both correct pGEX-myc and pGEX-myn were used for expression of fusion GST-Myc and Myn protein fragments in *E. coli.*

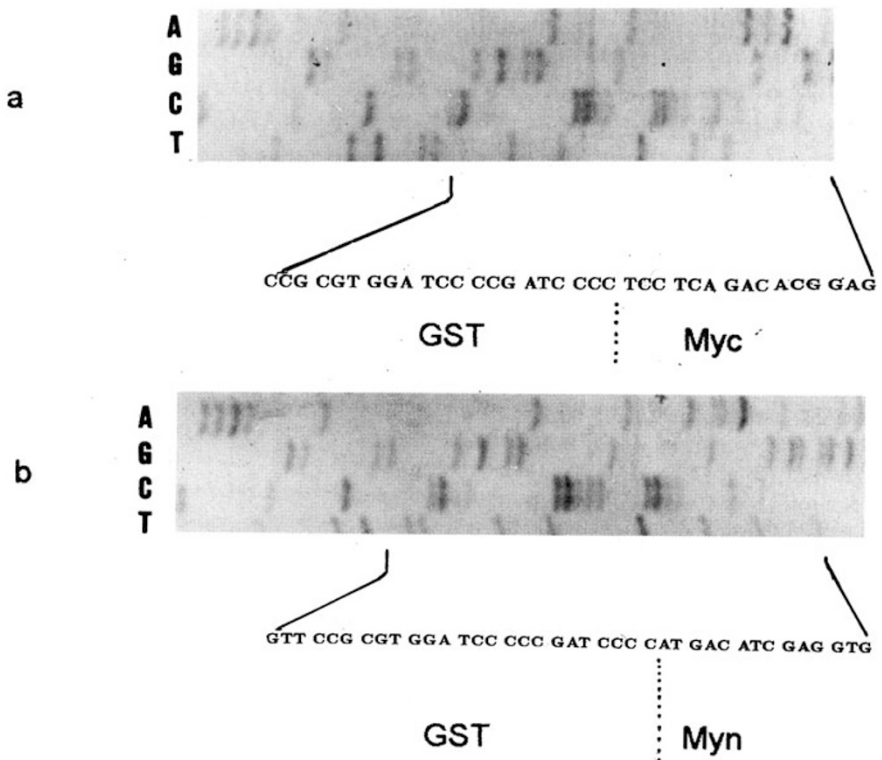


Fig 1. Linkage sequences shown correct in frame ligation of 3' end of GST gene and 5'ends of either myc or myn cDNA fragment in pGEX-myc or pGEX-myn respectively.
a. pGEX-myc, linkage sequence is 5' CTG GTT CCG CGT GGA TCC CCG ATC CCC TCC TCA GAC ACG GAG GAA3' ;
b. pGEX-myn, linkage sequence is 5'CTG GTT CCG CGT GGA TCC CCC GAT CCC CAT GAC ATC GAG GTG GAG3'' ;

Synthesis of Myc and Myn protein fragments in bacteria

pGEX-myc and pGEX-myn were transformed into competent JM109 and other hosts. GST-Myc or GST-Myn fusion proteins were synthesized in the cells and shown additional bands in the polyacrylamide gel indicating expression of fusion proteins (data not shown). After glutathione-agarose purification, both GST-Myc and GST-myn show about 40-42 kd single band in the gel respectively (Fig 2A, lane

3 and Fig 2B, lane 3). It should be noted here that the yield of both proteins in our system were not as high as expected. We noticed the instability of both proteins in some preparations, the phenomenon that may be due to low yield of the fusion protein in our experiment comparing to that of in original system[17]. Changing host could effect both yield and purity of the proteins. We observed that, besides from supernatants (as GST-myn show in Fig 2B, lane 3), both fusion proteins could also be extracted from bacterial pellets that may probably contain inclusive bodies with sarkosyl[23] after centrifugalization following sonication (as GST-Myc shown in Fig 2A, lane 3). Furthermore, excising of the stop codon in exon 3 of myc with Csp45 I could increase yield of intact soluble GST-Myc in cytoplasm significantly.

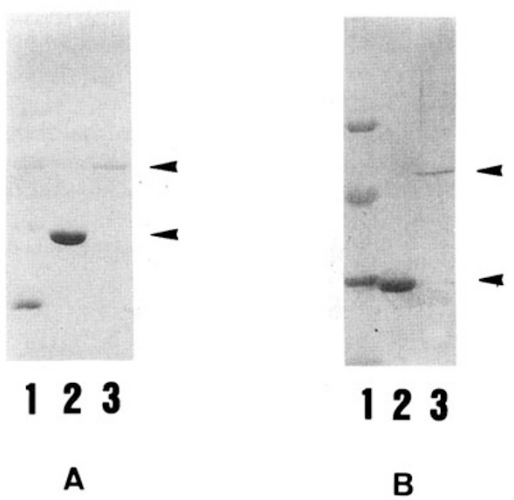


Fig 2. Glutathione-agarose beads purified GST (A. lane 2 and B. lane 2) GST-Myc (A. lane 3) and GST-Myn (B. lane 3). Protein molecular weight markers are 55.6, 39.2, 26.6 and 20.1 Kd from up to bottom respectively (A and B, lane 1). Samples were electrophoresed on 12 % SDS-polyaerylamide gel and stained with Coomassie Brilliant Blue (A) or Silver Stain (B).

Specific binding activity of GST-Myc and GST-Myn to E-box oligonucleotides

To testify if bacterially synthesized Myc/Myn protein fragments could be used for screening of DNA fragments of downstream regulated genes, we primarily checked the binding capacity of GST -Myc /GST-Myn to synthesized oligonucleotides NNNN-NNGACCACGTG containing CACGTG E-box consensus which can be specifically recognized by either Myc or Myn and complex of the both[3, 4]. Fig 3. shows binding of unpurified (A-D) and affinity purified (E-H) GST-Myc and/or GST-Myn to annealed filled oligos. We observed in several parallel experiments that either GST-Myc or GST-Myn alone and GST-Myc/GST-Myn complex show binding capacity whereas GST alone does not exhibit any activity although binding of GST-Myc alone is not strong in test presented here (Fig 3. lane F). However, as shown in lane H, Myc /Myn complex shows much more stronger activity than Myc or Myn alone even in case of only half amount of both GST-Myc and GST-Myn loaded (lane H).

This fact corresponds to the former description that the Myc /Myn heterodimer has much higher binding capacity than Myc homodimer[3, 4].

Affinity chromatography dependent selection (ACDS) of genomic DNA fragments exhibiting binding capacity to GST-Myc / Myn complex

To capture and enrich genomic DNA fragments which may specifically bind to Myc and Myn proteins, we designed a selection procedure named ACDS which combines steps of DNA-proteins interactions, affinity chromatography and PCR amplification. Whole genome PCR amplified DNA fragments were labelled and subjected to ACDS. During first round selection, only ~1% radioactive counts of DNA applied bound to the beads. Although being remained in the beads after entensive washing (4× 1 ml buffer), the DNA were readily eluted by as small a volume as only 50 μl elution buffer. The another interesting thing is that in the course of second round of selection, radioactive counts remained increased to 5%. It is therefore reasonable to speculate that bound counts in the beads may represent the existence of Myc /Myn-binding sequences (MBS).

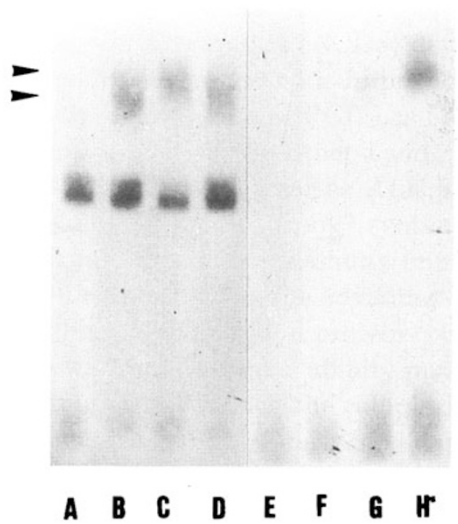


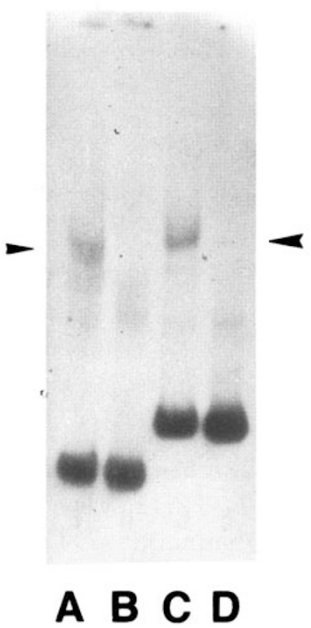
Fig 3. Specific binding of Myc and Myn protein fragments to E-box-containing oligonucleotides. 4% polyacrylamide gel for gel retardation assay using 1 μl each of unpurified total protein extracts from pGEX-2T (lane A), pGEX-Myc (lane B), pGEX-Myn (lane C), and 0.5 μl pGEX-Myc + 0.5 μl pGEX-Myn (lane D); 10 μl each of glutathione-agarose beads purified GST (lane E), GST-Myc (lane F), GST-Myn (lane G) and 5 μl GST-Myc+5μl GST-Myn (lane H) to 5μl labelled 24 bp E-box DNA respectively.

Gel retardation assay of chromatography selected DNA

PCR amplification of eluted DNA from second round selection was carried out. The amplified products were checked by 8% polyacrylamide gel electrophoresis. Fig 4. shows the products of several clear bands lying between 110-400 bps, but not in smear as found in original Hae III digested sample. The fact that product of reamplified single band cut from the gel was same as template (data not shown) testified

the appropriateness of PCR condition. Therefore results shown by the PAGE may reflect genuineness of the ACDS. Portions of individual bands cut from gel were radioactively labelled and used for gel retardation assay. As shown in Fig 5, DNA fragments No. 8 and 9 bound strongly to Myc/ Myn complex (lane A and C) but not to GST alone (lane B and D). Gel retardation assay for remaining bands are under way. The positive binding bands of fragments No. 8 and 9 have been cut down from the gel and used for further analysis. In these primary experiments we concentrated on DNA-binding of Myc /Myn complex, while leaving the check for binding of either Myc or Myn alone to further tests.

Fig 4. Amplification of Myc/Myn-bound genomic DNAs (MBS) enriched through ACDS technique. 8% polyacrylamide gel, ethidium bromide stain, lane 1. HaeIII digested pBR322 DNA markers; lane 2. Amplified DNA.



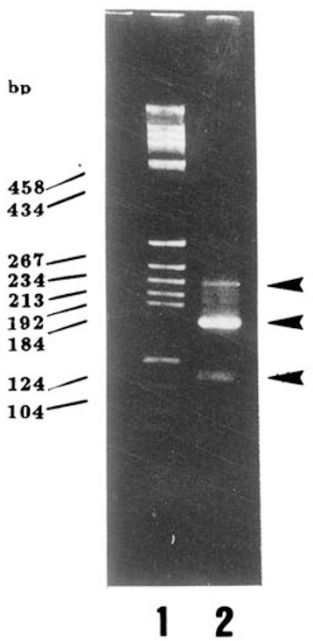


Fig 5. Specific binding of isolated MBS fragments to Myc and Myn proteins. Gel retardation assay using: lane A. 1/μl fragment No. 9 + 1μl GST-Myc/1 μl GST-Myn complex; lane B. 1 μl fragment No. 9 + 2 μl GST-alone; lane C. 0.5 μl fragment No. 8 + 1 μl GST-Myc/1 /μl GST-Myn complex; lane D. 0.5 μl fragment No. 8 + 2 μl GST-alone.

DISCUSSION

c-Myc, a BR /HLH/LZ protein together with its partner protein Myn (Max) possessing similiar structure, has been suggested to play important role in transcription regulation of genes[1-4]. Several candidate Myc regulated downstream genes have been proposed[9-12]. However, recent approach to this aspect discovered that Myc, as a component of transcription initiation complex, interacts with TATA-box binding protein and thereby may function in a general manner[13]. Further identification of Myc bound and regulated DNA elements is therefore still necessary.

Using pool of random synthetic oligonucleotides, SAAB selection was applied to determine binding sequences of Myc and Myn (Max) [7, 8]. Although important data have been accumulated in this respect, work concerning screening of Myc-binding sequences in whole genomic DNA populations has not yet reported.

This paper describes our approach using bacterially expressed mouse c-Myc and Myn fusion protein to enrich Myc specific binding sequences among DNA produced from whole-genome PCR. The results showed the success of obtaining several genomic DNA fragments bound to Myc-Myn complex as testified by gel retardion assay. After further subcloning, these DNA fragments, as expected, can be used as probes for locating the binding sites which may reside somewhere in Myc-regulated downstream genes.

As to methodology, affinity chromatography dependent selection (ACDS) developed in our work seems to be a simple, practical and sensitive method. In previous works, on the one hand, although not for Myc study, whole genomic PCR has been used for investigation of protein-DNA interaction[19, 20]; on the other hand, although not using genomic DNA, protein such as yeast GCN 4, murine ALF and so on were immobilized on sepharose or nitrocellulose paper to "hook" synthetic oligonucleotides they bind[21,22]. However, our procedure combines affinity immobilization and whole genome PCR together. This technique takes the advantage of the fact that only genomic DNA fragments which bind Myc and Myn protein could be left in the beads after absorption and entensive washing. Actually, affinity purification step plays a role just as anchorage for proteins. Considering the complexity of genome, we believe that it is still possible, to some extent, to get new candidates of Myc-binding DNA sequences through further screening with this technique.

ACKNOWLEDGEMENTS

We are grateful to Committee of Marx-Planck Guest-Laboratory for giving us laboratorial facilities. We thank Prof. U. Schwarz and Prof. W. Hennig for their kind gift of pGEX-2T vector; and Dr. M. Habernig for plasmid pSVc-myc-1.

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Received 20-12-1994. Revised 21-4-1995. Accepted 26-4-1995.