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c-Jun stimulates origin-dependent DNA unwinding by polyomavirus large T antigen

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The AP1 protein c-Jun has previously been shown to stimulate polyomavirus (Py) DNA replication *in vivo*. In order to define the mechanism, we added purified c-Jun protein to the origin-dependent and large T antigen (LT)-dependent *in vitro* DNA unwinding assay. c-Jun protein was found to stimulate by ~5-fold the unwinding of a 290 bp linear DNA fragment containing both the Py origin and the AP1 recognition sequence to which c-Jun binds. Efficient levels of stimulation were specifically observed at limiting concentrations of LT for unwinding. Under similar conditions, Py DNA replication was stimulated to a comparable extent by AP1 in a purified *in vitro* replication assay. Mobility shift and DNase I footprinting assays showed that c-Jun stimulates the ATP-dependent binding of LT to the origin core by ~7-fold. Furthermore, c-Jun was found to interact directly with LT, but not with replication protein A. The activities of c-Jun to stimulate unwinding and origin binding of LT were found to be harbored within the N-terminal region of c-Jun, which is distinct from the DNA binding domain. We speculate that certain transcription factors may possess specific DNA replication domains that function to stimulate the loading of replication factors at the origin during the initiation of DNA synthesis.

Keywords: c-Jun/c-Jun interaction with large T antigen/ DNA replication/DNA unwinding/polyomavirus large T antigen

Introduction

Most eukaryotic replication origins so far characterized generally have two basic components: the core components which determine where DNA synthesis begins and auxiliary components which facilitate initiation to an extent that depends on the nature of the core component and the cell environment. The core components [origin (*ori*) core] contain recognition sequences for initiator proteins and are absolutely required for initiation. The auxiliary components generally contain binding sites for specific transcription factors. The involvement of transcription factors in DNA replication is attractive, because they are key elements in the transduction of extracellular signals into the complex process of cell proliferation (DePamphilis, 1988, 1993).

Polyomavirus (Py) is a good model system to elucidate the role of transcription factors in replication. Py replicates in the nuclei of rodent cells and its replication depends on host replication proteins, with the exception of the viral protein large T antigen (LT). The transcription enhancer located on the late side of the *ori* core was shown to stimulate Py DNA replication by ~200- to 1000-fold (de Villiers *et al.*, 1984). The enhancer contains two cores, designated A and B or α and β (Muller *et al.*, 1983; Veldman *et al.*, 1985). Within the A core there exist binding sequences for the transcription factors AP1/PEA1 (Piette and Yaniv, 1987), PEA3 (an Ets family factor; Martin *et al.*, 1988; Wasylyk *et al.*, 1990a; Martin *et al.*, 1992; Xin *et al.*, 1992) /PEBP5 (Asano *et al.*, 1990) and PEA2 (Piette and Yaniv, 1987) /PEBP2 (Satake *et al.*, 1988; Yamaguchi *et al.*, 1989; Ogawa *et al.*, 1993), each of which contributes to both the stimulation of Py DNA replication and transcription (Muller *et al.*, 1988; Asano *et al.*, 1990; Murakami *et al.*, 1990; Wasylyk *et al.*, 1990b; Guo and DePamphilis, 1992). AP1 is made up of homo- or heterodimers of c-Fos, c-Jun and related factors (Bohmann *et al.*, 1987; Angel *et al.*, 1988; Curran and Franza, 1988). The c-Jun/c-Jun homodimer or c-Jun/c-Fos heterodimer by itself stimulates Py DNA replication through the AP1 binding sequence (Wasylyk *et al.*, 1990b; Murakami *et al.*, 1991; Guo and DePamphilis, 1992). Other transcription factors, such as GAL4 (Baru *et al.*, 1991; Bennett-Cook and Hassell, 1991), bovine papilloma virus E2 (Nilsson *et al.*, 1991), c-Rel (Ishikawa *et al.*, 1993) and p53 (Kanda *et al.*, 1994) have also been shown to stimulate Py DNA replication through their binding sites, indicating that at least some transcription factors have a dual function.

Transcription factors require an activation domain in addition to a DNA binding domain to stimulate transcription. To stimulate Py DNA replication, transcription factors also appear to require the activation domain. The activation domain for replication usually overlaps with that for transcription. For example, GAL4 requires the presence of the transcriptional activation domain to stimulate Py DNA replication and the domain could be replaced with transcriptional activation domains from other transcription factors, such as VP16 or c-Jun (Baru *et al.*, 1991; Bennett-Cook and Hassell, 1991). In some cases, however, both

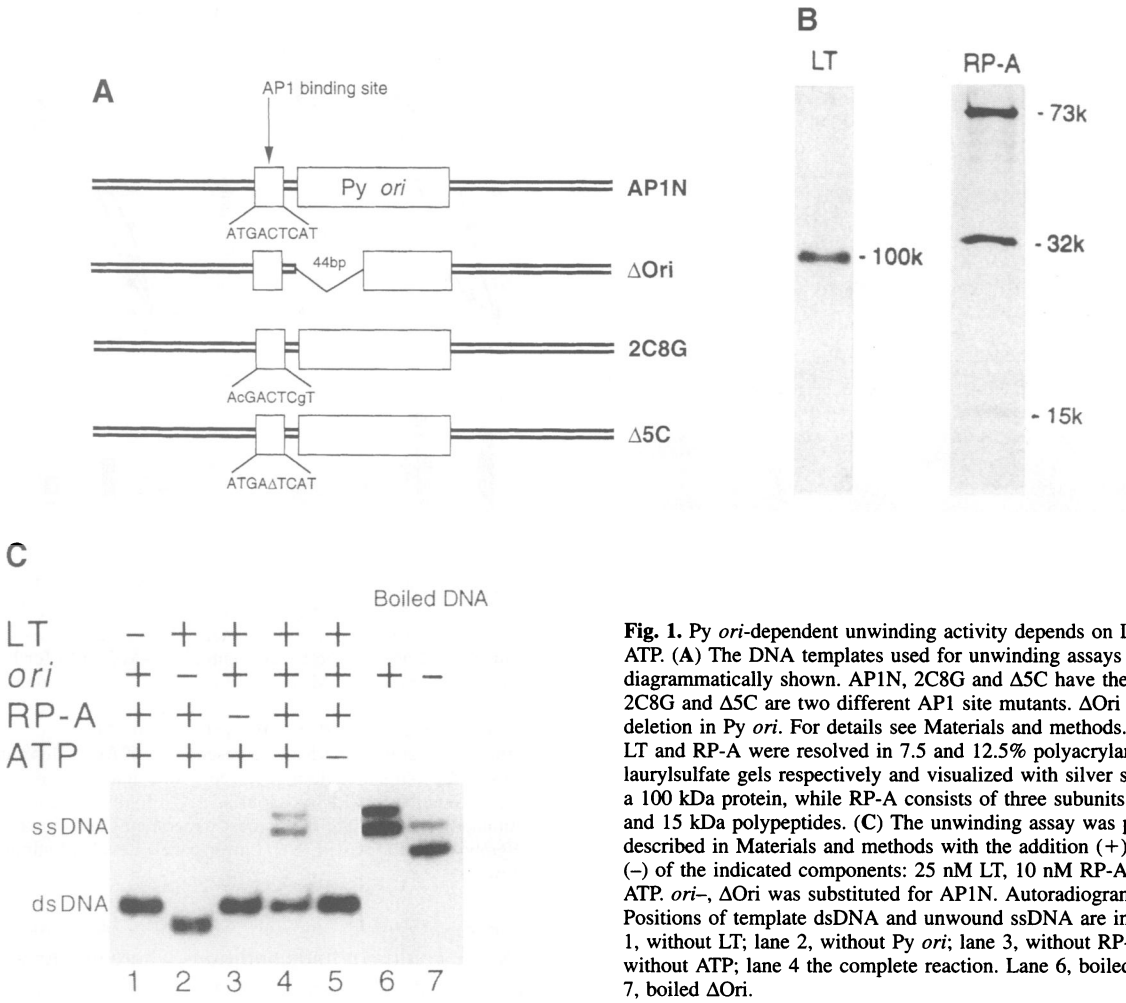

Fig. 1. Py *ori*-dependent unwinding activity depends on LT, RP-A and ATP. (**A**) The DNA templates used for unwinding assays are diagrammatically shown. AP1N, 2C8G and Δ5C have the Py *ori*. 2C8G and Δ5C are two different AP1 site mutants. ΔOri has a deletion in Py *ori*. For details see Materials and methods. (**B**) Purified LT and RP-A were resolved in 7.5 and 12.5% polyacrylamide–sodium laurylsulfate gels respectively and visualized with silver staining. LT is a 100 kDa protein, while RP-A consists of three subunits of 73, 32 and 15 kDa polypeptides. (**C**) The unwinding assay was performed as described in Materials and methods with the addition (+) or omission (–) of the indicated components: 25 nM LT, 10 nM RP-A and 4 mM ATP. *ori*–, ΔOri was substituted for AP1N. Autoradiograms are shown. Positions of template dsDNA and unwound ssDNA are indicated. Lane 1, without LT; lane 2, without Py *ori*; lane 3, without RP-A; lane 5, without ATP; lane 4 the complete reaction. Lane 6, boiled AP1N; lane 7, boiled ΔOri.

activities cannot be delimited to the same domain. Deletion analysis of the c-Jun transcription activation domain in the c-Jun–GAL4 fusion protein showed that the activities stimulating Py DNA replication and transcription did not correlate (Guo and DePamphilis, 1992). Moreover, we have identified domains in p53 (Kanda *et al.*, 1994) and c-Rel (Ishikawa *et al.*, 1993) which activate only replication.

The mechanism of transcription factor stimulation of Py DNA replication is still poorly understood, though it is apparently not dependent on transcription. Protein domains that stimulate replication may act like transcription activation domains to promote the assembly of an active multiprotein complex either through direct contacts with individual proteins or by facilitating interactions among components of the complex.

What might be the nature of the replication protein which acts as target for transcription factors? *In vitro* studies of Py and its closely related virus SV40 revealed the initial steps of DNA replication and the identity of the proteins involved in this reaction (Challberg and Kelly, 1989; Stillman, 1989; Borowiec *et al.*, 1990). First LT binds to the *ori* core and forms double hexamers in an ATP-dependent manner. In the presence of replication protein A [RP-A, a single-stranded DNA (ssDNA) binding protein] and topoisomerases, LT hexamers unwind the DNA bidirectionally. Nascent DNA is synthesized by the combined action of primase and DNA polymerases on the unwound ssDNA covered with RP-A. Recently, RP-A was shown to interact with the transcriptional activation domains of VP16, GAL4, p53 and E2 (Dutta *et al.*, 1993; He *et al.*, 1993; Li and Botchan, 1993). These factors are also known to stimulate Py DNA replication. In the case of p53, the replication-specific activation domains (Kanda *et al.*, 1994) also interact with RP-A (Dutta *et al.*, 1993). This suggests that RP-A is a target of the activation domain that stimulates Py DNA replication, although direct evidence in this area is still lacking. On the other hand, indirect evidence in other studies suggested that a subfamily of transcription factors stimulate replication, possibly by interacting with the LT through their activation domains (Guo and DePamphilis, 1992).

In this report, we have reconstructed the LT-dependent unwinding assay for the Py *ori* core using purified proteins and show that c-Jun and c-Fos can stimulate this process through the AP1 binding site when the amount of LT is limiting. The stimulation of unwinding was reflected in the stimulation of Py DNA replication *in vitro* by AP1 with purified proteins. This stimulatory effect is, at least in part, explained by c-Jun stimulation of LT–*ori* core complex formation.

Results

Py ori-dependent unwinding activity

In order to understand the mechanism of stimulation of Py DNA replication by AP1, we examined the effect of

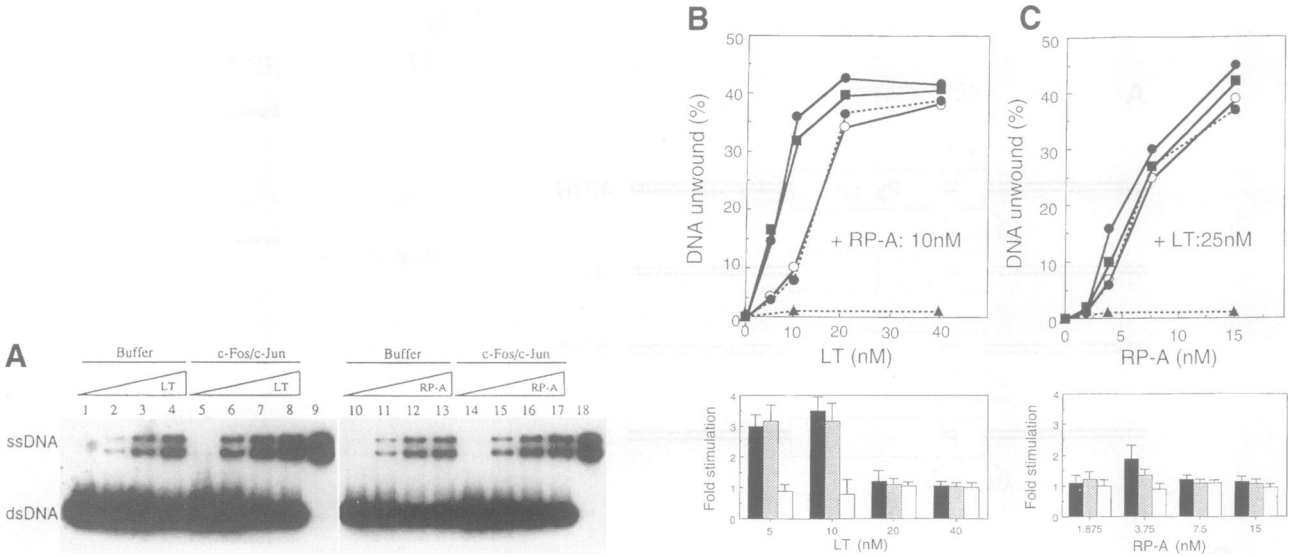

Fig. 2. Stimulation of Py *ori*-dependent DNA unwinding by AP1. (**A**) Unwinding assays were performed in the presence or absence of 0.1 U AP1 proteins using AP1N as a substrate (see text for a definition of AP1 protein activity). In this experiment, 0.1 U was equivalent to 40 nM c-Jun protein or 13 nM each of c-Jun and c-Fos as heterodimer. The RP-A concentration was 10 nM for lanes 1–8. LT concentrations were: lanes 1 and 5, none; lanes 2 and 6, 5 nM; lanes 3 and 7, 10 nM; lanes 4 and 8, 15 nM. Lane 9 shows boiled AP1N. The LT concentration was 25 nM for lanes 10–17. RP-A concentrations were: lanes 10 and 14, none; lanes 11 and 15, 2.5 nM; lanes 12 and 16, 5 nM; lanes 13 and 17, 7.5 nM. Boiled AP1N is shown in lane 18. (**B**) (Upper panel) Results shown in lanes 1–8 in (A) are quantified and plotted. Percentage unwinding was calculated from the ratio of ssDNA band intensity to total band intensity (dsDNA and ssDNA). –○–, no AP1 added, with AP1N; –●–, c-Jun/c-Fos, AP1N; –■–, c-Jun/c-Jun, AP1N; --●--, c-Jun/c-Fos, 2C8G; ---▲---, c-Jun/c-Fos, ΔOri. Values shown in this figure are representative of five independent experiments. (Lower panel) Levels of stimulation at each concentration of LT are shown. ■, fold stimulation observed with c-Jun/c-Fos and AP1N relative to AP1N without AP1; ▨, fold stimulation observed with c-Jun/c-Jun and AP1N relative to AP1N without AP1; □, fold stimulation observed with c-Jun/c-Fos and 2C8G relative to AP1N without AP1. The average stimulations from five independent experiments are shown. (**C**) (Upper panel) Results shown in lanes 10–17 in (A) are quantified and plotted. Symbols used are as in (B). (Lower panel) Levels of stimulation at each concentration of RP-A are shown. Symbols are as shown in the lower panel of (B).

AP1 on the LT-dependent unwinding reaction at the Py *ori* of replication. Purified LT and mouse RP-A proteins (Figure 1B) were able to unwind the double-stranded DNA (dsDNA) fragments containing Py *ori* core as previously reported (Wang and Prives, 1991). We prepared four types of unwinding substrates, AP1N, 2C8G, Δ5C and ΔOri (Figure 1A). We confirmed that the unwinding activity was dependent on LT, *ori*, ATP and RP-A (Figure 1C). Substrates containing point mutations in the AP1 site (2C8G and Δ5C) were also unwound as efficiently as AP1N (data not shown), showing that the AP1 recognition sequence alone does not influence LT unwinding at the Py *ori*.

Stimulation of Py ori-dependent unwinding and replication by AP1

Py *ori*-dependent unwinding activity requires the two replication proteins LT and RP-A and the reaction is dose dependent for these two proteins (Figure 2A, lanes 1–4 and 10–13), as previously reported in the analogous SV40 unwinding reaction (Goetz *et al.*, 1988). Full-length c-Fos and c-Jun were highly purified from *Escherichia coli* as described in Materials and methods (Figure 5B). When c-Jun/c-Fos heterodimers were added to the reaction mixtures, Py *ori*-dependent unwinding activity was noticeably stimulated, especially at low concentrations of LT (Figure 2A and B). The effect appears to be specific for LT, as no marked stimulation by AP1 was observed at any concentration of RP-A under saturating LT concentrations (Figure 2A and C). We also examined stimulation of unwinding by AP1 at lower concentrations of LT and RP-A. We could not find conditions where greater stimulation could be observed. Rather, we observed a gradual decrease in unwinding without seeing any stimulation when we lowered the concentration of RP-A at a given concentration of LT (data not shown). In addition, c-Jun/c-Jun homodimers were also effective. The stimulation of unwinding by AP1 protein was entirely dependent on a functional AP1 site, as the substrates containing point mutations in the AP1 site (2C8G) gave unwinding activities which were almost the same as those obtained when buffer replaced AP1 in the reaction (Figure 2B). Finally, the observed AP1 stimulatory effect requires the Py *ori*, as ΔOri showed no unwinding activity irrespective of RP-A or LT concentrations (Figure 2B and C). These experimental results demonstrate the following: (i) the stimulatory effect of AP1 depends specifically on low concentrations of LT; (ii) c-Jun homodimers are sufficient for the promotion of unwinding activity; (iii) the stimulatory effect is AP1 site dependent, as previously observed in the *in vivo* analysis (Murakami *et al.*, 1991).

In Figure 3A we examine the effect of increasing AP1 protein concentrations on Py DNA unwinding under conditions of limiting LT concentrations. The stimulation was dose dependent and up to a 6-fold stimulation could be obtained using c-Jun/c-Fos heterodimers. When c-Jun/c-Jun homodimers were employed, a 4.5-fold stimulation was detected, suggesting that the heterodimer is slightly more efficient than the homodimer for stimulating Py DNA unwinding (Figure 3B). Again, this activity depended entirely on a functional AP1 site and Py *ori* (Figure 3A and B).

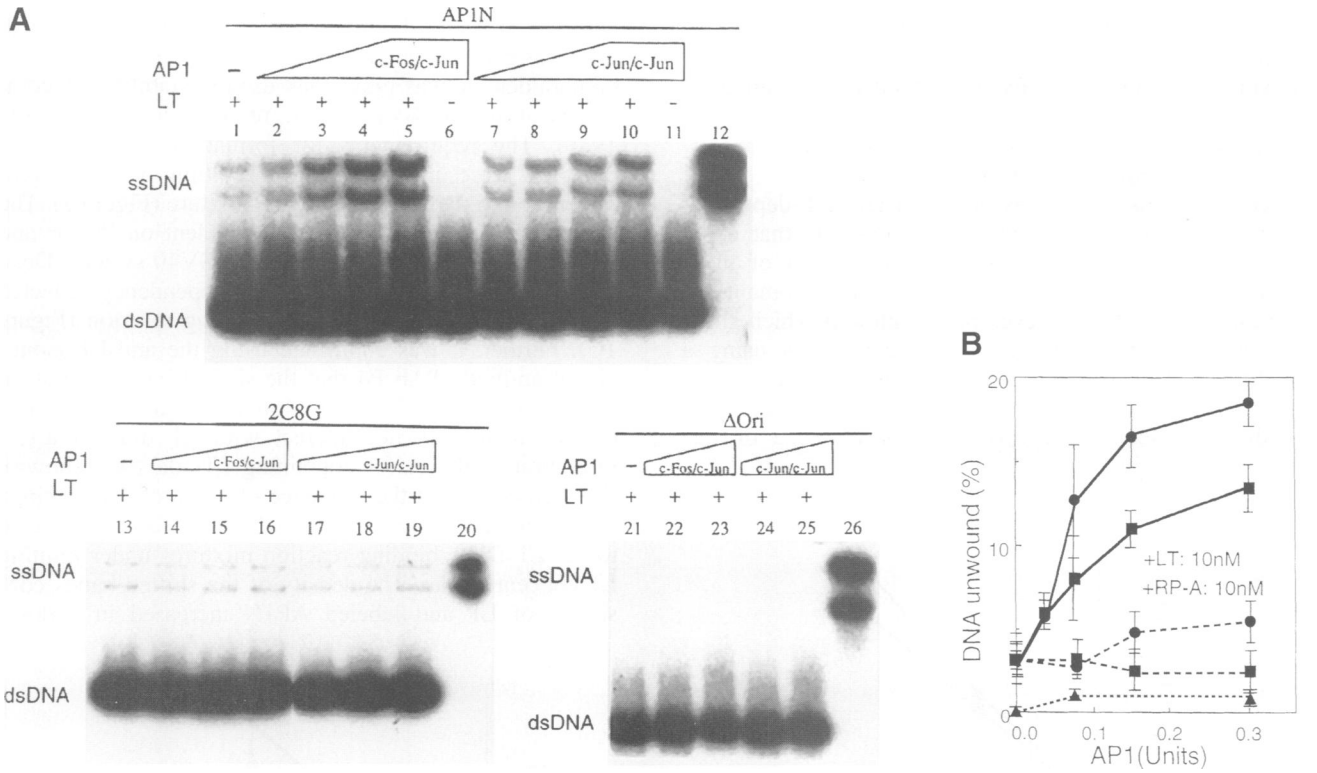

Fig. 3. Stimulation of Py *ori*-dependent unwinding by AP1 requires the AP1 binding site. (**A**) Unwinding assays were performed as described in Materials and methods and autoradiograms were made. Substrates used were AP1N (lanes 1–12), 2C8G (lanes 13–20) and ΔOri (lanes 21–26). The concentration of LT or RP-A was 10 nM each. The number of AP1 units per reaction in the case of c-Fos/c-Jun was: lane 2, 0.05; lanes 3,14 and 22, 0.1; lanes 4 and 15, 0.2; lanes 5, 6, 16 and 23, 0.4. The number of AP1 units per reaction in the case of c-Jun/c-Jun was: lane 7, 0.05; lanes 8, 17 and 24, 0.1; lanes 9 and 18, 0.2; lanes 10, 11, 19 and 25, 0.4. In lanes 1, 13 and 21, buffer was added in place of AP1 proteins. For a definition of AP1 protein units see text. LT was omitted in lanes 6 and 11. Boiled AP1N, 2C8G and ΔOri are shown in lanes 12, 20 and 26 respectively. Results are a representative of five independent experiments. (**B**) Percentage of unwound DNA is plotted against the number of AP1 units per reaction. The average unwinding values from five independent experiments are plotted against the number of AP1 units. –●–, c-Jun/c-Fos, AP1N; –■–, c-Jun/c-Jun, AP1N; ---▲---, c-Jun/c-Fos or c-Jun/c-Jun, ΔOri; --●--, c-Jun/c-Fos, 2C8G; --■--, c-Jun/c-Jun, 2C8G.

As the unwinding step is limiting for Py DNA replication, the increased unwinding by AP1 should be reflected in increased DNA synthesis. To test this possibility, we examined DNA synthesis in a monopolymerase system made up of four proteins or protein complexes; LT, RP-A, mouse DNA polymerase α–primase complex (Polα–pri) and mouse DNA topoisomerase I (Topo I) and various Py plasmid DNAs (Figure 4). The monopolymerase system is thought to mainly represent the initiation step of DNA replication (Lee *et al.*, 1989), although replication products representing the full-size template DNA were detected in denaturing gel electrophoresis in this system (data not shown). Under conditions of low LT concentrations, we found that AP1 could stimulate Py DNA replication in a dose-dependent manner, as was the case for the unwinding reactions. Up to a 4-fold and up to a 3-fold stimulation was observed using c-Jun/c-Fos heterodimers and c-Jun/c-Jun homodimers respectively. As was the case in the unwinding reactions, this activity depended entirely on a functional AP1 site, Py *ori* and LT (Figure 4). Thus, stimulation of Py *ori*-dependent unwinding by AP1 clearly results in enhanced initiation of DNA replication at the Py *ori*.

The region of c-Jun required for the stimulation of Py ori-dependent unwinding

As c-Jun/c-Jun homodimers alone were sufficient to observe stimulation of Py DNA unwinding at limiting concentrations of LT, we decided to determine the specific domain of c-Jun implicated in this activity as a first step to unraveling the reaction mechanism. Highly purified c-Jun and truncated c-Jun polypeptides (Figure 5A) were obtained by the procedures described in Materials and methods (Figure 5B). Electrophoretic mobility shift assays (EMSA) performed with a 25 bp oligonucleotide containing the AP1 site showed that all truncated c-Jun polypeptides had DNA binding activities (data not shown), as previously reported (Abate *et al.*, 1991). To examine the effect of truncated c-Jun derivatives on unwinding, we compared their activities on unwinding in relation to their DNA binding activities. One unit of AP1 was defined as the DNA binding activity which resulted in a displacement of 50% of the radioactivity of 0.25 nM ^{32}P-labeled oligonucleotide. Thus, 1 U c-Jun, ΔJ1, ΔJ2, ΔJ3, ΔJ4, c-Jun/c-Fos and ΔJ4/c-Fos contained 0.4, 0.6, 0.4, 0.3, 0.8, 0.13/0.13 μM and 0.25/0.13 μM protein respectively.

Under conditions of low LT concentrations, we examined the effect of truncated c-Jun polypeptide homodimers on the unwinding reaction (Figure 6). Full-length c-Jun and ΔJ1 (amino acids 90–334) showed nearly the same stimulatory effect on Py *ori*-dependent unwinding. However, ΔJ2 (amino acids 186–334) and ΔJ3 (amino acids 198–334) showed decreased stimulation of DNA unwinding and the shortest mutant, ΔJ4 (amino acids 224–334), even displayed some inhibitory activity. The results

clearly establish that a region outside the DNA binding domain in the N-terminal part of c-Jun is required for maximum stimulation of Py *ori*-dependent unwinding.

Stimulation of LT binding to Py ori by the N-terminal region of c-Jun

Since LT forms complexes at *ori* in an ATP-dependent manner (Lorimer *et al.* 1991), it is possible that c-Jun stimulates unwinding by enhancing formation of these complexes through its N-terminal activation domain. To test this possibility, we developed conditions which allow the detection of LT–Py *ori* DNA complexes using a modified EMSA. By analogy with SV40 LT, LT complexes at the Py *ori* would be in the form of a double hexamer in the presence of ATP. Because of the size of this complex, we could only observe mobility shifts using agarose gels after cross-linking with glutaraldehyde. Glutaraldehyde was previously used to stabilize LT complexes at the SV40 origin of replication (Dean *et al.*, 1987). The requirements for formation of the LT–*ori* complex were investigated by omitting various components from the binding reaction mixture (Figure 7). The formation of LT complex was dependent on Py *ori* and ATP, as previously described for the SV40 system (Dean *et al.*, 1987). The Py *ori* and ATP dependency reflected similar requirements for the unwinding reaction (Figure 1C). Further, it was confirmed using the anti-LT monoclonal antibody PAb F4 that the shifted bands contained LT (Figure 7, lanes 5 and 6). Monoclonal antibody against c-Fos which does not interact with LT did not affect migration of the bands containing LT (data not shown). We then examined the influence of c-Jun on LT complex formation at the *ori*. Purified c-Jun and ΔJ4 were added to the LT DNA binding reaction mixtures under limiting LT concentrations. The density of the shifted bands consisting of LT and labeled AP1N increased in a dose-

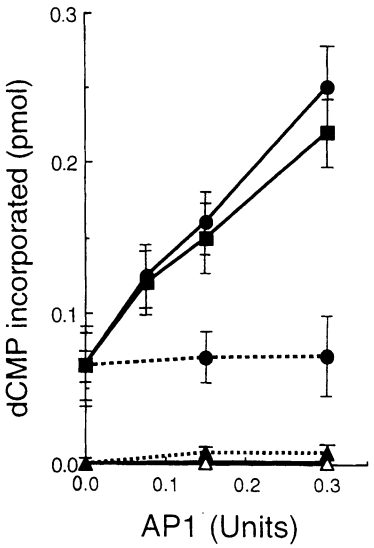

Fig. 4. Stimulation of Py *ori*-dependent *in vitro* replication by AP1. *In vitro* replication assays using purified proteins were performed as described in Materials and methods. The concentrations of LT and RP-A were both 10 nM, i.e. as used in the experiment shown in Figure 3. The average dCMP incorporated in five independent experiments is plotted against the number of AP1 units per reaction. For a definition of AP1 protein units see text. –●–, c-Jun/c-Fos, using the reporter, pPy(AP1)$_1$OICAT; –■–, c-Jun/c-Jun, pPy(AP1)$_1$OICAT; --●--, c-Jun/c-Fos, pPy(2C8G)$_1$OICAT; ----▲----, c-Jun/c-Fos or c-Jun/c-Jun, pPyΔAT(AP1)$_1$OICAT; –△–, c-Jun/c-Fos, pPy(AP1)$_1$OICAT, with no LT.

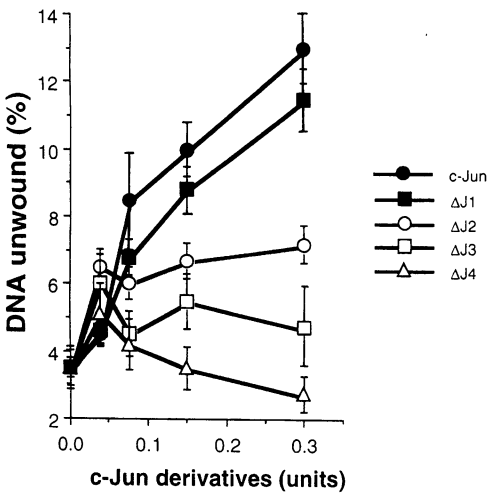

Fig. 6. Effects of truncated c-Jun on LT-dependent unwinding activity. Unwinding assays were performed with AP1N. The average unwinding values from five independent experiments are plotted against the number of AP1 units of c-Jun and truncated c-Jun. For a definition of AP1 protein units see text.

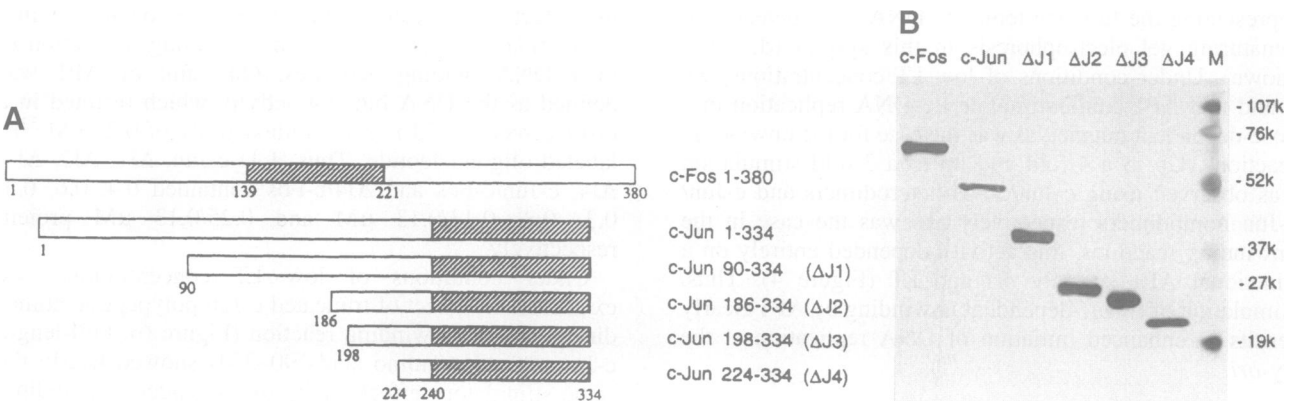

Fig. 5. Purified c-Jun and c-Fos. (**A**) The structure of truncated c-Jun. Shaded portions in the diagrams of c-Fos and c-Jun indicate the dimerization and DNA binding domains (b-Zip regions) (Abate *et al.*, 1991). Inclusive amino acid numbers of the deletion mutants of c-Jun, ΔJ1, ΔJ2, ΔJ3 and ΔJ4 are indicated. (**B**) The purified c-Fos, c-Jun and truncated c-Jun were resolved in 12.5% polyacrylamide–sodium laurylsulfate gels and visualized by silver staining. M, molecular weight standards (BioRad).

dependent fashion in response to increasing concentrations of full-length c-Jun (Figure 8A, lanes 1–4). At maximum a 7-fold stimulation of LT–DNA complex formation was observed (Figure 8B). The mobilities of the shifted bands also decreased in response to increasing amounts of c-Jun. We observed a similar decrease in mobilities when LT was titrated to higher levels (data not shown). Therefore, we suspect that this reflects the increase in the amount of LT present in the complexes. In the case of SV40, LT–*ori* complexes containing various multimers of LT have been detected (Mastrangelo *et al.*, 1989). However, in an experiment using the truncated derivative ΔJ4, which lacks the N-terminal region, very little stimulation of LT–DNA complexation was observed (Figure 8A, lanes 5–7, and B). Also, this stimulatory activity of c-Jun was almost entirely dependent on the presence of a functional AP1 site, as no stimulation was observed when Δ5C replaced AP1N (Figure 8A, lanes 8–11 and B). In this assay system, only LT–DNA complexes could be detected (Figure 8A, lanes 15 and 16). c-Jun–DNA complexes were not observed, probably because they are much smaller than LT–DNA complexes and are not resolved from uncomplexed DNA at the bottom of agarose gels.

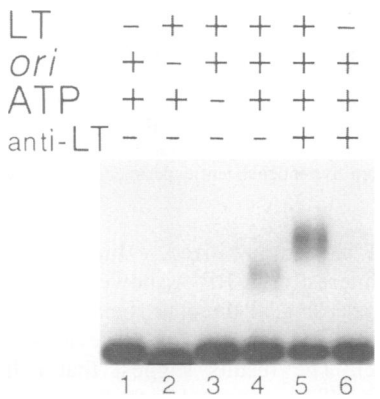

Fig. 7. Requirements for LT–*ori* complex formation as judged by EMSA. EMSA was performed as described in Materials and methods using AP1N as probe. (+) and (−) indicate the addition or omission of the indicated components: 15 nM LT, 4 mM ATP or 50 ng/μl anti-LT IgG1 monoclonal antibody. *ori*–, ΔOri was substituted for AP1N. Lanes 1 and 6, without LT; lane 2, without *ori*; lane 3, without ATP; lane 5, complete EMSA reactions with anti-LT antibody; lane 4, complete EMSA reactions without anti-LT antibody.

In order to examine whether the LT–DNA complexes observed in Figures 7 and 8 actually reflect a specific binding of LT to *ori* core, we performed DNase I footprinting analysis (Figure 9). End-labeled fragments containing the Py *ori* and the AP1 site (AP1N) or the Py *ori* and the AP1 site with a one base deletion (Δ5C) were used as substrates. A low concentration of LT weakly protected from DNase I digestion the regions where the consensus pentanucleotides for LT binding [5′-(G/T)(A/G)GGC-3′] are clustered (Figure 9, lane 2). Upon addition of full-length c-Jun, protection at the AP1 site was clearly observed. At the same time, the pentanucleotide regions were more strongly and more extensively protected as the amount of c-Jun was increased (Figure 9, lanes 2–4). In agreement with the results of EMSA, the stimulatory effect of c-Jun was entirely dependent on the N-terminal domain (compare lanes 8 and 9 with lanes 3 and 4 in Figure 9) and the presence of the AP1 site on the substrate DNA (compare lanes 10–12 with lanes 2–4 in Figure 9). The protection by LT at the *ori* was ATP-dependent (compare lane 2 with lane 7 in Figure 9), as reported before (Lorimer *et al.*, 1991). The protein c-Jun alone did not affect LT footprinting at the *ori* in the absence of ATP (compare lane 7 with lane 6 in Figure 9), indicating that the stimulatory effect of c-Jun was due to enhancement of the ATP-dependent binding of LT to the *ori*. As observed previously (Lorimer *et al.*, 1991), LT induced DNase I hypersensitive sites around the AP1 site in the absence of c-Jun (Figure 9, lanes 2 and 10–12).

Specific interaction between c-Jun and LT

Evidence that c-Jun physically interacts with LT was obtained using surface plasmon resonance measurement in a BIAcore instrument (Pharmacia Biosensor AB).

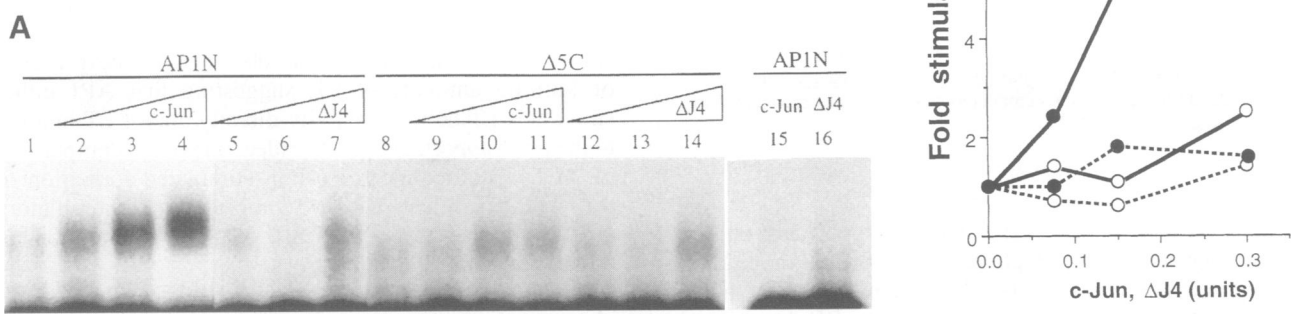

Fig. 8. Effects of c-Jun and ΔJ4 on LT–*ori* complex formation. (**A**) EMSA was performed with AP1N or Δ5C DNA probes. The LT concentration was 10 nM. The amounts of c-Jun or ΔJ4 used were as follows: lanes 2, 5, 9 and 12, 0.075 U; lanes 3, 6, 10 and 13, 0.15 U; lanes 4, 7, 11 and 14, 0.3 U. In lanes 15 and 16, 0.3 U each of c-Jun and ΔJ4 were added without LT. See text for a definition of AP1 protein units. (**B**) The results shown in (A) are plotted against the number of units of c-Jun or ΔJ4. Fold stimulation is the degree of stimulation observed in lanes 2–7 and 9–14 relative to lanes 1 and 8. –●–, c-Jun, AP1N; –○–, ΔJ4, AP1N; --●--, c-Jun, Δ5C; --○--, ΔJ4, Δ5C.

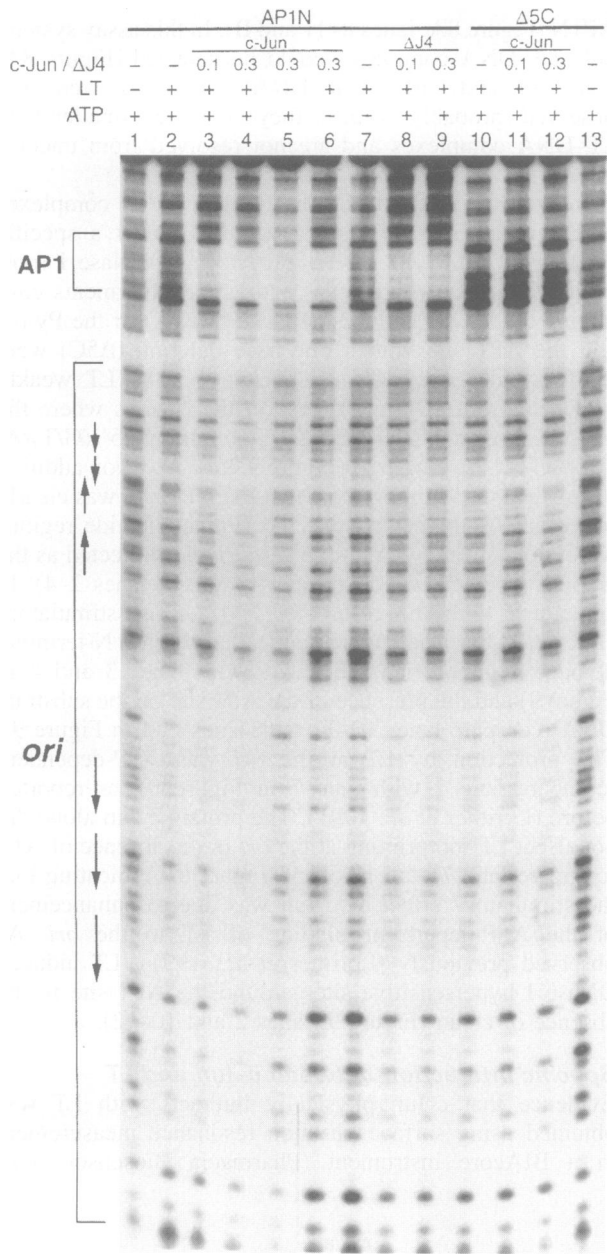

Fig. 9. DNase I footprinting analysis of the effects of c-Jun and ΔJ4 on LT–*ori* complex formation. DNase I footprinting was performed as described in Materials and methods using AP1N or Δ5C as DNA substrates. (+) and (−) indicate the addition or omission of the indicated components: 10 nM LT or 4 mM ATP. The amounts of c-Jun or ΔJ4 used were as follows: lanes 1, 2, 7, 10 and 13, none; lanes 3 and 11, 0.1 U c-Jun; lanes 4, 5, 6 and 12, 0.3 U c-Jun; lane 8, 0.1 U ΔJ4; lane 9, 0.3 U ΔJ4. See text for a definition of AP1 protein units. The position of the Py *ori* and the AP1 site are indicated. The arrows in *ori* indicate the consensus pentanucleotide sequences recognized by LT (Cowie and Kamen, 1984).

Highly purified c-Jun or LT were immobilized on the sensor surface, over which sample proteins in binding buffer were passed as described in Materials and methods. As shown in Figure 10A, LT, but not RP-A, showed significant binding to immobilized c-Jun under conditions that did not allow the binding of bovine serum albumin (BSA) or anti-LT IgG_1. The result was consistent with the observations shown in Figure 2. In the converse experiment

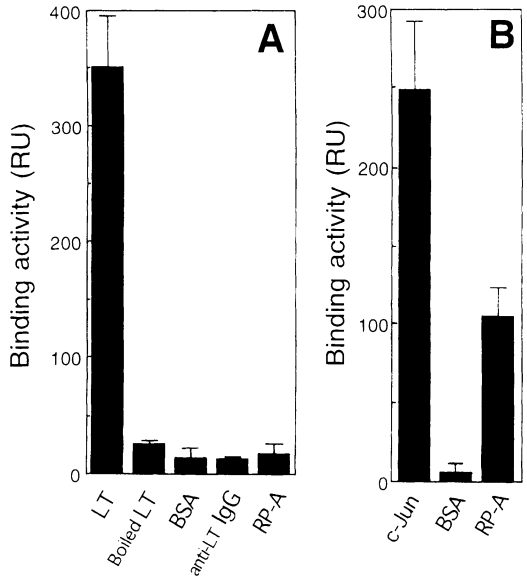

Fig. 10. Molecular interactions between LT, c-Jun and RP-A. Protein binding assays were performed as described in Materials and methods. Binding activities are defined as resonance units (RU). Concentrations of sample proteins are all 1.0 μM. (**A**) Binding activities to immobilized c-Jun. (**B**) Binding activities to immobilized LT. The average RU from five independent experiments are shown.

in which LT was immobilized, c-Jun was also found to bind to LT. Interestingly, RP-A showed significant binding to LT (Figure 10B). A direct association between SV40 LT and human RP-A was shown previously (Dornreiter *et al.*, 1992). The results suggest that c-Jun interacts directly with LT to recruit LT to the *ori* and that this novel activity of c-Jun is the basis for the stimulation of Py *ori*-dependent unwinding and replication.

Discussion

The binding of an initiator protein to its origin and the subsequent unwinding by DNA helicase are key steps in the initiation of DNA replication. In Py DNA replication, LT has both Py *ori* binding and helicase activities. Here we have demonstrated that the transcription factor AP1 stimulates LT-dependent unwinding of Py *ori*. AP1 also stimulated *in vitro* Py DNA replication with purified proteins under similar conditions. The extents of stimulation of unwinding and replication were comparable. The results suggest that stimulation of *in vitro* replication is a direct consequence of stimulation of unwinding. Greater stimulation of unwinding was observed in the presence of limiting amounts of LT, suggesting that AP1 either stimulates LT helicase activity directly and/or LT binding to the *ori*. Supporting the latter idea, one of the components of AP1, a homodimer of c-Jun, stimulated formation of the ATP-dependent LT–*ori* complex. This stimulatory effect could explain, at least in part, stimulation of unwinding and replication by AP1.

How does c-Jun stimulate binding of LT to the *ori*? We observed that c-Jun directly interacts with LT. Therefore, we assume that this direct interaction is the basis for stimulation of the formation of LT–*ori* complex by c-Jun. The precise mechanism by which c-Jun stimulates formation of the LT–*ori* complex is unclear. c-Jun could recruit

LT monomers to the *ori* to assemble LT into large comlexes that might finally result in the formation of double hexamers. This mechanism would be consistant with the observations of Mastrangelo *et al.* (1989) that various size classes of oligomers of SV40 LT are assembled at the origin, suggesting gradual formation of LT–*ori* complexes until double hexamers are eventually attained. Alternatively, c-Jun may stabilize preformed LT–*ori* complexes and thereby increase the chances of origin unwinding. This mechanism is analogous to that of the E2 protein in bovine papillomavirus (BPV) DNA replication. The E1 protein of BPV is an initiator possessing both origin binding and DNA helicase activities (Seo *et al.*, 1993a; Yang *et al.*, 1993). It is, therefore, functionally equivalent to LT. BPV DNA replication also requires the E2 protein, which is another virally encoded protein that can function as a transcription factor (Ustav *et al.*, 1991). E2 directly interacts with E1 (Mohr *et al.*, 1990; Lusky and Fontane, 1991). This interaction results in enhanced binding of E1 to the origin, with consequent stimulation of DNA unwinding and of DNA replication (Yang *et al.*, 1991; Seo *et al.*, 1993b; Sedman and Stenlund, 1995). These stimulatory effects were detected at limiting amounts of E1 (Yang *et al.*, 1991; Seo *et al.*, 1993b) and are, therefore, very similar to our observations.

Another possibility is that c-Jun induces structural changes in the DNA which would facilitate binding of LT to the neighboring *ori*. It has been shown that c-Jun induces DNA bending and that amino acids between 90 and 186 influence the extent of DNA bending by c-Jun (Kerppola and Curran, 1991a,b; Kerppola, 1996). This region is identical to the region that we identified to be important for maximum stimulation of DNA unwinding. It is possible that both protein–protein interactions and DNA bending are involved in c-Jun stimulation of LT–*ori* complex formation. Further study is needed to clarify this possibility.

Some transcription factors interact with RP-A through their activation domains (Dutta *et al.*, 1993; He *et al.*, 1993; Li and Botchan, 1993). It was suggested that this interaction was a common feature of transcription factors that activate Py DNA replication (He *et al.*, 1993). However, our observations show that the primary target of c-Jun is LT: c-Jun physically interacts with LT but not with RP-A. We cannot exclude the possibility, however, that an interaction between AP1 and RP-A plays some role in stimulation of unwinding. Indeed, we have some evidence to show that c-Fos interacts with RP-A under conditions where c-Jun is unable to do so (P.Hughes, unpublished observations).

Previous experiments using *in vitro* Py DNA replication with crude extract did not show any requirement for the transcriptional enhancer. A template DNA containing just the *ori* replicated as efficiently as that containing the enhancer (Prives *et al.*, 1987). This result could be explained by the low abundance of transcription factors such as AP1 in the crude extract used for the *in vitro* system (Y.Murakami, unpublished data).

Cheng and Kelly showed that the transcription factors NF1 and GAL4–VP16 stimulate SV40 DNA replication by preventing repression of replication by nucleosomes reassembled on the DNA template (Cheng and Kelly, 1989; Cheng *et al.*, 1992). Although the pre-binding of LT alone has been shown to be sufficient to prevent inhibition of initiation by nucleosome assembly (Ishimi, 1992), it is possible that the same mechanism is working for stimulation of Py DNA replication by transcription factors *in vivo*. Indeed, Li and Botchan showed that in BPV DNA replication, E2 or GAL4–VP16 could alleviate nucleosome repression (Li and Botchan, 1994). We suggest that one of the reasons why the extent of stimulation observed *in vitro* is lower than that *in vivo* (5-fold *in vitro* compared with 100-fold *in vivo*) is that the prevention of replication by chromatin lowers the basal level of replication *in vivo*. In the case of activation of transcription, it has also been suggested that prevention of chromatin inhibition could explain the low level of stimulation observed *in vitro* (also 5- to 10-fold versus >1000-fold *in vivo*) (Kornberg and Lorch, 1995).

Stimulation of formation of LT–*ori* complex was observed in the presence of a limiting amount of LT. This may reflect a role of transcription factors at early stages of viral DNA replication in viral infection, when the amount of LT available for replication may be limiting.

Since c-Jun is a cellular transcription factor, an interesting possibility is that c-Jun is involved in cellular DNA replication. Involvement of transcription factors in cellular DNA replication was clearly shown in the case of *Saccharomyces cerevisiae*. The B3 element of the ARS1 replication origin is a site for binding of the transcription factor Abf1 and is known to strongly stimulate initiation of DNA replication from this origin (Marahrens and Stillman, 1992). The site could be replaced by the GAL4 binding site. Interestingly, the activation domain of GAL4 is required for stimulation of ARS activity (Marahrens and Stillman, 1992). It is noteworthy that GAL4 also stimulates Py DNA replication and requires the activation domain for this stimulation (Baru *et al.*, 1991; Bennett-Cook and Hassell, 1991). Recently an origin recognition complex (ORC) was identified and suggested to function as a putative origin initiator protein in budding yeast (Bell and Stillman, 1992). However, Abf1 was shown not to effect the binding of ORC to the origin (Rao and Stillman, 1995). Also, ORC binds to the origin throughout the cell cycle (Diffley and Cocker, 1992). Other factors, including Cdc6 and Dbf4, were shown to interact with ORC, probably before initiation, in a step leading up to the formation of the pre-replicative complex (Dowell *et al.*, 1994; Liang *et al.*, 1995). It is possible that some of these factors or other unknown replication components could be a target for Abf1 activation of origin activity.

There still exists controversy over the precise nature of mammalian replication origins. However, in cases where putative origins have been identified, there nearly always exist binding sites for transcription factors. For example, the DHFR origin contains an AP1 binding site close to the putative initiation site (Caddle *et al.*, 1990). Furthermore, the long control region (LCR) of the β-globin gene, which regulates transcription of a cluster of the β-globin genes, was also shown to regulate the activity of a nearby origin (Aladjem *et al.*, 1995). We suspect that these binding sites and their associated binding factors could play a role in the regulation of DNA replication in a manner similar to that of Py DNA replication. We hope that further analysis will reveal the regulatory mechanisms of transcription factor activation of DNA replication.

Materials and methods

Proteins used in in vitro assays

LT was overexpressed in Sf9 cells using a baculovirus vector system and affinity purified using monoclonal antibody PAb F4 (Pallas *et al.*, 1986), as described previously (Eki *et al.*, 1991).

RP-A was purified from 4.0×10^{10} mouse FM3A cells. Cytosolic cell extracts were prepared from FM3A cells by lysing in hypotonic buffer [20 mM HEPES–KOH, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 0.25 mM phenylmethanesulfonyl fluoride (PMSF)]. The extracts were then centrifuged at 40 000 r.p.m. (Beckman 60Ti rotor) for 70 min at 2°C. The supernatant was dialyzed against H buffer (30 mM HEPES–KOH pH 7.5, 0.1 mM EDTA, 0.2 M NaCl, 10% glycerol, 0.01% Triton X, 1 mM DTT and 0.25 mM PMSF). The sample (15 mg/ml protein, 100 ml) was loaded onto a 100 ml phosphocellulose column equilibrated with H buffer. Flow-through fractions (7 mg/ml protein, 100 ml) were collected to which an equal volume of NaCl-free H buffer was added to adjust the NaCl concentration to 0.1 M. This was then applied to an 80 ml hydroxyapatite column equilibrated with H buffer containing 0.1 M KCl. The column was washed extensively with H buffer containing 0.1 M KCl. The protein was eluted with H buffer containing 0.1 M KCl and 80 mM potassium phosphate. The pooled fraction was dialyzed against H buffer containing 0.4 M NaCl. The sample (5 mg/ml protein, 50 ml) was loaded onto an 8 ml ssDNA–cellulose column (Sigma) equilibrated with H buffer containing 0.4 M NaCl. The column was washed extensively with H buffer containing 0.8 M NaCl first and then with 1.5 M NaCl. RP-A was eluted with H buffer containing 1.5 M NaCl and 50% ethylene glycol. RP-A was further purified by chromatography on Affi-Gel Blue columns. The eluted solution (0.2 mg/ml protein, 4 ml) from the ssDNA–cellulose column was dialyzed against T buffer (25 mM Tris–HCl pH 7.5, 0.2 mM EDTA, 0.1 M NaCl, 10% glycerol, 1 mM DTT) and applied to a 1 ml Affi-Gel Blue Gel column (BioRad) (Wold and Kelly, 1988) equilibrated with T buffer. The column was washed sequentially with T buffer containing 1 M NaCl and T buffer containing 0.5 M NaSCN. Highly purified RP-A (0.2 mg/ml, 2 ml) was eluted with T buffer containing 1.5 M NaSCN and dialyzed against NaSCN-free T buffer containing 0.1 M NaCl and 50% glycerol. Final yield of RP-A was 0.4 mg.

Polα–pri and Topo I were purified from whole cell extracts of a mouse FM3A cell line as described previously (Takada-Takayama *et al.*, 1990; Ishimi *et al.*, 1988).

c-Jun, truncated c-Jun and c-Fos were expressed in *E.coli* as hexa-histidine fusion proteins and purified by nickel affinity chromatography as described previously (Abate *et al.*, 1991). Proteins were renatured and further purified by 10–30% glycerol gradients.

DNA substrates for unwinding assay and replication assay

DNA fragments used in the unwinding assay were linear fragments designated AP1N (290 bp), 2C8G (290 bp), Δ5C (289 bp) and ΔOri (246 bp). All were cut with the restriction endonucleases *Nar*I and *Hin*dIII from pPy(AP1)$_1$OICAT, pPy(2C8G)$_1$OICAT, pPy(Δ5C)$_1$OICAT and pPyΔAT(AP1)$_1$OICAT respectively. The fragments were end-labeled with the Klenow fragment of DNA polymerase I using [α-^{32}P]dCTP. pPy(AP1)$_1$OICAT, pPy(2C8G)$_1$OICAT and pPy(Δ5C)$_1$OICAT contain respectively the 15 bp synthetic oligonucleotides representing the AP1 binding site (GATCCATGACTCATA), the AP1 site with two base changes at positions 2 and 8 (GATCCAcGACTCgTA) and the AP1 site with one base deletion at position 5 (GATCCATGAΔTCATA) (Nakabeppu *et al.*, 1988) at the *Bgl*II site of pPyOICAT (Murakami *et al.*, 1990), which is located on the late side of the *ori* (Figure 1A). pPyΔAT(AP1)$_1$OICAT was constructed by deleting a 44 bp region containing the A+T-rich region of the Py *ori* core sequence between the *Bgl*II and *Apa*I sites of pPy(AP1)$_1$OICAT.

pPy(AP1)$_1$OICAT, pPy(2C8G)$_1$OICAT and pPyΔAT(AP1)$_1$OICAT were highly purified by cesium chloride centrifugation. Superhelical circular duplex plasmid DNAs were used as templates in the replication assay.

Py ori-dependent unwinding assay

The unwinding reaction mixture (10 μl) contained 40 mM creatine phosphate (di-Tris salt, pH 7.8), 7 mM $MgCl_2$, 10 mM DTT, 0.3 μg/μl BSA, 4 mM ATP, 0.1 μg/μl creatine kinase, 0.25 nM ^{32}P-labeled dsDNA, indicated amounts of LT, RP-A and AP1 as described in the figure legends and 10 ng competitor DNA (*Hin*cII-digested λ DNA). Competitor DNA was required for *ori*-dependent unwinding. Reaction mixtures were incubated at 33°C for 60 min. The reaction was terminated by the addition of 3 μl 5% SDS, 100 mM EDTA, 0.1 mg/ml proteinase K, 0.5% bromophenol blue, 40% glycerol, followed by incubation at 37°C for 15 min. Reaction products were subjected to electrophoresis in 8% polyacrylamide gels in 89 mM Tris–borate and 2 mM EDTA (TBE) and autoradiograms were made. Relative amounts of ssDNA and dsDNA were quantified with a BAS2000 image analyzer (Fuji Photo Film Co. Ltd).

In vitro Py DNA replication assay

The reaction mixture (20 μl) contained 40 mM creatine phosphate (di-Tris salt, pH 7.8), 7 mM $MgCl_2$, 10 mM DTT, 0.3 μg/μl BSA, 4 mM ATP, 0.1 μg/μl creatine kinase, 200 μM each of CTP, UTP and GTP, 100 μM each of dATP, dGTP and dTTP, 20 μM [α-^{32}P]dCTP (10–20 c.p.m./fmol), 0.25 nM superhelical circular duplex plasmid DNAs, 1 ng/μl Polα–pri, 100 U Topo I and indicated amounts of LT, RP-A and AP1 as described in the figure legends. Reaction mixtures were incubated at 33°C for 60 min and acid-insoluble radioactivities were measured with a liquid scintillation counter (Aloka).

Electrophoretic mobility shift assay (EMSA) for detecting LT–DNA complex

The EMSA for detecting LT–Py *ori* complexes was performed using the same DNA fragments and reaction mixture as used for the unwinding reactions described above. Reaction mixtures were incubated at 33°C for 20 min. The protein–DNA complexes were cross-linked by the addition of glutaraldehyde to a final concentration of 0.1%, incubated for 5 min at room temperature and then analyzed by electrophoresis through a 1.9% agarose–7% glycerol horizontal gel in TBE for 2 h at a constant 150 V. Gels were dried on DE81 paper (Whatman) and autoradiograms were made. The relative amounts of the shifted bands were quantified with a BAS2000 image analyzer.

DNase I footprinting

Substrates for DNase I footprinting were generated by the polymerase chain reaction (PCR) in which pPy(AP1)$_1$OICAT or pPy(Δ5C)$_1$OICAT were used as template DNAs and an end-labeled oligonucleotide (5′-GGAAGGCGCCACAAGTTGCCTCTGGAAGCC-3′) and a non-labeled oligonucleotide (5′-GGGAATTCCGCCGGTACTGCCGGGCC-3′) were used as primers. The 5′-end of the former was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. As a result, AP1N (177 bp) and Δ5C (176 bp) fragments were obtained which contained respectively the Py *ori* and the AP1 site (AP1N) and Py *ori* with an AP1 site containing one base deletion (Δ5C). Binding of proteins to DNA was performed under the same condition as described previously for EMSA. The DNase I reaction was allowed to proceed for 1 min at 33°C and was then terminated with 10 μl DNase I stop solution (100 mM EDTA, 2 M ammonium acetate, 1% SDS, 2 mg/ml sheared salmon sperm DNA). The samples were extracted with phenol/chlorofrom (1:1 v/v) and precipitated by adding 2 vol. ethanol. The pellets were washed twice with 70% ethanol, dried, dissolved in 90% deionized formamide in TBE, boiled and electrophoresed in a 6.5% polyacrylamide–7 M urea sequencing gel in TBE. The gel was then dried and autoradiographed.

Protein binding assay

Surface plasmon resonance measurements were performed in a BIAcore instrument from Pharmacia Biosensor AB. Immobilization of purified LT and c-Jun to the sensor chip CM5 was performed by the ligand thiol method as described by the manufacturer (*BIAcore Methods Manual*, Pharmacia Biosensor AB). A continuous flow of HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Surfactant P20) passing over the sensor surface was maintained at 5 μl/min. The carboxylated dextran matrix was activated by injection of 35 μl of a 1:1 (v/v) mixture of *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) (Pharmacia). Aliquots of 40 μl 10 μg/ml LT or 15 μg/ml c-Jun in 20 mM sodium acetate, pH 5.0, were injected, followed by 35 μl 1.0 M ethanolamine–HCl, pH 8.5, to block unreacted NHS. The chip was then washed with 20 μl HBS buffer containing 2.0 M NaCl and 0.1% NP-40 to remove non-covalently bound proteins. Binding of sample proteins to the immobilized protein was performed at 25°C in HBS buffer. Aliquots of 1.0 μM of each sample protein in binding buffer (50 mM Tris–HCl, pH 8.2, 30 mM KCl, 7 mM $MgCl_2$, 10 mM DTT, 4 mM ATP) was passed over the immobilized protein for 90 s at a flow rate of 20 μl/min. Binding activities were expressed as resonance units (RU) (see *BIAcore Methods Manual*). Upon completion of application of the sample protein, dissociation of the bound protein begins. In order to obtain a specific value for molecular binding, the RU before the application of the sample

protein (baseline value) was subtracted from the RU at 10 s after initiation of dissociation. Identical wash cycles for 2 min with HBS buffer containing 2.0 M NaCl and 0.1% NP-40 were used to regenerate the chip between assays. Samples of 1000–2000 RU LT or 7000–9000 RU c-Jun was covalently linked to the sensor chip CM5.

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