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DNA replication origin and transcriptional enhancer in c-*myc* gene share the c-*myc* protein binding sequences

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We have previously reported that c-*myc* protein, or protein(s) complexed with c-*myc* protein, binds to the region upstream of the first exon of the c-*myc* gene and that this region contains an origin of cellular DNA replication (*ori*) and also a transcriptional enhancer. Here we show by Southwestern blotting that c-*myc* protein binds directly to a 7 bp sequence within the above region. Furthermore, we show that the c-*myc* protein binding sequences are indispensable for both *ori* and enhancer functions, but that additional sequences are required for maximal *ori* and enhancer activities. Thus, c-*myc* protein is a sequence specific factor which is apparently used both in initiation of DNA replication and in regulation of RNA transcription.

Key words: c-*myc* gene/enhancer/replication

Introduction

The function of c-*myc* protein, the product of the proto-oncogene c-*myc* (Sheiness and Bishop, 1979), has not yet been fully clarified. We have recently shown that one of the functions of the c-*myc* protein may be to promote cellular DNA replication, by binding to an origin of DNA replication. This idea was supported by demonstrating that a cloned human sequence which binds the c-*myc* protein, or protein(s) complexed with c-*myc* protein (Iguchi-Ariga *et al.*, 1987a), also functions as an autonomous replicating sequence (ARS). In this function, c-*myc* protein is somewhat similar to the large T antigens of SV40 and polyomavirus, which are necessary for the initiation of viral DNA replication and transcription (Tooze, 1980; DePamphilis and Bradly, 1986). Indeed, the c-*myc* protein can substitute for SV40 T antigen in an SV40 DNA replication system (Iguchi-Ariga *et al.*, 1987b). A similar observation was also reported by Classon *et al.* (1987). Furthermore, we have shown that the c-*myc* protein, or protein(s) complexed with c-*myc* protein, binds to a region ~2 kb upstream of the first exon of the c-*myc* gene itself and that this region contains a putative origin of cellular DNA replication (*ori*) and also a transcriptional enhancer, which suggests that the c-*myc* protein may be an enhancer binding protein which positively regulates its own transcription (Iguchi-Ariga *et al.*, 1988a). Cloned DNA containing this *Hin*dIII–*Pst*I region, termed the *myc* (H-P) region (Figure 1A), can replicate autonomously, is transmitted in transgenic mice at episomal state (K.Sudo, M.Ogata, Y.Sato, S.M.M.Iguchi-Ariga and H.Ariga, in preparation), and serves as an efficient template in a cell-free DNA replication system (Umekawa *et al.*, 1988).

Here we report that the *ori* and enhancer within the *myc* (H-P) region of the c-*myc* gene overlap and share sequences to which the c-*myc* protein binds, and that the c-*myc* protein has an essential function both in the initiation of DNA replication and for enhancer activity.

Results

Identification of the protein binding sequences in the c-myc upstream region

Nuclear extracts (NE) from human Burkitt lymphoma Raji cells (which contain high amounts of c-*myc* protein were prepared. The extracts were then incubated with the ^{32}P-labelled *Hin*dIII–*Pst*I fragment of the c-*myc* gene (Figure 1A) which contains the putative origin (*ori*) of DNA replication and the enhancer (Iguchi-Ariga *et al.*, 1988a). In DNase I footprinting analysis of this fragment with the Raji NE, nucleotides around positions 135–155 (see Figure 1B) were protected on both strands (Figure 2). An oligonucleotide designated *myc* (135–155) corresponding to the protected sequences (Table I) was chemically synthesized to examine the binding activity of c-*myc* protein. Band shift assay using Raji NE with the ^{32}P-labelled oligonucleotide *myc* (135–155) showed three shifted bands, denoted A, B and C (Figure 3). All three bands disappeared after addition of excess amounts of unlabelled *myc* (135–155), but not after addition of pBR322 *Hae*III fragments, indicating that the three bands were specifically

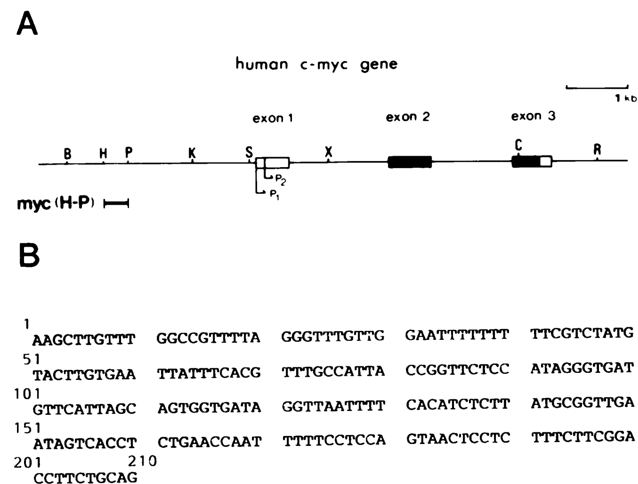

Fig. 1. Structure of human c-*myc* gene. **(A)** The open and filled-in boxes represent non-coding and coding exons respectively. B, *Bam*HI site; C, *Cla*I site; E, *Eco*RI site; H, *Hin*dIII site; K, *Kpn*I site; P, *Pst*I site; X, *Xba*I site; P1 and P2, promoters. **(B)** Nucleotide sequence of the region from the *Hin*dIII site to *Pst*I site, *myc* (H-P) region. Sequence of the 5'→3' oriented strand is shown.

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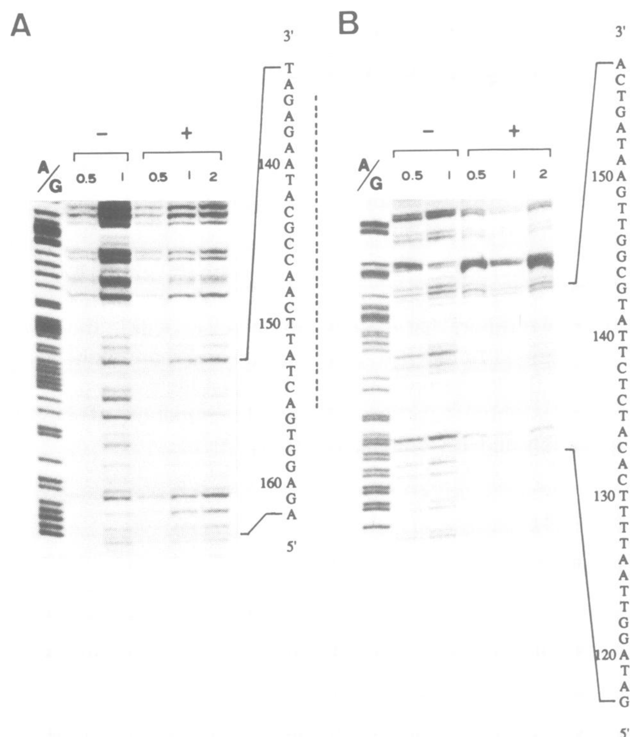

Fig. 2. DNase I protection analysis using cell nuclear extracts. The *myc* (H-P) fragment was 5′ end-labelled at either *Pst*I end (**A**) or *Hin*dIII end (**B**) and used for DNase I protection analysis as described in Materials and methods. + or − above the lanes indicates that the reaction was carried out in the presence or in the absence of the nuclear extract. The amount (μg) of added DNase I in the reaction was shown above each lane. The cleavage products of A+G sequencing reaction of the fragments were co-electrophoresed on the same gel in lanes A/G. Numbers beside the nucleotide sequence refer to the sequence of the *myc* (H-P) region shown in Figure 1. Dotted line indicates protected region.

complexed with protein(s) in the Raji NE. Nuclear extracts treated with polyclonal anti-c-*myc* antibody before the binding reaction gave rise to no shifted bands. The results indicate that c-*myc* protein, or protein(s) complexed with c-*myc* protein, binds to the sequences spanning nucleotides 135−155 of the *Hin*dIII−*Pst*I fragment of the c-*myc* gene and that three shifted bands are all due to the c-*myc* protein or its complexed partners.

To determine the exact binding sequence of c-*myc* protein, various oligonucleotides corresponding to deletion mutants of the *myc* (135−155) sequence were synthesized (Table I) and used in band shift assays (Figure 4). Oligo Δ(135−145/149−155) gave rise to three shifted bands as the intact *myc* (135−155) oligo does, while oligo Δ(135−149) showed two complexes B and C. Oligos Δ(135−141/146−149), Δ(135−145) and Δ(135−141) yielded only one band (complex C), and oligos Δ(142−149) and Δ(138−145) gave no specific band. A weak band seen with Δ(142−149) migrated slightly slower than complex C and may be due to unspecific single strand-binding complex. These results indicate that while the nucleotides protected from DNase digestion are from position 135 to 155, the minimum sequences necessary for c-*myc* protein binding are from nucleotides 135 to 141, TCTCTTA, and also suggest that sequences in addition to these are needed to facilitate binding to DNA of the c-*myc* protein and protein complexes involving c-*myc* protein: nucleotides 135−141 are essential for all of the shifted bands and enough to form complex C, but the formation of complex B requires nucleotides 142−145 besides 135−141, and the complex A can be formed only with the oligos containing nucleotides 149−155 in addition to 135−141 and 142−145.

When the NE was treated with phosphatase before binding reaction, the shifted bands A and B disappeared even with the intact *myc* (135−155) nucleotide (S.M.M.Iguchi-Ariga, Y.Negishi and H.Ariga, in preparation), suggesting that the A and B are due to the protein(s) complexed with c-*myc* protein and that the formation of the complexes are promoted by phosphorylation.

Southwestern blot analysis of the binding proteins

The proteins in Raji NE were separated in an SDS-polyacrylamide gel, transferred to a nitrocellulose filter, and reacted with monoclonal anti-c-*myc* antibody (Figure 5A). After visualization with ^{125}I-anti-mouse IgG, a protein of ~64 kd was detected, which is the expected size of the c-*myc* protein. When a blot run in parallel was reacted with ^{32}P-labelled *myc* (135−155) oligo, a protein of exactly the same size was detected. The NE immunoprecipitated with anti-c-*myc* protein antibody prior to electrophoresis showed the same band after hybridization with ^{32}P-labelled *myc* (135−155) oligo (Figure 5B), while ^{32}P-labelled pBR322 *Hae*III fragments, used as a negative control, did not bind to any polypeptides under these conditions (data not shown). These experiments suggest that the c-*myc* protein binds directly to DNA and the binding sequences of the c-*myc* protein are present within the *myc* (135−155). At present we cannot completely exclude the possibility that a protein(s) of the same mol. wt as c-*myc* protein binds directly to DNA, forming a complex with c-*myc* protein. However, we and other groups already have further supporting data that c-*myc* protein directly binds to DNA (discussed later). Lower band slightly observed in Western blotting might be of *myc* superfamily, like MyoD1 whose mol. wt is 45 kd (Tapscott *et al.*, 1988).

Replication and transcription activities within a 21 bp sequence

DNA replication assay was carried out as described in Materials and methods (Figure 6A). As a positive control, p*myc* (H-P) containing the 214 bp *myc* (H-P) region (Figure 1) of the c-*myc* gene could replicate in HeLa cells, whereas pUC19 could not. p*myc*-O, containing the *myc* (135−155) oligo, also replicated in HeLa cells, with almost the same efficiency of p*myc* (H-P). This experiment clearly showed that the 21 bp sequence containing the c-*myc* protein binding region is sufficient for initiation of DNA replication.

A CAT assay was performed to examine the transcriptional activity of the *myc* (135−155) oligo (Figure 7), which was inserted upstream of the SV40 promoter of pSVPCAT. Transcriptional activation of the CAT gene was slightly stronger with the *myc* (135−155) oligo (p*myc*-O-CAT) than with the *myc* (H-P) fragment of the c-*myc* gene [p(H-P)(+)PCAT]. Thus, the 21 bp oligonucleotide *myc* (135−155) contains sequences sufficient for transcriptional activity, for c-*myc* protein binding and for function as an origin of DNA replication.

Table I. Oligonucleotides used in these experiments

Oligonucleotide	Numbers above	Sequence (5′ → 3′)	Numbers below
myc(135−155)	135 ... 155	G A T C C T C T C T T A T G C G G T T G A A T A G T G	
		G A G A G A A T A C G C C A A C T T A T C A C C T A G	
(135−149)	135 ... 149	G A T C C T C T C T T A T G C G G T T G G	
		G A G A G A A T A C G C C A A C C C T A G	
(135−141/146−149)	135 ... 141	G A T C C T C T C T T A G T T G C	
		G A G A G A A T C A A C C C T A G	146 ... 149
(142−149)	142 ... 149	G A T C C T G C G G T T G G	
		G A C G C C A A C C C T A G	
(135−145/149−155)	135 ... 145	G A T C C T C T C T T A T G C G G A A T A G T G	
		G A G A G A A T A C G C C T T A T C A C C T A G	149 ... 155
(138−145)	138 ... 145	C C T T A T G C G G	
		T C G A G G A A T A C G C C C T A G	
(135−145)	135 ... 145	C T C T C T T A T G C G G	
		T C G A G A G A G A A T A C G C C C T A G	

Nucleotides in the c-*myc* gene are indicated in bold type. Numbers above and below the oligonucleotides refer to the nucleotide sequence of the *myc*(H-P) region shown in Figure 1.

Determination of the precise junction between ori and enhancer

The various oligonucleotides containing deletions of the *myc* (135−155) construct (Table I) were inserted into pUC19 or pSVPCAT, and replication and transcription activities were examined (Figure 8). All deletions, both within and outside of the c-*myc* protein binding sequences, abolished replication activity (Figure 8B). For transcriptional activity, however, somewhat different results were obtained (Figure 8C). Deletion of the c-*myc* protein binding core sequences 135−141 [oligos Δ(142−149) and Δ(138−145)] reduced transcriptional activity, indicating that they, too, are necessary for transcriptional activation and suggesting that the c-*myc* protein is a transcription factor. However, the presence of c-*myc* protein binding sequences alone was not sufficient to establish complete enhancer activity, since deletion of nucleotides 142−145 [oligo Δ(135−141/146−149)] resulted in reduced CAT activity, while the fragment from 135 to 145 [oligo Δ(135−145)] gave as strong CAT activity as the *myc* (135−155). This indicates that the entire sequence from 135 to 145 is needed for full transcriptional activity. Oligo Δ(138−145) which showed no shifted band in bandshift assay (Figure 4B) slightly activated transcription in CAT construct. There might not be enough 'space' for protein binding on DNA in this oligo although there are recognition sequences. When the oligo is cloned into CAT construct protein(s) necessary for transcriptional activation may become able to bind to the sequences.

To confirm the above results, a point mutation (C→A at nucleotide number 138) was introduced into the *myc* (135−155) oligo, and c-*myc* protein binding, DNA replication and transcriptional activities were measured

Fig. 3. Band shift analysis using cell nuclear extracts. Band shift assay was carried out as described in Materials and methods using end-labelled *myc* (135−155) (see Table I) as a probe. The underlined sequences are present in the c-*myc* gene. Antibodies were incubated with NE at 0°C for 10 min and the mixture was then subjected to band shift assay.

Fig. 4. Band shift assay with various oligonucleotides. (**A**) The oligonucleotides used as probes (see Table I). (**B**) Band shift assay was carried out with various oligonucleotides shown above. The oligonucleotide used as a probe is shown underneath each panel, and the components of the reaction mixtures in each lane are indicated above the figures. The shifted bands (A, B and C) and free DNA (F) are indicated by arrows.

(Figure 9). This mutation destroyed all three activities, indicating that the binding of the c-*myc* protein to this sequence is indispensable for its function both as an origin of DNA replication and as a transcriptional activator.

Discussion

Here we present data suggesting that the minimum sequence required for c-*myc* protein binding is TCTCTTA, and this sequence is essential for both DNA replication and transcriptional activation mediated by the *myc* (H-P) region. The sequences necessary for replication ori and enhancer activities overlap, sharing the c-*myc* protein binding region. There is some discrepancy, however, between the DNA binding and transcription assay for oligo Δ(138−145) which does not bind (Figure 3B) but has some CAT activity. A possible explanation for this point is that oligo Δ(138−145) is too small to give a shifted band in gel retention assay (there might not be enough 'space' for protein binding although there are recognition sequences). However, when the same oligo is cloned into a CAT construct, it may become able to be bound by protein(s) necessary for transcriptional activation. The critical point is whether or not the c-*myc* protein binds directly to the sequence. The results from Southwestern blotting analysis described here indicate that this is very likely. Furthermore, we have recently shown that purified c-*myc* protein expressed in *Escherichia coli* (Naoe *et al.*, 1988) binds directly to the TCTCTTA sequence (H.Ariga, unpublished data), and another group has purified c-*myc* protein from Raji NE by using affinity chromatography with an oligonucleotide containing this sequence (S.Izuta and S.Yoshida, in preparation). From this, we would like to conclude that c-*myc* protein binds directly to the DNA sequence.

The products of c-*jun* and c-*myb*, which are also nuclear oncogenes, have recently been shown to be sequence-specific DNA binding proteins (Bohmann *et al.*, 1987; Angel *et al.*, 1988; Bading *et al.*, 1987; Biedenkapp *et al.*, 1988). The function of the c-*jun* product, also referred to as AP-1, is transactivation of transcription through binding to an enhancer element, similarly to c-*myc* protein, as described in this and a previous paper (Iguchi-Ariga *et al.*, 1988a). It is quite possible that a common function of nuclear oncogene products is transcriptional regulation of various genes by binding to specific regulatory elements. To date, at least two well characterized transcription factors have turned out to be identical to cellular proteins required for viral DNA replication. CTF, a transcription factor responsible for specific recognition of the sequence CCAAT in eukaryotic promoters is indistinguishable from nuclear factor I (NF-I), a cellular DNA-binding protein that is essential for the

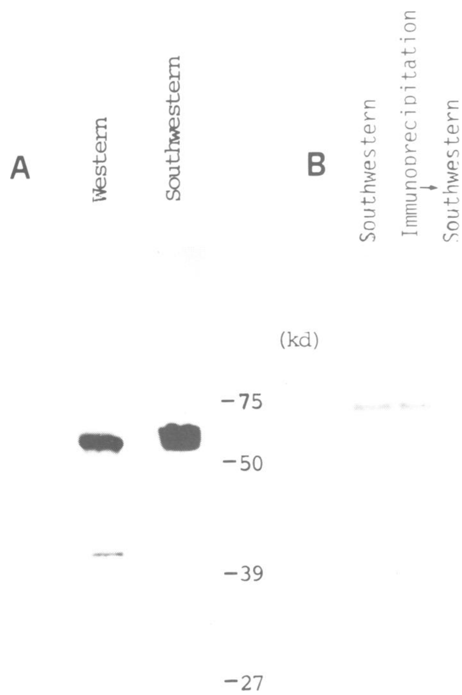

Fig. 5. Western and Southwestern blotting analyses. (**A**) 5 μg of Raji NE protein was separated in a 10% SDS–polyacrylamide gel and transferred to a nitrocellulose filter. One section was reacted with monoclonal anti-c-*myc* antibody (Miyamoto *et al.*, 1985), and proteins reacting with the antibody were visualized with ^{125}I-anti-mouse IgG. Another section of the same blot, run in parallel, was reacted with ^{32}P-labelled *myc* (135–155) as in Materials and methods. The sizes of mol. wt markers are shown beside the figure. (**B**) 5 μg of Raji NE protein was immunoprecipitated with an anti-human c-*myc* antibody. The precipitate (right) and the original Raji NE protein (left) were separated in a polyacrylamide gel and subjected to Southwestern analysis as in (**A**).

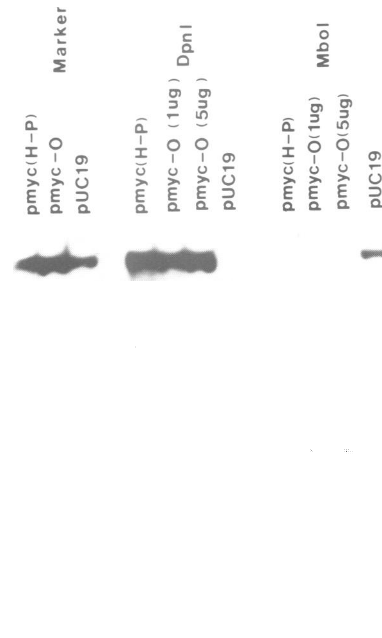

Fig. 6. DNA replication assay. p*myc*-O contains *myc* (135–155) (see Table I) inserted into *Bam*HI–*Sac*I site of pUC19. Replication assay was performed after the transfection of plasmid DNA into HeLa cells as described in Materials and methods. Plasmids and enzymes used in the experiments are shown above the figure. The equivalent of 20 copies of p*myc* (H-P), p*myc*-O or pUC19 per cell is shown as a copy number marker.

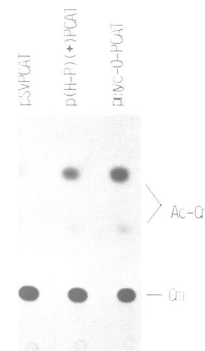

Fig. 7. Transcriptional activity of *myc* (135–155). CAT assay was carried out after the transfection of plasmid DNA into L cells in Materials and methods. The plasmids used are shown above the figures. pmyc-O-PCAT carrys the *myc* (135–155) inserted upstream of the SV40 promoter of pSVPCAT. Cm, chloramphenicol; Ac-Cm, acetylated chloramphenicol.

initiation of adenovirus DNA replication *in vitro* (Jones *et al.*, 1987). An octamer-binding transcription factor (OTF-1), which exists ubiquitously in mammalian cells, is physically and biologically identical to another cellular DNA-binding protein, nuclear factor III (NF-III) which is also required for adenovirus DNA replication (O'Neill *et al.*, 1988). CTF/NF-I and OTF-1/NF-III can serve both as transcription factors for RNA polymerase II and as initiation factors for viral DNA replication. The data shown in this report strongly suggest that c-*myc* protein is also a sequence-specific DNA binding protein which functions in both cellular DNA replication and transcription.

Band shift assay using Raji NE showed three shifted complexes, A, B and C. From the results using various oligonucleotides with deletion or point mutation, it is suggested that complexes A and B may be derived from the hetero- or homo-protein complexes with c-*myc* protein. Longer oligonucleotides form complexes more easily than oligonucleotides possessing only the minimum c-*myc* protein binding sequence of seven nucleotides. This suggests that complex formation needs the 'space' in DNA or, alternatively, that another protein containing non-specific DNA binding activity requires sequences besides the c-*myc* protein binding sequences to facilitate formation of protein–protein complexes. The formation of these complexes seems to be promoted by phosphorylation (Y.Negishi, S.M.M.Iguchi-Ariga and H.Ariga, in preparation) and to be important for the activities of the sequence in replication and transcription. Recently, phosphorylation of *myc* protein by casein kinase II has turned out to play a role in signal transduction to the nucleus (Luescher *et al.*, 1989). Phosphorylation by casein kinase II or by other kinase may be involved in complex formation we observed.

What kinds of protein might associate with c-*myc* protein? It is possible that c-*myc* protein binds to DNA replication protein(s) such as DNA polymerase, since c-*myc* protein is likely to function in initiation of DNA replication. p53, another nuclear oncogene product, which is also possibly a DNA replication protein (Iguchi-Ariga *et al.*, 1988b; Sturzbecher *et al.*, 1988), binds to SV40 T antigen associated

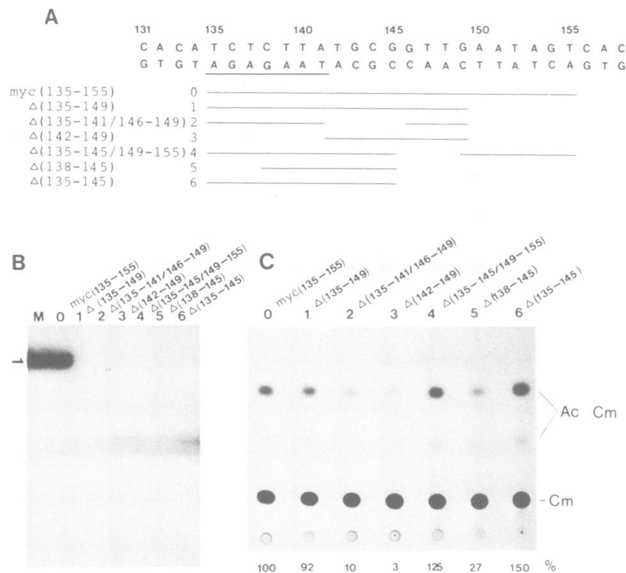

Fig. 8. Precise junction between sequences required for replication or transcriptional activity. The oligonucleotides (**A**) used in the binding assay shown in Figure 5 were inserted into *Bam*HI site or *Bam*HI–*Sac*I sites of pUC19 or pSVPCAT. Autonomous replication of pUC clones (**B**) and transcriptional activity of pSVPCAT clones (**C**) were measured as in Figures 6 and 7 respectively. The underlined sequences in A represent the minimum c-*myc* protein binding sequences. The arrow and M in B represent the positions of replicated plasmid DNA and pUC19 size marker respectively. Numbers below the lanes in C indicate the density, determined by densitometric scanning, of acetylated chloramphenicol spots. Cm, chloramphenicol; Ac-Cm, acetylated chloramphenicol.

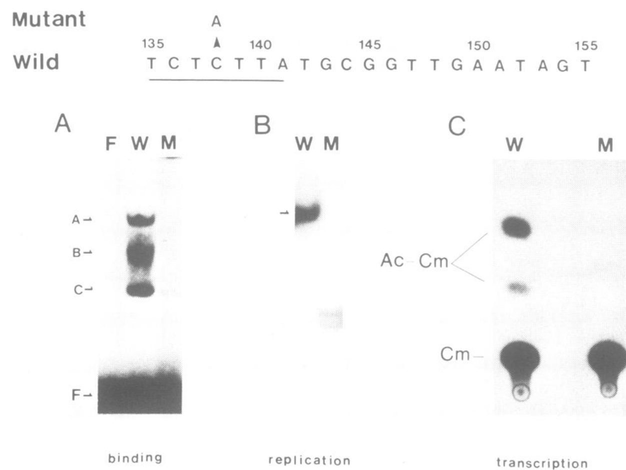

Fig. 9. Effect of point mutation within c-*myc* protein binding sequences on binding, autonomous replication and transcriptional activities. An oligonucleotide, designated as 'Mutant', containing an A/T pair instead of C/G at the position 138 in the *myc* (135–155) oligo (denoted 'Wild' in this figure) was used as a probe in band shift assay (**A**), inserted into *Bam*HI–*Sac*I sites of pUC19 or pSVPCAT and examined for autonomous replication activity (**B**) or transcriptional activity (**C**) respectively. The underlined sequence in the oligonucleotide shown above the figures represent the minimum c-*myc* protein binding sequences. W, 'Wild' type oligo *myc* (135–155); M, 'Mutant' oligo. The shifted bands in (**A**) are designated as A, B and C as in Figures 3 and 5, and F indicates free DNA. The arrow in (**B**) indicates the position of the replicated plasmid DNA. In (**C**) Cm and Ac-Cm represent the positions of chloramphenicol and acetylated chloramphenicol respectively.

with DNA polymerase alpha (Gannon and Lane, 1987; Braithwaite *et al.*, 1987). To investigate such a possibility for c-*myc* protein, we and others are now fractionating the cell-free c-*myc* dependent DNA replication system (Iguchi-Ariga *et al.*, 1987a; Umekawa *et al.*, 1988). c-*myc* protein may also bind to factors necessary for transcription. It has recently been demonstrated that the c-*fos* protein, another nuclear oncogene product, complexes with AP-1 to stimulate transcription (Rauscher *et al.*, 1988; Sassone-Corsi *et al.*, 1988; Chiu *et al.*, 1988). This interaction between AP-1 and *fos* protein is mediated by the 'leucine-zipper' (Landschulz *et al.*, 1988; Kouzarides and Ziff, 1988). These leucine-zippers are also present in the C-terminal region of the *myc* gene products suggesting the possibility of formation of complexes between c-*myc* proteins (as a homo-dimer) or between c-*myc* protein and other proteins with leucine-zippers. A recent report has shown that c-*myc* protein exists as a multimer *in vivo*, and that this requires the leucine-zipper (Dang *et al.*, 1989).

Now that purified biologically active c-*myc* protein will soon be available, the molecular mechanisms of initiation of DNA replication and enhancement of RNA transcription, mediated by c-*myc* protein, can be clarified.

Materials and methods

Cells

Human HeLa cells and mouse L cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Human Raji cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum.

Replication of plasmid DNA in HeLa cells

To examine autonomous replication of plasmid DNA, HeLa cells were transfected with DNA by the calcium phosphate method (Graham and van der Eb, 1973). 1×10^6 cells were transfected with 1 μg of DNA and cultured. Forty hours after transfection, low mol. wt DNAs were extracted by the Hirt procedure (Hirt, 1967), and digested with *Eco*RI to linearize the plasmid, and with *Dpn*I to eliminate the input plasmid DNA used for transfection, or with *Mbo*I to digest the replicated DNA. The digested DNA from the Hirt supernatant was then electrophoresed on a 2.0% agarose gel, blotted by the method of Southern (Southern, 1975) and hybridized with ^{32}P-labelled pUC19. Hybridization of the blotted filter with labelled probe was carried out as described previously (Ariga *et al.*, 1987).

CAT assay

Plasmid DNAs were transfected into mouse L cells by the calcium phosphate method (Graham and van der Eb, 1973). Two μg of DNA were used for 1×10^6 cells. Two days after transfection, the cells were harvested, suspended in 200 μl of 0.25 M Tris–HCl (pH 7.8), and disrupted by freeze–thawing three times prior to sonication. CAT assays were carried out with the cell lysate as described previously (Scholer and Gruss, 1984).

Band shift assay

Nuclear extract of Raji cells was prepared as described by Dignam *et al.* (1983) with minor modifications (Westin *et al.*, 1987). After $(NH_4)_2SO_4$ precipitation, the extract was resuspended in, and dialysed against, 20 mM HEPES–KOH (pH 7.9), 20% glycerol, 20 mM KCl, 2 mM $MgCl_2$, 0.2 mM EDTA and 0.5 mM dithiothreitol (DTT). In the band shift assay (Fried and Crothers, 1981; Garner and Revzin, 1981), binding reactions were carried out by incubating 2–4 fmol of end-labelled DNA (5000 c.p.m.) with 2–3 μg of nuclear extract proteins and 0.5 μg of poly(dI–dC) in a buffer containing 15 mM HEPES (pH 7.9), 15% glycerol, 2% polyvinyl-alcohol, 36 mM NaCl and 0.4 mM DTT at room temperature for 10 min in a final volume of 15 μl. After incubation, the reaction mixtures were loaded on a 4% polyacrylamide (29:1) gel in 0.25 × TBE buffer, and electrophoresed at 10 V/cm. For competition experiments, 2.5 pmol of double stranded annealed oligonucleotide was added to the reaction mixture prior to addition of the extract. In experiments with antibodies, 1 μg of murine polyclonal anti-human c-*myc* antibody (Naoe *et al.*, 1988) or murine anti-human IgG was incubated for 10 min at 0°C with NE, and the mixture was then subjected to band shift assay.

DNase I protection analysis

A 5′ end-labelled DNA fragment (1 fmol, 5000 c.p.m.) was incubated together with 10–20 μg of nuclear proteins for 15 min on ice in a final volume of 25 μl containing 60 mM KCl, 20 mM HEPES (pH 7.9), 8% glycerol, 1 mM DTT, 0.8 mM $MgCl_2$ and 1 μg of poly(dI-dC). 0.5–2 μg of DNase I was then added and allowed to digest for 80 s on ice, before the DNase I was inactivated by the addition of 0.6 M NaCl, 0.2% SDS, 10 mM EDTA, followed by phenol extraction and ethanol precipitation. The end-labelled DNA recovered was analysed on denaturing 8% polyacrylamide gels.

Western blotting analysis

5 μg of Raji NE protein was separated in a 10% SDS–polyacrylamide gel (Laemmli, 1970), blotted onto a nitrocellulose filter in a buffer containing 20 mM Tris and 150 mM glycine for 16 h at 15 V. The filter was subsequently blocked for 1 h at room temperature in PBS (130 mM NaCl, 7 mM Na_2HPO_4 and 3 mM NaH_2PO_4) containing 10% milk and 0.1% Tween 20, reacted with 1/50 dilution of 0.1 μg/μl of anti-c-*myc* antibody, IF7 (Miyamoto *et al.*, 1985) in the same buffer for 16 h at 4°C, and washed with PBS containing 0.1% Tween 20, and autoradiographed.

Southwestern blotting

A section of the same blot used in Western blotting as described above was probed according to the procedure of Miskimins *et al.* (1985) using ^{32}P-labelled oligo *myc* (135–155) (Table I).

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