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High efficiency polyoma DNA transfection of chloroquine treated cells

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ABSTRACT

Chloroquine treatment of rodent cells during the first hours of polyoma DNA transfection increase the fraction of cells expressing viral functions. The effect has been observed after DNA absorption using both the DEAE-dextran and calcium phosphate coprecipitation methods. Exposure to chloroquine increased the proportion of transfected mouse cells to approximately 40%. From a culture of one million such cells, microgram quantities of newly synthesized viral DNA could be isolated. Similarly, the transformation frequency of rat cells following polyoma DNA transfection was approximately 6-fold increased by chloroquine treatment. The effect of the compound was even more pronounced in transfections with linear forms of polyoma DNA, suggesting that chloroquine inhibits degradation of DNA absorbed by the cells.

INTRODUCTION

Polyoma DNA infectivity was first demonstrated using mouse cells, exposed to a hypertonic medium, as recipients (1,2). Subsequently, more efficient transfection methods have been described. Treatment of the recipient cells with DEAE-dextran (3) increased the infectivity of polyoma DNA approximately 100-fold (4). More recently, viral DNA coprecipitated with calcium phosphate was shown to be infectious when added to cell cultures (5).

Both the calcium phosphate coprecipitation and the DEAE-dextran method results in the transfection of only a few percent of the cells in a culture. Modifications of the methods have led to improvements (6,7,8,9). However, these modified procedures either result in a limited increase of the fraction of transfected cells, or are very toxic to many cell lines.

To achieve transfection of the majority of a cell population, microinjection of DNA (10), or fusion with bacterial spheroplasts,

carrying a recombinant plasmid, can be used (11,12). However, the microinjection technique is limited by the number of cells that can be manipulated, and the spheroplast fusion method is restricted to the use of recombinant DNA molecules which can be propagated in bacterial cells.

The cellular uptake of DNA mediated by DEAE-dextran and calcium phosphate precipitates seems to be quite efficient (5,13). However, most of the input DNA is degraded before it reaches the nucleus of the cells (14), where gene expression and DNA replication take place. Much of the degradation apparently occurs in the lysosomes. Lysosomal degradation of proteins, taken up by endocytosis, can be inhibited by lysosomotropic compounds such as $(NH)_4Cl$ and chloroquine diphosphate (15).

This communication describes the effect of chloroquine in increasing the infectivity of polyoma DNA.

MATERIALS AND METHODS

Cells and virus. The sources of mouse 3T6 cells and Fischer Rat-1 cells have been described (16). Cells were grown in Dulbecco's modification of minimum essential medium supplemented with 10% (v/v) newborn calf serum or 10% horse serum (3T6 cells).

Polyoma virus of the large plaque A-2 strain (17) was used for the preparation of DNA.

Transfection procedures. In analyses of transformation Rat-1 cultures containing approximately $5x10^5$ cells were transfected with polyoma DNA, using a modification of the calcium phosphate method described by Wigler et al. (18). The DNA precipitate, in a volume of 0.5 ml, was added to the growth medium of the cultures (5 ml). After 4 h at 37°C the precipitate was carefully removed from the cells by rinsing. The cultures were then re-incubated for another day. Following trypsinization, cells were seeded in growth medium containing 0.33% (w/v) agar. Visible colonies of transformed cells were counted after 3 weeks at 37°C.

In analyses of productive infection, cultures containing approximately $8x10^5$ 3T6 cells were used. Polyoma DNA dissolved in 0.2 ml of tris-buffered saline (19), containing 0.5 mg per ml of DEAE-dextran (molecular weight $5x10^5$; Pharmacia Fine Chemicals), was added to the cell monolayers which had previously been washed

twice with the saline solution. DNA absorption was for 20 min at room temperature. After rinsing, the cultures were overlaid with growth medium and incubated at 37°C.

Treatment of the cells with chloroquine diphosphate (Sigma Chemical Co.) was done by the addition to the growth medium of an aqueous solution of the compound, as indicated in the separate experiments.

T-antigen staining. The expression of polyoma T-antigen was analyzed by indirect immunofluorescence. Transfected cells, washed twice with tris-buffered saline, were trypsinized and transferred to a centrifuge tube. After sedimentation, the cells were suspended in a hypotonic medium consisting of 0.8 mM $MgCl_2$, 1.0 mM KCl, 30 mM glycerol and 10% calf serum, and then allowed to swell for 2-3 min. A drop of the cell suspension was placed on a microscope slide and air dried at room temperature. The cells were fixed in acetone:methanol (2:1) for 10 min at -20°C, followed by incubation for 1 h at 37°C with a monoclonal rat antibody directed against all three polyoma T-antigens (a generous gift of S. Dilworth). After careful rinsing with tris-buffered saline and re-incubation with fluorescein-labeled goat anti-rat IgG, the T-antigen positive cells were visualized by fluorescence microscopy.

DNA isolation. Polyoma DNA was selectively extracted from infected 3T6 cells. After incubation with proteinase K (E. Merck), extraction with phenol and precipitation with ethanol, the samples were treated with RNAase (16). For preparative purposes viral DNA was further purified by equilibrium centrifugation in CsCl-ethidium bromide gradients.

Electrophoresis in 0.8% (w/v) agarose gels and quantitation of DNA was performed as described earlier (20).

RESULTS

Effect of chloroquine on the activity of polyoma DNA in the transformation of rat cells. In an initial experiment, we tested the effect of chloroquine on the polyoma transformation of rat fibroblast cells. Rat-1 cells were transfected with polyoma DNA in the presence or absence of chloroquine diphosphate. DNA was coprecip-

itated with calcium phosphate and added to the culture medium together with the chloroquine. Four h later the cell monolayers were carefully rinsed and fresh medium was added. Transformation was analyzed by plating cells in soft agar-medium following trypsinization. Visible colonies of transformed cells were counted 3 weeks later. The result of the experiment (Fig. 1) shows that treatment with 100 µM chloroquine increased the transformation frequency approximately 6-fold at all DNA concentrations we tested.

The optimal chloroquine concentration was investigated, by transfecting Rat-1 cell cultures with 0.02 or 0.1 µg of polyoma DNA in the presence of increasing chloroquine concentrations. Figure 2 shows that the maximal enhancement of the transformation process was obtained using a concentration of compound of 100 µM. A further increase of the chloroquine concentration resulted in a significant loss of viable cells.

Analysis of polyoma T-antigen expression in transfected mouse cells. Transfection of mouse 3T6 cells with saturating amounts of polyoma DNA, using the DEAE-dextran procedure, leads to the expression of viral T-antigens in approximately 2% of the in-

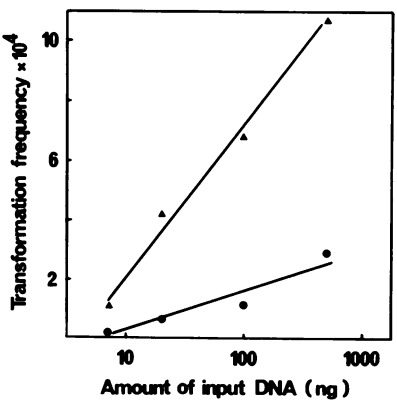

Figure 1. Effect on transformation frequency of DNA concentration and chloroquine treatment. Rat-1 cells were transfected in duplicate with the indicated quantities of polyoma DNA, using the calcium phosphate technique. One set of cultures were treated with 100 µM chloroquine diphosphate (▲—▲) during the 4 h period of DNA absorption. Another set was transfected under ordinary conditions (●—●). Transformation was analyzed by growth of transfected cells in semi-solid medium.

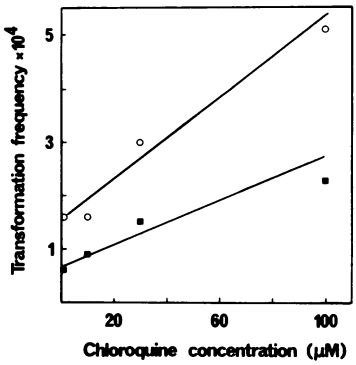

Figure 2. Effect of chloroquine concentration on transfection efficiency. Rat-1 cultures were transfected in duplicate by co-precipitating calcium phosphate and polyoma DNA, 20 ng (■—■) and 100 ng (o—o), respectively, onto the cells. During the 4 h absorption period chloroquine, at the indicated concentrations, was present in the growth medium. One day later the cells were seeded in soft agar-medium. Transformation was measured by counting visible cell colonies.

fected cells.

The effect of chloroquine on the number of T-antigen expressing cells was investigated by transfecting growing 3T6 cell cultures with normal polyoma DNA, or with DNA of a replication defective mutant, in the presence of DEAE-dextran. After absorption of DNA, the cells were rinsed and fed with growth medium containing 100 μM chloroquine. After 2-5 h the chloroquine containing medium was replaced with regular culture medium, and the incubation was continued. At 28 and 40 h post transfection, respectively, cultures were withdrawn. The cells were counted and analyzed for T-antigen expression by indirect immunofluorescence (Fig. 3). The results, summarized in Table 1, show that the fraction of T-antigen positive cells was proportional to the length of the chloroquine exposure. After 5 h as many as 40% of the cells expressed T-antigen. Furthermore, the fraction of fluorescent nuclei was relatively constant between 28 and 40 h post infection. The experiment also shows that 5 h of exposure to the compound is quite toxic to the 3T6 cells, as judged by the arrest of cell growth, and the morphology of the cells. However, in separate experiments 3T6 cell cultures have been

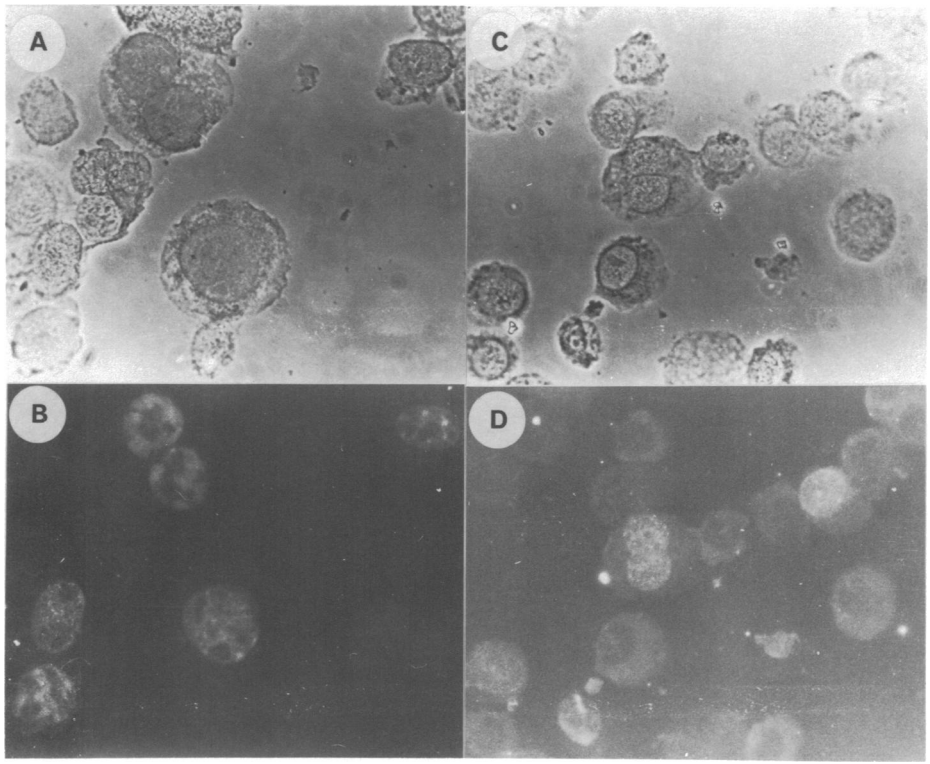

Figure 3. Effect of chloroquine on polyoma DNA transfection of mouse cells. Cultures of mouse 3T6 cells were transfected with 125 ng of normal polyoma DNA (A,B), or DNA of a replication defective mutant (C,D), in the presence of DEAE-dextran. After DNA absorption, the cells were overlaid with culture medium containing 100 µM of chloroquine. Five h later it was replaced by ordinary culture medium. At 40 h (A,B) or 28 h (C,D) post transfection cells were prepared for T-antigen analysis. Panels A and C show a microscopic view of two representative groups of cells. The T-antigen positive cells were visualized by their fluorescens (panels B and D).

maintained for 4-5 days after treatment with 100 µM chloroquine.

Polyoma DNA synthesis in mouse cells transfected with circular DNA. Chloroquine treatment increases the fraction of cells expressing viral functions approximately 20-fold. We were interested in investigating whether this transfection procedure would allow the convenient analysis of polyoma DNA synthesis in 3T6 cells.

For this purpose, cultures of growing 3T6 cells were trans-

Table 1. Effect of chloroquine on the fraction of transfected cells expressing T-antigen.[a]

Input DNA (ng)	Chloroquine treatment (h)	T-ag. positive cells (%) 28 h p.t.	40 h p.t.	No. of cells x 10^{-6} 40 h p.t.
0	0	-	-	4.0
0	2	-	-	2.4
0	3.5	-	-	1.7
0	5	-	-	1.2
125	0	3	2	4.4
125	2	11	7	2.2
125	3.5	31	31	1.1
125[b]	5	32 (27)	41	0.8

[a]Cultures of $8x10^5$ 3T6 cells were transfected with polyoma DNA in the presence of DEAE-dextran. Chloroquine treatment was performed as described in the legend to Fig. 6. At the indicated time points post transfection (p.t.) cells were harvested. T-antigen (T-ag.) positive cells were visualized by indirect immunofluorescence.

[b]The value in parenthesis denotes the result obtained with a replication defective mutant which has a deletion at the origin of DNA replication (16).

fected with 0.06-1.0 µg of polyoma DNA. Following DNA absorption, half of the cultures were incubated in the presence of 100 µM chloroquine, while the other half was incubated in normal growth medium. At 5, 28 and 40 h post transfection low-molecular-weight DNA was extracted from cells and analyzed by agarose gel electrophoresis (Fig. 4). At 5 h the input DNA from the cultures which had received the largest amounts was still visible. However, a few hours later no input DNA could be visualized using this method. Progeny DNA started to accumulate before 28 h, and had reached plateau levels by 40 h after transfection. Quantitation of the newly formed DNA (Fig. 5) showed that chloroquine did not influence the time course of the infection. The drop in the amount of viral progeny DNA isolated at 40 h post transfection, from the cultures which had received the largest amounts of DNA, was probably caused by the detachment of cells from the Petri dishes. As already apparent from Fig. 4, chloroquine treatment led to an approximately 5-fold increase in the amount of viral DNA produced in the cultures.

In the range of 0.06-1.0 µg, the amount of DNA used for the

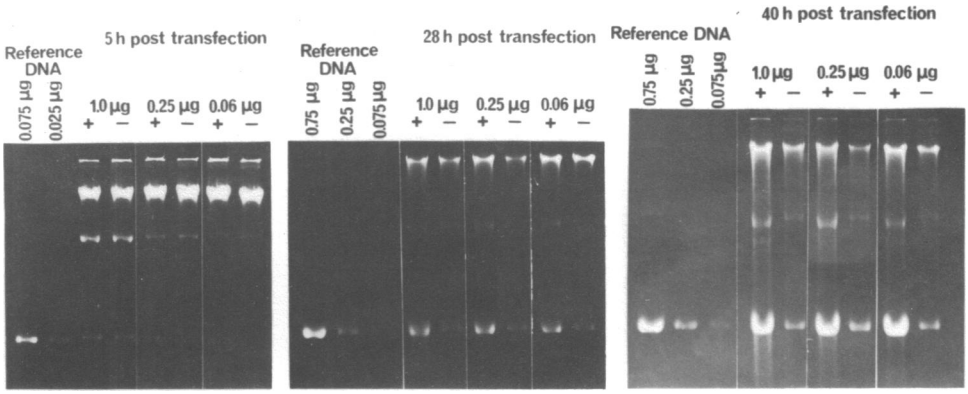

Figure 4. Effect on viral DNA synthesis of DNA concentration and chloroquine treatment at transfection. Mouse 3T6 cells were transfected, using the DEAE-dextran technique, with 0.06, 0.25 and 1.0 µg, respectively, of polyoma DNA. After DNA absorption, the cultures were either treated with 100 µM chloroquine for 5 h (+), or left untreated (-). At the indicated time points after transfection viral DNA was selectively extracted and analyzed by agarose gel electrophoresis. DNA was visualized by staining with ethidium bromide. Each lane contains DNA extracted from one cell culture (50 mm diameter). As references closed circular polyoma DNA was used.

transfection of a culture had little influence on the final amount of progeny DNA. However, other experiments have shown that a further decrease of the input DNA, to less than 0.05 µg, resulted in a reduction of viral DNA synthesis, particularly at relativley early times after transfection.

The chloroquine enhancement of the viral DNA synthesis (Fig. 5) seemed to be relatively less than the effect on T-antigen expression. The difference might be due to the toxic effects of chloroquine on the cells. To investigate this point further, the lenght of the chloroquine treatment was varied. In this experiment (Fig. 6), 3T6 cells were transfected with a saturating amount of viral DNA. Progeny DNA was extracted 40 h later and quantitated. The result confirmed that 4-5 h of chloroquine treatment led to a maximal stimulation of the viral DNA synthesis in the transfected cells. However, exposure to chloroquine for more than 6 h resulted in extensive cell death. This effect was also observed when the treatment was extended to include the periods before and during DNA absoption.

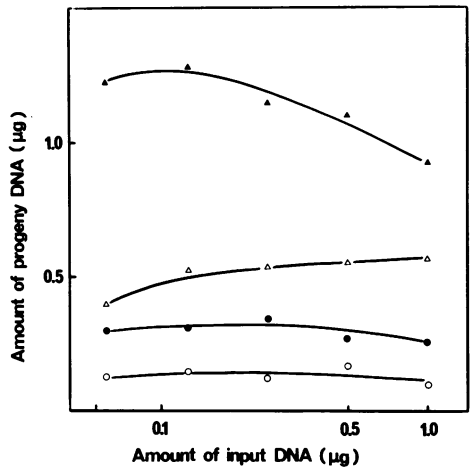

Figure 5. Quantitation of viral DNA synthesis in transfected mouse cells treated with chloroquine. DNA in the gels shown in Fig. 4, and in parallel gels, was quantitated by densitometer scanning of photographic negatives. The linear relationship between fluorescence intensity and DNA mass of the reference samples was used to determine the amount of closed circular polyoma DNA. Triangles represent DNA from chloroquine treated cells, and circles DNA from control cells, harvested 28 h (open symbols), or 40 h (filled symbols) after transfection.

Viral DNA synthesis in cells transfected with linear DNA. Chloroquine might facilitate the transfection process by inhibiting DNA degradation. Since linear DNA molecules are more susceptible to degradation than circular, the hypothesis was tested by an analysis of the influence of chloroquine on the infectivity of linear DNA.

Polyoma DNA was digested with restriction endonuclease BamHI and EcoRI, respectively. Both enzymes have single recognition sites in the polyoma genome. However, the EcoRI site, but not the BamHI site, is located in the early region of the genome (21). The linear DNA preparations were used for the transfection of 3T6 cells. Analysis of low-molecular-weight DNA isolated 40 h after transfection (Fig. 7) showed that chloroquine treatment of the cells increased the infectivity of linear DNA quite substantially. The effect was most obvious with BamHI linerized DNA, probably because loss of DNA sequences at the BamHI cleavage site, at the recircularization of the molecules, does not lead to the in-

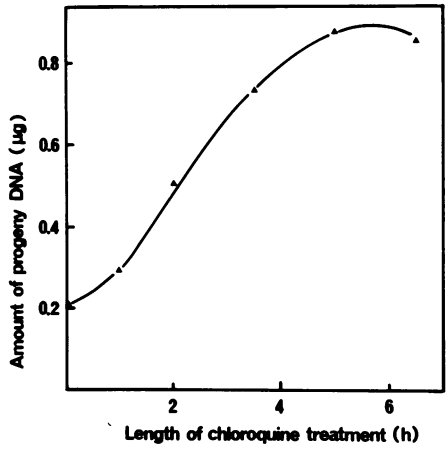

Figure 6. Effect on transfection of the length of chloroquine treatment. Cultures of 3T6 cells were transfected with 0.2 µg of polyoma DNA, using DEAE-dextran as a facilitator. After absorption of DNA, the cells were overlaid with chloroquine-containing medium which was replaced by ordinary medium after the indicated time periods. At 40 h after transfection viral DNA was extracted and quantitated as described in the legend to Fig. 5.

activation of gene functions required for the replication process. In fact, much of the DNA recircularized at the BamHI site had a reduced size, as judged from its electrophoretic mobility.

DISCUSSION

Polyoma DNA is infectious when it is added to rodent cells mixed with DEAE-dextran, or as a calcium phosphate coprecipitate. With limiting amounts of DNA, as in plaque assays, the DEAE-dextran method results in an approximately 10-fold higher DNA infectivity (10^3 plaques per ng of DNA), than does the calcium phosphate procedure. In transfections with larger quantities of of DNA, the two methods result in similar DNA infectivities. In this case, the number of competent cells in a culture appears to be the limiting factor, since not more than 2% of the recipients express viral functions. It is unclear what determines the competence of the cells. A large fraction of the input DNA is absorbed by the cells. However, little of the absorbed DNA reaches the cell nuclei, suggesting that degradation of DNA in the cyto-

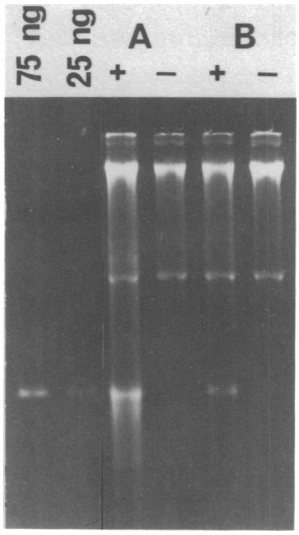

Figure 7. Effect of chloroquine on transfection with linear DNA. Cultures of 3T6 cells were transfected, in the presence of DEAE- dextran, with 0.5 µg of polyoma DNA linearized with BamHI (A) or EcoRI (B). Growth medium added to the cultures after DNA absorption contained 100 µM chloroquine (+). After 5 h it was replaced by ordinary medium. Control cultures (-) received no chloroquine treatment. At 40 h after transfection low-molecular-weight DNA was extracted and analyzed by gel electrophoresis. Closed circular polyoma DNA was used as a reference.

plasm might be a limiting factor of the transfection process.

Chloroquine has been shown to prevent protein degradation, particularly of proteins absorbed by endocytosis (15). The compound is taken up by the lysosomes, where it raises the pH, thereby inactivating the hydrolytic enzymes (22).

We found that chloroquine has an effect also on the efficiency of polyoma DNA transfection. Chloroquine treatment of mouse 3T6 cells, following absorption of polyoma DNA (Table 1), increased the fraction of T-antigen expressing cells approximately 20-fold. The enhanced DNA infectivity was independent of viral DNA amplification, since transfection with a replication defective deletion mutant resulted in a similar high proportion of T-antigen positive cells, although the fluorescence was less intense in this case (Fig. 3B)

Under optimal conditions chloroquine increases the amount of viral DNA formed in transfected cultures approximately 5-fold (Fig. 5). The apparent discrepancy between the effects of Chloroquine on T-antigen expression and on viral DNA synthesis is probably explained by the toxicity of the compound which, at least temporarily, arrests cell division. This conclusion is supported by a calculation of the number of viral DNA molecules per T-antigen positive cell. This was done by combining data of Table 1 and

Fig. 5. The result shows that both untreated and chloroquine-treated cultures yielded approximately $5x10^5$ DNA copies per cell, suggesting that chloroquine affects the uptake of DNA only.

The chloroquine enhanced transfection procedure provides a reliable and reproducible assay of viral DNA synthesis (16). Similar quantitative results were obtained whether polyoma DNA isolated from infected cells, or viral DNA excised from a recombinant plasmid and then recircularized, was used for the transfection of 3T6 cells.

The infectivity of polyoma DNA in plaque assays on 3T3 cells was not increased by chloroquine treatment. This observation suggests that different factors limit transfection of cells with picogram and microgram amounts of DNA, respectively.

Chloroquine increased the frequency of Rat-1 cell transformation, following transfection with polyoma DNA (Fig. 1), to about the same extent as it enhanced viral DNA synthesis in mouse cells. Since transformation was measured by the appearance of cell colonies, the experiment shows that the toxic effects of chloroquine can be reversible.

When chloroquine treatment is effective, it increases the fraction of cells that can be successfully transfected. The result is probably an effect of a decreased degradation of the DNA absorbed by the cells. This conclusion is supported by the result of experiments in which cells were transfected with linear forms of viral DNA (Fig. 7). In chloroquine treated cells the number of DNA molecules which had recircularized and were able to replicate, was much larger than in untreated cells. Since linear DNA molecules are very susceptible to degradation, a variable number of nucleotides are usually lost from their ends before recircularization (23). Chloroquine seems to partially inhibit this degradation, resulting in an increased infectivity of linearized polyoma DNA.

The chloroquine mediated inhibition of DNA degradation might be a result of an increased pH-value in the lysosomes. The concentrations of chloroquine required for increasing the infectivity of polyoma DNA are similar to those effective in raising the intralysosomal pH (22). However, chloroquine is also known to bind strongly to DNA (24), and might thereby protect DNA molecules

from nuclease degradation. Binding of the compound to DNA has previously been shown to inhibit the activities of bacterial DNA- and RNA-polymerases (25). In addition, chloroquine inhibits repair of DNA damage in mammalian cells (26).

In order to be effective, chloroquine has to be present during hours following DNA absorption. This extended time period presumably reflects the slow uptake of the DNA by the cell nucleus, where the biological activity of the viral genome can be expressed. Chloroquine treatment probably stimulates the transfection process by increasing the number of undegraded viral DNA molecules that reach the nuclei of the cells. It is likely that the method is applicable also for the transfer of non-viral genes to cells.

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