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# Letters to the Editor

## Autonomous Replication in Mouse Cells: A Correction

In the course of experiments designed to investigate the timing and mode of replication of the murine autonomously replicating sequence (muARS) plasmids reported by Holst et al. (Cell 52, 355–365, 1988), we found that the sequences identified by Dr. Friedrich Grummt and his colleagues as autonomously replicating were, in fact, integrated and were not capable of conferring high-frequency transformation as reported. Specifically, our results were as follows:

Southern blot analyses were performed using DNA isolated from cell lines muARS9, muARS4, and muARS5, provided by F. Grummt. DNA was physically sheared, digested with several restriction enzymes that do not cut within the plasmids, and also digested with enzymes that cut once within the plasmids. No monomeric supercoiled or nicked circular DNA could be identified. Different banding patterns were observed with different enzymes, indicating a significant amount of plasmid integration. About half of the signal migrated as linear monomer when treated with enzymes that cut once within the plasmid. A majority of the hybridizing signal in the DNA samples treated with enzymes that do not cut within the plasmid corresponded to fragments longer than 30 kb. In sheared samples, the hybridization pattern was similar to that of ethidium bromide–stained chromosomal DNA.

Hirt extractions were performed on the muARS9, muARS4, and muARS5 cell lines. Hybridization was found only to residual high molecular weight DNA, even after prolonged autoradiographic exposure. Ampicillin-resistant colonies could not be rescued to Escherichia coli.

Transient replication assays were performed on plasmids muARS1 and muARS4. Plasmids sensitive to digestion with MboI could not be generated in quantities greater than that with the plasmid lacking the muARS insert.

Two separate calcium phosphate transfections of Ltk$^-$ cell lines were carried out using muARS1 and muARS4. Although stable transfection frequencies in both experiments were on the order of $10^{-3}$ to $10^{-4}$ as measured by transfection with both herpesvirus and chicken thymidine kinase genes, no TK$^+$ colonies were generated with either the plasmid lacking the insert or the muARS plasmids, even though approximately $10^7$ cells were transfected with each plasmid DNA in each experiment.

Taken together, these data indicate that cell lines muARS9, muARS4, and muARS5 contain several hundred copies of the respective plasmids integrated in tandem clusters at many locations in the genome. We find no evidence for extrachromosomal replication of any of these constructs in these cell lines, and have obtained no evidence that plasmid insert muARS1 or muARS4 contains any replication activity, or activity associated with a high frequency of transformation of mouse Ltk$^-$ cells.

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We have previously reported on a series of mouse genomic DNA sequences, tentatively designated as muARSs, that confer on plasmid DNA the ability to persist at high copy numbers in mouse L fibroblasts (Holst et al., Cell 52, 355–365, 1988). The system used to screen for these sequences was based on the observation that plasmids containing a thymidine kinase gene with an inefficient, truncated promoter (McKnight et al., Cell 37, 253–262, 1984) were unable to transform mouse Ltk$^-$ cells to the TK$^+$ phenotype, since apparently only subthreshold levels of thymidine kinase were generated in transfected cells (Wigler et al., Cell 11, 223–232, 1977; Wilkie et al., NAR 7, 859–877, 1979). Recombinant ptk plasmids containing these "muARS" elements persist at high copy numbers in transfected mouse cells and hence compensate for the inefficient thymidine kinase gene promoter by a positive gene-dosage effect (Holst et al., op. cit.).

Single plasmid molecules can be rescued to E. coli by screening for ampicillin resistance 3 months after transfection. 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. The majority of the plasmid-specific hybridization signal was found to migrate close to the position of relaxed plasmid DNA.

However, subsequent analysis of cellular DNA by a variety of methods revealed that the plasmid-size DNA molecules were generated by shearing of DNA in the Hirt supernatant fraction. Inverse-field electrophoresis demonstrated that the vast majority of persisting plasmid DNA exists not extrachromosomally but as 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 leads to fragments indicative of head-to-tail tandem repeats.

Cytogenetic analysis of cell lines transformed by recombinant ptk plasmids revealed in situ hybridization signals of a long, homogeneously stained region (HSR) in one or

two chromosomes per cell nucleus. These results demonstrated that individual arrays of 50–300 kb long head-to-tail polymers are integrated as clusters into distinct chromosomal regions.

Therefore the designation of these murine *cis*-acting elements as muARSs is misleading. At present we do not know how these murine elements lead to the establishment of the high-copy-number, head-to-tail arrays of integrated plasmid DNA. Whether they act primarily as origins of (rolling circle?) replication in the step of establishment and/or as bona fide chromosomal origins is under investigation. The question of whether these elements are highly recombinogenic is also being investigated.

We recognize that this letter presents statements without supporting data. A manuscript is being prepared that includes the data for unpublished statements made here and will be available upon request.

We apologize for any inconvenience that the report of the episomal nature of plasmid DNA carrying these *cis*-acting mouse elements may have caused other workers.

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