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Induction of Circles of Heterogeneous Sizes in Carcinogen-Treated Cells: Two-Dimensional Gel Analysis of Circular DNA Molecules

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Extrachromosomal circular DNA molecules are associated with genomic instability, and circles containing inverted repeats were suggested to be the early amplification products. Here we present for the first time the use of neutral-neutral two-dimensional (2D) gel electrophoresis as a technique for the identification, isolation, and characterization of heterogeneous populations of circular molecules. Using this technique, we demonstrated that in *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine-treated simian virus 40-transformed Chinese hamster cells (CO60 cells), the viral sequences are amplified as circular molecules of various sizes. The supercoiled circular fraction was isolated and was shown to contain molecules with inverted repeats. 2D gel analysis of extrachromosomal DNA from CHO cells revealed circular molecules containing highly repetitive DNA which are similar in size to the simian virus 40-amplified molecules. Moreover, enhancement of the amount of circular DNA was observed upon *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine treatment of CHO cells. The implications of these findings regarding the processes of gene amplification and genomic instability and the possible use of the 2D gel technique to study these phenomena are discussed.

Genomic instability is a hallmark of tumor cells (17, 59) and can be enhanced by carcinogen treatment (8, 16, 25, 27, 38, 54, 65, 81). This phenomenon consists of translocations, aneuploidy, recombinations, deletions, gene amplification, and the appearance of small polydisperse circular DNA (spcDNA). Gene amplification is the most extensively studied process of genomic instability (for reviews, see references 72 and 89). It is a dynamic process, and the amplified sequences either are associated with the chromosome, as expanded chromosomal regions, or are extrachromosomal and represented as acentric circular structures known as double-minute chromosomes. Several mechanisms were proposed to explain the various phenomena of gene amplification, and, considering the diversity in size and molecular configuration of the amplified sequences, all of these mechanisms probably could exist in parallel (for reviews, see references 72 and 89). The two main explanations of gene amplifications involve either overreplication of specific sequences or unequal segregation of replication and recombination products. A combination of both possibilities was proposed as well.

Structural analysis showed that the amplified sequences are often organized as chromosomal or extrachromosomal inverted repeats (IRs). The possible involvement of inverted duplications in the generation of amplified DNA was suggested (19–21, 30, 35, 49, 50, 57, 62, 68, 69). In some cases the early amplification products were organized as extrachromosomal circles containing IRs. The formation of these circles was proposed to be one of the initial steps of gene amplification (11, 62, 68, 86, 88, 89). Chromosomal breaks, ligations, and recombinations could explain the formation of these extrachromosomal circles (9, 11, 68, 69, 88; for reviews, see references 86 and 89). However, the very early steps of the process could not be detected, since in all of these systems the amplified DNA was analyzed only upon selection, when the cells acquired a distinguishable phenotype, several generations after the initial amplification event.

An additional phenomenon, which appears in normal cells but was shown to increase in correlation with genomic instability, is the formation of spcDNA (for reviews, see references 24, 70, and 90). spcDNAs are found in cell lines from different organisms, such as *Drosophila melanogaster*, mice, hamsters, monkeys, and humans (2, 3, 5, 18, 22, 41–43, 52, 53, 56, 76, 77, 79, 80, 91). Their sequences represent the whole genome, and they are thought to be generated from the chromosomes. Enhanced amounts of spcDNA can be obtained following treatment with drugs arresting DNA replication (79, 80) and were correlated with genomic instability (55, 56, 86). The mechanism of spcDNA formation is still obscure; however, it may be similar to some of the mechanisms of gene amplification and to other phenomena which characterize genomic instability, such as recombinations, deletions, and translocations.

We are studying early events during carcinogen-induced gene amplification by using a model system consisting of simian virus 40 (SV40)-transformed Chinese hamster cells—the CO60 cells—in which the viral genome is integrated into the chromosome (44). Upon treatment with a wide variety of carcinogens, the SV40 origin of replication is activated and a massive viral DNA amplification is directly observed, without selection (44–46). This amplification is transient, reaching its peak at 72 to 96 h posttreatment (44). The mechanism controlling SV40 amplification was investigated in vivo in the living cells and in vitro by utilizing a cell-free system consisting of cytosolic extracts from carcinogen-treated and untreated CO60 cells and from HeLa cells (1, 4, 13, 47). The viral amplification products were analyzed both in vitro and in vivo.

Recently, we showed that IRs organized as hairpin or stem-loop structures, containing the SV40 origin of replication, were synthesized in vitro when cytosolic extracts from *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG)-treated CO60 cells were used (13). The in vivo-amplified SV40 sequences consisted mainly of extrachromosomal molecules, including molecules

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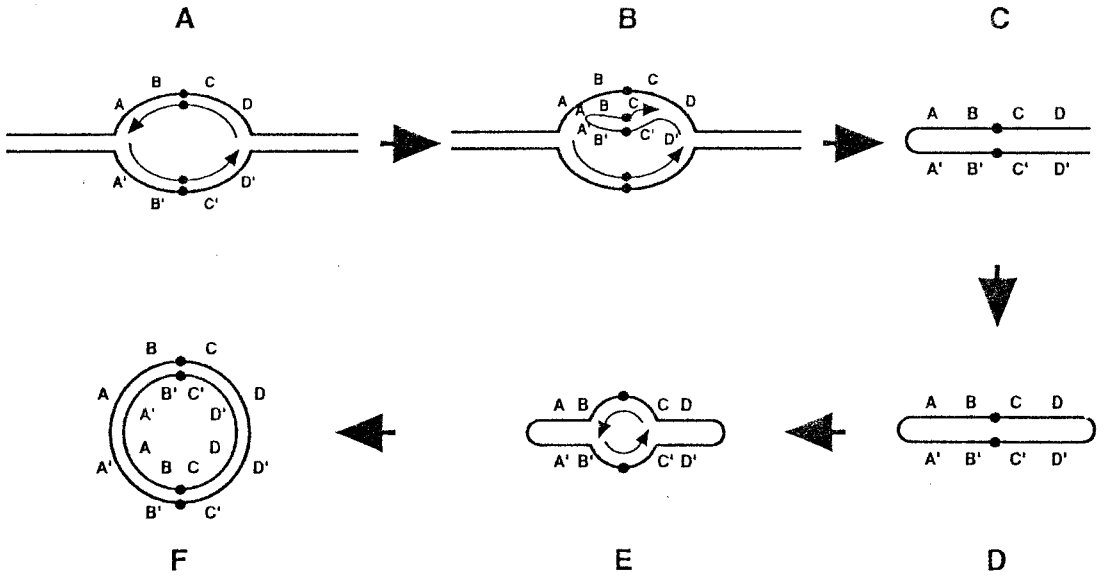

FIG. 1. The U-turn replication model, showing formation of hairpin structures at arrested replication forks and their conversion into circular inverted dimers (adapted from reference 13).

containing IRs. We proposed that the formation of IRs results from aberrant replication and that hairpin or stem-loop structures are synthesized at arrested replication forks by "U-turn" replication (Fig. 1). Thus, one of the events occurring at arrested forks is a template switch in which the DNA polymerase backtracks (U-turn), uses the newly synthesized strand as a template, and yields hairpin or stem-loop structures. These structures are displaced from the parental template upon their synthesis and might be ligated and rereplicated, generating circular inverted dimers (13). Similar molecules were suggested to serve as the early precursors of gene amplification by other mechanisms (9, 11, 68, 69, 86, 88).

In this paper we present the use of neutral-neutral two-dimensional (2D) gel analysis, which separates molecules according to their sizes and structures, as an easy and useful tool for the detection of circular molecules. Using this technique, we demonstrated that extrachromosomal circles with a wide size range are the major products of SV40 amplification in carcinogen-treated CO60 cells. A fraction of these circles contains IRs, as predicted by the U-turn replication model and consistent with the accumulating information about the early intermediates during cellular gene amplification. Circular DNA molecules similar in size to the SV40 amplified molecules were detected in CHO cells, and their amount was enhanced following MNNG treatment. Therefore, our studies demonstrate that the SV40 system can serve as a model to investigate the initial steps in gene amplification as well as other modes of genetic instability, such as the formation of spcDNA. A mechanistic linkage between the formation of spcDNA, gene amplification, and genomic instability is discussed.

MATERIALS AND METHODS

Cells and DNA preparation. CO60 cells (46), CHO cells, and BSC-1 cells were propagated in monolayer cultures in Dulbecco modified Eagle medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Biolabs, Jerusalem, Israel). Chromosomal (high-molecular-weight) and extrachromosomal (low-molecular-weight) cellular DNAs were prepared according to the procedure previously described by Hirt (32).

Generation of defective SV40 genomes. BSC-1 cells were plated at a density of 5×10^6/14-cm-diameter plate and were infected with wild-type SV40 strain 777. Defective viral stock was prepared according to a procedure previously described (48), in which SV40 was serially passaged in BSC-1 cells at a high multiplicity of infection. In the first few passages, a cytopathic effect appeared 4 to 5 days after infection. This time period increased with the next passages, suggesting the generation of defective virus particles. In the fifth passage, a cytopathic effect was detected after 11 days, and therefore cells were infected with the fourth-passage virus and harvested 6 days after infection. Viral DNA was prepared from the infected cells by the Hirt procedure (32).

MNNG treatment. CO60 and CHO cells were treated with MNNG 24 h after being seeded, as previously described (4). Log-phase cells were plated at a density of 5×10^6/14-cm-diameter plate. After 24 h, the cells were treated with 10 μg of MNNG (Aldrich) per ml, which had been freshly dissolved in dimethyl sulfoxide (Sigma), and added to the growth medium. One hour after the treatment, the medium was replaced with fresh medium, and the cells were allowed to grow in carcinogen-free medium until they were harvested (72 to 96 h post-treatment).

Neutral-neutral 2D gels. Separation of DNA on neutral-neutral 2D gels was performed according the procedure described by Brewer and Fangman (7) with the following modifications of the electrophoresis parameters: the first dimension was run in 0.4% agarose at 1 V/cm (for Fig. 4B and 8) or 0.5 V/cm (for Fig. 2, 3, 4A, 5, and 6) for 18 h, and the second dimension was run in 1% agarose at 5 V/cm for 3.5 h. The DNA was blotted onto nylon membranes (Hybond-N for Fig. 2 to 6 and Hybond-N^+ for Fig. 8) (Amersham, Amersham, England).

Supercoiled SV40 DNA purified with a CsCl gradient was used as a probe for amplified SV40 sequences. Hamster Cot-1 DNA (Bethesda Research Laboratories) served as a probe for the detection of CHO circular DNA (for Fig. 8). A plasmid containing the hamster mitochondrial genes for ATPase and cytochrome oxidase 3 derived from the λ-Zap library (kindly given by Y. Assaraf, Technion, Haifa, Israel) was used as a probe to detect mitochondrial DNA (data not shown) for normalization of the amount of circular DNA in Fig. 8. Hybridizations were carried out under standard conditions.

Quantitative analysis of the radioactive signals was done with a Fuji BAS1000 PhosphorImager and the Tina 2.07 program (Dinko & Renium, Bet-Nekofa, Israel).

Preparation of DNA for EM. Fifty micrograms of extrachromosomal DNA containing the defective SV40 genomes was separated on a 2D gel. The four arcs were visualized by ethidium bromide (EtBr) staining, and DNA from each of arcs 1 to 3 (not from arc 4, which contained the linear DNA) was extracted from the gel by using Elutip-D columns (Schleicher and Schuell, Dassel, Germany) according to the manufacturer's instructions. The DNA samples were resuspended in 50 μl of Tris-EDTA and examined by electron microscopy (EM) as previously described (39).

A sample from each eluted DNA was mixed with a λ *Hin*dIII linear size marker and rerun on a 2D gel to define its arc pattern following the extraction process. The gels were blotted onto a Hybond-N nylon membrane and hybridized to the SV40 probe and to a λ probe.

CsCl-EtBr density gradient. A CsCl-EtBr density gradient was used to separate the supercoiled circles from the linear DNA and from the relaxed circles according to the usual procedure, as described by Sambrook et al. (71). One

milligram of extrachromosomal DNA from MNNG-treated CO60 cells was loaded onto the gradient, which was run in a Beckman ultracentrifuge (Ty. 65 rotor) at 40,000 rpm at 20°C for 42 h.

The fast-migrating portion of the gradient, consisting of the supercoiled molecules, was collected in aliquots, and the DNA-containing fractions were pooled following determination of the amounts of DNA by UV illumination. The lower-density band containing the linear molecules and the relaxed circles was collected separately. The structural identity of the SV40 DNA in the two pools was determined by neutral-neutral 2D gel analysis as described in Results (see Fig. 6).

The "snap-back" assay: identification of IRs. Five micrograms of the supercoiled DNA fraction from the CsCl density gradient was linearized with *Bgl*I, mixed with 1 ng of *Alw*NI-digested p2-C (an IR-containing plasmid), and subjected to the snap-back assay as described by Ford et al. (19, 20). In brief, the linearized DNA was denatured at room temperature with 50 mM NaOH for 60 min, the volume was increased to threefold the initial volume with ice-cold water, and then the DNA was neutralized with 1 M HCl and 1 M Tris HCl (pH 7.8) on ice. The DNA was digested with S1 nuclease (10 U/μg of DNA) (Boehringer-Mannheim) in 50 mM NaCl–33 mM sodium acetate–0.03 mM $ZnSO_4$ (pH 4.5) for 30 min at 14°C, and the reaction was terminated with 10 mM EDTA and by phenol extraction. The S1 nuclease-resistant DNA was precipitated and analyzed with the restriction enzyme *Dpn*II. The DNA was separated on 1.3% agarose gels, blotted onto a nylon membrane (Hybond-N; Amersham), and hybridized to the SV40 probe under the standard conditions.

RESULTS

Extrachromosomal circles of heterogeneous sizes can be detected by neutral-neutral 2D gel electrophoresis. To determine whether circular molecules are included within the amplified SV40 DNA, we searched for an easy and convenient approach to detect and to analyze such molecules. The separation of circular DNA on a CsCl-EtBr density gradient is commonly used. However, this requires the preparation of huge amounts of extrachromosomal DNA and is inefficient because of the loss of a significant fraction of the circular molecules (the relaxed circles) which migrate with the linear DNA. Moreover, this approach does not facilitate further characterization of the circular molecules other than by cloning or EM.

We adapted the neutral-neutral 2D gel electrophoresis assay previously described by Brewer and Fangman (7) for the structural analysis of the amplified DNA accumulated in carcinogen-treated CO60 cells. This assay is widely used for the separation of branched DNA molecules from linear ones according to their differential migrations on these gels. In addition, it was previously shown that circular structures can be separated from the linear DNA on 2D gels, since covalently closed and nicked circles had retarded migrations in comparison with linear molecules with the same mass (60). However, in these experiments the DNA examined contained only one or a few species of extrachromosomal circles that migrated as discrete spots on the 2D gels (51, 60).

To study the pattern of migration of a heterogeneous population of circular DNA molecules, we chose to use defective SV40 genomes which were shown to be supercoiled and relaxed circular DNAs of heterogeneous sizes (48). Defective SV40 genomes were produced (as described in Materials and Methods) by serial passages of the virus in BSC-1 cells at high multiplicity of infection. The viral DNA was extracted from the infected cells by the Hirt procedure (32) and analyzed on 2D gels. Blotting followed by hybridization to SV40 probe revealed four arcs, designated 1 to 4 throughout this paper (Fig. 2B). Three of these arcs were detected by EtBr staining and are shown in Fig. 2A; the fourth arc (arc 2) is very faint and was invisible in Fig. 2A. Arc 4 comigrated with the λ *Hin*dIII linear size marker (Fig. 2A; see also Fig. 3B, E, and H) and thus represented linear DNA. The patterns of the other three arcs (arcs 1 to 3) were completely different from those formed by replicating intermediates of a single molecule or of a few molecules (7) as well as from the pattern of catanated molecules (7, 78), suggesting that these arcs represent populations of circular DNA.

To test whether such structures appear among the amplified SV40 DNA, a similar 2D gel analysis was performed with 10 μg of DNA from MNNG-treated CO60 cells prepared 96 h post-treatment. Only arc 4 (representing the linear DNA) could be detected by EtBr staining, since it consisted mainly of broken cellular sequences as determined by its strong hybridization to the hamster repetitive-DNA (Cot-1) probe (data not shown). However, upon hybridization to the SV40 probe, all four arcs appeared (Fig. 2C). The pattern of these arcs was similar to that of the defective SV40 circular genomes, but the arcs were much longer and thus represented a wider size distribution of circular DNA. Superimposition of Fig. 2B and C (in which the gels were run simultaneously in the same tank) verified the comigration of the defective SV40 genomes with the CO60 arcs (Fig. 2D). In further experiments, several plasmids in the size range of 2 to 8.2 kb were mixed with the CO60 extrachromosomal DNA. Upon 2D gel analysis, the plasmids comigrated with arcs 1 to 3, and hence the size spectrum of the amplified SV40 circular DNA is <2 to >10 kb (data not shown). Note that each of arcs 1 to 3 from the CO60 extrachromosomal SV40 DNA contains a single more intense spot which possibly represents a predominant extrachromosomal circular molecule existing in the CO60 cell line (for example, see Fig. 2C, 4B, and 5B).

EM analysis of "arc DNA." For further analysis, the DNAs from each of arcs 1 to 3 were gently purified from a preparative 2D gel of the defective SV40 circular genomes. A sample of each DNA preparation was mixed with the λ size marker and was rerun on the 2D gels to assess the yield and the integrity of the purified molecules. The blots were hybridized to the SV40 probe (Fig. 3A, D, and G) and then to the λ probe to reveal the position of the linear DNA (Fig. 3B, E, and H). The remaining purified arc DNA was analyzed by EM to determine the structures of the molecules (Fig. 3C, F, and I). EM analysis of the DNA eluted from arc 1 displayed nicked circles (Fig. 3C) and small amounts of linear DNA. This arc was reconstructed upon a second 2D gel analysis (Fig. 3A). In addition, there was a faint arc of linear DNA (arc 4), probably resulting from breaks in the circular DNA. We conclude that arc 1 represents the nicked circles.

Upon reanalysis on a 2D gel, arc 2 could not be reconstructed from the DNA purified from arc 2 (Fig. 3D). However, the arc and the EM picture were identical to those for arc 1 (Fig. 3D, E, and F), demonstrating that arc 2 consisted of nicked circular molecules like those in arc 1. As illustrated and discussed below (see Fig. 4), the open circles resulted from nicking of the closed circular molecules during the preparation of the second dimension of the gel.

The DNA eluted from arc 3 yielded on 2D gel analysis the original arc 3 as well as the three other arcs (Fig. 3G). EM analysis of this DNA revealed supercoiled molecules, open circles, and linear molecules (Fig. 3I). In an examination of 31 fields, 355 molecules were counted, of which 41% were supercoiled, 32% were relaxed, and 27% were linear. These numbers are in agreement with the data presented in Fig. 3G. Thus, we conclude that arc 3 originally contained covalently closed circles (supercoiled) which underwent nicking or breakage events during the purification procedure, probably because of the large amount of EtBr that intercalated into the DNA.

Analysis of the wild-type SV40 genome and of several plasmids on 2D gels demonstrated that every examined circular molecule migrated in the first dimension as two bands representing the nicked (relaxed) and the supercoiled (covalently closed) circles. However, upon electrophoresis on the second

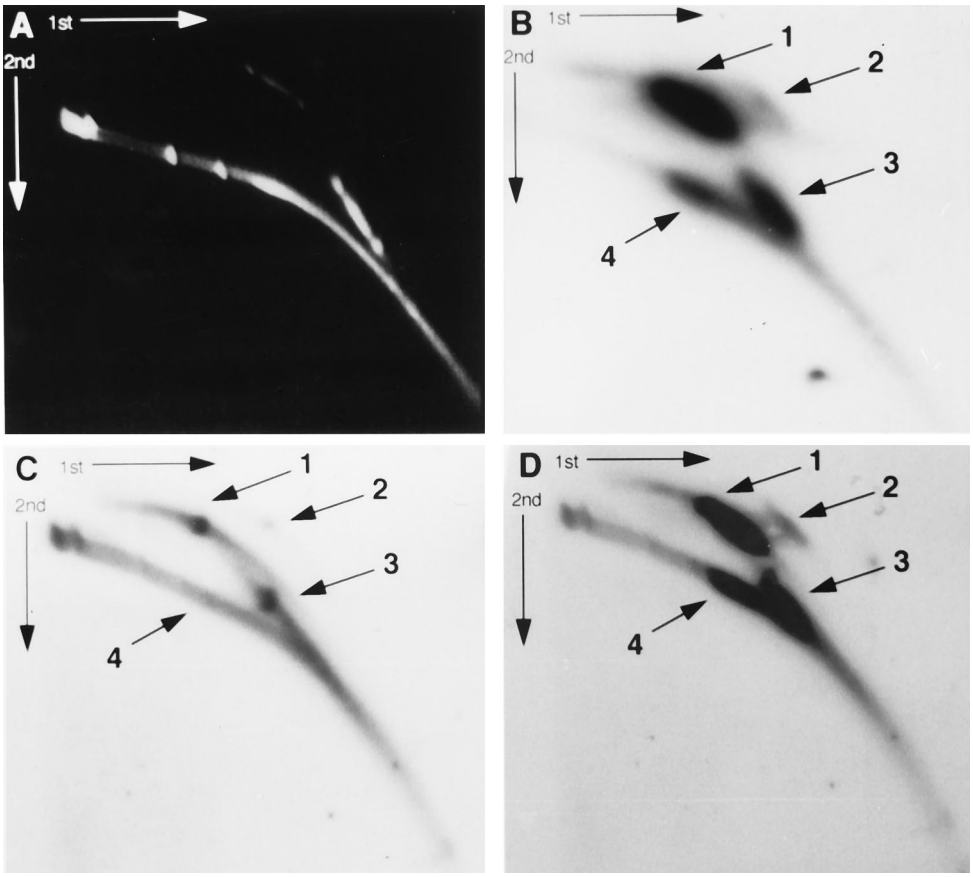

FIG. 2. Neutral-neutral 2D gel analysis of defective SV40 molecules shows an arc pattern similar to that of extrachromosomal MNNG-treated CO60 DNA. Defective SV40 genomes were extracted 6 days after infection from SV40-infected BSC-1 cells after the fifth serial passage (see Materials and Methods). Five micrograms was analyzed on the neutral-neutral 2D gel. (A) EtBr staining of the DNA. (B) A similar gel, containing 0.5 μg of defective SV40 DNA, following blotting and hybridization to the SV40 probe and 4 h of exposure. (C) Ten micrograms of extrachromosomal MNNG-treated CO60 DNA analyzed under the same 2D gel conditions, blotted, hybridized to the SV40 probe, and exposed for 24 h. (D) Superimposition of panels B and C demonstrates that the arcs migrated identically. The four arcs are designated 1 to 4.

dimension (Fig. 4A), the supercoiled DNA band separated into two spots: one (Fig. 4A, spot 3) migrated fast, as expected for supercoiled molecules, and the other (Fig. 4A, spot 2) migrated slowly to the same distance as did the relaxed circles (Fig. 4A, spot 1). This migration pattern suggests that the retarded spot (Fig. 4A, spot 2) represents a fraction of the original supercoiled circles that were nicked after the first-dimension electrophoresis. EtBr staining and UV illumination could have caused nicks in the supercoiled molecules during the preparation of the second dimension, resulting in the formation of open circular molecules. Heterogeneous populations of such DNA molecules will generate arc 2. Note that arc 2 is faint and in some cases is invisible (Fig. 3G; compare Fig. 4B and 5B), probably because of different nicking rates in the different experiments.

Amplified SV40 DNA is circular. Having established the technology, we used the 2D gel analysis to determine whether the circular SV40-containing molecules are amplified following MNNG treatment of CO60 cells. We compared the 2D gel patterns and the intensities of SV40 hybridization of 20-μg portions of extrachromosomal DNAs from MNNG-treated (Fig. 5B) and from untreated control (Fig. 5A) CO60 cells. The results show a dramatic increase in the extrachromosomal circular SV40 DNA in the treated CO60 cells.

Quantitative analysis of several blots containing low-molecular-weight DNA from MNNG-treated CO60 cells (including those in Fig. 2C and 5B) indicates that the circular DNA constitutes 30 to 50% of the total SV40 DNA. Thus, a significant fraction of the amplified SV40 DNA is organized in circles. Our findings clearly demonstrate that circular molecules are the primary SV40 amplification products in the MNNG-treated CO60 cells.

It should be noted that the circular fraction was relatively large when the DNA was tested either shortly after its preparation or shortly after being stored frozen and that it decreased with time (especially the supercoiled DNA) following storage at 4°C, suggesting that a portion of the linear molecules were originally circular.

The specific induction of circular DNA in response to MNNG treatment in our system raises two questions. One is whether these circles contain inverted repeats as predicted by the U-turn replication model. The second, which is probably more important, is whether circular molecules that contain cellular sequences are found in transformed cells and whether they are induced upon carcinogen treatment.

IRs are detected in the amplified extrachromosomal circular DNA. To test whether IRs are found within the amplified circular DNA population, we separated extrachromosomal DNA from MNNG-treated CO60 cells on a CsCl-EtBr density gradient. Two pools of fractions, one from the low-density

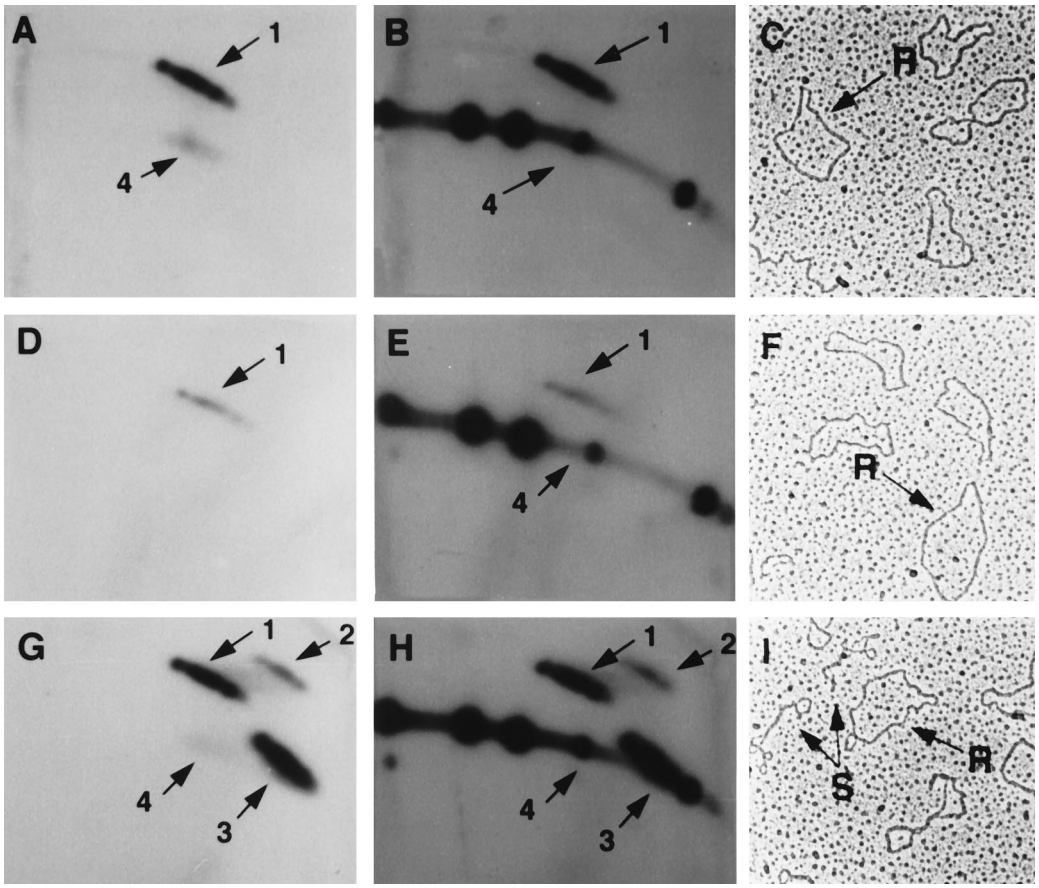

FIG. 3. Structural analysis of the arc DNA. Defective SV40 genomes were separated on 2D gels, and DNAs from arcs 1, 2, and 3 were extracted and examined by EM to reveal their structures (C, F, and I). A sample from each DNA was mixed with the linear size marker λ *Hin*dIII and rerun on 2D gels. The gels were blotted and were hybridized first to the SV40 probe (A, D, and G) and then to the λ probe for reference of the linear DNA migration (B, E, and H). (A, B, and C) Arc 1; (D, E, and F) arc 2; (G, H, and I) arc 3. S, supercoiled circles; R, relaxed circles.

portion and the second from the high-density portion of the gradient, were collected as described in Materials and Methods. The 2D gel electrophoresis served as a tool for structural identification of the DNA. Samples from each pool were mixed with the λ size marker, separated on 2D gels, and hybridized to the SV40 probe (Fig. 6). The first pool contained, as expected, arcs 1 and 4, which are the nicked (relaxed) circles and the linear DNA, respectively (Fig. 6A). The second pool, containing the supercoiled circular molecules, yielded the two other arcs: arc 3, representing the supercoiled molecules, and arc 2, displaying supercoiled molecules that were converted to relaxed forms during the preparation of the second dimension (Fig. 6B). The identities of these arcs in relation to linear DNA were verified by hybridization of the blot to the λ probe (Fig. 6C). These findings indicate that the SV40 DNA in the high-density pool consisted exclusively of supercoiled molecules.

The supercoiled fraction was examined for the presence of IR-containing SV40 sequences by using the snap-back assay (19, 20). This assay is based on the ability of inverted duplications to snap back into double-stranded forms following denaturation and rapid renaturation and thus to resist the S1 nuclease hydrolysis. Figure 7 shows the results of the snap-back assay with the closed circular fraction of extrachromosomal DNA from treated CO60 cells as well as with total extrachromosomal DNA from these cells.

As an internal control we used a plasmid (p2-C) (13, 40) containing a duplicated segment of SV40 sequences which is organized in an inverted order as determined by EM analysis and restriction mapping. Cleavage of p2-C with the restriction enzyme *Alw*NI generates three fragments (3.1, 1.8, and 1.55 kb), two of which (1.8 and 1.55 kb) contain SV40 sequences and therefore are visible following hybridization to an SV40 probe (Fig. 7, lane 1). In the snap-back assay the three original fragments disappeared and a new fragment of 365 bp, consisting of the folded IRs, appeared (Fig. 7, lane 5).

Being aware of the presence of a large fraction of covalently closed circular molecules among the amplified DNA, we linearized the supercoiled fraction with *Bgl*I, which cleaves at the SV40 origin of replication. Such digestion of the extrachromosomal DNA abolished SV40 hybridization to arcs 1 to 3, demonstrating that these arcs contained molecules with the viral origin which were linearized by *Bgl*I (data not shown). The linearized molecules were mixed with *Alw*NI-digested p2-C (the internal control) and then divided into two fractions. The first, containing 10% of the DNA, was directly digested with *Dpn*II to reveal the pattern of the SV40 DNA (Fig. 7, lane 4). Note that the plasmid DNA (p2-C) was methylated and therefore was *Dpn*II resistant. The remaining 90% of the DNA was subjected first to the snap-back assay and then to *Dpn*II digestion (Fig. 7, lane 8). As a result, the S1 nuclease-resistant DNA had the same SV40 pattern, although much weaker, as that in the non-S1 nuclease-digested control. In addition, a new p2-C fragment, containing the IRs, appeared. This result indicates that a fraction of the circular amplified SV40 DNA is arranged

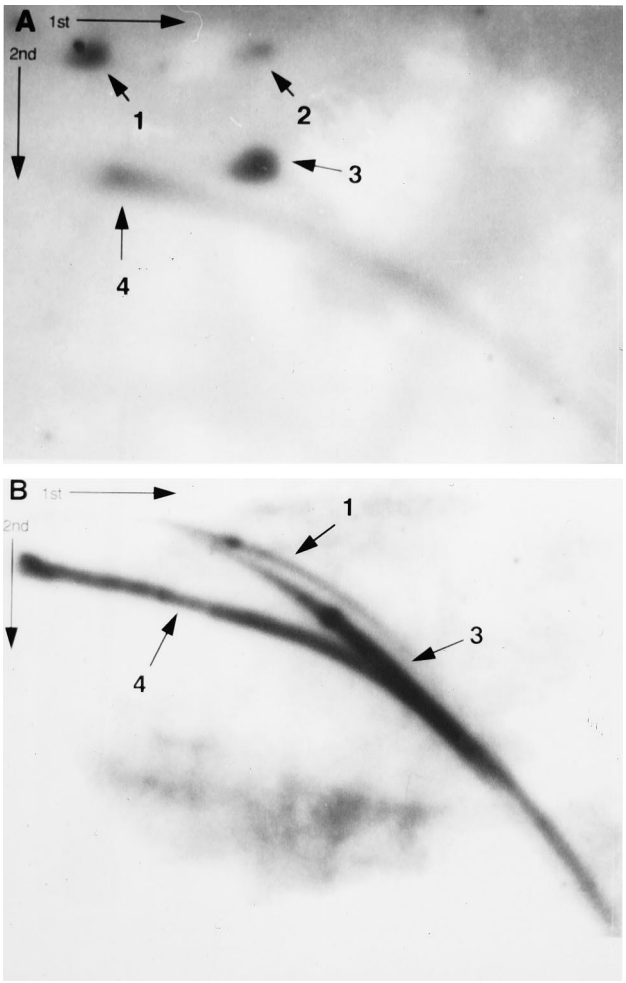

FIG. 4. Covalently closed circles form two arcs on 2D gels. (A) SV40 DNA extracted from viral particles and linear extrachromosomal DNA from treated CO60 cells were separated on a 2D gel, which was blotted and hybridized to the SV40 probe. The three spots (1, 2, and 3) represent the migration of the SV40 circular molecules, and the smooth line corresponds to linear DNA. Note that in the first dimension the faint spot (spot 2) migrated the same distance as the lower one (spot 3). In the second dimension, this spot migrated the same distance as the upper spot (spot 1). (B) 2D gel analysis of extrachromosomal DNA from MNNG-treated CO60 cells in which arc 2 is invisible, demonstrating that by careful preparation of the second dimension, the nicking of the closed circular DNA can be minimized.

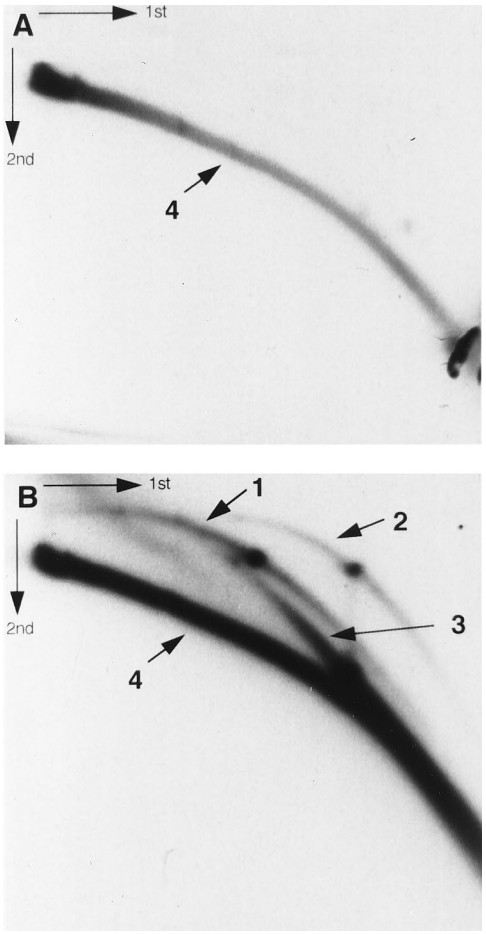

FIG. 5. Neutral-neutral 2D gel analysis of extrachromosomal DNAs from MNNG-treated and from untreated control CO60 cells. Extrachromosomal (Hirt supernatant) DNAs (20 μg) prepared from untreated control CO60 cells 48 h after seeding (A) and from MNNG-treated CO60 cells 96 h after treatment (B) were analyzed by 2D gel electrophoresis as described in Materials and Methods. The two DNA samples were run simultaneously in the same tank, blotted onto the same membrane, and hybridized together to the SV40 probe. The membrane was exposed for 17 h.

as IRs as predicted by the U-turn model. When the same test was performed with total low-molecular-weight DNA from MNNG-treated CO60 cells in the presence (Fig. 7, lanes 3 and 7) or the absence (Fig. 7, lanes 2 and 6) of the internal p2-C control, the same *Dpn*II pattern appeared following hybridization to the SV40 probe. The proportions of the S1 nuclease-resistant SV40 DNA in the supercoiled and in the nonfractionated extrachromosomal DNAs were similar, suggesting that the proportion of IRs in supercoiled DNA is similar to that in the total low-molecular-weight DNA.

We have previously shown that sequences representing the complete integrated SV40 genome were organized as IRs in the chromosomal and extrachromosomal DNAs from MNNG-treated CO60 cells (13). The identical *Dpn*II patterns of the total extrachromosomal DNA (Fig. 7, lanes 2 and 6) and of the supercoiled fraction (Fig. 7, lanes 4 and 8) before and after the snap-back assay demonstrate that the entire integrated SV40 genome is included in the amplified circular population and that at least a fraction of the circular viral molecules are organized as IRs.

Circular DNA molecules are enhanced upon MNNG treatment of CHO cells. To investigate circular molecules of cellular sequences, we examined CHO cells, which were reported to contain spcDNA that was detected by other methods (76). We asked whether spcDNA can be detected by using the 2D gels, whether it would be similar in size to the amplified SV40 circles, and whether MNNG treatment has any effect on this DNA.

Extrachromosomal DNA was prepared from MNNG-treated CHO cells 96 h posttreatment and from untreated control cells. Equal amounts of DNA from control and treated cells were analyzed on the 2D gel in the same tank as extrachromosomal DNA from MNNG-treated CO60 cells. The gels were blotted, and since the whole genomic sequences were reported to appear in spcDNA, we chose to use hamster Cot-1 DNA, which represents the genomic repetitive DNA, as the cellular probe to detect such circles in the CHO DNA. Figure 8 shows short and long exposures of the blots containing the DNAs from untreated (Fig. 8A and A′) and from MNNG-

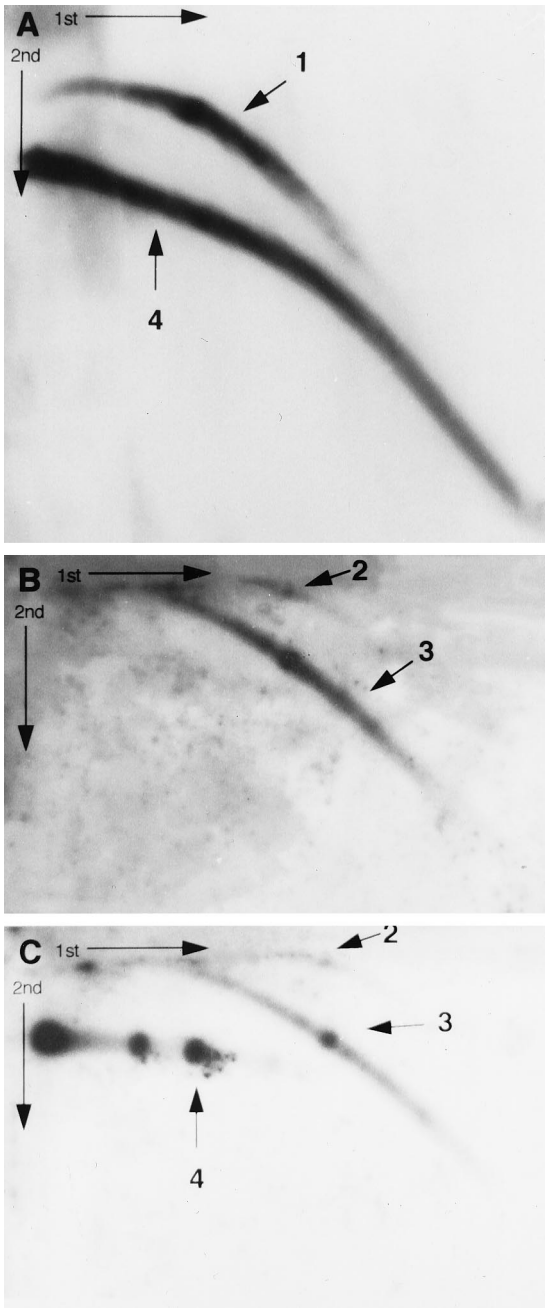

FIG. 6. Separation of covalently closed circular molecules on a CsCl-EtBr density gradient. One milligram of extrachromosomal DNA from MNNG-treated CO60 cells was separated on a CsCl-EtBr density gradient, and two fractions were collected as described in Materials and Methods. A sample of each fraction was mixed with the linear size marker λ *Hin*dIII and separated on 2D gels to determine the structure of the SV40 DNA. The gels containing the pool of the linear DNA and the nicked circles (A) and the pool of covalently closed circular DNA (B) were blotted and hybridized to the SV40 probe. The blot in panel B was also hybridized to λ DNA to visualize the migration of the linear DNA (C).

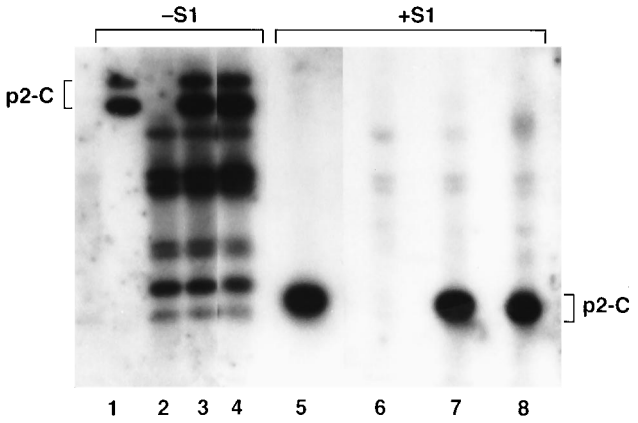

FIG. 7. Identification of IRs within the amplified circular SV40 DNA in MNNG-treated CO60 cells. Covalently closed circular DNA (lanes 4 and 8) and total extrachromosomal DNA (lanes 2, 3, 6, and 7) were prepared from MNNG-treated CO60 cells. These DNAs first were linearized with *Bgl*I (which cleaves at the SV40 origin) and then were either directly cleaved with *Dpn*II (−S1) or subjected to the snap-back assay prior to the *Dpn*II cleavage (+S1). One nanogram of the *Alw*NI-cut p2-C plasmid served as a control for the snap-back assay (lanes 1 and 5) and as an internal control in the reaction mixtures which contained the cellular DNA (lanes 3, 4, 7, and 8). The DNA was separated on 1.3% agarose, blotted, and hybridized to the SV40 probe. p2-C, plasmid fragments (1.8 and 1.55 kb) consisting of SV40 sequences (lanes 1, 3, and 4) and the 365-bp fragment containing IRs (lanes 5, 7, and 8).

treated (Fig. 8B and B′) CHO cells. The blot containing DNA from MNNG-treated CO60 cells was hybridized to the SV40 probe to serve as a reference for the migration positions of the arcs containing circular DNA (Fig. 8C). Comparison of the pattern of the amplified SV40 circular DNA (Fig. 8C) with the hybridization patterns in Fig. 8A and A′ shows that beside the massive arc of linear DNA (which corresponds to arc 4 in Fig. 8C), arcs of circular DNA (corresponding to arcs 1 and 2 in Fig. 8C) are clearly observed. The arc that corresponds to arc 3 is masked by the heavy hybridization to the linear DNA. The arrows point to arc 1 as a representative for comparison between the panels. These results demonstrate the presence of a significant amount of circular DNA molecules in the low-molecular-weight DNA fraction of CHO cells and that they are characterized by a size range similar to that of the amplified SV40 circles. The level of the circular molecules in the CHO cells is lower than that of SV40 circles observed in treated CO60 cells, and most of the hybridization to the Cot-1 probe was to the linear DNA arc. This was, in fact, the expected result, since most of the extrachromosomal DNA is linear and since the Cot-1 hybridization represents the repetitive genomic DNA. On the other hand, SV40 hybridization in CO60 DNA represents only the viral DNA, which constitutes a small fraction of the total extrachromosomal DNA. However, the SV40 sequences are very common in the circular population because of its extremely massive amplification.

Enhancement of the circular DNA population was observed following carcinogen treatment of CHO cells. This is indicated by the increased hybridization to the arcs of circular DNA in Fig. 8B and B′ in comparison to the arcs in Fig. 8A and A′, which contain DNA from the untreated cells. To quantitate the enhancement of the circular DNA in the carcinogen-treated CHO cells in comparison with the control, the blots were reprobed with hamster mitochondrial DNA (data not shown). Mitochondrial DNA is extracted with the low-molecular-weight DNA and was previously shown to remain unchanged following carcinogen treatment (79). Thus, the mitochondrial signals were used to normalize the circular DNA signals in Fig. 8. Quantitative analysis revealed that the circular DNA in carcinogen-treated CHO cells was enhanced 3.85- to 4.11-fold (for the lower part of arc 2 and for the entire arc 1, respectively) in comparison with the circular DNA from the control cells. It should be noted that induction of circles containing

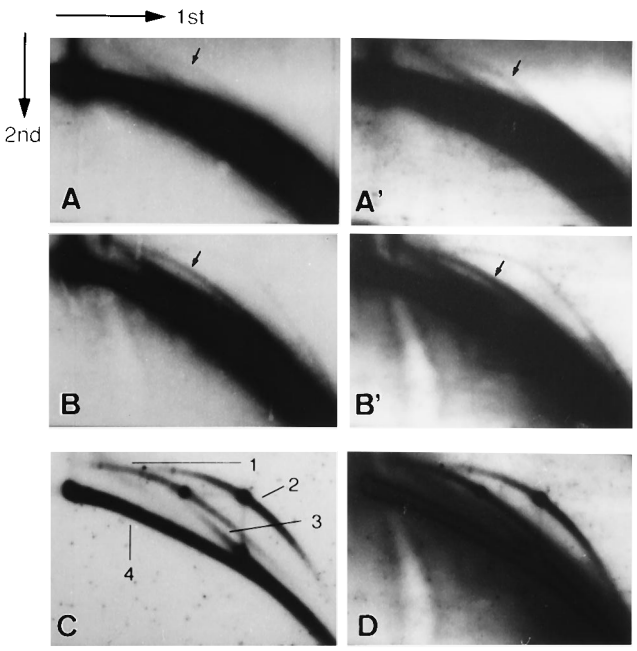

FIG. 8. Neutral-neutral 2D gel analysis of extrachromosomal DNAs from MNNG-treated and from untreated control CHO cells. Extrachromosomal (Hirt supernatant) DNAs (30 μg) prepared from untreated control CHO cells (A and A′) and from MNNG-treated CHO cells (B and B′) were analyzed by 2D gel electrophoresis. Following blotting, the DNA was hybridized to the hamster Cot-1 probe. Short (16-h) (A and B) and long (48-h) (A′ and B′) exposures of the membranes demonstrate the enhancement of circular molecules in the MNNG-treated CHO cells. The arrows point to the arcs of relaxed circles (corresponding to arc 1 of SV40 DNA). Amplified extrachromosomal SV40 DNA from MNNG-treated CO60 cells was separated simultaneously in the same electrophoresis tank and served as a reference for the migration positions of the circle arcs on the 2D gel following hybridization to the SV40 probe (C). The typical arcs 1 to 4 are indicated. Superimposition of panels B′ and C indicates that the CHO arcs indeed migrated as supercoiled and relaxed circles (D).

Cot-1 DNA was observed in MNNG-treated CO60 cells (data not shown).

Superimposition of Fig. 8C on Fig. 8B′ confirmed that the arcs in the CHO blots migrated to exactly the same position as did the SV40 circles (Fig. 8D). Thus, we conclude that a significant amount of circular DNA is found in the hamster-transformed CHO cell line and might represent a characteristic feature of transformed cells, which are known to be unstable. Furthermore, the circular DNA is enhanced in the carcinogen-treated CHO cells in a mode similar to the SV40 amplification in the treated CO60 cells.

DISCUSSION

Supercoiled circles, relaxed circles, and linear molecules migrate in distinct arcs on 2D gel electrophoresis. To study circular structures, we adapted the neutral-neutral 2D gel electrophoresis that is usually used for the detection of replication intermediates and for the identification of origins of replication (7). As a model for circular DNA, we used a heterogeneous population of defective SV40 circular genomes. Their migration pattern on the 2D gels revealed four distinct arcs containing the SV40 sequences (Fig. 2B). The patterns of the arcs were different from those reported for replication intermediates of linear or circular molecules (7, 78) and were assumed to represent different forms of a heterogeneous circular population.

The large yield of the defective viral genomes enabled the detection of these arcs by EtBr staining of the gel (Fig. 2A) and their elution for further identification by EM. EM analysis clearly showed that the arc DNA represented different forms of circular molecules. The DNA eluted from arcs 1 and 2 contained mainly relaxed circles and small amounts of linear DNA that probably resulted from molecules which broke during the DNA preparation (Fig. 3C and F). Reanalysis on 2D gels of the arc DNA eluted from both arcs 1 and 2 reconstructed arc 1 (Fig. 3A and D), demonstrating that both arcs 1 and 2 contained relaxed circles. Further analysis, presented in Fig. 4, clearly demonstrated that arc 2 represented molecules that migrated in the first dimension as supercoiled molecules and were nicked and converted into relaxed forms during the preparation of the second dimension. According to the EM analysis, the DNA eluted from arc 3 contained supercoiled circles as well as relaxed circles and linear molecules (Fig. 3I). This finding was confirmed by 2D gel analysis of the eluted DNA, in which all four arcs appeared (Fig. 3G). Since arcs 1 and 2 were shown to contain relaxed circles, we conclude that arc 3 consisted of supercoiled circles and that the other forms were generated from nicking and breakage events. This interpretation was supported by the finding that DNA from arc 3 could be converted to form the three other arcs but that the opposite (i.e., the conversion of DNA from arc 1 or 2 to arc 3) never occurred. In addition, similar observations that partially in situ-digested supercoiled pBR322 molecules (separated in the first dimension) were converted into the relaxed form (75) are in accordance with our interpretation.

Amplification of extrachromosomal circles is induced by MNNG treatment of CO60 cells. The separation of the treated CO60 extrachromosomal DNA on the 2D gels revealed an arc pattern similar to that of the defective SV40 genomes (Fig. 2C and D). Quantitative analysis of several 2D gels showed that a significant fraction (ca. 30 to 50%) of the extrachromosomal SV40 sequences is found in the circle arcs.

Comparison of the 2D patterns of identical amounts of extrachromosomal DNAs from MNNG-treated and from untreated control CO60 cells revealed a dramatic increase in the circular SV40 molecules following carcinogen treatment. This is a heterogeneous population of molecules with a wide size spectrum that was estimated to be <2 to >10 kb. We suggest that the carcinogen treatment might activate mechanisms associated with the production of circular molecules.

The majority of the amplified SV40 sequences were previously shown to be associated with the extrachromosomal DNA fraction (13). When the high-molecular-weight fraction (Hirt pellet) from the treated CO60 cells was analyzed with the 2D gels, an arc pattern typical of that of circular molecules was observed, thus demonstrating that the high-molecular-weight DNA fraction was contaminated with low-molecular-weight circular SV40 DNA (data not shown). This finding further supports the idea that the SV40 amplification products are mainly extrachromosomal circles.

The circles might be produced by different mechanisms, as outlined below. However, we can not rule out the possibility that some of the circular amplified molecules were derived from preexisting circles. The intense spots that were always observed on the SV40 arcs (Fig. 2C and 5B) might represent such molecules. The newly synthesized circles might arise from overinitiation at the SV40 origin of replication, as described by the "onion skin" model (73; for reviews, see references 72 and 89), followed by recombination, breakage, and fusion events. In addition, several modes of recombination events at the replication bubble could give rise to these molecules (for reviews, see references 86 and 89).

Breakage events at arrested replication forks followed by ligations were suggested to generate circular molecules containing IRs as early amplification products (88). Using the in vitro amplification system, we showed arrested replication forks during SV40 replication (13, 85). The formation of hairpin and stem-loop structures in this system led us to propose the U-turn replication model for their synthesis at arrested forks. Further secondary events might convert them into circular dimers containing IRs. Therefore, we searched for IRs within the circular SV40 DNA population in the treated CO60 cells.

Extrachromosomal circles containing IRs are found among the SV40 amplification products. CsCl-EtBr density gradient analysis of DNA from MNNG-treated CO60 cells was used to separate supercoiled circles from relaxed circles and linear extrachromosomal DNA. Using the 2D gel analysis, we showed that the isolated DNA contained supercoiled molecules (Fig. 6B). A fraction of this DNA harbored IRs as demonstrated by the snap-back assay and hybridization to the SV40 probe (Fig. 7). The S1 nuclease-resistant DNA represented only a small fraction of the circular molecules, suggesting that this fraction could be generated by the U-turn replication. Possibly a larger fraction of the circles contain IRs; however, in these molecules the IRs might compose only a part of each circular molecule. Such a configuration could be formed if the original U-turn product was in the structure of a stem-loop. In this case only the stem region appears in IRs, while the loop region is unique and would be digested following the snap-back assay. This molecular organization can explain the reduction in the intensity of SV40 hybridization to the S1 nuclease-resistant DNA in comparison with the input DNA and in comparison with p2-C, in which IRs are present in each molecule (Fig. 7, +S1 lanes compared with −S1 lanes). However, as stated above, we could not determine whether the primary amplification products in vitro are hairpins or stem-loop structures.

Using model hairpin and stem-loop molecules, we found that on neutral-neutral 2D gels hairpins migrate in the position of linear double-stranded DNA, while stem-loop molecules migrate faster, in the second dimension, forming an arc below the linear double-stranded DNA (85). Such an arc was not detected under our assay conditions. A better approach for the detection of hairpin and stem-loop structures is the use of a neutral-alkali 2D gel, which was previously described (13). In preliminary experiments we failed to detect such molecules in the DNA used in our experiments (preparations from 96 h posttreatment). However, positive results were obtained for DNA prepared at short times after treatment. If synthesized as in the cell-free system, hairpins and stem-loop structures do not seem to accumulate in the cells but rather undergo further processes, including circularization and degradation, and therefore cannot be easily detected. Currently, we are studying the configuration of the IRs by cloning of the isolated circular DNA. Note that p2-C was cloned from carcinogen-treated CO60 cells and contains a stem-loop structure.

The amounts of IRs described in this paper were smaller than those reported earlier (13). Previously the DNA was cleaved with *Bgl*II prior to the snap-back assay. This enzyme was initially chosen to reduce the length of the chromosomal and extrachromosomal DNAs without altering the SV40 pattern, as there is no *Bgl*II site in the SV40 genome. At that time the amplified DNA was assumed to be linear, especially considering the facts that SV40 amplification is transient and that the DNA was examined shortly after its induction and was not expected to undergo additional processes.

However, following the 2D gel analysis in this work, it became apparent that a very substantial fraction of the amplified DNA molecules were circular. Therefore, it was essential to linearize the molecules prior to the snap-back assay to prevent reannealing of supercoiled molecules.

In an additional experiment we compared the S1 nuclease-resistant DNAs that were obtained from *Bgl*I-linearized DNA and *Bgl*II-cut DNA. This experiment clearly showed the reduction in the appearance of S1 nuclease-resistant DNA following linearization (data not shown). Probably the *Bgl*II-linearized DNA contained covalently closed circular molecules that reannealed in the snap-back assay, although they did not contain IRs. On the other hand, in this study, all of the SV40-containing circles were linearized, only IRs could reanneal, and therefore, the signal decreased. Thus, the actual amount of IRs in this system is reflected by the results shown in Fig. 7 and probably was previously overestimated.

Our main conclusion from this experiment is that the entire viral genome was represented in IRs and displayed the same pattern and relative amount as did the total extrachromosomal fraction. Thus, the U-turn model could be one of the mechanisms for circle formation.

spcDNA and genomic instability. Small extrachromosomal circles, named spcDNA, are very common in a large variety of eukaryotic cells and were present in every cell line examined (for reviews, see references 24, 70, and 90). For example, spcDNA was found in and cloned from human cells (2, 3, 41–43, 52, 53, 56), monkey BSC-1 cells (5, 74), CHO cells (77), mouse cells (18, 22, 79, 80, 91), and *D. melanogaster* cells (76). Therefore, spcDNA may represent a universal phenomenon in higher organisms. It is well accepted that these circles are derived from preexisting chromosomal sequences and that they interact with each other (for a review, see reference 24). The diversity in the size, amount, sequence content, and organization of spcDNA implies the existence of several modes for its formation. It was suggested to be the by-product of specific chromosomal events reflecting genetic plasticity, such as recombinations (36, 64, 80), transpositions (for a review, see reference 66), or autonomously replicating DNA (67, 72, 86). The size of spcDNA ranges generally between a few hundred base pairs and few kilobases, and cloning experiments showed a wide representation of repetitive sequences as well as unique chromosomal sequences (for reviews, see references 24, 70, and 90).

Analysis of an spcDNA library derived from angiofibromas in patients with tuberous sclerosis revealed some unique cellular sequences that were proposed to be involved in duplications and rearrangements in the patients' genomes (2). Furthermore, a transposon-like sequence, which contains the R repeats organized in an inverted orientation, was found in a clone from an spcDNA library of mouse thymocytes (22). A study on the DNA repair defect syndrome Fanconi's anemia showed the association of genetic instability and elevated levels of spcDNA molecules (56).

The 2D gel as a tool for the study of spcDNA and genomic instability. We analyzed DNA from CHO cells by using 2D gel electrophoresis in an attempt to identify spcDNA by this technique and to compare the cellular circles with the SV40 amplified circular DNA. On the basis of previous reports on spcDNA, we chose to use the Cot-1 DNA as a probe for the identification of circles containing highly repetitive sequences. Indeed, arcs that represent the circular DNA were detected following hybridization, as was a massive arc of linear DNA (Fig. 8A and A′). These results indicate the presence of a significant basal level of circular molecules in the CHO cells which can be detected by the 2D gels. The circular population in the CHO cells has a size range identical to that of the

amplified circular SV40 DNA, according to their similar arc lengths.

Analysis of spcDNA libraries from 3T6 cells (80), mouse thymocytes (22), and human cells (3, 33, 36) revealed the enrichment of specific repetitive DNA families within the spcDNA relative to their chromosomal abundance. Hence, these findings suggest the possible role of specific repetitive sequences in the formation and selection of the circular molecules. The use of other probes, such as specific families of mid-repetitive sequences or gene families, for hybridization to the DNA separated on the 2D gels might show interesting hybridization patterns and illuminate the features of the spcDNA phenomenon in the aspects of both size and sequence content.

To test the effect of carcinogens, we treated the CHO cells with MNNG under the same conditions as we used for the CO60 cells (for the detection of SV40 amplification). The DNA from the treated CHO cells displayed elevated levels of circular molecules (Fig. 8B and B′) in comparison with the untreated cells, as determined by the stronger hybridization to the circular-DNA arcs. The rate of enhancement was lower than that of the SV40 molecules in the CO60 cells, but it is similar in principle. It should be noted that we did not examine the effects of several different treatment conditions on CHO cells, which could possibly give different or more sharp results regarding the induction of circles. The SV40 system serves as an extreme model, and the viral origin, which is activated and could initiate in a non-cell-cycle-dependent manner, could play a role in the amplification. However, the increase in the circular population is observed also in the cellular DNA of CHO cells upon carcinogen treatment, and this does not depend on a viral origin. Thus, we propose that SV40 can serve as a model for this cellular phenomenon. We conclude that the MNNG treatment enhanced the formation of the circular molecules, similar to its effect on the SV40 sequences in the CO60 cells.

It has been reported that treatment with cycloheximide and drugs arresting DNA replication, such as hydroxyurea and 7,1-dimethylbenzanthracene plus nalidixic acid, enhanced the formation of spcDNA (79, 80). Similar conditions were found to enhance SV40 amplification in CO60 cells (45, 46) and were reported to induce cellular gene amplification in different cell lines (72). It has been observed that higher degrees of genetic instability and increased levels of spcDNA generally parallel each other (55, 56, 64, 86).

Little is known about the abundance of circular molecules in tumor cells, and we would like, in the future, to compare spcDNAs from a tumor and a healthy tissue of the same donor. It is also tempting to perform a wide study, using the 2D gel assay, on spcDNAs in different tumors and in different stages of the malignancy and to determine whether the size, amount, or sequence content of spcDNA could serve as a marker for malignancy.

Using the 2D gel electrophoresis technique, we recently detected extrachromosomal circles containing genomic sequences in other Chinese hamster lines (CO60 and OD4 cells) and in HeLa cells. In addition, spcDNA was easily detected in cells from Fanconi's anemia patients, while we failed to detect it in fibroblasts from healthy donors. Upon carcinogen treatment, an induction of spcDNA was observed in the normal fibroblasts, and a clear enhancement in the circular-DNA population was detected in the Fanconi's anemia cells (14).

We propose that the 2D gel analysis presented here can serve as a very useful tool for the detection, isolation, and analysis of spcDNAs in various cell lines and in normal and tumor cells under different growth conditions. This technique facilitates the direct characterization of the DNA included in the circular population and the detection of the sequences that are enriched and might be involved in the generation and selection of this population.

Extrachromosomal circles and gene amplification. Extrachromosomal circles were shown to be involved in early events of gene amplification both in cancerous mammalian cells (9, 15, 58, 62, 67, 68, 83, 84, 86, 88, 89) and in lower eukaryotes (6, 23, 26, 29, 31, 34, 37, 61, 63, 82, 87). These are usually large circles containing amplicons ranging in size from a few tens to a few hundreds of kilobase pairs, which are often organized in either an inverted or a direct order. A well-examined example is the amplification of the H circle in several species of *Leishmania*, which accompanies the amplification of the dihydrofolate reductase gene (6, 26, 37, 61, 63, 87). These circles are usually about 30 kb long (37) and can be found in wild-type strains (31) or can arise in response to methotrexate selection (61). The 4,300-fold amplification of the adenosine deaminase gene in circular molecules in the mouse fibroblast line B-1/50 was extensively characterized (58). This is a large circle of 500 kb that is organized as an imperfect IR.

In our system the IR-containing circles are smaller than those described above; however, no selection was used and we analyzed the total crude amplification products, which might include a minor fraction of larger molecules. These molecules might not be detected under our experimental conditions. Alternatively, the small circles might evolve to larger molecules by secondary recombination events (9, 15, 28, 58, 67, 68, 83, 84, 86).

A mechanistic linkage between gene amplification and the formation of spcDNA. Our model system seems to relate both to DNA amplification and to circular DNA formation. Gene amplification is well accepted as a marker for genomic instability characterizing transformed cells (as opposed to normal cells) and as part of tumorgenesis. spcDNA is a naturally occurring phenomenon which demonstrates the flexibility of the genome, although its level is enhanced in situations of loss of genomic stability which is either endogenous, such as in cancer and aging, or exogenous, such as in exposure to carcinogens.

These two phenomena are not necessarily separated. One might lead to the other, or there could be common mechanisms which are responsible for both. Our model system is not the only one that seems to link gene amplification and spcDNA. Common mechanistic principles led to previous proposals that spcDNA, which carries a selective advantage and contains an origin of replication, would remain in the cells, similarly to amplified sequences. As a working hypothesis, Rush and Misra (70) proposed that some of the spcDNA represents amplified genes whose replication and expression are favored under appropriate conditions. An implication associated with this proposal is that the initial stages of the extrachromosomal amplification are not rare, but the detection of the amplified sequences requires selection to increase these molecules above the low levels normally observed in the cells. This is exactly what we suggest for the SV40 amplification system: the circular molecules which are generated will disappear if they do not confer a selective advantage. DNA amplification as revealed by drug selection of resistant cultured mammalian cells can involve extrachromosomal forms (72) and may appear initially as unstable circular intermediates (6). Cellular oncogenes are also often amplified in extrachromosomal forms and are maintained in the tumor cells because of the growth advantage conferred to the cells with such amplification. As mentioned above, these amplified sequences were probably slightly different from the original amplicon.

Gene amplification and spcDNA might arise from U-turn

replication or a replication-driven onion skin, which represents successive initiation events from a single origin. Alternatively, breakage and ligation events at arrested replication forks could also occur. The amplification of the circular molecules could result from unequal mitotic segregation coupled with selection or a relaxed type of episomal replication, provided that an origin was captured in the circular molecules. Not only the fact that the spcDNA formation reflects genomic instability, but also further processing of spcDNA, such as autonomous replication and recombination events with the spcDNA itself or with the chromosomes, might contribute to additional genomic changes. Autonomous replication of spcDNA still has to be proven, and it is clear that these molecules have to contain origin features or other features, such as telomeric sequences, that will enable replication. However, there is some evidence that outside the chromosomal context, any human sequence included in a large enough viral circle (which is replication defective itself) could autonomously replicate (41a, 41b). Moreover, even without autonomous replication, the presence of such a heterogeneous reservoir of extrachromosomal circles is prone to undergo secondary interactions, such as homologous and nonhomologous recombination events with the chromosomes, leading to clastogenic effects, and thus it influences genomic fluidity. It should be noted that analysis of the integrated SV40 in subclones which were derived from MNNG-treated CO60 cells revealed new unique bands which differed from one clone to another. This finding suggests rearrangements of the viral sequences which could arise from reintegrations of extrachromosomal molecules (46a).

spcDNA formation may be a mechanism of gene amplification that has been maintained in evolution to respond to selective changes in the environment. The SV40 amplification system, which generates molecules with the same size and structure as spcDNA, strongly suggests that these processes are highly related.

The 2D gel analysis presented in this paper may serve as a powerful tool for the identification of chromosomal plasticity and for the characterization of sequences facilitating autonomous replication. In addition, other circular molecules, such as mitochondrial DNA, plasmids, and viruses, can be studied by using this approach.

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