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Isolation and Characterization of Sequences from Mouse Chromosomal DNA with *ARS* Function in Yeasts

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Fragments of chromosomal DNA from a variety of eucaryotes can act as *ARS*s (autonomously replicating sequence) in yeasts. *ARS*s enable plasmids to be maintained in extrachromosomal form, presumably because they function as initiation sites for DNA replication. We isolated eight different sequences from mouse chromosomal DNA which function as *ARS*s in *Saccharomyces cerevisiae* (bakers' yeast). Although the replication efficiency of the different mouse *ARS*s in yeasts appears to vary widely, about one-half of them functions as well as the yeast chromosomal sequence *ARS1*. Moreover, five of the *ARS*s also promote self replication of plasmids in *Schizosaccharomyces pombe* (fission yeast). Each of the *ARS*s was cloned into plasmids suitable for transformation of mouse tissue culture cells. Plasmids were introduced into thymidine kinase (TK)-deficient mouse L cells by the calcium phosphate precipitation technique in the absence of carrier DNA. In some experiments, the *ARS* plasmid contained the herpes simplex virus type 1 TK gene; in other experiments (cotransformations), the TK gene was carried on a separate plasmid used in the same transformation. In contrast to their behavior in yeasts, none of the *ARS* plasmids displayed a significant increase in transformation frequency in mouse cells compared with control plasmids. Moreover, only 1 of over 100 cell lines contained the original plasmid in extrachromosomal form. The majority of cell lines produced by transformation with an *ARS* TK plasmid contained multiple copies of plasmid integrated into chromosomal DNA. In most cases, results with plasmids used in cotransformations were similar to those for plasmids carrying TK. However, cell lines produced by cotransformations with plasmids containing any one of three of the *ARS*s (m24, m25, or m26) often contained extrachromosomal DNAs.

Replication of many bacterial chromosomal, plasmid, and viral DNAs begins at a single unique site on the DNA molecule. Small segments of DNA corresponding to these procaryotic origins can support replication of nonreplicating plasmids in bacterial cells (for a review, see reference 22). In contrast, replication of eucaryotic chromosomal DNA begins at multiple sites along the DNA molecule, and there is little direct evidence that these sites represent specific, fixed genetic loci. Yeast cells can be transformed by recombinant DNA plasmids containing a selectable gene (16). A subset of sequences from yeast chromosomal DNA promotes high-frequency transformation of yeasts, and plasmids containing these sequences are found in extrachromosomal form in transformed cells (18, 33). From analogy with procaryotic systems, these *ARS* (autonomously replicating sequence) elements are hypothesized to serve as initiation sites for yeast DNA replication. Results from experiments with *ARS* plasmids in vivo (5, 7, 13, 39) and in vitro (6, 21) support this hypothesis.

Although *ARS* plasmids are found in multiple copies in transformed yeast cells, they are usually present in only a fraction of the cells growing under selection for the plasmid (31) and are lost from the population when cells are grown without selection (18, 33). These properties are due to nonrandom segregation rather than defective replication (38). Sequences from the centromere region (*CEN*) of yeast chromosomes promote the equal segregation of an *ARS* plasmid. Plasmids containing *ARS* and *CEN* DNAs are found in about one copy per cell in cells growing under selection for the plasmid and are stably transmitted during mitosis and meiosis (8). Plasmids containing *CEN* sequences are rarely found integrated into yeast chromosomal DNA. When integration of *CEN* plasmids into chromosomes

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can be detected, it is accompanied by deletion or rearrangement of the resident centromere (R. Surosky and B.-K. Tye, manuscript in preparation).

In addition to yeast DNA, a subset of DNA sequences from a wide variety of eucaryotes, but not from *Escherichia coli*, can function as *ARS*s in yeasts (32). Moreover, at least three DNA fragments which contain known origins of replication for eucaryotic extrachromosomal DNAs function as *ARS*s in yeasts (20, 24, 36). These data are consistent with the hypothesis that there are specific conserved sequences utilized for initiation of DNA replication in eucaryotes. We tested this hypothesis by isolating sequences from mouse chromosomal DNA which function as *ARS*s in yeasts and then reintroducing these sequences on plasmids into mouse tissue culture cells. Some of the plasmids used in these experiments also contained yeast centromere DNA. None of the *ARS* plasmids reproducibly promotes high-frequency transformation of mouse cells. However, some *ARS* plasmids were often found in extrachromosomal form in transformed cells, but with one exception, the structure of these extrachromosomal plasmids was always different from that of the original DNA.

MATERIALS AND METHODS

Strains. *E. coli* RR1 and HB101 were used for transformations (4). For yeast transformations, *Saccharomyces cerevisiae* 689 (**a** *leu2-3 leu2-112 ura3-50 can1-101*; provided by D. Botstein) and *Schizosaccharomyces pombe* h ura4-294 (*ura4-294*; provided by J. Kohli) were used. Mouse cell transformations were carried out with the thymidine kinase-deficient (TK^-) L cell line B82 (provided by J. McDougall).

DNAs and recombinant DNA manipulations. YIp5 consists of pBR322 and 1.1 kilobase pairs (kb) of yeast chromosomal DNA containing the *URA3* gene (33). YRp12 contains a 1.4-kb *Eco*RI fragment from yeast chromosomal DNA which contains the *TRP1* gene and the *ARS1* locus inserted in YIp5 (32). pML2 is a derivative of pBR322 with a deletion between 1,120 and 2,490 base pairs (bp) (provided by M. Lusky and M. Botchan). The deleted stretch of DNA has been shown to have an inhibitory effect on the replication of hybrid plasmids consisting of pBR322 and the simian virus 40 origin of replication in simian cells (25). The thymidine kinase (TK) gene of herpes simplex virus type 1 (HSV-1) was isolated as a 3.4-kb *Bam*HI restriction fragment from the pBR322 derivative LE576 (obtained from L. Enquist). pYE (*CEN3*)3 contains a 627-bp *Sau*3A fragment which carries the yeast chromosome III (C3) centromere function inserted into pBR322 (14; provided by K. Bloom). To obtain high-efficiency transformation, the method of Dagert and Ehrlich was used with *E. coli* RR1 (10). Restriction endonucleases and T4 ligase (Bethesda Research Laboratories) were used as directed by the manufacturer.

Isolation of DNAs. Plasmid DNA for restriction enzyme analysis was isolated from a few milliliters of *E. coli* cells by the method of Birnboim and Doly (2). Plasmid DNA for yeast transformations were isolated by the quick-screening procedure described previously (28). For large-scale plasmid preparations, DNA was extracted by the procedure of Birnboim and Doly (2) and purified by CsCl-ethidium bromide equilibrium density centrifugation. Mouse DNA from untransformed or transformed cell lines was isolated as follows. Confluent cells grown on 100-mm plates were rinsed two times with 20 ml of 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate; pH 7.0), and the cells were then lysed on the plate with 3 ml of SET (1% sodium dodecyl sulfate, 10 mM Tris, 5 mM EDTA; pH 8.0). The resulting lysate was scraped into a tube and digested with proteinase K (100 μg/ml) for 5 to 20 h at 37°C. The DNA was extracted with phenol-chloroform and precipitated with ethanol. Separation of high- and low-molecular-weight DNA was done according to Hirt (17).

Construction of plasmids for mouse cell transformation. A variety of plasmids was constructed for use in mouse tissue culture cells (Fig. 1). For P-mx-TK plasmids, the sequences m4, m8, m11, m20, m21, m25, and m26 were cloned as *Hin*dIII-*Bam*HI fragments into *Hin*dIII–*Bam*HI-digested, agarose gel-purified pML2. The 5.5-kb *Hin*dIII fragment of m24 was ligated into *Hin*dIII-digested pML2. These plasmids were then digested with *Bam*HI, and the 3.4-kb *Bam*HI fragment with the HSV-1 TK gene ligated into them. For P-mx-C3-TK plasmids, an 8.4-kb *Bam*HI fragment containing centromere sequences from yeast chromosome III was isolated from YRp16-Sc4301 (30; provided by R. Davis and D. Stinchcomb) and cloned into pBR322. From this plasmid, *CEN3* was isolated as a 6-kb *Hin*dIII fragment, a fragment which also includes the 346-bp *Hin*dIII-*Bam*HI fragment from pBR322. This fragment was inserted into *Hin*dIII-cut P-mx-TK plasmids. Plasmids containing m11 or m21, *CEN4*, and TK were constructed by ligating the 2.25-kb *Eco*RI-*Hin*dIII *CEN4* fragment isolated from YCp19 (30; provided by R. Davis and D. Stinchcomb) into plasmids containing the *ARS* and then by ligating the *Bam*HI TK fragment into these plasmids.

Mouse cell transformation. Mouse L TK^- cells were transformed by the calcium phosphate method of Graham and van der Eb (15). Cells were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum. Twenty-four hours before addition of the DNA, the cells were seeded onto 60-mm plates at a density of 5×10^5 cells per plate. The donor DNA was sterilized by chloroform extraction and adjusted to 250 mM $CaCl_2$. The amount of plasmid DNA per plate was 0.5 to 2 μg. No carrier DNA was added. Under constant agitation, an equal volume of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (0.3 M NaCl, 10 mM KCl, 1 mM Na_2HPO_4, 10 mM glucose, 40 mM HEPES; pH 7.12) was added. The DNA-calcium phosphate coprecipitate was kept at room temperature for 30 min then added to the cells. After 6 h, the medium was changed. Twenty-four hours after addition of the DNA precipitate, the medium was changed to HAT (Dulbecco modified Eagle medium, 10% calf serum, 14 μg of hypoxanthine per ml, 2 μg of aminopterine per ml, and 4 μg of thymidine per ml). The cells were fed every 3 days, and independent colonies were isolated after about 10 days.

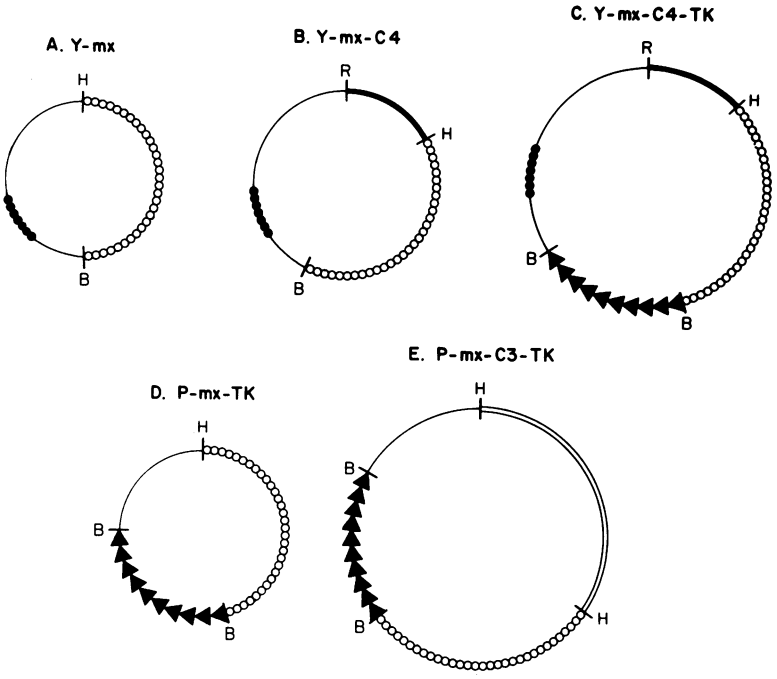

FIG. 1. Plasmid types used in yeast or mouse cell transformations or both. (A) Y-mx plasmids consist of YIp5 (33) plus a *Hin*dIII (H)-*Bam*HI (B) fragment from mouse DNA which acts as an *ARS* in *S. cerevisiae*. (B) Y-mx-C4 plasmids are like the Y-mx plasmids, except they also contain a 2.2-kb *Eco*RI (R)-*Hin*dIII fragment from yeast chromosome IV (C4) which functions as a centromere. (C) Y-mx-C4-TK plasmids are like the Y-mx-C4 plasmids except they also contain a 3.4-kb *Bam*HI fragment from HSV-1 which carries the gene for TK. (D) P-mx-TK plasmids consist of pML2 (25) plus a *Hin*dIII-*Bam*HI *ARS* fragment from mouse DNA and the *Bam*HI fragment with the TK gene of HSV-1. P-mx plasmids lack the *Bam*HI TK fragment. (E) P-mx-C3-TK plasmids are like the P-mx-TK plasmids, except they also contain a 6.3-kb *Hin*dIII fragment. This fragment is comprised of a 6.0-kb *Hin*dIII-*Bam*HI fragment from yeast chromosome III (C3) as well as 346-bps of pBR322 DNA. Restriction sites used for insertion of fragments are indicated. Symbols: ○, mouse fragment with *ARS* activity in yeast; ●, the yeast *URA*3 gene; ——, pBR322 DNA; ◀, 3.4-kb TK gene of herpes simplex 1; ■, yeast centromere IV DNA; □, yeast centromere III DNA (includes 346 bp from pBR322).

Miscellaneous methods. *S. cerevisiae* was transformed with minor modifications of the method of Beggs (1), and *S. pombe* was transformed by the method of Russell (27). Yeast cells were grown either in Y complete media minus uracil (38; hereafter called YC minus uracil) or in the same media to which was added 20 μg of uracil per ml. Stability tests were carried out as described previously (38).

Conditions for horizontal agarose gel electrophoresis have been described previously (37). DNA fragments used in nick translations or ligations were isolated from agarose gels by the diethylaminoethyl paper method (11). Before transfer of DNA from agarose gels to the nitrocellulose filters (29), the gels usually were treated with acid (35). Radioactive hybridization probes were removed from blots by washing for at least 30 min at 65°C in hybridization solution (35) without dextran sulfate.

RESULTS

Isolation of *ARS*s from mouse DNA. Total mouse DNA was digested with *Bam*HI and *Hin*dIII restriction enzymes, and fragments of 4 to 6 kb were isolated by agarose gel electrophoresis. These fragments were ligated to gel-purified *Hin*dIII–*Bam*HI-digested YIp5 DNA and then used to transform *E. coli*. Thirty different plasmid DNAs, containing inserts with an average size of 4 kb and representing a total of 120 kb of mouse DNA, were isolated, and each was used to transform yeasts. Plasmids containing five different inserts (m4, m8, m11, m20, and m21) transformed yeasts at a high frequency (Table 1). These data suggest that 5 of the 30 mouse fragments function as *ARS*s in *S. cerevisiae*. Assuming one *ARS* per fragment, the density of *ARS*s in mouse DNA is about 1 in 24 kb.

Three additional mouse *ARS*s were isolated with a plasmid containing *CEN4* DNA in an attempt to identify sequences which function as well as the yeast sequence *ARS1* in conjunction with yeast centromere DNA. The plasmid was constructed by inserting the 2.2-kb *Eco*RI-*Hin*dIII *CEN4* fragment from YCp19 (30) into *Eco*RI–*Hin*dIII-digested YIp5. Plasmids containing centromere DNA transform yeasts only if they also contain an *ARS* (8). Furthermore,

TABLE 1. Properties of mouse ARSs[a]

Plasmid	A Size insert (kb)	B Complexity	Transformants/μg		E dt (h)	F % Cells with plasmids	G % Loss/generation	H % Relative stability with *CEN*
			C *S. cerevisiae*	D *S. pombe*				
Y-m4	5.0	SC	2,100	2,500	4.5	20	18	60
Y-m8	6.3	REP	2,300	2,700	4.6	20	12	100
Y-m11	2.4	SC	1,210	—	17	0.15	[b]	8
Y-m20	6.3	REP	1,500	—	10	8	20	9
Y-m21	4.8	SC	830	5,500	4	4	22	15
Y-m24	5.5	REP	1,770	8,300	5	26	ND[c]	70
Y-m25	5.5	REP	1,130	—	6	32	ND	24
Y-m26	5.0	REP	1,120	8,300	5.5	29	ND	70
YRp12	1.45	SC (yeast)	1,900	—	4.0	25	21	100

[a] The sizes of the *ARS* fragments (column A) and their complexity in mouse DNA (column B; SC, single copy; REP, repetitive) are indicated. The number of yeast transformants is based on 5×10^7 viable cells (columns C and D). (YIp5 produces <1 transformant per μg with strain 689.) Y-m11, Y-m20, Y-m25, and YRp12 did not transform *S. pombe* at high frequency. Information in columns E, F, G, and H refers to *S. cerevisiae* cells carrying plasmid DNAs. The doubling time (dt; column E) and percentage of cells with plasmid (column F) were determined in YC minus uracil (689 cells grow with a 2 h doubling time in YC plus uracil). The percent loss per generation (columns G and H) was determined for cells growing in YC plus uracil. The rate of loss of mouse *ARS* centromere plasmids (column H) was compared with that of YCp19 (1 to 4% loss per cell per generation in the same experiment.

[b] Y-m11 was found in fewer than 1 in 3×10^4 cells after six generations in nonselective medium.

[c] ND, Not determined.

cells carrying the centromere plasmid grow more quickly under selective growth, and in the presence of uracil, the centromere plasmid is lost more slowly than YRp12 from transformed cells (Table 1). *Hin*dIII–*Bam*HI-digested, size-selected mouse DNA was ligated into *Hin*dIII–*Bam*HI-digested YIp5 *CEN4*. The ligation mixture was used directly to transform yeast strain 689, and Ura$^+$ transformants were selected. About 20 of the fastest growing transformants were tested for stability by three successive replica platings on nonselective plates. From these experiments, three plasmids (Y-m24-C4, Y-m25-C4, and Y-m26-C4) were identified which appeared to be as stable mitotically as YCp19. One of these plasmids, Y-m24-C4, contained two inserts, one 5.5-kb fragment with *Hin*dIII sites at both ends and a 7.5-kb fragment with *Hin*dIII-*Bam*HI ends. The 5.5-kb *Hin*dIII fragment had *ARS* activity and was used in subsequent experiments.

Properties of mouse *ARS*s in *S. cerevisiae*. All eight of the mouse sequences promoted high-frequency transformation of *S. cerevisiae* (Table 1). In addition, cells transformed by these plasmids exhibited the following properties characteristic of cells carrying an extrachromosomal plasmid (Table 1). (i) The doubling time of each of the transformed strains in YC minus uracil was greater than that of strain 689 in YC plus uracil. (ii) Only a fraction of the transformed cells growing in YC minus uracil had a Ura$^+$ phenotype (i.e., contained plasmid DNA), and the Ura$^+$ phenotype was rapidly lost when cells were grown under nonselective conditions. (iii) The original plasmid could be recovered by using DNA extracts from transformed yeast cells to transform *E. coli* to ampicillin resistance (data not shown). (iv) Each *ARS* could provide replication function during mitotic growth for *CEN4* DNA. By the criteria listed in Table 1, some of the mouse *ARS*s (m4, m8, m24, and m26) function about as well in yeast mitotic cells as the yeast sequence *ARS1*.

We also determined whether the mouse *ARS*s could promote extrachromosomal replication of plasmids in a second distantly related yeast, *S. pombe*. Since defects in the *URA4* gene of *S. pombe* can be complemented by a wild-type copy of the *URA3* gene from *S. cerevisiae*, the YIp5 *ARS* vectors were tested directly in a *ura4* strain of *S. pombe*. Five of the eight mouse sequences (m4, m8, m21, m24, and m26) transformed *S. pombe* at a high frequency (Table 1). Southern blot analysis indicated that the plasmids exist in the extrachromosomal form in transformed cells. Therefore, five of the mouse sequences function as *ARS*s in both yeast strains. These five *ARA*s include the four mouse sequences which function as well as *ARS1* and *S. cerevisiae*.

Characterization of mouse *ARS*s. To verify that the isolated *ARS*s were mouse DNA, each of the *ARS* fragments was isolated by agarose gel electrophoresis, nick translated, and hybridized to mouse DNA. The hybridization patterns indicated that m4, m11, and m21 are single copy sequences (for example, see Fig. 3), whereas

m8, m20, m24, m25, and m26 hybridize to multiple bands (Fig. 2) and therefore contain middle repetitive DNA. None of the *ARS*s hybridized to low-molecular-weight DNA preparations made from mouse cells by the Hirt method (17; data not shown). Since these preparations are enriched in mitochondrial DNA, none of the mouse *ARS*s is derived from mitochondrial DNA. Moreover, none of the eight fragments hybridized either to human or yeast DNAs or to any of the other seven mouse *ARS*s (data not shown).

Behavior of plasmids in mouse tissue culture cells. Plasmids containing each of the *ARS*s and appropriate for transformation of mouse cells were constructed (Fig. 1). The different plasmid DNAs were introduced into the mouse TK$^-$ cell line B82 by the calcium phosphate precipitation technique (15) in the absence of carrier DNA (Table 2). In those cases where the *ARS* plasmid did not carry the TK gene, the cells were "co-transformed" with pML2-TK (1:3 [wt/wt] ratio of pML2-TK to *ARS* plasmid). Each plasmid was used in at least two different transformations. The efficiencies of transformation by *ARS* plasmids containing TK were in the range obtained for the control plasmids pBR322-TK and pML2-TK (i.e., between 2 and 80 transformants per μg of TK sequences). Thus, the efficiency of transformation of mouse cells by plasmids containing TK is not markedly affected by the presence of a mouse *ARS*.

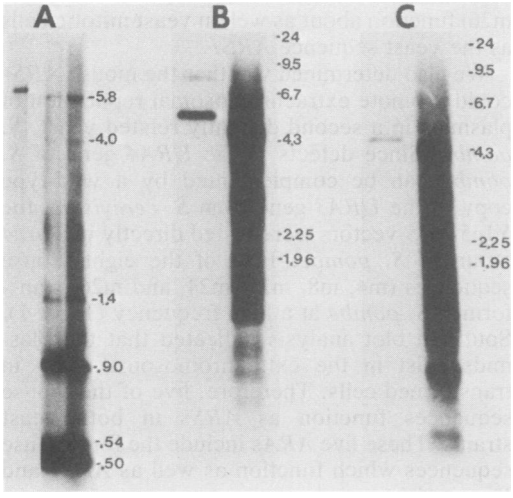

FIG. 2. Hybridization of mouse *ARS*s to mouse DNA. Fragments containing mouse *ARS*s were isolated from recombinant DNA plasmids and nick translated. Plasmids containing the *ARS*s (first lanes in A, B, and C) and total mouse DNA (second lanes in A, B, and C) were digested with *Bam*HI and *Hin*dIII and subjected to electrophoresis in 0.7% agarose gels. In all figures, sizes are in kb. (A) m20. (B) m24. (C) m26.

TABLE 2. Plasmids used to transform TK$^-$ mouse L cells[a]

ARS	Plasmid	No. of lines analyzed
4	P-m4-TK	2
	P-m4-C3-TK	3
8	P-m8-TK	5
	P-m8-C3-TK	6
11	P-m11-TK	1
	P-m11-C3-TK	3
	Y-m11-TK	5
	Y-m11-C4-TK	16
	P-m11-C4-TK	3
	P-m11-C4 + pML2-TK	6
	Y-m11-C4-TK (3/1)	3
20	P-m20-TK	2
	P-m20-C3-TK	3
21	P-m21-TK	2
	P-m21-C3-TK	8
	P-m21-C4 + pML2-TK	6
24	P-m24 + pML2-TK	4
	Y-m24-C4 + pML2-TK	5
25	P-m25 + pML2-TK	6
26	Y-m26 + pML2-TK	2
	Y-m26-C4 + pML2-TK	2
	P-m26 + pML2-TK	20
	P-m26-C4 + pML2-TK	3

[a] Mouse fragments with *ARS* activity in yeasts (Table 1) were inserted into vectors suitable for transformation of TK$^-$ mouse cells. Fragments were inserted into either pML2 (25; designated P) or into YIp5 (33; designated Y). In some plasmids a 3.4-kb *Bam*HI fragment containing the HSV-1 TK gene was also inserted into the vector. In those cases where TK was not included in the vector itself, pML2-TK was used in the transformation. Some vectors also carried fragments of centromere DNA from yeast chromosome III (C3) or IV (C4). Y-m11-C4-TK (3/1) is the plasmid recovered through *E. coli* from mouse cell line 3/1, a line produced by transformation with Y-m11-C4-TK. Each cell line was derived from an independent TK$^+$-transformed colony and analyzed by Southern blot hybridization for the presence and arrangement of plasmid DNA.

DNA was extracted from each of the transformed cell lines and analyzed for the content and form of plasmid DNAs by Southern blot hybridization. The first cell line analyzed, called 3/1, had been transformed with plasmid Y-m11-C4-TK. This plasmid contains a single copy mouse sequence (m11) as well as *CEN4* DNA. Total DNA was isolated from 3/1 and probed with nick-translated Y-m11-C4-TK DNA (Fig. 3, lanes 4 through 7). A DNA species which mi-

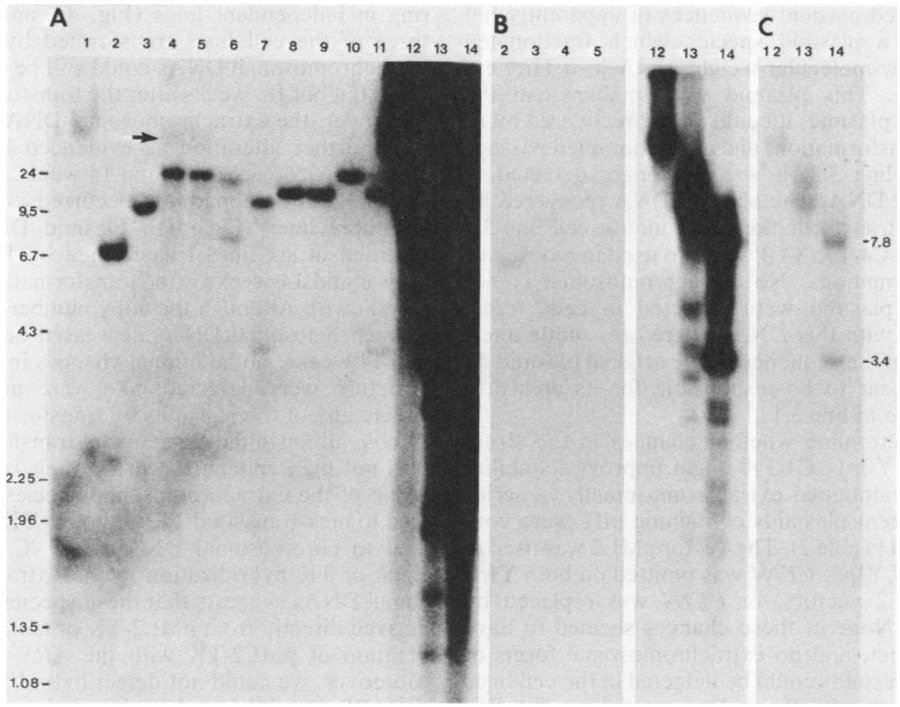

FIG. 3. Hybridization pattern of two mouse cell lines transformed by plasmids containing the mouse *ARS* m11. DNA was restricted and subjected to electrophoresis in 0.7% agarose gels. Cellular DNA (10 μg) or purified plasmid DNA (10 pg) was used in each lane. DNA was transferred to nitrocellulose and hybridized for 2 days with 5×10^6 cpm of nick translated Y-m11-C4-TK DNA. This blot was not treated with acid, and thus, transfer of undigested chromosomal DNA was inefficient. Lanes 1 through 3 contain DNA from untransformed mouse cells (lane 1, undigested; lane 2, *Hin*dIII-digested; lane 3, *Bam*HI-digested). Lanes 4 through 7 contain DNA from cell line 3/1 which was transformed by Y-m11-C4-TK (lanes 4 and 5, undigested; lane 6, *Hin*dIII-digested; lane 7, *Bam*HI-digested). The arrow indicates the position of chromosomal DNA from the ethidium bromide-stained profile of the gel. The *Bam*HI fragment containing the endogenous copy of m11 has a size of about 10 kb and is not resolved from the plasmid fragment (lane 7). Lanes 8 through 11 contain Y-m11-C4-TK plasmid DNA (lanes 8 and 9, undigested; lane 10, *Hin*dIII-digested; lane 11, *Bam*HI-digested). The mobility of plasmid sequences is altered by the presence of 10 μg of mouse DNA (compare lanes 4 and 5 with lanes 8 and 9). Lanes 12 through 14 contain DNA from a cell line transformed by Y-m11-TK (lane 12, undigested; lane 13, *Hin*dIII-digested; lane 14, *Bam*HI-digested). Panels (A), (B), and (C) represent different exposures of the same blot.

grates faster than chromosomal DNA was detected in unrestricted DNA (Fig. 3, lanes 4 and 5). When DNA was digested with *Hin*dIII, which cuts the original plasmid one time (Fig. 3, lane 6), *Bam*HI (Fig. 3, lane 7), or *Bam*HI-*Hin*dIII (data not shown), only bands which corresponded to the endogenous copy of the mouse *ARS* and the original plasmid were observed; no junction fragments were visible. From the relative intensities of the bands created by the endogenous copy of m11 and the *Hin*dIII-linearized plasmid sequences, the copy number of the plasmid must average about one per cell. By using total DNA from the 3/1 transformed cell line to transform *E. coli*, a plasmid was isolated whose pattern of digestion with three different restriction enzymes was identical to that of Y-m11-C4-TK (data not shown). The data from cell line 3/1 are best explained if the cell line contains about one copy per cell of an extrachromosomal plasmid similar (or identical) to the plasmid used in the transformation. From analogy with the yeast system, a plasmid containing functioning *ARS* and *CEN* sequences would be expected to exhibit this behavior.

However, cell line 3/1 was the only transformed cell line in which the original plasmid could be detected in the extrachromosomal form. DNA from 15 additional cell lines produced by transformation with Y-m11-C4-TK was examined by Southern blot hybridization of both total DNA and DNA which was size fractionated by the Hirt method. All of these lines contained a variable number of plasmid sequences (estimated at 1 to 20 copies per cell) integrated into high-molecular-weight DNA. Only one of the lines (3/2) carried, in addition to

integrated plasmid sequences of apparently full length, a plasmid species which fractionated with low-molecular-weight DNA in a Hirt extraction. This plasmid was smaller than the original plasmid, it could not be recovered by *E. coli* transformation, and after continued passage of cell line 3/2, it was no longer detected in isolated DNA. The plasmid DNA recovered by *E. coli* transformation from mouse cell line 3/1 [Y-m11-C4-TK(3/1)] was also used in mouse cell transformations. No extrachromosomal copies of the plasmid were detected in cells transformed with this DNA. Therefore, subtle alterations in the sequence of the original plasmid do not appear to be responsible for its ability to replicate in line 3/1.

To determine whether changes in the structure of Y-m11-C4-TK might improve its ability to be maintained extrachromosomally, a series of different plasmids containing m11 were constructed (Table 2). The vector pML2 was used in place of YIp5, *CEN4* was omitted on both YIp5 or pML2 vectors, or *CEN4* was replaced by *CEN3*. None of these changes seemed to have any effect, and no extrachromosomal forms of these plasmids could be detected in the cell lines transformed by them. For example, a cell line transformed by Y-m11-TK contained ~50 copies of the plasmid, all of which seemed to be integrated into chromosomal DNA (Fig. 3, lanes 12 through 14). A series of cell lines transformed by similar plasmids containing the other mouse *ARS*s were also analyzed (Table 2). One of the lines transformed with P-m21-TK contained, in addition to integrated plasmid sequences, an extrachromosomal plasmid. The structure of this extrachromosomal plasmid was different from that of the original plasmid, it was not possible to recover it by *E. coli* transformation, and the extrachromosomal plasmid was no longer detectable in DNA from cells grown an additional 10 weeks in culture.

Likewise, in many of the cell lines produced by cotransformation, extrachromosomal DNAs were not detected in transformed cells. However, many of the cell lines produced by cotransformations with pML2-TK and P-m24 (4 of 4 lines), P-m25 (6 of 6 lines), or P-m26 (8 of 20 lines) contained extrachromosomal species which were detected by hybridization to nick-translated pML2 (Fig. 4A and D). These species were found in low copy number and were fractionated preferentially with low-molecular-weight DNA (Fig. 4A and D). In all cases, the extrachromosomal DNAs we detected were different from the original plasmid in that they no longer contained a *Hin*dIII site. However, different cell lines transformed by the same plasmid displayed similar patterns of hybridization to pML2, as if the same alteration were occurring in independent lines (Fig. 4A and D). In three of the cell lines transformed by P-m26, extrachromosomal DNAs could still be detected at 14, but not 18, weeks after the transformation. Moreover, the extrachromosomal DNAs underwent further alteration as evidenced by a decrease in size between 5 and 14 weeks, and the same alteration apparently occurred in each of the three lines (Fig. 4D). Plasmid DNA was detected in six lines transformed by P-m25 at both 6 and 14 weeks after transformation (data not shown). Although the copy number of these extrachromosomal DNAs decreased between 6 and 14 weeks, no additional changes in plasmid structure were detected. We were unable to isolate any of the plasmids by transformation of *E. coli*, although the efficiency of transformation was not high enough to ensure their recovery. None of the extrachromosomal species hybridized to nick-translated TK (although TK hybridized to chromosomal DNA) (Fig. 4C and F). Lack of TK hybridization to the extrachromosomal DNAs suggests that these species are not derived directly from pML2-TK or from recombination of pML2-TK with the *ARS* plasmid. Moreover, we could not detect hybridization of the *ARS* contained on the original plasmid to the extrachromosomal species (Fig. 4B and E). However, since m24, m25, and m26 are all middle-repetitive sequences, it is likely that their hybridization to these low-copy-number plasmids would have been obscured by their hybridization to mouse chromosomal DNA. It seems unlikely that the extrachromosomal DNAs are due to contamination of the mouse cells or the DNA preparations with an extraneous plasmid with homology to pML2. Cell lines with extrachromosomal plasmids came from transformations carried out on three different dates, and other transformed cell lines produced on the same days contained no species which hybridized to pML2. Moreover, 10 independent cell lines produced by cotransformation with pML2 and pML2-TK contained no extrachromosomal species with homology to pML2 (data not shown).

Most of the 71 cell lines produced by transformation with a single plasmid (i.e., not by cotransformation) contain multiple copies of integrated plasmid. Only 4 lines appear to contain a single integrated copy. Moreover, in most cell lines (60%), the plasmid probably exists in head-to-tail configuration in high-molecular-weight DNA since restriction digestion of DNA from these cells produced bands which are identical to those produced by digestion of the plasmid itself (Fig. 3C, lane 14). From comparison of the hybridization signal either from known amounts of plasmid DNA or from the endogenous *ARS*, some of these cell lines probably contain 50 or

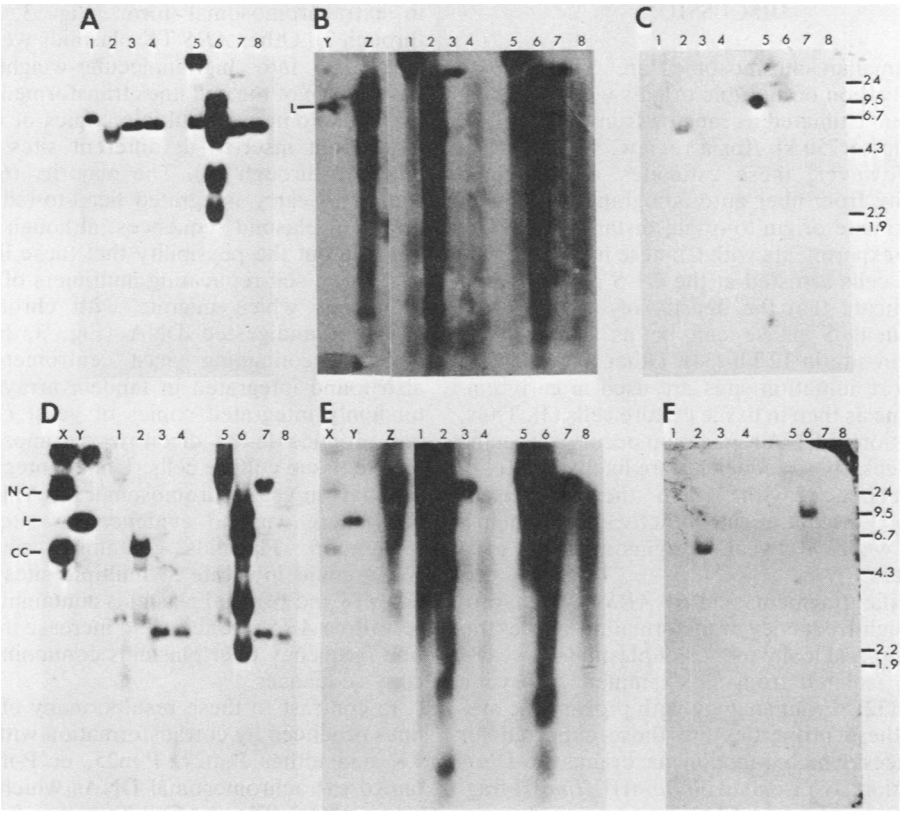

FIG. 4. Hybridization pattern of mouse cell lines cotransformed with pML2-TK and P-m26. Amounts of DNA and conditions are those described for Fig. 3, except the gel was acid treated. Panels (A), (B), and (C), which contain DNA isolated 5 weeks after transformation, were probed successively with nick-translated pML2 (A), m26 (B), and TK (C). Lanes 1 through 4 and 5 through 8 are from two independent cell lines. The lanes contain undigested total DNA (lanes 1 and 5), *Hin*dIII-digested total DNA (lanes 2 and 6), undigested supernatant from a Hirt extraction (lanes 3 and 7), and *Hin*dIII-digested Hirt supernatants (lanes 4 and 8). The position of undigested chromosomal DNA can be determined from the position of hybridization of nick-translated m26. The average molecular weight of chromosomal DNA is less in a Hirt supernatant than in total DNA (for example, compare lanes 1 and 3 [B]) because the latter procedure is selective for low-molecular-weight DNA. Lane Y contains *Hin*dIII-digested P-m26 in an amount equal to roughly one copy per cell, and lane Z contains *Hin*dIII-digested B82 mouse DNA. Panels (D), (E), and (F), which contain DNA isolated from the same lines shown in panels (A), (B), and (C) 14 weeks after transformation, were probed successively with nick-translated pML2 (D), m26 (E), and TK (F). The DNAs in the lanes were treated as described in (A), (B), and (C). Lane X contains undigested P-m26 in an amount roughly equal to one molecule per cell (NC, nicked circle; L, linear; CC, covalently closed forms of the plasmid). Hybridization of TK (F) is seen to *Hin*dIII fragments of the same size as those detected in DNA isolated at 5 weeks (C), but the extrachromosomal DNA is smaller in both cell lines (D).

more tandemly integrated copies of plasmid DNA (Fig. 3B, lanes 12 through 14). In addition to bands attributed to full-length plasmid DNA, there were usually bands of varying sizes which hybridized to the plasmid probes (Fig. 3B, lanes 13 and 14). These bands most likely represent junction fragments created by the insertion of plasmid DNA into chromosomal sequences. Ninety percent of the cell lines appear to have more than one site of integration of plasmid DNA. The number and pattern of integration sites for a plasmid do not seem to be influenced either by copy number of the *ARS* on the plasmid (i.e., single-copy versus middle-repetitive) or by the presence or absence of centromere DNA. In a number of cell lines, multiple tandem copies of plasmids containing yeast centromere sequences were detected. DNA from one such line hybridized to a nick-translated, 627-bp fragment isolated from pYe(*CEN3*)3 which contains the portion of *CEN3* required for centromere activity (data not shown).

DISCUSSION

Mammalian chromosomes are replicated by the activation of multiple origins whose spacing has been estimated as ranging from less than 30 kb to up to 750 kb (for a review, see reference 12). However, these estimates, which come primarily from fiber autoradiography, probably overestimate origin-to-origin distances. For example, experiments with Chinese hamster tissue culture cells arrested at the G1-S phase boundary indicate that the density of origins in the subsequent S phase can be as high as one initiation site in 12 kb (34). Other data indicate that more initiation sites are used in early embryogenesis than in tissue culture cells (3). Thus, if initiation of DNA replication occurs at specific sequences, these sequences are likely to differ in the frequency with which they are used; "strong" origins might be activated in each S phase, whereas "weak" origins might be used less often.

Specific fragments called *ARS*s which promote high-frequency transformation and extrachromosomal maintenance of plasmids in yeasts can be isolated from all examined eucaryotic DNAs (32). From analogy with procaryotic systems, these properties are those expected for sequences which function as origins of DNA replication. We isolated 30 *Bam*HI-*Hin*dIII fragments (average size, 4 kb) from mouse chromosomal DNA and tested them for *ARS* activity in yeasts. Five of these fragments exhibited *ARS* function; thus, for the mouse sequences tested, *ARS*s are found about once in every 24 kb. If *ARS*s are origins of replication, this spacing is consistent with the detection by the yeast transformation system of all or most sequences in mouse DNA with the potential for serving as initiation sites. To increase our chances of identifying strong *ARS*s, we used a second isolation scheme which was based on methods developed for isolating yeast centromeres (19). In the second selection, three additional mouse sequences were identified. By a number of criteria, one-half of the mouse *ARS*s functions about as well in yeast mitotic cells as the yeast chromosomal sequence *ARS1* (Table 1). Five of the eight sequences also function as *ARS*s in a different species of yeast.

Each of the mouse *ARS*s was inserted into plasmids suitable for transformation of TK^- mouse tissue culture cells. The different plasmids exhibited no marked differences in transformation efficiency compared with control plasmids. Over 100 independent TK^+-transformed cell lines were examined for structure and content of plasmid DNAs. A cell line produced by transformation with Y-m11-C4-TK was the only one in which the original plasmid was detected in extrachromosomal form (Fig. 3, lanes 4 through 7). Other *ARS* TK plasmids were found integrated into high-molecular-weight DNA; most (90%) of the cell lines transformed by such plasmids contained multiple copies of the plasmid, often inserted at different sites (Fig. 3, lanes 12 through 14). The majority (60%) appeared to carry integrated head-to-tail tandem arrays of plasmid sequences, although we cannot rule out the possibility that these lines carried large, self-replicating multimers of plasmid sequences which migrate with chromosomal DNA in undigested DNA (Fig. 3, lane 12). Plasmids containing yeast centromeres were also found integrated in tandem arrays. Thus, tandemly integrated copies of yeast *CEN*s do not produce lethal dicentric chromosomes in mouse tissue culture cells. Unlike integration of plasmids in yeast chromosomal DNA, in mouse cells there was no evidence for site-specific integration. Plasmids containing single-copy *ARS*s could integrate at multiple sites (Fig. 3, lanes 13 and 14), and plasmids containing middle repetitive *ARS*s exhibited no increase in integration frequency over plasmids containing single-copy sequences.

In contrast to these results, many of the cell lines produced by cotransformation with pML2-TK and either P-m24, P-m25, or P-m26 contained extrachromosomal DNAs which hybridize to pML2 (Fig. 4). Cell lines transformed by these plasmids are difficult to analyze because the plasmids contain no sequence unique to the *ARS* plasmid. pML2 sequences are also on the cotransforming plasmid, and each of the *ARS*s exists in multiple copies in mouse DNA. These plasmids are found in low copy number, fractionate preferentially with low-molecular-weight DNA, and are altered by passage in mouse cells.

What are the implications of these data in terms of DNA replication of mammalian chromosomes? It is clear that none of the sequences with an *ARS* phenotype in yeasts displays comparable behavior in mouse cells. None of the sequences promotes elevated levels of transformation, and none reproducibly enables plasmids to be maintained unaltered in transformed cells. These data are consistent with other studies which have also failed to identify sequences from mammalian chromosomes capable of maintaining plasmids in extrachromosomal form (9). Certain characteristics of mammalian cells may explain the failure to identify sequences which function as replication origins in mouse cells.

(i). It is possible that high levels of recombination in mouse cells mask the ability of plasmids to replicate. In *S. cerevisiae*, integration of plasmid DNAs occurs only through homologous recombination. If recombination in yeasts were promiscuous, as it seems to be in mammalian

cells, integration would presumably occur at a much higher rate and would probably obscure the ARS$^+$ phenotype. In an attempt to select against integration, *CEN* DNA was included on some of our plasmids. If yeast *CEN*s functioned in mouse cells, integration might produce dicentric chromosomes and thereby cause cell death. However, many of the cell lines produced by transformation with plasmids with centromere DNA contained multiple copies of plasmid, often in tandem arrays. These results indicate that mouse chromosomes can tolerate multiple, integrated copies of yeast centromere DNA.

(ii). It is possible that our *ARS* plasmids replicate in mouse cells but that plasmid DNA is usually lost when the nuclear membrane breaks down. In contrast, the nuclear membrane remains intact during yeast mitosis which presumably facilitates maintenance of self-replicating plasmids. Maintenance of extrachromosomal DNAs in mouse cells might require both a replication origin and a sequence that prevents plasmid loss. If so, yeast *CEN*s apparently can not provide this function.

(iii). It is possible that use of TK has selected against maintenance of plasmids in extrachromosomal form. This hypothesis assumes that extrachromosomal copies of TK are more likely to be transcriptionally active than integrated copies and that excess phosphorylated thymidine is harmful to cells. If so, multiple extrachromosomal copies of an *ARS* TK plasmid could be lethal to cells. This hypothesis is supported by observations showing that cell lines with many (~50) integrated copies of TK exhibit levels of TK transcription and enzyme activity which are similar to that of cell lines with one or a few copies of TK (R. Kucherlapati, personal communication). In an attempt to circumvent this potential problem, cotransformations in which the TK gene was not physically joined to the *ARS* plasmid were performed. Indeed, extrachromosomal DNA detected by homology to pML2 DNA was found in many of the cell lines produced by cotransformation with pML2-TK and either P-m24, P-m25, or P-m26 (Fig. 4). These results may reflect the ability of m24, m25, and m26 to serve as initiation sites for DNA replication in mouse cells. These three *ARS*s were selected by a procedure designed to isolate strong *ARS*s. By a number of criteria, m24 and m26 (but not m25) function as efficiently as *ARS1* in *S. cerevisiae* and, in addition, display *ARS* activity in *S. pombe* (Table 1). However, even plasmids containing these *ARS*s are not found unaltered in transformed cells. Moreover, since we could not demonstrate hybridization of the *ARS* to the extrachromosomal DNAs, it is difficult to conclude that the *ARS* itself promotes self-replication of pML2 DNA.

In summary, we isolated eight sequences from mouse chromosomal DNA which function as *ARS*s in yeasts. At least four of these mouse *ARS*s function as well in yeasts as the yeast chromosomal sequence *ARS1*. These data suggest that whatever sequences are important for efficient *ARS* function in *S. cerevisiae*, they are unlikely to be unique to yeast DNA. In contrast, we find no compelling evidence for a replicative role for mouse *ARS*s in mouse tissue culture cells. Indeed, the only sequences which consistently support self-replication of recombinant DNA plasmids in mammalian cells are those from viral DNAs such as simian virus 40 or bovine papillomavirus (23, 26). These results may reflect fundamental differences in nuclear structure, replication, or recombination between lower and higher eucaryotes.

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