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IN VITRO TRANSFORMATION AND MUTATION OF CHINESE HAMSTER CELLS BY DIFFERENT SV40 NUCLEOPROTEIN COMPLEXES

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Summary. — SV40 minichromosomes (MCH) either isolated from SV40 infected CV-I monkey cells (native MCH) or reconstituted in vitro from viral DNA and the H1 depleted calf thymus histone fraction could transform and mutate Chinese hamster (CH) cells in vitro. Whereas reconstituted MCH transformed and mutated CH cells with about the same efficiency as purified SV40 DNA, approximately 10—200-fold increase in the transforming activity had been demonstrated for native MCH. All transformed cell colonies and a major part of the isolated mutant cell clones recovered after inoculation of CH cells with SV40 MHC expressed the SV40 T antigen. Addition of H1 to both purified SV40 DNA and reconstituted MHC drastically diminished the transforming capacities of both agents. Possible reason(s) for the inhibition effect of H1 histone is discussed.

Key words: *Simian virus 40 minichromosomes*; *virus transformation*; *SV40 induced mutation*; *histones*; *SV40 nucleoprotein complexes*

Introduction

Simian virus 40 (SV40) transforms a variety of cells of different species and has become an important tool in the search for mechanisms of tumour formation in higher organisms and in regulation of eukaryotic gene expression (Geissler *et al.*, 1980; Tooze, 1980). It has also been shown that SV40 induces mutations in infected Chinese hamster, mouse and human cells at different loci and that SV40 induced mutation and transformation are, at least to a certain extent, related phenomena (Theile *et al.*, 1980*a*). Stable transformation is a relatively inefficient process and several methods have been developed to enhance the transformation frequency (e.g. Tooze, 1980). It has also been demonstrated that purified SV40 DNA can transform and mutate different animal and human cells in vitro (Abrahams and van der Eb, 1975; Theile *et al.*, 1980*b*).

The intracellular viral DNA is complexed with cellular histones in a manner which closely resembles to the structure of cellular chromatin (Kornberg, 1977). Histone H1 appears to be essential for keeping the chromatin structure in a condensed, stabilized state (Müller *et al.*, 1978; Böttger *et al.*, 1979). However, the nucleoproteins (NP) enclosed in the mature virion are H1 depleted. This led to the speculation that replacement of histone H1 by VP3 is necessary to condense SV40 DNA inside the virion and to stabilize the interactions between capsid shell and the SV40 DNA-histone complex (Christiansen *et al.*, 1977; Coca-Prados and Hsu, 1979).

In contrast to cellular histones, virion and NP complex histones enriched in H3 and H4 histones by 15%—40% showed a higher level of acetylation (Chen *et al.*, 1979; La Bella and Vesco, 1980). Obviously such modifications are correlated with changes in transcriptional, structural and replicative properties of the MCH (Chen *et al.*, 1979). Furthermore it has been shown that reconstitution in vitro of DNA and the four nucleosomal histones H2A, H2B, H3 and H4 leads to formation of chromatin-like structures which retain the circular headed appearance of relaxed MCH (Oudet *et al.*, 1975; Christiansen *et al.*, 1977). The question how closely the reconstituted complexes resemble to the native MCH in their biological behaviour has remained open.

In a previous study we found that both native and reconstituted SV40 MCH can transform primary CH cells in vitro (Waehlte and Scherneck, 1979). Whereas the transforming capacity of reconstituted minichromosome was only slightly raised, the transforming capacity of native MCH was about 200-fold increased in comparison to purified SV40 DNA. This communication presents investigations confirming the ability of different SV40--histone complexes to transform and to mutate CH cells in vitro.

Materials and Methods

SV40 DNA and SV40 MCH. SV40 DNA was extracted from infected CV-I monkey cells (m.o.i. of 10^{-4} PFU/cell) using the Hirt procedure (Hirt, 1967) and purified as described (Böttger *et al.*, 1976). Native MCH were prepared according to Christiansen and Griffith (1977) from the nuclei of SV40 infected CV-I cells.

The reconstitution of histone/DNA complexes has been carried out using a modification of the procedure of Oudet *et al.* (1975) as described by Waehlte and Scherneck (1979) using SV40 form I DNA and the four calf thymus histones H2A, H2B, H3 and H4. H1-DNA and H1-reconstituted SV40 MCH complexes were prepared according to Böttger *et al.* (1976, 1981). Sterile NP complexes were reconstituted by prefiltering solutions of SV40 form I DNA and histones through a sterilized 0.45 μm nitrocellulose Millipore filter.

Cell cultures. CV-I monkey cells and primary cultures of Chinese hamster lung (CHL) cells were used (for details see Waehlte and Scherneck, 1979) as well as Chinese hamster (CHO-K1; V79) cells. They were grown in Eagle's minimal essential medium (MEM) supplemented with 8 per cent calf serum and 2 per cent foetal bovine serum (CHL cells were grown in 15% foetal bovine serum only), non-essential amino acids, sodium pyruvate and antibiotics (for details see Waehlte and Scherneck, 1979 and Theile *et al.*, 1980*b*). Selective media, in addition, contained aminopterin (0.55×10^{-2} μg/ml) or 6-thioguanine (1.25 μg/ml).

Transformation of CHL cells by SV40 DNA and SV40 NP complexes. Subconfluent monolayers of CHL cells in the first passage were infected with SV40 or SV40 NP complexes for 2 hr at 37 °C at concentrations indicated in the legends to Tables 1—3 in 0.5 ml HEPES buffered saline pH 7.5

containing 5 μg salmon sperm DNA. In analogous experiments, 1.25 mmol/l $CaCl_2$ was added to the transformation mixture. Transformation assay was performed with an agar suspension technique (Waehlte and Scherneck, 1979).

Mutagenesis. Subconfluent cell monolayers were exposed for 90 min at 37 °C to SV40 DNA or to SV40 MCH. In some experiments the cells were washed twice with phosphatbuffered saline (PBS) and treated at room temperature with DEAE-dextran (m.w. 2×10^6, diluted in PBS to 150 μg/ml) for 40 min prior to incubation with DNA. Mutant selection, estimation of the plating efficiency as well as mutant frequences and isolation of clones were performed as described by Theile *et al.* (1980*a*).

Detection of SV40 T-antigen. Cells selected by growth in soft agar and by focus formation were grown on slides in 50 mm Petri dishes. Cells were analysed for the presence of SV40 T antigen by indirect immunofluorescence (IF) using hamster anti-T sera and FITC-conjugated rabbit anti--hamster gamma globulin as described (Scherneck *et al.*, 1979).

Electron microscopy. Electron microscopy was performed according to von Mickwitz *et al.* (1979). The samples taken from the centrifuged cells were diluted to 5.0 μg/ml final DNA concentration.

Results

Transformation of Chinese hamster lung (CHL) cells by SV40 DNA and SV40 MCH

Nonpermissive cells can be most efficiently transformed by SV40 DNA using the calcium technique (Abrahams and van der Eb, 1975). As demonstrated by these authors, the transformation efficiency is influenced additionally by the DNA concentration used, being the highest at low viral DNA concentration and leveling off to an almost constant value above 1 μg per 50 mm dish. In the following experiments not more than 3 μg SV40 DNA per 1×10^5 cells or an equivalent amount of SV40 MCH was used for transformation of CHL cells (exceptions are indicated in the legend to Tables).

Table 1 shows the result of a transformation experiment in which the specific transformation efficiency of SV40 DNA or MCH was determined by growth of CHL cells in soft agar at different times after inoculation. By seeding 1×10^5 cells in soft agar 12 colonies could be detected 27 days after infection of cells with SV40 DNA using the calcium technique. When comparing these results with the data obtained by Abrahams and van der Eb (1975) an about 50-fold decreased transforming ability of SV40 DNA for CHL-cells was found. However, in some other experiments the transformation frequencies were similar to those described by the authors mentioned above (see also Table 3). As pointed out by Waehlte and Scherneck (1979) there could be differences in the average specific transformation activity of SV40 DNA for cells of different species. Analogous experiments but using both native and reconstituted MCH (histone/DNA ratio 1.5 : 1) instead of purified DNA demonstrated transforming ability of both components. Whereas 29 colonies could be detected in soft agar 27 days after inoculation of CHL cells with reconstituted MCH, about 300 colonies were visible after inoculation of native MCH. On the other hand, only 12 colonies could be found after inoculation of purified SV40 DNA, indicating a 2.5 and 10 times higher transforming ability of reconstituted and native SV40 MCH, respectively, than that of purified SV40 DNA. In other sets of experiments we did not

Table 1. Transformation of CHL cells by SV40 DNA and SV40 MCH

Days after inoculation	SV40 DNA		Reconstituted		Native	
			SV40	MCH**	SV40	MCH
	$+Ca^{++}$	$-Ca^{++}$	$+Ca^{++}$	$-Ca^{++}$	$+Ca^{++}$	$-Ca^{++}$
8	1*	0	30	0	< 1	< 1
27	12	0	29	0	~ 300	19

* Number of colonies in soft agar (per 1×10^5 cells).
** Histone/DNA w/w ratio 1 : 5 : 1.

find any significant difference in the transformation efficiencies of reconstituted MCH and purified DNA (compare Table 3). Under our conditions, the MCH reconstituted at a histone/DNA ratio of 1.5 : 1 roughly corresponded in the histone composition to H1 depleted native MCH. Saturation binding according to Voordouw *et al.* (1977) did not reach the histone/DNA ratio of 1.75 : 1.

Mutagenic effect of SV40 DNA and SV40 MCH reconstituted in vitro

To detect the possible mutagenic action of SV40 DNA and SV40 MCH in non-permissive CH cells, these were inoculated with either the SV40 DNA or the MCH and subsequently exposed to selective medium containing

Table 2. Mutagenic action of SV40 DNA and SV40 MCH reconstituted in vitro (Remi)

Infecting agent	Histone/DNA ratio (w/w)	$\mu g/10^5$ cells	Mutation frequency ($\times 10^{-5}$)[a] selection	
			V79-TG	CHO-AP
No infection*	—	—	5[b]	12
SV40 DNA*	—	2	22	61
SV40 Remi*	1.5	2	27	78
SV40 Remi*	4	2	19	n.t.
SV40 Remi**	1.5	2	21	n.t.
SV40 Remi**	1.5	10	6	n.t.

* Pretreated with DEAE-dextran (150 μg/ml)
** Not pretreated.
[a]) Mutation expression time 7 days.
[b]) The plating efficiency of mock infected and that of infected cells was determined in each experiment and ranged from 65 to 80 per cent.

either 6-thioguanine (TG) or aminopterin (AP) and adjusted for selection of TG- and AP-resistant mutants (Theile *et al.*, 1980*a*, *b*). As shown in Table 2, the TG-resistant mutants occurred with a frequency of 27×10^{-5} (histone/DNA ratio 1.5 : 1). After mock infection or inoculation of V79 cells with the purified SV40 DNA mutant, frequencies of 5×10^{-5} and 22×10^{-5} were observed. On the other hand, inoculation of CHO cells with MCH reconstituted with a histone/DNA ratio of 4 : 1 resulted in a mutation frequency of 19×10^{-5}. Obviously, the amount of histones under these conditions bound to SV40 DNA was higher than that in the native MCH (Voordouw *et al.*, 1977).

In a second set of experiments, infected CHO-cells were selected for AP resistant mutants. Similarly to the results observed with the TG-selection system, both SV40 DNA and reconstituted MCH (histone/DNA ratio 1.5 : 1) induce AP resistant mutants; the mutant frequency obtained after inoculation of CHO cells with MCH was only slightly higher (78×10^{-5}) than after inoculation of purified DNA (61×10^{-5}). These frequencies are 5 times and 6.5 times higher, respectively, than the mutant frequencies obtained after mock infection of CHO cells.

The complex polycation DEAE-dextran has been found to be more advantageous for the mutagenic effect of SV40 DNA than other helper agents or simple application of SV40 DNA (Theile *et al.*, 1980*b*). In contrast to these results, we did not find any increase in the yield of mutants when DEAE-dextran and other helper agents were used before inoculation of CHO cells with SV40 MCH. As shown in Table 2, inoculation of V79 cells with MCH was not effective when concentration of 10 μg/ml was used instead of 2 μg/ml. This result is in accordance with results obtained when high concentrations of purified SV40 DNA were inoculated (Theile *et al.*, 1980*b*).

Influence of H1 histone on the transformation of CHL cells by SV40 DNA-H1 or by SV40 MCH-H1 complexes

SV40 DNA-H1 complexes were obtained by direct mixing in 0.1 × SSC buffer corresponding to 0.02 mol/l Na^+ (Böttger *et al.*, 1981). Depending on the molar input H1/DNA ratio (167 Mol H1/Mol DNA corresponded to a w/w ratio of roughly achieving a complete neutralization of DNA phosphate) three components of sedimenting species were obtained (1) component 25S sedimenting similarly to superhelical DNA, sedimentation coefficient $s_{20,w}$ (observable up to ratio 335 Mol H1/Mol DNA); (2) component 120 S appearing at ratio of 135 Mol H1/Mol DNA only and (3) growing amounts of > 1000S heterogeneous aggregates present above the 135 Mol H1/Mol DNA ratio. Electron microscopic analysis revealed that the 25S component consisted of double fibres without recognizable superhelix turns while the 120S component consisted of several bundles of such fibres. The aggregates represented cable-like structures (Fig. 1). Addition of ethidium bromide to the complexes effects a redistribution of H1 molecules and shows that H1 is responsible for bundle formation (Böttger *et al.*, 1981).

Table 3. Transformation of CHL cells by SV40 DNA/H1 — and SV40 reconstituted MCH (Remi)/H1 complexes

Days after inoculation	SV40 DNA (3 μg) without H1[b])	SV40 DNA + 60 Mol H1/ Mol DNA[b])	SV40 DNA + 135 Mol H1/ Mol DNA[b])	SV40 Remi[a] (3 μg) without H1f	SV40 Remi[a] (3 μg) + 11 Mol H1/ Mol DNA[c])	SV40 Remi[a] (1.5 μg) without H1[c])	SV40 Remi[a] (1.5 μg) + 11 Mol H1/ Mol DNA[c])
6–8	29*	2	5	29	30	136	30
14	> 300	< 1	< 1	> 200	10	1730	273

* Number of colonies in soft agar (per 10^5 cells).
[a]) Histone/DNA ratio 1.5 : 1 (w/w)
[b]) In 0.02 mol/l Na^+
[c]) In 0.06 mol/l Na^+

Both, 25S and 120S complexes were tested for their ability to transform CHL-cells. As demonstrated in Table 3, neither complexes significantly transformed CHL-cells by 6—8 days or by 14 days after inoculation.

Reconstitution of MCH by stepwise dialysis against decreasing concentrations of NaCl (Böttger *et al.*, 1976) showed the presence of beaded circles in solutions of low salt concentration (0.015 mol/l NaCl, 2×10^{-3} mol/l Na citrate, 2×10^{-4} mol/l EDTA). Up to 24 nucleosomes with diameters of about 10 nm connected by short DNA filaments could be observed at a histone/DNA weight input of 1.5 : 1. The complexes were undistinguishable by electron microscope and by sedimentation analysis from the H1 depleted native MCH. At higher salt concentrations (0.15 mol/l NaCl) these complexes were more or less condensed and again very similar to H1 depleted native MCH in "physiological" salt concentrations. To reconstitute the H1 containing "native" MCH like complexes, H1 was added (input ratio of 11 Mol H1/Mol DNA to the reconstituted MCH (in 0.15 mol/l NaCl); the complexes were brought to lower salt concentrations by dialysis in order to stabilize the reconstitutes against electrostatic expansion.

Several authors have shown that native H1-containing MCH are able to adapt a still more compact conformation at the same salt concentration than the depleted ones (Christiansen and Griffith, 1977). Therefore, we tested the sedimentation coefficient of the H1 containing complexes at 0.06 mol/l NaCl. We have found the value 86S for the H1-containing and the value 62S for the H1-depleted reconstitutes. Below 0.06 mol/l NaCl the complexes proved unstable.

Testing the imitated H1-containing "native" MCH for their transformation ability in CHL cells revealed that the H1-depleted reconstitutes (3 μg/10^5 cells) transformed CHL-cells by the nearly same rate as did the purified SV40 DNA (compare Table 3 and Table 1). On the other hand the ability of H-1-icontaining MCH to transform CHL cells was drastically reduced when the colonies were counted 14 days after inoculation. After application of 1.5 μg MCH this effect was even more pronounced: whereas 1730 colonies could be detected with H1 depleted complexes, only 273 colonies have been found after addition of H1-containing MCH.

Expression of SV40 related T antigen

Foci of CHO cell mutants as well as colonies of CHL cells grown in soft agar after inoculation of purified SV40 DNA or each MCH types were used to establish cell lines. Twenty cell lines were tested for the presence of SV40 T antigen by the indirect IF technique. All cell lines established from colonies growing in soft agar contained T antigen in 90—100% of their cells. The percentage of T antigen positive cells was not changed in cells with high passage numbers ($\sim$ 80) and there was no difference in the T antigen pattern in neither cell line tested (Fig. 2). The majority of CHL cell mutant clones also proved to be T antigen positive and 50—100% of the cells contained T antigen in the nuclei (Fig. 2). Only 1 out of 5 cell lines tested has remained negative so far.

Discussion

The data in this report strongly suggest that different forms of SV40 NP complexes reconstituted in vitro or isolated from SV40 infected cells can induce cellular transformation and mutation. However, there are considerable distinctions between these complexes in respect to their induction capacities.

MCH reconstituted in vitro using SV40 DNA and the four nucleosomal histones had a transforming capacity similar to that of purified SV40 DNA. However, significant activity was ascertained only by the use of calcium technique yielding frequencies of about 0.03% – 0.3%. We were also able to demonstrate the mutagenic action of reconstituted MCH and to isolate CH cell mutants resistant to both AP and TG. In analogy to the transformation test, the mutation frequencies of SV40 MCH and purified DNA were similar and more than 6-fold higher than the spontaneous mutation frequency. Reconstruction experiments have excluded the possibility that mutations appearing in inoculated cultures merely reflect a favoured growth of preexisting mutants (Theile and Strauss, 1977).

Recently Cohen *et al.* (1980) did not find transformation of mouse BALB/ /3T3 cells inoculated with reconstituted SV40 MCH. Altough the reconstitution method used by the authors was the same as employed in our study, their results should be regarded different for several reasons: (1) they did not use any helper agent for the uptake of viral MCH and (2) the cell densities used for calculation of transforming efficiencies in methylcellulose were very low (5—5000 cells per dish instead of 1×10^5 cells in our test system). As follows from our results, transformation frequencies of about 0.03% can be expected. In a sharp contrast to reconstituted MCH, infection of CHL cells with native SV40 MCH isolated from productively infected monkey cells resulted in 10- to 200-fold increase in transformed cells. These differences in the transforming capacities might be due to the biochemical and/or structural peculiarities of the NP complexes.

As demonstrated in our previous study (Waehlte and Scherneck, 1979), the NP H1-containing complexes may be favoured in their penetration. Determining the proportion of DNA actually reaching the cell nucleus we found 0.3% of total radioactivity of the SV40 DNA and 0.85% that of reconstituted H1-depleted MHC in the nucleus. However, about 12-fold more radioactivity was observed with native MCH. The nuclear radioactivity was precipitable by trichloroacetic acid. It seems unlikely that a breakdown and reincorporation of viral DNA took place since confluent monolayers with strongly decreased DNA synthesis were used. In comparison to purified DNA or H1-depleted reconstituted MCH, a slightly decreased uptake was found for both SV40 DNA/H1 complexes (60 Mol H1/Mol DNA) and reconstituted MCH/H1 complexes by the cells. This was true probably because aggregate formation prevented an exact estimation of the uptake process data not shown). The data presented in Table 3 do not support the suggestion that for the enhanced transformation efficiency of native MCH the enhanced uptake of SV40 NP H1-containing complexes would be mainly responsible.

It seems more likely that structural aspects may play a crucial role in these processes.

Histone H1 plays a fundamental role in compacting the 10 nm chromatin fibre into higher order structures; there are reports on a crosslink function of H1 between different oligosome chains (Strätling, 1979; Allen *et al.*, 1980). We could show that this crosslink function of H1 can also be observed in H1/SV40 DNA complexes (Böttger *et al.*, 1981). Depending on the molar input ratio Mol H1/Mol DNA we obtained 3 different components, 2 of which, the 25S and the 120S ones were tested in the transformation test. One can only speculate about the mode of operation of such complexes; a mechanism(s) directly involving H1 seems to be included. Neither the 25S nor the 120S components showed any significant transforming capacity in CHL cells. We therefore suggest that the H1-induced altered DNA conformation may prevent the expression of early SV40 gene functions necessary for transformation.

Reconstituted SV40 MCH stabilized by the addition of 11 Mol H1/Mol DNA also showed a significantly reduced transforming ability (an about 6-fold decrease as compared to H1 depleted MCH). Obviously, the full degree of condensation and the full stabilization against relaxation as seen with native MCH can not be reached in this system. The reason(s) are possibly connected with incorrect phasing of nucleosomes involving erroneous histone-DNA interaction which may have harmful consequences for the expression of viral gene functions (Hiwasa *et al.*, 1981).

One of the mechanisms proposed to operate in gene activation are histone modifications such as acetylation and phosphorylation, especially acetylation of histones H3 and H4 (La Bella and Vesco, 1980). Acetylation does not affect the histone-DNA interaction noticeably but it does weaken the histone--histone interaction (Shewmaker and Wagner, 1980). The viral chromatin, soon after synthesis, does not contain histones with a high degree of acetylation but ends up in virions with highly acetylated histones. The increase in histone acetylation may represent an advantage in facilitating gene transcription for the infecting virus (La Bella and Vesco, 1980). A correlation between histone acetylation and gene expression has been previously described for both SV40 and polyoma virus (Schaffhausen and Benjamin, 1976; MacGregor *et al.*, 1978). It has been shown that in the non-trasforming host range of the polyoma mutants, the histones H3 and H4 lack of high degree acetylation (Schaffhausen and Benjamin, 1976). Recently Cohen *et al.* (1980) failed to detect cellular transformation of BALB/3T3 cells with reconstituted SV40 MCH but he succeeded in transforming the same cells with reconstituted SV40 MCH containing in vitro hyperacetylated forms of histones H3 and H4. The transformation efficiency in this test system (non selective analysis and growth in methylcellulose) was surprisingly as high as 19%. On the other hand, the transforming efficiency of purified SV40 DNA was $< 0.02\%$, i.e. reconstituted MCH containing hyperacetylated histones H3 and H4 enhance the transformation frequency about 1000-fold. In comparison, the highest transformation efficiency induced by native MCH in CHL cells was about 3.5%, i.e. 200 times higher than with purified SV40 DNA.

In the light of these results it seems that rather the histone modification than structural changes may provide the SV40 MCH with a high transformation ability. Thus, they represent a very sensitive sstem for studying the processes involved in cellular transformation.

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Explanation of Micrographs (Plates I—II):

Fig. 1. Electron micrographs of DNA/histone complexes in 0.1 SSC buffer, pH 7.0

I — SV40-DNA form I lacking histones.

II — SV40-DNA/histone H1 complexes at an input ratio of 1 Mol DNA/146 Mol H1; regular double fibrillar structurres induced by histone H1.

III — SV40-DNA/histone H1 complexes at an input ratio of 1 Mol DNA/234 Mol H1; loosely or dense twisted cable-like bundles consisting of several double fibrillar SV40-DNA molecules.

IV — SV40 MCH reconstituted in vitro at an input ratio histones/DNA of 1.5/1.0 w/w; all MCH are well spread.

V — SV40 MCH as in (IV) after addition of histone H1 with an input ratio of 1 Mol DNA/11 Mol histone H1. Nearly all reconstituted MCH are visible as compact struct es resembling "native" MCH.

Bars indicate 100 nm.

Fig. 2. Demonstration of SV40 T-anigen in CHL cells, transformed with in vitro reconstituted SV40 MCH (I) and in cells of an AP resistant CHO mutant which was selected after mutagenesis with in vitro reconstituted SV40 MCH (II).