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A Novel DNA Replication Origin Identified in the Human Heat Shock Protein 70 Gene Promoter

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A general and sensitive method for the mapping of initiation sites of DNA replication in vivo, developed by Vassilev and Johnson, has revealed replication origins in the region of simian virus 40 *ori*, in the regions upstream from the human c-*myc* gene and downstream from the Chinese hamster dihydrofolate reductase gene, and in the enhancer region of the mouse immunoglobulin heavy-chain gene. Here we report that the region containing the promoter of the human heat shock protein 70 (*hsp70*) gene was identified as a DNA replication origin in HeLa cells by this method. Several segments of the region were cloned into pUC19 and examined for autonomously replicating sequence (ARS) activity. The plasmids carrying the segments replicated episomally and semiconservatively when transfected into HeLa cells. The segments of ARS activity contained the sequences previously identified as binding sequences for a c-*myc* protein complex (T. Taira, Y. Negishi, F. Kihara, S. M. M. Iguchi-Ariga, and H. Ariga, Biochem. Biophys. Acta 1130:166–174, 1992). Mutations introduced within the c-*myc* protein complex binding sequences abolished the ARS activity. Moreover, the ARS plasmids stably replicated at episomal state for a long time in established cell lines. The results suggest that the promoter region of the human *hsp70* gene plays a role in DNA replication as well as in transcription.

In order to study DNA replication in higher eukaryotes, identification and mapping of DNA replication origins in chromosomes are required. Although origins in mammalian chromosomes still remain enigmatic, several mammalian sequences which promote autonomous replication of plasmids in mammalian cells have been reported. A method for mapping origins that work in vivo (an origin mapping method) has been developed (32). This method has the following advantages: it avoids the use of metabolic inhibitors, does not require synchronized cells, and can detect replication origins even in single-copy sequences (31). This is thus at present the most advanced technique to identify replication initiation sites under the conditions reflecting living cells. Several replication origins were determined by this method in mammalian genes, including the region upstream from the human c-*myc* gene (33), the region downstream from the Chinese hamster dihydrofolate reductase gene (31), and the enhancer region of the mouse immunoglobulin heavy-chain gene (3, 15).

We have previously shown that a protein complex including the c-*myc* protein (or proteins with c-*myc* protein-like epitopes) specifically binds to two sites in the promoter region of the human heat shock protein 70 (*hsp70*) gene (Fig. 1) (29). The two binding sites contain seven nucleotides, CCTCTCA (HSP-MYC A) or CCTCTGA (HSP-MYC B), homologous to the core sequence for c-*myc* protein complex binding, TCTCTTA, identified in the region upstream from the human c-*myc* gene (1, 23). Furthermore, the sequence of the c-*myc* gene has shown to possess autonomously replicating sequence (ARS) activity as well as transcriptional enhancer activity (1). The ARS/enhancer sequence was located in the region identified as an origin of DNA replication in vivo in the c-*myc* gene (33). In this study, we examined the promoter region of the human *hsp70* gene for replication activity. A replication origin was mapped in the region in vivo, and the segments of the region, cloned in pUC19, showed ARS activity in transfected cells. Several cell lines in which the plasmids replicated stably at episomal state were established.

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MATERIALS AND METHODS

In vivo mapping of DNA replication origin. All procedures were performed as previously described (31–33), except that HeLa cells were used. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. For each experiment, 10 dishes (diameter, 15 cm) containing 50 to 60% confluent cells were labeled with 5-bromodeoxyuridine (BrdU) for 15 min at 37°C. Total DNA was extracted from the cells after three washes with cold phosphate-buffered saline. The DNA was denatured with NaOH (final concentration, 0.2 M) and applied on 5 to 20% (wt/vol) linear sucrose gradients containing 0.2 M NaOH and 2 mM EDTA over a 60% sucrose cushion. The gradients were centrifuged at 24,000 rpm for 18 h at 15°C in a Hitachi SRP28S rotor. The gradients were divided into 15 fractions. The top fraction with the shortest DNA fragments was discarded, because the fragments were too short to serve as a template in the following PCR amplification. The last three fractions at the bottom were also discarded, because of their contamination with viscous DNA of high molecular weight, which gives rise to poor resolution in the gradients. Fractions 2 to 12 were thus renumbered as 1 to 11. Each fraction was neutralized with 2 M HCl, and the DNA was ethanol precipitated. Of the DNA from each fraction, 1/100 was end labeled with terminal deoxynucleotidyl transferase and [α-^{32}P]CTP and electrophoresed through a 0.8% alkaline agarose gel, in parallel with λ DNA, digested with *Hin*dIII and ^{32}P end labeled, as size markers in the same gel. The gel was dried and subjected to autoradiography, and the total count of the each fraction was calculated by using a bioimage analyzer (BAS 2000; Fuji Film Co. Ltd.,

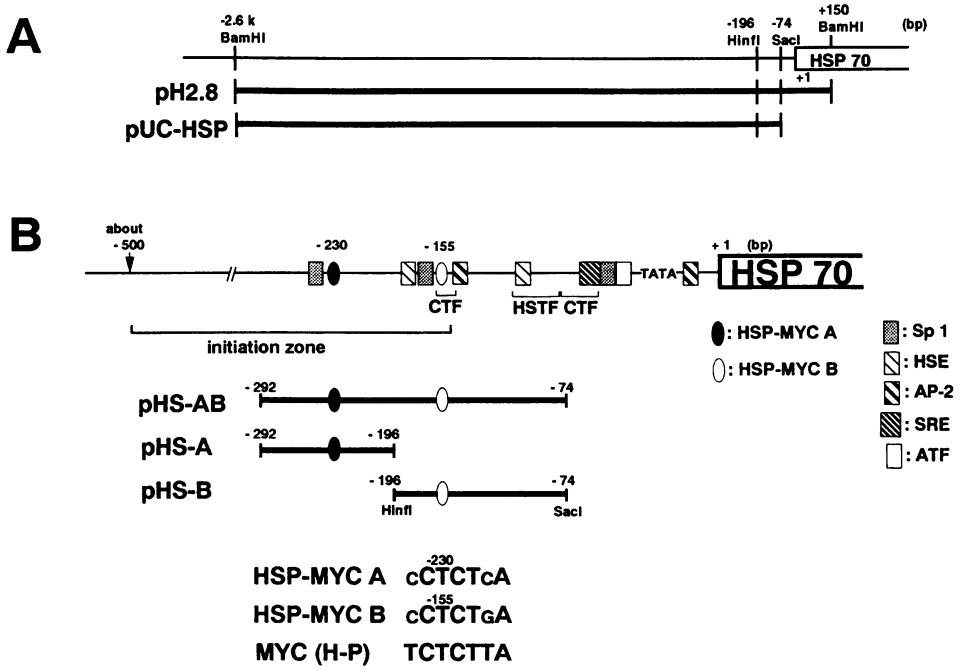

FIG. 1. Schematic drawing of the promoter region of the human *hsp70* gene. (A) pH2.8 and pUC-HSP contain the region upstream from the human *hsp70* gene. Numbers are nucleotide positions relative to the transcription initiation site. (B) Various transcriptional elements exist in the promoter region of the *hsp70* gene. Numbers are nucleotide positions relative to the transcription initiation site. The positions of the Sp1 binding sites, the CTF (CCAAT box-binding factor) binding site, HSE (heat shock element), SRE (serum-responsive element), the ATF (activating transcription factor) binding site, the AP-2 binding site, and TATA box in addition to the HSP-MYC boxes are indicated. The location of the initiation zone determined in the in vivo mapping (see Fig. 2B) and the fragments inserted in pHS-AB, pHS-A, and pHS-B are also shown. Two different sequences in the *hsp70* promoter region, HSP-MYC A and HSP-MYC B, and MYC(H-P), which is identified in the region upstream from the human c-*myc* gene as the core sequence for binding of a c-*myc* protein complex, are shown in the lower panel.

Tokyo, Japan). The rest of the DNA from each fraction was dissolved in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA (TE buffer), denatured at 95°C for 3 min, rapidly cooled on ice, and incubated with 2 μg of mouse anti-BrdU monoclonal antibody (Bio Cell Consulting) for 20 min at room temperature in a buffer containing 10 mM sodium phosphate (pH 7.0), 0.14 M NaCl, and 0.05% Triton X-100. One hundred micrograms of rabbit immunoglobulin G against mouse immunoglobulin G was then added to the BrdU-DNA-antibody complexes. After incubation at room temperature for 60 min, the immunoprecipitates were recovered by centrifugation for 5 min at 12,000 rpm, washed once with the reaction buffer as described above, and resuspended in a 50 mM Tris-HCl (pH 8.0) buffer containing 10 mM EDTA and 0.5% sodium dodecyl sulfate (SDS) in addition to 250 μg of proteinase K per ml. After overnight digestion at 37°C, the DNA was extracted from the reaction mixtures twice with phenol-chloroform (1:1) and then ethanol precipitated together with 100 μg of salmon sperm DNA (sonicated) as a carrier. The DNA was dissolved in TE buffer and used for PCR amplification. Four segments referred to as A, B, C, and D were selected at approximately −4.0 to −3.3 kb, −1.2 to −0.8 kb, −164 to +150 bases, and +1,650 to +2,135 bases, respectively (see Fig. 2C), and PCR primers were chemically synthesized in either direction for each segment. PCR amplifications were carried out with the nascent DNA; the products were fractionated and purified as described above. PCR conditions were as follows. The template DNA was denatured at 94°C for 1 min and annealed at 52°C for 2 min. The extension reaction was carried out at 72°C for 1 min with 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus). These reactions were repeated for 30 cycles in a thermal cycler (Perkin-Elmer Cetus). The nucleotide sequences of primers A, B, C, and D are as follows: A (upper), 5′-ACACCGCTGT GATTGAACTC-3′; A (lower), 5′-TCCTGCCGATCAGAC GTT-3′; B (upper), 5′-TGCAGTGAGCCAAGATCG-3′; B (lower), 5′-GATCTGATTGATTGTGAA-3′; C (upper), 5′-TCTGGCCTCTGATTG-3′; C (lower), 5′-TGAGCCGCTCG GTGTCCGTG-3′; D (upper), 5′-GAGGGCCATGACGAA AG-3′; and D (lower), 5′-CTAATCTACCTCCTCAATG GT-3′. One-third of the PCR products was blotted to nitrocellulose filters with a Minifold II slot-blot set (Schleicher & Schuell) and hybridized with ^{32}P-end-labeled oligonucleotide probes at 52°C for 16 h in a buffer containing 6× SSC (1× SSC contains 0.3 M NaCl and 0.03 M sodium citrate), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, and 0.1% SDS. The filters were washed at room temperature twice with 6× SSC–0.1% SDS for 10 min and twice with 0.1× SSC–0.1% SDS for 10 min and subjected to autoradiography.

Plasmid construction and assay of autonomously replicating activity. Plasmid pH2.8 contains the *Bam*HI fragment of about 2.8 kb, −2.6 kb to +150 bases of the human *hsp70* gene (37) (Fig. 1A). The *Bam*HI-*Sac*I fragment of pH2.8 was subcloned into *Bam*HI-*Sac*I sites of pUC19 (pUC-HSP [Fig. 1A]). pUC-HSP was treated with exonuclease III after digestion with *Bam*HI and *Sph*I, blunt ended with mung bean nuclease and Klenow fragment (12, 38), and self-ligated. pHS-AB, a deletion mutant of pUS-HSP, was thus obtained (see Fig. 3B). pHS-A and pHS-B were subcloned from pHS-AB by partial digestion with *Hin*fI. Oligonucleotides

corresponding to the HSP-MYC sequences were synthesized with or without mutations and inserted, respectively, into the *Hin*dIII-*Sal*I sites (for pwt-A and pMu-A) or the *Eco*RI-*Bam*HI sites (for pwt-B and pMu-B) of pUC19 (see Fig. 5, upper panel). An internal control plasmid, pUC-cibiPCR, is a pUC19 subclone lacking the region of 420 bp from *Ssp*I (at position 2501) to *Bbe*I (at position 235) of pUC19. The plasmids were prepared either in a *dam*$^+$ strain of *Escherichia coli* C600 or in a *dam* mutant strain of *E. coli* GM119. Autonomously replicating activity of the plasmids in HeLa cells was assayed by PCR (36) or by standard Southern blotting. Five micrograms of a test plasmid was transfected to HeLa cells (40 to 50% confluent in a 6-cm-diameter dish) by the calcium phosphate method together with *dam*$^+$ or *dam* mutant pUC-cibiPCR (10). Seventy-two hours after being boosted by 25% dimethyl sulfoxide for 3 min, low-molecular-weight DNAs were extracted from the cells as described by Hirt (13). One-third of the DNAs was digested with 10 U of *Dpn*I (New England Biolabs) for more than 6 h, extracted with phenol and chloroform, and precipitated with ethanol. The inserts of the *Dpn*I-resistant pUC clones were amplified by PCR with 0.1 μg of L and R primers under the same conditions as in the origin mapping. L and R primers correspond to the sequences adjacent to both ends of the multicloning sites of pUC plasmids, which are 5′-CAGGAAACAGCTATGAC-3′ and 5′-TTCGATGTAACCCACTCGTG-3′, respectively (see Fig. 3A). After PCR amplification, the DNAs were extracted and electrophoresed through a 1.2% agarose gel containing 0.5 μg of ethidium bromide per ml. The PCR products from use of 1.0, 0.1, and 0.01 pg of pUC19 or pUC-cibiPCR as templates were run on the same gel in parallel as copy number controls. For Southern blot analysis, 10 μg of a test plasmid was similarly transfected to HeLa cells in a 10-cm-diameter dish. Low-molecular-weight DNAs were digested with *Dpn*I and *Hin*dIII, separated in a 1.0% agarose gel, blotted to a nitrocellulose filter, and hybridized with a ^{32}P-labeled pUC19- or *hsp*-derived fragment probe, as described previously (12).

BrdU labeling and isopycnic centrifugation. The HeLa cells transfected with plasmid DNAs, or the cell lines of 3Y1 containing the ARS plasmids (see below), were cultured in the presence of 40 μM BrdU for 30 h before being harvested at 60 h after boosting. Low-molecular-weight DNAs were extracted and precipitated as described above. The density of the DNA samples, in a buffer containing 25 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 100 mM NaCl, was adjusted to 1.73 g/cm^3. The samples were centrifuged in an SW50.1 rotor at 43,000 rpm for 48 h at 25°C. After fractionation, the DNA was precipitated, slot blotted to a nitrocellulose filter, and hybridized with a ^{32}P-labeled pUC19 probe. As for the similar experiments using the 3Y1 cell lines, total DNAs were extracted after BrdU labeling for 20 or 40 h. Before blotting and hybridization with labeled probes, the DNAs were digested with *Hin*dIII, extracted with phenol, precipitated with ethanol, and separated in a 1.0% agarose gel. The DNA from each fraction was slot blotted in parallel, as in the origin mapping, and hybridized with a ^{32}P-labeled probe of B-2 (M-2, a mouse type 2 *Alu* sequence) (19) to see the replication of chromosomal DNA. The positions of LL (light-light; unsubstituted), HL (heavy-light; hybrid), and HH (heavy-heavy; fully substituted) fractions were determined by refraction indexes of the fractions.

Establishment of cell lines. Rat 3Y1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. Five micrograms of a test plasmid, together with 0.25 μg of pSVHg (an expression vector for the hygromycin B resistance gene), was transfected to 3Y1 cells 40% confluent in a 6-cm-diameter dish by the calcium phosphate method (10). Four hours posttransfection, the cells were boosted by 20% dimethyl sulfoxide. The cells were cultured for 48 h, replated into two dishes (diameter, 10 cm), and cultured further in a medium containing 70 to 100 μg of hygromycin B (Wako Pure Chemicals Indust. Ltd., Osaka, Japan) per ml, which was changed every 3 days. Drug-resistant clones were isolated and cultured in a drug-free medium after the cell number exceeded 10^4.

Southern blot analysis. Total DNA was extracted from about 10^6 cells by following the standard protocol, digested with restriction enzymes, separated in a 1.2 or 0.8% agarose gel, and subjected to Southern blotting. The ~220-bp *Hin*dIII-*Eco*RI fragment of pHS-AB, containing both HSP-MYC A and HSP-MYC B elements, was ^{32}P labeled and used as a probe for hybridization, as described previously (2). The blotted filters were then autoradiographed and analyzed by a bioimage analyzer (BAS 2000; Fuji Film Co.).

Labeling of de novo-synthesized DNA. The 3Y-AB-6 cells were cultured for 20 h in medium without serum and then in medium containing 1.0 mCi of both [^{3}H]thymidine (71 Ci/mmol) and [^{3}H]deoxycytidine (20 Ci/mmol) per ml in addition to 10% serum. After 10, 20, 60, or 120 min, low-molecular-weight DNA was extracted from the cells. The DNA was mixed with 1.5 μg of nonlabeled pHS-AB, digested with *Eco*RI and *Hin*dIII, and separated in a 1.2% agarose gel containing 0.5 μg of ethidium bromide per ml. The bands of the two fragments were excised under UV light, melted at 80°C in 0.5 ml of H_2O_2 overnight, and counted for radioactivity by a liquid scintillation counter.

RESULTS

Mapping of the origins of DNA replication in the *hsp70* gene. The principles and the procedure of the in vivo origin mapping have been described before (31–33). Nascent DNA labeled with BrdU was recovered and size fractionated by alkaline sucrose density gradient centrifugation. To determine the average length of the DNAs in each fraction, an aliquot of each fraction was end labeled with ^{32}P and separated in an alkaline agarose gel (Fig. 2A). Four reference segments, A, B, C, and D, were selected at fixed positions in the *hsp70* gene spanning about 6 kb (Fig. 2C), and a pair of primers for each segment were chemically synthesized (see Materials and Methods). The nascent DNAs in the fractions were thereby amplified by PCR, after immunoprecipitation with an anti-BrdU monoclonal antibody. The PCR products were blotted and hybridized with probes specific for segment A, B, C, or D (Fig. 2B, upper panel). To confirm the specificity of each probe for hybridization, 1 μg of total DNA from HeLa cells was amplified in parallel with a pair of primers and hybridized with a corresponding probe. The results show that the probes specifically hybridize with each segment (Fig. 2B, lower panel). The nascent DNA fragments covering segment C were present in fraction 1 and those with longer DNAs. For segments A, B, and D, nascent fragments were detected in fractions 8, 4, and 7, respectively. The results suggest that segment C is closest to the replication origin, because the shortest fragments among the four were hybridized with probe C. Segment B is hence suggested to be closer to the origin than segments A and D, since the DNA fragments hybridized with probe B were shorter than those with A and D. The average size of the shortest fragment hybridized with each segment corresponds to the distance between the replication origin and the segment (Fig. 2A). The location of the replication origin, or initiation

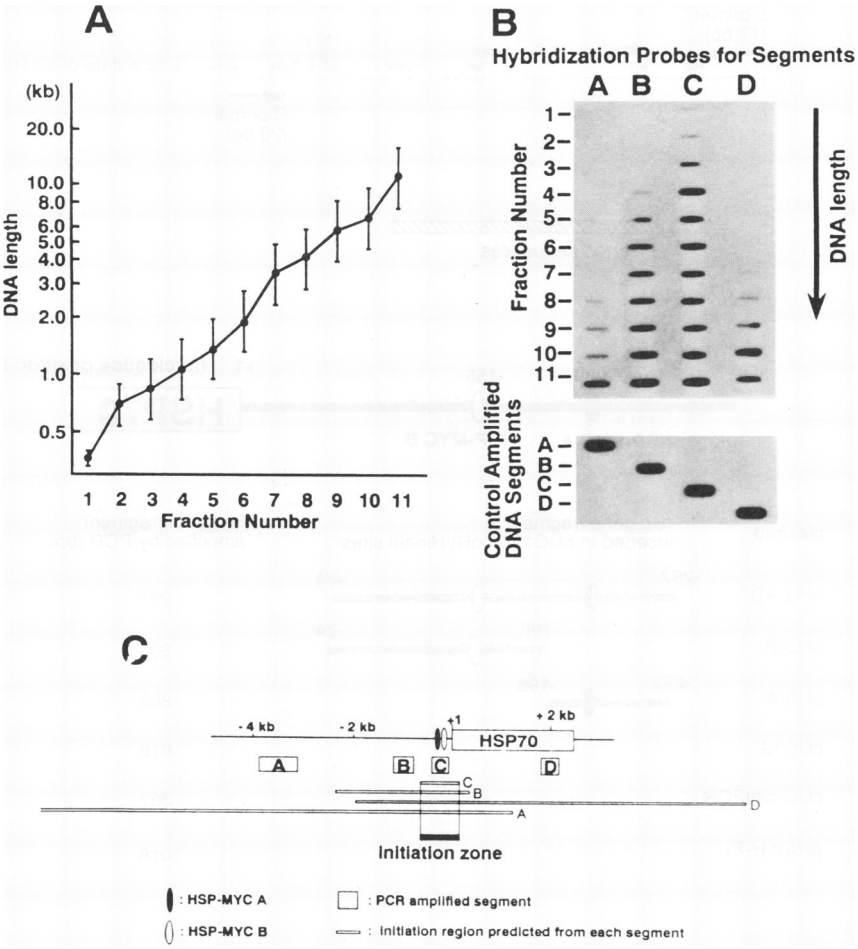

FIG. 2. In vivo mapping of DNA replication origin. (A) Determination of the lengths of DNAs separated in alkaline sucrose gradients. DNA recovered from each fraction of the alkaline sucrose density gradients was end labeled with ^{32}P and separated in a 0.8% alkaline agarose gel. The gel was subjected to autoradiography, and the total radioactivity of each fraction was calculated by using an imaging analyzer (Fuji BAS 2000). The average lengths of the DNAs in each fraction are displayed in a diagram, and the numbers on the left indicate lengths (in kilobases). (B) Slot blot hybridization with probes for segments A, B, C, and D. BrdU-labeled HeLa DNA was fractionated by alkaline sucrose gradient centrifugation. The DNAs recovered from the 11 fractions (see Materials and Methods) were precipitated with an anti-BrdU monoclonal antibody and subjected to PCR with a pair of primers for each segment. The PCR products were blotted to a nitrocellulose filter and independently hybridized with ^{32}P-oligonucleotide probes specific for segments A, B, C, or D. In addition to the BrdU-labeled DNAs from the gradients, 1 μg of total DNA of HeLa cells, as a control, was used in the PCR with the same primers. (C) Location of an initiation site of DNA replication mapped in the region upstream from the human *hsp70* gene in HeLa cells. The white box represents the *hsp70* coding sequences. HSP-MYC A (black oval) and HSP-MYC B (white oval) are homologous to the core sequence for binding of a c-*myc* protein complex. Shaded boxes, A, B, C, and D, represent the segments amplified by PCR. Initiation regions predicted from each segment and the initiation zone identified in the overlapping region are indicated.

zone, is thus determined at the overlapping region of the regions predicted from four segments (Fig. 2C).

Autonomous replication of the plasmid containing the *hsp70* promoter in HeLa cells. In order to check whether the *hsp70* promoter region, which overlaps the initiation zone identified above, works as an ARS in mammalian cells, we constructed three plasmids carrying the sequences of the region (Fig. 3B). pHS-AB contains the nucleotides from position −74 (relative to the transcription initiation site) to −292 in the promoter region of human *hsp70*, pHS-B contains nucleotides −74 to −196, and pHS-A contains nucleotides −196 to −292. We have previously identified two binding sites for protein complexes including the c-*myc* product in the region (29). The two sites, termed HSP-MYC A and HSP-MYC B, are located at −230 and −155, respectively. The plasmids, pHS-A, pHS-B, and pHS-AB, hence contain either or both of the HSP-MYC A and B sequences. The test plasmids were transfected to HeLa cells by the $CaPO_4$ method together with an internal control plasmid, and low-molecular-weight DNAs were extracted from the cells 72 h after transfection. The DNAs were completely digested with *Dpn*I, and PCR of the residual *Dpn*I-resistant DNAs was carried out with L and R primers (Fig. 3A). With the primers, the internal control (pUC-cibi PCR) gives rise to an amplified fragment of 397 bp, and the replicated test plasmids give rise to fragments of 800 to 900 bp. The test plasmids prepared in *dam*$^+$ *E. coli* were thus methylated, so that the input material was sensitive to *Dpn*I digestion. Plasmids replicated in mammalian cells, which lack *dam* methylase, are hemimethylated or unmethylated and therefore become resistant to *Dpn*I digestion (25). An internal control plasmid, pUC-cibiPCR prepared in a *dam* mutant strain of *E. coli*, also survived *Dpn*I digestion and thereby cotransfected to the cells

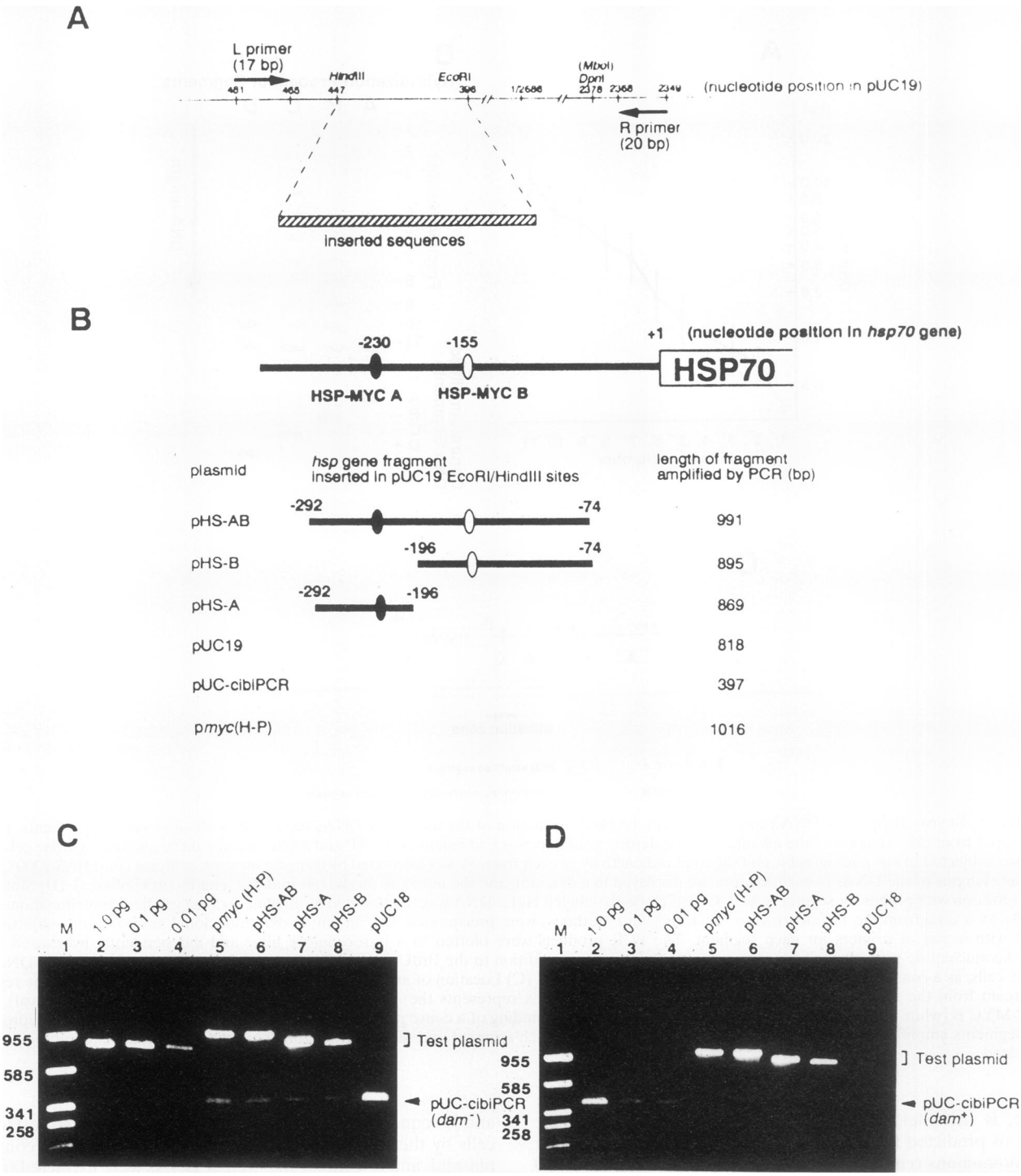

FIG. 3. Autonomous replication in HeLa cells of plasmids carrying the segments upstream from the human *hsp70* gene. (A) The locations of two primers used for PCR are shown. L primer, from nucleotide position 481 to 465, and R primer, from 2368 to 2349, were prepared in order to amplify the sequences inserted in the multicloning sites of pUC18 or -19. There exists a *Dpn*I restriction site between R primer and the multicloning sites. (B) The lengths of the fragment amplified by PCR are shown for the plasmids used for assay. The *hsp70* gene fragments cloned in pUC19, as well as the map of the promoter region, are also shown; numbers are nucleotide positions relative to the transcription initiation site. (C) The fragments amplified by PCR on the plasmids replicated in HeLa cells were separated in an agarose gel containing ethidium bromide. pUC-cibiPCR prepared in *dam* mutant *E. coli* was cotransfected with test plasmids at a 1:25 ratio. p*myc*(H-P) (lane 5) and pUC18 (lane 9) are positive and negative controls, respectively. Lanes 2, 3, and 4 are copy number controls, which are the PCR-amplified products of 1.0, 0.1, and 0.01 pg of pUC19. M (lane 1) contains *Sau*3AI-digested pUC19 fragments as size markers. Positions expected for the bands due to test plasmids and pUC-cibiPCR are indicated on the right. (D) The fragments amplified by PCR of the plasmids replicated in HeLa cells were separated in an agarose gel containing ethidium bromide as in panel C. pUC-cibiPCR prepared in *dam*$^+$ *E. coli* was cotransfected with test plasmids at a 1:1 ratio. p*myc*(H-P) (lane 5) and pUC18 (lane 9) are a positive and a negative control, respectively. Lanes 2, 3, and 4 are copy number controls, which are the PCR-amplified products of 1.0, 0.1, and 0.01 pg of pUC19. M (lane 1) contains *Sau*3AI-digested pUC19 fragments as size markers. Positions expected for the bands due to test plasmids and pUC-cibiPCR are indicated on the right.

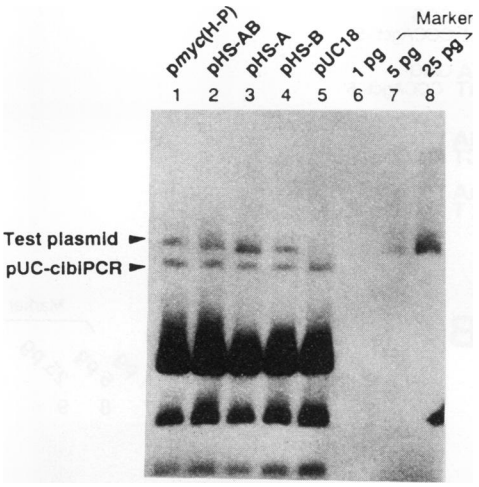

FIG. 4. Autonomous replication in HeLa cells of plasmids carrying the segments upstream from the human *hsp70* gene (Southern blotting method). Low-molecular-weight DNAs were extracted from the HeLa cells transfected with test plasmids and *dam* mutant pUC-cibiPCR, digested with *Dpn*I and *Hin*dIII, and analyzed by Southern blot hybridization. pUC19 labeled with ^{32}P was used as a probe. The positions of bands expected for test plasmids and pUC-cibiPCR are indicated on the left. One, 5, or 25 pg of pUC19 digested with *Hin*dIII was separated and blotted in parallel as markers (lanes 6 to 8).

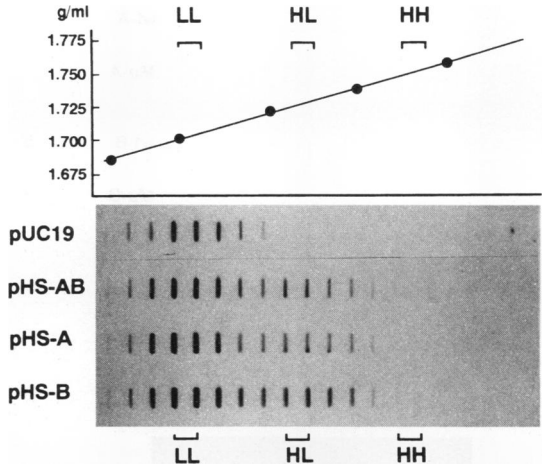

FIG. 5. Isopycnic CsCl centrifugation at neutral pH of DNA extracted from HeLa cells. HeLa cells were transfected with pHS-AB, pHS-A, pHS-B, or pUC19 and labeled with BrdU for 30 h prior to being harvested at 60 h after boosting. The DNA extracted from the cells was fractionated by CsCl equilibrium centrifugation, blotted to a nitrocellulose filter, and hybridized with a ^{32}P-labeled probe of pUC19. LL, HL, and HH indicate the positions expected for unsubstituted (LL), hybrid (HL), and fully substituted (HH) DNAs, respectively. The densities of several fractions are shown in the upper panel. DNA molecules that replicated in the cells are expected to be detected in the HL and HH regions.

at a ratio of 1:25 (number of cells per number of test plasmids) in order to standardize the transfection efficiencies among the samples. p*myc*(H-P), which contains the region of 200 bp upstream from the human c-*myc* gene and possesses ARS activity (1, 16), and pUC18 were used as a positive control and a negative control, respectively. No amplification was observed with pUC18, while clear bands of amplified fragments were detected with p*myc*(H-P), pHS-AB, pHS-B, and pHS-A (Fig. 3C). The results show that pHS-AB, pHS-B, and pHS-A, as well as p*myc*(H-P), replicated in HeLa cells. It was difficult to prove completion of *Dpn*I digestion, on which the results depend. We therefore cotransfected test plasmids with the same amount of a negative control plasmid, pUC-cibiPCR, prepared in *dam*$^+$ *E. coli*. As shown in Fig. 3D, only test plasmids, and not pUC-cibiPCR, yielded bands of amplified fragments. The *Dpn*I digestion was therefore suggested to be adequate to distinguish replicated from nonreplicated molecules in the assays. Replication of the pHS plasmids was also detected by the standard Southern method (Fig. 4). Low-molecular-weight DNAs were extracted from the HeLa cells transfected with test plasmids, digested with *Dpn*I and *Hin*dIII, and blotted. pUC19 digested with *Hin*dIII and labeled with ^{32}P was used as a probe for hybridization. In addition to intense bands of 924 and 585 bp due to *Dpn*I-digested fragments of nonreplicated molecules, pHS-AB, pHS-A, pHS-B, and p*myc*(H-P) gave rise to bands of expected sizes, 2.9, 2.8, 2.8, and 2.9 kb, respectively. The results also suggest that the pHS plasmids replicated in HeLa cells. Similar results were obtained with monkey CV-1 cells, mouse L cells, and rat 3Y1 cells (data not shown).

To confirm that the replication of the plasmids containing the *hsp70* promoter in HeLa cells was due to semiconservative replication, not to random repair synthesis, the transfected cells were incubated with BrdU for 30 h (from 30 to 60 h after transfection), and the low-molecular-weight DNA was analyzed by neutral CsCl equilibrium centrifugation (Fig. 5). The DNAs extracted from the cells transfected with pHS-AB, pHS-A, and pHS-B gave signals at the position corresponding to the density of HL molecules as well as at that of LL molecules. The DNAs from the cells transfected with pUC19, on the other hand, appear only at the density of LL molecules and not of HL molecules. These results suggest that the plasmids containing the human *hsp70* promoter replicated semiconservatively in HeLa cells.

As mentioned above, pHS-AB, pHS-A, and pHS-B contain either one or two of the binding sites for a c-*myc* protein complex(es) (HSP-MYC A and HSP-MYC B) (Fig. 3B) (29). Since the corresponding sequence located upstream from the human c-*myc* gene has ARS activity (1), it was conceivable that the ARS activities of the pHS plasmids were due to these sequences. To examine this possibility, oligonucleotides synthesized corresponding to HSP-MYC A and HSP-MYC B, with or without mutations, were cloned into pUC19 (Fig. 6, upper panel) and examined for ARS activity in HeLa cells. By both the PCR (Fig. 6A) and the Southern (Fig. 6B) methods, molecules which replicated in transfected cells were detected for pwt-A and pwt-B, which contain the wild-type oligonucleotides of HSP-MYC A and HSP-MYC B, respectively, as well as for pHS-A and pHS-B. By contrast, pMu-A and pMu-B, carrying the mutated sequences, did not replicate in the cells. These results indicate that the HSP-MYC sequences are responsible for the ARS activity of the *hsp70* promoter region.

Stable replication of the ARS plasmids containing the *hsp70* sequences in established cell lines. The pHS plasmids, carrying the segments in the region upstream from the human *hsp70* gene, transiently replicated at the episomal state in various mammalian cells. The plasmids containing the oligonucleotides corresponding to the binding elements for c-*myc* protein complexes therein similarly replicated in the same systems. These results suggest that the *hsp70* sequences contain ARS activity but are still not fully convincing, because the results were obtained in transient experiments for 2 to 3 days after

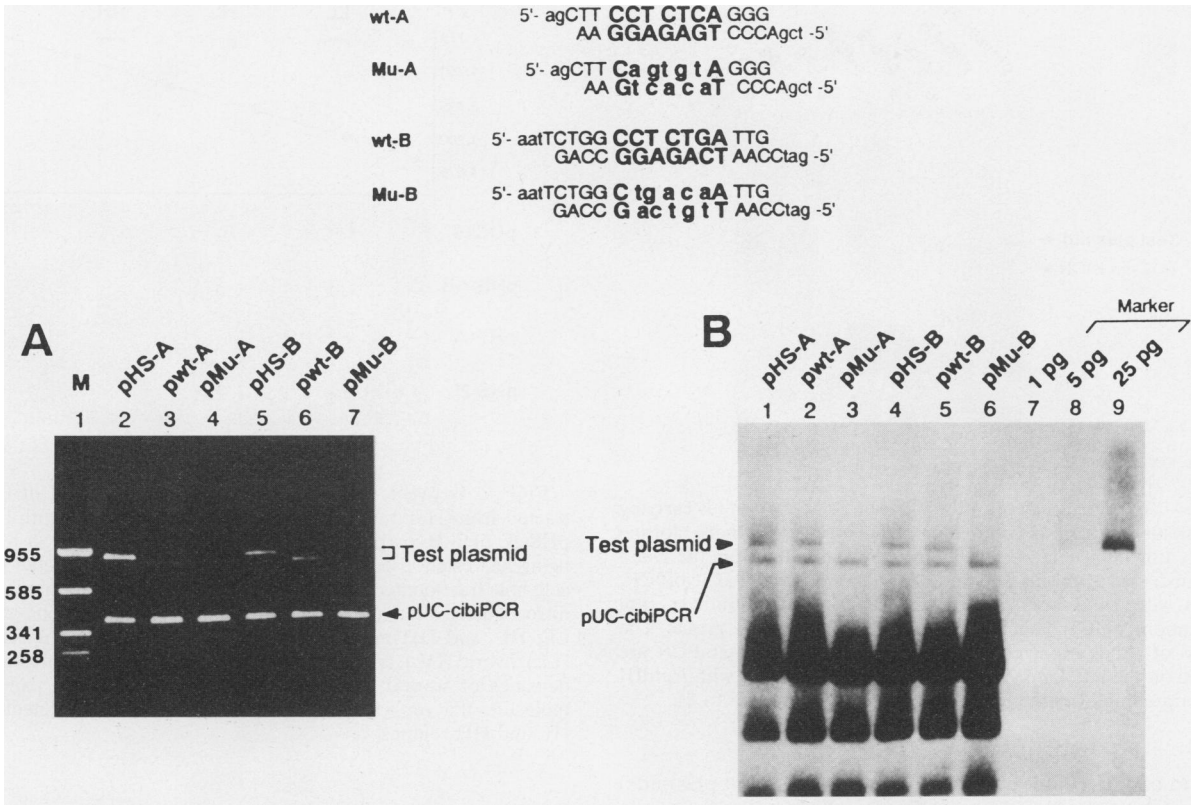

FIG. 6. Autonomous replication in HeLa cells of plasmids containing the oligonucleotides corresponding to HSP-MYC A and HSP-MYC B. The sequences of wild types (wt-A or wt-B) and mutants (Mu-A or Mu-B) of HSP-MYC A and HSP-MYC B oligonucleotides are shown in the upper panel. The sequences derived from the *hsp70* gene are in capital letters, while the sequences of the linker's restriction sites are in lowercase letters. Capital boldface letters indicate the core sequences for the binding of a c-*myc* protein complex (or a c-*myc* protein-like protein complex) identified previously (29). The nucleotides exchanged within the core sequences are shown in lowercase boldface letters. (A) The fragments amplified by PCR of the plasmids that replicated in HeLa cells were separated in an agarose gel containing ethidium bromide. pHS-A (lane 1) and pHS-B (lane 4) (see Fig. 3 and 4) are positive controls. pwt-A or pwt-B contains a wild-type oligonucleotide of HSP-MYC A or HSP-MYC B, respectively, while pMu-A or pMu-B contains a mutated oligonucleotide. M (lane 1), *Sau*3AI-digested pUC19 fragments as size markers. Positions expected for the bands due to test plasmids and pUC-cibiPCR are indicated on the right. (B) Low-molecular-weight DNAs were extracted from the HeLa cells transfected with test plasmids and *dam* mutant-pUC-cibiPCR, digested with *Dpn*I and *Hin*dIII, and analyzed by Southern blot hybridization. pUC19 labeled with ^{32}P was used as a probe. The positions of bands expected for test plasmids and pUC-cibiPCR are indicated on the left. One, 5, or 25 pg of pUC19 digested with *Hin*dIII was separated and blotted in parallel as markers (lanes 7 to 9).

transfection into cells. To confirm the observations, we established cell lines stably carrying ARS plasmids in episomes. The various plasmids used in the transient experiments described above were transfected to rat 3Y1 cells together with pSVHg, which expresses the hygromycin B resistance gene under the control of the simian virus 40 promoter. Rat 3Y1 cells, known to resemble normal (untransformed) cells in various aspects, can be easily synchronized by serum starvation. The transfected cells were cultured in the presence of hygromycin B to select resistant cell lines (see Materials and Methods). Total DNAs or low-molecular-weight DNAs were extracted from the drug-resistant cell lines and examined for the presence of the plasmids by Southern blotting with the *hsp70* sequences inserted in the respective plasmids. The results are summarized in Table 1. None of the resistant lines transfected with pMu-A, pMu-B, or pUC19 showed signals at the position of free plasmids, but all did at that of chromosomal DNA. The result suggested that the plasmids did not exist in episomes but were lost or integrated in chromosomal DNA of host cells. For the cell lines transfected with pHS-AB, pHS-A, and pwt-B, on the other hand, some of the lines gave rise to signals at the positions corresponding to the respective plasmids used for transfection and are therefore suggested to carry the plasmids in an episomal state. The plasmid-carrying lines were designated 3Y-AB, 3Y-A, and 3Y-wtB, respectively, and were further examined.

The DNA extracted from 3Y-AB-6, a clone of the 3Y-AB line, or 3Y-A-5, a clone of the 3Y-A line, was subjected to

TABLE 1. Summary of the established cell lines

Plasmid used for transfection	No. of established cell lines (plasmid-carrying lines[a]/total)	Recovery of plasmid by back-transformation of bacteria
pHS-AB	3/6	+
pHS-A	3/6	+
pHS-B	0/7	−
pwt-A	0/4	−
pwt-B	3/6	+
pMu-A	0/4	−
pMu-B	0/4	−
pUC19	0/7	−

[a] Free plasmids were detected by Southern blot analysis.

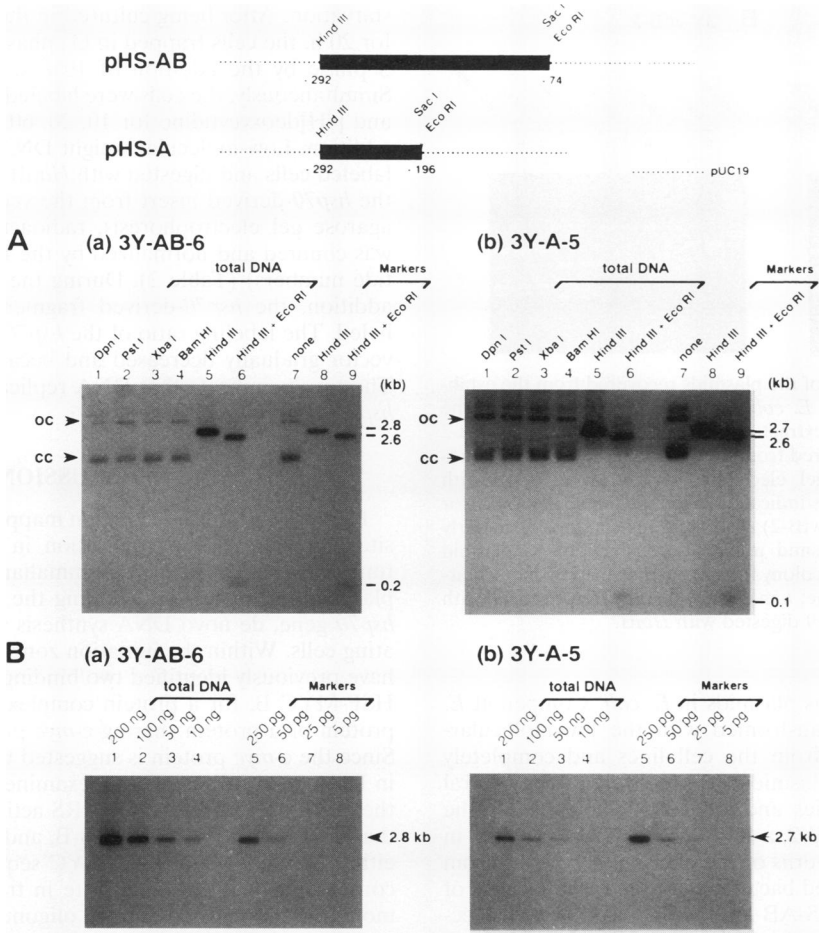

FIG. 7. Autonomous replication of the plasmids carrying the segments upstream from the human *hsp70* gene in established cell lines. (A) Total DNA was extracted from 3Y-AB-6 (a) or 3Y-A-5 (b) cells and subjected to Southern blot analyses after digestion with restriction enzymes (as indicated above the lanes) in addition to *Dpn*I. The *Hin*dIII-*Eco*RI fragment of pHS-AB was used as a probe. pHS-AB (for 3Y-AB-6) or pHS-A (for 3Y-A-5) (see upper panel) were similarly analyzed in parallel as size markers. (B) Different amounts of the total DNA extracted from 3Y-AB-6 (a) or 3Y-A-5 (b) were subjected to Southern blot analyses after digestion with *Hin*dIII and *Dpn*I as for panel A. Several amounts of pHS-AB (for 3Y-AB-6) or pHS-A (for 3Y-A-5) were digested with *Hin*dIII and similarly analyzed in parallel as size and quantitative markers.

Southern blot analyses after digestion with various restriction enzymes in addition to *Dpn*I (Fig. 7). *Pst*I, *Xba*I, and *Bam*HI (noncut enzymes) do not cleave the original plasmids, pHS-AB and pHS-A, used for transfection, while *Hin*dIII has one recognition site therein and linearizes the plasmids. Digestion with both *Hin*dIII and *Eco*RI yields the inserted *hsp70* segments in addition to the vector fragment (Fig. 7, upper panel). The samples digested with *Dpn*I alone (Fig. 7, lane 1) or with noncut enzymes (Fig. 7, lanes 2 to 4) in addition gave rise to signals at open and closed circular forms of the plasmids (Fig. 7Aa and b). After digestion with *Hin*dIII, a single band was detected at the same positions as that detected for the *Hin*dIII-digested original plasmids (Fig. 7Aa and b, lanes 5 and 8). The digestion with both *Hin*dIII and *Eco*RI gave rise to two signals, similarly observed for both the DNAs from cell lines and the original plasmids (Fig. 7Aa and b, lanes 6 and 9). With probes of *hsp70*-derived fragments, vector fragments were detected as well, probably because they contain homologous sequences. The cotransfected pSVHg was probably integrated in chromosomal DNA and was not detected under the conditions used here. After a long exposure of the autoradiographs, we observed bands due to the endogenous *hsp70* gene at the expected size in all the cell lines examined, including the lines without ARSs. These results suggest that the signals observed in the Southern analyses were due to the plasmid DNA in episomes and not to fragments derived from chromosomal DNA, and so that the plasmids, indistinguishable from the original plasmids used for transfection, replicated at episomal state in 3Y-AB-6 and 3Y-A-5 cells. To estimate the copy numbers of the plasmids in these cells, Southern blotting was carried out after titration of the DNA extracted from the cells (Fig. 7B). Various amounts (10 to 200 ng) of the total DNA were separated in an agarose gel after digestion with *Hin*dIII and subjected to Southern blotting. The *Hin*dIII digests of 5 to 250 pg of pHS-AB or pHS-A were electrophoresed in parallel in the same gel as the markers. The intensities of the signals in an autoradiograph were measured by a bioimage analyzer (BAS 2000; Fuji Film Co.). The copy numbers of the plasmids that replicated in 3Y-AB-6 and 3Y-A-5 were thus estimated as about 300 and 50 to 100 copies per cell, respectively. Similar results were obtained for the 3Y-wtB line, in which the plasmid replicated at 50 to 100 copies per cell, as well as for the rest of the 3Y-AB and 3Y-A clones (data not shown).

The episomes in these cell lines were examined for the

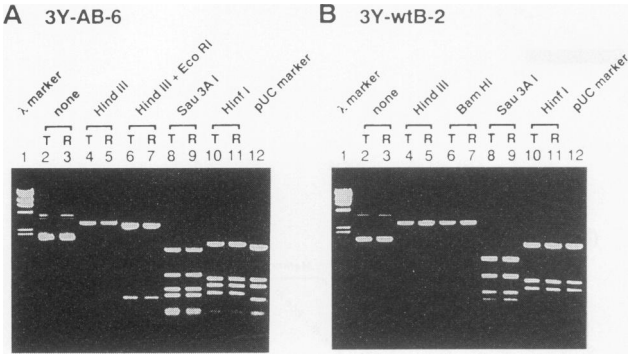

FIG. 8. Characterization of the plasmids recovered from the established cell lines. Competent *E. coli* cells were transformed with the low-molecular-weight DNA extracted from 3Y-AB-6 (A) or 3Y-wtB-2 (B). The plasmid was recovered from the ampicillin-resistant colonies and analyzed by agarose gel electrophoresis after digestion with various restriction enzymes as indicated above the lanes. pHS-AB (for 3Y-AB-6) or pwt-B (for 3Y-wtB-2) used for transfection was similarly analyzed in parallel. T, plasmid used for transfection; R, plasmid recovered from the bacterial colony transformed by the low-molecular-weight DNA from the cell line; λ marker, λ phage DNA digested with *Hin*dIII; pUC marker, pUC19 digested with *Hin*fI.

ability to be propagated as plasmids in *E. coli*. Competent *E. coli* DH5α cells were transformed with the low-molecular-weight DNAs extracted from the cell lines and completely digested with *Dpn*I. The plasmids were recovered from several ampicillin-resistant colonies and analyzed. The data for the lines 3Y-AB-6 and 3Y-wtB-2, a clone of 3Y-wtB, are shown in Fig. 8. The restriction patterns of the plasmids recovered from the colonies of transformed bacteria were identical to those of the original plasmids, pHS-AB and pwt-B, used for transfection. Sequence analysis around the inserts of the plasmids from three independent colonies per cell line revealed that the *hsp70*-derived sequence and the junctions to the vector were preserved intact (data not shown). Similar results were obtained for the plasmids derived from the 3Y-A clones in addition to all the 3Y-AB and 3Y-wtB clones. pHS-AB, pHS-A, and pwt-B are thus suggested to stably replicate for a long time in the cell lines without rearrangement.

To examine whether the ARS plasmids in the cell lines replicate semiconservatively, 3Y-AB-6 cells were subjected to BrdU labeling experiments. The cells were cultured for 20 or 40 h in the presence of BrdU. Total DNAs were extracted, digested with *Hin*dIII, and fractionated according to the density in CsCl gradients. The DNA recovered in each fraction was separated in an agarose gel, blotted, and hybridized with a ^{32}P-labeled pUC18 probe (Fig. 9). The band of 2.8 kb due to the episomal pHS-AB, linearized by *Hin*dIII, was detected in the fractions of LL density before BrdU labeling. After 20 or 40 h of labeling, the band was observed in the fractions of HL or HH density. The results suggest that pHS-AB in episome of 3Y-AB-6 cells replicated semiconservatively. Moreover, we analyzed the same DNA samples for B-2, a repetitive sequence found in mouse genes (19). The DNAs that hybridized with a B-2 probe were similarly detected in the fractions of LL, HL, or HH density after 0, 20, or 40 h of BrdU labeling. The results indicate that pHS-AB replicated semiconservatively at the same timing of chromosomal DNA replication in 3Y-AB-6 cells.

To examine where replication of the pHS-AB in episomes of 3Y-AB-6 cells initiates in the *hsp70*-derived sequences, not in the vector sequences, the cells were synchronized by serum starvation. After being cultured in the medium without serum for 20 h, the cells trapped in G_0 phase were triggered to enter S phase by the addition of 10% serum-containing medium. Simultaneously, the cells were labeled with both [^{3}H]thymidine and [^{3}H]deoxycytidine for 10, 20, 60, or 120 min after serum addition. Low-molecular-weight DNA was extracted from the labeled cells and digested with *Hin*dIII and *Eco*RI to separate the *hsp70*-derived insert from the vector. After separation by agarose gel electrophoresis, radioactivity of either fragment was counted and normalized by the fragment length (nucleotide numbers) (Table 2). During the first 10 min after serum addition, the *hsp70*-derived fragment was preferentially labeled. The labeling ratio of the *hsp70*-derived fragment to the vector gradually decreased and became nearly 1 in 120 min. The results indicate that DNA replication initiated within the *hsp70*-derived HS-AB region.

DISCUSSION

Here we show that the region mapped in vivo as an initiation site of cellular DNA replication in the human *hsp70* gene functioned as an ARS in mammalian cells when cloned in a plasmid. In the region including the promoter of the human *hsp70* gene, de novo DNA synthesis was detected in proliferating cells. Within the initiation zone thus mapped in vivo, we have previously identified two binding sites, HSP-MYC A and HSP-MYC B, for a protein complex that includes the c-*myc* protein or a protein sharing c-*myc* protein-like epitopes (29). Since the c-*myc* protein is suggested to play an important role in DNA replication, we then examined the segments covering the HSP-MYC A and B for ARS activity. The results indicate that the plasmids pHS-A, pHS-B, and pHS-AB, which include either or both of the HSP-MYC sequences, replicated semiconservatively at episomal state in transfected cells. Furthermore, the plasmids containing oligonucleotides corresponding to either of the HSP-MYC sequences also functioned as an ARS. It is therefore suggested that ARS activity of the *hsp70* promoter region is due to the HSP-MYC sequences. The idea was supported by the finding that mutations within the HSP-MYC sequences abolished ARS activity of the plasmids. The ARS activity due to the *hsp70* sequences was observed not only in transiently transfected cells but also in established cell lines. Stable replication of mammalian ARSs from the known genes for a long term has scarcely been confirmed, except for a human c-*myc*-derived ARS in transgenic mice (28) and in tissue culture cell lines (21), a murine adenosine deaminase (6), a mouse immunoglobulin heavy chain (23a), and a human N-*myc*-derived ARS (22a) in culture cell lines and the human *hsp70*-derived ones in the cell lines reported here. Replication of the pHS-AB in episomes of 3Y-AB-6 cell line initiated within the *hsp70*-derived sequences, not in those of the vector. The results of these long-term experiments strongly suggest that the *hsp70* fragment contains a replication initiation site and that the ARSs include sequences required for stable transmission in addition to core *ori* sequences.

Several observations have suggested that DNA replication and transcription are concertedly regulated. Two possibilities are considered: *cis*- or *trans*-transcriptional elements directly regulate DNA replication, or vice versa (7, 11, 14). Replication of polyomavirus DNA requires domain A of polyomavirus enhancers (8, 34) and is decreased by introducing mutations in the enhancer (20). Furthermore, activation of initiation of polyomavirus DNA replication depends on the space between the enhancer and the origin (18, 22). Replication origins of mammalian cells identified so far are located near, or overlapped with, transcriptional regulatory regions. Binding sites

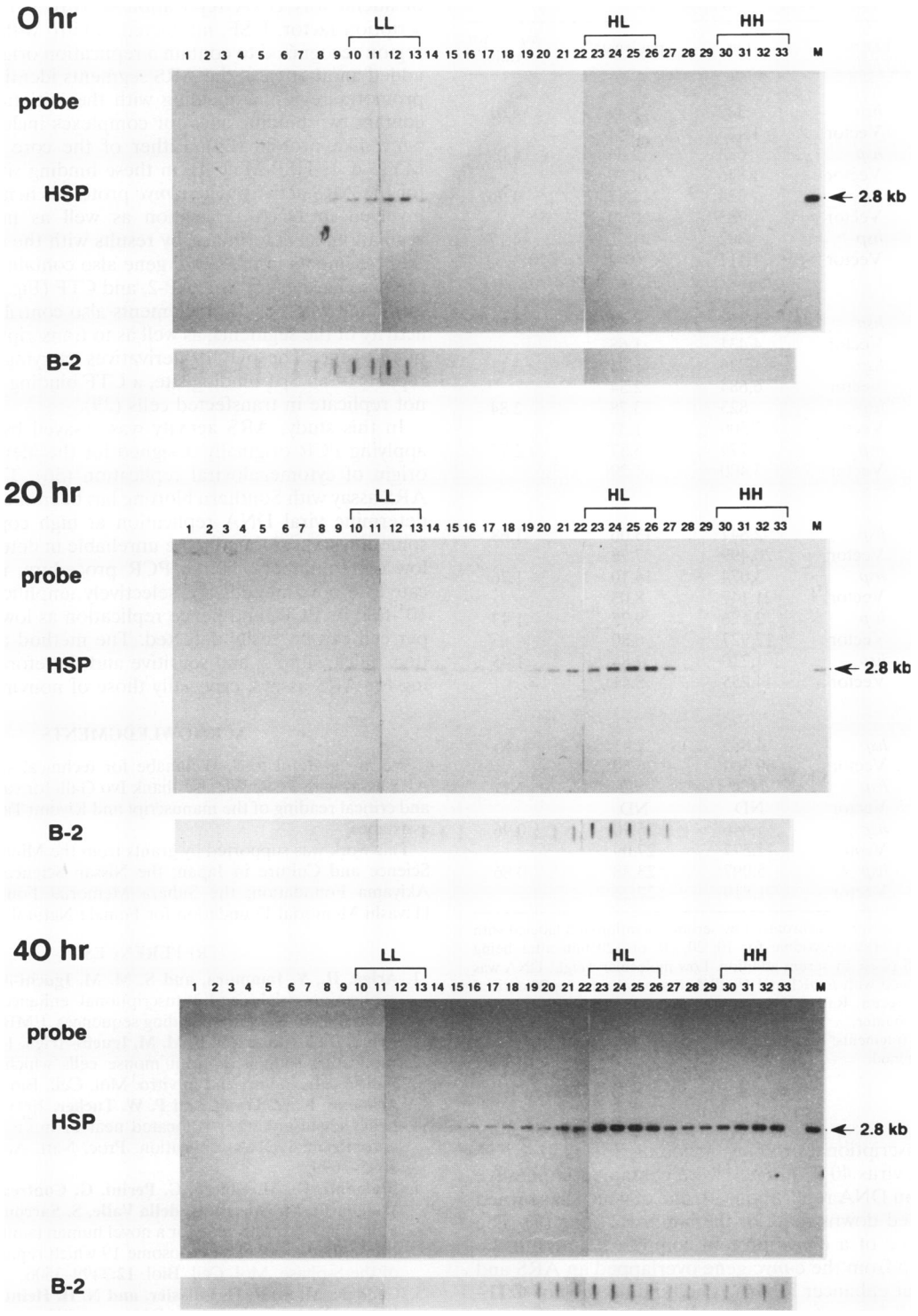

FIG. 9. Semiconservative replication of pHS-AB in 3Y-AB-6 cells. 3Y-AB-6 cells were cultured for 0, 20, or 40 h in the presence of BrdU. Total DNAs were extracted, digested with *Hin*dIII, and fractionated by a neutral CsCl equilibrium centrifugation. The DNAs were separated in an agarose gel and analyzed by Southern blotting. The *hsp*-derived *Hin*dIII-*Eco*RI fragment of pHS-AB (Fig. 3B) was labeled with ^{32}P and used as a probe (probe HSP). LL, HL, and HH indicate the positions expected for unsubstituted, hybrid, and fully substituted DNAs, respectively. DNA molecules replicated in the cells are expected to be detected in the HL and HH regions. Lane M contained 50 pg of pHS-AB linearized by *Hin*dIII digestion (2.8 kb) as a marker. Below the Southern blots, the results of slot blot hybridization of the same DNA samples with a mouse repetitive sequence probe (probe B-2) are shown.

TABLE 2. Initiation of replication of the pHS-AB in the HSP-MYC-containing fragment

Labeling time (min)	Fragment[a]	cpm	cpm/length[b]	*hsp*/vector ratio
10				
Expt 1	*hsp*	542	2.49	4.61
	Vector	1,423	0.54	
Expt 2	*hsp*	623	2.86	4.09
	Vector	1,846	0.70	
Expt 3	*hsp*	324	1.47	4.90
	Vector	783	0.30	
Expt 4	*hsp*	382	1.75	4.17
	Vector	1,111	0.42	
20				
Expt 1	*hsp*	1,425	6.54	3.89
	Vector	4,431	1.68	
Expt 2	*hsp*	1,725	7.91	3.11
	Vector	6,684	2.54	
Expt 3	*hsp*	823	3.78	2.84
	Vector	3,500	1.33	
Expt 4	*hsp*	779	3.57	2.77
	Vector	3,400	1.29	
60				
Expt 1	*hsp*	2,847	13.06	1.68
	Vector	20,499	7.78	
Expt 2	*hsp*	3,074	14.10	1.76
	Vector	21,147	8.03	
Expt 3	*hsp*	2,126	9.75	1.43
	Vector	17,927	6.80	
Expt 4	*hsp*	1,710	7.84	1.45
	Vector	14,255	5.41	
120				
Expt 1	*hsp*	4,885	22.41	0.85
	Vector	69,300	26.30	
Expt 2	*hsp*	ND[d]	ND	ND
	Vector	ND	ND	
Expt 3	*hsp*	5,646	25.90	0.96
	Vector	71,377	27.09	
Expt 4	*hsp*	5,097	23.38	0.86
	Vector	71,819	27.26	

[a] The 3Y-AB-6 cells were synchronized by serum starvation and labeled with [^{3}H]thymidine and [^{3}H]deoxycytidine for 10, 20, 60, or 120 min after being triggered to enter S phase by serum addition. Low-molecular-weight DNA was extracted and digested with *Eco*RI and *Hin*dIII to separate the *hsp70*-derived fragment from the vector. Radioactivity of each fragment was counted with a liquid scintillation counter.

[b] Lengths of the fragments: *hsp*, 218 bp; vector, 2,635 bp.

[c] ND, not determined.

for several transcriptional proteins, including Sp1, OTF-1/NF-III, and simian virus 40 T antigen, were contained in putative origins of human DNA replication (9), one of which has turned out to be located downstream of the lamin B2 gene (4). The binding sequence of a c-*myc* protein complex defined in the region upstream from the c-*myc* gene overlapped an ARS and a transcriptional enhancer (1, 16). The binding sites of OTF-1/NF-III and AP-1 were found in the region near a replication origin downstream of the Chinese hamster dihydrofolate reductase gene (5). As for protein factors, OTF-1 (Oct 1), first identified as a ubiquitous transcription factor recognizing the octamer sequence, has been shown to be physiologically and biologically indistinguishable from nuclear factor III (NF-III), a cellular protein required for adenovirus DNA replication in vitro (24, 26, 27, 35). CTF is responsible for specific recognition of the CCAAT sequence in eukaryotic promoters, is indistinguishable from NF-1, and is essential for the initiation of adenovirus DNA replication in vitro (17). Another transcription factor, USF, interacted in vitro with a human DNA region recognized to contain a replication origin (30). Here we added another case: the ARS segments identified in the *hsp70* promoter region, coinciding with the initiation zone in vivo, contain two binding sites for complexes including c-*myc* or a c-*myc*-like protein (29). Either of the core elements, HSP-MYC A or HSP-MYC B, in these binding sites was required for the ARS activity. The c-*myc* protein is hence implied to be involved in DNA replication as well as in transcriptional regulation, as determined by results with the c-*myc* gene. The ARS segments in the *hsp70* gene also contain HSE, SRE, and the binding sites for Sp1, AP-2, and CTF (Fig. 1). We have not examined whether these elements also contribute to the ARS activity of the segments, as well as to transcriptional regulation of the gene. The pUC19 derivatives carrying one or tandem repeats of an Sp1 binding site, a CTF binding site, or SRE did not replicate in transfected cells (39).

In this study, ARS activity was assayed by a new method applying PCR originally designed for the identification of the origin of cytomegaloviral replication (36). The conventional ARS assay with Southern blotting has been successfully used to determine viral DNA replication at high copy numbers but sometimes was claimed to be unreliable in detection of ARS at low copy numbers. In the PCR procedure, molecules replicated de novo in cells are selectively amplified to more than 10^4-fold by PCR, and hence replication as low as at 10 copies per cell can be easily detected. The method applying PCR is thus quick, simple, and sensitive and therefore highly promising for ARS assays, especially those of nonviral sequences.

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REFERENCES

1. **Ariga, H., Y. Imamura, and S. M. M. Iguchi-Ariga.** 1989. DNA replication origin and transcriptional enhancer in c-*myc* gene share the c-*myc* protein binding sequences. EMBO J. **8**:4273–4279.
2. **Ariga, H., T. Itani, and S. M. M. Iguchi-Ariga.** 1987. Autonomous replicating sequences from mouse cells which can replicate in mouse cells in vivo and in vitro. Mol. Cell. Biol. **7**:1–6.
3. **Ariizumi, K., Z. Wang, and P. W. Tucker.** 1993. Immunoglobulin heavy chain enhancer is located near or in an initiation zone of chromosomal DNA replication. Proc. Natl. Acad. Sci. USA **90:** 3695–3699.
4. **Biamonti, G., M. Giacca, G. Perini, G. Contreas, L. Zentilin, F. Weighardt, M. Guerra, G. della Valle, S. Saccone, S. Riva, and A. Falaschi.** 1992. The gene for a novel human lamin maps at a highly transcribed locus of chromosome 19 which replicates at the onset of the S-phase. Mol. Cell. Biol. **12**:3499–3506.
5. **Caddele, M. S., R. H. Luissier, and N. H. Heintz.** 1990. Intramolecular DNA triplexes, bent DNA and DNA unwinding elements in the initiation region of a amplified dihydrofolate reductase replication. J. Mol. Biol. **211**:19–33.
6. **Carroll, S. M., M. L. DeRose, J. L. Kolman, G. H. Nonet, R. E. Kelly, and G. M. Wahl.** 1993. Localization of a bidirectional DNA replication origin in the native locus and in episomally amplified murine adenosine deaminase loci. Mol. Cell. Biol. **13**:2971–2981.
7. **Depamphilis, M. L.** 1988. Transcriptional elements as components of eukaryotic origins of DNA replication. Cell **52**:635–638.
8. **de Villiers, J., W. Schaffner, C. Tyndall, S. Lupton, and R. Kamen.**

1984. Polyoma virus DNA replication requires an enhancer. Nature (London) **312**:242–246.
9. **Falaschi, A., G. Biamonti, F. Cobianchi, E. Csorda-Toth, G. Faulkner, M. Giacca, D. Pedacchia, G. Perini, S. Riva, and C. Tribioli.** 1988. Presence of transcription signals in two putative DNA replication origins of human cells. Biochim. Biophys. Acta **951**:430–442.
10. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
11. **Held, P. G., and N. H. Heintz.** 1992. Eukaryotic replication origins. Biochim. Biophys. Acta **1130**:235–246.
12. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28**:351–359.
13. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
14. **Iguchi-Ariga, S. M. M., and H. Ariga.** 1989. Concerted mechanism of DNA replication and transcription. Cell Struct. Funct. **14**:649–651.
15. **Iguchi-Ariga, S. M. M., N. Ogawa, and H. Ariga.** 1993. Identification of the initiation region of DNA replication in the murine immunoglobulin heavy chain gene and possible function of the octamer motif as a putative DNA replication origin in mammalian cells. Biochim. Biophys. Acta **1172**:73–81.
16. **Iguchi-Ariga, S. M. M., T. Okazaki, T. Itani, T., M. Ogata, Y. Sato, and H. Ariga.** 1988. An initiation site of DNA replication with transcriptional enhancer activity present upstream of the c-*myc* gene. EMBO J. **7**:3135–3142.
17. **Jones, K. A., J. T. Kadonaga, P. J. Rosenfeld, T. J. Kelly, and R. Tjian.** 1987. A cellular DNA-binding protein that activates eukaryotic transcription and DNA replication. Cell **48**:79–89.
18. **Kolluri, R., T. A. Torrey, and A. J. Kinniburgh.** 1992. A CT promoter element binding protein: definition of a double-strand and a novel single-strand DNA binding motif. Nucleic Acids Res. **20**:111–116.
19. **Kominami, R., M. Muramatsu, and K. Moriwaki.** 1983. A mouse type 2 Alu sequence (M2) is mobile in the genome. Nature (London) **301**:87–89.
20. **Martin, M. E., J. Piette, M. Yaniv, W.-J. Tang, and W. R. Folk.** 1988. Activation of the polyomavirus enhancer by a murine activator protein 1 (AP1) homologue and two contiguous proteins. Proc. Natl. Acad. Sci. USA **85**:5839–5843.
21. **McWhinney, C., and M. Leffak.** 1990. Autonomous replication of a DNA fragment containing the chromosomal replication origin of the human c-*myc* gene. Nucleic Acids Res. **18**:1233–1242.
22. **Murakami, Y., M. Satake, Y. Yamaguchi-Iwai, M. Sakai, M. Muramatsu, and Y. Ito.** 1991. The nuclear protooncogene c-*jun* and c-*fos* as regulators of DNA replication. Proc. Natl. Sci. Acad. USA **88**:3947–3951.
22a. **Nakagawa, T., Y. Imamura, S. M. M. Iguchi-Ariga, and H. Ariga.** Unpublished data.
23. **Negishi, Y., S. M. M. Iguchi-Ariga, and H. Ariga.** 1992. Protein complexes bearing *myc*-like antigenicity recognize two distinct DNA sequences. Oncogene **7**:543–548.
23a. **Ogawa, N., S. M. M. Iguchi-Ariga, and H. Ariga.** Unpublished data.
24. **O'Neil, E. A., C. Fletcher, C. R. Burrow, N. Heintz, R. G. Roeder, and T. J. Kelly.** 1988. Transcription factor OTF-1 is functionally identical to the DNA replication factor NF-III. Science **241**:1210–1213.
25. **Peden, K. W. C., J. M. Pipas, S. Pearson-White, and D. Nathans.** 1980. Isolation of mutants of an animal virus in bacteria. Science **290**:1392–1396.
26. **Prujin, G. J. M., W. van Driel, and P. C. van der Vliet.** 1986. Nuclear factor III, a novel sequence-specific DNA-binding protein from HeLa cells stimulating adenovirus DNA replication. Nature (London) **322**:656–659.
27. **Prujin, G. J. M., W. van Driel, R. T. van Miltenburg, and P. C. van der Vliet.** 1987. Promoter and enhancer elements containing a conserved sequence motif are recognized by nuclear factor III, a protein stimulating adenovirus DNA replication. EMBO J. **6**:3771–3778.
28. **Sudo, K., M. Ogata, Y. Sato, S. M. M. Iguchi-Ariga, and H. Ariga.** 1990. Cloned origin of DNA replication in c-*myc* gene can function and be transmitted in transgenic mice in an episomal state. Nucleic Acids Res. **18**:5425–5432.
29. **Taira, T., Y. Negishi, F. Kihara, S. M. M. Iguchi-Ariga, and H. Ariga.** 1992. c-*myc* protein complex binds to two sites in human *hsp70* promoter region. Biochim. Biophys. Acta **1130**:166–174.
30. **Tóth, É. C., L. Marusic, A. Ochem, A. Patthy, S. Pongor, M. Giacca, and A. Falaschi.** 1993. Interactions of USF and Ku antigen with a human DNA region containing a replication origin. Nucleic Acids Res. **21**:3257–3263.
31. **Vassilev, L. T., W. C. Burhans, and M. L. DePamphilis.** 1990. Mapping an origin of DNA replication at a single-copy locus in exponentially proliferating mammalian cells. Mol. Cell. Biol. **10**: 4685–4689.
32. **Vassilev, L. T., and E. M. Johnson.** 1989. Mapping initiation sites of DNA replication in vivo using polymerase chain reaction amplification of nascent strand segments. Nucleic Acids Res. **17**:7693–7705.
33. **Vassilev, L. T., and E. M. Johnson.** 1990. An initiation zone of chromosomal DNA replication located upstream of the c-*myc* gene in proliferating HeLa cells. Mol. Cell. Biol. **10**:4899–4904.
34. **Veldman, G. M., S. Lupton, and R. Kamen.** 1985. Polyomavirus enhancer contains multiple redundant sequence elements that activate both DNA replication and gene expression. Mol. Cell. Biol. **5**:449–658.
35. **Verrizer, C. P., A. J. Kal, and P. C. van der Vliet.** 1990. The DNA binding domain (POU domain) of transcription factor oct-1 suffies for stimulation of DNA replication. EMBO J. **9**:1883–1888.
36. **Watanabe, S., and N. Yamaguchi.** 1993. Deletion analysis of a replication origin of human cytomegalovirus by a novel assay system with a combination of microinjection and polymerase chain reaction. Virology **192**:332–335.
37. **Wu, B. J., C. Hunt, and R. I. Morimoto.** 1985. Structure and expression of the human gene encoding major heat shock protein HSP70. Mol. Cell. Biol. **5**:330–341.
38. **Yanisch-Perron, C. J., Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
39. **Yokoyama, M., H. Ariga, and S. M. M. Iguchi-Ariga.** Submitted for publication.