Topoisomerases provide the unlinking activity necessary for replication fork movement during DNA replication. It is uncertain, however, whether topoisomerases are also required for the initiation of replication. To investigate this point, we have performed pulse-chase experiments with SV40 minichromosomes as template to distinguish between the initiation and the elongation of replication. Using an unfractionated cytosolic extract as a source of replication functions, we found that the addition of topoisomerases at the initiation step significantly increased the number of active chromatin templates, whereas addition of topoisomerases at the elongation step had only minor effects. Minichromosomes with an extended chromatin structure as well as protein-free DNA required less topoisomerase for effective replication initiation. We could exclude the possibility that topoisomerases enhance the origin binding of T antigen, the SV40 replication initiator, and propose instead that the arrangement of nucleosomes influences the diffusion of supercoils during initial DNA unwinding. Efficient initiation therefore requires a high local concentration of topoisomerases to relax the torsional stress.

DNA replication is initiated by the helicase-promoted unwinding of the parental strands. This process generates positive superhelical stress that must be relaxed by DNA topoisomerases to allow continued replication fork movement (1). Eukaryotic DNA topoisomerases I and II relax both positive and negative supercoiled DNA by transiently inducing single-stranded or double-stranded DNA breaks, respectively (2).

Genetic studies with yeast topoisomerase mutants have indicated that either topoisomerase I or II can serve as a swivel for DNA replication (3–5). By using selective inhibitors or antibodies against the topoisomerases, the roles of these enzymes during SV40 minichromosome replication have been further elucidated. Thus, topoisomerase I was found to be specifically associated with replicating SV40 minichromosomes (6–8), and biochemical and electron microscopic methods further revealed that topoisomerase I acts close to the replication forks, presumably by relieving supercoiling tension in front of the advancing replication forks (9, 10). Whereas either topoisomerase I or II can provide the swivel activity necessary for the efficient synthesis of progeny SV40 DNA molecules (11), topoisomerase II is uniquely required as a swivelase in the late stages of minichromosome replication and has an essential role during segregation of the daughter molecules after DNA replication (9; 11–15).

The in vivo substrate for DNA replication is chromatin, and thus, topoisomerases have to relieve the torsional stress arising during replication on nucleosomally organized DNA. Analysis of topoisomerase cleavage sites in nucleosomal DNA has shown that the nucleosome positioning is a critical determinant for the activity of topoisomerases. Thus, topoisomerase II requires a free access to DNA, either through nucleosome-free regions or through the linker DNA between adjacent nucleosomes (16, 17). In fact, we could previously show that the accessibility to topoisomerases and consequently the efficiency of replication are regulated by the chromatin structure. Accordingly, we found that minichromosomes with a relaxed chromatin structure need less topoisomerase for an efficient replication compared with minichromosomes with a compact structure (18).

However, recent studies have shown that neither topoisomerase I nor topoisomerase II was able to relieve the superhelical tension of covalently closed circular minichromosomes as generated by the unwinding by the SV40 T antigen (T-Ag)1 in a purified system. Only very early unwinding intermediates were found, suggesting that relaxation by topoisomerases is a critical step at the early stages of replication. In contrast, linear chromatin molecules, which do not accumulate torsional stress, were clearly unwound in a T-Ag-dependent manner (19).

To further elucidate the roles of topoisomerases during chromatin replication and to discriminate between their effects on initiation and elongation, we performed pulse-chase experiments. We found that the complementation of a replication-competent cytosolic extract by the addition of an excess of topoisomerases increases the number of initiation events but not the efficiency of chain elongation. To demonstrate that indeed the organization of the chromatin determines the requirement for an excess of topoisomerases, we used SV40 minichromosome templates that had been treated with trypsin to remove the amino-terminal histone domains. This leads to a more extended chromatin structure (20) and reduces the amount of topoisomerases needed for an efficient initiation, indicating that nucleosome organization regulates the efficiency of topoisomerase activity and, thus, the efficiency of replication initiation.

EXPERIMENTAL PROCEDURES

Preparation of SV40 Minichromosomes and SV40 DNA—Salt-treated SV40 minichromosomes were isolated from CV1 cells 24 h post-infection and purified on 5 to 30% sucrose gradients containing 500 mM salt exactly as described (21). For the studies with BglI-digested chromatin, SV40 minichromosomes were incubated with BglI (10 units/1 μg) for 1 h at 25 °C in the corresponding buffer. To remove the restriction enzyme, BglI-digested chromatin was purified in parallel.

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‡ The abbreviations used are: T-Ag, T antigen; MNase, micrococcal nuclease; bp, base pair(s).
with undigested chromatin on a 5-ml Sepharose 4B column (Amersham Pharmacia Biotech). Trypsinized minichromosomes were obtained by incubating 1 µg of salt-treated SV40 minichromosomes with 50 ng of trypsin for 10 min at 25 °C (18, 20). SV40 DNA was prepared from CV1 cells 60 h post-infection according to the procedure of Hirt (22) and purified twice over CsCl density gradients.

Preparation of the SV40 T-Ag and Cytosolic S100 Replication Extract—SV40 T-Ag was isolated from infected Sf9 cells and purified by immunoaffinity chromatography (20). Cytosolic S100 extracts were prepared from HeLa cells exactly as described previously (24). For pulse-chase analysis, the extract was dialyzed immediately before use on Millipore filters (type VS, 0.25 µm) for 90 min at 4 °C against LS-buffer (20 mM Hepe-KOH, pH 7.8, 5 mM potassium acetate, 0.5 mM MgCl₂, and 0.5 mM dithiothreitol), to remove the endogenous nucleotides.

In Vitro Replication of SV40 DNA and Minichromosomes—For standard in vitro replication assays, 500 ng of SV40 minichromosomes or 125 ng of SV40 DNA were incubated with 1 µg of T-Ag and 175 µg of cytosolic S100 extract in the presence or absence of additional topoisomerases (topoisomerase I, Biozym; topoisomerase II, TopoGen) for 2 h at 37 °C as described earlier (18). Different amounts of protein-free SV40 DNA and minichromosomes were used to account for the fact that only 25% of minichromosomes possess a nucleosome-free origin (25–27) and are able to replicate under the conditions used (28–30).

Pulse-Chase Analysis—Elongation Studies—For pulse-chase analysis, SV40 in vitro replication assays were performed in a two-step procedure as described by Foteder et al. (31) with the following modifications. In the first step, 1 µg of T-Ag was incubated with 500 ng of SV40 chromatin or 125 ng of SV40 DNA in 175 µg of dialyzed S100 replication extract for 30 min to allow the formation of initiation complexes on the different templates. This preincubation was done in the presence of 3 mM Mg²⁺/ATP, 30 mM Hepes-KOH, pH 7.8, 0.5 mM dithiothreitol, 40 mM creatine phosphate, and 0.24 µg/ml creatine kinase at 26 °C. After initiation complexes were assembled, elongation was initiated as the second step by the addition of the remaining three ribonucleotides (80 µM each CTP, GTP, and UTP), 100 µM dGTP and dCTP, and 5 µCi of [α-³²P]dATP to label the newly synthesized DNA. During this step, elongation was limited by the absence of dTTP. After 30 s, a chase was initiated by the addition of a 1000-fold excess of cold dATP and 100 µM dTTP to allow further elongation. All steps were carried out at 26 °C. After the indicated time points (15 s to 10 min), aliquots of the different reactions were removed and stopped by the addition of 0.6 volumes of stop-mix solution (2.0% SDS, 60 mM EDTA). After proteinase K digestion, phenol extraction, and ethanol precipitation, purified products were resuspended in 50 mM NaOH, 1 mM EDTA and separated by 1.2% alkaline agarose gel electrophoresis. The sizes of the ³²P-labeled fragments of the molecular weight marker are as indicated (R). DNA synthesis was quantitated by trichloroacetic acid precipitation. C, the average lengths (b) of the newly synthesized DNA fragments were determined by densitometric scanning of equally exposed autoradiograms (A), and the values were blotted against the elongation time (control open squares; +topo I (pre) closed circles; +topo I (post) open circles).

FIG. 1. Influence of topoisomerase I on the initiation and elongation of chromatin replication. The effect of topoisomerase I on chromatin replication was investigated by incubating SV40 minichromosomes under replicational conditions in the absence (control) or presence (+topo I) of additional 30 units of topoisomerase I. To allow the formation of initiation complexes, 500 ng of salt-treated SV40 minichromosomes were preincubated in cytosolic S100 replication extract in the presence of 1 µg of SV40 T-Ag, 3 mM Mg²⁺/ATP, and /+ topoisomerase I for 30 min at 26 °C. After initiation complexes were assembled, elongation was initiated by the addition of the remaining ribonucleotides, dGTP, dCTP, and [α-³²P]dATP. After 30 s, the pulse-labeling was terminated by the addition of dTTP and a 1000-fold excess of cold dATP. To discriminate between initiation and elongation, topoisomerase I was either present from the very beginning (pre) or added after initiation complex formation (post), immediately before the elongation process was started. At the indicated time points (15 s to 10 min), aliquots were removed, and the isolated DNA was analyzed by 1.2% alkaline agarose gel electrophoresis and autoradiography. The sizes of the ³²P-labeled fragments of the molecular weight marker are as indicated (R). DNA synthesis was quantitated by trichloroacetic acid precipitation.
extract, which was used as a source for replication functions, was complemented with an excess of topoisomerases. We performed pulse-chase experiments to investigate whether additional topoisomerases stimulate the initiation or the elongation reaction. To this end initiation complexes were formed using salt-treated SV40 minichromosomes in the presence of the SV40 T-Ag, dialyzed cytosolic S100 replication extract and ATP/Mg$^{2+}$. During this preincubation step no T-Ag-dependent DNA synthesis was measured (data not shown). DNA elongation was initiated by the addition of ribonucleoside triphosphates, dGTP and dCTP, to preincubated reactions and pulse-labeled with [$\alpha$-$^{32}$P]dATP. After 30 s, dTTP was added, and the elongation reactions were chased with an excess of unlabeled dATP (Fig. 1). Topoisomerase I was either present during the initiation step (Fig. 1A, pre) or added just before the beginning of the elongation step (Fig. 1A, post). The products were then analyzed under denaturing conditions by alkaline gel electrophoresis (Fig. 1A), and the incorporated nucleotides were determined by trichloroacetic acid precipitation (Fig. 1B). The average lengths of the replication products were evaluated by densitometric scanning of the autoradiograms (Fig. 1C). This kind of pulse-chase analysis allows two different measurements. Thus, the intensities of the autoradiographic signal and the efficiencies of nucleotide incorporation correspond to the number of replicating DNA intermediates. Comparison of the lengths of the replication products gives the rate of DNA chain elongation.

When topoisomerase I was present at the beginning of initiation, incorporation of nucleotides was around 3-fold higher compared with the control as visualized by the intensity of the autoradiographic signal (Fig. 1A) and the trichloroacetic acid precipitation data (Fig. 1B). Comparison of the nascent DNA produced within the first minute shows no differences in length (about 400 nucleotides, which corresponds to the length of protein-free DNA within the nucleosome-free origin (25)), indicating that the higher incorporation of nucleotides in the presence of additional topoisomerase I is solely because of a stimulation of the initiation reaction. In accordance, the addition of topoisomerase I to the elongation step did not stimulate the incorporation of nucleotides compared with the control (Fig. 1, A and B, post).

After 10 min of chase, we measured a 1.6-fold difference in the product lengths of salt-treated minichromosomes incubated with and without topoisomerase during the initiation step (Fig. 1C). This supports the conclusion that the overall stimulation of replication efficiency by additional topoisomerase I, which was measured in previous experiments (18), is mainly because of an effect on initiation. The increase in product lengths was even less pronounced when topoisomerases were added after initiation (Fig. 1C), indicating that in this case topoisomerase I did not become part of the replication elongation apparatus.

To further exclude that the increased incorporation is because of 3-fold longer nascent DNA produced during the limited initiation step, we did the pulse-chase analysis in the absence of dTTP (Fig. 2). Without dTTP during the elongation step, the product lengths are the same for control, +topoI/pre and +topoI/post (around 125 bases) (Fig. 2B). The difference in intensity between control and +topoI/pre is 3-fold (Fig. 2A), as observed in the presence of dTTP, demonstrating that the observed differences in the incorporation of nucleotides are indeed because of a different number of active molecules and not to different elongation rates during the preincubation step.

It is known that either topoisomerase I or topoisomerase II can provide the unlinking activity necessary for fork propagation during SV40 DNA replication. However, topoisomerase II is uniquely required for the segregation of newly synthesized DNA daughter strands (11). To determine whether topoisomerase II has the same stimulatory effect on the initiation of chromatin replication as observed for topoisomerase I, we repeated the pulse-chase experiment of Fig. 1 in the presence of topoisomerase II added either before or after the initiation step...
Again, we observed a significant increase in the incorporation of nucleotides only when topoisomerase II was present from the beginning of the initiation step and not when added to the elongation reaction (Fig. 3, A and B). The increase in the product lengths after 10 min of elongation was slightly less than observed with topoisomerase I. The data of Figs. 1–3 demonstrate that both topoisomerase I and II have a clear stimulatory effect on the initiation but not on the elongation of chromatin replication.

We repeated the experiment with protein-free SV40 DNA as template, to determine whether the stimulation of replication efficiency was specific for chromatin. Topoisomerase I or topoisomerase II were either added to the initiation step or during elongation. In contrast to the observed stimulation of replication of chromatin templates, neither topoisomerase I (data not shown) nor topoisomerase II (Fig. 4) increased the incorporation of nucleotides (Fig. 4, A and B). An effect on the elongation of DNA replication, when protein-free DNA was used as template, was not observed (Fig. 4C). Thus, the amounts of topoisomerases present in the S100 replication extract are sufficient for the efficient replication initiation of protein-free DNA but not of chromatin molecules.

Why are chromatin templates more active when the replication mixture is complemented by an excess of topoisomerases? One possibility is that topoisomerases could enhance the binding of the SV40 T-Ag to the origin and thereby increase the number of molecules that initiate replication. Alternatively, mechanistic differences in the replication of protein-free DNA and chromatin might be responsible for the higher amounts of topoisomerases required during chromatin replication. We have investigated these possibilities.

Topoisomerase Does Not Enhance the Binding of the SV40 T-Ag—First, we performed replication experiments with increasing amounts of T-Ag and determined that chromatin and DNA templates respond similar to the amount of T-Ag added. Both templates replicated optimally at a T-Ag concentration of

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**Fig. 3. Influence of topoisomerase II on the replication of chromatin.**

The effects of topoisomerase II on the initiation and elongation of chromatin replication were analyzed exactly as described for topoisomerase I (see Fig. 1), except that 15 units of purified topoisomerase II (+ topo II) were added to the pulse-chase reactions (A). After elongation was started, aliquots of the individual reactions were removed at the indicated time (15 s to 10 min) and analyzed by 1.2% alkaline agarose gel electrophoresis followed by autoradiography. B, incorporation of nucleotides during pulse-labeling with [α-32P]dATP was measured by trichloroacetic acid precipitation. C, the average lengths of the newly synthesized DNA daughter strands in (A) were blotted against the time of elongation (control (open squares); + topo I (pre) (closed circles); + topo I (post) (open circles)).
A characteristic repeat length of digestion indicated that the DNA surrounding the SV40 origin to nucleosome oligomers seen at intermediate stages of MNase SV40 origin (Fig. 5). Because nucleosomes protect associated DNA from nuclease (MNase) to investigate whether topoisomerase I promotes more efficiently than minichromosomes as described previously (18, 35). We then performed digestion experiments with micrococcal nuclease (MNase) to determine whether topoisomerase I promotes the binding of T-Ag to the SV40 origin sequences. We digested salt-treated minichromosomes after formation of the initiation complex in the absence or presence of topoisomerase I (Fig. 5). Because nucleosomes protect associated DNA from MNase digestion, DNA fragments of nucleosome and oligonucleosome-sized DNA can be visualized by agarose gel electrophoresis. The MNase pattern at specific sites was revealed by Southern blotting and hybridization with oligonucleotides complementary to the origin sequences (Fig. 5A) or to a sequence outside the SV40 origin (Fig. 5B) (30). The ladder of fragments corresponding to nucleosome oligomers seen at intermediate stages of MNase digestion indicated that the DNA surrounding the SV40 origin was organized in a regular spaced array of nucleosomes with a characteristic repeat length of ~180 bp. Upon extensive digestion with MNase, a subnucleosomal fragment of around 80 bp became evident. This fragment consisted of origin DNA protected from MNase digestion by bound T-Ag. This can be concluded because rehybridization of the same DNA blot with oligonucleotides complementary to a sequence outside the origin (Fig. 5B) showed no subnucleosomal fragments, but only the regular-spaced nucleosomal ladder. Furthermore by MNase digestion of protein-free SV40 DNA incubated in the presence or absence of T-Ag (Fig. 5B, right panel), we found in the presence of T-Ag a fragment of 80 bp that was protected from MNase digestion when hybridized with the origin-specific oligonucleotide, whereas the rest of the plasmid was degraded. In the absence of T-Ag this 80-bp fragment could not be detected. Importantly, the same amount of DNA was protected by T-Ag from MNase digestion when initiation complex formation was done in the presence or absence of additional topoisomerase I, demonstrating that topoisomerase does not stimulate the binding of T-Ag to further SV40 minichromosome molecules.

Previous experiments have shown that a nucleosome-free origin is a prerequisite for the initiation of replication of chromatin molecules (28, 29, 34, 35). However, CHRAC, a chromatin remodeling complex containing topoisomerase II as one sub-unit (36), can overcome this repression (30). To address the question of whether topoisomerases alone are capable of stimulating replication from a nucleosomal origin, we eliminated those templates with accessible origins (about 40–50%) by linearization with BglII, a restriction endonuclease that cuts SV40 DNA once within the origin sequences. To exclude the possibility that BglII digests the templates made accessible to the nuclease by topoisomerase-mediated chromatin remodeling, BglII was separated from the chromatin on a Sepharose 4B column (data not shown). BglII-digested minichromosomes were then used as the template in the SV40 in vitro replication system in the absence or presence of additional topoisomerases and investigated by neutral gel electrophoresis (Fig. 6A) and trichloroacetic acid precipitation (Fig. 6B). Whereas topoisomerase I stimulates the replication of undigested chromatin by a factor of 3–4, no significant incorporation of nucleotides was measured with BglII-digested chromatin in the presence of either topoisomerase I (Fig. 6) or topoisomerase II (30), indicating that topoisomerases alone cannot activate molecules with a nucleosomal origin.

Chromatin Structure Influences the Rate of Replication Initiation—Recent experiments have shown that the chromatin structure has a profound effect on the replication efficiency of chromatin molecules. Thus, molecules with a modified chromatin structure, such as acetylated minichromosomes (37), chromatin-containing high mobility group proteins (38), or trypsinized minichromosomes (20) show a higher rate of replication than control chromatin. In addition it has been shown that the chromatin structure influences the accessibility for topoisomerases and thus the replication efficiency of the templates (18). To address the question of whether the chromatin structure also influences the efficiency of initiation, molecules with a different chromatin structure were used as template in the pulse-chase experiment. To this end, the amino-terminal histone domains of salt-treated (control) minichromosomes were removed by trypsin treatment, which results in an opening of chromatin structure of these molecules. Control and trypsinized minichromosomes as well as protein-free DNA were then incubated in the two-step procedure in the cytosolic S100 replication extract (Fig. 7). Replication products were analyzed under denaturing condition by alkaline gel electrophoresis (Fig. 7A), and the incorporation of nucleotides was determined by trichloroacetic acid precipitation (Fig. 7B), as described above. The product lengths were calculated by densitometric scanning of the autoradiogram (Fig. 7C).

We observed a significant difference in the intensity of the autoradiographic signal obtained from control and trypsinized minichromosomes, which was around 3–4-fold, as determined from the incorporated nucleotides. Tryptinized minichromo-
somes show almost the same incorporation as protein-free DNA. Calculation of the product lengths of the reactions stopped at different time points revealed that the difference between DNA and control minichromosomes was 1.5-fold and around 1.2-fold between control and trypsinized minichromosomes (Fig. 7C). Thus, the major difference measured in the incorporation rates of control and trypsinized minichromosomes is because of a difference in the initiation rates. These differences can be overcome by the addition of topoisomerases (compare Figs. 1 and 3 with Halmer and Gruss (18)), indicating that the nucleosomal organization is responsible for the efficiency of topoisomerase activity during the initiation of replication of chromatin molecules.

**DISCUSSION**

A first step during the initiation of replication is the recognition and binding of origin sequences by initiator proteins such as the *Escherichia coli* DnaA protein, the phage λO protein, the SV40 T-Ag, or the eucaryotic origin recognition complex (ORC) (reviewed in Ref. 39). Once bound to the origin, initiator proteins recruit additional factors involved in the assembly and function of the replication machinery. A critical step in fork assembly is the initial separation of the two DNA strands by disruption of the hydrogen bonds, which is required for loading of the replication machinery and for providing the template for DNA synthesis. This step is performed by DNA helicases, such as the *E. coli* DnaB helicase, the SV40 T-Ag, or possibly the MCM proteins,
which may function as DNA helicases in eucaryotic cells (40). Unwinding of the DNA template and the progression of the replication fork create topological stress, which is eliminated by DNA topoisomerases. Helicases and topoisomerases may be part of a large replication complex (41), and indeed, a direct interaction between eucaryotic topoisomerase I and the SV40 T-Ag has recently been described (42–45).

We present evidence, that in addition to their known roles during the progression of the replication fork and the segregation of the daughter molecules, topoisomerases are a critical determinant for the efficiency of initiation of chromatin replication. We found by pulse-chase analysis that more chromatin molecules can start replication by increasing the amount of either topoisomerase I or II in the SV40 in vitro replication system. Thus, the nucleosomal structure itself can significantly influence the amount of topoisomerases needed for an efficient initiation.

The influence of topoisomerases on the efficiency of chromatin replication are not entirely understood, but several possibilities may be considered. One possibility is that topoisomerases stimulate further binding of T-Ag to the SV40 origin sequences. This should, however, also increase the replication efficiency of protein-free DNA templates. This was not observed. Furthermore, by micrococcal nuclease footprinting we could demonstrate that the addition of topoisomerases did not increase the number of molecules containing bound T-Ag.

A second possibility could be the activation of additional chromatin molecules with a nucleosomally organized origin. These molecules are otherwise inactive for replication in this system (28, 29, 35). However, they can be activated through specific remodeling of the chromatin structure at the origin sequences by the chromatin remodeling complex CHRAC (30). This complex contains topoisomerase II (36). We show here that topoisomerases alone are not able to remodel the chromatin structure of a nucleosomally organized origin nor to activate these molecules for the initiation of replication.

Our most interesting observation was that additional topoisomerases stimulate the initiation of replication from chromatin but not from protein-free DNA molecules. Thus a higher amount of topoisomerases seems to be a logical consequence for the efficient removal of the tails by trypsinization occur through post-translational modifications of the histone proteins as the acetylation of the core histones and the phosphorylation of histone H1 or the association with non-histone proteins like HMGI (high mobility group)-17. In all these cases an increase in the replication efficiency was measured in vitro in comparison with control chromatin (21, 37, 38). This indicates that the nucleosomal organization is a critical determinant for replication initiation.

Changes in nucleosomal structure can induce a more extended chromatin structure and, thus, a higher accessibility for topoisomerases. Furthermore these changes might facilitate the diffusion of supercoils, which otherwise accumulate in front of the replication machinery at the first nucleosome encountered. Thus by either modifying the nucleosomal structure, e.g. through histone modifications, or by increasing the amount of topoisomerases, the cell might be able to regulate the efficiency of the initiation of chromatin replication.

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Involvement of Topoisomerases in the Initiation of Simian Virus 40 Minichromosome Replication
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