***In vitro* chromatin remodelling by chromatin accessibility complex (CHRAC) at the SV40 origin of DNA replication**

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DNA replication is initiated by binding of initiation factors to the origin of replication. Nucleosomes are known to inhibit the access of the replication machinery to origin sequences. Recently, nucleosome remodelling factors have been identified that increase the accessibility of nucleosomal DNA to transcription regulators. To test whether the initiation of DNA replication from an origin covered by nucleosomes would also benefit from the action of nucleosome remodelling factors, we reconstituted SV40 DNA into chromatin in *Drosophila* embryo extracts. In the presence of T-antigen and ATP, a chromatin-associated cofactor allowed efficient replication from a nucleosomal origin *in vitro*. In search of the energy-dependent cofactor responsible we found that purified ‘chromatin accessibility complex’ (CHRAC) was able to alter the nucleosomal structure at the origin allowing the binding of T-antigen and efficient initiation of replication. These experiments provide evidence for the involvement of a nucleosome remodelling machine in structural changes at the SV40 origin of DNA replication *in vitro*.

Keywords: CHRAC/chromatin remodelling/chromatin replication/nucleosomal origin

Introduction

Little is known about how the replication machinery is able to recognize and initiate replication from an origin packaged into chromatin (Wang and Li, 1995). Nucleosomes covering the origin will efficiently inhibit replication *in vivo*, best illustrated by studies in *Saccharomyces cerevisiae*, where a positioning of a nucleosome on the ARS origin resulted in a marked decrease in replication efficiency (Simpson, 1990).

Also, the temporal regulation of cellular origin function during S phase is dictated by the chromosomal context; transcriptionally inert domains of mammalian chromosomes are replicated late, while active domains are replicated early in S phase (reviewed in Diffley and Stillman, 1990). Goldman *et al.* (1984) have shown that tissue-specific genes replicate early in cells in which they are expressed, while they replicate late during S phase in cells that do not express the genes. Furthermore, a yeast origin of DNA replication that normally functions early in S

phase replicates late when placed near telomere sequences, which are normally late-replicating (Ferguson and Fangman, 1992). Since correlations between gene activity and the degree of packaging in chromatin are well established it appears that the activity of replication origins is also modulated by the chromatin environment. It is conceivable that, comparable with the different chromatin structures found in promoters (Wallrath *et al.*, 1994), different classes of origins might exist. ‘Preset’ origins which are activated early in S phase might either be free of nucleosomes or constantly covered by initiation complexes such as ORC (Diffley and Cocker, 1992). In contrast, those which are activated late during S phase might first require remodelling by dedicated factors. This could be triggered, for example, through binding of transcription factors in the proximity of origin sequences. Transcription factors could then activate replication either by direct interaction with the replication machinery (He *et al.*, 1993; Li and Botchan, 1993) or by an indirect mechanism involving alterations of chromatin structure (Cheng and Kelly, 1989).

Cell-free replication systems have served as useful tools for analysis of the factors required for replication (Fairman and Stillman, 1988; Stillman, 1989; Tsurimoto and Stillman, 1991). Due to its simplicity, the SV40 origin of replication has served as a paradigm for eukaryotic replication (DePamphilis, 1993). Replication at this origin is initiated by the T-antigen (T-Ag), a virus-encoded, multifunctional factor whose binding to the origin is the first step in a series of events leading to replication (reviewed in Fanning and Knippers, 1992). T-Ag also functions as a helicase that unwinds the DNA ahead of the replication machinery (Stahl *et al.*, 1986). The SV40 system also provided the first information about replication in the context of chromatin. Reconstitution of SV40 minichromosomes with high nucleosome densities in *Xenopus* oocyte extracts efficiently inhibits replication *in vitro* (Cheng and Kelly, 1989). Prior binding of the transcription factor NF-I perturbs the distribution of nucleosomes at the origin locally, thereby increasing the accessibility of the origin region to initiation factors (Cheng and Kelly, 1989). Pre-binding the T-Ag alone does not create a replication-competent origin during assembly in extracts from *Xenopus* oocytes (Cheng and Kelly, 1989; Gruss *et al.*, 1993). In contrast, when the assembly reaction is performed in mammalian cell extracts, pre-bound T-Ag is able to keep the origin free of nucleosomes and competent for replication (Ishimi, 1992). It is conceivable that the successful interaction of T-Ag with a chromatin substrate requires cofactors present in some extracts but not others.

The chromatin structure of active origins *in vivo* is usually in a perturbed, DNase I hypersensitive state, phenomenologically similar to that at transcription pro-

motors and enhancers. It is, therefore, quite possible that transcription and replication in chromatin are governed by similar principles which ensure that initiating factors gain access to their binding site. Biochemical and genetic studies have identified various macromolecular complexes which affect chromatin structure to facilitate interactions of transcription factors with regulatory elements. These include the SWI/SNF and RSC complexes from yeast (Peterson and Tamkun, 1995; Cairns *et al.*, 1996) and related activities in human cells (Wang *et al.*, 1996), the nucleosome remodelling factor (NURF; Tsukiyama and Wu, 1995) and the ATP-utilizing chromatin assembly and remodeling factor (ACF; Ito *et al.*, 1997) from *Drosophila*. Recently another activity, the chromatin accessibility complex (CHRAC), has been identified and purified to apparent homogeneity from *Drosophila* extracts (Varga-Weisz *et al.*, 1997). This complex combines enzymes that modulate both nucleosome structure and DNA topology. Two of the five CHRAC subunits have been identified as the nucleosome-stimulated ATPase ISWI (Elfring *et al.*, 1994) and topoisomerase II (Varga-Weisz *et al.*, 1997). ISWI is also an integral subunit of NURF (Tsukiyama *et al.*, 1995); however, CHRAC and NURF appear to be quite different. While NURF mediates transcription-factor dependent nucleosome disruption *in vitro*, which can lead to the association of transcription factors with chromatin templates followed by transcription from chromatin templates, CHRAC was unable to catalyze the same reactions (Mizuguchi *et al.*, 1997; Varga-Weisz *et al.*, 1997). While differences in these cell-free reactions cannot be taken as an indicator of the physiological roles of remodelling complexes, the presence of topoisomerase II in CHRAC suggests that it might be involved in the dynamic changes of chromosome structure during cell cycle or replication. *In vitro* CHRAC not only causes a general ATP-dependent increase in chromatin accessibility, but also functions as a chromatin assembly factor. CHRAC is able to catalyze the energy-dependent alignment of nucleosomes into regular arrays from irregular chromatin resulting from nucleosome assembly in the absence of ATP. Such a 'nucleosome spacing activity' may be important for nucleosome assembly concomitant with replication.

To assess whether replication could be initiated from a nucleosomal origin in a system that allows dynamic chromatin transitions, we reconstituted SV40 DNA into chromatin in *Drosophila* S150 embryo extracts (Becker and Wu, 1992). We found that in the presence of the SV40 T-Ag, ATP and a chromatin-associated cofactor, replication occurred efficiently from a nucleosomal origin. In the search for the required chromatin remodelling activity we found that purified CHRAC specifically remodelled the chromatin structure at the SV40 origin allowing the binding of T-Ag and efficient initiation of DNA replication.

Results

A chromatin-associated activity mediates initiation of DNA replication from a nucleosomal origin

Chromatin assembly extracts derived from preblastoderm *Drosophila* embryos that replicate their genomes at the highest rates contain a wealth of activities that are able to alter chromatin structure in an energy-dependent manner

(Pazin *et al.*, 1994; Tsukiyama *et al.*, 1994; Varga-Weisz *et al.*, 1995, 1997; Ito *et al.*, 1997). To test whether any of those activities might be able to open the chromatin structure at a nucleosomal origin of replication and thus to trigger the initiation of DNA replication, we reconstituted SV40 DNA into chromatin in *Drosophila* S150 embryo extracts (Figure 1A). In order to control for the presence of ATP we routinely removed the ATP after the chromatin assembly reaction by gel filtration of the chromatin through Sephacryl S300 columns. Since we specifically wanted to focus on nucleosomal origins we eliminated those templates with accessible origins (~40–50%) by linearization with *Bgl*II, a restriction endonuclease which cuts SV40 DNA once within the origin sequences (Figure 1A). NURF and CHRAC activities are sensitive to the detergent sarkosyl (Tsukiyama and Wu, 1995; Varga-Weisz *et al.*, 1997). To remove these chromatin-associated activities, one half of the reconstituted minichromosomes was treated with sarkosyl (0.075%) before purification by spin column centrifugation. Purified minichromosomes were incubated with or without ATP and the SV40 T-Ag for 45 min at 26°C. Equal amounts of reconstituted chromatin were then used as template in the SV40 *in vitro* replication system (Li and Kelly, 1984). Purified replication products were analyzed by agarose gel electrophoresis and autoradiography (Figure 1B) and replication efficiencies of the different templates were determined by TCA precipitation (Figure 1C). Replication of minichromosomes depended entirely on the presence of T-Ag (Figure 1B and C, lanes 1, 3, 5 and 7). Without *Bgl*II digestion of the chromatin templates the replication products and efficiencies were very similar for sarkosyl-treated and untreated chromatin (Figure 1B and C, lanes 2 and 4) (see below). However, digesting the reconstituted templates with *Bgl*II and thus eliminating molecules with a nucleosome-free origin, led to a significant difference between the two templates. Whereas the replication efficiency of the sarkosyl-treated chromatin was drastically reduced (Figure 1B and C, lane 8), the untreated chromatin was replicated 2.5- to 4-fold higher (Figure 1B and C, lane 6). Determination of the incorporated nucleotides (see legend to Figure 1) showed that ~50% of the circular, non-*Bgl*II-linearized chromatin replicated under these conditions. These data show that a sarkosyl-sensitive activity was associated with the reconstituted chromatin, which facilitated the initiation of replication from a nucleosomal origin in a reaction that depended on T-Ag and ATP.

Specific remodelling of the chromatin structure at the SV40 origin

In order to visualize the presumed alterations of nucleosome structure at the SV40 origin sequences SV40 DNA was assembled into chromatin, treated with *Bgl*II and sarkosyl as described above (Figure 1A) and digested with micrococcal nuclease (MNase). Since nucleosomes protect associated DNA from MNase digestion, DNA fragments of nucleosome and oligonucleosome-sized DNA arose which were separated on an agarose gel. The MNase pattern at specific sites was revealed by Southern blotting and hybridization with oligonucleotides complementary to the origin sequences (Figure 2A) or to a sequence outside the origin (Figure 2B) (Tsukiyama *et al.*, 1994).

The presence of a 146 bp fragment containing the origin

Fig. 1. A chromatin-associated activity allows the replication of SV40 chromatin from a nucleosomal origin. **(A)** Schematic representation of the experimental procedure. **(B)** Reconstituted chromatin was incubated in the absence or presence of *Bgl*I. Sarkosyl (Sark.) was used to remove chromatin-associated activities. Two hundred ng of chromatin were replicated for 2 h at 37°C in the SV40 *in vitro* replication system in the absence or presence of SV40 T-Ag. Replication products were purified and separated on a 0.8% agarose gel. The dried gel was autoradiographed. The position of replicative intermediates (RI), superhelical DNA (I), relaxed, closed circular and nicked molecules (II) are indicated. **(C)** One tenth of the replication assay was precipitated with TCA to determine the incorporated nucleotides. The replication efficiency of the samples in **(B)** is standardized against the replication efficiency of non-*Bgl*I-digested and non-sarkosyl-treated chromatin (lane 2) and shown as percentage incorporation. The standard deviation is determined from three independent experiments. (Incorporation in pmoles dNTP was as follows: 7, 183, 8, 195, 8, 87, 11 and 28 for lanes 1–8, respectively.)

region which was protected from MNase digestion showed that nucleosomes had been assembled at the origin (Figure 2A, lane 1). 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 an array of nucleosomes with a characteristic repeat length of ~180 bp. A different cleavage pattern at the origin was seen when the chromatin was incubated with SV40 T-Ag in the presence of ATP (Figure 2A, lane 4). Upon extensive digestion with MNase the abundance of the 146 bp fragment, diagnostic for the intact nucleosome, decreased drastically, but shorter subnucleosomal fragments became evident. These fragments (~100 bp) consist of origin DNA protected from MNase digestion by bound T-Ag; MNase digestion of SV40 complexes formed on naked DNA in the presence of ATP yielded a similar pattern of small fragments protected from MNase digestion when hybridized with the origin-specific oligonucleotide while the rest of the plasmid was degraded (data not shown). These data indicate that the nucleosome at the origin had been lost.

Nucleosome remodelling at the SV40 origin depended on the presence of ATP (Figure 2A, lanes 3 and 4), indicating the involvement of an energy-dependent remodelling activity. This activity was sensitive to sarkosyl treatment (Figure 2A, lane 8). Addition of *Drosophila* extract to the sarkosyl-treated chromatin recreated, to some extent only, in an ATP and T-Ag-dependent manner,

the MNase digestion pattern characteristic of the 're-modelled' origin (Figure 2A, lane 9). The observed nucleosome remodelling was specific for origin sequences. Rehybridization of the same DNA blot with oligonucleotides complementary to a sequence outside the origin showed that T-Ag and ATP did not affect the global MNase digestion pattern non-specifically (Figure 2B). Collectively, these data show that a sarkosyl-sensitive activity, which was associated with the reconstituted chromatin, allowed the binding of T-Ag to the origin in an ATP-dependent reaction.

CHRAC facilitates initiation of replication from a nucleosomal origin by remodelling nucleosomes at the origin

Drosophila embryo extracts contain several activities able to remodel chromatin structure, thereby facilitating access to DNA-binding proteins (Pazin *et al.*, 1994; Tsukiyama and Wu, 1995; Varga-Weisz *et al.*, 1995; Wall *et al.*, 1995; Ito *et al.*, 1997). One of these factors is the CHRAC, a five subunit complex containing the ATPases ISWI and topoisomerase II. This complex increases the accessibility of DNA in chromatin to binding proteins in an energy-dependent manner (Varga-Weisz *et al.*, 1997). To test for an effect of CHRAC on the initiation of DNA replication from a nucleosomal origin, reconstituted SV40 chromatin, with or without sarkosyl treatment, was incubated with CHRAC, purified to homogeneity (Varga-Weisz *et al.*,

Fig. 2. T-Ag-binding to a nucleosomal SV40 origin depends on a sarkosyl-sensitive activity present in the *Drosophila* extract. (A and B) Micrococcal nuclease analysis of *Bgl*II-digested SV40 chromatin. Experimental procedure is outlined in Figure 1A (1–6). Sarkosyl-treated or untreated chromatin was pre-incubated in the absence or presence of ATP and/or T-Ag for 45 min at 26°C, as indicated. *Drosophila* extract (DREX) was added to sample 9 after sarkosyl-wash and spin column centrifugation, incubated for 30 min at 26°C and then further treated as samples 1–8. Chromatin was digested with MNase for 30, 60 or 120 s. Purified DNA was analyzed by Southern blotting with oligonucleotides: (A) complementary to the SV40 origin sequence; (B) complementary to a sequence outside the SV40 origin, respectively. Numbers to the left indicate size markers in bp.

1997), for 30 min at 26°C before addition of T-Ag and ATP. These minichromosomes were then used as templates for replication (Figure 3A and B). Remarkably, the very poor replication efficiency of sarkosyl-treated, *Bgl*II-digested chromatin (Figure 3A and B, lane 7) was stimulated 5- to 8-fold if chromatin was pre-incubated with CHRAC, T-Ag and ATP (Figure 3A and B, lane 8), indicating that CHRAC enabled the initiation from a nucleosomal origin. Remodelling by CHRAC stimulated the replication of sarkosyl-treated or untreated templates that had not been digested with *Bgl*II 1.5- to 3.5-fold (Figure 3A and B, lanes 2 and 4). Those templates have a higher basal level of replication due to the presence of endogenous CHRAC or nucleosome-free origins, respectively.

The reason for the poor stimulation by CHRAC of chromatin without the sarkosyl wash is currently unknown. The observed stimulation of replication by CHRAC was specific for chromatin, since identical amounts of CHRAC did not affect the efficiency of *in vitro* replication of protein-free DNA significantly (Figure 3C and D). Deter-

mination of the incorporated nucleotides (Figure 3B and D) revealed that after addition of CHRAC ~90% of the circular chromatin initiated DNA replication, which is almost identical to the initiation of protein-free DNA (100%). The higher incorporation of nucleotides into protein-free DNA compared with chromatin (see legend to Figure 3) is due to re-replication of DNA in this system (Krude *et al.*, 1994), which does not occur with chromatin.

Since topoisomerase II is an integral part of CHRAC (Varga-Weisz *et al.*, 1997) and topoisomerases stimulate the elongation of replication of SV40 minichromosomes *in vitro* (Halmer and Gruss, 1997), we tested whether the observed stimulation of initiation was due to the topoisomerase II activity in the CHRAC complex. An amount of purified *Drosophila* topoisomerase II (USB) identical to the amount added with CHRAC, as determined by etoposide-mediated cleavage and relaxation assays, was pre-incubated with *Bgl*II-digested, sarkosyl-treated chromatin. When these minichromosomes were used as template in the SV40 *in vitro* replication system, no significant incorporation of nucleotides was measured, indicating that topoisomerase II alone was not responsible for the stimulation of chromatin replication (data not shown).

The stimulation of chromatin replication by CHRAC was accompanied by nucleosome remodelling of the kind previously observed in the crude embryo extract. *Bgl*II-digested, sarkosyl-treated chromatin was incubated in the absence or presence of CHRAC, ATP and T-Ag followed by MNase treatment and Southern blot analysis as before (Figure 4). While the regular nucleosomal array was unperturbed in the absence of CHRAC or ATP (Figure 4A and B, lanes 1–4), in the presence of CHRAC a similar T-Ag and ATP-dependent structural change at the origin was observed that had been seen in the crude system (Figure 4A, lane 8).

To confirm that CHRAC mediates the binding of the SV40 T-Ag to the origin sequences, *Bgl*II-digested, sarkosyl-treated chromatin was analyzed by DNase I footprinting (Figure 5). To this end the reconstituted chromatin was incubated with increasing amounts of T-Ag in the absence or presence of CHRAC. Samples were treated with DNase I and purified products were analyzed by primer extension. In the absence of CHRAC, T-Ag was not able to bind to the nucleosomally organized origin sequences (Figure 5, lanes 2 and 3). In contrast, in the presence of CHRAC, T-Ag gained access to the origin as revealed by the protection of T-Ag binding site I and II from DNase I digestion (Figure 5, lanes 4–6). On protein-free DNA the same sites were bound by T-Ag (data not shown). The same footprinting pattern was also observed with *Bgl*II-digested, non-sarkosyl-treated chromatin in the presence of T-Ag and ATP (data not shown), suggesting that chromatin-associated CHRAC (or other alike activities) are able to facilitate T-Ag binding.

To address the question of whether the promotion of T-Ag binding to the origin sequences by CHRAC was necessary and sufficient to enhance replication efficiency, T-Ag was bound to the DNA template prior to chromatin assembly (Figure 6B). Because these templates elongate efficiently during the subsequent chromatin assembly in *Drosophila* extracts, as can be seen by the appearance of labeled form I DNA (Figure 6A, lane 1), the assembly

Fig. 3. CHRAC stimulates the initiation of SV40 chromatin replication from a nucleosomal origin but not of protein-free DNA. (A) Chromatin preparation was as in Figure 1A except that prior to the incubation with T-Ag and ATP the chromatin was incubated with 1 μ l purified CHRAC (250 ng/ μ l). Two hundred ng of chromatin were replicated for 2 h at 37°C in the SV40 *in vitro* replication system. Replication products were analyzed as in Figure 1. (B) Replication efficiency of the samples in (A) is standardized against the replication efficiency of non-*Bgl*I-digested and non-sarkosyl-treated chromatin (lane 1) and shown as percentage incorporation. The standard deviation is taken from three independent experiments. (Incorporation in pmoles dNTP was as follows: 145, 225, 144, 512, 77, 95, 29 and 168 for lanes 1–8, respectively.) (C) Protein-free SV40 DNA was pre-incubated in the absence or presence of 1 μ l purified CHRAC (250 ng/ μ l) and used as template in the SV40 *in vitro* replication system. Autoradiography of the purified replication products is shown. (D) Replication efficiency of the samples in (C) is standardized against the replication efficiency of protein-free SV40 DNA without pre-incubation with CHRAC (lane 1) and shown as percentage incorporation. The standard deviation is taken from three independent experiments. (Incorporation in pmoles dNTP was as follows: 1058 and 1120 for lanes 1 and 2, respectively.)

was performed in the presence of aphidicolin (Gruss *et al.*, 1993). In this case no further elongation of pre-initiated molecules occurred and no completely replicated molecules could be detected (Figure 6A, lanes 2 and 3). The aphidicolin is removed during the gel filtration step after the sarkosyl treatment. Reconstituted chromatin with pre-bound T-Ag was able to replicate efficiently in the SV40 *in vitro* replication system (Figure 6C, lane 1). Addition of CHRAC to these templates had no stimulatory effect on replication efficiency (Figure 6C, lane 2), demonstrating that the binding of T-Ag to the origin, mediated by the remodelling of nucleosomes by CHRAC, caused the replication stimulation.

Purified CHRAC has no effect on the elongation of DNA and chromatin replication

To determine whether CHRAC is involved in the elongation phase of DNA replication, we undertook pulse-chase experiments (Figure 7). For the formation of initiation complexes sarkosyl-treated chromatin or protein-free DNA were incubated in the absence or presence of purified CHRAC with T-Ag, ATP and dialyzed S100 replication extract. DNA elongation was initiated by the addition of ribonucleoside triphosphates, dGTP and dCTP to pre-incubated reactions and pulsed with [α - 32 P]dATP. After

30 s, dTTP was added, and the elongation products were chased with an excess of unlabeled dATP. A comparison of the replication elongation products reveals the intensity of CHRAC induction whereas no difference in size of the elongation products throughout the time course was detected. This clearly demonstrates that the CHRAC effect was entirely due to initiation and elongation was unaffected (Figure 7, left panel). The intensity and the size of the elongation products of protein-free DNA are the same in the absence and presence of CHRAC, which supports the conclusion that CHRAC has no influence on the initiation and elongation of protein-free DNA (Figure 7, right panel).

Taken together, these results demonstrate that the selective remodelling of a nucleosomal origin by CHRAC leads to T-Ag binding and efficient initiation of replication from a nucleosomal origin.

CHRAC stimulates the replication efficiency of histone H1 containing chromatin and SV40 minichromosomes

Chromatin reconstituted with the early *Drosophila* embryo extract is deficient in histone H1. Exogenous H1 can be incorporated into the chromatin and its association with nucleosomes can be monitored by the increase in the nucleosomal repeat length (Becker and Wu, 1992;

Fig. 4. CHRAC-dependent binding of T-Ag to a nucleosomal origin of replication. Reconstituted SV40 chromatin was digested with *Bgl*I and treated with sarkosyl as described in Figure 1A. After spin column purification the chromatin was pre-incubated in the absence or presence of ATP, the SV40 T-Ag and 1 μ l of purified CHRAC (250 ng/ μ l) for 30 min at 26°C. Treatment with MNase and subsequent Southern blot hybridization was exactly as described in Figure 2: (A) complementary to the SV40 origin; (B) complementary to a sequence outside the SV40 origin, respectively. Filled triangles indicate a 123 bp marker.

Sandaltzopoulos *et al.*, 1994; Varga-Weisz *et al.*, 1995) (Figure 8A). Thus, incorporation of increasing amounts of histone H1 causes an expanded repeat length of 20–30 bp (Becker and Wu, 1992; Hayes and Wolffe, 1993) (Figure 8A, lanes 2 and 3). To ensure the stability of the H1 containing templates during *in vitro* replication, RNA, which otherwise works as a competitor for histone H1 (Ilyin *et al.*, 1971; Halmer and Gruss, 1995), was removed from S100 replication extracts by treatment with RNase A. Under these conditions CHRAC stimulates the replication of histone H1-containing chromatin as efficiently as the replication of H1-deficient chromatin (Figure 8B), which is in agreement with the results of Varga-Weisz *et al.* (1995), who found that the accessibility for restriction enzymes was not restrained by the presence of histone H1 in chromatin.

We examined further whether CHRAC can stimulate replication in heterologous chromatin. To this end SV40 minichromosomes were isolated from infected CV1 cells, and treated with 500 mM salt to remove non-histone proteins (Gruss and Knippers, 1995). To eliminate the

Fig. 5. DNase I footprint analysis of CHRAC-dependent binding of T-Ag to the origin of replication. *Bgl*I-digested, sarkosyl-treated chromatin was incubated with increasing amounts of T-Ag (lane 2 and 3: 3 and 4.5 μ g; lane 4, 5 and 6: 1.2, 3 and 4.5 μ g, respectively) in the absence or presence of CHRAC and 3 mM ATP. Samples were treated with DNase I and after primer extension the fragments were resolved on an 8% sequencing gel and mapped with respect to dideoxy sequencing reactions using the same primer. Binding of T-Ag is revealed by protection of T-Ag-binding sites I and II from DNase I digestion (shadowed boxes). I, binding site I; IR, inverted repeat; PEN, pentanucleotide domain; AT, AT-rich domain (Parsons *et al.*, 1990). The *Bgl*I-cutting site is labeled by an asterisk.

population with a nucleosome free origin (Jakobovits *et al.*, 1980; Saragosti *et al.*, 1980; Sogo *et al.*, 1986) these salt-treated minichromosomes were digested with *Bgl*I and used as substrate in the SV40 *in vitro* replication system. Whereas in the absence of CHRAC very little replication occurred (Figure 9A and B, lane 2), in the presence of ATP and increasing amounts of CHRAC replication was 3- to 4-fold induced (Figure 9A and B, lanes 3–5). The same results were obtained with native SV40 minichromosomes containing histone H1 and numerous non-histone proteins (data not shown).

Taken together, these results demonstrate that CHRAC

Fig. 6. Pre-binding of T-Ag abolishes the effect of CHRAC on the replication of assembled chromatin. (A) SV40 DNA was pre-incubated with T-Ag and then used for chromatin assembly in the absence or presence of aphidicolin and [α - ^{32}P]dATP. The replication products after incubation for 6 h at 26°C are shown. The positions of replicative intermediates (RI), superhelical DNA (I), relaxed closed circular and nicked molecules (II) are indicated. (B) Schematic representation of the experimental procedure. (C) The effect of CHRAC on the replication of T-Ag containing templates is shown, experimental procedure is as outlined in (B). The autoradiography of the replication products is shown. The position of replicative intermediates (RI), superhelical DNA (I), relaxed closed circular and nicked molecules (II) is indicated.

can also remodel the chromatin structure of templates containing histone H1 as well as *in vivo* assembled chromatin allowing the binding of T-Ag and most probably other factors to their cognate binding sites.

Discussion

Initiation of SV40 DNA replication requires binding of the SV40 T-Ag to the origin sequences, the localized unwinding of the DNA and recruitment of cellular replication proteins (Stillman, 1989). Ramsperger and Stahl (1995) suggested that the main limiting step in replication initiation is the binding of the T-Ag to nucleosomal DNA which does not occur at physiological ionic conditions. The process of DNA unwinding by the T-Ag helicase, once initiated, is then not inhibited by nucleosomes (Ramsperger and Stahl, 1995). This nucleosomal inhibition of replication was also observed when we reconstituted SV40 DNA into chromatin in *Drosophila* S150 extracts in the absence of activities capable of altering chromatin structures. Thus, a nucleosome at the origin strictly inhibits the initiation of chromatin replication in this system. Inhibition of T-Ag binding will prevent initiation of replication in chromatin (Cheng and Kelly, 1989; Ishimi, 1992; Gruss *et al.*, 1993). Consistent with these notions, the stimulatory effect of CHRAC on replication from a nucleosomal origin was due to its capacity to alter the nucleosome structure to render the origin sequences accessible to T-Ag.

While we cannot exclude the fact that other nucleosome remodelling activities present in the crude chromatin assembly extract, such as NURF and ACF (Tsukiyama

and Wu, 1995; Ito *et al.*, 1997), may affect replication from a nucleosomal origin, we definitely demonstrate that CHRAC is able to do so. A role for CHRAC in replication is plausible, since it is abundant in early *Drosophila* embryos when there is no transcription but extremely rapid DNA replication (Varga-Weisz *et al.*, 1995). Remarkably, the replication initiation function of T-Ag is compatible with the replication machinery present in *Drosophila* embryo extracts, such that replication is initiated from SV40 origins in *Drosophila* embryo extracts if T-Ag is added during chromatin assembly (Kamakaka *et al.*, 1994). It is possible that the abundance of CHRAC in these extracts is required for this efficient replication.

So far, remodelling of chromatin structure has only been suggested to be involved in the transcriptional activation of nucleosomal promotor or enhancer elements (reviewed in Peterson and Tamkun, 1995; Felsenfeld, 1996; Kingston *et al.*, 1996; Peterson, 1996). In these cases re-organization of the chromatin structure enables efficient access of transcription factors to their target sequences. The experiments shown here demonstrate for the first time a direct effect of a chromatin remodelling factor on the initiation of DNA replication from a nucleosomal origin *in vitro*.

Both *in vitro* studies in *Xenopus* extracts and *in vivo* studies in *S.cerevisiae* indicated that the initiation of DNA replication operates through a two-step process (reviewed in Dutta and Bell, 1997; Rowles and Blow, 1997; Toone *et al.*, 1997). The first step occurs during mitosis of the previous cell cycle, where a so-called pre-replicative complex (pre-RC) forms over the origin of replication. Thereby, the Cdc6 protein and the MCM proteins are

Fig. 7. Effect of CHRAC on the elongation of chromatin and DNA. Initiation complexes were formed on chromatin and DNA in the presence of T-Ag, ATP, S100 replication extracts in the absence or presence of CHRAC (see Materials and methods). After a 30 s pulse in the presence of [α - 32 P]dATP, the remaining ribonucleotides, dGTP and dCTP, the pulse was terminated by adding a 100-fold excess of cold dATP, together with dTTP to allow elongation during the chase. Aliquots were removed at the indicated time points during the chase, and the DNA was isolated and analyzed on a 1.2% alkaline agarose gel by autoradiography. The position of size markers is indicated.

assembled in an ordered fashion onto a chromatin bound ORC-origin complex prior to the initiation of DNA synthesis. After passage through the G_1 decision point (START), a rise in cyclin-dependent kinase activity and the following activation of downstream protein kinases triggers the initiation from the pre-formed initiation complexes by phosphorylating targets within the pre-RC (reviewed in Dutta and Bell, 1997; Rowles and Blow, 1997; Toone *et al.*, 1997). Whether ORC is able to bind to nucleosomally organized origin sequences by remodelling the chromatin structure by itself or whether CHRAC or related activities are involved in these processes remains to be explored.

Recent experiments indicate that profound alterations in nucleosome structure are required to permit efficient replication initiation. Post-translational acetylations of the core histones, which are frequently enriched in transcriptionally active, DNase I sensitive chromatin (reviewed in Csordas, 1990; Davie and Hendzel, 1994; Turner and O'Neill, 1995) are not sufficient to allow the initiation of DNA replication from a nucleosomally organized origin; thus after removal of molecules with a nucleosome-free origin, SV40 minichromosomes containing hyperacetylated histones were unable to replicate in the cell-free system (Alexiadis *et al.*, 1997). Even the complete removal of the N-terminal histone domains by trypsinization did not relieve nucleosomal repression of replication initiation (Quintini *et al.*, 1996). Like SV40 minichromosomes

Fig. 8. CHRAC activity is not inhibited by the presence of H1 on chromatin. (A) Chromatin assembly reactions were carried out with increasing amounts of histone H1 for 6 h at 26°C. Aliquots (500 ng) of the assembled chromatin were treated with MNase for 30, 60 or 120 s. The ethidium bromide stained agarose gel of the purified DNA fragments is shown. The position of the trimer is labeled (•). M indicates the position of a 123 bp marker. (B) The rest of the assembled chromatin (1 μ g) was treated with sarkosyl and after pre-incubation with ATP, T-Ag and $\pm$ CHRAC, used in an *in vitro* replication assay with an RNase-treated cytosolic S100 extract (see Materials and methods). Autoradiography of the purified replication products is shown. The positions of replicative intermediates (RI), superhelical DNA (I), relaxed closed circular and nicked molecules (II) are indicated.

containing the non-histone protein HMG-17 (Vestner *et al.*, 1998), minichromosomes with modified histone N-termini have a more extended chromatin structure compared with control chromatin, which facilitates the elongation through chromatin but not initiation. More rigorous changes of the chromatin structure at the origin such as the displacement of the entire nucleosome, the removal of histone H2A/H2B dimers or the translational repositioning of the nucleosome (reviewed in Steger and Workman, 1996) seem to be required for the binding of initiation factors.

The mechanism of 'nucleosome remodelling' and the

Fig. 9. CHRAC stimulates the replication of SV40 minichromosomes. (A) Two hundred and fifty ng salt-treated SV40 minichromosomes were digested with *Bgl*I, pre-incubated with T-Ag and ATP in the absence or presence of increasing amounts of purified CHRAC (lane 3, 250 ng; lane 4, 375 ng; lane 5, 500 ng) and used as template in an *in vitro* replication assay. Autoradiography of the replication products is shown. The positions of replicative intermediates (RI), superhelical DNA (I), relaxed closed circular and nicked molecules (II) are indicated. (B) One tenth of the replication assay was precipitated with TCA to determine the incorporated nucleotides.

precise nature of the change in nucleosome structure induced by ATP-dependent enzymes that facilitates the binding of regulatory factors to nucleosomal DNA are unclear at present. However, the few facts known about the three ISWI-containing remodelling machines in *Drosophila* embryo extracts indicate that there may be more than one strategy. While NURF, ACF and CHRAC all facilitate the interaction of proteins with nucleosomal DNA (Tsukiyama and Wu, 1995; Ito *et al.*, 1997; Varga-Weisz *et al.*, 1997), the differences between the three remodelling complexes are instructive. While NURF will perturb the regular chromatin structure in an undirected, non-specific manner when added in stoichiometric amounts with respect to the nucleosomal substrate (Tsukiyama and Wu, 1995), CHRAC and ACF will not only not cause such a general decay of chromatin regularity but will, in contrast, catalyze the ATP-dependent alignment of irregularly spaced nucleosomes into a regular array (Ito *et al.*, 1997; Varga-Weisz *et al.*, 1997). ACF has been purified following an assay for chromatin assembly, rather than disassembly. Therefore, and in apparent contrast to NURF, ACF and CHRAC are functional as chromatin assembly factors as well as nucleosome remodelling factors. We have speculated that these apparently opposing effects of CHRAC (general increase in accessibility, but improvement of regularity of nucleosomal arrays) might be achieved through a general 'mobilization' of nucleosomes (Varga-Weisz *et al.*, 1997).

Alternatively, CHRAC may influence the equilibrium between canonical nucleosome structures and somehow altered particles of increased accessibility in favour of the latter. The intrinsic dynamic properties of nucleosomes (Polach and Widom, 1995) may be enhanced by a re-

modelling factor such as CHRAC and a DNA-binding protein would benefit from a general increase in dynamic transitions in nucleosome structure. The observed specificity of remodelling at the origin of replication may be due to the independent action of T-Ag which, by binding to the origin during a 'window of opportunity', prevents a reversion of the transiently remodelled structure to the canonical nucleosome. CHRAC may be confined to highly active chromosomal domains *in vivo* which may be characterized by their enhanced accessibility during gene activity and early replication.

Alternatively, a specific synergism between CHRAC and the helicase function of T-Ag may lead to a selective destabilization of nucleosomes at the origin, an interesting possibility that remains to be explored further. CHRAC contains topoisomerase II, an enzyme required for efficient replication in the cell-free system (Halmer and Gruss, 1997). So far we have been unable to document an involvement of the topoisomerase II activity in CHRAC function (Varga-Weisz *et al.*, 1997). Whether topoisomerase II, due to its known preference for particular chromosomal sites (Adachi *et al.*, 1989; Käs and Laemmli, 1992), is able to target CHRAC to particular sequences remains to be explored.

Materials and methods

Chromatin assembly, *Bgl*I-digestion and sarkosyl treatment

SV40 DNA was assembled into chromatin in *Drosophila* embryo extract for 6 h at 26°C, exactly as described (Becker *et al.*, 1994). For the pre-binding experiment 4 µg of T-Ag were incubated with 500 ng SV40 DNA in EX120 (10 mM HEPES-KOH pH 7.6, 120 mM KCl, 10% glycerol, 5 mM MgCl₂, 0.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM DTT) with 3 mM ATP for 30 min at 26°C prior to chromatin assembly. In this case the assembly reaction was performed in the presence of aphidicolin (10 µg/ml). When the assembly was carried out in the presence of histone H1, 500 ng SV40 DNA were assembled into chromatin in the presence of 167 or 334 ng histone H1 (CalBiochem). After assembly, ATP was removed by spinning the assembly reactions (135 µl/column) through 1.8 ml settled Sephacryl S-300HR spin columns which were equilibrated with EX120 and pre-spun at 1100 g for 2 min. The chromatin was then incubated with either 10 U *Bgl*I/µg chromatin or the respective buffer for 1 h at 26°C. Approximately 50% of the molecules were linearized by *Bgl*I digestion. Sarkosyl-treatment (0.075%) was carried out for 10 min at RT and sarkosyl was removed by spin column centrifugation through 1.8 ml settled Sephacryl S-300HR spin columns which were equilibrated with EX120 and pre-spun at 1100 g for 2 min.

Preparation of cytosolic S100 replication extracts and the SV40 T-Ag

Cytosolic S100 extracts were prepared from HeLa S3 cells exactly as described (Stillman and Gluzman, 1985). SV40 T-Ag was purified from insect cells (Sf9), infected with a recombinant baculovirus containing the SV40 T-Ag coding region (Lanford, 1988). Purification was done by immunoaffinity chromatography as described (Simanis and Lane, 1985).

CHRAC purification

CHRAC was purified to apparent homogeneity as described (Varga-Weisz *et al.*, 1997). Protein concentration was determined by a modified Bradford assay (Bio-Rad). When not stated otherwise, 3 mM ATP were included in all reactions where CHRAC is incubated with chromatin.

Replication of reconstituted SV40 chromatin and protein-free DNA

Sixty µl (=200 ng) of reconstituted chromatin or protein-free SV40 DNA were pre-incubated in the absence or presence of ATP (3 mM) and 2 µg purified T-Ag for 45 min at 26°C and then used as templates in the SV40 *in vitro* replication system. *In vitro* replication was carried out with 350 µg cytosolic S100 extract from HeLa cells and another

2 µg T-Ag (where indicated) in a total volume of 190 µl with phosphoenolpyruvate (10 mM) and pyruvatekinase (1.8 µg) as ATP-regenerating system, exactly as described (Gruss *et al.*, 1993). The radioactive nucleotide used was [α - 32 P]dATP (Amersham). For *in vitro* replication of H1-containing templates 400 µg cytosolic S100 extract were incubated with 20 µl immobilized Ribonuclease A (Sigma) for 30 min at 37°C and used exactly as described previously (Halmer and Gruss, 1995). Replication products were deproteinized, phenol-extracted, precipitated and run on a 0.8% agarose gel in TBE buffer (14 h, 3.5 V/cm). One tenth of the replication assay was precipitated with TCA to determine the incorporated nucleotides (Gruss *et al.*, 1993). In all experiments where CHRAC is included, purified chromatin or DNA were pre-incubated for 30 min at 26°C with 1 µl highly purified CHRAC (250 ng/µl) determined by a modified Bradford assay (Bio-Rad) with BSA as a standard and then treated as described above.

Elongation assays

Elongation assays were essentially done as described (Fotedar *et al.*, 1996). Sixty µl (=200 ng) of sarkosyl-treated chromatin or DNA were incubated for 30 min at 26°C with 2.4 µg T-Ag and 3 mM ATP in the absence or presence of CHRAC. Cytosolic S100 extract was dialyzed on Millipore filters (Type VS, 0.025 µm) against LS-buffer (20 mM HEPES-KOH, pH 7.8, 5 mM potassium acetate, 0.5 mM MgCl₂, 0.5 mM DTT) and 650 µg of extract proteins were incubated for 30 min at 26°C under replication conditions with the pre-incubated chromatin. The pulse was for 30 s at 26°C with [α - 32 P]dATP, the remaining ribonucleosides, dGTP and dCTP. The pulse was terminated by adding a 100-fold excess of cold dATP, and dTTP was added to allow elongation during the chase at 26°C. Aliquots were removed after various time points and DNA was extracted as described (Gruss *et al.*, 1993). Purified products were resuspended in 50 mM NaOH, 1 mM EDTA and then analyzed on a 1.2% agarose gel in 30 mM NaOH, 1 mM EDTA. The gel was fixed for 20 min in 10% methanol, 10% TCA, dried on DE81 paper (Whatmann) and autoradiographed.

Micrococcal nuclease digestion and Southern blot analysis

Assembled chromatin was treated as described for the *in vitro* replication assay (Figure 1A, 1–6) and then incubated with 100 U micrococcal nuclease (Pharmacia) for 30, 60 or 120 s. DNA purification and Southern blot analysis was as described (Varga-Weisz *et al.*, 1995). Oligonucleotides used for Southern blot analysis were either complementary to the origin sequences (nucleotides 5217–5242, lower strand of the SV40 genome) or complementary to a sequence outside the origin (nucleotides 1744–1768, upper strand of the SV40 genome).

DNase I footprinting

For DNase I digestions 30 µl (100 ng) of assembled chromatin were incubated with 0.8 U DNase I (Boehringer Mannheim) in a total volume of 65 µl, for 1 min at RT, before adding 30 µl stop mixture (64 mM EDTA, pH 8.0; 1.6% sarkosyl, 0.7% SDS). DNA was purified by treatment with RNaseA and Proteinase K as described (Becker *et al.*, 1994) followed by organic extractions and ethanol precipitation in the presence of ammonium acetate. Purified DNase I digestion products were analyzed by primer extension with [α - 32 P]-labeled primer (CAATTAGTCAGCAACCATAG, lower strand). Primer extension was performed with TAQ polymerase (Perkin-Elmer) for 10 min at 70°C, and precipitated fragments were loaded on an 8% sequencing gel in formamide loading buffer (Sambrook *et al.*, 1989). The gel was fixed, dried and autoradiographed.

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