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Distinct mouse DNA sequences enable establishment and persistence of plasmid DNA polymers in mouse cells

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ABSTRACT

Distinct elements isolated from mouse genomic DNA confer on plasmid DNA the ability to persist at high copy numbers in mouse L fibroblasts (1). Field inversion gel electrophoresis demonstrated that - in contrast to our previous assumption - the persisting plasmid DNA does not exist extrachromosomally but as clusters of tandem repeats integrated into genomic DNA. Digestion with restriction endonucleases that do not cut within the plasmid DNA results in fragments of 50-300 kb in length indicating reiteration of 10-50 plasmid DNA molecules. Restriction with several enzymes that cut once or twice within the plasmid sequences lead to fragment(s) indicative for head-to-tail tandem repeats. In situ hybridization revealed signals for a long homogeneously staining region (HSR) in one or two chromosomes per cell nucleus. Possibilities how these elements could act in the establishment and/or maintenance of the head-to-tail polymers of plasmid DNA in mouse cells are discussed.

INTRODUCTION

Gene transfer may be used to converse thymidine kinase-deficient mouse fibroblasts (Ltk^-) to the tk^+ phenotype. The herpes simplex virus 1 (HSV-1) thymidine kinase gene is widely used as donor gene (1, 2) and transformed cells are selected by growth in hypoxanthine-aminopterin-thymidine (HAT) medium (3). Integration of only one or a few copies of the marker gene into the genome is sufficient to allow survival under selective conditions as long as the transfected DNA contains the complete promoter structure (4). Usually several hundred HAT resistent Ltk^+ colonies can be obtained after transfection of 10^6 L cells with one microgram of plasmid DNA containing a HSV-1 tk gene with a full length promoter (1, 2, 4).

If, however, promoter-less HSV-1 tk genes are used for transfection the transformation rate drops by two to four orders of magnitude. Under these conditions tk^+ subclones were shown to result from 20 to 50 fold amplification of the linked plasmid along with significant lengths of adjacent carrier DNA (5-8). These variants produced aberrant tk transcripts such that multiple tk genes are required to produce sufficient thymidine kinase to convert the

cell to the tk^+ phenotype. Analysis of the physical structure of these amplified promoter-less tk genes revealed that the arrays are not regularly repeated structures but are heterogeneous both in size and in sequence content (6). It was concluded that gene amplification with promoter-less tk genes involves multiple cycles of unscheduled DNA replication at a single locus, followed by multiple recombination events (6). Low transformation efficiencies were also reported when the tk gene promoter is not completely truncated but only partially disrupted, i.e., if the second distal SP 1 binding site is deleted by Eco RI digestion of the HSV-1 tk gene (1, 9). We have recently exploited this observation to screen for DNA sequences which confer on plasmid DNA the ability to persist at high copy numbers in mouse L fibroblasts (10, 11). The system to screen for these sequences was based on the observation that disruption or truncation of the second distal SP 1 binding site of the tk gene promoter decreases the transcriptional efficiency of the tk gene by 95 % (12). This observation together with previous (1, 9) and our own reports (10) demonstrating that such a truncated tk gene produce none (1, 9) or only very few (10) surviving L cell colonies in HAT medium suggests that the truncated promoter allows only subthreshold levels of thymidine kinase to be synthesized in transfected cells. Recombinant transfection vectors containing these elements leading to persistance of multiple copies of plasmid DNA could compensate for the inefficient tk gene promoter by a positive gene dosage effect. By this screening method 21 distinct DNA sequences could be isolated from genomic mouse cell DNA. These elements were tentatively designated as "murine autonomously replicating sequences" ("mu-ARS") since we concluded from our previous data that the plasmid DNA persists episomally in transformed mouse L cells (10, 11).

Subsequent analysis described in this report revealed, however, that the vast majority of the amplified persisting plasmid DNA does not exist extrachromosomally but as head-to-tail tandem repeats integrated into the chromosome.

MATERIALS AND METHODS

Cell lines and plasmids, growth of mouse L fibroblasts, radioactive labelling blot hybridization were performed as described in a previous paper (10).

Isolation of cellular DNA.

Monolayer cells were trypsinized, washed in ice-cold phosphate-buffered saline (PBS), and suspended in a small volume of 10 mM EDTA, 10 mM Tris-HCl, pH 7.5. Cell lysis upon addition of 0.25 vol of 3% sodium dodecyl sulfate

in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was followed by RNAse treatment (100 µg/ml) for 60 min at 30 °C and proteinase K (Serva-Heidelberg; 1 mg/ml final concentration) digestion overnight at 55 °C. The mixture was gently extracted with phenol and chloroform-isoamyl alcohol (24:1, vol/vol), the DNA ethanol precipitated and dissolved in TE.

Sucrose gradient centrifugation.

For analytical separation and preparative enrichment of plasmid DNA polymers were applied to preformed linear sucrose gradients (10 to 40%, wt/vol) in TE, and centrifuged for 8 hr at 20 °C in a swing-out rotor (Beckman SW 41) at 38.000 rpm. After fractionation, aliquots from individual fractions were applied to nitrocellulose membranes with a filtration manifold (Minifold, Schleicher & Schüll). The relative distribution of different DNA species within the gradient was analyzed by separate hybridization probes.

Field inversion gel electrophoresis (FIGE).

Pulse field gel analysis (13) of plasmid DNA polymers was carried out as described in a published protocol (14, 15). Mouse L fibroblasts were trypsinized, washed once in PBS and resuspended at room temperature at a concentration of 0.8×10^6 cells per 40 µl PBS. An equal volume of 1% LMP agarose (UltraPure, BRL Laboratories) prepared in PBS was added, and the solution was dispensed in 80 µl aliquots into 7x6x2 mm spaces of a mold chilled on ice. The agarose blocks were placed in 0.5 M EDTA (pH 8), 1% Sarkosyl, 2 mg/ml proteinase K and then incubated at 50 °C for 48 hrs. The blocks were rinsed twice at 50 °C in 50 ml TE plus 40 µg/ml PMSF (phenylmethylsulfonylfluoride, Sigma). For enzyme digestion the agarose blocks were placed in 120 µl containing appropriate restriction endonuclease and buffer, and incubated for 4-8 hrs with 8-20 units of enzyme per 80 µl block. After digestion proteinase K was added to a final concentration of 1 mg/ml, and incubation continued for 30 min at 50 °C. The blocks were loaded into the wells of a 1% agarose gel and sealed in place. Pulse field electrophoresis was carried out using a Consort microcomputer electrophoresis power supplier E654. The conditions are: 7.5 V/cm voltage gradient for pulse 1 and 6.2 V/cm for pulse 2, ramp slope 50 min, starting pulse times: t_1 = 9 sec, t_2 = 3 sec; 0.4 x TBE buffer (1x is 0.089 M Tris base, 0.089 M boric acid, 0.002 M EDTA). The gel and buffer were cooled to 10 °C, and electrophoresis continued for 18 hr.

Cytogenetic analysis and in situ hybridization.

Cytogenetic analysis was performed on metaphase chromosome spreads. Colcemid (0.05 µg/ml) was added 2 hr before harvesting the cells. Hypotonic treatment with 0.075 M KCl took 20 min at 37 °C. The cells were fixed in 3:1 me-

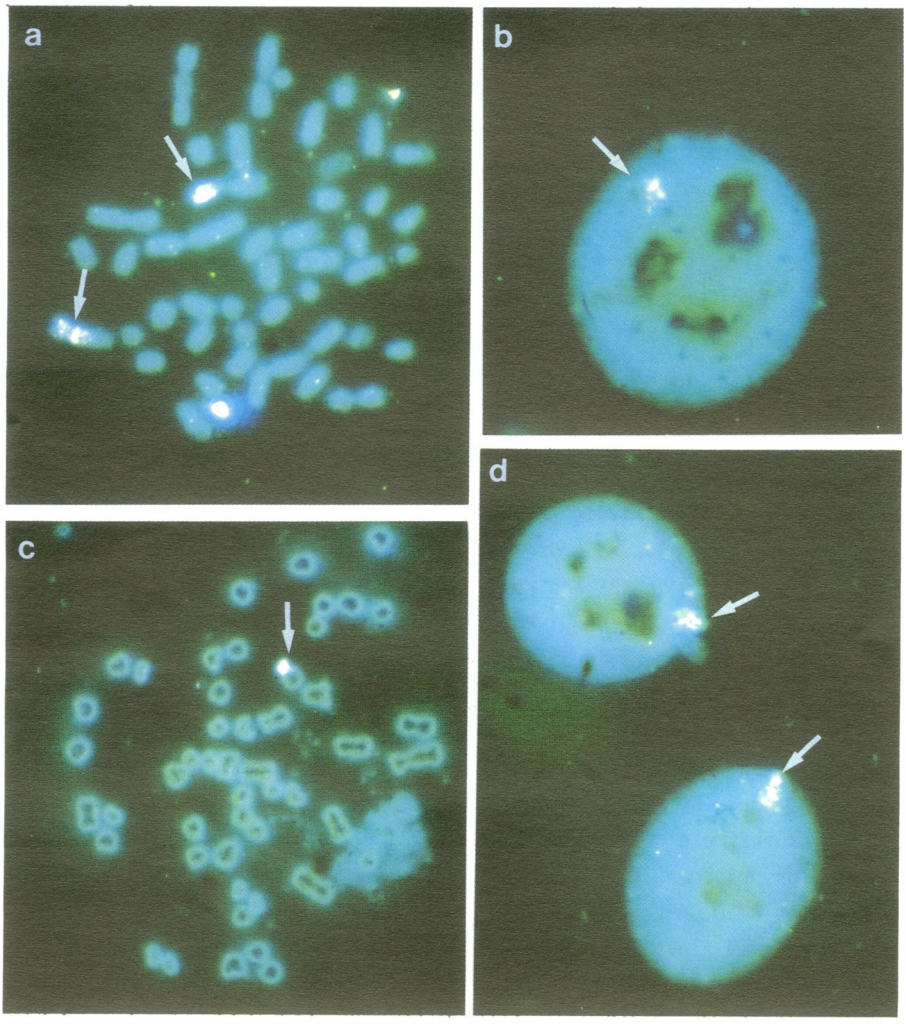

Figure 1. In situ hybridization of a biotin-labelled plasmid (ptk) probe to metaphase chromosomes and interphase nuclei from mouse L cells transformed by ptk-muARS-1 and ptk-muARS-4. The photographs on the left (a, c) show representative metaphase chromosome spreads from ptk-muARS-1-(a) or ptk-muARS-4-transformed L cells following hybridization in situ with the respective biotinylated plasmid probe. The photographs on the right (b, d) show interphase nuclei of ptk-muARS-1-(b) or ptk-muARS-4-transformed L cells.

thanol:acetic acid for 45 min and were washed 3x in the same solution before dropping them on slides.

In situ hybridization followed a published procedure (16). The probes were non-radioactive labelled by random priming according to the supplier of the DNA labelling and detection kit, nonradioactive, (Boehringer Mannheim) with Bio-11-dUTP (ENZO). To minimize non-specific binding of the probe, slides were treated with 100 µl DNase-free RNase (100 µg/ml) and sealed with a coverslip. After 1 hr incubation at 37 °C, coverslips were removed and slides were washed with water and dehydrated with ethanol. 50 µl of the hybridization mixture were placed on the slides under a coverslip and sealed with rubber cement. The probe and the chromosomes were denaturated together at 72-74 °C for 10 min. The hybridization mixture consists of 50% formamide, 10% dextran sulfate, 0.5 mg/ml sonicated salmon sperm DNA and 1 µg/ml of biotinylated probe all in 2xSSC. Hybridization was at 37 °C for 12 hr. Slides were then washed thoroughly three times with 2xSSC at room temperature and three times at 37 °C, once with PBS, 0.1% Triton X-100 and three times with PBS. Detection of the hybridized probe was according to the manual for the DETEC I - hrp Bio-Probe-System (EBP-820-1, ENZO) by incubation with streptavidin conjugated with horse-raddish peroxidase. Diaminobenzidine (DAB) development was for 15 min at room temperature. When using aminoethylcarbazole (AEC) for the staining reaction, development was for 30 min to 1 hr at room temperature in the dark. Slides were rinsed in water and then stained with DAPI (50 µg/ml) for 1 hr.

For microscopic analysis of the hybridization signal a Leitz photomicroscope equiped with reflection contrast (17) for DAPI fluorescence was used. Metaphases on slides without coverslip were first analysed by DAPI fluorescence with low magnification (250x) to localize metaphase spreads and then with reflection contrast with 40x and 100x objectives.

RESULTS

In situ hybridization of plasmid DNA

To determine whether the plasmid DNA persists in transformed mouse cells episomally or integrated into the genome, metaphase chromosomes were prepared and hybridized in situ to plasmid DNA probes labelled with biotin by nick translation. Following the in situ hybridization reaction, the biotin-labelled nucleotides were detected by binding peroxidase-conjugated streptavidin, and then developing with diaminobenzidine (DAB) (16). The precipitate that formed at the site of hybridization after the DAB reaction was visualized using reflection-contrast microscopy (17).

The results of the in situ hybridization experiments are shown in Figure

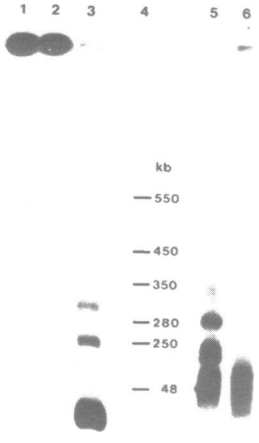

Figure 2. Field inversion gel analysis of plasmid DNA from transformed mouse cells. Total DNA from mouse cells transformed with ptk-muARS-1 (lane 1 and 5) and ptk-muARS-4 (lanes 2 and 6) was separated by inversed field gel electrophoresis as described in Material and Methods, photographed after staining in ethidium bromide, transfered to Gene Screen membrane, and hybridized with nick-translated ptk plasmid probe.

No signals corresponding to free, extrachromosomal plasmid DNA (lane 3) can be detected if whole cell DNA is fractionated by inversed field gel electrophoresis (lane 1, 2). All DNA remains at the origin of the gel (lane 1, 2) indicating the integration of the chimeric ptk molecules into the mouse genome. After incubation of DNA blugs with restriction enzymes Kpn I and Xba I which do not cut within the chimeric ptk vectors, ptk hybridizable material comigrates to a size corresponding to 50-60 kb (lane 5, 6). Isolated DNA of a cell clone of muARS1 (lane 5) reveals distinct bands up to 250 kb.

1 a-d. Representative photographs of chromosome spreads from a Ltk^+ cell line obtained by transformation with ptk-muARS-1 (Fig. 1a) or ptk-muARS-4 (Fig. 1c), are presented. The plasmid DNA-specific signals are indicated in the photographs by the arrows. The signals obtained with the biotin-labelled plasmid DNA probes were visible as dots on a single chromosome (Fig. 1c) or on two chromosomes (Fig. 1a). Interphase nuclei of both cell lines show labelling in distinct regions (Fig. 1b, and d). This hybridization pattern indicates the amplification and integration of the transfected plasmid DNA into the host genome.

Copy number determination for the cell lines analyzed here revealed that 600-700 plasmid DNA units persist per transformed mouse L cell (data not shown).

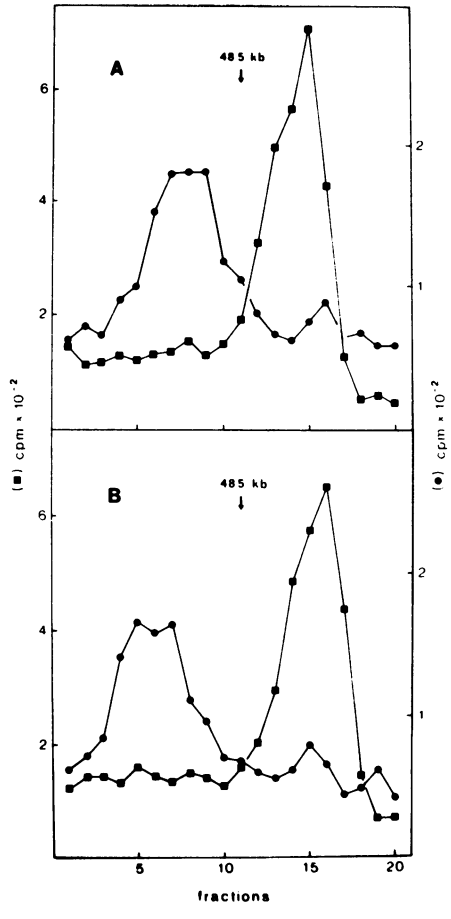

Figure 3. Sedimentation profile and preparative enrichment of amplified plasmid DNA from transformed mouse L cells. (A) DNA from ptk-muARS-1 and (B) from ptk-muARS-4-transformed L cells. DNA was multiply digested with an excess of restriction enzymes Kpn I, Sac I, Xba I, and Xho I, mixed with lambda LDNA, and centrifuged. Aliquotes of the fractions were hybridized with nick-translated ptk DNA (●), mouse cell DNA (■), and lambda DNA (arrow). Radioactivity on individual spots was determined by scintillation counting.

Size of multicopy plasmid integrates

We have used inversed field gel analysis to determine whether persisting plasmid DNA in transformed mouse cells exists in the form of unit length circular episomes or whether it is arranged in some kind of a polymerized conformation. Figure 2, lane 1, shows that undigested DNA from transformed L cells remains at the start of the gel. No DNA recognized by the plasmid probe was

found in positions of free plasmids (lanes 1, and 2). Treatment of total DNA from transformed mouse L cells with restriction endonucleases which do not cut in the recombinant ptk plasmids (KpnI and XbaI), however, leads to multiple bands reflecting linear polymeric plasmid DNA molecules (Fig. 2, lanes 5, 6). The average length of these hybridizing DNA bands range from approximately 60 to 300 kb corresponding to 10-50 times unit length of the recombinant ptk plasmids used for transfecting mouse L fibroblasts. The length of these arrays does not support the assumption that all persisting plasmid units are arranged in one large single tandem repeat. Apparently blocks of 10-50 unit length repeats are separated by stretches of cellular DNA at the chromosomal locus visible as a homogeneously staining region (HSR).

Digestion of total DNA with the noncutting enzymes KpnI and XbaI and additionally with Hind III (a single cutter for the plasmid DNA) revealed a shift of the hybridization signals for plasmid DNA from the region (50-300 kb) to the unit length position of approximately 6 kb.

Enrichment of concatameric plasmid DNA

A selective large-scale enrichment of plasmid DNA polymers was approached by sedimentation in neutral sucrose gradients. Total cellular DNA from L fibroblasts transformed by ptk-muARS-1 (Figure 3A) and -4 (Figure 3B), respectiely, was treated with four restriction enzymes being non-cutters for plasmid DNA (Kpn I, Sac I, Xba I, and Xho I). After purification by phenol extraction and ethanol precipitation, the DNA was applied to linear sucrose gradients. After spinning, the gradients were fractionated and analyzed by dot hybridization with different probes. The distribution of different DNA species was analyzed by counting of the membrane-bound radioactivity. Inclusion of an intact lambda marker DNA into the sucrose gradients demonstrates that the majority of plasmid DNA sediments as high-molecular-weight DNA. The bulk of digested cellular DNA can be separated from plasmid DNA polymers as small fragments at the top of the gradient (Figure 3A and B).

Structural organization of multicopy plasmid DNA in mouse cells.

We analyzed the structural organization of ptk DNA polymers by restricting the DNA with various enzymes cutting once or twice in ptk. To investigate the arrangement of the monomers in these tandem repeats we have digested total cellular DNA of mouse L fibroblasts transformed by ptk-muARS1 and -4, respectively, both with the noncutting enzymes for plasmid DNA, Kpn I, Xba I, and Xho I, as well as a single-cut enzyme (Cla I, Hind III, Pvu II, and Sal I, respectively). After cleavage DNA samples were separated on 0.6% agarose gels blotted and probed with nick-translated ptk DNA. Figure 4 demonstrates that

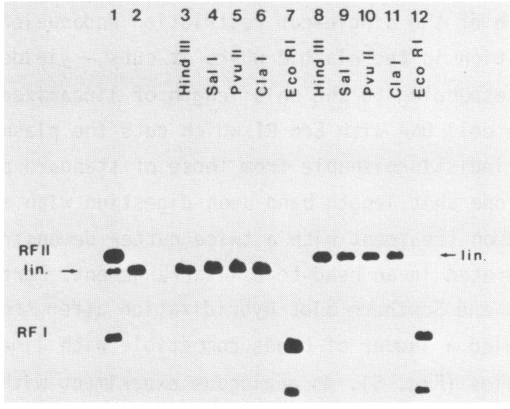

Figure 4. Southern blot analysis of plasmid DNA maintained in transformed mouse L cell lines. Total cell DNA extracted from ptk-muARS-1 (lanes 3-7) and ptk-muARS-4 (lanes 8-12) transformed L cells ($2x10^6$ per lane) was digested, as indicated, and electrophoresed on a 0.6% agarose gel. Nick-translated ptk DNA ($5x10^7$ cpm/µg) was used as a probe. Lane 1 and 2, ptk-muARS-1 DNA isolated from E. coli, undigested (lane 1) or digested with Hind III (lane 2).

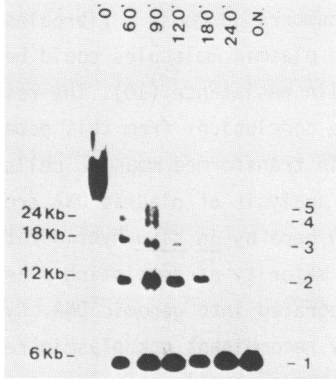

Figure 5. Partial hydrolysis of plasmid DNA from transformed mouse L cells with a single-cutting restriction enzyme. A 3 µg portion of total DNA isolated from Ltk^+ cells, transformed by ptk-muARS-1, pretreated with proteinase K was incubated at 37 °C with 2 U of Hind III in a total volume of 20 µl. After 60, 90, 120, 180, 240 min, and overnight the reaction mixture was heated to 60 °C for 10 min. Samples from the reaction kinetics were electrophoresed in a 0.4% agarose gel, blotted and hybridized with nick-translated ptk DNA as a probe. The apparent molecular weights of partial digestion products were determined by using a ladder of oligomerized standard ptk plasmid DNA and lambda DNA restricted with Sma I and Xba I as marker (not shown). The observed mobility of the five smallest ptk-specific partial digest fragments (1 through 5) is compatible with the theoretical mobility of linear mono-, di-, tri-, tetra- and pentameric ptk-muARS molecules.

digestion with each of the single-cut restriction endonucleases - independently of the position in the plasmid where it cuts - yields a band of the same mobility corresponding to the unit length of linearized plasmid DNA. Digestion of mouse cell DNA with Eco RI which cuts the plasmid DNA twice yielded two fragments indistinguishable from those of standard plasmid DNA. The occurence of only one unit length band upon digestion with single-cut enzymes and of two bands upon treatment with a twice cutter demonstrates that plasmid monomers are reiterated in an head-to-tail arrangement. Partial hydrolysis of the concatamers and Southern blot hybridization after separation on 0.4% agarose gels revealed a ladder of bands compatible with linear oligomeric plasmid DNA molecules (Fig. 5). An analogous experiment with Pvu II produced an identical fragmentation pattern (not shown). The most plausible interpretation of these data is the persistence of plasmid DNA in transformed mouse L cells as concatamers of unit length monomers.

DISCUSSION

We have previously reported on a series of mouse genomic DNA sequences, tentatively designated as "muARS", which confer on plasmid DNA the ability to persist at high copy numbers in mouse L fibroblasts (10, 11). Three months after transfection single plasmid molecules could be rescued into E. coli by screening for ampicillin resistance (10). The rescue rate, however, is extremely low. One of the conclusions from this paper was that the plasmid DNA persists episomally in transformed mouse L cells. This conclusion was drawn from Southern blot analysis of plasmid DNA present in the Hirt supernatant fraction. As shown here by *in situ* hybridization and inversed field electrophoresis the vast majority of persisting plasmid DNA exists not extrachromosomally but is integrated into genomic DNA. Cytogenetic analysis of cell lines transformed by recombinant ptk plasmid revealed *in situ* hybridization signals of a long homogeneously staining region (HSR) in one or two chromosomes per cell nucleus. Digestion with restriction endonucleases that do not cut within the plasmid DNA results in fragments of 50-300 kb in length indicating reiteration of 10-50 plasmid DNA molecules. Restriction with several enzymes that cut once or twice within the plasmid sequences lead to fragment(s) indicative for head-to-tail tandem repeats. Since these results demonstrate that polymers of plasmid DNA are integrated as clusters into distinct chromosomal regions the designation of these murine elements as "muARS" is inadequate. At present we do not know how these murine elements lead to the establishment of multiple head-to-tail arrays of plasmid DNA. Transfor-

mation frequencies of the ptk vectors containing such elements are two to three orders of magnitude lower than those observed with a vector containing a full length promoter, but still significantly higher than those obtained with ptk alone. All of the Ltk^+ cell lines obtained with ptk containing these murine sequences are characterized by high copy numbers of plasmid DNA units (ca. 300-2000) (10).

The fact that these sequences lead to persistence of plasmid molecules at high copy numbers was recently exploited to construct high-copy-number expression vectors for heterologous proteins. Based on the murine DNA element 4 ("muARS-4") tissue-type plasminogen activator could be expressed at high yield in mouse cells (20). Using the same approach distinct DNA elements with sequence homology to the murine consensus box ($CTA\frac{A}{T}GAGA\frac{GG}{CC}AA$) could be isolated from human immunoglobulin V_k gene upstream regions (21). These human sequences lead also to the persistence of plasmid DNA at high copy numbers in transformed mouse cells.

Various functions are conceivable for these murine DNA elements, e.g., the elements might act in replication, they could lead to integration, and/or they could be recombinogenic. At the present stage of investigation we cannot rule out any of these possibilities. Interestingly, plasmid polymers have been observed before, after transfection of HSV-tk gene segments without insertion of cellular DNA elements into the transfecting vector (5-7, 22, 23). In these cases donor DNA appears to be converted first to long concatameric forms, including plasmid and carrier DNA, before the entire block is integrated into the genome of the recipient cell. Under these circumstances a mosaic of head-to-tail, head-to-head, and tail-to-tail arrangements interrupted by carrier DNA is obtained.

In contrast, we observe in our system exclusively an uninterrupted head-to-tail arrangement of the individual plasmid DNA polymers even if carrier DNA is added in excess and therefore a different mechanism of amplification. We favour the notion that the murine DNA elements primarily act in DNA replication. One model would predict that they enable a rolling-circle type of DNA synthesis at the initial step of the establishment of tandem repeats which later become integrated. Individual head-to-tail integrates could then independently be amplified either in the chromosomal context or after excision. A similar mechanism has been suggested for the amplification of single integrated viral sequences (SV 40, polyoma, HSV) in the mammalian cells upon treatment with carcinogenic agents or by superinfection (24-27). In these

cases cis-acting origins and trans-acting initiator proteins are required for rolling circle replication leading to uniform head-to-tail amplicons. Whether the murine DNA elements act primiarily as origins of (rolling circle?) replication in the step of establishment and/or as bona fide chromosomal origins is under investigation.

Alternatively the inserts described here may function as hot spots of recombination leading to long head-to-tail concatemers. This possibility is also being investigated.

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