Preferential association of a transcriptionally active gene with the nuclear matrix of rat fibroblasts transformed by a simian-virus-40–pBR322 recombinant plasmid

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To study the relationship between the structural organization and function of the eukaryotic genome, DNA associated with nuclear matrix was analysed by using a transformed rat fibroblast cell line. The nuclear matrices were prepared from the isolated nuclei of pSV1-FR, a rat fibroblast cell line transformed by a pBR322-based recombinant plasmid containing an early gene region, which codes for large T-antigen, of simian virus 40. This transformed cell contained a single copy of the plasmid sequence integrated into the chromosomal DNA of the host cell. The early gene of this plasmid was constitutively expressed, as demonstrated by positive immunofluorescence staining of the cell for large T-antigen and by RNA-blot analysis for its specific mRNA. DNAs were extracted from whole isolated nuclei and nuclear-matrix preparations of the cells, and the relative amounts of the sequence similar to that of the plasmid were compared between these DNA preparations. By employing dot hybridization and Southern-blot analyses we found that the plasmid sequence was more enriched in the DNA extracted from the nuclear matrices than in the DNA extracted from the whole nuclei. When an albumin gene sequence that was not transcribed in this cell line was compared similarly as a control, we found no significant enrichment of this sequence in the DNA associated with the nuclear matrix. Our results strongly support the concept that a transcriptionally active gene is preferentially associated with the nuclear matrix.

INTRODUCTION

A large body of evidence suggests that the interphase chromatin fibres of eukaryotic cells are organized into supercoiled loops of about 50–100 kbp of DNA (Cook & Brazell, 1975; Benyajati & Worcel, 1976; Pinon & Salts, 1977; Igo-Kemenes & Zachau, 1978). Although the precise mechanism by which this integrity of chromatin fibres is maintained is not fully understood, it has recently been proposed that a special nuclear structure, i.e., 'nuclear matrix' or 'nuclear cage', may constitute a skeleton or a framework to which some segment of this DNA loop is anchored (Vogelstein *et al.*, 1980; Berezney & Buchholtz, 1981). This special structure is composed primarily of non-histone nuclear proteins and some tightly bound DNA (Berezney & Coffey, 1975a; Long *et al.*, 1979) and is assumed to be identical with, or almost equivalent to, the 'chromosome scaffold' of the metaphase chromosome (Laemmli *et al.*, 1977). The nuclear matrix is obtained by treating isolated nuclei with a high-concentration salt solution and nuclease, and is presently believed to play important roles in DNA replication (Wood & Collins, 1986; Aguinaga *et al.*, 1987; Tubo & Berezney, 1987; Ciampor, 1988), gene expression (Robinson *et al.*, 1982; Jost & Seldran, 1984; Neuer & Werner, 1985; Birch & Schreiber, 1986; Keppel, 1986; Buttyan & Olsson, 1986; Smith & Rothblum, 1987) and processing of pre-mRNA (Schröder *et al.*, 1987; Zeitlin *et al.*, 1987). More recently it has also been suggested that some proteins tightly bound to the nuclear matrix are related to regulatory elements of gene expression (Bekhor & Mirell, 1979; Gates & Bekhor, 1979; Pumo *et al.*, 1980).

Since some DNA segments are closely associated with the nuclear matrix, it is tempting to hypothesize some possible correlations between this association and gene expression. One experimental strategy to test this possibility is to see whether the transcriptionally active genes are preferentially enriched in the fraction of DNA associated with the nuclear matrix. In fact, Nelkin *et al.* (1980) and Cook *et al.* (1982) have found preferential enrichment of a transcriptionally active integrated viral sequence in the nuclear-matrix-associated DNA obtained from cells

transformed by simian virus 40 or polyoma virus. Similarly, Robinson *et al.* (1982) found enrichment of an ovalbumin gene sequence in the DNA associated with the nuclear matrix of oviduct cells. On the other hand, Kirov & Tsanev (1986) and Basler *et al.* (1981) have found essentially opposite results. There are also conflicting results with regard to DNA replication (Djondjurov *et al.*, 1986).

In an attempt to clarify these controversial results, we have analysed a transcriptionally active gene of a well-characterized rat fibroblast cell line transformed by a recombinant plasmid containing an early gene region, which codes for large T-antigen protein, of simian virus 40. The particular advantage of this cell line is that it contains only a single copy of the active gene in question, and this gene is constitutively expressed at a high level. These features make the analysis very simple and reliable. The present study clearly demonstrates that the integrated and transcriptionally active plasmid sequence is highly enriched in the nuclear-matrix-associated DNA, whereas a transcriptionally inactive albumin gene, used as a control, is not, supporting the idea that the active genes are preferentially associated with the nuclear matrix.

MATERIALS AND METHODS

Materials

A cell line, pSV1-FR, was established (Benoist & Chambon, 1980) from a single transformation focus that grew after transfection of a Fisher-rat fibroblast cell line, FR3T3 (Seif & Cuzin, 1977), with the recombinant plasmid pSV1. This plasmid was constructed by inserting an entire early gene region of simian virus 40 (large *HpaII*–*BamHI* fragment, 0.73–0.15 map unit) into an *EcoRI* site of pBR322 (see also Fig. 1 below) by a calcium phosphate co-precipitation method (Benoist & Chambon, 1980). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) calf serum and 40 µg of gentamycin/ml, and were regularly split in a 1:4 dilution in every 4 days for propagation.

Preparation of nuclear matrix and matrix-associated DNA

Confluent pSV1-FR cells (5×10^8 cells) were harvested by trypsin treatment and washed once with phosphate-buffered saline (10 mM-sodium phosphate/140 mM-NaCl, pH 7.3). The cells were next suspended in 10 ml of Buffer A (100 mM-NaCl/50 mM-KCl/4 mM-MgCl₂/20 mM-Tris/HCl, pH 7.5) supplemented with 0.5% (w/v) Nonidet P40, and homogenized by 50 strokes of a Dounce homogenizer with a tight-fitting pestle. Cells were monitored by microscopic observations of samples until complete lysis was achieved. The lysed cell suspension was centrifuged at 500 *g* for 5 min, washed twice with Buffer A, and finally suspended in 30 ml of the same buffer. The suspension was transferred to a 500 ml glass beaker, and 20 ml of 5 M-NaCl was added dropwise over a period of 10 min while the beaker was swirled gently and continuously to avoid local high-salt concentration. This step is of crucial importance to obtain a homogeneous and reproducible nuclear-matrix preparation. The mixture was left for 20 min and was then mixed with 15 μ l of DNAase I (Worthington; code DPFF; 3400 units/mg) solution (5 mg/ml) and incubated at 37 °C, for 5–30 min. Large (12 ml) and small (100 μ l) samples were taken after appropriate time intervals and chilled immediately on ice. The small samples were subjected to fluorescence-microscopic analysis after the addition of a 1 mg/ml solution of ethidium bromide to a final concentration of 100 μ g/ml, to monitor the extent of DNA degradation. The large samples were centrifuged at 8000 *g* for 20 min at 2 °C. Each pellet was next gently suspended in 10 ml of Buffer B (200 μ M-MgCl₂/10 mM-Tris/HCl, pH 7.2), centrifuged as described above, and resuspended in 350 μ l of the same buffer. All operations were carried out at 0–2 °C unless otherwise specified, and all working solutions, except that used for the final step, were supplemented with 500 μ M-phenylmethanesulphonyl fluoride from an ethanolic 100 mM solution immediately before use. DNA was prepared from the whole nuclei and from the nuclear matrix as described by Gross-Bellard *et al.* (1973). The final DNA preparations were dissolved in Buffer C (1 mM-EDTA/10 mM-Tris/HCl, pH 8.0) and their DNA concentrations were determined by using diphenylamine as described by Burton (1956). Calf thymus DNA (Sigma, type I) was used as a standard.

Southern-blot analysis

A portion (10 μ g) of each DNA preparation obtained from the whole nuclei or the nuclear matrices was electrophoresed either under non-denaturing conditions (500 μ M-EDTA/20 mM-Tris/10 mM-acetic acid, pH 7.8) or under denaturing conditions (30 mM-NaOH/2 mM-EDTA) on a 1%-(w/v)-agarose gel (Sigma, type II). The gel was then stained with ethidium bromide (0.5 μ g/ml) and photographed through a red filter. The DNA species in the gel were transferred by blotting on to a strip (10 cm $\times$ 15 cm) of nitrocellulose filter (BA 85; Schleicher and Schüll) as described by Southern (1975). The filter strip was air-dried and then baked in a vacuum oven at 80 °C for 2.5 h. It was next prehybridized in 50% (w/v) formamide/75 mM-sodium citrate buffer (pH 7.0)/50 mM-sodium phosphate buffer (pH 7.0)/750 mM-NaCl/0.1% (w/v) BSA/0.1% (w/v) polyvinylpyrrolidone/0.1% (w/v) Ficoll 400 (Pharmacia)/1% (w/v) glycine/denatured and sheared salmon sperm DNA (Sigma, type III; 500 μ g/ml) for 4 h at 42 °C with constant shaking. Hybridization was next performed in a prehybridization solution supplemented with 10% (w/v) dextran sulphate (sodium salt; Pharmacia) and 10^7 c.p.m. of denatured ³²P-labelled (by nick-translation) plasmid DNA or its fragment for 24 h at 42 °C with constant shaking in a reaction volume of 30 ml. After hybridization the filter strips were washed twice for 5 min at room temperature with 300 mM-NaCl/30 mM-sodium citrate buffer (pH 7.0)/0.1% (w/v) SDS/0.1% sodium pyrophosphate

over 2 h at 50 °C in a shaking water bath. Finally the filter strips were dried and exposed for 24–72 h to a Kodak XAR-5 film with du Pont Lightening Plus intensifying screens at –70 °C.

RNA-blot analysis

Total RNA was extracted from 10^8 pSV1-FR cells by using guanidinium thiocyanate as described by Chirgwin *et al.* (1979). A 10 μ g portion of RNA was next denatured in 16 μ l of deionized 1 M-glyoxal containing 50% (w/v) dimethyl sulphoxide and 10 mM-sodium phosphate buffer, pH 7.0, at 50 °C for 1 h, then mixed with 4 μ l of loading buffer, i.e. 50% (w/v) glycerol/10 mM-sodium phosphate buffer (pH 7.0)/0.4% (w/v) Bromophenol Blue, and electrophoresed through a 1%-(w/v)-agarose gel in 10 mM-sodium phosphate buffer, pH 7.0 (Thomas, 1980). The gel was then blotted immediately on a strip of nitrocellulose filter and hybridized with ³²P-labelled pSV1 fragment (1.1 kbp *Hpa*I fragment; see Fig. 1b below) or a rat albumin genomic DNA fragment of 2.7 kbp spanning the seventh to ninth exons (*Eco*RI fragment) (Sargent *et al.*, 1981) as a probe.

Dot hybridization

Various amounts of DNA in 1 μ l of 10 mM-Tris/HCl, pH 7.5, were spotted on a sheet of nitrocellulose filter (BA 85; 85 mm diameter), suspended in air, and then dried at 80 °C for 2 h under vacuum. The filter was sequentially soaked at room temperature in 2 ml each of 0.5 M-NaOH (30 min), 1 M-Tris/HCl, pH 7.4 (1 min), 1 M-Tris/HCl, pH 7.4 (1 min), 1.5 M-NaCl (5 min), 300 mM-NaCl/30 mM-sodium citrate/2 mM-EDTA/25 mM-sodium phosphate buffer, pH 7.4 (1 min). The filter strip was then briefly dried on a filter paper, dried again at 80 °C for 2 h under vacuum, hybridized with nick-translated (Norman & Bekhor, 1981) ³²P-labelled DNA, and exposed to an X-ray film as described above. The exposed film was then scanned by a densitometer (Beckman DU-40) to quantify the signal intensity of the autoradiogram.

Other methods

Immunofluorescence detection of large T-antigen was carried out as described (Benoist & Chambon, 1980), using serum from a control hamster or that of a simian-virus-40-induced-tumour-bearing hamster, directly or after absorption with D2 protein (kindly provided by Dr. R. Tjan), a protein that shares its major structure and antigenicity in common with large T-antigen (Tooze, 1980), to remove anti-(large T-antigen)-specific antibody. The hamster serum was next detected by fluorescein isothiocyanate-labelled donkey anti-hamster antibody.

RESULTS

Characterization of pSV1-FR

pSV1-FR was established by transforming a rat fibroblast cell line FR3T3 (Seif & Cuzin, 1977) with a plasmid pSV1 (Fig. 1), which bears an entire early-gene region of simian virus 40 (Benoist & Chambon, 1980). This cell line possesses very stable morphological and growth characteristics for at least 2 years of regular passages. It is well established that constitutive expression of early gene, i.e., the large T-antigen gene, is necessary and sufficient for initiation and maintenance of cell transformation (Tooze, 1980). Indeed, the cell line pSV1-FR constitutively expresses the large T-antigen, as demonstrated by indirect immunofluorescence using an anti-(large T-antigen) serum (Fig. 2b). Control as well as preabsorbed serum gave no fluorescence under the same conditions (Figs. 2a and 2c). The expression of the large T-antigen was further confirmed by demonstrating that there was a large-T-antigen-specific mRNA in the total cellular RNA that hybridized with an early-gene-region DNA of simian

Fig. 1. Structure of the pSV1 plasmid

The restriction map of a circular plasmid, pSV1, is shown as linear for convenience (b). The solid and the open bars indicate simian-virus-40 and pBR322 origins respectively. The abbreviations for the restriction sites are as follows: A, *AvaI*; B, *BglI*; Bm, *BamHI*; H, *HindIII*; E, *EcoRI*; Hp, *HpaI*; P, *PstI*; Bc, *BclI*. The numbers 1-7 with arrows shown in (a) are fragments used as DNA probes for dot hybridization (see Fig. 10). The fragments shown in (c) are those detected by Southern-blot analysis (see Fig. 4).

virus 40 (Fig. 3a). The above experiments unambiguously demonstrate that the early gene region in pSV1-FR is constitutively transcribed. The transformed phenotype of pSV1-FR was also confirmed by formation of colonies in soft agar and by tumour formation in an athymic nude mouse (results not shown).

The structure of the pSV1 gene in pSV1-FR cells was examined by Southern (1975)-blot analysis for total cellular DNA of pSV1-FR digested with various restriction enzymes, using the ³²P-labelled pSV1 sequence as a probe (Fig. 4). When digested with *XhoI*, *XbaI* and *AvaI*, which have no cleavage site inside the pSV1 sequence, a single band of more than 20 kbp was detected, indicating that the pSV1 sequence was integrated into the chromosomal DNA of pSV1-FR. When digested with *EcoRI*, *BclI* or *BamHI*, which have a single cleavage site in the pSV1 sequence, two bands of 8.0 and 5.0, 9.4 and 6.2, or 6.5 and 4.4 kbp respectively were detected, indicating the presence of a single copy of the pSV1 sequence integrated into the chromosomal DNA of pSV1-FR cell, and this interpretation is consistent with the above results. By using other enzymes that have more than one cleavage site, several fragments were detected, among which some were consistent with the expected structure of pSV1, i.e. 1.4-kbp fragment for *PstI*, 1.1-kbp fragment for *HpaI*, 3.6-kbp fragment for *BglI*, and 1.2-, 0.53- and 0.44-kbp fragments for *HindIII* (see also Fig. 1c). Taken together, I conclude that there is a single and almost intact copy of the pSV1 sequence integrated into the pSV1-FR chromosomal DNA, and that the entire early region plus some of the pBR322 region, i.e. the *HindIII* site (1.4 kbp from the *AvaI* site) to the *BglI* site (5.4 kbp from the

Fig. 2. Immunofluorescence detection of large T-antigen

The fixed pSV1-FR cells were incubated either with control serum (a), anti-(large T-antigen) serum (b) or anti-(large T-antigen) serum preabsorbed with D2 protein, which shares its major structure in common with large T-antigen (c).

Fig. 3. Detection of large-T-antigen-specific mRNA by RNA-blot analysis

Portions (10 μ g) of the total cellular RNA were electrophoresed in each lane of agarose gel, transferred to a nitrocellulose filter, and hybridized with ³²P-labelled 1.1-kbp *HpaI* fragment of pSV1 (a) or 2.7-kbp *EcoRI* fragment of rat albumin genomic sequence covering the seventh to ninth exons (b) as a probe.

AvaI site), is preserved after integration. It is also concluded that integration had occurred at some point in the pBR322 region.

The copy number per cell of the pSV1 sequence was next determined by an independent method, i.e. dot hybridization, for two preparations of the total cellular DNA of pSV1-FR (Fig. 5, upper part) in parallel with a known amount of pSV1 DNA as a standard (Fig. 5, lower part). In this experiment, 2 μ g of pSV1-FR DNA gave a signal equivalent to 1.8 ± 0.2 pg of pSV1 DNA, which is equivalent to 0.75 ± 0.08 copies of the pSV1 sequence per cell. Therefore we conclude that the pSV1-FR cell contains one

Fig. 4. Southern-blot analysis of total cellular DNA of pSV1-FR

Portions (10 μ g each) of pSV1-FR DNA were digested with various restriction enzymes as indicated, electrophoresed on 1%-agarose gel, transferred to a nitrocellulose filter, and hybridized with ³²P-labelled pSV1 DNA as a probe. Size markers (lane M) are also shown.

Fig. 5. Determination of pSV1 copy number by dot hybridization

A portion (2 µg) of total cellular DNA obtained from pSV1-FR cells (upper part) or various amounts of pSV1 plasmid DNA (lower part) were spotted on a strip of nitrocellulose filter and hybridized with ^{32}P -labelled pSV1.

nearly complete copy of the pSV1 sequence. It should be noted that the dot-hybridization method employed in the present study is fairly accurate, as shown by a low level of experimental error. Thus this estimation of the copy number appears to be very reliable.

Preparation of nuclear matrix and matrix-bound DNA

The nuclear matrix was prepared from the isolated nuclei by treating them first with a buffer containing 2 M-NaCl and then with DNAase I. When the nuclei are treated with the buffer containing 2 M-NaCl, histones and most other nuclear proteins are removed (Benyajati & Worcel, 1976) and the chromosomal DNA is released as loops from the matrix structure (Pinon & Saltz, 1977; Igo-Kemenes & Zachau, 1978). These loops are revealed, by ethidium bromide staining, as a halo surrounding a residual structure, the nuclear matrix (Fig. 6a). Subsequent nuclease digestion progressively removes chromosomal DNA loops from the nuclei, and the halo gradually disappears (Figs. 6b–6d), whereas the background fluorescence increases, suggesting an increase in the DNA concentration in the surrounding solution. The DNA was extracted from these nuclear-matrix preparations or from whole nuclei as described by Gross-Bellard *et al.* (1973). The relative amounts of DNA obtained were 100, 59, 34, 16 and 4.3% for 0, 5, 10, 20 and 30 min-incubated nuclear-matrix preparations respectively. It is possible to decrease the amount of the nuclear-matrix-bound DNA to 0.5% of the total nuclear DNA by exhaustive digestion with DNAase I. The DNA preparation thus obtained may represent DNA tightly associated with the nuclear matrix. However, such a DNA preparation was too short, i.e., less than 0.5 kbp, to be retained efficiently by a nitrocellulose filter (Southern, 1975) and was not suitable for the subsequent analyses. The sizes of the DNA species were 50–100 kbp by non-denaturing (Fig. 7a) and 1.5–50 kb by alkali-denaturing-gel

Fig. 6. DNAase I digestion of pSV1-FR nuclei treated with a buffer containing 2 M-NaCl

The pSV1-FR nuclei treated with a buffer containing 2 M-NaCl were incubated with DNAase I, and aliquots were removed after periods of 0 (a), 5 (b), 10 (c), and 30 (d) min. They were then stained with ethidium bromide and observed under a fluorescence microscope.

Fig. 7. Characterization of whole nuclear and nuclear-matrix-associated DNA species on denaturing and non-denaturing agarose-gel electrophoresis

A 10 µg portion of each DNA obtained from the whole nuclei (W) or from the nuclear matrices containing 59, 34 or 16% of the total nuclear DNA were electrophoresed on 1%-(w/v)-agarose gel under non-denaturing (a) or alkaline-denaturing (c) conditions and stained with ethidium bromide. The non-denaturing gel was then subjected to Southern-blot analysis using ^{32}P -labelled pSV1 as a probe (b). Positions of size markers are also shown.

electrophoresis (Fig. 7c). The pSV1-derived sequence also showed the same distribution as the bulk DNA, as demonstrated by Southern blotting (Fig. 7b). These results suggest that all DNA preparations can be retained sufficiently (quantitatively) by a nitrocellulose filter, since they are larger than 0.5 kb (Southern, 1975), and hence a quantitative determination of a specific DNA sequence by dot hybridization would be justified.

Quantification of a sequence similar to that of pSV1 in the nuclear-matrix-associated DNA by dot hybridization

To elucidate whether the pSV1-derived sequence is attached to the nuclear matrix, the relative amounts of a sequence similar to that of pSV1 in the nuclear-matrix-associated DNAs were compared by spotting equal amounts of DNAs on a strip of

A 1 µg portion of each DNA obtained from the whole nuclei (W) or from the nuclear matrices containing 59, 34 or 16% of the total nuclear DNA was spotted on to a nitrocellulose filter and was hybridized with ^{32}P -labelled pSV1 (upper panel) or albumin gene (lower panel) as a probe. Values in parentheses indicate relative intensities of the signals of the autoradiograms with respect to that of the whole nuclear DNA.

Fig. 9. Southern-blot analysis of whole nuclear and nuclear-matrix-associated DNA species

A 10 μ g portion of each DNA obtained from the whole nuclei (W) or from the nuclear matrices containing 59, 34 or 16% of the total nuclear DNA were digested with *Hind*III and subjected to Southern-blot analysis using ^{32} P-labelled pSV1 (a) or albumin gene (b) as a probe. Size markers (M) are also shown.

nitrocellulose filter and hybridizing them with ^{32} P-labelled pSV1 sequence as a probe. The relative amounts of pSV1-hybridizing sequence increased with longer DNAase I digestion, and after 30 min incubation, at which time 16% of the total DNA is still associated with the nuclear matrix, the relative amount of pSV1-derived sequence increased 5.7-fold as compared with that of the total cellular DNA (Fig. 8, upper part). As a control, a transcriptionally inactive albumin gene was used as a hybridization probe and the relative amounts of the albumin gene sequence in the same DNA preparations were similarly compared. As shown in Fig. 8 (lower part), there was no significant difference between the total cellular and the nuclear-matrix-associated DNAs. Essentially similar results were obtained for the same DNA preparations subjected to Southern-blot analysis after *Hind*III digestion (Fig. 9). Although it is difficult to exclude completely the possibility that the albumin gene is transcriptionally active, such exclusion might be justified on the basis of the fact that fibroblasts do not synthesize albumin and that I could not, indeed, detect any albumin-specific transcript by RNA-blot analysis (Fig. 3b). By employing various subfragments of the pSV1 sequence (fragments 1–7) as hybridization probes (see Fig. 1a for subfragments of the pSV1 sequence), we questioned whether there is any difference with respect to sub-regions of the pSV1 sequence in the association with the nuclear matrix. As Fig. 10 shows, it would seem that there are essentially no significant differences.

DISCUSSION

We have demonstrated that a transformed rat fibroblast cell line, pSV1-FR, which had been transformed by transfection with

Fig. 10. Dot-blot analysis of nuclear-matrix-associated DNA using subfragments of pSV1 as probes

A 1 μ g portion of each DNA obtained from the whole nuclei (W) or from the nuclear matrices containing 61, 44 or 27% of the total nuclear DNA was spotted on to a nitrocellulose filter and was hybridized with ^{32} P-labelled fragments 1–7 of pSV1 (see Fig. 1a). The values in parentheses indicate relative intensities of the signals with respect to that of the whole nuclear DNA.

pSV1 plasmid, contains one almost complete copy of the pSV1 sequence integrated into chromosomal DNA of the cell. Judging from the results of Southern-blot analysis (Fig. 4), the site of integration with respect to the pSV1 sequence is somewhere in the pBR322-derived sequence of the plasmid, but the exact site of integration was unclear from the present experiments. The cell line pSV1-FR is very useful for the study of gene expression and gene structure analyses, since it contains a single, almost complete, copy of the pSV1 sequence, and one can easily demonstrate the expression of this gene, since it is well known that large T-antigen, an early gene product of simian virus 40, must be constitutively expressed to maintain the transformed phenotype (Tooze, 1980).

I have shown that the transcriptionally active and integrated pSV1 sequence is enriched in the nuclear-matrix-associated DNA. Jackson & Cook (1985) demonstrated association of transcriptionally active genes with the 'nuclear matrix' prepared under iso-osmotic conditions, but they claimed that their preparation, which they termed 'nucleoskeleton', was distinct from conventional high-salt-extracted nuclear matrix, implying that the latter might be an artifact of preparation. Therefore their results cannot directly be compared with mine. Nelkin *et al.* (1980) reported that the simian-virus-40 sequence was enriched in the matrix-bound DNA of a simian-virus-40-transformed cell. This cell contains more than one copy of the simian-virus-40 genome integrated into the chromosomal DNA. As the copy number per cell is more than one, it remains ambiguous to correlate directly the expression and the association of DNA to the nuclear matrix, since the copy of simian virus 40 that was associated with the nuclear matrix might be the one which was not transcribed. On the other hand, pSV1-FR contains only one

plasmid copy per cell, and hence it is evident that the nuclear-matrix-associated pSV1 sequence itself is actively transcribed.

The relative enrichment of the pSV1 sequence in the nuclear-matrix-associated DNA was demonstrated by two different methods, i.e. Southern-blot analysis and dot hybridization. Although these two methods are based on similar principles, dot hybridization appears to be more accurate in terms of quantification of specific DNA sequences, since it involves no DNA-transfer step. Therefore I consider that the result of the dot hybridization is more reliable than that of Southern-blot analysis in terms of quantification of the specific DNA sequence.

It is suggested that DNA in the genome of higher organisms is arranged in loops of about 50–100 kbp and that, during nuclease digestion, the DNA is gradually removed from the distal portion to the proximal portion, with respect to the nuclear-matrix-attachment site (Cook & Brazell, 1975; Benyajati & Worcel, 1976; Pinon & Salts, 1977; Igo-Kemenes & Zachau, 1978). If this is the case, the present results indicate that the pSV1 sequence is close to the nuclear-matrix-attachment site. However, some caution must be exercised with this interpretation. For instance, there may be special structures in the transcribed DNA, such as the presence of a single-stranded region or of DNA-associated RNA polymerase. These special structures might cause 'artificial enrichment' of this sequence during preparation of the nuclear matrix if there is some binding between them. A possible example of this is that DNA associated with RNA polymerase, once released from the nuclei, may reassociate with the nuclear matrix if the RNA polymerase has an affinity for the nuclear matrix, as pointed out by Cataldi *et al.* (1987). It is also possible that the apparent enrichment of the pSV1 sequence in the nuclear-matrix-associated DNA is merely a consequence of the plasmid integration itself. For instance, integration might have occurred at a segment of cellular DNA that was already associated with the nuclear matrix. Another possibility is that the pSV1 sequence might have associated with its replication origin derived from simian virus 40 to the nuclear matrix, since it is suggested that DNA replication occurs at the nuclear matrix (Wood & Collins, 1986; Aguinaga *et al.*, 1987; Tubo & Berezney, 1987; Ciampor, 1988), and consequently the pSV1 sequence might well be associated with the nuclear matrix via its replication origin [1.76 kbp to the right of the *Aval* site (see Fig. 1b)].

By employing several different pSV1 fragments as hybridization probes, I mapped the difference in the extent of attachment to the nuclear matrix (Fig. 10). To obtain a significant difference, it is important to consider the relative size of the DNA fragments. It is possible to observe the difference if the DNA size of the sample, i.e. the nuclear-matrix-associated DNA, is sufficiently small. In this regard, the DNA size in this experiment might be too large to detect any local difference present. Therefore my results cannot completely exclude the possibility of some minor local difference in the gene in attachment to the nuclear matrix. Cook *et al.* (1982) found enrichment of a polyoma sequence in the nuclear-matrix-associated DNA using a cell line transformed by polyoma virus. Those authors claimed that the flanking cellular sequences are induced to become associated with the nuclear matrix, since it is also enriched to the same extent as the polyoma sequence. They did not show the accurate size of the flanking cellular DNA associated with the nuclear matrix; they simply claimed that it was 'very large'.

It is tempting to assume that all the active genes are enriched in the nuclear matrix. In this respect the work of Robinson *et al.* (1982) is worthy of mention. Using chicken oviduct cells, those authors demonstrated that the ovalbumin gene, which was actively transcribed in these cells, was enriched in the nuclear-matrix-associated DNA, whereas the globin gene, which was not

transcribed in these cells, was not enriched. Although it is too early to generalize the above hypothesis, these results may give us some insight concerning the relationship between the regulation of gene expression and the structural integrity of the eukaryotic cell nucleus.

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