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Circular DNA of 3T6R50 double minute chromosomes

Alexander M.van der Bliek, Carsten R.Lincke[1] and Piet Borst*

Division of Molecular Biology and The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam and [1]Department of Biochemistry, University of Amsterdam, Academisch Medisch Centrum, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

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ABSTRACT

In pulsed field gradient gel electrophoresis (PFGE) the intact deproteinized circular DNA of Mycoplasma (800 kb) and Escherichia coli (4700 kb) remains trapped in the slot. We show here that γ-irradiation of the DNA in agarose plugs is a convenient method to partially convert these circles into full-length linears, migrating with the expected mobility in PFGE. We have used this method to study the structure of Double Minute chromosomes (DMs) from the methotrexate (MTX)-resistant mouse cell line 3T6R50. Intact deproteinized DM DNA is immobile in these gels, but is converted into a single band of about 2500 kb by either γ-irradiation, DNaseI in the presence of Mn^{2+}, or restriction enzymes. We conclude that the DM DNA in 3T6R50 cells consists of a homogeneous population of 2500-kb circles.

INTRODUCTION

Gene amplification underlies many forms of resistance to cytotoxic drugs (1). The amplified DNA is either inserted in pre-existing chromosomes or present in separate entities. These range in size from submicroscopic elements (plasmids)(2, 3), through double minute chromosomes (DMs) visible under the light microscope (1, 4, 5), to large ring chromosomes (6). The plasmids identified in mammalian cells were shown to be 300 or 650 kb circles (2, 3). DNA circles ranging from 30 to 120 kb and containing amplified DNA have also been observed in the protozoan genus Leishmania (7, 8). On the basis of these data one may expect that the much larger DNA in DMs will also turn out to be circular. Indeed, no free ends were observed in DM chromatin (5) by electron microscopy (EM).

Nevertheless, more convenient and conclusive analytical procedures are required for the analysis of DM DNA. EM of chromatin is a specialized technique, it cannot exclude the possibility that the ends of a linear DNA are held together by protein, and it does not provide precise quantitative information on the size and size homogeneity of DM DNA. Moreover, not all amplification events lead to DNA circles. For instance, developmental gene

amplification may lead either to stable forked molecules or to linear mini-chromosomes (9). We have therefore tried to adapt available gel techniques to analyse DM DNA.

We have previously shown that the intact DNA molecules present in DMs are trapped in the slots of PFGE gels (10). This is the behaviour expected of large circles (11, 12), but also of very large linear DNA molecules. It should be possible to discriminate between these alternatives by introducing single double-strand breaks in the DM DNA followed by size-fractionation of the products with recently improved PFGE methods (13, 14, 15) that resolve linear duplex DNAs up to 7 megabasepair (Mb). We have found that γ-irradiation of DNA immobilized in gels provides a convenient method to introduce single double-strand breaks breaks in large DNA molecules. This method was first tested with bacteria of known genome size (16, 17), and then applied to DNA of the mouse cell line 3T6R50, which has amplified dihydrofolate reductase DHFR-genes on DMs (18).

MATERIALS AND METHODS

Sample preparation. Bacterial cultures were grown to stationary phase, then pelleted and resuspended in phosphate-buffered saline, after which DNA embedded in agarose was prepared as described previously (11), except that lysis of E. coli samples was preceded by a 4 h incubation with 1 mg lysozyme per ml at 30 °C. The Mycoplasma samples, M. hyorhinis strain DBS1050 and M. hominis strain VKL312 (obtained from Dr. A.F. Angulo, Utrecht), contained DNA from 1.6 X 10^9 cells in 50 µl and the E. coli samples (strain DH5) contained DNA from 2.5 X 10^7 cells in 50 µl. Schizosaccharomyces pombe strain 972 h^- (obtained from Dr. J. Klootwijk, Amsterdam) was pelleted and resuspended in phosphate-buffered saline and DNA was prepared after zymolyase treatment in agarose (19); each 50 µl sample contained 2.5 X 10^7 cells. The MTX-resistant mouse cell line 3T6R50 (obtained from Dr. R.T. Schimke, Stanford) was cultured as described previously with 50 µM MTX (18). We obtained a revertant cell line by culturing 3T6R50 for 3 months without methotrexate. Genomic DNA embedded in agarose was prepared as described above with 10^6 cells per sample of 50 µl. DNA samples of Saccharomyces cerevisiae strain DBY702 were obtained from Dr. P.J. Johnson. Phage lambda DNA was embedded in agarose and annealed in 10 mM sodium EDTA and 10 mM Tris/Cl, pH 7.5, at room temperature with 1 µg DNA per sample.

Controlled degradation of DNA. Samples were γ-irradiated, immersed in 10 mM sodium EDTA (ethylene-diamine-tetra-actetate) and 10 mM Tris/Cl, pH 7.5,

by placing them between two ^{137}Cs sources (1.0 Gray/min). Alternatively, 3T6R50 DNA was degraded with pancreatic DNaseI (Boehringer GmbH, Mannheim) after a preincubation with enzyme but without divalent cations to allow the enzyme to diffuse into the agarose before the reaction was started when $MnCl_2$ was added to 0.6 mM (10). Partial NotI digests were obtained by incubating for 1 h with 10 u enzyme (New England BioLabs, Inc., Beverly, Mass.) at 37 °C in the buffer recommended by the manufacturer. The reactions were stopped with washes in 0.1% SDS, 10 mM sodium EDTA and 10 mM Tris/Cl pH 7.5.

Pulsed field gradient gel electrophoresis. DNA was size-fractionated with a PFGE-apparatus (13), which is a hybrid of the two original designs (11, 19), except for the experiment presented in Fig. 2, which shows fractionation with a Pulsaphor apparatus (L.K.B., Bromma). Electrophoresis was in 90 mM Tris, 90 mM Borate and 1 mM EDTA pH 7.0 at 15 °C with 0.5 or 0.7% agarose gels and with voltages, switch frequencies and run-times as specified in the figure legends. After the run the gels were stained with ethidium bromide, destained, UV-fluorescence was photographed and the DNA was blotted to nitrocellulose (20). Pre-run samples had been placed in dialysis tubes and subjected to alternating electric fields as in the final fractionation.

Hybridization conditions. The following probes were used: pR400-12 (20; DHFR cDNA obtained from Dr. A.D. Levinson, San Francisco), p5-248R72H20 (21; M-43.2 genomic DNA obtained from Dr. R.T. Schimke, Stanford) and pAct-1 (22; hamster actin cDNA obtained from Dr. H. Bloemendal, Nijmegen). Plasmid DNA was prepared by alkaline lysis and the inserts were gel-purified and nick-translated (23) for hybridization in 3 X SSC (1 X SSC = 150 mM NaCl, 15 mM Na-citrate pH 7.0) and 0.1% SDS (sodium-dodecyl-sulphate), 5 X Denhardt-solution, 10% Dextran-sulfate and 50 µg denatured salmon sperm DNA per ml at 65 °C (24). The final washes for the DHFR and actin cDNA probes were in 0.1 X SSC and 0.1% SDS at 65 °C, or in 0.03 X SSC and 0.1% SDS at 65 °C for the M-43.2 probe. Before re-hybridization the previous probe was removed by washing in 0.1% SDS at 65 °C and the blot checked with a test exposure.

RESULTS

Linearization of bacterial genomes by γ-irradiation. We have tested linearization of circular DNA by γ-irradiation on well-defined large circular molecules: genomic DNA from Mycoplasma bacteria (Fig. 1). Even without treatment, a band of 800 kb corresponding to the EM-measured genome

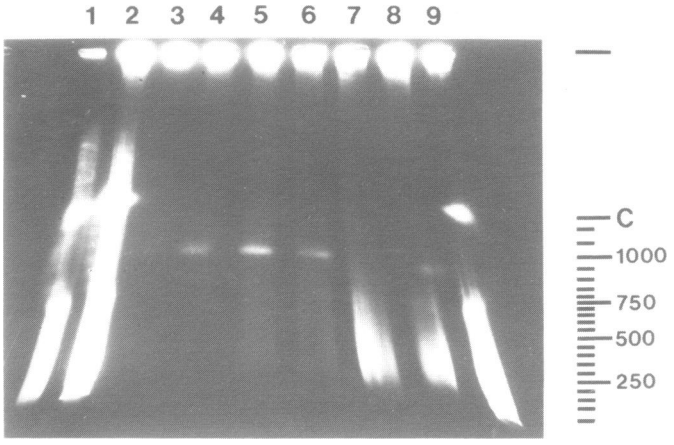

Fig. 1. Mycoplasma DNA linearized by γ-irradiation.
Ethidium bromide stained PFGE gel with untreated M. hominis DNA in lane 8, pre-run M. hominis DNA in lane 3, pre-run and γ-irradiated M. hominis DNA in lanes 4-7 with doses of 10, 30, 60 and 180 Gray, S. cerevisiae DNA as size-marker in lane 1, and multimers of phage lambda DNA as size-marker in lanes 2 and 9. The compression zone is marked by C. The samples were size-fractionated on a 0.7% agarose gel with 200 V fields alternating every 75 sec for 24 h.

size for M. hominis (16) was visible in ethidium-stained gels after PFGE (Fig. 1, lane 8). Similar results were obtained with the slightly larger DNA of M. hyorhinis (17), which banded at 850 kb (not shown). Since a large part of the DNA remained trapped in the slot, we attribute the band and smear below it to degradation and removed this with a pre-run under PFGE-conditions (Fig. 1, lane 3). With subsequent γ-irradiation, the 800-kb fragments reappeared in a dose-dependent fashion, i.e. at low doses the fraction of linearized circles increases and at higher doses these are in turn converted to smaller fragments (Fig. 1, lanes 4-7). Part of the DNA, however, is released only at the highest dose apparently without banding at 800 kb. Such trapping is either an artefact common to PFGE gels, or represents aberrant structures in the Mycoplasma DNA. γ-Irradiation may cause up to 100 times more single-strand breaks than double-strand breaks (25). However, single-strand breaks have no known effect on the mobility of linear DNA in agarose-gels nor do we expect it to influence trapping in these experiments. Theta-structures from replicating bacteria, for example, would not be expected to run as a discrete band. Nevertheless, this experiment demonstrates that γ-irradiation effectively linearizes large circular DNA molecules, which can then be size-fractionated by PFGE.

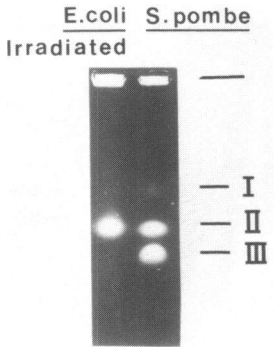

Fig. 2. E. coli genome linearized by γ-irradiation.
Ethidium bromide stained PFGE gel with γ-irradiated (1 Gray) E. coli DNA and S. pombe DNA. I-III indicate chromosomes I-III from S. pombe, which were used as markers. The samples were size-fractionated on a 0.7% agarose gel with 90 V fields alternating every 75 min for 150 h.

Recently conditions have been described that allow the size fractionation of DNA fragments in the order of Megabasepairs. The chromosomes of S. pombe were used as markers with sizes estimated to be: 3, 6 and 9 Mb (14) or 3, 5 and 7 Mb (15) (chromosomes III, II and I, respectively). To get an additional reliable marker in this size range, we have used linearized E. coli DNA, known to be 4.7 Mb on the basis of restriction enzyme mapping (26, 27). Fig. 2 shows that γ-irradiated E. coli DNA runs slightly slower than chromosome II of S. pombe under our PFGE conditions, suggesting that the previous estimates for the size of this chromosome may have been too high. It should be kept in mind, however, that mobility of large DNAs may not be totally independent of DNA base composition and sequence, as noted by Bernards et al. (28).

Banding of 3T6R50 DMs after γ-irradiation. The 3T6R50 cell line contains small DMs homogeneous in size (18 and our observations); the DMs harbour the 50-fold amplified dihydrofolate reductase (DHFR) gene (18); DHFR overproduction is the cause of MTX resistance in this cell line. We exposed 3T6R50 DNA to γ-irradiation and size-fractionated the samples with a resolution of up to 3000 kb (Fig. 3a). Increasing the radiation dose (lanes 3-6) led to increased degradation of bulk DNA, which was otherwise trapped in the slot (lane 2). Fig. 3b shows that the irradiation released a discrete band that hybridized with a DHFR cDNA probe. This band migrates at about 2500 kb, it is faintly visible in the ethidium-stained gel (Fig. 3a) and it is also detected by a second DNA probe (M-43.2, see Fig. 3c), which maps 43 kb upstream of the DHFR gene promoter (21). As the latter probe contains repetitive sequences, it gives a high background of non-specific hybridization. When the dose of γ-irradiation was increased to 100 Gray, the 2500-kb band was replaced by a smear of degraded DNA (Fig. 3b, lane 6).

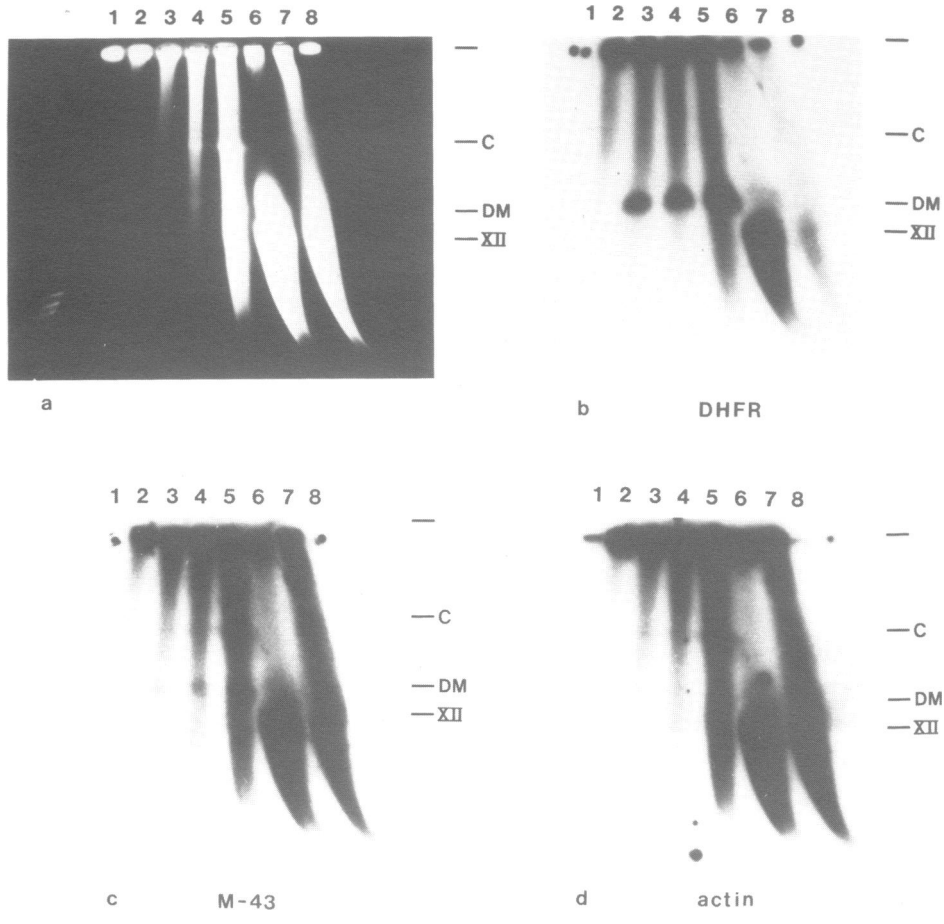

Fig. 3. 3T6R50 DMs linearized by γ-irradiation.
a. Ethidium bromide stained gel with DNA size-fractionated by PFGE; b. blot of the gel in a. hybridized with DHFR cDNA; c. as b. but hybridized with probe M-43.2 (a genomic fragment mapped 43.2 kb from the DHFR gene); d. as b. but hybridized with an actin cDNA. The samples were as follows: untreated 3T6R50 DNA in lane 2, γ-irradiated 3T6R50 DNA in lanes 3-6 (doses of 3, 10, 30 and 100 Gray), γ-irradiated 3T6R50-revertant DNA in lane 7 (dose of 30 Gray) and S. cerevisiae DNA in lanes 1 and 8 as size-marker. The compression zone is marked by C, the position of linearized DM DNA by DM and S. cerevisiae chromosome XII by XII. This latter chromosome was identified with a rDNA probe (not shown). The samples were size-fractionated on a 0.5% agarose gel with 200 V fields alternating every 240 sec for 24 hr.

Random breaks in circles fit a Poisson distribution with a maximum of 38% (= 1/e) singly hit DMs at the optimal dose (1 break per circle), while 10 times more irradiation reduces this to 0.05%, which explains the sudden drop at

100 Gray. As with the Mycoplasma samples, we attribute the remaining slot-hybridization to trapping inherent to PFGE or to replication intermediates. Cells in S-phase presumably make up a sizeable fraction of our sample, because the cultures were harvested before reaching confluency to avoid degradation from cell-death. The 2500-kb band is not a compression artefact, as it was not detected by an actin gene probe (Fig. 3d). It is also dependent on the presence of specific DMs, as the band was absent from a 3T6R50 revertant cell line, obtained by culturing 3T6R50 for 3 months without MTX (Fig. 3b, lane 7). Hence, we conclude that the 2500-kb band represents full-length DM DNA linearized by γ-irradiation.

To test whether multimers of the 2500-kb DM DNA exist, we have also fractionated partial NotI-digests and γ-irradiated samples of 3T6R50 DNA under conditions shown to seperate the S. pombe chromosomes. The ethidium stain shows that 3T6R50 DNA can also be size-fractionated in this range (Fig. 4a). In the NotI-digested sample the array of rDNA repeats forms a discrete band, but not in the irradiated samples. We detect no multimers of DM DNA in this size range, however (Fig. 4b). We provisionally estimate the size of linearized DM DNA to be about 2500 kb, since it migrates much faster than the 3-Mb chromosome III of S. pombe, but somewhat slower than chromosome XII of S. cerevisiae, recently reported to be 2.5 Mb (15). Clearly, the size estimates in this range are still very approximate.

Enzymatic degradation of 3T6R50 DMs. To exclude the possibility that DM DNA contains sites hypersensitive to γ-irradiation that would yield discrete fragments irrespective of DM structure, we also introduced double-strand breaks with enzymes. Partial digests were obtained with DNaseI in the presence of Mn^{2+}, which produces double strand breakage (10, 29), and with the infrequently cutting restriction enzyme NotI. These treatments yielded a 2500 kb band hybridizing to the DHFR cDNA (Fig. 5). Progressive NotI digestion yields a series of partials and the end-product, a 300-kb fragment detected by the DHFR probe. Although the intensity of the 1.6-Mb NotI partial suggests that the DMs comprise two amplicons (dimers), we have not yet succeeded in mapping the DMs unequivocally. We do, however, find that other infrequently cutting restriction enzymes (MluI, NaeI, SmaI, and SfiI) also yield comigrating 2500-kb partials without any larger products detected by the DHFR probe (not shown).

A larger fraction of hybridizing material was present in the 2500-kb band with γ-irradiation than with enzyme digestion under all conditions tested. This was not unexpected, since nucleases are not homogeneously

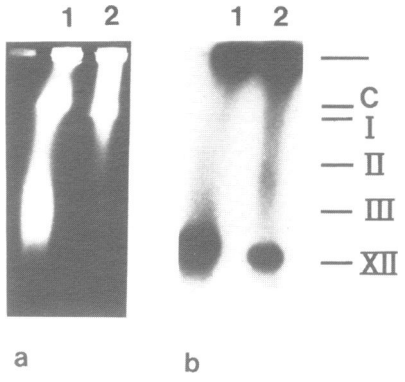

Fig. 4. 3T6R50 DM DNA size-fractionated in the range up to 7 Mb.
a. Ethidium bromide stained gel with DNA size-fractionated by PFGE; b. blot of the gel in a. hybridized with the DHFR cDNA. The 3T6R50 samples were γ-irradiated (20 Gray) or partially digested with NotI. C marks the position of the compression zone, I-III of S. pombe chromosomes I-III and XII of S. cerevisiae chromosome XII. This latter chromosome was identified with a rDNA probe (not shown). The samples were size-fractionated on a 0.5% agarose gel with 55 V electric fields alternating every 45 min for 150 hr.

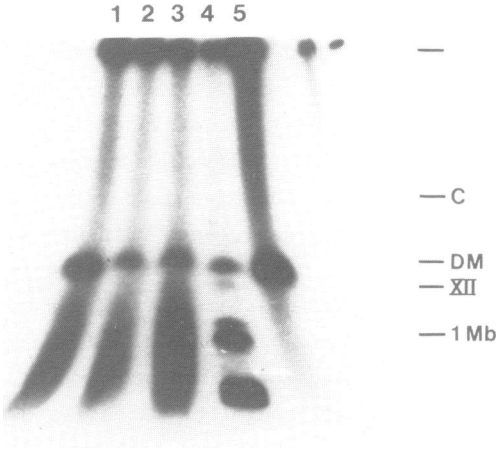

Fig. 5. 3T6R50 DMs linearized enzymatically.
Samples of 3T6R50 DNA were size-fractionated by PFGE, blotted to nitrocellulose and hybridized with a DHFR cDNA. The DNA in lanes 1-3 had been digested with DNaseI (0.5, 1 and 2 µg enzyme per ml), partially digested with NotI in lane 4 and γ-irradiated in lane 5 (30 Gray). C indicates the position of the compression zone, DM of linearized DM DNA and XII of S. cerevisiae chromosome XII. The samples were size-fractionated on a 0.5% agarose gel with 200 V fields alternating every 240 sec for 24 hr.

distributed throughout the gel plug in partial digestion experiments, whereas the γ-irradiation dose is evenly distributed through the gel. This makes γ-irradiation the method of choice for the measurement of large circular DNA molecules and for generating size-markers from well-characterized bacterial genomes.

DISCUSSION

Our experiments show that γ-irradiation provides a convenient method to linearize large DNA circles in agarose plugs. In combination with the rapidly improving PFGE procedures for size fractionation of large DNA, γ-irradiation should allow a rapid determination of the size and size heterogeneity of any circular DNA in the range of 0.1 to 7 Mb. This range covers most circular DNA molecules of biological interest that cannot be easily studied by other methods, i.e. bacterial DNAs and large plasmids. Given that radiation damage follows a Poisson distribution, the optimal dose is proportional to the inverse of the target size. Linearized molecules are, however, visible with a broad range of doses (Fig. 1). The main limitation of the method is its low yield. The Poisson distribution predicts that breakage by random-hits maximally yields 38% full-length linears; moreover, some of these long linears are trapped in the slot in all currently available PFGE systems. Hence, the method is less suitable for quantitating the relative amounts of circular DNA molecules or finding minor circular species.

Our results with the 3T6R50 cells lead us to conclude that the DMs in this cell line contain 2.5-Mb circles. Although only a minority of the DHFR genes end up in the 2.5-Mb band after irradiation or nuclease digestion, we think that all the DMs contain 2.5-Mb circles. In unirradiated plugs all DM DNA remains in the slot; DNA breakage only results in 2.5-Mb linears without other discrete bands in the 0.5-7 Mb range; and although a substantial fraction of the DNA remains in the slot in all irradiation doses, the same holds for chromosomal DNA, as represented by the actin genes.

To test whether the circularity and homogeneous size of DM DNA in 3T6R50 cells is representative of DMs in general, we have tested two other cell lines (10 and unpublished results). In both lines the intact DM DNA also remained trapped in the slot during PFGE. One of the lines, yielded a sharp 3-Mb band hybridizing with probes for the amplified DNA after γ-irradiation. The other line, which contains sub-amplicons of 250, 350 and 550 kb linked in large arrays (10), yielded only broad smears after irradiation. Such

heterogeneity of DM DNA size has also been inferred from EM of chromatin from another mouse cell (3T3R500) containing DMs (5).

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*To whom correspondence should be addressed

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