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Possible function of the c-*myc* product: promotion of cellular DNA replication

Sanae M.M.Iguchi-Ariga, Teru Itani, Yukiko Kiji and Hiroyoshi Ariga

The Institute of Medical Science, The University of Tokyo 4-6-1, Shirokanedai, Minato-ku, Tokyo 108, Japan

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We have recently cloned a plasmid, pARS65, containing the sequences derived from mouse liver DNA which can autonomously replicate in mouse and human cells (Ariga *et al.*, 1987). In this report, we show that replication of pARS65 in HL-60 cells can be inhibited by co-transfection with anti-c-*myc* antibody. In an *in-vitro* replication system using HL-60 nuclear extract, pARS65 functioned as a template. This *in-vitro* replication was also blocked by addition of anti-c-*myc* antibody. Specific binding activity of the c-*myc* product to pARS65 was detected by an immunobinding assay, suggesting that the c-*myc* protein promotes DNA replication through binding to the initiation site of replication. This has been substantiated using the antibody to help isolate a human DNA segment that can autonomously replicate in the cells.

Key words: c-*myc* protein/DNA replication/autonomous replicating sequence

Introduction

The c-*myc* gene, first identified as a cellular counterpart of the transforming gene of avian myelocytomatosis virus MC29 (v-*myc*) (Sheiness and Bishop, 1979), exists in a variety of eukaryotes (Shilo and Weinberg, 1981; Bishop, 1983) and is expressed in most tissues (Gonda *et al.*, 1982). Abnormal expression of c-*myc* is often observed in various tumors, and it has been suggested that over-expression of c-*myc* gives rise to 'immortalization' in two steps of cell transformation (Land *et al.*, 1983). Since the predicted amino acid sequence of the c-*myc* protein has homology with the sequence of adenovirus E1A protein (Ralston and Bishop, 1983), it was suggested that a common function of these proteins in cell transformation is 'immortalization'. Expression levels of c-*myc* are high in actively proliferating tissues and low in non-proliferating tissues (Pfeifer-Ohlsson *et al.*, 1984; Slamon and Cline, 1984), and can be quickly induced when quiescent cells are induced to proliferate ($G_0 \rightarrow G_1$) by growth factors or mitogens (Kelly *et al.*, 1983). Therefore c-*myc* may be a fundamental gene closely involved with cell proliferation and the c-*myc* protein may play some part in cellular DNA replication. The function of the c-*myc* protein, however, has not been clarified to date, although the protein localizes in cell nuclei and has DNA-binding activity (Donner *et al.*, 1982; Persson and Leder, 1984).

We have recently cloned a plasmid pARS65 containing sequences derived from mouse liver DNA which can autonomously replicate in both mouse and human cells (Ariga *et al.*, 1987). pARS65 replicated semiconservatively, and initiation of DNA replication started from the mouse DNA sequences when the replicating activity of the plasmid was studied in an *in-vitro* replication system developed from mouse FM3A cells. Here we report that replication of pARS65 is inhibited by an antibody to the c-*myc* gene product both *in vivo* (intact cells) and *in vitro* (cell-free system). We also show that the c-*myc* product specifically binds to the ARS sequence of pARS65, suggesting that the c-*myc* product promotes cellular DNA replication by binding to the *ori* region. In order to substantiate this, we tried to isolate autonomously replicating sequences (ARS) of human DNA as the binding sequences of the c-*myc* product. Nearly 90% of the clones obtained could autonomously replicate in mouse and human cells. Nucleotide sequences and possible higher structures of a cloned human ARS are shown.

Results

Effect of anti-c-myc antibody on cellular DNA synthesis

HL-60 cells were transfected with anti-human c-*myc* antibodies by a liposome-mediated transfection technique. This novel techni-

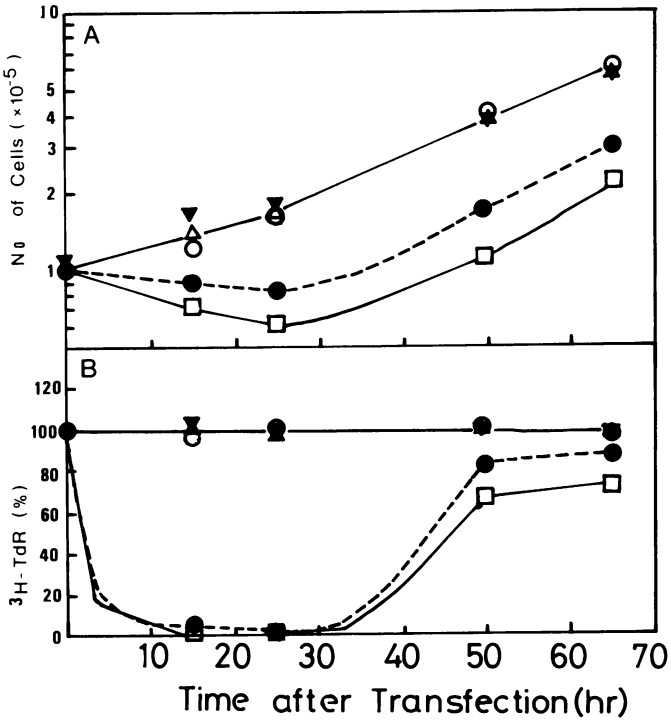

Fig. 1. Inhibition of DNA replication in HL-60 cells by anti-human c-*myc* antibody. HL-60 cells were transfected with anti-human c-*myc* antibody (Oncor, Inc.) or non-specific anti-human IgG (Itani *et al.*, 1987). At various times, the cell number was determined. The incorporation of [^{3}H]thymidine into the cells was assayed at each time after 1 h incubation with [^{3}H]thymidine at 25 μCi/ml. The amount of incorporation was expressed as the percentage of that of 10^5 cells immediately after transfection.
A, Proliferation of HL-60 cells after transfection with antibodies.
B, Incorporation of [^{3}H]thymidine into HL-60 cells after transfection with antibodies. ○, non-transfected; ●, transfected with anti-human c-*myc* antibody (Oncor, Inc.); □, transfected with c-*myc* protein specific monoclonal antibody, IF7; ▼, transfected with anti-human IgG; △, mock-transfected with empty liposomes.

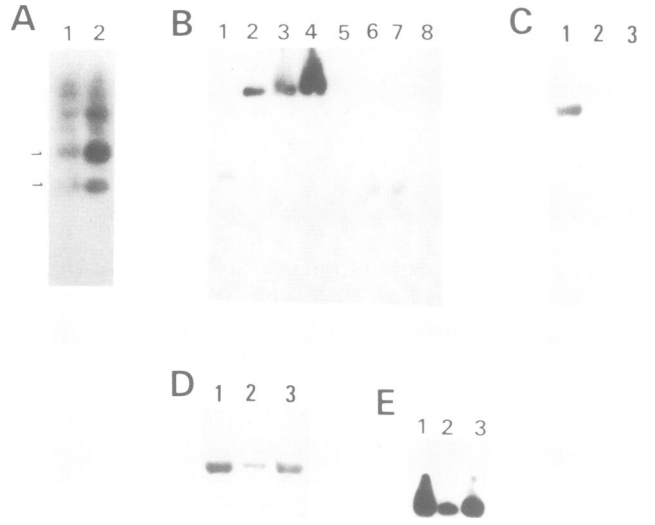

Fig. 2. Replication of pARS65 in HL-60 cells. **A,** Replication of pARS65 in HL-60 cells. Low molecular weight DNA was extracted at 0 (lane 1) and 2 days (lane 2) after transfection, and analyzed by the Southern method without any digestion by restriction enzymes. The arrows indicate the positions of forms II and I of pARS65 from the top respectively. **B,** Time course experiment. The HL-60 cells were transfected with pARS65 (lanes 1–4) or pUC19 (lanes 5–8). The DNA was extracted from the cells at 0 (lanes 1 and 5), 1 (lanes 2 and 6), 2 (lanes 3 and 7), and 3 days (lanes 4 and 8) after transfection, and analyzed as in A. **C,** Replication of pARS65 (lane 1), pMU111 (lane 2), and pUC19 (lane 3) in HL-60 cells. **D,** Inhibition of replication of pARS65 in HL-60 cells by anti-human c-*myc* antibody. Lane 1, pARS65 only; lane 2, pARS65 with anti-human c-*myc* antibody; lane 3, pARS65 with sheep anti-human IgG. E, Inhibition of replication of pARS65 in HL-60 cells by c-*myc* specific monoclonal antibody IF7. Lane 1, pARS65 only; lane 2, pARS65 with IF7; lane 3, pARS65 with mouse anti-human IgG. For experiments in C, D, and E, DNA was extracted 3 days after transfection.

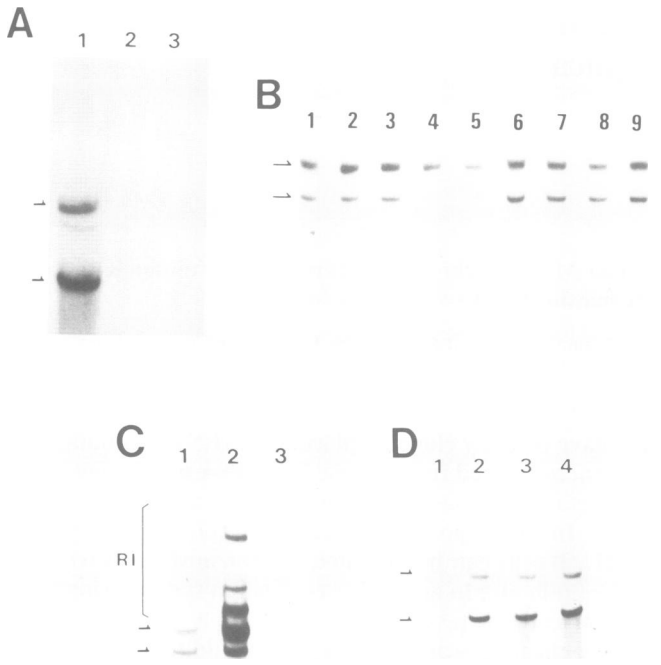

Fig. 3. *In-vitro* replication of pARS65 in nuclear extract of HL-60 cells. **A,** *in-vitro* replication of pARS65 (lane 1), pMU111 (lane 2), and pUC19 (lane 3) in HL-60 NE. **B,** inhibition of *in-vitro* replication of pARS65 in HL-60 NE by anti-human c-*myc* antibody. Anti-human c-*myc* antibody was added to the reaction mixture at the protein concentration of 0.1 (lane 2), 0.2 (lane 3), 0.3 (lane 4), or 0.4 (lane 5) μg/100 μl. To other mixtures, anti-human IgG was added instead at 0.1 (lane 6), 0.2 (lane 7), 0.3 (lane 8), or 0.4 (lane 9) μg/100 μl. Lane 1, no antibody was added. **C,** *in vitro* replication of pARS65 in NE of various cells. Lane 1, FM3A; lane 2, HL-60; lane 3, U937. **D,** Complementation of defect of replication activity in U937 NE. The reaction mixture for U937 NE (lane 1) was added with 5 μl (lane 2) or 10 μl (lane 3) of HL-60 NE and reacted. Lane 4 indicates the product synthesized in HL-60 NE. The arrows indicate the positions of form II and I of plasmid DNA from the top, and RI indicates replicative intermediates. Approximately 150 pmol of [^{32}P]dCMP/μg of the added pARS65 were incorporated in 1 h of incubation with HL-60 nuclear extract, indicating that about 10% of added DNA were used as a template.

que (Itani *et al.*, 1987) allows introduction of DNA as well as protein into more than 95% of cells in suspension culture without toxicity. After transfection with either of two anti-c-*myc* antibodies, (polyclonal and monoclonal antibodies, see Materials and methods) cell growth was inhibited (Figure 1A) and incorporation of [^{3}H]thymidine was almost completely blocked (Figure 1B). Transfection with non-specific anti-human IgG or empty liposomes had no effect on cell growth or DNA synthesis, indicating that the inhibition was due to the specific inactivation of the c-*myc* product. Inhibition of cell growth and DNA synthesis was observed for more than 40 h following transfection, and then gradually the cells began to grow again. These results suggest that the c-*myc* protein is necessary for, or closely related to, cellular DNA replication.

Replication of ARS plasmid

We next transfected HL-60 cells with pARS65 containing a mouse-derived ARS which can autonomously replicate in mouse and human cells (Ariga *et al.*, 1987). Two days after transfection, DNA in a Hirt supernatant (Hirt, 1967) was extracted and visualized on agarose gel by Southern blotting (Southern, 1975) (Figure 2A). Approximately five times more pARS65 DNA was recovered after 2 days than at day zero. The structures of recovered DNA were the same as those at day zero [closed circular (form I), open circular (form II) and a concatemer migrating above (form II)]. To determine if pARS65 actually replicated in the transfected cells, the recovered pARS65 DNA was digested with *Dpn*I to eliminate the DNA used for transfection and also with *Bam*HI to linearize before electrophoresis. The plasmids used for transfection were grown in a dam$^+$ *E. coli* strain and were methylated and sensitive to cleavage by *Dpn*I. DNA replicated in mammalian cells, on the other hand, are hemimethylated or unmethylated and are insensitive to *Dpn*I digestion, since dam methylase is lacking in mammalian cells. As shown in Figure 2B, the amount of *Dpn*I-resistant pARS65 increased with time, while no *Dpn*I-resistant pUC19 (control plasmid) was detected. From the autoradiography, it has been calculated that pARS65 replicates episomally and there are about 10 000 copies per cell. Other transfected plasmids, pMU111 and pUC19, were not detected in Hirt supernatants of cells on the third day after transfection (Figure 2C). Plasmid pMU111 also carries mouse-derived sequences but cannot replicate in mouse FM3A cells (Ariga *et al.*, 1987). To examine the effect of the c-*myc* product upon replication of pARS65, two different anti-human c-*myc* antibodies as well as non-specific anti-human IgG were co-transfected with pARS65 into HL-60 cells. Replication of pARS65 was inhibited by the specific antibodies, but not by the non-specific control (Figure 2D,E). This suggests that the c-*myc* protein may participate in replication of pARS65 in these cells.

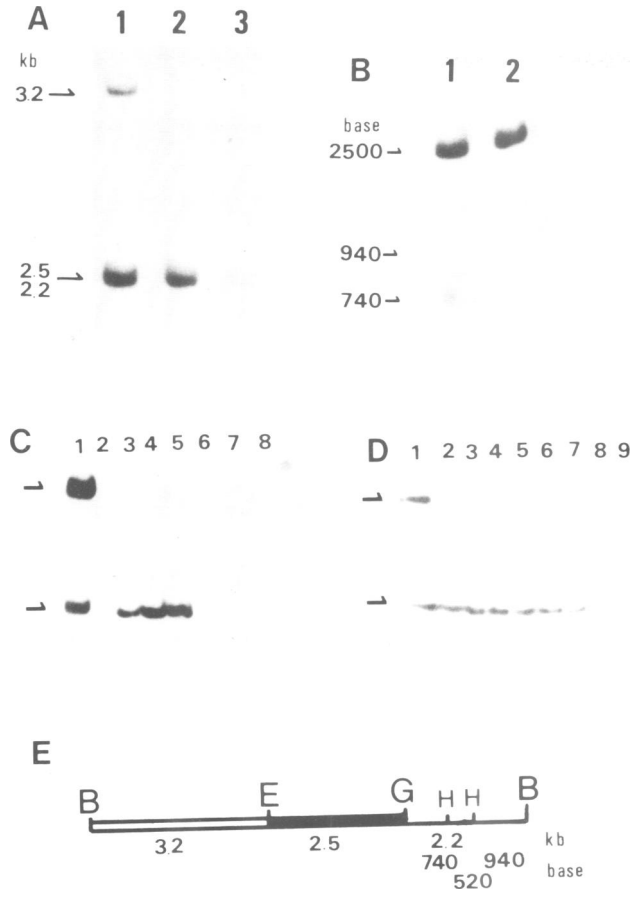

Fig. 4. Binding of c-*myc* product of HL-60 to sequences in pARS65. pARS65 was digested with *Eco*RI, *Bgl*II, and *Bam*HI, and end-labelled with [^{32}P]. **A,** The ^{32}P-labelled pARS65 fragments (lane 1) were incubated with HL-60 NE and then precipitated as immunecomplex with 2 μg of anti-human c-*myc* antibody (lane 2) or anti-human IgG (lane 3). **B,** The ^{32}P-labelled pARS65 fragments recovered from immunecomplex precipitated with anti-human c-*myc* antibody (lane 1) were digested with *Hin*dIII (lane 2). The arrows indicate the size of the fragments. **C,** The ^{32}P-labelled pARS65 fragments (lane 1) were incubated with HL-60 NE and then precipitated with 0, 0.5, 1, and 2 μg of anti-human c-*myc* antibody (lanes 2–5, respectively) or 0.5, 1, and 2 μg of anti-human IgG (lanes 6–8, respectively). **D,** The ^{32} P-labelled pARS65 fragments (lane 1) were incubated with HL-60 NE in addition to cold 3.2 kb of vector DNA (lanes 3 and 4), 2.2 kb of SV40 DNA (lanes 5 and 6), and 2.5 kb of mouse DNA (lanes 7–9) (see Figure 4E). The amounts of DNA added in the reaction were 0 μg (lane 2), 0.5 μg (lane 7), 2 μg (lanes 3, 5 and 8), and 8 μg (lanes 4, 6 and 9). **E,** Structure of pARS65 cut with *Bam*HI. B, E, G, and H indicate the sites of *Bam*HI, *Eco*RI, *Bgl*II, and *Hin*dIII respectively. □, pKSV10; ■, mouse DNA; —, SV40 DNA.

Effect of anti-c-myc antibody on DNA replication in-vitro

To confirm the dependence of DNA replication upon the c-*myc* product, *in-vitro* replication of pARS65 was studied in HL-60 nuclear extract. As shown in Figure 3A, pARS65 functioned as a template in this assay and yielded two major bands, form I and form II, as well as replicative intermediates seen above form II. We confirmed that *in-vitro* DNA products were produced by semiconservative replication and not by repair synthesis in HL-60 nuclear extract as in the FM3A system (Ariga *et al.*, 1987). Approximately 98% of the *in-vitro* products in the reaction containing BudUTP, instead of dTTP, with pARS65 as a template were found in heavy-light and heavy-heavy DNA regions by isopycnic centrifugation (data not shown). Therefore, the *in-vitro* products visualized on agarose gels, even without digestion by *Dpn*I, are actually the replicated molecules. It was also confirmed in this system that the initiation point of *in-vitro* replication of pARS65 exists in the 2.5 kb *Eco*RI–*Bgl*II fragment derived from mouse DNA (see Figure 4E) and replication moves bidirectionally as previously described (Ariga *et al.*, 1987). Other plasmids, pMU111 and pUC19, gave rise to no detectable band of newly synthesized DNA, suggesting that they did not replicate in the system (Figure 3A, lanes 2 and 3 respectively). The *in-vitro* replication of pARS65 was also inhibited when the polyclonal anti-human c-*myc* antibody was added to the system (Figure 3B). The extent of the inhibition was dependent upon the amount of antibody. Replication was not inhibited by sheep anti-human IgG. This suggested that the c-*myc* product may be necessary for replication of pARS65. Replication of pARS65 was tested in three different cell lines, HL-60, FM3A and U937. In U937 cells, the c-*myc* gene was not amplified and its expression was much lower than in HL-60, while in FM3A cells, c-*myc* was expressed at a moderate level (data not shown). The *in-vitro* replication of pARS65 using nuclear extract was found to be most active with extract from HL-60, less with FM3A and undetectable with U937 nuclear extract (Figure 3C). Thus the level of replication of pARS65 may be co-related to the level of c-*myc* expression. Similar results were obtained for replication of pARS65 in intact cells (Ariga *et al.*, 1987). Furthermore, the defect in *in-vitro* replication with U937 nuclear extract was complemented by addition of the HL-60 nuclear extract (Figure 3D). These results suggest two possibilities: either the c-*myc* product may be necessary for replication of pARS65, or HL-60 nuclear extract does not contain inhibitors of DNA synthesis.

Binding of c-myc protein to mouse ARS

Since our results suggest the presence of an interaction between pARS65 DNA and c-*myc* protein, we examined the binding of c-*myc* protein to pARS65 by an immunobinding assay. The c-*myc* product has a strong affinity to DNA but has no sequence specificity (Watt *et al.*, 1985). Since the immunobinding assay is very sensitive, we could detect specific binding over a low background of non-specific binding (Prives *et al.*, 1983). pARS65 DNA was digested with *Eco*RI, *Bgl*II and *Bam*HI, labelled with [γ-^{32}P]ATP, and used in the immunobinding assay. A fragment of ~2.2–2.5 kb of pARS65 was specifically precipitated with anti-human c-*myc* antibody, while no labelled fragment was precipitated by anti-human IgG (Figure 4A). These results suggest that the c-*myc* protein specifically binds to sequences of the 2.2 kb- and/or 2.5-kb fragment of pARS65. A 3.2-kb *Bam*HI–*Eco*RI fragment, derived from pKSV10, was not precipitated. Since it is difficult to resolve the 2.5- and 2.2-kb fragments on a gel, the DNA fragments were recovered from the immunecomplexes by phenol extraction and electrophoresed following digestion by *Hin*dIII (Figure 4B). A similar electrophoretic pattern with a single band of ~2.5 kb was observed before and after the digestion. This indicates that the specific binding site of the c-*myc* protein is present in the 2.5-kb *Eco*RI–*Bgl*II fragment of pARS65, which is derived from mouse DNA and contains the initiation site of autonomous replication (see Figure 4E). To confirm this result, the same immunobinding assay was carried out using various amount of antibody (Figure 4C). Only the 2.5-kb fragment was precipitated with various amounts of anti-c-*myc* antibody, but not with anti-human IgG. Furthermore, the precipitation of the 2.5-kb fragment by anti-c-*myc* antibody was inhibited by addition of cold 2.5-kb fragment the immunobinding reaction, while neither the vector 3.2-kb fragment nor a 2.2-kb SV40 fragment inhibited the precipitation (Figure 4D).

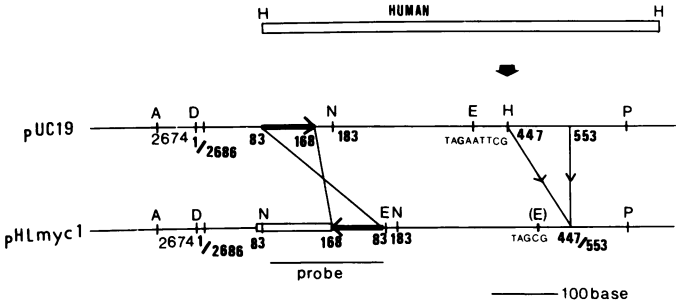

Fig. 5. Structure of pHLmyc1. The structure of pHLmyc1 was determined after restriction enzyme analysis and nucleotide sequencing analysis. The open boxes represent human sequences. The thick arrows in pUC19 indicate the direction of the sequences. **A,** *Aat*III site; **D,** *Dra*II site; **E,** *Eco*RI site; **H,** *Hin*dIII site; **N,** *Nde*I site; **P,** *Pvu*II site. The number indicates the position of sequences in pUC19.

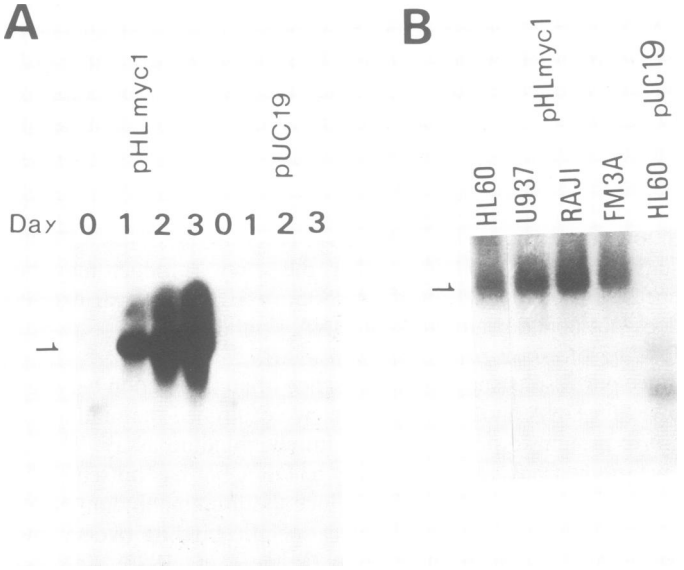

Fig. 6. Replication of pHLmyc1 in various cells. **A,** pHLmyc1 or pUC19 was transfected into HL-60 cells. On various days after transfection, the low molecular weight DNAs were extracted from the cells by Hirt procedure (Hirt, 1967), electrophoresed after enzyme digestion, Southern-blotted (Southern, 1975), and hybridized with ^{32}P-labelled pUC19. **B,** pHLmyc1 was transfected into HL-60, U937, Raji, and FM3A cells. pUC19 was transfected into HL-60 cells as a negative control. Two days after transfection, the DNA was analyzed as above. The arrow represents the position of the replicated plasmid DNA.

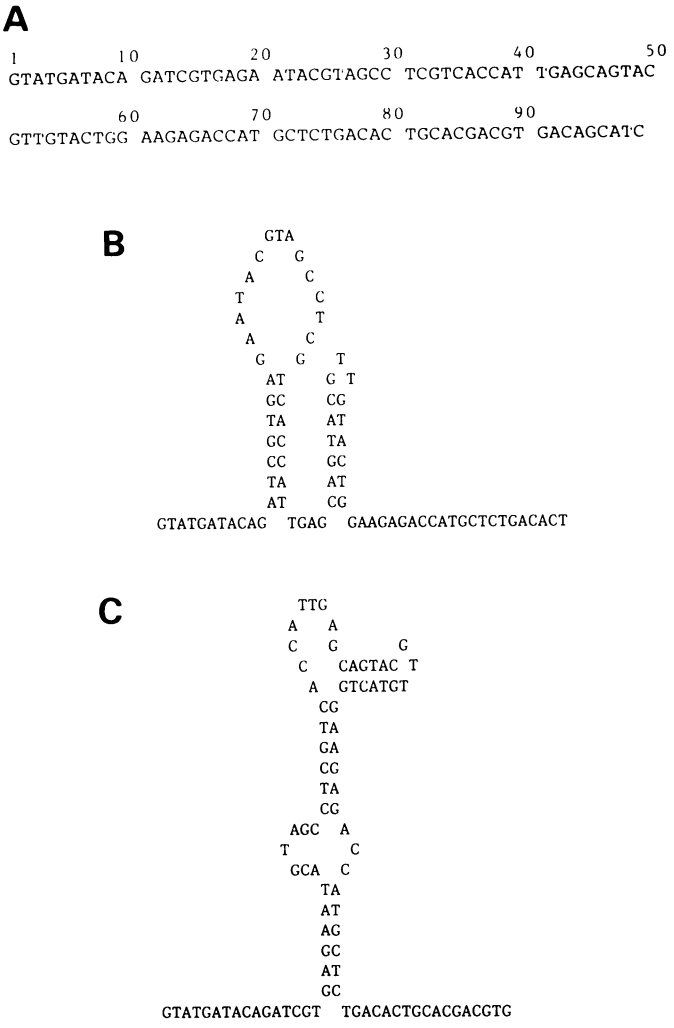

Fig. 7. Sequence and possible higher structure of the cloned human ARS fragment in pHLmyc1. **A,** The nucleotide sequence of the human DNA-derived fragment in pHLmyc1 was determined by dideoxy chain termination method (Messing, 1983). **B** and **C**, a possible secondary structure of the fragment was speculated by computer-assisted sequence analysis.

Isolation of ARS derived from human DNA

The results described above suggest that the c-*myc* product promotes cellular DNA replication by binding at or near the initiation site of replication (*ori*). Thus, human DNA fragments selected as containing binding sequence for c-*myc* protein should contain ARS derived from human DNA. Isolation of human ARS was, therefore, tried as described in Materials and methods. Among several thousand colonies obtained, 10 clones were examined for their ARS activity in HL-60 cells. The plasmid DNAs, designated pHLmyc1, pHLmyc2, pHLmyc3, etc. respectively, were prepared on a large scale with CsCl centrifugation, and then transfected into HL-60 cells (Itani *et al.*, 1987). Two days after transfection, a large amount of pUC19-hybridizable DNA was recovered from cells by the Hirt procedure (Hirt, 1967). The structures of the recovered DNA were closed circular and open circular DNAs and concatemeric form as in the case of pARS65 replicated in HL-60 cells (data not shown). The recovered DNAs in the Hirt supernatant were digested with *Dpn*I before electrophoresis to eliminate the plasmids originally used for transfection. Since the carrier vector, pUC19, did not replicate in HL-60 cells, the cloned fragments derived from HL-60 DNA are likely to be due to the ARS activity of pHLmyc's (Figure 6). The pHLmyc clones replicated episomally at about 500−10 000 copies/cell. Of the ten clones tested, nine clones had ARS activity in HL-60 cells. These clones lacked homology with either the *Alu* or the *Kpn*I family as analyzed by Southern blotting. Furthermore, all the replicated pHLmyc's were sensitive to digestion by *Mho*I which cuts only unmethylated DNA, suggesting that re-initiation of pHLmyc DNA replication occurred in the transfected HL-60 cells (Iguchi-Ariga *et al.*, manuscript in preparation). pHLmyc1 and the other eight clones showed ARS activity in U937, Raji, and FM3A cells as well as in HL-60 cells (Figure 6), suggesting that the DNA fragment of pHLmyc, derived from HL-60 DNA, can generally function as an ARS in mammalian cells.

We also noted that gene rearrangement occurred in all the pHLmyc clones chosen, resulting in loss of the *Hin*dIII sites at

the junctions of the pUC19 gene and the HL-60 DNA-derived sequences, shortening of the HL-60 fragments and deletion (~100 bp) and rearrangement including inversion of sequences of pUC19 (Figure 5). The fine structure of the recovered pHLmyc clones will be described in detail elsewhere (Y.Kiji, S.M.M.Iguchi-Ariga and H.Ariga, in preparation). The other pHLmyc clones with replicating activity also had the same structure of rearranged pUC19 sequences with small differences in the inserted human sequences (described in detail below). The reason for such rearrangement is not clear, but it is obvious that it has been caused by insertion of the human sequences. pHLmyc1, which replicated to the highest copy number in HL-60 cells was used for further experiments.

An immunobinding assay of pHLmyc clones after digestion with *Eco*RI and *Nde*I revealed that only a fragment of about 0.2 kb of pHLmyc1 was precipitated in a dose-dependent fashion by polyclonal anti-human c-*myc* antibody, while no labelled fragments were precipitated by non-specific anti-human IgG (Iguchi-Ariga *et al.*, in preparation). Precipitation of ^{32}P-labelled 0.2-kb fragment with anti-human c-*myc* antibody was inhibited by addition of cold 0.2-kb fragment, but not by addition of 2.4-kb fragment. Hence the specific binding site for the c-*myc* protein is present in the 0.2-kb *Eco*RI–*Nde*I fragment of all these pHLmyc clones. Therefore, the specific binding sequences of the c-*myc* product are related to the ARS activity of these clones.

pHLmyc1 had rearranged sequences of parental pUC19 in addition to human sequences (Figure 5). Therefore sequences necessary for replication in mammalian cells might be derived from rearranged pUC19 sequences, or from both human and rearranged pUC19 sequences. To clarify this question, the 0.2-kb *Eco*RI–*Nde*I fragment of pHLmyc1, containing human sequences, was introduced into the *Eco*RI site of pUC19 or pBR322 after the *Nde*I site was changed to *Eco*RI site. The replicating activities of the new clones named pUCHLmyc or pBRHLmyc respectively were examined two days after transfection into HL-60 cells. Both the clones replicated well at copy numbers as high as pHLmyc1 (Iguchi-Ariga *et al.*, in preparation), suggesting that only the human sequences are enough for pHLmyc1 to replicate in HL-60 cells. However, we cannot completely rule out the possibility that pUC19 sequences spanning from Nn 83 to Nn 168 were also necessary.

Southern blot analysis revealed that sequences similar to the *Eco*RI–*Nde*I fragment of pHLmyc1 are common in the genomic DNAs of human cells ($1-5 \times 10^4$ copies/haploid genome). These results support the idea that pHLmyc1 contains human ARS of DNA replication, which are present throughout the human genomic DNA. These will be described in detail elsewhere (Iguchi-Ariga *et al.*, in preparation).

Structural features of cloned human replication origin

The nucleotide sequence of the cloned human DNA fragment in pHLmyc1 (~100 nucleotides) obtained by the dideoxy chain termination method (Messing, 1983) is shown in Figure 7. Other pHLmyc clones, containing replicating activity, have exactly the same sequences as shown in Figure 7. pHLmyc4, which has less replicating activity than pHLmyc1, had a slightly different sequence as follows: CAGTA from Nn 45 to 49 were changed to ACTCC, giving rise to a non-loop structure as shown in Figure 7B. Computer-assisted analysis revealed that the sequence of the pHLmyc1 fragment is a unique sequence without homology to any sequence listed to date in GENBANK and EMBL DNA data bases. No significant homology was found between the pHLmyc1 sequence and the initiation sites of DNA replication so far reported for *E. coli oriC* (Kornberg, 1980), SV40 *ori*, polyoma virus *ori*, left-end of adenovirus DNA, *Saccharomyces cerevisiae ars1* and *ars2* (Stinchcomb *et al.*, 1981; Tschumper and Carbon, 1981), *Xenopus* ARS (Hiraga *et al.*, 1982), human mitochondrial DNA *ori* (Attardi *et al.*, 1978) and human sequences necessary for replication in yeast (Montiel *et al.*, 1984). There are several inverted repeats in the cloned sequence which can form hairpin structures, and two such possible higher structures are shown in Figures 7B,C.

Discussion

Using a mouse-derived ARS clone, we present evidence suggesting that one of the functions of the c-*myc* product is to promote cellular DNA replication by binding to the initiation site of replication. Our conclusion that c-*myc* product promotes cellular DNA replication is highly dependent upon the specificity of anti-c-*myc* antibodies used. As described in Materials and methods, the antibodies reacted with only one 60 000-dalton protein in HL-60 cells as well as insect cells infected with baculovirus vector which specifically express human c-*myc* protein, suggesting that it was specific for the c-*myc* protein. Data showing that copy numbers of pARS65 replicated in various cells are parallel to the level of c-*myc* expression in these cells both *in vivo* and *in vitro* (Figure 4C) (Ariga *et al.*, 1987) support our conclusion, although there is the possibility that another protein with a similar size is recognized by the antibody and is involved in promotion of replication. On the basis of these experiments, we cloned an autonomously replicating sequence from human DNA as reported here. This supports the view that the c-*myc* product probably works on *ori* and promotes cellular DNA replication. However, we cannot rule out the possibility that other proteins complexed with c-*myc* protein are the actual replication proteins.

Based on our data as well as numerous published reports, the c-*myc* product may function like bacterial *dnaA* protein and SV40 large T antigen. In the *E. coli* genome or the SV40 genome, which are single replicons, *dnaA* protein or T antigen bind to the initiation site of DNA replication (*oriC* or SV40 *ori*) and promote replication (Reed *et al.*, 1975; Tooze, 1980; Kornberg, 1982; Fuller *et al.*, 1984). SV40 T antigen also initiates the cell cycle ($G_0 \rightarrow G_1 \rightarrow S$) although direct interaction between T antigen and cellular DNA has not been observed (Tooze, 1980). Besides SV40 T antigen, adenovirus E1A protein and the c-*myc* product also initiate the cell cycle. All of these are phosphoproteins localized in nuclei. One common function of these proteins may be to promote cellular DNA replication. Adenovirus E1A was recently revealed to be involved in viral DNA replication (Ohsima and Shiroki, 1985). SV40 T antigen has effects on both the initiation and elongation steps of replication, while bacterial *dnaA* protein participates only in initiation. It is not clear at present how the c-*myc* product promotes DNA replication. During the preparation of our manuscript, data were reported suggesting participation of the c-*myc* protein in the elongation phase of DNA replication (Studzinski *et al.*, 1986). Since the SV40 T antigen is involved in functions other than DNA replication, including initiation and maintenance of transformation, regulation of gene expression, stimulation of rDNA transcription, etc., it may be that the c-*myc* product also has a number of functions.

The cellular DNA of eukaryotes starts to replicate at many points on the chromosome, and there are approximately 10^4-10^5 initiation sites of DNA replication in each mammalian cell (Kornberg, 1980). Cloning of the DNA fragments contain-

ing the initiation site of chromosomal DNA replication has been attempted by many groups. A number of plasmids containing inserts of DNA fragments of yeast chromosome have been isolated which can autonomously replicate in yeast cells (Stinchcomb *et al.*, 1981; Tschumper and Carbon, 1981). Other DNA fragments with ARS activity in yeast cells have also been cloned from the DNAs of other eukaryotes (Stinchcomb *et al.*, 1980; Roth *et al.*, 1983; Montiel *et al.*, 1984). It has not been proven, however, that the DNA sequences which function as ARS in yeast cells also function as initiation sites of replication in the cells of their original species. On the other hand, it has been reported that plasmids carrying the *Alu* family, one of the human polydispersed repetitive sequences, have no ARS activity in yeast cells (Zakian, 1981), although it is supposed that there are initiation-sites of replication in these sequences.

It has not been proven that all of the *ori*s of DNA replication in an organism share common sequences. Assuming that specific origins exist, it is unlikely that they are all the same (Campbell, 1986). In *Drosophila*, initiation sites of replication in the embryo, where DNA replicates at high speed, were observed every 8 kb, whereas they occur every 40 kb in more slowly-dividing tissues (Kreigstein and Hogness, 1974). Random DNAs do not replicate when injected into various cells, but do replicate under cell cycle control in *Xenopus* and sea urchin eggs (Harland and Lasky, 1980; Chambers *et al.*, 1982; Hines and Benbow, 1982; Mechali and Kearsey, 1984). Thus different classes of origins may be activated at different times of development or differentiation by specific proteins. Rates of replication also change depending upon the nutritional environment. It has been proposed that this regulation depends upon the number of initiation sites within the genome (Edenberg and Huberman, 1975; Hand, 1978). The human ARS we have cloned may involve an initiation site of human chromosomal DNA. The *Alu* family, one of the human repetitive sequences (supposed to be a replication origin) can function as *ori* in the SV40 T antigen-dependent cell-free system (Ariga, 1984) and also in monkey Cos cells (Johnson and Jelinek, 1986). *Alu* family sequences appear at a frequency of 3×10^5 copies/haploid genome (Jelinek and Schmid, 1982), and that number nearly meets the maximum initiation sites of replication expected. We propose that the *ori* we have cloned may be of the adult type, and that repetitive sequences such as *Alu* family may function as *ori* at some specific stages of development or differentiation when the rate of replication is very high.

Materials and methods

Cells

HL-60 (human promyelocytic leukemia cell line), U937 (human histiocytic lymphoma cell line), Raji (human Burkitt's lymphoma cell line), and FM3A (mouse mammary carcinoma cell line) cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Raji and FM3A cells were obtained from the Japanese Cancer Research Resources Bank.

Characterization of the antibody

Two independent anti-human c-*myc* antibodies used in this study were characterized as follows. The c-*myc* specific monoclonal antibody, IF7, was well characterized previously and precipitated human c-*myc* protein (Miyamoto *et al.*, 1985a, b). The other antibody, a sheep polyclonal one, was prepared by Oncor, Inc., USA, using a synthetic oligopeptide specific for human c-*myc* protein and it was confirmed by the company that the antibody reacts with purified bacterially expressed human c-*myc* protein. HL-60 nuclear extract (NE) was prepared as described previously (Ariga and Sugano, 1983) and used as a source of c-*myc* protein. Two μg of anti-human c-*myc* antibody or sheep anti-human IgG (Cooper Biomedical, Inc., USA) were incubated with 20 μl of HL-60 NE at 0°C for 30 min. The immunecomplexes were precipitated by a suspension of formamide-fixed *Staphylococcus aureus*, redissolved with 20 mM Tris−HCl (pH 8.5), 0.25 M 2-mercaptoethanol, 1% $NaDodSO_4$, separated on 10% polyacrylamide gel (Laemmli, 1970), and transferred to a nitrocellulose filter. The filter was incubated with anti-c-*myc* antibody at 37°C for 1 h, and further incubated with biotinylated anti-sheep IgG at 37°C for 1 h. After incubation of the filter with 0.5 μCi/ml of ^{125}I-labelled streptavidin at room temperature for 30 min, the filter was washed twice with phosphate buffered saline (PBS) containing 0.05% nonidet P-40 for 20 min and further washed five times with PBS for 10 min at room temperature. The filter was dried and autoradiographed at −80°C. Only one band of 60 000-dalton protein in HL-60 NE precipitated with the commercial polyclonal anti-c-*myc* antibody of Oncor, Inc. was clearly detected on the filter, while no band was detectable with non-specific IgG. To confirm these results, a whole cell extract of [^{35}S]methionine-labelled HL-60 cells was immunoprecipitated with the anti-c-*myc* antibody or non-specific anti-human IgG. In addition, the recombinant c-*myc* protein expressed in insect cells infected with a baculovirus expression vector was used (Miyamoto *et al.*, 1985b). 2×10^6 HL-60 cells or 2×10^6 *S. frugiperda* cells infected with Ac377/hc-*myc* at 40 h post-infection were labelled with 100 μCi of [^{35}S]methionine for 3 h and the proteins were extracted by the buffer containing nonidet P-40 (Sugano and Yamaguchi, 1984). The extracted proteins were immunoprecipitaed with commercial anti-human c-*myc* antibody of Oncor, Inc., the c-*myc* specific monoclonal antibody IF7 or anti-human IgG as described above, separated on 10% polyacrylamide gel, and visualized by fluorography. Only one 60 000-dalton protein band was precipitated with anti-c-*myc* antibody, while no band was seen with control non-specific IgG. Both the commercial polyclonal anti-c-*myc* antibody and the monoclonal anti-c-*myc* antibody IF7 precipitated an ~60 000-dalton protein. These data indicated that the commercial anti-human c-*myc* antibody, as well as the monoclonal antibody IF7, specifically react with the 60K protein which must be the c-*myc* product and that the antibody immunoprecipitates the specific c-*myc* protein in HL-60 NE. Furthermore, this anti-human c-*myc* antibody of Oncor, Inc., could not inhibit the activities of DNA or RNA polymerase, or DNA primase in HL-60 cells (data not shown).

Transfection of antibody into HL-60 cells

HL-60 cells were transfected with antibodies by a liposome-mediated transfection technique (Itani *et al.*, 1987). Briefly 10^6 cells were transfected with 2 μg protein of antibody in liposomes composed of 1 μmol of phosphatidylserine and then cultured at 10^5 cells/ml.

Analysis of replicating DNA after transfection of plasmid DNA into HL-60 cells

HL-60 cells were transfected with plasmid DNAs as described above using plasmids instead of antibodies (8 μg of the DNA were used respectively) (Itani *et al.*, 1987). Three days after transfection, low molecular weight DNAs were extracted from the cells by the Hirt procedure (Hirt, 1967), and digested with *Bam*HI to linearize the plasmid and with *Dpn*I to eliminate the input plasmid used for transfection. The digested DNA from the Hirt supernatant was then electrophoresed on 0.8% agarose gel, blotted by the method of Southern (1975) and hybridized with ^{32}P-labelled pKSV10 (*ori* sequence and β-lactamase gene of pKSV10 were common in pARS65, pMU111, and pUC19). ^{32}P-labelled pUC19 was used as a probe for analysis of pHLmyc clones.

Conditions for in-vitro reaction

Nuclear extract (NE) of HL-60, U937, and FM3A cells was prepared as described previously (Ariga and Sugano, 1983). The reaction mixture and all the procedures used in this experiment were as described previously (Ariga and Sugano, 1983; Ariga *et al.*, 1987).

Immunobinding assay

The ^{32}P-labelled DNA fragments were incubated with 20 μl of HL-60 NE at 0°C for 1 h in 25 mM Tris−acetate buffer solution (pH 6.5) containing 150 mM NaCl and 0.5% nonidet-P40, and then incubated with the added polyclonal anti-human c-*myc* antibody or anti-human IgG (2 μg protein respectively) at 0°C for 30 min. The following procedures were the same as described previously (Ariga, 1984).

Cloning of c-myc binding sequences from HL-60 DNA

Whole genomic DNA extracted from 1×10^6 HL-60 cells was completely digested with *Hin*dIII, and 5 μg were incubated with 20 μl of HL-60 NE. The anti-human c-*myc* antibody (Oncor, Inc.) was added to the mixture, and the immunecomplexes were precipitated as described for immunobinding assay. The precipitate was dissolved with 20 mM Tris−HCl (pH 8.5), 0.25 M 2-mercaptoethanol, 1% SDS, and incubated for 30 min at 37°C. Then, the mixture was centrifuged at 10 000 × *g* for 10 min. DNA was extracted with phenol from the supernatant, precipitated with ethanol, and cloned into the *Hin*dIII site of pUC19.

Conditions for hybridization

The hybridization of the blotted filter with labelled probe was carried out as described previously (Ariga *et al.*, 1987).

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