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Evidence for binding of extrachromosomal DNA sequences to nuclear matrix proteins in multidrug-resistant KB-V1 cells

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Multidrug-resistant KB-V1 cells carry amplified mdr1 gene sequences located in an extrachromosomal compartment (on episomes). Since episomes do not contain centromeric or telomeric sequences it is unclear whether they are able to bind to nuclear matrix proteins that may regulate episomal gene expression. Using high salt treatments followed by in situ hybridization and dot blot analyses we found evidence for direct binding of episomal DNA to nuclear matrix proteins. This binding could only be reversed after incubation with trypsin or proteinase K as determined by contour-clamped homogeneous electric field (CHEF) electrophoresis. Our findings are consistent with the concept that circular extrachromosomal DNA may not only reintegrate into nuclear DNA but may also be subject to functional control by regulatory proteins within the nuclear matrix.

Episome; Plasmid; Nuclear matrix; Drug resistance; KB-V1 cell

1. INTRODUCTION

Gene amplification has been reported for a variety of human neoplasms. Amplified DNA sequences may not only be found as part of chromosomal DNA but may also be present as elements of submicroscopic circular extrachromosomal DNA (episomes) or microscopically visible double minutes [1,2]. It has been proposed that submicroscpic episomal sequences are derived from nuclear DNA by deletion events that are induced by various forms of molecular stress [3]. After several amplification steps they may multimerize into extrachromosomally located double minutes [4,5]. It has also been proposed that double minutes are capable of reintegration into chromosomal DNA forming homogeneously staining regions [5–7].

Eukaryotic cells contain non-histone proteins that form an insoluble skeletal framework of the nucleus [8–10]. Using appropriate experimental conditions, intact loops of supercoiled eukaryotic DNA can be visualized as a halo surrounding these nuclear matrix proteins [11]. The intimate contact of DNA with its nuclear matrix may serve an organizational purpose. In addition, the nuclear matrix has also been reported to be involved in DNA replication and transcription [8]. There is evidence that the active site of replication requires attachment to nuclear matrix proteins [12].

Vinblastine-resistant human KB-V1 cells contain amplified mdr1 gene sequences of approximately 750 kb as circular supercoiled episomes [13,14]. Overexpression of this gene results in resistance to a large group of lipophilic agents including anthracyclines and vinca alkaloids [15]. Multidrug-resistant KB-V1 cells that contain a large number of episomes have been reported to divide more slowly than the drug-sensitive parent line without episomes suggesting that the presence of episomes may increase cell cycling time [16].

In the current study, we have investigated the possibility that episomal DNA sequences bind to nuclear matrix proteins, a prerequisite for a possible functional interference with replication-controlling units. The KB-V1 model was chosen because it has been useful in a number of studies of mdr1 gene amplification and expression and mdr1 sequences can conveniently be used as marker for amplified episomal DNA.

2. MATERIALS AND METHODS

2.1. *Cells*

Vinblastine-resistant KB-V1 cells and their drug-sensitive parent line KB3-1 were cultivated in Dulbeccos's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 200 mM glutamine, 2000 U/ml penicillin/streptomycin. KB-V1 cells were grown in the presence of vinblastine (1 μg/ml) and contain amplified copies of mdr1 on episomal DNA. KB3-1 cells do not contain amplified copies of mdr1.

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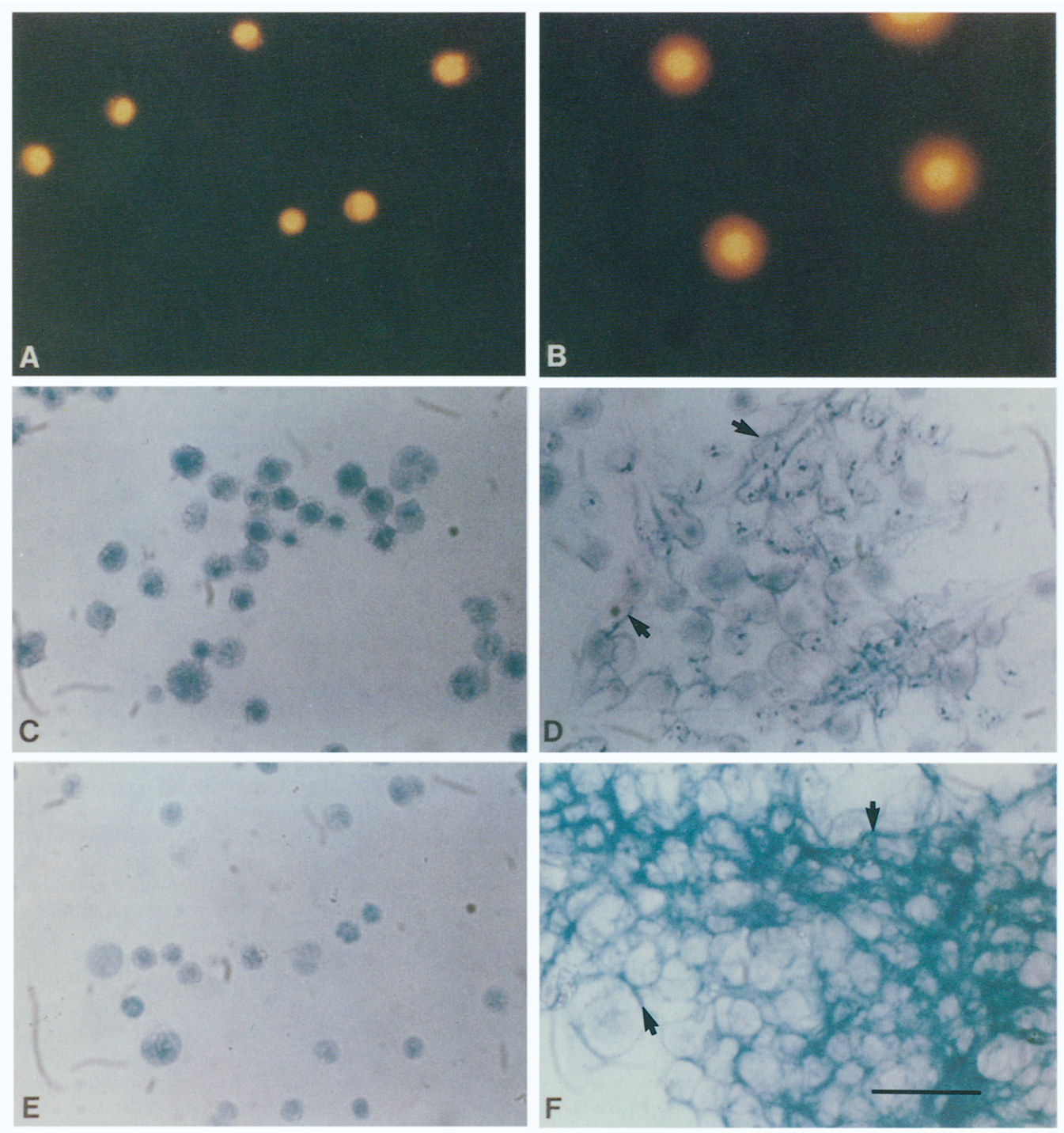

Fig. 1. Production of DNA halos in isolated nuclei of KB-V1 cells. A, fluorescence microscopy – controls with low salt concentration. B, flourescence microscopy – treatment with 2 M NaCl. Note flourescent halo around nuclei. C, Giemsa stain – controls with low salt concentration. D, Giemsa stain – treatment with 2 M NaCl. E, Methyl green stain – controls with low salt concentration. F, Methyl green stain – treatment with 2 M NaCl. Note that at low salt concentrations nuclear matrix and DNA are intimately associated. Treatment with 2 M NaCl induced stretching of supercoiled DNA loops to form halos surrounding the nuclear matrix (arrows). In some cells nucleolar remnants are visualised within the amorphous appearing nuclear matrix. Bar: 4.5 μm.

2.2. *Halo preparation*

Preparation of DNA halos was performed as published by Vogelstein et al. with several modifications [11]. Briefly, nuclei were isolated using 50 mM HEPES, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.22 M sucrose, 0.5% NP40 and transferred into 10 mM Tris containing 0.2 mM $MgCl_2$. Separation of nuclear matrix and DNA was achieved by adding 10 mM Tris, 0.2 mM $MgCl_2$, 2 M NaCl. After addition of 2 μl ethidium bromide (10 mg/ml) an aliquot was examined using a Leitz fluorescence microscope. Another aliquot was irradiated with UV light (10^6 J·cm^{-2}) using a Stratagen UV Stratalinker 2400. Halo prepa-

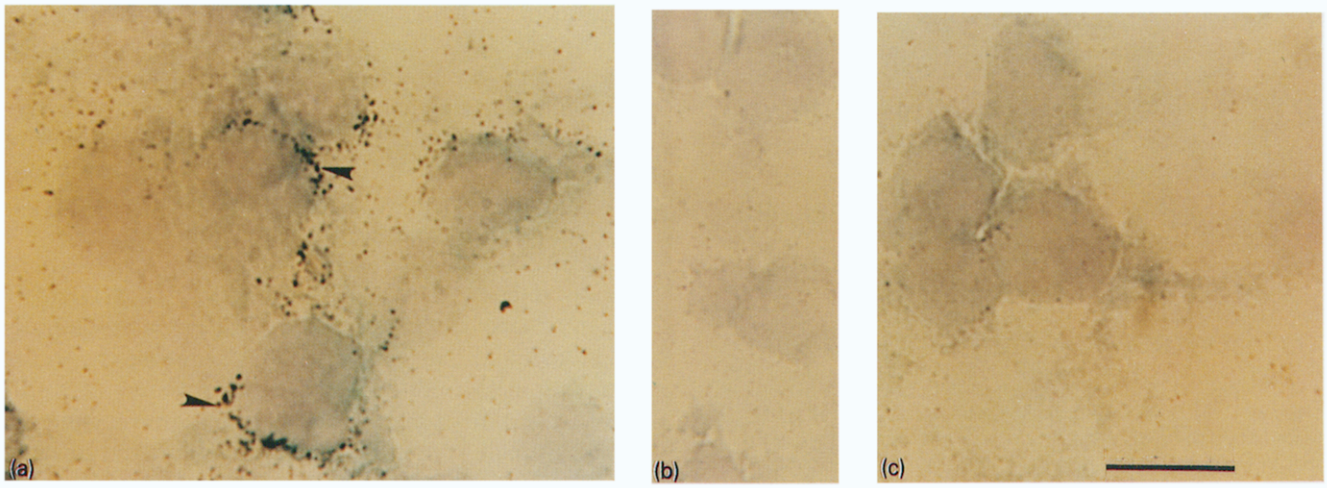

Fig. 2. In situ hybridization of nuclei from KB-V1 and KB3–1 cells after treatment with 2 M NaCl. Hybridization was performed with a biotinylated MP64MDR1 probe after RNAase digestion followed by a two-step gold method using a goat anti-biotin antibody-1 nm gold complex. The signal was amplified (× 4) with silver. A, nuclei from mdr-1 positive KB-V1 cells. Note intense labeling of nuclear matrix (arrows). B, nuclei from mdr-1 positive KB-V1 cells after treatment with DNase. Note the absence of label. C, nuclei from mdr1-negative KB3–1 cells. Note the absence of label.

rations were made from both irradiated and unirradiated samples. Aliquots of the preparations were further processed for staining (Giemsa, Methyl green) and for in situ hybridization using standard techniques [17].

2.3. *In situ hybridization and peroxidase reaction*

The MP64MDR1 probe was prepared using a kit from QIAGEN Inc. (Chatsworth, CA) and was labeled with biotinylated UTP using a kit from Boehringer Mannheim Co. (Indianapolis, IN). After incubation with RNase (25,000 U/mg, 100 μg/ml) or DNase (2 000 U/ml, 20 μl) the slides were immersed in 70% formamide, 2 × SSC (70°C, 10 min) and rapidly cooled on ice. Ten microliters of hybridization mixture was placed on each slide, covered with a plastic cover slip and incubated over night in a moist chamber at 37°C. The slides were then washed six times in 50% formamide/2 × SSC, 3 times 2 × SSC followed by phosphate-buffered saline, pH 7.2. The peroxidase reaction was carried out using Streptavidin-Peroxidase (Jackson Immunoresearch, West Grove, PA; 2 μg/ml, 30 min). After removal of the complex, the slides were incubated for 10 min with diaminobenzidine and H_2O_2 and stained with Methyl green.

2.4. *Colloidal gold and silver amplification*

Slides were incubated with 20 μg/ml goat-gold complex anti-biotin (Goldmark, Phillipsburg,NJ) in PBS/10% BSA for 4 h. After removal of the gold complex, the slides were fixed in 3.7% formaldehyde/PBS. Signal enhancement was achieved with a silver enhancer kit from Goldmark.

2.5. *DNA extraction, dot blot analysis, and CHEF electrophoresis*

DNA extraction and dot blotting was done using standard techniques. Contour-clamped homogeneous electric field (CHEF) electrophoresis was performed as described earlier [4]. Experimental conditions were chosen for optimal resolution in the range of 200–2000 kbp. After staining with ethidium bromide the gels were photographed and blotted on nylon membranes for Southern blot hybridization with the MP64MDR1 probe. For controls, rehybridization was performed with p11A2 (kindly provided by Dr. G. Wahl, San Diego, CA) and with MP64MDR1 after preincubation of the CHEF gel blots with RNase (100 μg/ml).

3. RESULTS

Production of DNA halos was accomplished by exposing the nuclei preparation of KB-V1 cells to increasing concentrations of NaCl. This treatment forced DNA loops to stretch outside the nuclear matrix. As shown in Fig. 1A–F, fluorescence micrsocopy, as well as Giemsa and Methyl green stains, of cell nuclei revealed halos that contain filamentous material representing DNA while the nuclear matrix appears as more amorphous material in the stained preparations. Some nucleolar remnants were noted inside the matrix with more intensive staining. The same result was obtained when the nuclei were either incubated with ethidium bromide followed by flourescence microscopy or when methyl green was used for DNA staining. In situ hybridization with MP64MDR1 followed by amplification of the signal with colloidal gold and silver enhancement showed binding of the label to the nuclear matrix (Fig. 2). Incubation with RNAase prior to hybridization decreased the signal intensity indicating that some of the mdr1 sequences were present within the RNA pool. No labeling was observed in drug-sensitive KB3–1 cells that lack the mdr1 gene. Labeling was abolished by pretreatment with DNase.

Total DNA was extracted from matrix pellets and from intact nuclei with or without pretreatment with 2 M NaCl, in an attempt to further provide evidence for binding of episomal DNA to nuclear matrix proteins. No DNA was found in the supernatant. Probing with MP64MDR1 showed that pretreatment with 2 M NaCl did not influence the amount of mdr1 sequences detected (Fig. 3). Densitometry of dot blots gave the following MP64MDR1/p11A2 average ratios: Control: 2.61 ± 0.82, 2 M NaCl-treatment: 2.31 ± 0.54. CHEF electrophoresis was performed on KB-V1 and KB3–1 cells using a resolution range of 200–2000 kbp. As shown in Fig. 4, pretreatment with proteinase K or trypsin was required to release episomes into the gel and

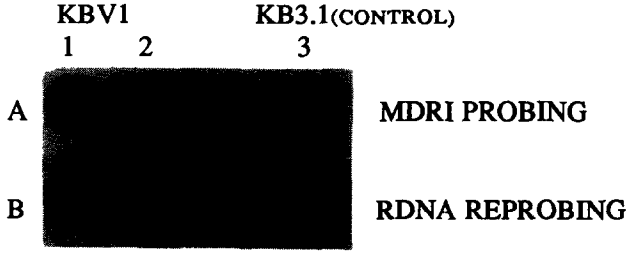

Fig. 3. Dot blots of DNA extracts form KB-V1 and KB3–1 cells after treatment with 2 M NaCl using MP64MDR1 and P11A2 probes. The amount of mdr1 sequences detected was not decreased after exposure to 2 M NaCl suggesting that episomal DNA may be bound to nuclear matrix proteins.

to generate bands at 560 kbp and 750 kbp. Exposure to gamma-irradiation was ineffective. RNase digestion prior to reprobing yielded identical bands. Reprobing with p11A2 was negative. This result further supports the notion that episomal DNA sequences may bind to nuclear matrix proteins.

4. DISCUSSION

Replication and transcription of chromosomal DNA has been reported to be associated with binding of DNA to nuclear matrix proteins [12,18]. These observations have led to the hypothesis that the nuclear matrix may have a role in the regulation of replication or transcription. Episomes are submicroscopic circular pieces of extrachromosomal DNA within the nucleus that may carry amplified oncogenes and multidrug-resistance genes. Episomes are devoid of centromeric or telomeric sequences that are required for binding of chromosomal DNA to the nuclear matrix. It has therefore been unclear whether episomes can bind to matrix proteins and how replication or transcription of episomal DNA sequences is regulated.

We now present evidence for binding of episomes that carry amplified mdr1 sequences in KB-V1 epidermoid cancer cells to nuclear matrix proteins. The MDR1/KB-V1 system was chosen since MDR1 is a convenient marker for episomal DNA and the drug-sensitive parental cell lines KB3–1 can be used for controls. Our first experiment was performed under the assumption that forcing nuclear DNA loops outside the nuclear matrix by treatment with 2 M NaCl will solubilize smaller pieces of DNA not tightly attached to the matrix. We were unable to find any episomal DNA in the supernatant of our preparations indicating that most if not all extrachromosomal DNA is attached to the nuclear matrix. Detection of MDR1 sequences that remain attached to the nuclear matrix was achieved using silver amplification of the hybridization signal after RNase treatment. Potential limitations of this approach are that some MDR1 sequences might have been reintegrated into chromosomal DNA as homogenously staining regions or that extrachromosomal DNA might have been non-specifically trapped by the excess amount of chromosomal DNA. To address this possibility dot blotting experiments were performed on nuclear preparations with or without pretreatment with 2 M NaCl and on MDR1-negative KB3–1 nuclei. These experiments indicate that no significant loss of MDR1 sequences occurs. In addition, CHEF electrophoresis demonstrated that episomal DNA sequences hybridizing with the MDR1 probe were only released after protease digestion of the nuclear matrix. However, some of the episomal sequences remained within the agarose blocks and failed to migrate into the gel even after protease treatment. The reason for this observation is not clear but may include trapping of episomal DNA by genomic DNA. Also, the size of some episomes may have been beyond the resolution of the CHEF electrophoresis. Thirdly, if incorporation of MDR1 sequences

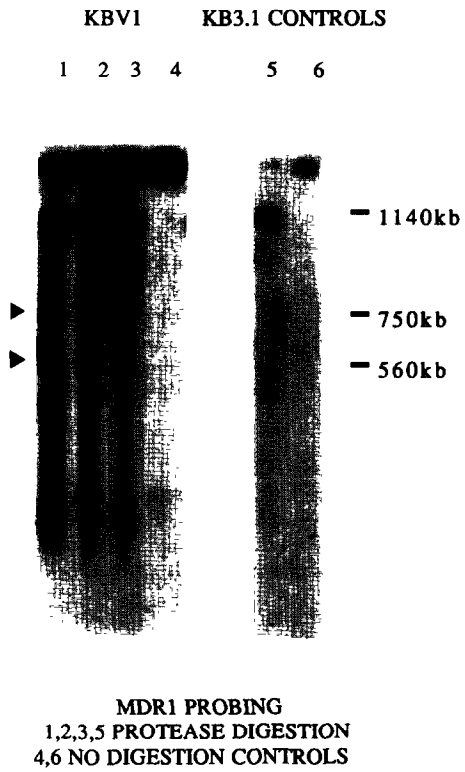

Fig. 4. Southern blots of CHEF electrophoresis using KB-V1 and KB3–1 cells. Hybridization was performed using the MP64MDR1 probe. Pretreatment with proteases was required to release episomal DNA sequences into the gel whereas gamma-irradiation alone was ineffective. Lane 1, KB-V1 cells – pretreatment with proteinase K and gamma-irradiation; lane 2, KB-V1 cells – pretreatment with proteinase K; lane 3, KB-V1 cells – pretreatment with trypsin, lane 4, KB-V1 cells – pretreatment with gamma-irradiation; lane 5, KB3–1 cells – pretreatment with proteinase K and gamma-irradiation; lane 6, KB3–1 cells – pretreatment with gamma-irradiation.

into chromosomal DNA had occurred, migration into the gel would not be possible.

In summary, we have provided evidence that episomal DNA may bind to nuclear matrix proteins. Future research will be required to address the specificity and functional significance of this association. Recent evidence suggest that presence of episomal DNA may increase cell cycling time and thus may slow tumor growth in vitro [16]. It is tempting to hypothesize that this may be the result of interference (by episomal DNA) with the orderly progression of replication of chromosomal DNA in vitro. By binding to sites within the nuclear matrix that are involved in the regulation of transcription and/or replication episomal DNA may compete with genomic DNA for further processing. This concept would predict increased cell cycling times in cells that carry extrachromosomal DNA. Identification of the exact binding sites could be important for the development of new therapeutic agents.

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