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Production of a truncated human c-*myc* protein which binds to DNA

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Two kinds of truncated human c-*myc* proteins were produced in *Escherichia coli*. The human c-*myc* gene is composed of three exons, exons 2 and 3 having coding capacity for a protein of 439 amino acids. 252 N-terminal amino acids are encoded by exon 2, the C-terminal 187 amino acids being encoded by exon 3. One of the proteins (p42) produced in *E. coli* corresponds to 342 amino acids from the 98th Gln to the C-terminus, plus 21 amino acids derived from the H-*ras* gene at the N-terminus. The other (p23) corresponds to 155 amino acids from the 98th Gln to the 252nd Ser, plus five amino acids (Gly-Gly-Thr-Arg-Arg) at the C-terminus, plus 21 amino acids from the H-*ras* gene at the N-terminus. The p23 protein was produced by using cDNA in which a frame shift occurred at the boundary between exons 2 and 3. We investigated the DNA-binding activity in p42 and p23 proteins. DNA-cellulose column chromatography showed that p42 binds to DNA, whereas p23 does not. This DNA-binding activity of p42 was inhibited by antiserum prepared against p42 but not by antiserum against p23. This indicates that the DNA-binding activity of c-*myc* protein is localized in the portion encoded by exon 3.

recombinant DNA; c-*myc* protein; DNA-binding activity

1. INTRODUCTION

The c-*myc* gene, first identified as a cellular counterpart of the transforming gene of avian myelocytomatosis virus MC29 [1], is found in all vertebrate animals [2]. The c-*myc* protein is localized in the nucleus [3], and is associated with the nuclear matrix [4]. The c-*myc* protein produced in *E. coli* exhibits a high sequence-nonspecific affinity for double-stranded DNA [5]. The human c-*myc* gene is composed of three exons, exons 2 and 3 having coding capacity for a protein of 439 amino acids [6]. The N-terminal 252 amino acids are encoded by exon 2, the C-terminal 187 amino acids being encoded by exon 3. Recent analyses using deletion and insertion mutants of c-*myc*-coding domains by Sarid et al. [7] and Stone et al. [8] defined the functional regions in c-*myc* protein for subcellular localization and transforming ability. Here, we produced two kinds of truncated human c-*myc* proteins in *E. coli*, prepared two kinds of antisera against both proteins, and show that the DNA-binding activity of c-*myc* protein is localized in the exon 3-encoded region.

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Abbreviations: ABC, avidin-biotin complex; DTT, dithiothreitol; FCS, fetal calf serum; NP40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethylsulphonyl fluoride

2. MATERIALS AND METHODS

2.1. *Bacteria, plasmids and cells*

E. coli 803 (rk^-,mk^-,SuIII$^+$), obtained from K. Murray (University of Edinburgh), was used as the host for preparation

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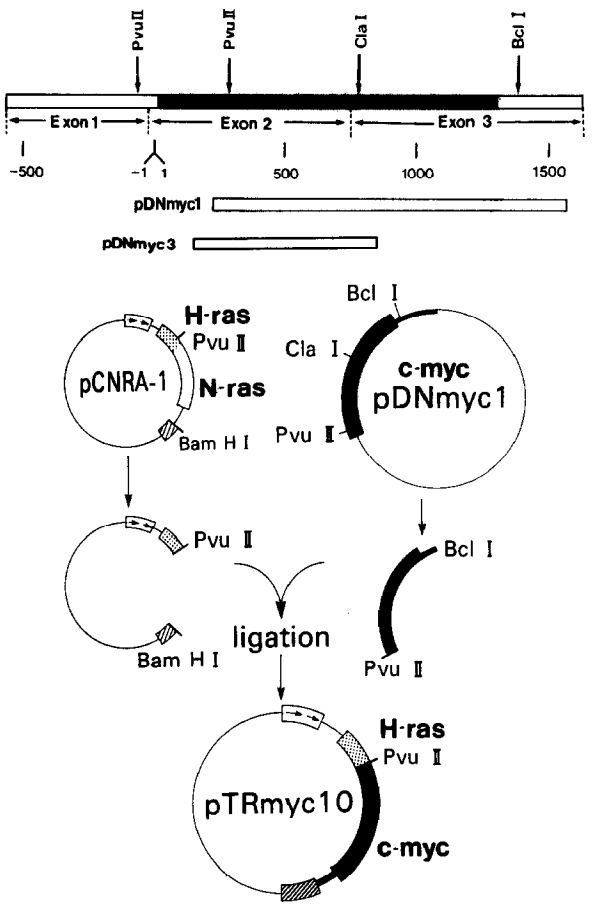

Fig.1. Construction of pTRmyc10. Physical map of the full-length cDNA of c-*myc* gene is shown at the top. We constructed a cDNA library from Daudi mRNA by the ordinary dG-dC tailing method [20] in pBR322, and four independent cDNA clones (pDNmyc1–4) were obtained by colony hybridization [20] with v-*myc* probe [9]. The longest clone, pDNmyc1, covers the range from the 5′-side of the *Pvu*II site in exon 2 to the 3′-end of the gene. The *Pvu*II-*Bam*HI fragment of pCNRA1 which directs the expression of the fused H-*ras* and human N-*ras* genes in *E. coli* [10] was replaced by the *Pvu*II-*Bcl*I fragment of pDNmyc1, resulting in plasmid pTRmyc10. The *Pvu*II-*Cla*I fragment of pTRmyc10 was replaced by the *Pvu*II-*Cla*I fragment of pDNmyc3, resulting in pTRmyc22. Its primary structure is the same as that of pTRmyc10 except for the boundary between exons 2 and 3 (see section 3).

of plasmid DNAs, and grown in L-broth. *E. coli* W3110 was used as the host for production of truncated c-*myc* proteins. A Burkitt lymphoma cell line, Daudi, was obtained from L.J. Old (Memorial Sloan-Kettering Cancer Center, New York). Plasmid pMC3 [9] containing the v-*myc* gene was supplied by Y. Taya (National Cancer Center, Tokyo). The expression vectors, pKYP200 and pCNRA1, have been described previously [10].

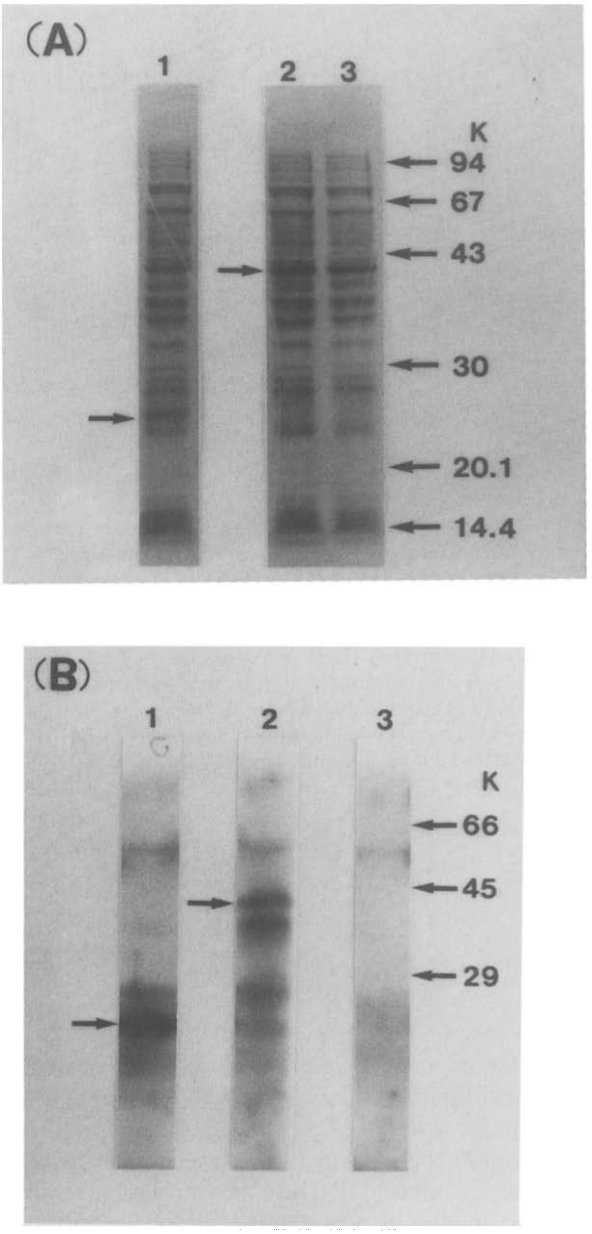

Fig.2. (A) Expression of truncated fused c-*myc* gene in *E. coli*. (lane 1) pTRmyc10-, (lane 2) pTRmyc22-, (lane 3) pKYP200-transfected W3110 cells were grown to stationary phase in M9 medium supplemented with 0.5% casamino acids and 25 μg ampicillin/ml, harvested by centrifugation, and suspended in Laemmli buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.002% bromophenol blue). Total proteins were analyzed by SDS-PAGE [21]. The proteins were stained with Coomassie brilliant blue. (B) Western blots of p23 and p42. Lanes: 1, pTRmyc10-, 2, pTRmyc22-, 3, pKYP200-transfected W3110. ^{125}I-labeled antibody was used.

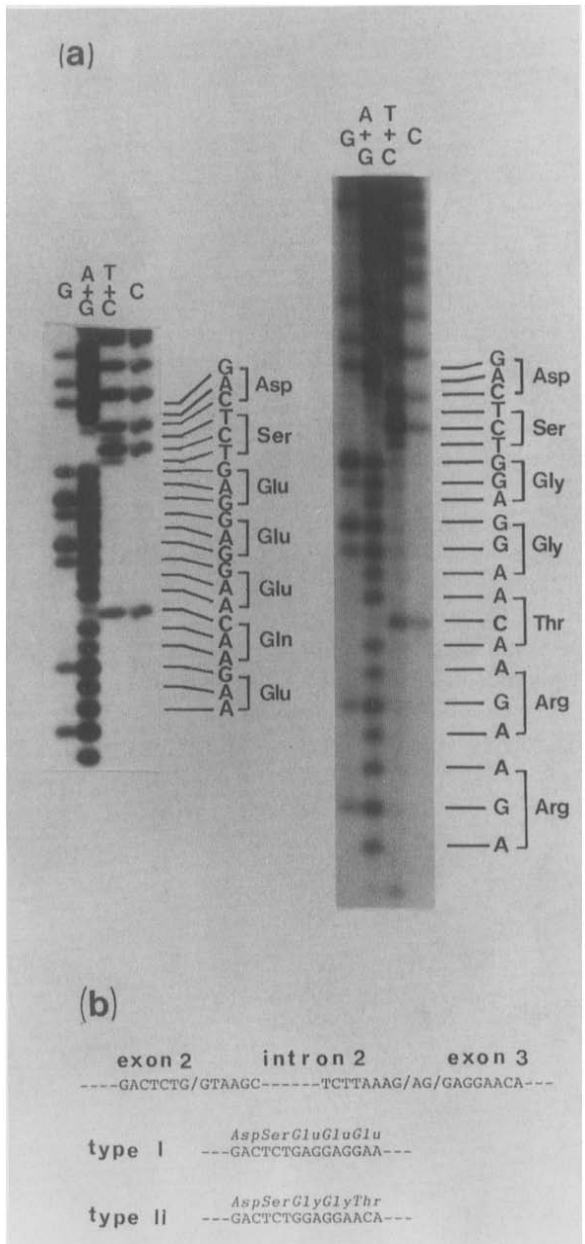

Fig.3. DNA sequence of two kinds of cDNA clones and RNA splicing pathways. (a) DNA sequence ladder of the boundary between exons 2 and 3. DNA sequencing was performed according to Maxam and Gilbert [22]. (Left) pDNmyc3, (right) pDNmyc1. (b) Scheme of alternative RNA splicing pathways.

2.2. *Purification of truncated* c-myc *proteins*

pTRmyc10-transfected W3110 was grown in 6 l of M9 medium supplemented with casamino acids. The inclusion bodies containing p23 were isolated as in [10]. The final pellets were dissolved in 20 ml of 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.1 mM DTT, 5% glycerol, 0.1 mM PMSF and 8 M urea. This fraction contained 250 mg proteins of which p23 was approx. 60%. The sample was further purified by SDS-PAGE. The gel was sliced and the portion corresponding to p23 was retained. The proteins were electrically eluted from the gel (Isco model 1750 sample collector) and dialyzed against 10 mM Tris-HCl (pH 8.0). SDS was removed from the protein sample using the ion-pair extraction method [11]. p42 was similarly purified from pTRmyc22-infected W3110 according to the same protocol.

2.3. *Preparation of mouse antisera against p23 and p42*

(BALB/c × C57BL/6)F_1 female mice were hyperimmunized with purified p23 or p42 at 2-week intervals. For the initial immunization, 50 μg c-*myc* proteins were inoculated subcutaneously with complete Freund's adjuvant. Subsequent immunizations were carried out by intraperitoneal inoculations of 100 μg c-*myc* proteins. The titer of antibodies against p23 or p42 was monitored by enzyme-linked immunosorbent assay. Mouse sera immunized more than 4 times were collected and pooled. The characterization of the anti-p23 and anti-p42 sera will be published elsewhere. The sera were used for Western blots at 100-times dilution, and for assaying inhibition of DNA binding without dilution.

2.4. *Western blots*

Proteins separated by SDS-PAGE were electroblotted onto a nitrocellulose membrane (Scheicher & Schull BA85) as described by Towbin et al. [12]. The membrane was preincubated in 2% non-fat dry milk-containing PBS for 1 h, then incubated with anti-p23 antiserum diluted to 1/100 in buffer A (PBS containing 2% FCS and 0.5% Tween 20) for 1 h at room temperature. The membrane was washed several times with buffer A, incubated with ^{125}I-labeled sheep anti-mouse immunoglobulin antibodies (Amersham, 0.4 μCi/ml) in 5 ml buffer A for 1 h at room temperature, washed several times with buffer A, dried and exposed to X-ray film. Avidin-biotin complex (ABC) techniques were also employed (Vector Labs, USA).

3. RESULTS AND DISCUSSION

3.1. *Construction of recombinant plasmids that direct the expression of truncated human* c-myc *proteins in* E. coli

We isolated c-*myc* cDNA clones in an attempt to produce human c-*myc* protein in *E. coli*, and succeeded in producing a truncated molecule encoded by pTRmyc10 (fig.1). It includes double *trp* promoters, the N-terminal 21 amino acids derived from the H-*ras* gene, the truncated c-*myc* gene from the *Pvu*II site in exon 2 to the C-terminus and a terminator. When total proteins from pTRmyc10- and pKYP200-transfected *E. coli*

bacteria were analyzed by SDS-PAGE, an extra band of high intensity was observed at 23 kDa in the former preparation (fig.2A). The 23 kDa polypeptide (p23) was purified and its primary structure was analyzed. The C-terminal amino acid was arginine, which does not correspond to that of normal c-*myc* protein, alanine. Since the molecular mass of p23 protein was much less than the expected 42 kDa, we determined the nucleotide sequence of the cDNA clones. We found an unexpected feature at the boundary between exons 2 and 3. Fig.3a shows two typical examples of the sequence ladder. The sequence in pDNmyc3 is GACTCTG/AGGAGGAA (type I), the same as reported in [13]. However, the sequence in pDNmyc1, which was used to construct pTRmyc10, is GACTCTG/GAGGAA (type II), resulting in a frame shift of c-*myc* protein. The primary structure of the p23 protein deduced from the nucleotide sequence of the constructed plasmid was as follows: the N-terminal 21 amino acids derived from the H-*ras* gene, and 155 amino acids encoded by a part of the c-*myc* gene from the *Pvu*II site to the 3′-end of exon 2, plus 5 amino acids (Gly-Gly-Thr-Arg-Arg).

To obtain a c-*myc* protein including the exon 3-encoded portion, we constructed plasmid pTRmyc22 as described in the legend to fig.1. Analysis of total proteins from pTRmyc22-infected bacteria showed a thick band at 42 kDa (fig.2A). Since pKYP200-infected bacteria also showed a thick band at the same size, immunoblottings were carried out with the murine antiserum prepared against p23 to determine whether p42 observed in pTRmyc22-infected bacteria contains a c-*myc* protein. Fig.2B clearly shows that the p42 fraction in pTRmyc22-transfected bacteria contains a large amount of c-*myc* proteins. The weak bands observed at lower molecular masses (panel B, lane 2) might be degradation products.

3.2. *DNA-binding activity of* c-myc *protein is localized in the exon 3-encoded region*

To ascertain whether p42 and p23 bind to DNA, they were purified from pTRmyc22- and pTRmyc10-transfected bacteria, respectively, and analyzed on a DNA-cellulose column (fig.4) [14]. The protein band was observed at 42 kDa in the 0.5 M NaCl eluate in the case of p42 being applied. No p23 was retained on the DNA-cellulose column

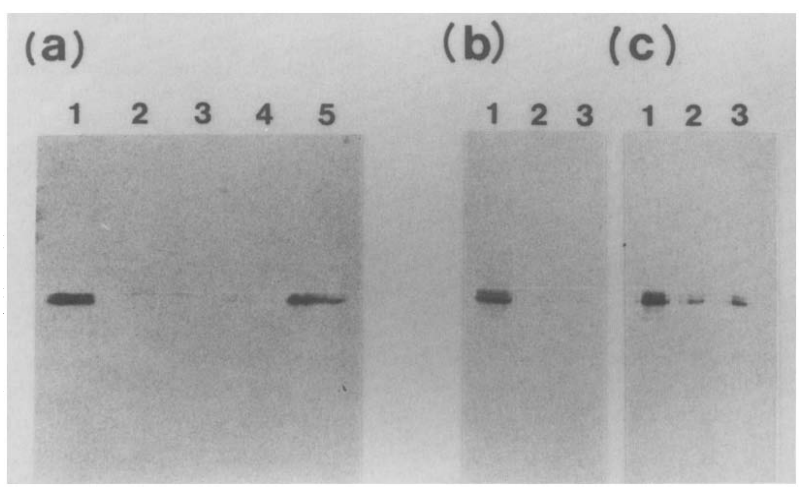

Fig.4. DNA-cellulose column chromatography of p42. p42 proteins were purified as described in section 2. (a) 2 μg p42 were dissolved in 10 μl buffer B (50 mM Tris-HCl, pH 6.8, 0.5% NP40), and applied to double-stranded calf thymus DNA-cellulose (Pharmacia) columns (bed volume 0.2 ml) which had been equilibrated with buffer B. Columns were then washed with 5 bed volumes of buffer B. Proteins bound to the columns were eluted by increasing salt concentrations in buffer B. Proteins in each fraction were precipitated in 5% trichloroacetic acid, resolved in Laemmli buffer, and analyzed by SDS-PAGE [lanes: (1) flow through; (2) 0 mM NaCl wash; (3) 1 bed volume, 100 mM NaCl; (4) 5 bed volumes, 100 mM NaCl; (5) 1 bed volume, 500 mM NaCl]. Proteins were identified on Western blots using the ABC system. (b) The flow-through fraction was re-applied to the DNA-cellulose column and eluted as above (1, 0 mM NaCl; 2, 100 mM NaCl; 3, 500 mM NaCl). (c) The 500 mM NaCl eluate (lane 5) was re-applied to the DNA-cellulose column after dialysis against buffer B, and eluted (1, 0 mM NaCl; 2, 100 mM NaCl; 3, 500 mM NaCl).

(not shown). Since more than half of p42 was observed in the flow-through fraction, both the flow-through and retained fractions of p42 were recovered and applied again to the DNA-cellulose column. One-third of the retained fraction was bound to DNA once more. The reason why a portion of p42 was observed in the flow-through fraction may be that the renaturation of p42 was incomplete in our preparation and/or p42 was unstable. This suggests that p42 has DNA-binding activity. To determine whether the DNA-binding activity of p42 is localized in the exon 3-encoded region, which p23 does not contain, we prepared two kinds of antisera against p42 and p23. As shown in fig.5, anti-p23 did not exert any influence on the DNA-binding site in p42 (lane 2) although binding of p42 to the DNA column was completely blocked by anti-p42 serum (lane 1). This indicates that DNA-binding activity is localized in the portion encoded by exon 3.

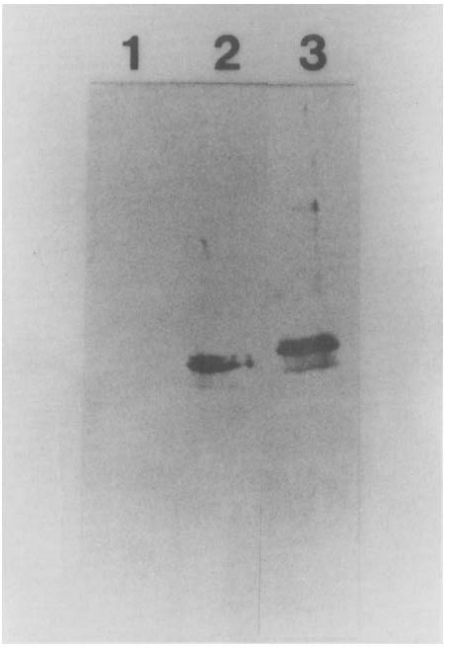

Fig.5. Effects of antisera on DNA-binding activity of p42. 2 μg p42 and 2 μl antiserum were incubated overnight at 4°C, p42-antibody complex was applied to the DNA-cellulose column, then washed with 5 bed volumes of buffer B and successively 5 bed volumes of buffer B containing 100 mM NaCl. p42 bound to the DNA-cellulose column was eluted by 500 mM NaCl in buffer B. Proteins eluted by 500 mM NaCl were precipitated in 5% trichloroacetic acid and analysed on SDS-PAGE and Western blots using the ABC system (1, anti-p42 antiserum against p42; 2, antiserum against p23; 3, non-immunized mouse antiserum).

The DNA-binding region of c-*myc* protein has been believed to be localized in the 3′-domain of the molecule, based on the following observations. First, the specific antibody against this domain strongly inhibits binding of the molecule to DNA [15]. Second, homology between the amino acid sequence of c-*myc* protein and those of other DNA-binding proteins such as *myb*, E1a, *fos* and *jun* has been found mainly in the C-terminal region of c-*myc* [16,17]. There are several examples of DNA binding mediated through a helix-turn-helix structure [18], and computer analyses using the program of Chou and Fasman [19] predict that the secondary structure of the C-terminal region of c-*myc* would prefer an α-helical conformation. Here, we prepared two kinds of truncated c-*myc* molecules in *E. coli*, of which p42 contains an exon 3-encoded region whereas p23 does not, and directly showed that the exon 3-encoded portion has DNA-binding activity.

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