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Comparative analysis of DNA loop length in nontransformed and transformed hamster cells

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Summary

The size of the looped, matrix-attached DNA domains was estimated in both nontransformed and transformed hamster cells. In BHK cells it was observed that in interphase as well as during mitosis, transformed cells, on the average, have shorter loops than nontransformed cells. In CHO cells, however, no alteration of the length of the looped DNA domains was found to accompany transformation. The implications of these observations are discussed in relation to the process of transformation.

Throughout the cell-cycle chromosomal DNA in eukaryotic cells appears to be constrained into looped domains, 20–100 kilobases in size. These loops result from periodic attachment of DNA to non-histone protein scaffolding, referred to as nuclear matrix (Berezney and Coffey, 1974; Wanka et al., 1977; Vogelstein et al., 1980) or nucleoid (Cook and Brazell, 1976) in interphase nuclei or as chromosomal scaffold (Paulson and Laemmli, 1977) in metaphase chromosomes.

From a score of studies aimed at assessing its functional implications, it has become clear that the looped DNA arrangement is of great relevance to DNA replication and to transcription. Both processes have been shown to occur at the nuclear matrix (Berezney and Coffey, 1975; Dijkwel et al., 1979; Pardoll et al., 1980; McCready et al., 1980; Jackson et al., 1981; Robinson et al., 1982). In line with the observation that DNA loop length correlates positively with replicon size (Buongiorno-Nardelli et al., 1982), it was recently shown that the bases of the loops, i.e. the attachment sites of DNA to the matrix and scaffold, act as replication origins (Aelen et al., 1983; Van der Velden et al., 1984; Dijkwel et al., 1986).

Several studies suggest that replicon size is under functional control (Blumenthal et al., 1973; Callan, 1972; Van't Hof, 1976; Taylor, 1977). This is confirmed by a recent study, in which the observed increase of the size of DNA loops in the course of differentiation (Buongiorno-Nardelli et al., 1982), appears to support the concept of the switching off of certain classes of replication origins during differentiation. A mechanism, in which detachment from the scaffolding structures is the main feature, can be thought of in this respect.

As evidence exists for a change of replicon size

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accompanying transformation (Martin and Oppenheim, 1977; Kapp et al., 1979; Hartwig, 1982; Hanaoka et al., 1983) and as alterations at the origin level can be considered to have important bearing on the regulation of DNA replication, we initiated a study to establish whether DNA loop length changes in the process of transformation.

Materials and methods

Cell culture and labeling procedures

Baby Hamster Kidney (BHK) cells strain A3 (nontransformed) and strain T33 (transformed; derived from A3 by ethylnitrosourea treatment and selection by repeated focal growth in soft agar) (Cupido and Simons, 1984; Dijkwel and Wanka, 1985) and Chinese Hamster Ovary (CHO) cells strain A5 (nontransformed) and strain C13 (transformed; derived from A5 by treatment with 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea) were kind gifts of Dr. J.W.I.M. Simons from the State University at Leiden.

All cell strains were grown as monolayers on plastic (Nunclon) in media supplemented with 8% foetal calf serum (Grand Island Biological Company). BHK cells were maintained in Minimal Essential Medium (Flow Laboratories) and CHO cells were propagated in Ham F-10 medium (Flow Laboratories).

For experiments, in which the DNA arrangement was analysed, one of the BHK or CHO strains was labeled for 3–5 generations with 1 μCi/ml [*Me*-^{3}H]thymidine (spec. act. 20 Ci/mmole; Amersham) while the other strain was labeled with 0.04 μCi/ml [2-^{14}C]thymidine (spec. act. 52.8 Ci/mole; Amersham). To label protein, cells were grown for 3–5 generations in medium containing either 1 μCi/ml L-[4,5-^{3}H]leucine (spec. act. 45 Ci/mmole; Amersham) or 0.5 μCi/ml [^{35}S]methionine (spec. act. 1480 Ci/mmole; Amersham).

Preparation of nuclear matrices

Monolayers of appropriate cells were rinsed twice with 0.9% NaCl in 5 mM Tris–HCl pH 8.0. Then cells of one of each pair of cell strains were washed from the plastic surface with TT-buffer (i.e. 0.1% Triton X-100 in 5 mM Tris–HCl pH 8.0). The suspension was then poured onto the monolayer of the other strain. Nuclei were isolated by forcing the suspension twice through a hypodermic needle (0.8 mm diameter) and centrifuging the suspension for 3 min at 800 × *g*. Nuclei were resuspended subsequently in 50 mM Tris–HCl pH 8.0 and extracted with high salt by addition of one volume of 4 M NaCl in 50 mM Tris–HCl pH 8.0.

For some experiments the procedure was altered. Cells were removed from the plastic surface by trypsination, collected by centrifugation and suspended in fresh growth medium. After approximately 1 h, cells of the two types were mixed, harvested by centrifugation and rinsed twice with 0.9% NaCl. The cells were then suspended in TT buffer, to which an equal volume of 4 M NaCl was added immediately.

Preparation of chromosomal scaffolds

Chromosomal scaffolds were prepared essentially as described before (Pieck et al., 1985). 0.5 μg/ml nocodazole (methyl-5(2-thienyl)-1*H*-benzimidazol-2-yl-carbamate; Sigma) was added to cell cultures in log-phase. After several hours mitotic cells were removed from the plastic surface by gentle shaking and collected by centrifugation. Mitotic indices of the sample were determined by light microscopy after staining with crystal violet. Occasionally, flow cytofluorometry was used. Samples with indices below 95% were discarded.

Mitotic cells from cell-strain pairs were subsequently mixed, rinsed with 0.9% NaCl and resuspended in hypotonic buffer (5 mM Tris–HCl pH 8.0; 0.5 mM spermine; 0.15 mM spermidine). In this medium cells were allowed to swell for 5 min at 0 °C, after which the suspension was slowly added to the lysis mixture (0.2% Triton X-100; 2 M NaCl; 5 mM Tris–HCl pH 8.0; 0.5 mM spermine; 0.15 mM spermidine: final concentrations).

DNAaseI digestion and sucrose-gradient centrifugation

To samples containing nuclear matrices for chromosomal scaffolds, suspended in 2 M NaCl, $MgCl_2$ was added to a concentration of 7.5 mM. Then 10 U/ml DNAase I (Sigma) were added and the samples were incubated at 37 °C. Aliquots of 5 ml were withdrawn from the moment of enzyme addition ($t = 0$) onward at 5-min intervals. Di-

gestion was stopped by chilling on ice and addition of EDTA till 15 mM. Subsequently, the aliquots were layered on 10–30% neutral sucrose gradients containing 2 M NaCl, with a wide-bore plastic pipette. Centrifugation was performed in Beckman SW-28 rotors spun at 22 000 rpm at 20 °C till w^2t values of between 2 and 4×10^{10} were attained. Gradients were fractionated and radioactivity was determined as described before (Wanka, 1974).

In experiments in which fragment lengths were estimated, only one half of the rapidly sedimenting material was used for determination of radioactivity. The remainder of the (usually 3) bottom fractions of the gradient was pooled and one volume of distilled water was added to reduce sucrose and salt concentrations. DNA was precipitated by adding 2.5 volumes of 96% ethanol containing 1% K-acetate and storage overnight at −20 °C. The DNA was collected by centrifugation and dissolved in 0.5% SDS in 50 mM Tris–HCl pH 8.0. Samples were then layered on 5–25% sucrose gradients and centrifuged in SW-28I rotors at 20 000 rpm till w^2t values of 2.5×10^{11} were reached.

Alternatively, the samples were analysed on 0.8% neutral agarose gels run overnight at 1 V/cm.

Halo-micrography

To prepare halo-ed matrices the method of Vogelstein et al. (1980) was used. Briefly, cells grown on coverslips were treated for 30 sec with 0.5% Nonidet P-40 in 50 mM Hepes pH 7.8; 10 mM $MgCl_2$; 0.5 mM $CaCl_2$; 0.22 M sucrose. Then the permeabilized cells were extracted sequentially with 0.0, 0.2, 0.4 ..... 1.8 M NaCl in 10 mM Tris–HCl pH 7.4; 0.2 mM $MgCl_2$. Finally the extracted cells were transferred to 2.0 M NaCl containing either 4 μg/ml or 100 μg/ml ethidium bromide. In the microscope a phase-contrast photograph was first made, after which, from the same structure, a fluorescent image was recorded. To estimate loop length, the diameter of the matrix, obtained from the first micrograph, was subtracted from that of the matrix + halo, obtained from the fluorescent image. When 100 μg/ml ethidium bromide had been used, the salt extracted structures were UV-irradiated for 45 sec prior to photography.

Results

(1) Background of the experimental approach

DNA is attched to skeletal structures in arrays of loops. To establish whether average loop length is altered during transformation, skeletal structures of nontransformed and transformed cells are mixed and the mixtures are then subjected to the action of nucleases.

Assuming transformation is accompanied by a change of the length of the DNA loops, in mixtures obtained as described above, skeletal structures with small loops (X) (Fig. 1A) will exist along with structures with loops of a larger size (Y) (Fig. 1B). Nucleases, such as DNAase I, introduce nicks randomly and as digestion of X and Y is performed under identical conditions, the DNA fragments generated will be of the same average length (Figs. 1C and 1D). As shown in Figs. 1E and 1F, the proportion of DNA remaining associated with the skeletal structures is expected to increase with increasing numbers of attachment sites per unit length of DNA, i.e. with decreasing loop length.

When the DNA detachment approaches 100%, the ratio of the proportions of DNA bound to structures of types X and Y will reflect the relative difference of the size of the DNA loops.

Experiments described in the following section were essentially performed along these lines. Separation of matrix or scaffold attached DNA from detached DNA was attained by centrifugation, employing the rapidly sedimenting nature of the skeletal structures.

(2) Comparative analysis of DNA loop length

DNA loops, attached to the nuclear matrix as depicted in Figs. 1A and 1B, can be visualized as an ethidium bromide stainable "halo". An initial indication that DNA loop length in nontransformed BHK cells differed from that in transformed cells, was obtained by estimating the halo-radii of both cell strains. To that purpose, halo-ed residual nuclei were prepared (Vogelstein et al., 1980). Prior to measurement the DNA loops were relaxed either by addition of 4 μg/ml ethidium bromide or by a combination of 100 μg/ml ethidium bromide and UV-irradiation. From the micrographs, in both cases, a mean loop

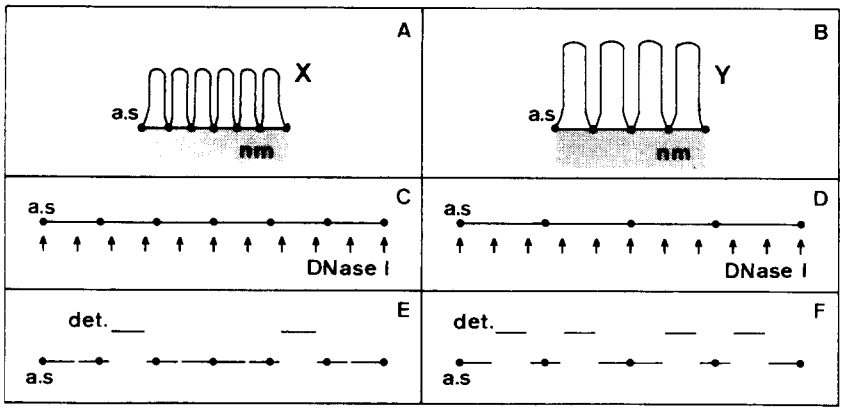

Fig. 1. Experimental approach to assess DNA loop length differences. Mixtures of nuclei of nontransformed and transformed cells are extracted with 2 M NaCl. Consequently, matrices of type X, to which DNA is attached in small loops (A), will exist along with matrices of type Y, to which DNA is attached in larger loops (B). DNAase I is added to the mixtures and nicks are introduced into the DNA associated with either matrix type with similar frequency (C–D). When matrices are subsequently centrifuged over neutral sucrose gradients, part of the DNA (designated det.) will no longer be attached to the matrices and will not enter the gradient (E–F). The fraction of DNA cosedimenting with the matrices is directly related to the number of attachment sites per unit length of DNA. a.s., attachment site of DNA to nuclear matrix or chromosomal scaffold. n.m., nuclear matrix or chromosomal scaffold.

length of 26.1 ± 2.6 μm ($n = 20$) was calculated for the nontransformed A3 strain, whereas the loops of the transformed T33 cells averaged to 21.0 ± 2.5 μm ($n = 23$).

Separate analysis on neutral sucrose gradients of A3 and T33 nuclear matrices, of which the DNA had been labeled randomly with [^{14}C]thymidine and the protein with [^{3}H]leucine, gave results compatible with those presented above. Fig. 2 shows that A3 matrices (2A) sediment at lower rates than T33 matrices (2B). As the matrices both contain about 20% of total nuclear protein and 80% of the DNA, the most plausible reason for the differing sedimentation behaviour is a different particle size. Previous studies (Cook and Brazell, 1976) have demonstrated the dramatic

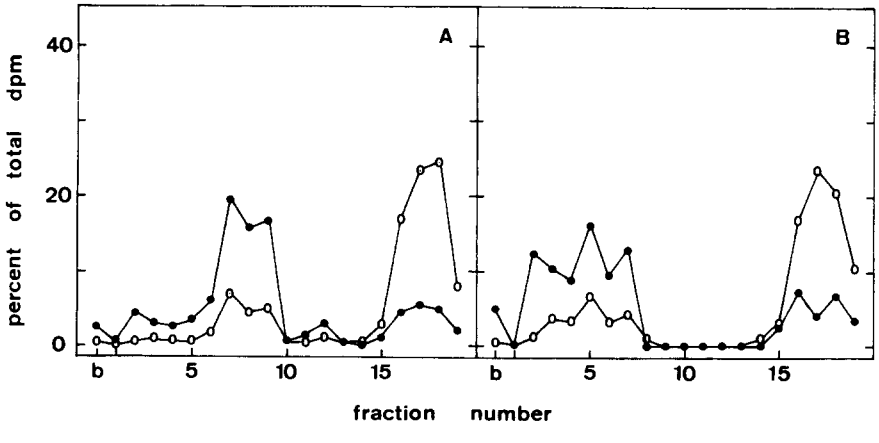

Fig. 2. Sedimentation analysis for A3- and T33-nuclear matrices. A3 and T33 cells were continuously labeled with both [^{3}H]leucine and [^{14}C]thymidine. Nuclei were isolated and extracted with 2 M NaCl. (A) Sedimentation of A3 matrices, centrifuged for 50 min at 5000 rpm. Sedimentation is from right to left. Fraction b represents the label sedimented to the bottom of the tube. ○, [^{3}H]leucine: 55100 dpm; ●, [^{14}C]thymidine: 23400 dpm. (B) Sedimentation of T33-matrices (as under A). ○, [^{3}H]leucine: 48600 dpm; ●, [^{14}C]thymidine: 2600 dpm.

effect of a change of the radius of the halo on sedimentation rates. Most probably, therefore, the differences between A3 and T33 are caused by different DNA loop sizes. Note that T33 cells incorporate thymidine inefficiently compared to A3. Consequently, in subsequent experiments, T33 cells were labeled with rather high (1 μCi/ml) concentrations of [^{3}H]thymidine.

For further comparative analysis of loop size, DNA of the nontransformed cells was randomly labeled with [^{14}C]thymidine, while transformed cells were labeled with [^{3}H]thymidine. The cell cultures were then split. Part was treated with nocodazole to obtain mitotic cells, from which chromosomal scaffolds were isolated. From the other part of the cell cultures nuclei were isolated and extracted with 2 M NaCl to obtain nuclear matrices. Mixtures of chromosomal scaffolds of each pair of cell strains, as well as mixtures of nuclear matrices, were then digested with DNAase I for increasing periods of time to obtain a graded release of DNA from the skeletal structures. The results of subsequent analysis on sucrose gradients for a typical experiment with BHK cells are depicted in Fig. 3.

Both nuclear matrices (Fig. 3A) and chromosomal scaffolds (Fig. 3B), not treated with DNAase I, were found to be associated with most of the chromosomal DNA. Upon progressive DNAase I digestion, DNA was released from the structures increasingly, and differentially (Figs. 3B–3F). Differential detachment is reflected in an increase of the relative ratio of T33- over A3-DNA in the rapidly sedimenting fraction upon increasing detachment. From this observation it might be inferred that, on the average, the transformed strain has smaller loops than the nontransformed strain.

Results of 3 independent experiments with interphase as well as metaphase A3- and T33-cells are summarized in Figs. 4A and 4B. In these figures the relative ratio of the amount of attached

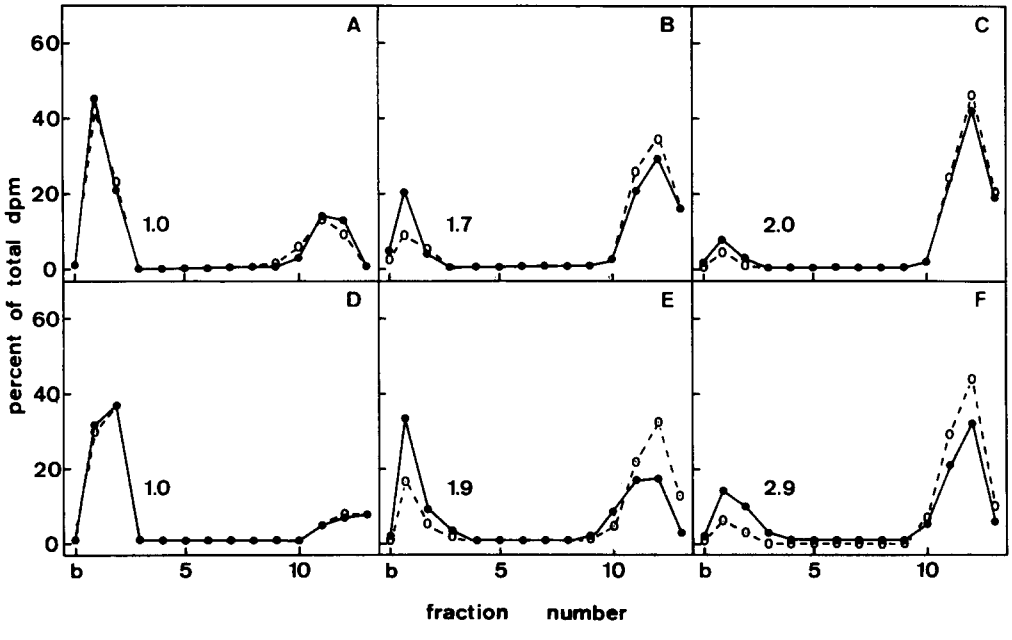

Fig. 3. Effect of DNAase I digestion on the DNA-label distribution in mixtures of A3- and T33-nuclear matrices and A3- and T33-chromosomal scaffolds. BHK A3 and T33 cells were continuously labeled with [^{14}C]thymidine and [^{3}H]thymidine respectively. From mixtures of A3- and T33-nuclei or A3- and T33-mitotic cells nuclear matrices respectively chromosomal scaffolds were isolated. After incubation with 10 U/ml DNAase I for 0 (A and D), 10 (B and E) and 25 (C and F) minutes, the mixtures were analysed on sucrose gradients. Nuclear matrix samples (A, B and C) contained, on the average, 120 000 ^{3}H-dpm (●) and 175 000 ^{14}C-dpm (○). Chromosomal scaffold samples (D, E and F) contained, on the average, 70 000 ^{3}H-dpm (●) and 11 000 ^{14}C-dpm (○). The numbers in each panel represent the ratios of the percentages of ^{3}H- and ^{14}C-label residing in the rapidly sedimenting material.

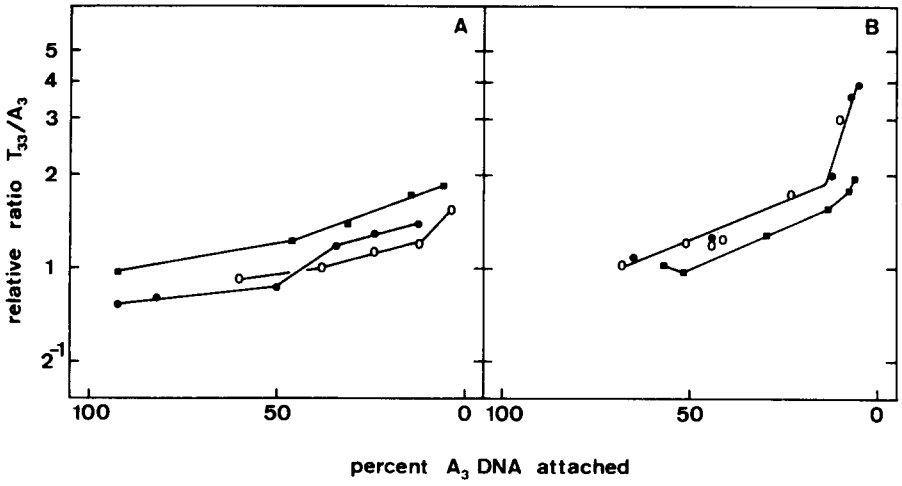

Fig. 4. Differential effect of progressive DNAase I digestion on attached DNA in mixtures of A3- and T33-nuclear matrices (A) and A3- and T33-chromosomal scaffolds. For 3 Expts. the ratio of the relative amounts of T33-DNA over A3-DNA attached to the rapidly sedimenting structures is plotted as a function of the DNA detachment from the A3 structures.

T33-DNA over attached A3-DNA is plotted versus the proportion of attached A3-DNA. It is observed that initially matrices and scaffolds of the nontransformed A3-cells are associated with a larger proportion of the DNA than the skeletal structures of the T33 cells. This might be the consequence of enhanced nulease sensitivity of the chromatin of T33 cells compared to that of A3 cells (Dijkwel and Wanka, 1985). As a result, endogenous nucleases will degrade T33-DNA during the isolation procedure to a somewhat greater extent than A3-DNA.

Alternatively, transformed cells might have a larger fraction of very large DNA loops.

Upon progressive DNAase I digestion it was consistently found that a greater proportion of DNA was detached from A3- than from T33-matrices (Fig. 4A).

Final ratios appeared to cluster at values between 1.4 and 1.9. Experiments in which DNA organization relative to the chromosomal scaffold was analysed (Fig. 4B) also indicate that loops in nontransformed cells are larger than in transformed cells. Upon increasing DNA detachment from the scaffolds, ratios steadily increased to attain final values exceeding 2. These observations rule out the possibility that loop length differences between A3 and T33 are primarily caused by different distributions over the cell cycle. However, as during mitosis a greater difference exists than during interphase, some effect of inequal fractions of cells in S-phase might be discernable.

Comparative loop length analyses were also done with CHO cells. As for BHK cells, DNA of the transformed strain C13 was randomly labeled with [^{3}H]thymidine, while the nontransformed strain A5 was grown in medium containing [^{14}C]thymidine. Mixtures of C13- and A5-matrices as well as scaffolds were then prepared and subjected to graded DNAase I digestion as described before. Fig. 5 shows that from both matrices and scaffolds differential release of DNA did not occur. Consequently, in CHO cells transformation does not appear to affect average length of the looped DNA domains.

(3) Estimation of DNA loop size in BHK cells

It is apparent on theoretical grounds that the ratios of the relative amounts of A3- and T33-DNA in the gradient fractions containing the skeletal structures can be related to loop length differences only if it is demonstrated that the length of the attached DNA fragments is equal for both cell strains. Therefore, after DNAase I digestion, DNA was released from the proteinaceous skeletal structures by SDS. The size of the fragments was

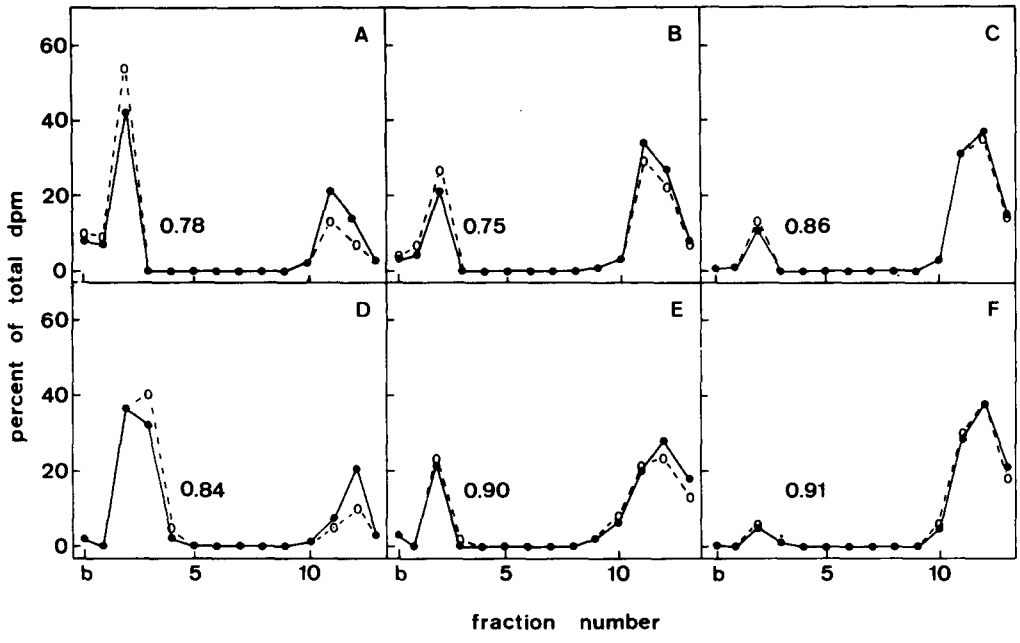

Fig. 5. Effect of DNAase I digestion on the DNA-label distribution in mixtures of A5- and C13-nuclear matrices and A5- and C13-chromosomal scaffolds. CHO A5 and C13 cells were continuously labeled with [^{14}C]thymidine and [^{3}H]thymidine respectively. For further details see legend to Fig. 2. Nuclear matrix samples (A, B and C) contained, on the average, 820000 ^{3}H-dpm (●) and 145000 ^{14}C-dpm (○). Chromosomal scaffold samples (D, E and F) contained, on the average, 11500 ^{3}H-dpm (●) and 4700 ^{14}C-dpm (○).

subsequently determined either by sucrose gradient centrifugation or by agarose gel-electrophoresis.

Fig. 6 shows the size distribution of matrix-attached DNA on neutral sucrose gradients. Since at all extents of detachment assayed, the positions of

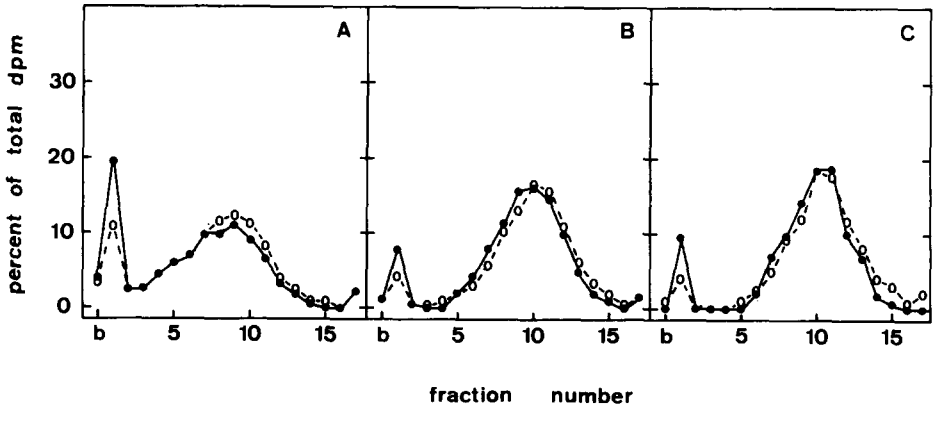

Fig. 6. Analysis of the size distribution of matrix-attached DNA fragments. Mixtures of A3- and T33-nuclear matrices were digested with DNAase I to different extents and centrifuged over sucrose gradients. From the gradient fractions containing the matrices DNA was collected. The size distribution of this DNA fraction was analysed on neutral 5–20% sucrose gradients. A3 DNA: ○; T33 DNA: ●. (A) A3 DNA matrix attached after DNAase I digestion: 28% (6600 dpm); T33 DNA matrix attached after DNAase I digestion: 36% (11000 dpm). (B) A3 DNA, 13.5% (4050 dpm); T33 DNA, 22.5% (6400 dpm). (C) A3 DNA, 5.0% (1200 dpm); T33 DNA, 8.5% (1900 dpm).

the ^{3}H- and ^{14}C-peaks were found to coincide, it can be concluded that the ratio variations observed upon DNAase I digestion reflect differences of the number of attachment sites per unit length of DNA or, alternatively phrased, differences of loop size. Analysis of chromosomal scaffold attached DNA yielded similar results (data not shown).

The length of the DNA loops in A3 and T33 cells was estimated by converting the label distribution found on sucrose gradients and in agarose gels to a fragment number distribution. From this the average fragment length was calculated and multiplied by the appropriate factor, i.e. 100/% DNA attached, to obtain the average DNA loop length. Tables 1 and 2 summarize the most relevant data.

(4) Absence of differential stability of skeletal structures

For BHK cells our data indicate that, upon progressive detachment of DNA from mixtures of A3 and T33 nuclear matrices and chromosomal scaffolds, the ratio of attached T33-DNA over A3-DNA increases. This can be considered evidence for a difference of average loop length only if it can be ruled out that the ratio change is the consequence of the relative instability of the skeletal structures obtained from one of the BHK strains upon prolonged incubation at 37°C in 2 M NaCl. To assess whether such a difference exists, the following experiments were performed. T33 cells were continuously labeled with [^{35}S]methionine while A3 cells were grown in medium containing [^{3}H]leucine. From mixtures of A3 and T33 nuclei matrices were isolated, which were subjected to graded DNAase I digestion. On sucrose gradients it was subsequently analysed whether, during incubation, the $^{35}S/^{3}H$-ratio in the rapidly sedimenting material changed.

Fig. 7 shows that during the DNAase I digestion the proportion of protein residing in the matrices of the T33 as well as A3 cells did not change significantly and amounted to approximately 10% of total nuclear protein. Consequently, the relative ratio of the two protein labels remained essentially constant during detachment of DNA from the matrices. This indicates that the observed differential detachment of DNA by DNAase I is real and cannot be ascribed to the relative instability of A3 matrices compared to T33 matrices.

Moreover, the polypeptide composition of A3 and T33 matrices was found not to differ, with as sole exception the presence in the nontransformed cells of a protein of high molecular weight (data not shown). This polypeptide, however, might consist of a complex of matrix proteins, as its presence is much less prominent in matrices isolated in the presence of thiol-agents (Dijkwel and Wenink, 1986).

Comparable results were also found for chromosomal scaffolds. However, contrary to matrices, which were found to contain a constant proportion of total nuclear protein, the relative amount residing in the scaffolds was observed to increase during DNAase I treatment. This renders interpretation of these data rather complex. Nevertheless, as the ratio of the two labels in the rapidly sedimenting material remained constant through-

TABLE 1

ESTIMATION OF THE SIZE OF THE DNA LOOPS ATTACHED TO THE NUCLEAR MATRICES OF A3- AND T33-CELLS

Strain	Average length of the DNA fragments	Percent of DNA matrix attached	Average loop length	
			kb	μm
A3	9000 bp	13.5	67	22
T33		22.5	41	14
A3	4000 bp	5.0	80	27
T33		8.5	48	16
A3	2500 bp	4.0	63	21
T33		6.5	40	13

TABLE 2

ESTIMATION OF THE SIZE OF THE DNA LOOPS ATTACHED TO THE CHROMOSOMAL SCAFFOLDS OF A3 AND T33 CELLS

Strain	Average length of the DNA-fragments	Percent of DNA scaffold attached	Average loop length	
			kb	μm
A3	21000	25.0	80	27
T33		48.0	45	15
A3	13000	13.0	97	32
T33		21.0	63	21
A3	6000	7.0	84	28
T33		12.0	51	17

out the DNAase I incubation, the results are also taken to indicate that in mixtures of chromosomal scaffolds differential effects at the protein level do not occur.

(5) Similarity of loop size in interphase and during mitosis

Several authors have presented evidence favouring the idea of a constant size of the looped DNA domains throughout the cell cycle (McCready et al., 1980; Georgiev et al., 1983). Data presented by us so far indicate that final relative ratios in chromosomal scaffolds attain higher values than those in interphase nuclear matrices, hereby contradicting previous evidence.

To further substantiate our findings, one culture of T33 cells was continuously labeled with [^{3}H]thymidine. This culture was treated with nocodazole and mitotic cells were isolated. From another culture of T33 cells, of which the DNA had been labeled randomly with [^{14}C]thymidine, nuclei were isolated. These nuclei were added to hypotonically treated mitotic cells, after which the mixture was extracted with 2 M NaCl in the presence of Triton X-100. Subsequently, the mixture was subjected to graded DNAase I digestion.

Fig. 8 shows that, initially, and most probably as the consequence of the isolation procedure, DNA is detached from the chromosomal scaffolds to a greater extent than from the nuclear matrices.

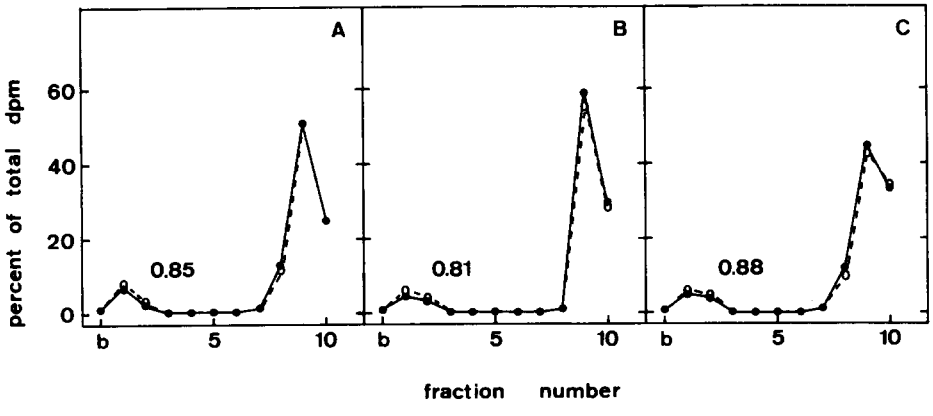

Fig. 7. Effect of DNAase I digestion on the protein-label distribution in mixtures of A3- and T33-nuclear matrices. BHK A3 and T33 cells were continuously labeled with [^{3}H]leucine and [^{35}S]methionine respectively. From mixtures of A3- and T33-nuclei matrices were isolated and treated with 10 U/ml DNAase I for 0 (A), 15 (B) and 30 (C) min. Profiles show the protein-label distribution on neutral sucrose gradients. Samples analysed contained, on the average, 6000 ^{35}S-dpm (●) and 50 000 ^{3}H-dpm (○). The numbers in each panel represent the ratios of the percentages of ^{35}S- and ^{3}H-label residing in the rapidly sedimenting material.

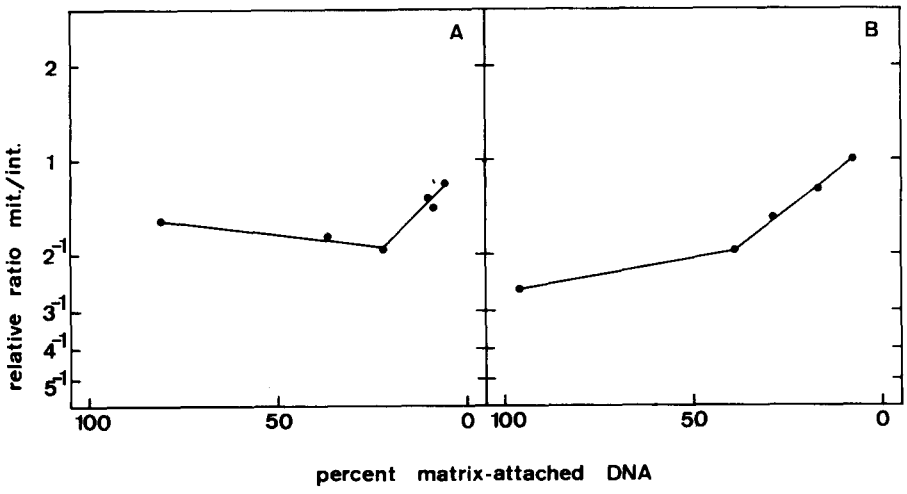

Fig. 8. Effect of progressive DNAase I digestion on attached DNA in mixtures of T33-nuclear matrices and chromosomal scaffolds (A) and A3-nuclear matrices and chromosomal scaffolds (B). For each strain the ratio of the relative amounts of DNA attached to the scaffolds and matrices is plotted as a function of DNA-detachment from the nuclear matrices.

However, upon increasing detachment by DNAase I, the ratio is observed to approach the value of 1, indicating that during mitosis average DNA loop length does not differ from that during interphase in a significant way. Similar observations were made for A3 cells (data not shown).

Discussion

One of the main features of higher-order organization of the eukaryotic genome is the existence of supercoiled domains. These domains arise as the consequence of constrainment of the chromatin fiber by periodic attachment to proteinaceous skeletal structures, i.e. the nuclear matrix during interphase and the chromosomal scaffold during mitosis. Due to the employed isolation procedures, which generally are based on extractions with high concentrations of NaCl, the existence in vivo of these structures is still somewhat controversial (Bouteille et al., 1983; Wunderli et al., 1983). However, biochemical (Pieck et al., 1985), immunological (Earnshaw and Heck, 1985) and functional (Dijkwel et al., 1979; Mirkovitch et al., 1984) evidence argues against the skeletal structures being total artefacts and indicates that these structures very probably have an important relation to in vivo structures in the nucleus and in the chromosome.

In this study, in which the nuclear matrix and the chromosomal scaffold are operationally defined as structures remaining after extraction with 2 M NaCl, we analysed whether tranformation is in some way related to alterations of higher order DNA organization. Results presented indicate that in BHK cells transformation, induced by chemical mutagens, is accompanied by a reduction of the average length of the looped DNA domains. In CHO cells, however, no change of the size of the DNA loops was observed to accompany transformation. These observations, apparently contradictory, can be related to the growth characteristics of the two cell types studied. Of the BHK cells only T33 can be propagated in soft agar, while both CHO strains grow efficiently in this medium. The transformed CHO strain C13 differs from the A5 strain, from which it was derived, only in its enhanced ability to form tumors upon inoculation into nude mice (J.W.I.M. Simons, personal communication). Consequently, only in the case of the BHK cells, transformed cells have been compared with nontransformed cells.

In view of the involvement of the nuclear matrix in DNA replication, the change of the size of the looped DNA domains observed to accompany transformation, potentially is of great interest. It has been shown that loop size and replicon size are related (Buongiorno-Nardelli et al., 1982). Re-

cently, by both biochemical (Aelen et al., 1983; Van der Velden et al., 1984) and autoradiographic (Dijkwel et al., 1986) methods it has been shown that matrix attachment sites exist on the DNA in the proximity of the origins of replication. Consequently, decrease of the size of the DNA loops might reflect an increase of the number of operational origins, occurring during transformation in BHK cells. Since regulation of DNA replication at the origin level can be considered to be an essential process in cell proliferation, acquisition by transformed cells of (a) set(s) of replication origins, silent in normal cells, might lead to disturbance of regulatory patterns operative in nontransformed cells.

Recently, data were also presented indicating rearrangements of higher order structure of nuclear DNA, resulting in a decrease of the size of the looped DNA domains, might be related to malignancy (Hartwig, 1982). In view of the reported relation between replicon and loop size (Buongiorno-Nardelli et al., 1982), results indicating that a reduction of replicon size occurs upon SV40-induced transformation (Martin and Oppenheim, 1977; Hanaoka et al., 1983), might also be considered to be in support of a hypothesis relating malignancy to an alteration of DNA loop length. Other authors, however, have presented evidence for an increase of replicon size upon SV40-induced transformation (Kapp et al., 1979), which contradicts the hypothesis outlined above. At present an explanation for these contradictory results cannot be provided. Further systematic comparison of nontransformed with transformed cells along the lines of this study will be required.

Higher order DNA structure and malignancy might also be related in another way. It has been shown that actively transcribed genes are located in the DNA loops at positions close to or at the nuclear matrix (Robinson et al., 1982; Ciejek et al., 1983). Both for multiple attachment of active genes to the matrix (Small et al., 1985) and for a unique attachment site (Mirkovitch et al., 1984) evidence has been presented. Interestingly, the sites are located upstream of the gene, possibly in regulatory sequences.

We have shown that transformation of BHK cells is accompanied by an increase of the number of matrix attachment sites per unit length of DNA. It might therefore be conceived that in transformed cells a (number of the) additional attachment site(s) is located close to (a) gene(s) silent in nontransformed cells. As a consequence, the gene, which might be a proto-oncogene, will be activated. In this view activation is the consequence of either disturbance of the regular chromatin repeat structure in regions flanking a gene as the consequence of binding by non-histone proteins (Emerson and Felsenfeld, 1984) or of the fact that the gene is displaced to the matrix compartment in the nucleus, which can be considered to be highly enriched in transcription factors (Mirkovitch et al., 1984).

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References

Aelen, J.M.A., R.J.G. Opstelten and F. Wanka (1983) Organization of DNA replication in Physarum, Attachment of the origins of replication and replication forks to the nuclear matrix, Nucleic Acids Res., 11, 1181–1195.

Berezney, R., and D.S. Coffey (1974) Identification of a nuclear protein matrix, Biochem. Biophys. Res. Commun., 60, 1410–1419.

Berezney, R., and D.S. Coffey (1975) Nuclear protein matrix: association with newly synthesized DNA, Science, 189, 291–293.

Blumenthal, A.B., H.J. Kriegstein and D.S. Hogness (1973) Units of DNA replication in *Drosophila melanogaster* chromosomes, Cold Spring Harbor Symp. Quant. Biol., 38, 205–223.

Bouteille, M., D. Bouvier and A.P. Seve (1983) Heterogeneity and territorial organization of the nuclear matrix and related structures, Int. Rev. Cytol., 83, 134–182.

Buongiorno-Nardelli, M., G. Micheli, M.T. Carri and M. Marilley (1982) A relationship between replicon size and supercoiled loop domains in the eukaryotic genome, Nature (London), 298, 100–102.

Callan, H.G. (1972) Replication of DNA in chromosomes of eukaryotes, Proc. Roy. Soc. (London), 181, 19.

Ciejek, E.M., M. Tsai and B. O'Malley (1983) Actively transcribed genes are associated with the nuclear matrix, Nature (London), 306, 607–609.

Cook, P.R., and I.A. Brazell (1976) Conformational constraints in nuclear DNA, J. Cell Sci., 22, 287–302.

Cupido, M., and J.W.I.M. Simons (1984) A reappraisal of the BHK-21 clone 12 cell transformation system with respect to assay conditions and the mutational origin of the transformed phenotype, Carcinogenesis, 5, 857–862.

Dijkwel, P.A., and F. Wanka (1985) Chromatin degradation in isolated nuclei of normal and transformed BHK-cells, Cancer Res., 45, 2046–2049.

Dijkwel, P.A., and P.W. Wenink (1986) Structural integrity of the nuclear matrix, Differential effects of thiol-agents and metal-chelators, J. Cell Sci., 84, 53–67.

Dijkwel, P.A., L.H.F. Mullenders and F. Wanka (1979) Analysis of the attachment of replicating DNA to a nuclear matrix in mammalian interphase cells, Nucleic Acids Res., 6, 219–230.

Dijkwel, P.A., P.W. Wenink and J. Poddighe (1986) Permanent attachment of active replication origins to the nuclear matrix in BHK-cells, Nucleic Acids Res., 14, 3241–3249.

Earnshaw, W.C., and M.M.S. Heck (1985) Localization of topoisomerase II in mitotic chromosomes, J. Cell Biol., 100, 1716–1725.

Emerson, B.M., and G. Felsenfeld (1984) Specific factor conferring nuclease hypersensitivity at the 5′-end of the chicken adult β-globin gene, Proc. Natl. Acad. Sci. (U.S.A.), 81, 95–99.

Hanaoka, F., F. Takeuchi, T. Matsumara, M. Goto, T. Miyamoto and M. Yamada (1983) Decrease of the average size of replicons in a Werner syndrome cell-line by simian virus 40 infection, Exp. Cell Res., 144, 463–467.

Hartwig, M. (1982) The size of independently supercoiled domains in nuclear DNA from normal human lymphocytes and leukemic lymphoblasts, Biochim. Biophys. Acta, 698, 214–217.

Jackson, D.A., S.J. McCready and P.R. Cook (1981) RNA is synthesized at the nuclear cage, Nature (London), 292, 552–555.

Kapp, L.N., S.D. Park and J.E. Cleaver (1979) Replicon sizes in nontransformed and SV-40 transformed cells as estimated by a bromodeoxyuridine photolysis method, Exp. Cell Res. 123, 375–377.

Martin, R.G., and A. Oppenheim (1977) Initiation points for DNA-replication in nontransformed and SV-40 transformed Chinese hamster lung cells, Cell, 11, 859–869.

McCready, S.J., J. Godwin, D.W. Mason, I.A. Brazell and P.R. Cook (1980) DNA is replicated at the nuclear cage, J. Cell Sci., 46, 365–386.

Mirkovitch, J., M.E. Mirault and U.K. Laemmli (1984) Organization of the higher order chromatin loop: specific DNA attachment sites on the nuclear scaffold, Cell, 39, 223–232.

Pardoll, D.M., B. Vogelstein and D.S. Coffey (1980) A fixed site of DNA-replication in eukaryotic cells, Cell, 19, 527–536.

Paulson, J., and U.K. Laemmli (1977) The structure of histone-depleted metaphase chromosomes, Cell, 12, 817–828.

Pieck, A.C.M., H.M.W. Van der Velden, A.A.M. Rijken, J.M. Neis and F. Wanka (1985) Protein composition of the chromosomal scaffold and interphase nuclear matrix, Chromosoma, 91, 127–144.

Robinson, S.I., B.D. Nelkin and B. Vogelstein (1982) The ovalbumin gene is associated with the nuclear matrix in chicken oviduct cells, Cell, 28, 99–106.

Small, D., B.D. Nelkin and B. Vogelstein (1985) The association of transcribed genes with the nuclear matrix of Drosophila cells during heat shock, Nucleic Acids Res., 13, 2413–2431.

Taylor, J.H. (1977) Increase in DNA replication sites in cells held at the beginning of S-phase, Chromosoma, 62, 291–300.

Van der Velden, H.M.W., G. Van Willigen, R.H.W. Wetzels and F. Wanka (1984) Attachment of origins of replication to the nuclear matrix and the chromosomal scaffold, FEBS Lett., 171, 13–16.

Van't Hof, J. (1976) Replicon size and rate of fork movement in early S of higher plant cells (*Pisum sativum*), Exp. Cell Res., 103, 395–403.

Vogelstein, B., D.M. Pardoll and D.S. Coffey (1980) Supercoiled loops and eukaryotic DNA-replication, Cell, 22, 79–85.

Wanka, F. (1974) Decreased DNA-synthesis in mammalian cells after exposure to deoxyadenosine, Exp. Cell Res., 85, 409–414.

Wanka, F., L.H.F. Mullenders, A.G.M. Bekers, L.J. Pennings, J.M.A. Aelen and J. Eijgensteijn (1977) Association of nuclear DNA with a rapidly sedimenting structure, Biochem. Biophys. Res. Commun., 74, 739–747.

Wunderli, H., M. Westphal, B. Armbrust and P. Labhart (1983) Studies on the structural organization of membrane-depleted nuclei and metaphase chromosomes, Chromosoma, 88, 241–248.

Zieve, G., D. Turnbull, J.M. Mullins and J.R. McIntosh (1980) Production of large numbers of mitotic cells by the use of the reversible mitotic inhibitor Nocodazole, Exp. Cell Res., 126, 397–405.