SV40 chromatin structure is not essential for viral gene expression

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The biological activity and the fate of SV40 DNA (minichromosomes, DNA I, DNA II, DNA III) were tested in culture cells by immunofluorescence staining and blot analysis. Following microinjection of 2–4 circular SV40 molecules (minichromosomes, DNA I, DNA II) into the cytoplasm or the nuclei of monkey and rat cells, T- and V-antigen synthesis was demonstrable in nearly every recipient cell. Only linear DNA induced T-antigen synthesis with a very low efficiency after cytoplasmic injection. This low activity correlates with a rapid degradation of DNA III in the recipient cells. Further modifications observed immediately after injection are relaxation of superhelical molecules and formation of high-M_r DNA. Assembly of the injected DNA into SV40 chromatin-like structure, however, occured only late after early viral gene expression.

Microinjection *SV40 gene expression* *DNA modification* *SV40 chromatin formation*

1. INTRODUCTION

Simian virus 40 (SV40) is a DNA tumor virus which infects monkey cells productively and transforms a variety of different kinds of tissue culture cells. During the normal life cycle and in the transformed cells the SV40 DNA exists only in a chromatin-like structure. Although a significant portion of the DNA molecules contain a nucleosomal free region around the origin of replication and the putative early and late promotor region, it is still unclear whether the chromatin structure is essential for viral gene expression [1]. Efficient SV40 gene expression is observed after microinjection of purified SV40 DNA or subgenomic DNA fragments into mammalian cells [2]. In these experiments however, it was not tested whether SV40 chromatin assembly preceded expression of the injected DNA.

Here, we analysed the biological activity and the fate of different configurated DNA molecules after injection into the cytoplasm or nuclei of monkey and rat cells. These experiments have shown that circular DNA molecules (SV40 chromatin, DNA I and DNA II) are equally efficiently expressed. Only liner DNA was less biologically active following injection into the cytoplasm. Degradation of the DNA inside the cytoplasm is at least in part responsible for the observed low rate of T-antigen synthesis. Blot analysis of the reextracted DNA revealed that the injected DNA undergoes different modification steps shortly after the transfer. This includes relaxation of superhelical DNA molecules and formation of high-M_r DNA. However, assembly of the injected DNA into chromatin structure occurs only after the commencement of early SV40 gene expression. This was demonstrated by blot analysis and electronmicroscopical examination of the reextracted DNA.

2. MATERIALS AND METHODS

2.1. *Cell culture and microinjection*

Monolayers of TC7 cells (a subline of the CV1 monkey cells) and rat cells (Ref 52) were cultivated on glass slides in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum

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(Gibco). Details of the microinjetion technique and immunofluorescence staining are described elsewhere [3].

2.2. *Isolation of SV40 DNA*

SV40 DNA was isolated from virus infected TC7 cells by the method of [4]. Purification of the DNA I, DNA II and preparation of linear DNA III and subgenomic DNA fragments are described in [5].

2.3. *Reextraction of injected SV40 DNA and chromatin from the recipient cells*

For reextraction experiments cells were grown on small glass slides (2 × 2 mm). Different hours after injection glass slides with the cells were removed from the culture dish, washed with PBS and transferred into Eppendorf test tubes filled with 100 μl lysis buffer (10 mM Tris–HCl, pH 7.6, 1 mM EDTA, 0.6% sodium dodecyl sulfate). The Hirt-extracted DNA was separated on 1.2% agarose gels, blotted to nitrocellulose filter and hybridized to nick-translated SV40 DNA (spec. act. $1–2 \times 10^8$ cpm/μg) [6].

SV40 chromatin was isolated from the injected cells in accordance to the procedure in [7] with some modifications. In short: Cells were lysed with Triton X-100, 10 mM Tris–HCl (pH 6.8), 10 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), the isolated nuclei were washed in 0.25% Triton X-100, 100 mM NaCl, 10 mM EDTA, 0.1 mM PMSF, 10 mM Tris–HCl (pH 6.8) and further gently agitated for 3 h at 4°C in extraction buffer (0.25% Triton X-100, 200 mM NaCl, 10 mM EDTA, 10 mM Tris–HCl (pH 8.0) and 0.1 mM PMSF). Following removal of the Triton X-100 by SM-2 Bio-beads treatment (Bio-Rad) samples were diluted with borate buffer (0.1 mM borate, pH 9.0) [8], and processed for electron microscopy as in [9].

3. RESULTS AND DISCUSSION

3.1. *Biological activity and fate of injected SV40 DNA*

To test if the topological state of the SV40 DNA is of importance for the efficiency of early SV40 gene expression, we microinjected SV40 minichromosomes, superhelical SV40 DNA I, relaxed DNA II and linear DNA III molecules into the cytoplasm and into the nuclei of monkey (TC7) and rat cells (Ref 52). The DNA concentration in the injection buffer was such that 2–4 DNA molecules were always transferred per recipient cell. Different hours after injection cells were fixed and stained for T-antigen. These experiments showed that T-antigen synthesis (and also late SV40 gene expression) occurs with the same efficiency after either intranuclear or cytoplasmic injection of SV40 chromatin, DNA I and DNA II molecules (table 1). In contrast, a significant lower number of T-antigen positive cells were obtained after cytoplasmic injection of DNA III. As shown in table 1, only 1–2% of the recipient cells were T-antigen positive 20 h after injection, while nearly all nuclear injected cells supported expression of the linear SV40 DNA. To test if degradation of the DNA III inside the cytoplasm may be the reason for the low rate of T-antigen synthesis, the stability of SV40 DNA III was analysed after intranuclear and cytoplasmic injection. To answer this question, we microinjected 100 TC7 cells for each test point and reextracted the DNA by the method of [4]. Following separation by standard agarose gel

Table 1

Biological activity (%) of SV40 chromatin and DNA after microinjection into the nuclei or cytoplasm of TC7 cells

Injection	Injected into			
	Nucleus		Cytoplasm	
	T	V	T	V
SV40 chromatin[a]	99	99	95	90
SV40 DNA I	99	99	90	80
SV40 DNA II	95	90	80	75
SV40 DNA III	95	80	1	0.2
SV40 DNA fragment (*HpaII/Bam*)	80	0.0	<1	0.0
SV40 DNA I + PV DNA[b]	99	99	95	85

[a] SV40 chromatin was isolated from virus infected TC7 cells [7] and from intact virus particles [10]. TC7 cells were microinjected with a multiplicity of 2–4 DNA molecules and fixed and stained for T- and V-antigen 20 h after injection

[b] In these injection experiments 2–4 SV40 DNA and 2000–4000 polyoma virus DNA molecules were transferred per recipient cell

electrophoresis, the DNA was transferred to nitrocellulose filter and hybridized with nick-translated SV40 DNA.

Fig.1 demonstrates that the DNA III is progressively degradated in the cytoplasm of the recipient cells. This is indicated by the continuous loss of DNA III molecules and the appearance of a DNA smear, which migrates faster than the DNA III. However, a small number of the injected DNA III molecules also survive after cytoplasmic injection and give rise to the production of wild type virus particles (not shown). After intranuclear injection the rate of DNA degradation is less (fig.1B). In *Xenopus laevis* oocytes, degradation of the injected DNA is not the reason for the low biological activity of linear DNA molecules, but rather by the low rate of transcription [11]. This indicates that data obtained from the frog system are not necessarily transferable to mammalian culture cells.

Circular DNA molecules (minichromosomes, DNA I and DNA II) are better protected against degradation than the linear DNA. This supports the assumption that the degradation of DNA III is caused by exonucleolytic digestion.

DNA I molecules also undergo different modifications after injection into either the cytoplasm or the nuclei of mammalian cells. These include: (i) conversion of superhelical DNA into relaxed circular form, and (ii) formation of higher-M_r DNA.

(i) We found from DNA reextraction experiments that conversion of superhelical DNA into relaxed circular molecules occurs immediately after injection. Extracts obtained directly after intranuclear injection of DNA I were nearly free of superhelical molecules (fig.2). Conversion of DNA I into DNA II was also demonstrable in cells injected at 4°C. For these low temperature experiments TC7 cells were transferred into precooled medium 30 min before injection and remained at this temperature until DNA reextraction.

Relaxation of the DNA I is catalysed by a cellular nicking-closing enzyme. DNA extracts,

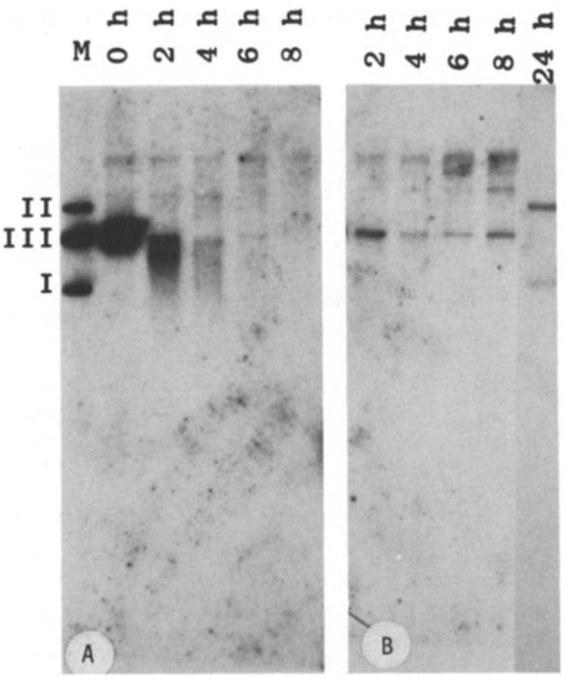

Fig.1. DNA blot of reextracted SV40 DNA III molecules: (a) after microinjection into the cytoplasm of TC7 cells, (b) extracts obtained from nuclear injected TC7 cells. M: marker SV40, DNA I, DNA II, DNA III. For all DNA blotting experiments (fig.1–4) DNA concentration in injection buffer was 1 mg/ml.

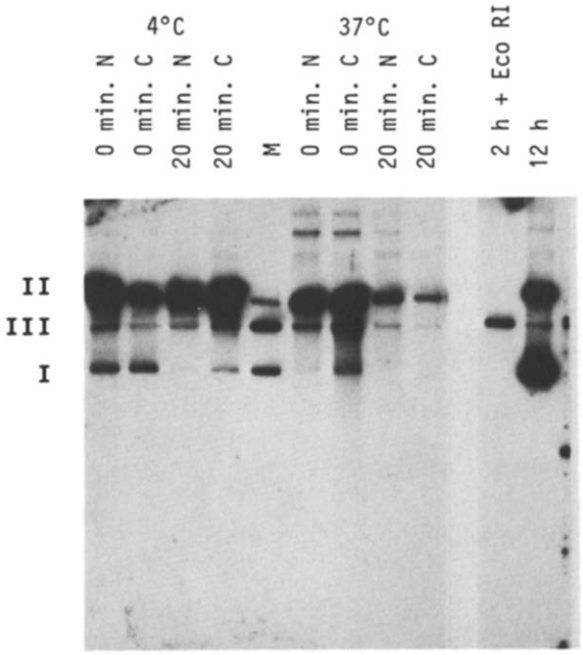

Fig.2. DNA blot of SV40 DNA I after intranuclear (N) or cytoplasmic (C) injection at low (4°C) and normal temperature (37°C).

subjected to ethidium bromide agarose gels [12] contain molecules with different degrees of superhelical turns which migrate on the gel between the DNA I and DNA II (fig.3A). This stepwise relaxation process occurs very rapidly after injection and is also demonstrable at the low temperature of 4°C. These intermediates are also found after incubation of SV40 DNA I with a crude TC7 cell protein extract (fig.3C). These intermediates are not found after microinjection of SV40 minichromosomes (fig.3B) or after in vitro incubation of the minichromosomes with the TC7 protein extract (fig.3C). So far, the biological relevance of the DNA relaxation is unclear, but it may be that the superhelical turns have to be removed in order to allow transcription of the DNA.

(ii) The second phenomenon observed was the formation of higher-M_r forms of the injected DNA. Nuclear or cytoplasmic extracts obtained directly after injection of SV40 DNA (minichromosomes, DNA I, DNA II and DNA III) contained SV40 DNA molecules with a lower mobility on agarose gels than the DNA II (fig.2). This DNA is converted into SV40 DNA III upon cleavage with the *Eco*RI endonuclease. This enzyme cleaves SV40 DNA only once at the map position 00. Oligomerisation of the injected DNA occurs with a low efficiency at 4°C.

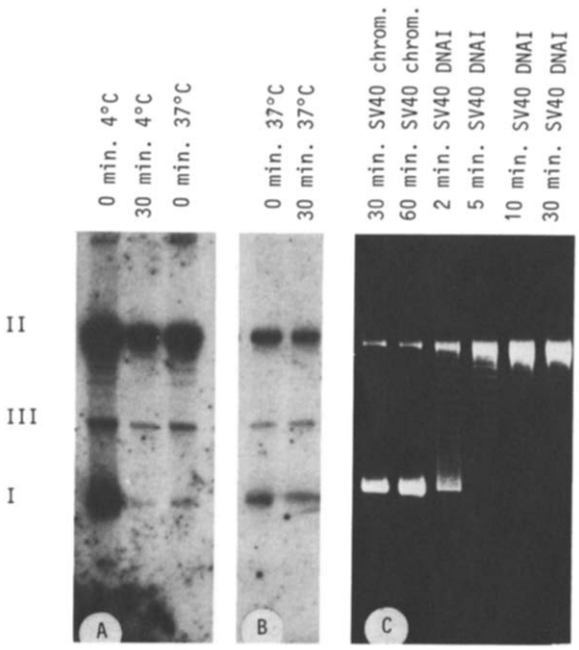

Fig.3. DNA blot after separation of SV40 DNA and SV40 chromatin on EdtBr agarose gels. (A) DNA reextracted from microinjected TC7 cells. Time point of reextraction after microinjection is indicated as well as the temperature at which the injection was performed. (B) Extracts obtained after microinjection of SV40 chromatin. (C) EdtBr-agarose gel picture of SV40 chromatin and SV40 DNA I after incubation with the crude TC7 cell protein extract.

3.2. *Formation of SV40 chromatin is not required for early SV40 gene expression*

We further asked when, after SV40 DNA injection, formation of minichromosomes will take place. As an indirect marker reformation of superhelical DNA was analysed. Reappearance of superhelical DNA molecules reflects chromatin assembling [13]. As shown in fig.4, DNA I molecules are again demonstrable in extracts isolated 6–8 h after microinjection. T-antigen synthesis however was demonstrable as early as 2–3 h

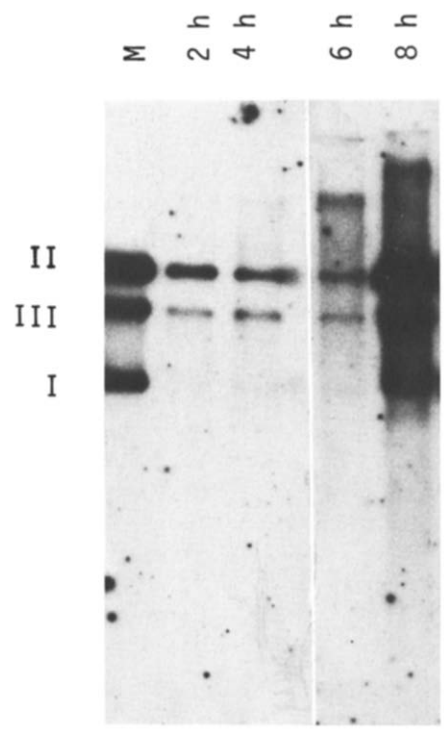

Fig.4. DNA blot of reextracted SV40 DNA I molecules. M, the SV40 DNA before microinjection. The positions of the DNA I, DNA II, and DNA III are indicated.

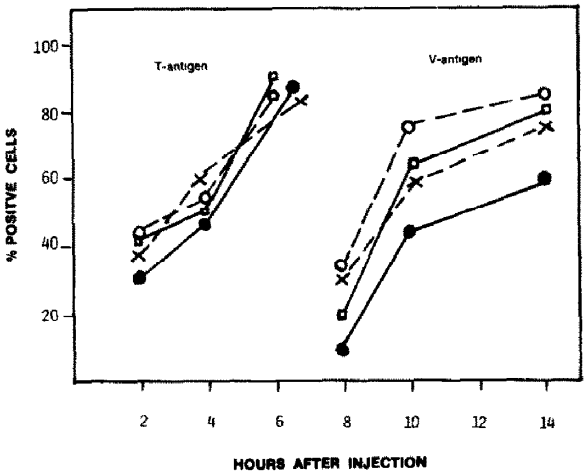

Fig.5. Time course of T-antigen and V-antigen synthesis in TC7 cells after intranuclear injection of: (○—○) SV40 DNA I; (×—×) SV40 DNA II; (●—●) SV40 DNA III; (□—□) SV40 DNA I (0.001 mg/ml) + polyoma virus DNA I (1 mg/ml). SV40 DNA concentration in injection buffer was 0.001 mg/ml, equivalent to 2–4 injected DNA molecules/cell.

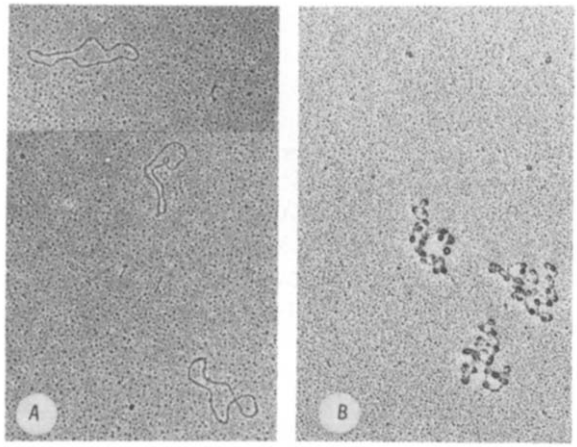

Fig.6. Electron micrography of nuclear extracts isolated from TC7 cells injected with SV40 DNA I: isolated (A) 6 h, and (B) 10 h after intranuclear injection.

after injection of either DNA I or DNA II (fig.5). This observation supports the assumption that early SV40 gene expression precedes formation of SV40 chromatin. To exclude the possibility that a very small number of the DNA molecules are assembled into chromatin early after injection, we coinjected 2–4 SV40 DNA molecules with 2000–4000 polyoma virus DNA molecules per cell and followed the time course of SV40 T-antigen synthesis. These competition experiments showed that the excess of polyoma virus DNA did not affect SV40 T-antigen synthesis (fig.5). More direct evidence that SV40 chromatin formation occurs late after DNA injection was obtained from electron microscopical examination of the extracts. Up to 8 h after injection isolates were always free of SV40 chromatin. Only nuclear extracts obtained at later time points contained SV40 chromatin like structures as shown in fig.6. Similar results were obtained after digestion of the reextracted molecules with micrococcal endonuclease (not shown).

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