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Replication of yeast DNA and novel chromosome formation in mouse cells

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ABSTRACT

To determine whether yeast DNA can replicate or segregate in mammalian cells, we have transferred genomic DNA from the yeast *Saccharomyces cerevisiae* into mouse cells. Most of the lines contained stably integrated yeast DNA. However, in two of the lines, the yeast DNA was maintained as numerous small extrachromosomal elements which were still present after 26 cell divisions in selection but which were lost rapidly out of selection. This indicates that, although yeast DNA can replicate in mouse cells, the yeast centromere does not function to give segregation. In one cell line we observed a large novel chromosome consisting almost entirely of yeast DNA. This chromosome segregates well and contains mouse centromeric minor satellite DNA and variable amounts of major satellite DNA which probably comprise the functional centromere. The yeast DNA in the novel chromosome has a compacted chromatin structure which may be responsible for the efficient formation of anaphase bridges. Furthermore, yeast DNA integrated into mouse chromosomes forms constrictions at the point of integration. These features have previously been presumed to be hallmarks of centromeric function in transfection assays aimed at identifying putative centromeric DNA. Hence our results suggest caution be exercised in the interpretation of such assays.

INTRODUCTION

The DNA sequences necessary for replication and segregation in mammalian cells are poorly understood. In contrast, replication origins and centromeric sequences in the yeast *Saccharomyces cerevisiae* are well defined. Replication origins were isolated by their ability to confer replication on a plasmid and are called 'autonomously replicating sequences' (ARSs) (reviewed in 1). Plasmids containing an ARS element are maintained extrachromosomally if selection is maintained but are rapidly lost from the culture if selection is dropped, due to unequal segregation. DNA with ARS activity, as determined by the plasmid assay, generally correlates with replication origins located in the yeast chromosomes. Centromeric DNA stabilizes ARS plasmids in yeast by conferring equal segregation at mitosis (reviewed in 1).

Similar experiments involving transfection of plasmid- or cosmid-cloned fragments of mammalian DNA into mammalian cells have not led to the functional characterization of either origins of replication or centromeres. A number of different origins of replication have been located within mammalian chromosomes by a variety of methods. In particular, one located ~17 kb downstream of the dihydrofolate reductase (*Dhfr*) gene in Chinese hamster chromosomes has been well characterized by a number of different investigators (reviewed in 2,3). However, when the DNA encompassing this putative origin of replication was introduced back into mammalian cells it was not found to replicate any more efficiently than neighbouring DNA known not to contain a chromosomal replication origin (4) and it does not form extrachromosomal elements which can be maintained under selection. One explanation for the observation that DNA containing a known mammalian chromosomal replication origin does not generally form extrachromosomal replicating elements in mammalian cells (similar to ARS plasmids in yeast) is that a nuclear localization signal is needed in addition to the ability to replicate (5). Thus, a plasmid carrying a 13.3 kb fragment containing the *Dhfr* replication origin replicates efficiently in a transient replication assay over 4 days, but is not stable over 15 days (6). However, if a nuclear retention signal (but not a replication origin) is added from the latent origin of replication (OriP) of Epstein Barr virus, along with the viral protein EBNA1, the plasmid is stable over 15 days (6).

Transient replication assays have been used to show that replication of introduced fragments of DNA is dependent on the size of the DNA, with fragments of human DNA larger than ~12 kb replicating efficiently (7). In addition, yeast DNA has been shown to replicate only marginally less efficiently than human DNA, while more CG-rich bacterial DNA replicates significantly less efficiently (8). This has led to the general hypothesis that replication in mammalian cells is determined by chromatin context in the chromosomes but that extrachromosomal DNA is replicated in a way that is dependent on size, but independent of sequence (references above and reviewed in 9).

DNA cloned in yeast artificial chromosomes (YACs) has been transferred into mammalian cells and has given rise to extrachromosomal replicating elements in mammalian cells. When the YAC yHPRT, which contains 660 kb of human DNA, was introduced into mouse L A-9 cells, about half the resulting cell lines contained yeast and YAC DNA as extrachromosomal elements in some of the cells (10). Similarly, when a YAC

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containing ~70 kb near the adenosine deaminase gene was transferred to either a Chinese hamster ovary cell line or another mouse fibroblast line, extrachromosomal elements were observed in two out of nine cell lines (11). However, in each case, the extrachromosomal elements contained both human DNA and yeast genomic DNA, making it impossible to conclude which DNA was responsible for replication of the DNA in the mouse cells.

Transfection experiments aimed at determining whether putative centromeric DNA can function in mammalian cells have also led to results that are difficult to interpret. The mammalian centromere is located at the primary constriction where several megabases of repetitive DNA are generally located and this has precluded the cloning of the centromeric regions in an intact and unrearranged form. Interest has centred on the α satellite DNA in man and the sequence which is thought to be functionally equivalent in mouse, minor satellite. The α and minor satellites are tandemly repeated sequences located at the centromeres of all human or mouse chromosomes. Alphoid DNA is always retained when truncations of the human Y chromosome are selected by retention of a functional centromere (12). Also, natural deletions of the Y chromosome always carry alphoid DNA at the functioning centromere (13), indicating that these sequences are probably necessary for Y centromere function. Although transfection of alphoid DNA into mammalian cells has not led to efficient centromere formation, it does give several features of centromeres. In one set of experiments, alphoid DNA was introduced into African green monkey cells (14). After integration into a host chromosome, anaphase bridges were observed which could be due to a dicentric chromosome. In addition, *de novo* chromosomes were observed which consisted largely of the input alphoid DNA, not host cell alphoid DNA, and had a single functional centromere (14). In another set of experiments, alphoid DNA integrated into human chromosomes was observed to form constrictions and anaphase bridges (15). A *de novo* chromosome has also been observed after transfection of a λ clone containing putative centromere DNA (16).

DNA cloned in YACs has been transferred into mammalian cells to assay both replication ability and centromere function. As some yeast genomic DNA is often transferred along with the cloned DNA, it is important to know whether yeast genomic DNA alone can replicate and whether the yeast centromeres function in mammalian cells. It is also important to know how heterologous non-specific DNA behaves in mouse cells so that one can compare this with the behaviour of DNA with putative replication and centromere function. In this paper we have transferred yeast genomic DNA to mouse cells and determined the fate of the yeast DNA.

MATERIALS AND METHODS

Yeast culture, transformation and DNA preparation

The yeast strain F9 is derived from the strain AB1380 (17) by integration of a mammalian selectable marker, neomycin resistance (*neo*^{r}), into the *ura3* gene on yeast chromosome V. First, a yeast strain with a YAC carrying the human factor IX gene was isolated from the Washington University library (18). The retrofitting vector pLUNA was then introduced and integrated into the chromosomal copy of the *ura3* gene rather than the *URA3* gene on the YAC (19). This strain was grown overnight without selection and single colonies grown up and checked for loss of the YAC. F9 is a resulting colony which carries the *neo*^{r} marker on chromosome V and contains no human DNA. Agarose blocks of high molecular weight DNA were made by a previously published protocol (20) using the modifications previously described (21).

Mammalian cell culture and fusion with yeast spheroplasts

Mouse cell line L A-9 (GM00346B), which is negative for hypoxanthine phosphoribosytransferase (HPRT) activity, was obtained from the NIGMS Human Genetic Mutant Cell Repository. The chromosomes in this mouse cell line are mostly metacentric and there are also chromosomes with multiple centromeres. L A-9 cells were cultured in DMEM containing 10% fetal bovine serum at 37°C with 5% CO_2. Fusion between L A-9 cells and yeast spheroplasts was carried out as previously described (10). After fusion, cells were grown with 600 μg/ml active G418 (Gibco BRL) in order to select for the *neo*^{r} gene. Colonies arose at a frequency of ~2×10^{-6} and only one colony was picked from each plate. Cells were tested regularly for mycoplasma and were always found to be negative.

Each fusion colony was expanded to ~10^8 cells, at which point the rate of loss of the selectable marker was determined (Table 1), DNA was made (Fig. 1) and cells were stored under liquid nitrogen. The cells were subsequently thawed and grown with or without selection for all analyses by fluorescence *in situ* hybridization (FISH) and for Figure 5.

Loss of selectable marker

To measure the rate of loss of the selectable marker, cells were grown without selection for a number of days. Then, ~1000 cells were plated onto two 10 cm dishes and allowed to settle for 3 days. The medium was replaced on one of the plates with selective medium and the cells grown until colonies were visible by eye. The colonies were stained with crystal violet (0.5% crystal violet, 50% ethanol) and the number of colonies which had grown with and without selection were counted. L A-9 cells without selection grow at ~1 cell division/24 h. To calculate the rate of loss, we used an exponential decay equation where $\ln(N_t/N_0) = -\lambda t$, where N_t is the number of resistant cells at time t, N_0 is the number of resistant cells at time 0, t is time in days and λ is the fraction of cells which lose all yeast DNA in 1 day or one cell division.

Preparation of mammalian DNA in agarose plugs

Cells were washed once in 1× PBS and then resuspended at 2×10^7 cells/ml in 1× PBS. They were warmed briefly to 37°C and then an equal volume of 2% SeaPlaque low melting point agarose (FMC) in 1× PBS at 40°C was added and the mixture pipetted into a chilled plug mould. Plugs were incubated in LDS solution (1% lithium dodecylsulfate, 100 mM EDTA, 10 mM Tris, pH 8.0, 10 ml per 1 ml plug) for 1 h at 37°C with agitation and then with fresh LDS solution overnight. Plugs were washed twice in NDS solution (0.2% lauryl sarcosine, 100 mM EDTA, 2 mM Tris, pH 9.0, 10 ml per 1 ml plug) for 2 h at room temperature before being stored in NDS solution at 4°C. Slices of plug were equilibrated twice for 30 min with TE and then twice for 1 h with restriction enzyme buffer before digestion.

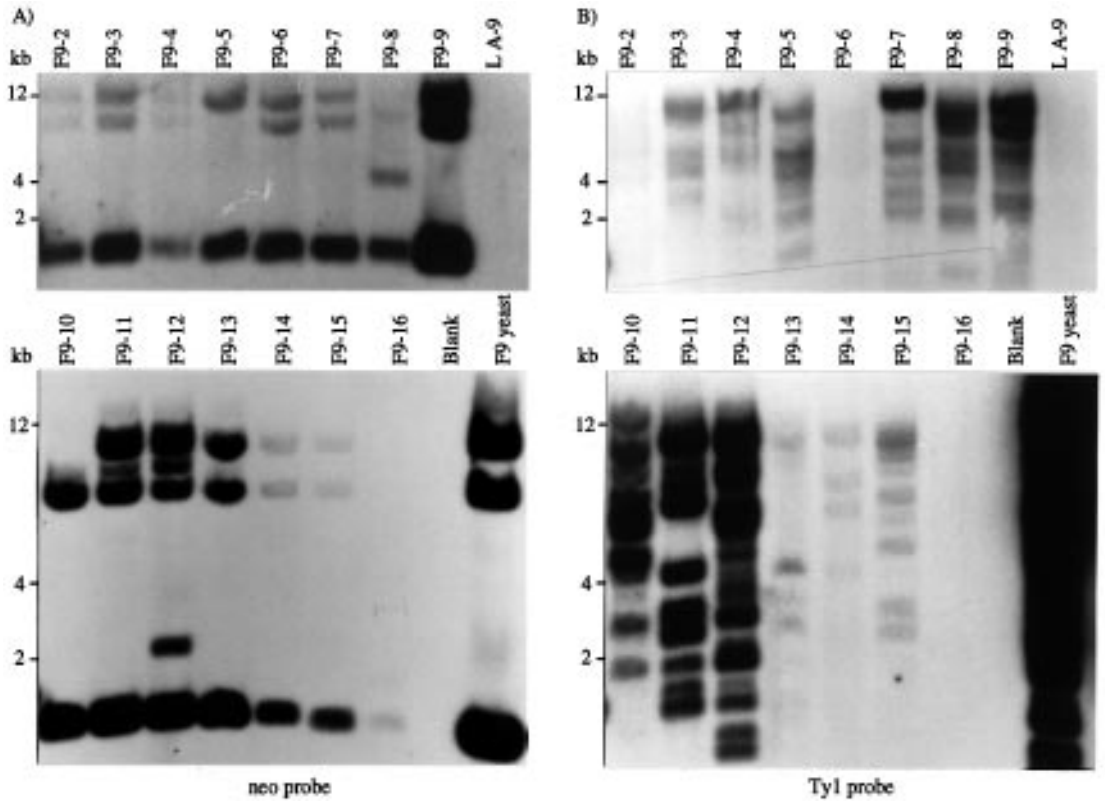

Figure 1. DNA content of the F9 fusion cell lines. DNA from each cell line was digested with *Eco*RI, separated on an agarose gel and blotted. (**A**) The filters were hybridized with *neo*. (**B**) The filters were stripped and then hybridized with *Ty1*. The cell line is indicated above each lane and the positions of size markers are indicated on the left.

Table 1. Cell lines generated by fusion between mouse L A-9 cells and the yeast strain F9

Cell line	*neo*[a]	*Ty1*[a]	Days in no selection[b]	Per cent loss[b]	FISH
F9-1	+	+	26	40	nd
F9-2	+	+	24	35	nd
F9-3	+	+	25	49	nd
F9-4	+	+	nd	nd	nd
F9-5	+	+	27	23	Integrated
F9-6	+	–	nd	nd	nd
F9-7	+	+	nd	nd	nd
F9-8	+	+	nd	nd	nd
F9-9	+	+	18	14	nd
F9-10	+	+	23	19	nd
F9-11	+	+	19	99	Extrachromosomal
F9-12	+	+	22	98	Extrachromosomal
F9-13	+	+	22	0	Integrated
F9-14	+	+	32	12	nd
F9-15	+	+	20	12	Integrated
F9-16	+	–	34	9	nd
F9-17	+	+	23	35	nd

nd indicates not done.

[a]The presence of the *neo* gene and yeast *Ty1* DNA was determined by Southern blotting (see Fig. 1).

[b]Cells were grown for the given number of days without selection before the percentage of cells which had lost the selectable marker was determined as described in Materials and Methods.

Gels, DNA transfer and hybridization

DNA was transferred from agarose gels to Hybond N (Amersham) as recommended by the manufacturer. DNA probes were labelled using the Megaprime kit (Amersham) and purified using commercially available push columns (Stratagene NucTrap probe purification columns).

Prehybridization and hybridization of Southern blots was carried out in a modified version of Church buffer (22) (16.8 g/l $NaH_2PO_4 \cdot H_2O$, 54.1 g/l $Na_2HPO_4 \cdot 12H_2O$, 7% SDS, 100 µg/ml denatured salmon sperm DNA) at 65°C. Filters were washed in 0.5× SSC, 0.1% SDS at 65°C for 30 min. To strip the filters, 500 ml 0.1× SSC, 0.01 M EDTA, pH 8.0, was boiled and then SDS added to 0.1%. This mixture was added to the filter and left shaking at room temperature until cool.

Probes

The following probes were used on Southern blots. The *neo*^{r} probe is a 1.1 kb *Xho*I–*Hin*dIII fragment from the plasmid PMC1Neo Poly A (23). The *Ty1* probe is a 1.2 kb *Xho*I–*Hin*dIII fragment from the plasmid pCS-X (24). The single copy mouse probe is a 1.7 kb *Bam*HI–*Sst*I fragment of the mouse *Utrophin* gene (a gift of Ms U. Gangadharan).

The following probes were used for FISH. Total yeast DNA was prepared from the strain AB1380. Mouse minor satellite was prepared by PCR from total mouse DNA using the primers 5′-AAATCCCGTTTCCAACGAATGTG-3′ and 5′-GTAGAAC-AGTGTATATCAATGAG-3′. The major satellite probe was a 200 bp *Pst*I fragment excised from the plasmid R531 (a gift of David Kipling). Total mouse DNA was prepared from C57BL/6 tissue.

Fluorescence *in situ* hybridization

Rapidly dividing cells were incubated with a low concentration of colcemid (final concentration 0.01 µg/ml) for 1 h before fixation. Generally this gave a reasonable proportion of cells in metaphase. In one pellet of cells from the line F9-12 (4 days in selection and 4 days with no selection; see Table 2) there was quite a high percentage of anaphase cells in addition to metaphase cells.

Preparation of slides and fluorescence *in situ* hybridization (FISH) were carried out largely as described elsewhere (25). Labelling with biotin-14-dATP was carried out by two methods. Yeast genomic DNA and mouse genomic DNA were labelled using a Bionick kit (Gibco BRL), whereas the mouse minor PCR product and the plasmid-derived fragment of major satellite DNA were labelled using a Bioprime kit (Gibco BRL). For double detections, total yeast DNA was labelled with digoxigenin-dUTP using a DIG DNA labelling kit (Boehringer Mannheim).

Aliquots of 100 ng probe, 10 µg sonicated and denatured salmon sperm DNA and 10 µg *Escherichia coli* tRNA were hybridized per slide. Washing and detection for single detection of biotin-labelled yeast DNA was carried out as follows. Slides were washed for 20 min in 50% formamide, 2× SSC at 37°C, then for 20 min in 2× SSC at 37°C and then for 20 min in 1× SSC at room temperature. Avidin/FITC mixture (200 µl, 5 µg/ml avidin/FITC, 4× SSC, 1% Marvel) was then added and a cover slip placed over the solution followed by incubation in a moist chamber at 37°C for 1 h. The slides were then washed three times for 5 min in 4× SSC, 0.1% Tween-20 at 42°C and then placed in a propidium iodide counterstain solution (0.2 µg/ml propidium iodide, 2× SSC) for 15 min at room temperature and then destained (2× SSC, 0.05% Tween-20) for 1 min at room temperature.

Double detection of digoxigenin-labelled yeast DNA and another biotin-labelled probe was carried out as follows. The biotin was detected as above up to the three washes in 4× SSC, 0.1% Tween-20 at 42°C. Then 200 µl mouse monoclonal anti-digoxigenin antibody (Sigma, D8156) mixture (5 µl/ml in 4× SSC, 1% Marvel) was added and the slides incubated in a moist chamber at 37°C for 1 h. The slides were then washed three times for 5 min in 4× SSC, 0.1% Tween-20 at 42°C. Anti-mouse IgG TRITC conjugate (200 µl) (Sigma, T2402) mixture (1 µl/ml in 4× SSC, 1% Marvel) was then added and incubated at 37°C for 1 h. The slides were then washed three times for 5 min in 4× SSC, 0.1% Tween-20 at 42°C. DAPI (25 µl, 0.2 µg/ml) antifade solution was applied to the slide.

In the case of the total mouse probe, 2 µg of probe were used and the signal was amplified. The slide was washed with 50% formamide, 2× SSC for 20 min at 42°C, then 2× SSC for 20 min at 42°C and then 1× SSC for 20 min at room temperature. The biotin was detected as above up to the three washes in 4× SSC, 0.1% Tween-20 at 42°C. Then 200 µl of a mixture of 5 µl/ml mouse anti-digoxigenin antibody (Sigma, D8156) and 5 µg/ml Biotinylated Anti-Avidin D (Vector Laboratories) in 4× SSC, 1% Marvel was added and incubated at 37°C for 1 h. The slides were then washed three times for 5 min in 4× SSC, 0.1% Tween-20 at 42°C. Then 200 µl of a mixture of 5 µg/ml avidin/FITC and 1 µl/ml anti-mouse IgG–TRITC conjugate in 4× SSC, 1% Marvel was added and incubated at 37°C for 1 h. The slides were washed once more and stained with DAPI as above. Slides were visualized on a Leitz Aristoplan Microscope and photographed using Fujichrome ASA 1600 slide film.

RESULTS

Transfer of yeast genomic DNA into mouse L A-9 cells by fusion with yeast spheroplasts

The yeast strain F9 used for these experiments is a derivative of AB1380. It carries a mammalian selectable marker, resistance to the drug G418 (*neo*^{r}), integrated into the *ura3* gene on chromosome V. The yeast genomic DNA from the strain F9 was transferred to mouse L A-9 cells (an established mouse fibroblast line) by fusion with yeast spheroplasts followed by selection with G418 to select for cells which had taken up yeast DNA. Seventeen independent cell lines (called F9-1 to F9-17) were grown up from colonies on separate plates.

The DNA content of the cell lines was determined by Southern blotting. All of the cell lines were found to carry the *neo*^{r} gene, though at widely varying copy number, as shown in Figure 1A (data for lines F9-1 and F9-17 not shown). They were also analysed for the yeast repetitive element *Ty1* DNA, which is present at ~30 copies spread throughout the yeast genome. *Ty1* DNA was present in all the cell lines except F9-6 and F9-16, as shown in Figure 1B (data for lines F9-1 and F9-17 not shown). Finally the blots were hybridized with a single copy mouse probe to confirm roughly equal loading of the mouse DNA in each lane (data not shown). These results are summarized in Table 1.

Fluorescence *in situ* hybridization (FISH) was then used to determine the fate of the yeast DNA in the mouse cells. The probe was total yeast DNA labelled with FITC (green) and the

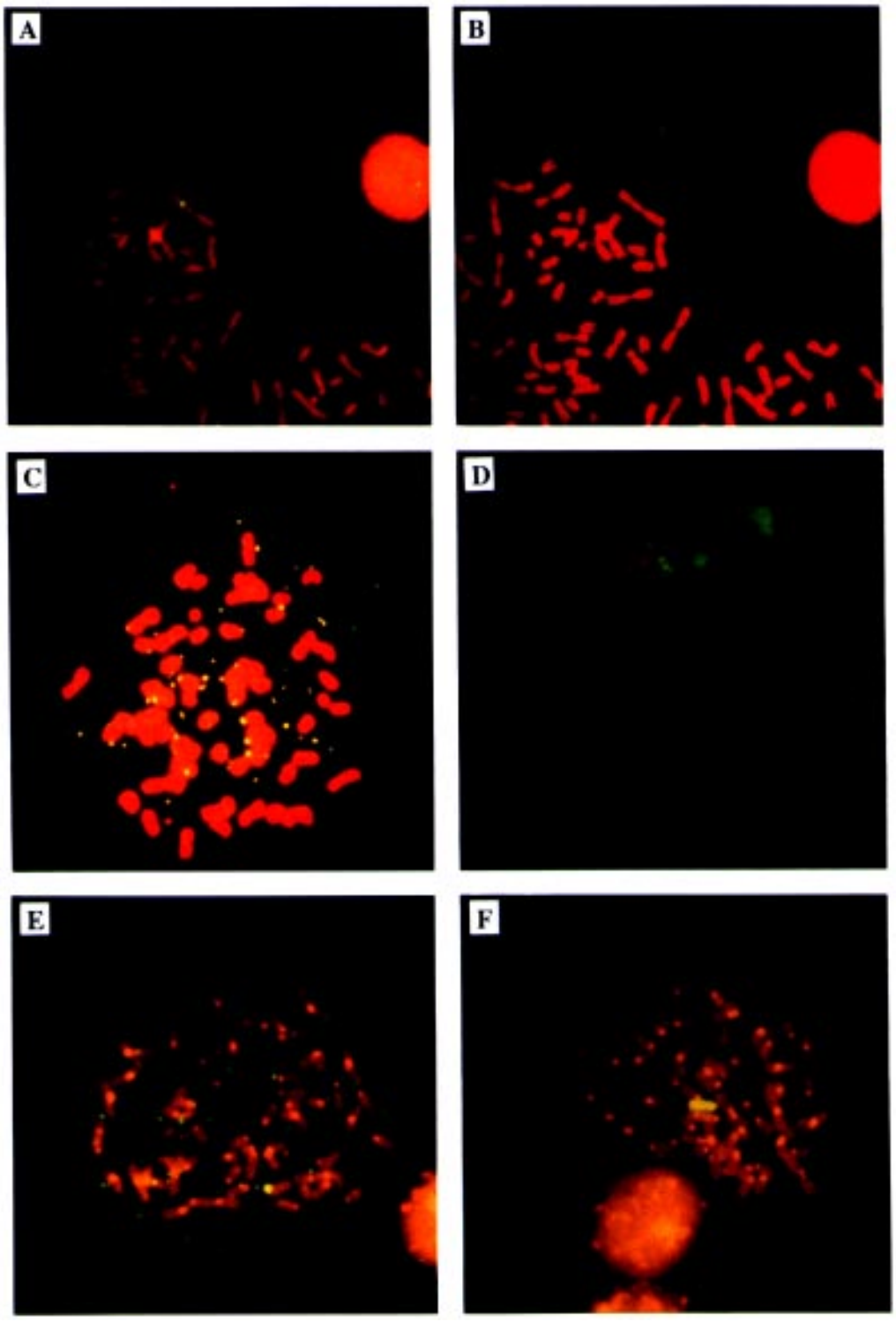

Figure 2. Yeast DNA in lines F9-11, F9-12 and F9-13. FISH was carried out with yeast DNA detected with FITC (green) and the chromosomes are counterstained with propidium iodide (red). (**A**) Integration of yeast DNA (green) in cell line F9-13. (**B**) The same metaphase spread with chromosomes stained with propidium iodide showing the constriction at the position of integration. (**C**) Extrachromosomal elements in a metaphase spread from F9-11. (**D**) An interphase nucleus (red and green) and several micronuclei containing yeast DNA (green) in line F9-11. (**E**) Extrachromosomal elements in line F9-12. (**F**) Novel chromosome in F9-12.

chromosomes were counterstained with propidium iodide (red), as shown in Figure 2. The cell lines F9-5, F9-13 and F9-15 were all found to contain a single, small integration of yeast DNA into a mouse chromosome (Fig. 2A and data not shown). In two of the cell lines the yeast DNA was found in a number of different forms in different cells. Cell line F9-11 was found to be a mixture of cells

containing small integrations of yeast DNA (27% of cells), cells containing large numbers of small extrachromosomal elements (32% of cells, Fig. 2C) and cells containing no yeast DNA (40% of cells). Cell line F9-12 was found to contain cells with small integrations of yeast DNA (23% of cells), cells with large numbers of small extrachromosomal elements (17% of cells, Fig. 2E), cells with very large integrations of yeast DNA (18% of cells), cells with a large novel chromosome (14% of cells, Fig. 2F) and cells with no signal (28% of cells). The results for these two cell lines are summarized in Table 2.

Integrations of yeast DNA are stable and form a constriction

In three cell lines, F9-5, F9-13 and F9-15, the yeast DNA was found as integrations into a mouse chromosome. The position of integration of the yeast DNA could generally be seen on the propidium iodide or DAPI stained chromosomes as a constriction of the chromosome, as shown in Figure 2B. The stability of the yeast DNA in the integrations was investigated by growing the cells without selection for ~25 days and then determining the percentage of cells still resistant to G418. The G418 resistance in the fusion cell lines F9-5, F9-13 and F9-15 was found to be fairly stable, with <23% of cells losing the G418 resistance in ~25 days of growth without selection (Table 1). These cells divide every ~24 hours out of selection, so for F9-5, where 23% of cells lost G418 resistance over 27 divisions, this would correspond to 1% of cells losing the G418 marker per cell division. Most of the other cell lines were not analysed by FISH. However, in 11 of the 17 cell lines, the G418 resistance was lost from <50% of cells in ~25 days of growth without selection (Table 1), which would correspond to 3% per cell division. These cell lines probably have stable integrations of the yeast DNA.

Extrachromosomal elements consisting of yeast DNA replicate, but segregate poorly

In two of the fusion cell lines, F9-11 and F9-12, the yeast DNA in some of the cells was present as extrachromosomal elements (Fig. 2C and E). The elements occur at quite high copy number, several hundred per cell, and are scattered amongst the chromosomes. The elements are present in the cells after the cell lines had been expanded to ~10^8 cells (at least 26 cell divisions), suggesting that the yeast DNA can replicate efficiently in the mouse cells. It is possible that the extrachromosomal elements have picked up DNA from the mouse host cells and it is this DNA which allows them to replicate. However, when total mouse DNA was used as a probe in FISH, it hybridized to the mouse chromosomes very strongly, but no signal was seen over the extrachromosomal elements (Fig. 4C and D). Mouse minor and major satellites were also investigated and neither were detected on the extrachromosomal elements (Fig. 3A and C).

The ability of the elements to segregate at cell division was investigated by growing the cell lines without selection for 19–22 days followed by determination of the number of cells still resistant to G418. Ninety nine per cent of cells of F9-11 and 98% of cells of F9-12 had lost resistance to G418 after 19 and 22 days respectively of growth out of selection (Table 1), which would correspond to at least 24 or 18% of cells losing the G418 resistance per cell division respectively. Filter hybridization confirmed that the loss of resistance to G418 was due to loss of the *neo*^{r} gene rather than inactivation of the gene (Fig. 5). This loss in the absence of selection was analysed in more detail for F9-12, which was grown for 13 days without selection and analysed by FISH at various times (Table 2). Over this time the percentage of cells with extrachromosomal elements fell from 17 to 0%, and this is largely matched by the rise in the percentage of cells with no detectable yeast DNA, which rose from 28 to 39%. Going from 17 to 3% in 11 days (Table 2) would correspond to 16% of cells losing the elements per cell division. Clearly these extrachromosomal elements are maintained inefficiently in the mouse cells.

When F9-11 was grown for an additional 22 days with selection, the percentage of cells containing extrachromosomal elements fell from 32 to 1%, while the percentage of cells with small integrations rose from 27 to 93% (Table 2). Clearly there is a selective advantage for cells with an integration of the yeast DNA, as these cells are taking over the population. This would explain why unstable extrachromosomal elements were not observed in all the cell lines; a random early integration event would lead to a cell line with stably integrated yeast DNA.

Table 2. Stability of yeast genomic DNA as determined by FISH

Cell line	Days in selection[a]	Days out of selection[a]	Number of metaphases scored	Per cent small integration	Per cent extra chromosomal elements	Per cent large integration	Per cent novel chromosomes	Per cent no signal
F9-11	9	0	62	27	32	0	0	40
F9-11	12	0	53	34	28	0	0	38
F9-11	18	0	55	55	5	0	0	40
F9-11	31	0	69	93	1	0	0	6
F9-12	5	0	153	23	17	18	14	28
F9-12	4	4	120	28	13	19	14	26
F9-12	4	8	75	35	6	21	11	27
F9-12	4	11	123	33	3	17	11	36
F9-12	4	13	100	32	0	19	10	39

[a]Cells were brought up from liquid nitrogen and grown first in selection and then out of selection for the number of days shown before analysis by FISH.

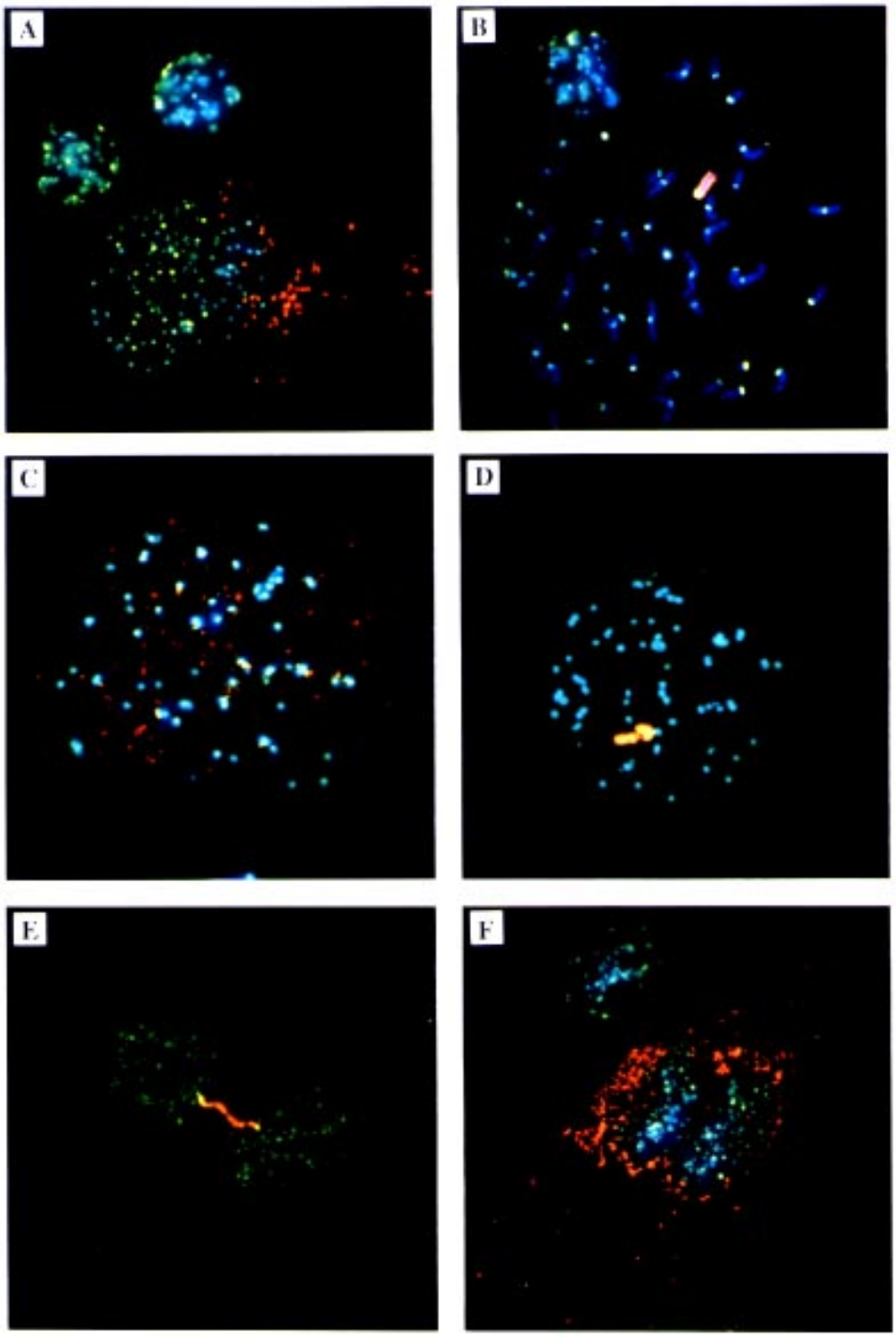

Figure 3. FISH analysis of cell line F9-12. Yeast DNA is labelled red, minor or major satellite green and the chromosomes are counterstained blue with DAPI. (**A**) Extrachromosomal elements in F9-12 contain yeast DNA (red) but no detectable minor satellite (green). (**B**) Novel chromosome in F9-12 contains yeast DNA (red) and a block of minor satellite (green). (**C**) Extrachromosomal elements in F9-12 contain yeast DNA (red) but no detectable major satellite (green). (**D**) Novel chromosomes in F9-12 contain yeast DNA (red) but no detectable major satellite (green). (**E**) Anaphase bridge in F9-12 consists of yeast DNA spread between the two clusters of mouse chromosomes with minor satellite (green) at the two ends of the anaphase bridge and at the centromeres of the mouse chromosomes (chromosomes not counterstained). (**F**) Extrachromosomal elements in an anaphase cell of F9-12; the elements contain yeast DNA (red) and are not clustered with the chromosomes (blue); the mouse minor satellite is labelled green.

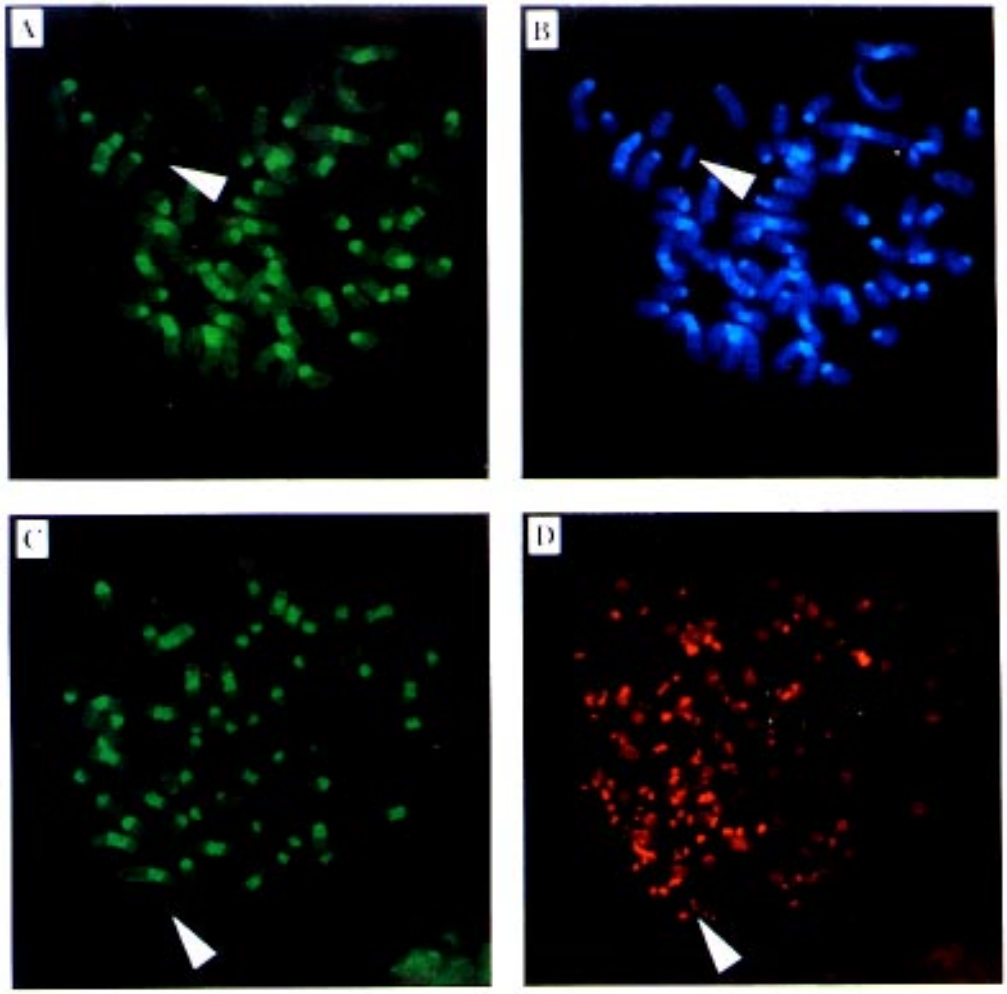

Figure 4. FISH analysis with total mouse DNA probe. (**A**) The novel chromosome in F9-12 (arrow) has a small amount of mouse DNA at one end but none along the length of the chromosome. (**B**) The same metaphase spread stained with DAPI shows the extent of the novel chromosome and the compacted chromatin structure of the yeast DNA. (**C** and **D**) Extrachromosomal elements in F9-12 (arrow) contain yeast DNA (red) but do not contain any detectable mouse DNA (green). The very strong signal from the total mouse probe comes through the red filter and shows up at the centromeres in (D).

A possible mechanism for loss of the extrachromosomal elements during growth without selection can be visualized by FISH. At anaphase the extrachromosomal elements are more widely spread through the cytoplasm than the chromosomes (Fig. 3F). During interphase, micronuclei containing large amounts of yeast DNA were frequently seen close to the main nucleus of a cell (which also contains yeast DNA), as shown in Figure 2D. The micronuclei could be caused by packaging into distinct micronuclei of elements which are distant from cellular chromosomes at anaphase. Micronuclei containing yeast DNA were present in ~15% of cells in F9-11 and loss of these from the cells could account for the rapid loss of the yeast DNA.

The novel chromosomes segregate and have picked up mouse centromeric DNA

FISH analysis revealed that 14% of cells in the line F9-12 contained novel chromosomes which were large and occurred at one or two copies per cell, as shown in Figure 2F. The low copy number of the chromosomes suggests that, unlike the high copy number extrachromosomal elements, the novel chromosomes are segregating. The ability of the chromosomes to segregate was measured by growing the cell line for a period of time without selection followed by FISH analysis to determine how many of the cells carried the chromosome. The percentage of cells carrying the novel chromosome went down from 14 to 10% during 13 days growth without selection (Table 2), which would correspond to only ~3% loss per cell division. This slow rate of loss is in marked contrast to the small extrachromosomal elements, which went from 17 to 0% during the same experiment, or ~16% loss per cell division. Thus, although not completely stable, the novel chromosomes segregate well.

The ability of the novel chromosomes to segregate, while the extrachromosomal elements do not, could be due to the larger size of the chromosomes or to the acquisition of mouse DNA from the host cell. FISH analysis with mouse minor satellite DNA as the probe showed that the novel chromosomes carry a region of mouse minor satellite DNA at one end (Fig. 3B). In contrast, no

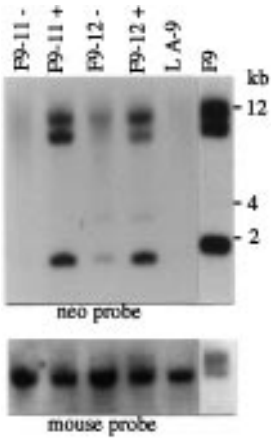

Figure 5. Loss of the *neo*^{r} gene from F9-11 and F9-12 in the absence of selection. DNA was prepared from cells which were grown in G418 selection (F9-11+, F9-12+) and from cells which had been grown without selection for 19 days (F9-11–) or 22 days (F9-12–), as shown in Table 1. The DNA was digested with *Eco*RI, separated on an agarose gel, blotted and hybridized with *neo* (top panel) and a single copy mouse probe (bottom panel) to show that the loading is equivalent in each lane. The cell line and growth conditions are indicated above each lane and the positions of size markers are indicated on the right.

minor satellite DNA was detected on the extrachromosomal elements, which do not segregate (Fig. 3A). Major satellite was also observed on some of the novel chromosomes, but this was present in widely varying amounts and was often not detectable (Fig. 3D), and it was also not detectable on the extrachromosomal elements (Fig. 3C). Finally, total mouse DNA was used as a probe and a small region could be detected corresponding to the minor satellite DNA, but none was detected along the bulk of the novel chromosome, indicating that the arms of this chromosome consist almost entirely of yeast DNA (Fig. 4A and B). Figure 4 also shows the compacted structure of the yeast DNA in the novel chromosome.

The minor satellite DNA on the novel chromosome is probably located at the functional centromere, as it is on normal mouse chromosomes. This was tested by observing the novel chromosome at anaphase. Out of 167 anaphase spreads of F9-12 examined, 10 (6%) had bridges consisting of mouse DNA, while 18 (11%) had bridges which consisted of yeast DNA. When the bridges were observed after hybridization with both yeast and minor satellite probes, it was found that the mouse minor satellite had separated into the two anaphase clusters along with the normal mouse centromeres, while the yeast DNA formed the bridge (Fig. 3E). This indicates that the minor satellite is located at a functional centromere which has segregated, while the yeast DNA is holding the two chromatids together, forming an anaphase bridge.

DISCUSSION

We have analysed the fate of *S.cerevisiae* genomic DNA present in a number of cell lines formed by fusion of mouse L A-9 cells with spheroplasts of a yeast strain which carries the *neo*^{r} gene on yeast chromosome V. In the majority of the cell lines, the yeast DNA has probably stably integrated into a mouse chromosome. However, in two of the cell lines, the yeast DNA was found to be maintained as extrachromosomal elements or novel chromosomes.

Our observations on the behaviour of extrachromosomal elements suggest that *S.cerevisiae* DNA can replicate in mouse cells. Efficient replication of the yeast DNA is supported by the presence of the elements after the cell lines had been expanded for at least 26 cell divisions. It is possible that the yeast DNA is being repeatedly excised from the chromosomes, but this is not likely, as there is selection for integration rather than excision on prolonged growth in selection. It is also possible that the extrachromosomal elements had picked up small amounts of mouse DNA, though none was detected by FISH. The conclusion that *S.cerevisiae* DNA can replicate in mammalian cells is consistent with the previous observation that a chromosome from *Schizosaccharomyces pombe* is capable of forming similar unstable extrachromosomal elements in mouse cells (26). Also, in a transient replication assay over 4 days, fragments of *S.cerevisiae* DNA have been shown to replicate in human cells almost as efficiently as human DNA (8). However, this is unlikely to be due to use of the yeast replication origins directly, as yeast ARS sequences are not preferentially utilized to initiate replication in mammalian cells (8) or *Xenopus* oocytes (27). This suggests that yeast origins of replication were not mediating replication in the extrachromosomal elements described here.

The elements occurred at a high copy number per cell and they were lost rapidly in the absence of selection (~16% of cells lost the elements per cell division), suggesting that they do not segregate. This is very similar to ARS plasmids in *S.cerevisiae*, which are known to replicate efficiently but to segregate poorly, and also to double minutes (DMs) in cancer and drug-resistant cell lines (28), which replicate efficiently but segregate with variable efficiency and generally do not have a functional centromere. Small (between ~12 and 40 kb in size) fragments of eukaryotic DNA replicate efficiently in human cells but do not form stable elements over longer periods unless viral elements for nuclear localization are provided (5,7,8). The yeast-derived elements seen here appear to segregate well enough to be maintained in the presence of selection and this could be due to yeast sequences which increase nuclear retention or to the relatively large size of the elements.

A novel chromosome formed almost entirely of yeast DNA was observed in a proportion of the cells of line F9-12. These chromosomes are far larger than the unstable extrachromosomal elements described above and they appear to have a functioning centromere, as they occur at one or two copies per cell and segregate quite well in the absence of selection (~3% loss per cell division). Indeed, on FISH analysis they were always found to carry mouse centromeric minor satellite DNA, whereas the unstable extrachromosomal elements did not. The mouse minor sequences and the functioning centromere have clearly come from the mouse host during formation of the chromosome, which has also involved some form of amplification or co-ligation of the yeast DNA. Novel chromosome formation thus appears to be a feature of heterologous DNA introduced into mammalian cells.

The *S.cerevisiae* DNA in the mouse cells forms a compacted chromatin structure in comparison with the mouse chromosomes. This can be observed in the constrictions formed at the site of integration of the yeast DNA and also in the narrow structure of the novel chromosome, which could be detected even without FISH analysis. Constrictions have previously been observed at the position of integration of alphoid DNA (15), of non-centromeric YACs (10,11) and of *S.pombe* DNA (29). The compacted chromatin may also be responsible for the anaphase bridges

formed by the yeast DNA in the novel chromosomes. A considerable amount of work has been carried out to try and understand the basis of the constrictions formed by *S.pombe* DNA and it appears that the frequent attachment of the yeast DNA to the rodent cell nucleoskeleton may be responsible (29).

A number of other investigators have reported the transfer of YAC and yeast genomic DNA to rodent cells by fusion. Generally, the YAC DNA, with varying amounts of yeast genomic DNA, has been observed by FISH to be integrated into the mouse genome (30–32). We have previously transferred YAC DNA to the mouse LA-9 cell line used in this report and have observed extrachromosomal elements in some of the cells in ~50% of the cell lines and these elements behaved very similarly to the elements described in this paper (10). Similarly, extrachromosomal elements have been observed by Nonet and Wahl (11) in Chinese hamster cells and mouse cells. The fact that most other investigators have not observed extrachromosomal elements could be due to the use of other cell lines which do not support such elements efficiently or to the fact that fast growing cell lines have been preferentially investigated, which would select with stable integrations.

YACs can carry large inserts and may be useful for cloning the functional elements of mammalian chromosomes, including replication origins and centromeres. In this paper we have introduced *S.cerevisiae* genomic DNA into mouse cells. We find that yeast DNA is able to replicate in mouse cells. This means that care must be taken when assaying DNA cloned in YACs for ability to replicate to make sure that yeast genomic DNA is not responsible for the replication. The yeast DNA is also involved in the formation of constrictions, novel chromosomes and anaphase bridges. As there is no evidence of centromeric activity of yeast DNA in mammalian cells, these features should be interpreted as possibly being non-specific results of the introduction of exogenous DNA. Thus these observations are of importance in interpreting the results of assays for centromere function.

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