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Circular YAC Vectors Containing a Small Mammalian Origin Sequence Can Associate With the Nuclear Matrix

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Abstract Three different mammalian origins of DNA replication, 343, S3, and X24, have been cloned into a 15.8 kb circular yeast vector pYAC*neo*. Subsequent transfection into HeLa cells resulted in the isolation of several stably maintained clones. Two cell lines, C343e2 and CS3e1, were found to have sequences maintained as episomes in long-term culture with a stability per generation of approximately 80%. Both episomes also contain matrix attachment region (MAR) sequences which mediate the binding of DNA to the nuclear skeleton and are thought to play a role in DNA replication. Using high salt extraction of the nucleus and fluorescent in situ hybridization, we were able to demonstrate an association of the 343 episome with the nuclear matrix, most probably through functional MAR sequences that allow an association with the nuclear matrix and associated regions containing essential replication proteins. The presence of functional MARs in small episomal sequences may facilitate the replication and maintenance of transfected DNA as an episome and improve their utility as small episomal constructs, potential microchromosomes. J. Cell. Biochem. 67:439–450, 1997. © 1997 Wiley-Liss, Inc.

Key words: artificial chromosome; episome; YAC; nuclear matrix attachment region; MAR; replication origin; DNA replication; fluorescent in situ hybridization

In addition to a replication origin, telomeres, and a centromere, a human artificial chromosome (HAC) might also require other cis-acting elements for optimum function. The first step in the construction of a HAC has already been accomplished with the cloning of three different mammalian replication origin sequences into the circular 15.8 kb yeast vector pYAC*neo* [Nielsen et al., submitted]. These include 343, a sequence isolated from an actively transcribed region on human chromosome 6q [Shihab-El-Deen et al., 1993; Wu et al., 1993b] S3, a human autonomously replicating sequence [Nielsen et al., 1994], and X24, a fragment which contains the hamster oriβ replication initiation site associated with the dihydrofolate reductase gene locus [Burhans et al., 1990]. These versatile constructs (Y.343, YAC.S3, Y.X24, respectively) have the advantage of being well-characterized, relatively small DNA sequences, which can be easily transfected into human cells. Studies performed on HeLa cells transfected with these constructs by calcium phosphate coprecipitation and selected by G418 resistance resulted in the isolation of several stably maintained clones [Nielsen et al., submitted], two of which (C343e2 and CS3e1) maintained the episomes (Y.343 and YAC.S3, respectively) for at least 80–90 days, with a stability per generation of approximately 80% [Nielsen et al., submitted]. DNA matrix attachment regions (MARs), which mediate the binding of DNA to the nuclear skeleton, are thought to play an important role in DNA replication. Origins of DNA replication are associated with the nuclear matrix [Berezney and Coffey, 1975; Mah et al., 1993, and references therein], and MARs are frequently found as intrinsic features of replication origin sequences [Boulikas, 1993]. As both Y.343 and YAC.S3 contain MAR sequences [Wu et al., 1993a, unpublished data], we wanted to

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determine whether the episomal DNA sequences were associating with the HeLa cell nuclear matrix in vivo. To test this association, we performed high salt extraction [Vogelstein et al., 1980] on the cell clones followed by in situ hybridization and signal quantification.

MATERIALS AND METHODS

Vector Constructs

YAC vectors were constructed as previously described [Nielsen et al., submitted]. Briefly, three different mammalian replication origin sequences were cloned into the EcoRI site of the circular 15.8 kb yeast vector pYAC*neo*. These include a 450 bp EcoRI-HincII sequence (343; GenBank accession number L08443) isolated from an actively transcribed region on human chromosome 6q [Shihab-El-Deen et al., 1993; Wu et al., 1993b], S3, a 1.1 kb human autonomously replicating sequence and origin [Nielsen et al., 1994; Tao et al., submitted], and X24 (GenBank accession number X52034), a 4.3 kb XbaI fragment which contains the hamster replication initiation site, oriβ, of the dihydrofolate reductase gene [Burhans et al., 1990; Zannis-Hadjopoulos et al., 1994]. YAC plasmids containing these origin sequences were designated Y.343, YAC.S3, and Y.X24, respectively. Transfected clonal cell lines were designated as C followed by the name of the transfected construct, then a letter designating the integrated (i) or episomal (e) state of the DNA, and finally a number indicating the clonal cell line.

Cell Culture and Transfections

Cell culture and transfections were performed using HeLa cells. Briefly, log phase HeLa cells (10^4 cells/cm^2), grown in alpha MEM, 10% fetal calf serum (FCS), were transfected with one of the origin-containing YAC constructs (1 μg/cm^2) by calcium phosphate coprecipitation [Graham and Van der Eb, 1973]. At 2 days posttransfection, cells were transferred to medium containing 400 μg/ml G418, and growth continued until 20 days posttransfection, when dishes were scored for visible drug-resistant colonies. Individual drug-resistant clonal subpopulations were picked from 60 mm dishes using a sterile pasteur pipette, expanded into increasingly larger size dishes, and propagated in long-term culture under G418 selection in 80 cm^2 flasks. Several clones with different growth rates in the presence of G418 were picked for further analysis. Cultures were maintained in 400 μg/ml G418 for a minimum of 45 cell doublings between initial transfection and subsequent analysis. In addition, transiently transfected cell lines were also prepared using HeLa cells transfected by calcium phosphate coprecipitation as described above using plasmids Y.343, Y.X24, and pYAC*neo*.

Fluorescent In Situ Hybridization (FISH)

Nuclei were prepared from the clonal HeLa cell lines C343e2 and CS3e1 as follows. After each cell line had been stably maintained in culture for 99 and 84 days, respectively, log phase cultures were further accumulated in metaphase with 0.06 μg/ml colcemid for 3 h. Methanol/acetic acid–fixed nuclei were prepared by standard techniques. Samples were stored in fixative at −20°C until use. In addition, preparations were made from HeLa cells which had been transiently transfected with Y.X24, which does not contain a MAR, and harvested 2 days later; similarly a HeLa cell clone containing an integrated form of pYAC-*neo* (CYACi2) served as a comparative example. Stored samples were centrifuged (150*g*, 5 min), and nuclei were resuspended in fresh 3:1 methanol:acetic acid. The resuspended nuclei were then dropped onto clean microscope slides and left to air-dry. The nuclei were denatured by incubating the slides in 70% formamide, 2× SSC, pH 7.0, at 73°C for 3 min and briefly rinsed in ice-cold 70% ethanol before immersing sequentially in ice-cold 70%, 90%, and 100% ethanol for 3 min each and drying. pYAC*neo* labeled with biotin-14-dATP using the BioNick labeling system (Gibco-BRL, Gaithersburg, MD) was used as a probe. The hybridization mixture, containing 50% formamide, 10% dextran sulphate, 2× SSC, 2 μg human COT-1 DNA (Gibco-BRL), 10 μg sonicated salmon sperm DNA (Stratagene, La Jolla, CA) and 100 ng probe DNA, was denatured at 95°C for 3 min and then preannealed for 1.5 h at 37°C. A 10 μl aliquot of the hybridization mixture was placed on each slide, which was then covered with a glass coverslip and sealed with rubber cement. Slides were left to hybridize overnight in a moist chamber at 37°C. The slides were then unsealed, immersed in 2× SSC at 42°C for 5 min, and washed in 50% formamide, 2× SSC at 42°C three times for 5 min each. Slides were subsequently washed in 0.1× SSC at 60°C three times for 5 min each, rinsed with 0.05%

Tween-20 in phosphate-buffered saline (PBS), and then blocked with 3% BSA, 4× SSC for 30 min at 37°C. Twenty-five microliters of mouse antibiotin (Sigma, St. Louis, MO), diluted 1:500 in 4× SSC, 0.05% Tween-20, was added to each slide and a plastic coverslip placed on top. After incubation at 37°C for 60 min, slides were rinsed with 0.05% Tween-20 in PBS three times for 5 min each at room temperature (r.t.). The biotin signal was then amplified using a biotinylated tyramine deposition technique as detailed in Kerstens et al. [1995]. Briefly, 25 µl of biotinylated antimouse antibody, diluted 1:200 in PBS with 1.5% blocking serum (ABC Immunostain system; Santa Cruz Biotechnology, Santa Cruz, CA), was added to the slides and incubated for 30 min at 37°C. Samples were then washed in 0.05% Tween-20 in PBS three times for 5 min each at r.t. Twenty-five microliters of biotinylated HRP-avidin (ABC Immunostain system) diluted in PBS according to the manufacturer's instructions, was then added and the slides incubated for 30 min at r.t. Samples were then rinsed, first in 0.05% Tween-20 in PBS and then in PBS. Biotinylated tyramine (BT), prepared as in Kerstens et al. [1995], was then diluted 1:10 in PBS, and hydrogen peroxide was added to a final concentration of 0.01%. After incubation for 10 min at r.t. with this mixture, slides were rinsed in 4× SSC, 0.05% Tween-20. Twenty-five microliters of FITC-avidin (Oncor, Gaithersburg, MD) diluted 1:50 in 4× SSC, 0.05% Tween-20 was added to each slide and incubated at 37°C for 30 min. Slides were then rinsed twice with PBS and mounted with 0.03 µg/ml propidium iodide/antifade (Oncor). Fluorescent signals were visualized using a Leitz (Wetzlar, Germany) Fluovert FS microscope, and photomicrographs were taken with Kodak (Rochester, NY) Tmax 400 black-and-white film.

Ammonium Salt Extraction

HeLa cells were grown on coverslips and then dipped into 0.5% NP-40, after which the nuclei remain attached to the coverslip [Vogelstein et al., 1980]. The coverslips were then fixed in 3:1 mixture of freshly prepared methanol:acetic acid prior to ammonium sulphate extraction, as described in Fey et al. [1986]. Briefly, nuclei fixed to slides were incubated in cytoskeleton buffer (100 mM NaCl, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM $MgCl_2$, 1 mM EGTA, 1.2 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM vanadyl adenosine, and 0.5% Triton X-100) at 4°C for 5 min. Slides were then transferred into extraction buffer containing 250 mM ammonium sulphate, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM $MgCl_2$, 1 mM EGTA, 1.2 mM PMSF, 2 mM vanadyl adenosine, and 0.5% Triton X-100 for 10 min at 4°C. Slides were then rinsed twice in ice-cold PBS and DNA-visualized by staining with 0.03 mg/ml propidium iodide/antifade.

High Salt Extraction

For comparison between different techniques of extraction, cells were grown on coverslips, permeabilized, fixed, and then extracted with 2 M NaCl as described below. For high salt extraction of the stably maintained cell clones, nuclei were prepared as described in the FISH method and the number of signals counted. After visualization, coverslips were removed, and nuclei on the slides were permeabilized by incubation in 10 mM Tris-Cl, pH 7.4, 5 mM $MgCl_2$, 150 mM NaCl, 0.5 mM PMSF, and 0.5% Triton X-100 for 10 min at room temperature. Samples were then subjected to high salt extraction as described in Vogelstein et al. [1980]. Briefly, slides were dipped sequentially in 0.2 mM $MgCl_2$, 10 mM Tris-Cl, pH 7.4, containing 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, and 1.8 M NaCl, at 4°C for 30 s each. Slides were then placed in 0.2 mM $MgCl_2$, 10 mM Tris-Cl, pH 7.4, 2 M NaCl at 4°C for 10 min. After being washed twice in ice-cold PBS, samples were relabeled according to the protocol for FISH described above.

Statistical Analysis

The sampling distribution of data was subjected to statistical analysis using a simple chi-square test in a 2×2 contingency table with 1 degree of freedom [Spiegel, 1994]. All comparisons were done at the 0.05 level of significance. The Null hypothesis states that the proportion of cells retaining the episome (and thus FISH signal) is unchanged following salt or ammonium sulphate extraction.

RESULTS

In this study, we tested whether stably maintained episomal sequences, which contain functional replication origins and MAR sequences, were associated with the nuclear matrix on the basis of resistance to high salt extraction.

Various methods have been used to extract histones and other soluble components during isolation of intact nuclear matrices, including extraction with ammonium sulphate [Capco et al., 1982; Fey et al., 1986], LIS (lithium 3,5-diiodosalicylate) [Mirkovitch et al., 1984], or sodium chloride [Berezney and Coffey, 1974, 1975; Bouvier et al., 1984; Kaufman et al., 1981]. For our purposes, we wanted to use the most stringent technique available to solubilize episomal DNA whilst leaving the nuclear matrix insoluble and undigested.

Low salt extraction using LIS was originally proposed as a milder alternative to high salt extraction. However, it was found that LIS-treated matrices had a significantly higher amount of associated DNA and histones [Belgrader et al., 1991], and this technique was not further considered for this study, due to the probability of artefactual association of episomal DNA with the nuclear matrix. In high salt–extracted nuclei, previous studies have shown that the DNA protrudes outwards to form a characteristic halo structure consisting of supercoiled loops of DNA attached at their bases to the residual nuclear matrix [Vogelstein et al., 1980]. Extraction with high (2 M) NaCl depletes soluble proteins and histones together with up to 90% of the soluble DNA component, leaving behind the insoluble nuclear matrix proteins [Berezney and Coffey, 1975]. As the preparations used in this study were not subjected to DNAse I digestion prior to extraction, the majority of the nuclear DNA would be expected to remain in the nucleus following salt treatment. However, small soluble fragments of DNA, such as episomes, will be removed under these conditions unless they are associated with the nuclear matrix [Thiebaut et al., 1993]. Thus, this method would be more suited to solubilizing episomal DNA and has already been used to demonstrate the association of episomal DNA with the nuclear matrix [Thiebaut et al., 1993]. On the other hand, ammonium sulphate extraction is thought to preserve the nuclear morphology more precisely [Fey et al., 1986], but is a milder extraction than that obtained with high salt treatment [Belgrader et al., 1991].

For the purpose of demonstrating the association of episomal DNA sequences with the nuclear matrix, we required a method that would maintain the nuclear matrix structure but solubilize the unassociated DNA to the maximum extent, as extrusion and solubilization of episomal DNA would be crucial in decreasing any artefactual association of these sequences with the matrix. Although both high salt and ammonium sulphate extraction maintain the integrity of the nuclear matrix [Ivanchenko and Avramova, 1992], it was not clear if both would be equally effective at removing any unattached episomal DNA. Thus, an initial experiment was performed to compare the effects of high salt vs. ammonium sulphate extraction on the nuclei of fixed HeLa cells (Fig. 1).

By comparison to unextracted nuclei (Fig. 1A), extraction with 2 M salt gave the characteristic halo structure [Vogelstein et al., 1980] caused by DNA looping out from the nucleus (Fig. 1B). On the other hand, extraction with 250 mM ammonium sulphate did not extrude the nuclear DNA to any significant extent and produced a nucleus with a distinct intracellular structure (Fig. 1C). Therefore, whilst the use of ammonium sulphate may be desired in systems requiring milder isolation of the nuclear matrix in order to preserve fibrogranular networks for morphological studies, extraction with 2 M salt appeared to be the more suitable method for efficiently solubilizing episomal DNA. This is in agreement with previous studies on the isola-

Fig. 1. HeLa cell nuclei following extraction with ammonium sulphate or high salt. **A:** Fixed HeLa cell nucleus, unextracted. **B:** Fixed HeLa cell nucleus, extracted with up to 2 M NaCl. **C:** Fixed HeLa cell nucleus, extracted with 250 mM ammonium sulphate. DNA was visualized with 0.03 mg/ml propidium iodide in antifade.

tion of HeLa cell matrices, which showed that the 2 M salt extraction method is the best suited for study of matrix-adjacent DNA sequences [Fey et al., 1986].

To confirm this observation and to determine if the high salt extraction method could effectively solubilize unattached episomal sequences in fixed HeLa nuclei, we used both extraction methods to compare the nuclear matrix association of episomes in transiently transfected cell lines. HeLa cells were transiently transfected either with Y.343, a plasmid which contains a functional MAR sequence previously shown to efficiently bind to the nuclear matrix, or with pYAC*neo*, the parental vector which does not contain a MAR sequence.

HeLa cells transiently transfected with either pYAC*neo* or Y.343 were harvested and nuclei prepared according to standard techniques. FISH was performed using pYAC*neo* as a probe and the number of signals per nucleus determined. The fixed nuclei were then subjected to extraction with either high salt or ammonium sulphate and relabeled by FISH. In nuclei extracted with high salt, there was a significant decrease, following treatment, in the number of episomes detected in cells transfected with pYAC*neo* (Fig. 2) $\chi^2 = 4.02$, 0.05 significance level), while no significant effect was seen with Y.343 transfected cells (Fig. 2) ($\chi^2 = 0.26$, 0.05 significance level). In contrast, nuclei which were extracted using 250 mM ammonium sulphate did not show any significant alteration in episome signal following extraction in either the Y.343 or pYAC*neo* transiently transfected cell lines ($\chi^2 = 0.67$ and 1.81, respectively, 0.05 significance level).

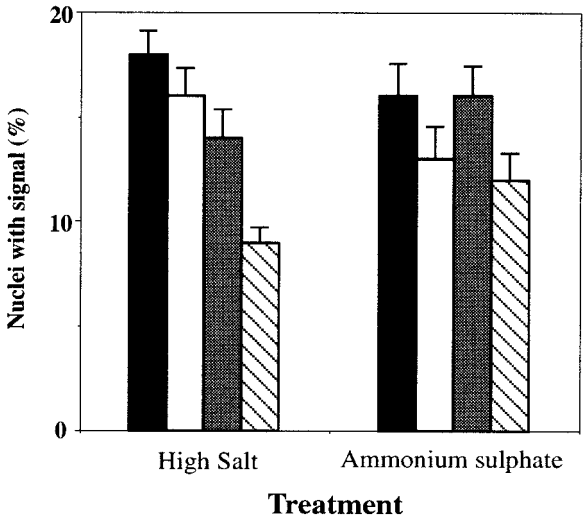

Fig. 2. Differential association with the nuclear matrix of episomes transiently transfected into HeLa cells. Nuclei of HeLa cells transiently transfected with either pYAC*neo* (grey and hatched bars) or Y.343 (black or white bars) were subjected to analysis by FISH using biotin-labeled pYAC*neo* as a probe. Samples were then extracted either with high (2 M) salt or with ammonium sulphate and FISH analysis repeated. The number of fluorescent signals per nucleus before (black or grey bars) and after (white or hatched bars) extraction is shown following either high salt or ammonium sulphate treatment. Bars indicate the standard deviation of the sample.

Although a significant proportion of the pYAC*neo* plasmid was removed following high salt extraction, some of the plasmid was retained following treatment. Reduced efficiency of extraction may be due to the potential collapse of the nuclear architecture after high salt extraction and the subsequent trapping of a small proportion of the episomes in the matrix of insoluble fibers.

While Y.343 showed an association with the nuclear matrix using either extraction technique, pYAC*neo* was efficiently solubilized only following high salt extraction. Previous data had already suggested that high salt was a more efficient method of extruding DNA (Fig. 1) [Vogelstein et al., 1980], and these data further suggest that it is also the most efficient method for solubilizing unattached episomal DNA sequences in fixed nuclei. It should also be noted that ammonium sulphate extraction did show a similar trend in discriminating between matrix-associated and unassociated sequences, and it is likely that with a larger sample number this will become statistically significant.

Subsequently, using the high salt extraction method, we tested four different cell lines for the association of the transfected constructs with the nuclear matrix: two HeLa cell lines which had been stably transfected with Y.343 (cell line C343e2) or YAC.S3 (cell line CS3e1) and which had maintained the sequences episomally for approximately 45 cell generations, cells transiently transfected with Y.X24 (no long-term Y.X24 transfected HeLa cell clone which did not have the DNA integrated into genomic DNA was found), and a cell line which had stably integrated the pYAC*neo* vector (CYACi2). For comparison, untransfected HeLa cells were also tested. Both the Y.343 and YAC.S3 constructs contain the SAR-T consensus sequence [Gasser and Laemmli, 1986]; however, only the MAR sequence in Y.343 has been previously shown to be functional [Wu et al., 1993a]. The Y.X24 construct does not include a MAR se-

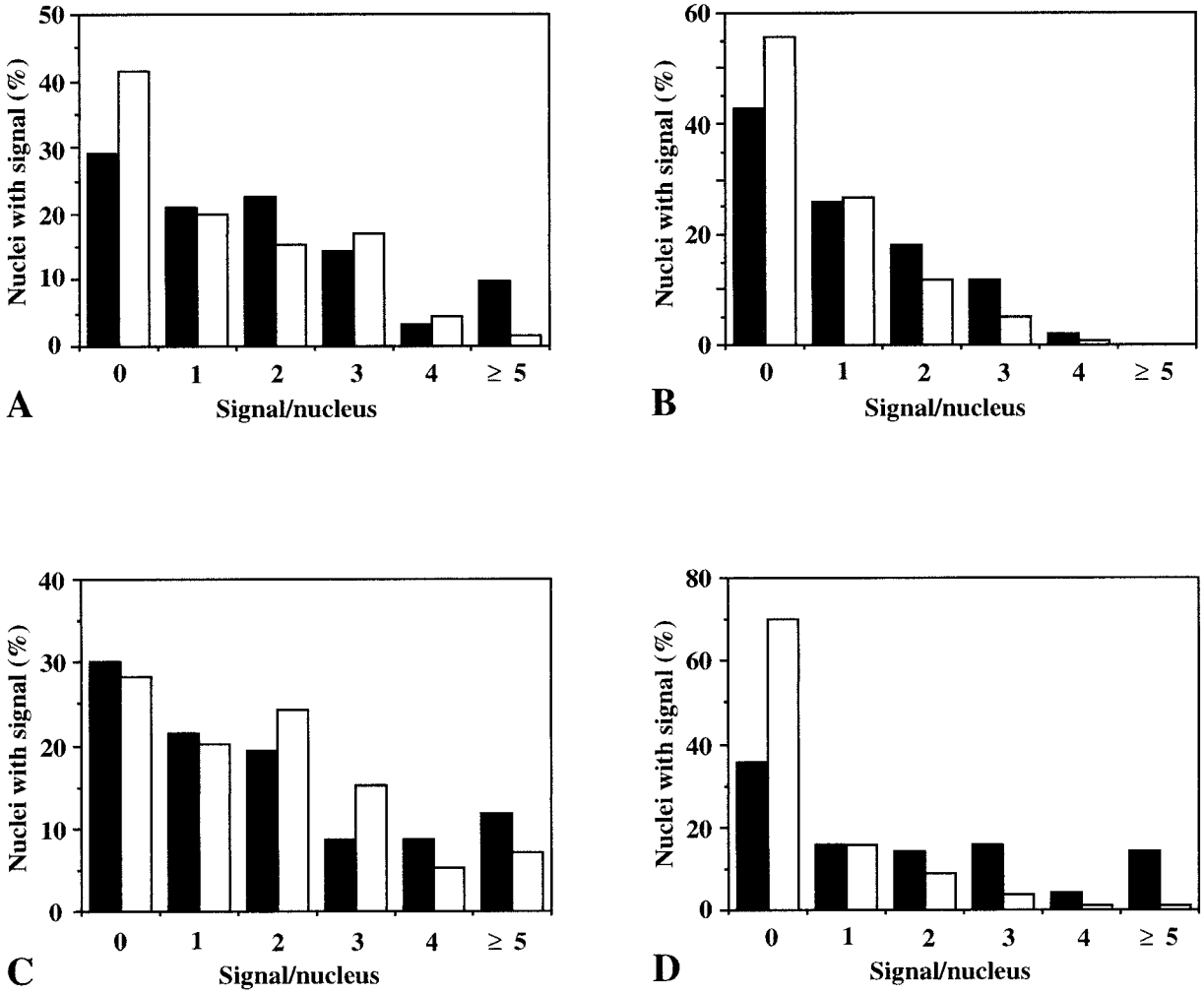

Fig. 3. MAR sequence–containing episomes show no significant alteration in distribution after high salt extraction: in situ hybridization signal distribution in nuclei of HeLa cells. Two cell lines with episomally maintained DNA (C343e2 and CS3e1), a cell line transiently transfected with Y.X24, and a stable integrated (CYACi2) cell line were examined for the presence of transfected DNA. Transfected YAC DNA was detected in methanol/acetic acid–fixed nuclei using fluorescent in situ hybridization with FITC (see Materials and Methods). The number of fluorescent signals per nucleus before salt extraction (black bars) and after salt extraction (white bars) is shown. **A:** HeLa cell line C343e2 (episomal). **B:** HeLa cell line CS3e1 (episomal). **C:** HeLa cell line CYACi2 (integrated). **D:** HeLa cell line transiently transfected with Y.X24 (episomal).

quence [Burhans et al., 1990; DePamphilis, 1993].

Greater than 70% of the labeled nuclei in the C343e2 cell line showed more than one signal per nucleus (Figs. 3A, 4A; Table I), consistent with the presence of multiple copies dispersed at more than one distinct site in the majority of nuclei. A similar pattern was seen with CS3e1 (Figs. 3B, 4C; Table I), although fewer nuclei (54%) showed more than one signal. The integrated cell line (CYACi2) showed a signal distribution pattern similar to that seen in the 343 cell line, with approximately 40% of the nuclei containing three or more signals per nucleus

Fig. 4. Episomes containing a functional MAR sequence remain associated with the nuclear matrix after high salt extraction. Photomicrographs of fluorescent in situ hybridization of HeLa cell nuclei. Cell lines were C343e2 (**A,B**), CS3e1 (**C,D**), CYACi2 (**E,F**), cells transiently transfected with Y.X24 (**G,H**), or cells mock transfected with no DNA (**I,J**). Samples were tested before (A,C,E,G,I) and after (B,D,F,H,J) high salt extraction for the presence of transfected DNA using FITC fluorescent in situ hybridization. In some labeled samples, there was an increase in the intensity of counterstaining with propidium iodide in the nucleolar region of the nucleus, which may be attributed to differences in compaction. However, the green fluorescent signal was clearly distinguished against this red background. ×1,000.

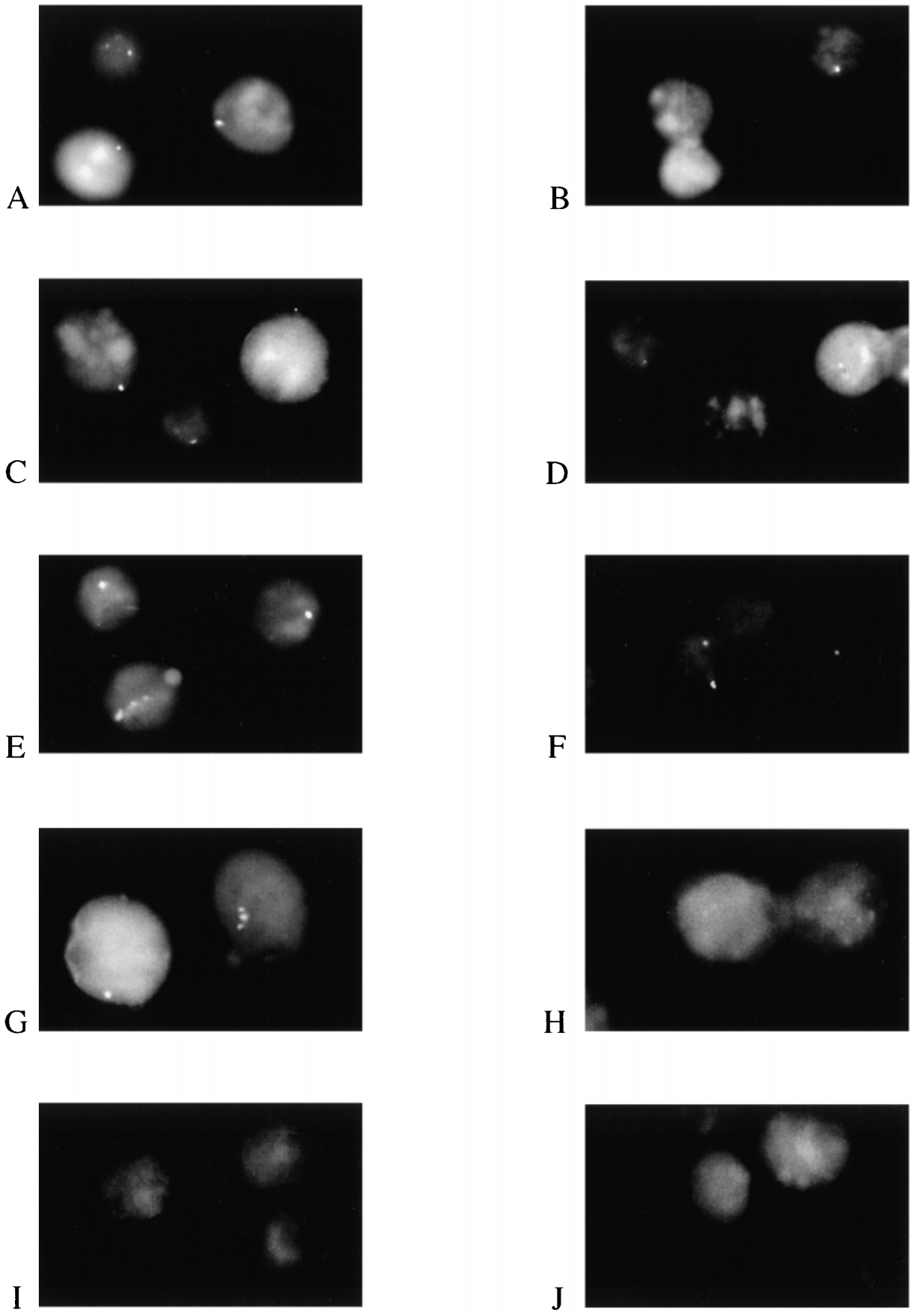

Figure 4

TABLE I. Proportion of Sequences Demonstrating a Specific Association With the Nuclear Matrix*

		Number of nuclei		Percentage of nuclei		Statistical analysis[c]	
Sample[a]	Treatment[b]	Total counted	Total with signal	Total with signal	Total without signal	Groups compared	Significance (0.05 level)
C343e2	None	62	44	71	29	None vs. salt	n.s.
	Salt	65	38	58	42		
CS3e1	None	112	64	57	43	None vs. salt	s.
	Salt	120	53	45	55		
CYACi2	None	103	72	70	30	None vs. salt	n.s.
	Salt	99	71	72	28		
X24 transiently transfected	None	70	45	64	36	None vs. salt	s.
	Salt	110	33	30	70		

*To determine if circular YAC vectors containing replication origin sequences were associated with the nuclear matrix, nuclei from transfected HeLa cells were subjected to high salt extraction and the proportion of nuclei retaining a signal determined using FITC fluorescent in situ hybridization. In situ hybridization was performed on methanol:acetic acid–fixed nuclei from transfected HeLa cells (see Materials and Methods).

[a]Sample indicates HeLa cell lines C343e2 (episomal), CS3e1 (episomal), CYACi2 (integrated), and HeLa cells transiently transfected with Y.X24, as described in Materials and Methods.

[b]Treatment indicates the extraction of the sample in increasing concentrations of NaCl up to 2 M, as described in Materials and Methods. None, samples before treatment; salt, samples after treatment.

[c]Chi-square analysis was performed for all groups comparing sampling distribution with and without signal before (none) and after (salt) high salt extraction. n.s., not significant; s., significant at the 0.05 level.

(Figs. 3C, 4E; Table I). This could indicate either multiple sites of integration or the presence of episomal copies in addition to the integrated copies of the pYAC*neo* vector. HeLa cells transiently transfected with Y.X24 (Figs. 3D, 4G; Table I) showed the majority of nuclei with multiple signals (>75% of nuclei with signal), consistent with cells taking up multiple copies of the plasmid during transfection. A wide range in the number of signals per nucleus for both the episomal and integrated cell lines would be expected even in these clonally derived populations of cells since the cells were grown for at least 45 cell doublings before chromosome preparations were made. During this time, rearrangements and unequal segregation at mitosis would give rise to a more diverse population of cells with varying episome number [Featherstone and Huxley, 1993].

After quantification, the nuclei on the slides were then subjected to 2 M salt extraction. The cells transiently transfected with Y.X24 showed a significant decrease in signal level ($\chi^2 = 20.48$, 0.05 significance level) and a shift from multiple to zero copy signal after treatment (Figs. 3D, 4H; Table I), indicating a lack of association of the transfected DNA with nuclear matrix proteins. In contrast, results obtained with C343e2 showed no significant difference in signal level ($\chi^2 = 2.17$, 0.05 significance level) after treatment with 2 M NaCl (Figs. 3A, 4B; Table I), indicating that the Y.343 episome was associated with the nuclear matrix. However, the CS3e1 cell line showed a significant loss following extraction (Figs. 3B, 4D; Table I) ($\chi^2 = 3.90$, 0.05 significance level), indicating that, despite the presence of a MAR sequence, the YAC.S3 episome was associating only weakly with the nuclear matrix.

The CYACi2 cell line also showed no significant alteration in signal with salt extraction (Figs. 3C, Fig. 4F) ($\chi^2 = 0.08$, 0.05 significance level). This would be expected in the absence of DNase I treatment, as the DNA loops would remain anchored to the matrix by MARs present on the chromosomes [Dijkwel and Hamlin, 1995], and integrated copies of the episome would not be lost. The untransfected HeLa cells (negative control) showed no notable labeling of nuclei either before or after salt treatment (Fig. 4I,J).

Digestion of nuclei with proteinase K (1 mg/ml, 30 min at 37°C) resulted in the collapse of the nuclear skeleton (data not shown) and release of the probe signal. No episomal signal was detected in excess of 62 nuclei, consistent with the attachment of the sequence to the protein component of the nuclear matrix. This was in agreement with work done by Thiebaut et al. [1993], who found that extrachromosomal DNA sequences bound to nuclear matrix proteins were released after protease digestion of the nuclear matrix.

In situ hybridization of metaphase spreads of the cell lines confirmed that in the majority of cells containing multiple signals, the signal was dispersed and not clustered together at one distinct site (Fig. 5). In both C343e2 and CS3e1, the signals were not associated with the chromosomes but sometimes seen as paired spots (e.g., C343e2) (Fig. 5A, arrows) or at the perimeter of the spreads as single spots (e.g. CS3e1) (Fig. 5B, arrows). In the integrated cell line, generally a signal could be seen to be located on one or more chromosomes (Fig. 5C, arrow). In contrast, the transiently transfected cell line (48 h) showed multiple dispersed signals, some of which were associated with chromosomes (Fig. 5D, arrows). The untransfected HeLa cell line showed no labeling (Fig. 5E).

DISCUSSION

Although no strict consensus sequence describing all MARs has been derived, the majority may be classified into related groups according to certain common sequences or structures [Boulikas, 1993]. The SAR-T consensus sequence, originally found in association with developmentally regulated *Drosophila* genes, is closely related to other sequences from an AT-rich class of MARs which include 100–1,000 bp of AT-rich sequences and the DNA sequence motifs ATTA and ATTTA [Gasser and Laemmli, 1986; Boulikas, 1993]. However, a 4–20 nucleotide AT-rich tract alone is generally not sufficient to confer MAR activity, and other factors such as motif spacing, curved or kinked DNA, or other topological characteristics are also important.

Analysis of the stably maintained HeLa cell line (C343e2) after extraction with 2 M NaCl indicated that the episome (Y.343) was associating with the nuclear matrix. The 343 origin sequence contains a SAR-T consensus sequence (TWWTDTTWWW) [Gasser and Laemmli, 1986; Wu et al., 1993a] and had previously demonstrated a specific association with the nuclear

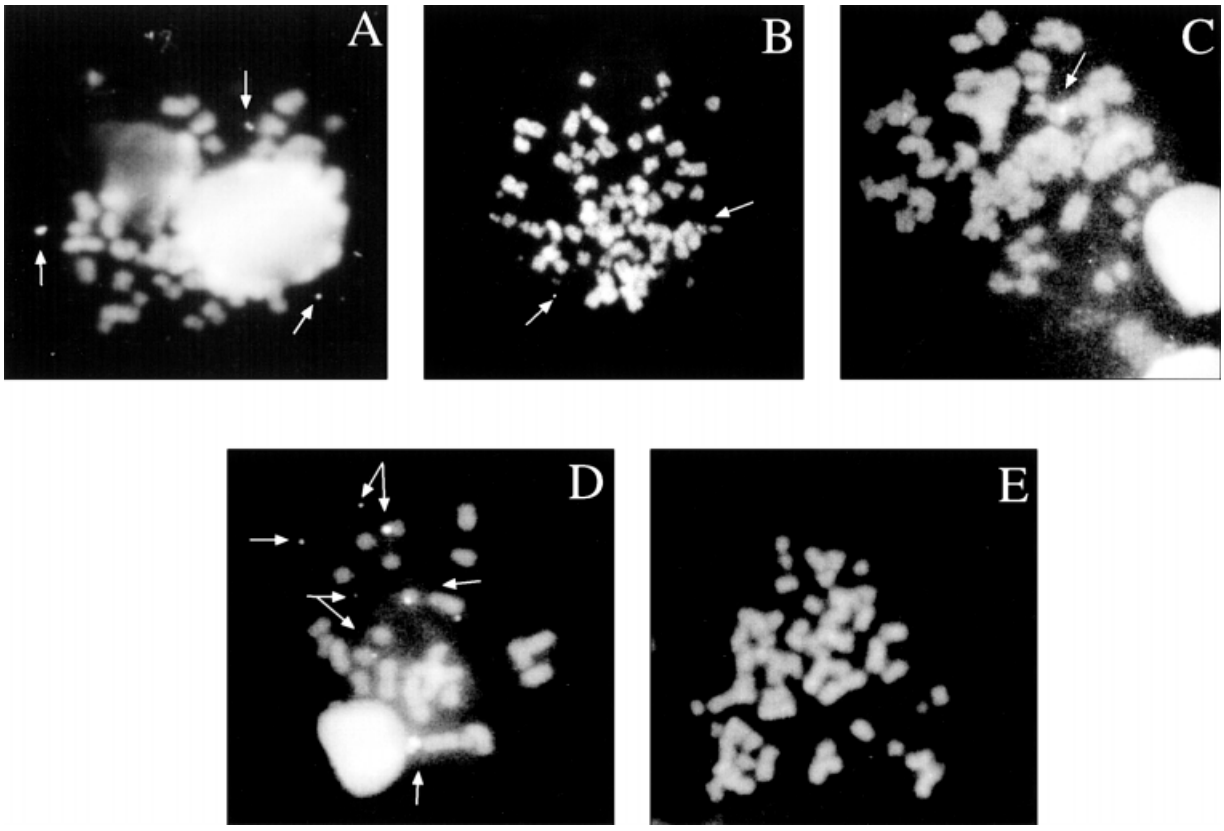

Fig. 5. Fluorescent in situ hybridization of metaphase spreads of stably maintained HeLa cell clones. Two cell lines with episomally maintained DNA (C343e2 and CS3e1), a cell line transiently transfected with Y.X24, and a stable integrated (CYACi2) cell line were examined for the presence of episomal elements. Episomal DNA was detected by fluorescent in situ hybridization, using pYAC*neo* as a probe. Arrows indicate the presence of a signal. **A:** HeLa cell line C343e2 (episomal). **B:** HeLa cell line CS3e1 (episomal). **C:** HeLa cell line CYACi2 (integrated). **D:** HeLa cells transiently transfected with Y.X24 (episomal). **E:** Untransfected HeLa cell line.

matrix [Wu et al., 1993a]. The X24 episome did not show any association with the nuclear matrix, consistent with the absence of a MAR sequence in this portion of the *dhfr* gene locus [Burhans et al., 1990; DePamphilis, 1993]. However, the cell line containing the YAC.S3 episome (CS3e1) also did not show a significant association with the nuclear matrix despite the presence of a SAR-T consensus sequence.

This suggested, firstly, that the presence of an origin alone did not result in nuclear matrix association and, secondly, that the inclusion of a simple MAR sequence within the origin region may not be enough to confer matrix binding activity or may not result in matrix binding demonstrable by these extraction methods. This is consistent with results obtained by Amati et al. [1990], who found that the presence of a MAR sequence alone was not sufficient for MAR activity in yeast containing *Drosophila* SAR sequences. Fine mapping of SAR regions revealed that there was a requirement for a critical minimum length of SAR DNA for optimal binding activity and that there were several AT-rich stretches within this region which cooperatively enhanced MAR binding. Interestingly, the 343 sequence contains three such blocks of >80% AT-rich sequence (57–95 nts in size), juxtapositioned close to the MAR sequence, and has already shown MAR binding activity [Wu et al., 1993a]. However, the S3 origin, while it did contain a MAR sequence, did not contain any similar AT-rich sequence motifs, which may account for a lack of function.

It is still not clear what effect a functional MAR would have on DNA replication and how this would contribute to episome maintenance. MAR sequences are common characteristics of replication origins [Boulikas, 1993], and it is therefore likely that they play a role, albeit not an essential one, in regulating origin activity. In fact, although a functional MAR is not necessary for origin activity, its presence can significantly stimulate DNA replication [Amati and Gasser, 1990]. Thus, it is possible that Y.343 replicates more efficiently due to the presence of a functional MAR, a notion which is consistent with the observation that Y.343 gave a five times higher G418 transformation activity by comparison to YAC.S3 in transfection assays [Nielsen et al., submitted].

The presence of a functional MAR sequence may also increase the retention of the plasmid. Experiments upon the retention of plasmids containing MARs in yeast found that plasmid loss was significantly decreased when a functional MAR sequence was present [Amati and Gasser, 1990]. It is possible that a similar effect is responsible for transformation and maintenance of Y.343; in the initial screening of transfected HeLa cell clones, no stably maintained episomal clones were detected for either the Y.X24 or the pYAC*neo* construct (containing no MAR sequences) out of five clones tested, whereas two (YAC.S3 and Y.343) out of four clones containing MAR sequences showed stably maintained episomal copies [Nielsen et al., submitted]. Subsequently, we have studied another five stably transfected Y.343 HeLa cell clones, of which at least three contain episomal Y.343 ($\chi^2 = 4.32$, significant at the 0.05 level, comparing sequences with and without MAR sequences). It is possible, therefore, that the presence of a MAR sequence enhances the stability of the episome (perhaps by virtue of its attachment to the nuclear matrix), by assisting in its persistence as an episome and/or reducing the likelihood of an integration event. For example, the association of episomal DNA sequences with the nuclear matrix may more effectively replicate episomal DNA [Thiebaut et al., 1993], as replication factories are thought to be fixed at specific sites along the nuclear matrix, replicating DNA by spooling it through the machinery [Hozak et al., 1993]. Thus, MAR sequence-containing episomes would have an increased probability of replicating and would be selectively favored.

Previous work on EBNA-1–expressing human cells with stably maintained Epstein-Barr virus origin (*ori*P)–containing constructs demonstrated that the episomal elements were always associated with the host cell chromosomes [Simpson et al., 1996], and it was suggested from these and other [Rawlins et al., 1985; Harris et al., 1985] data that the viral EBNA-1 protein acts as an intermediary in targeting the movement of the DNA to the host cell nucleus and in attachment of the episomes to the host cell chromosomes and thus is important in long-term episomal maintenance [Krysan et al., 1989].

We were interested in seeing if there was any evidence in our cell clones for a similar specific attachment of the episomes to the host cell chromosomes. FISH studies performed on metaphase chromosomes in the stably maintained cell lines C343e2 and CS3e1 showed the epi-

somes to be in the form of low numbers of extrachromosomal elements, dispersed throughout the chromosomes, similar to the distribution pattern of extrachromosomal elements seen with YACs containing the hypoxanthine phosphoribosyltransferase gene in mouse cells [Featherstone and Huxley, 1993]. Thus, although no evidence was found to support nuclear localization signal targeting of the episomes to the nucleus per se, it is possible that the MAR sequences may play an equivalent role by contributing to the maintenance of episome stability (e.g., by altering replication, retention, or segregation properties, etc).

It is still not clear whether or not there is a minimum size for a stable mammalian chromosome. While some estimates are between 50 and 100 kb for a YAC, a stable human X-derived minichromosome of <10 Mb has been generated using chromosome fragmentation [Farr et al., 1995]. Our study discusses the behavior of a potential "microchromosome" which can be stably maintained as an episome. The maintenance of episomes (i.e., nonintegration) may be enhanced by the presence of a MAR sequence. MAR sequences, therefore, may represent an important auxiliary component in the construction of microchromosomes and human artificial chromosomes, necessary for maintaining the partition of structures with functional domains in replication.

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