The Histone-interacting Domain of Nuclear Factor I Activates Simian Virus 40 DNA Replication in Vivo*

(Received for publication, July 13, 1999, and in revised form, October 26, 1999)

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Efficient initiation of SV40 DNA replication requires transcription factors that bind auxiliary sequences flanking the minimally required origin. To evaluate the possibility that transcription factors may activate SV40 replication by acting on the chromatin structure of the origin, we used an in vivo replication system in which we targeted GAL4 fusion proteins to the minimally required origin. We found that the proline-rich transcriptional activation domain of nuclear factor I (NF-I), which has been previously shown to interact with histone H3, specifically activates replication. Evaluation of a series of deletion and point mutants of NF-I indicates that the H3-binding domain and the replication activity coincide perfectly. Assays with other replication factors, such as Sp1, confirmed the correlation between the interaction with H3 and the activation of replication. These findings imply that transcription factors such as NF-I can activate SV40 replication via direct interaction with chromatin components, thereby contributing to the relief of nucleosomal repression at the SV40 origin.

Transcription factor binding sites are often found in sequences adjacent to viral origins of replication. They stimulate replication by modulating the efficiency of replication, but not the basic mechanism of replication. Different mechanisms for the stimulation of replication by transcription factors have been proposed. For instance, on the adenovirus origin of replication, cellular transcription factors such as NF-I, 1 a member of the CAAT box-binding transcription factor CTF/NF-I family of DNA-binding proteins, activate replication by recruiting the viral DNA polymerase to the origin (1–3). In other cases, replication depends on complex arrays of transcription factor binding sites, such as in polyoma and SV40. SV40 DNA replication initiates at a well-defined origin (SV40 ori) that is flanked by two auxiliary sequences. The binding motifs for the Sp1 transcription factor contained in the auxiliary sequences are required for efficient SV40 replication (4). SV40 is a relatively simple and widely used model to study the activation of cellular DNA replication because its initiation requires only one viral protein, the large T-antigen; all the other proteins needed for initiation and elongation are provided by the host cell (5). Furthermore, SV40 circular DNA is covered with nucleosomes, analogous to cellular chromatin (6).

The mechanisms allowing SV40 replication activation by transcription factors remain poorly understood. In vitro studies have suggested that transcription factors might recruit replication factors such as Replication Factor A to the origin (7, 8). Alternatively, it has been suggested that transcription factors prevent the chromatin structure from interfering with the binding of initiation factors to the replication origin, analogous to their probable role on promoters. For instance, polyomavirus and SV40 auxiliary sequences are only required in in vitro replication assays in the presence of repressive chromatin structures (9). In infected cells, the SV40 ori is free of nucleosomes in a fraction of the minichromosomes, which is thought to represent the actively replicating fraction. It was suggested that Sp1, which is necessary for efficient initiation of SV40 replication, is involved in these changes in chromatin structure (10). Interestingly, both Sp1 and NF-I have been reported to bind histone H3, suggesting an involvement of these transcription factors in chromatin remodeling (11). NF-I has been implicated in the regulation of several steroid-sensitive promoters subjected to chromatin remodeling, and was shown to mediate reconfiguration of reconstituted chromatin in vitro (11–13). In addition, NF-I binding sites adjacent to the SV40 ori core have been shown to prevent chromatin-mediated repression of DNA replication in vitro (14). Thus, NF-I and other transcription factors might function by perturbing the local distribution of nucleosomes, thereby increasing the accessibility of the origin to the replication machinery.

In this study we investigated whether transcription factors, namely NF-I, can activate SV40 replication through their interaction with chromatin components. We show that the activation domains of several transcription factors previously shown to interact with H3 also mediate SV40 replication activation. Both H3 interaction and replication were found to be similarly reduced by a single amino acid substitution in NF-I, thus strongly correlating the interaction with histone H3 with the activation of replication. This study supports the role of chromatin remodeling activities of transcription factors in the activation of SV40 replication.

MATERIALS AND METHODS

Plasmid Constructions—The replication standard plasmid pUCSV40ori was constructed by inserting the SalI-HindIII fragment of pSVori Maricarmen (J. Sogo) into pUC19, cleaved with HindIII and AflIII, after filling in the SalI and AflIII site with the Klenow enzyme. For pSGAL4ori, the replication reporter plasmid, the Neol HindIII fragment from pSVori Maricarmen, was inserted into pCTF1 (15), cleaved with Neol and HindIII. This intermediate vector was cut with BamHI and XbeI, and five GAL4 binding sites were inserted as a BglII-XbaI fragment from pG5BCAT (16). The sequences encoding the GAL4 fusion proteins of NF-I (11) (GAL399–499), GAL438–499, and GAL4(486–499) were introduced as HindIII-EcoRV fragments from the respective pHGAL fusion expression vectors into pHSV BXB (17), cut with XbaI.

* This work was supported by the Swiss National Science Foundation, the Etat de Vaud, and the University of Lausanne 450th Anniversary Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: NF-I, nuclear factor I; RSV, Rous sarcoma virus; CAT, chloramphenicol acetyltransferase; CTF, CAAT box-binding transcription factor.
and HindIII, with the XbaI site filled in with the Klenow enzyme. The coding sequences for GAL399–438, GAL399–472, GAL399–486, and GAL438–472 were transferred from their pSG424 backbone as HindIII-XbaI fragments into pRSV BXB cleaved with the same enzyme. The vectors with RSV promoter-driven expression of the GAL4-NF-1 fusions carrying a point mutation of the GAL4-NF-1 site filled in with the Klenow enzyme. The coding sequences for the fusions were electroporated with a DNA mixture containing 1 μg of pUCori replication standard plasmid, 10 μg of pSGAL4ori replication reporter, and 10 μg of RSV expression vector. Sonicated salmon sperm DNA (Sigma) was added to a total of 50 μg of transfected DNA. The medium was supplemented with 50 μCi/ml [3H]thymidine. Cells were harvested 36 h after transfection, and plasmid DNA was extracted using the QIAQuick plasmid extraction kit according to the supplier’s instructions (QIAGEN). DNA was eluted from the columns with 50 μl of H2O. Extracted plasmids were linearized by BamHI and separated on a 0.6% agarose gel. Plasmid bands were excised and subjected to scintillation counting in Hionic Fluor R (Packard) to quantify the incorporated radiolabeled DNA. The value obtained for the reporter plasmid was normalized to that obtained for the standard plasmid. For CAT assays, COS7 cells were co-transfected with three plasmids: (a) an expression vector for the indicated GAL4 fusions, (b) an expression vector for VP16-transcriptional activation domain alone (VP) or an expression vector for the indicated GAL4 fusions, (c) the G5BCAT reporter construct. Cell extracts were prepared with lysis buffer (Promega), and the assays were performed as described previously (11). All figures display the mean of at least three experiments.

Gel Mobility Shift Assays—For bandshift analysis of GAL fusion proteins, transiently transfected COS7 cells were harvested 36 h after transfection, washed once in 1× phosphate-buffered saline, and lysed in 100 μl of extraction buffer (20 mM Tris, pH 7.5, 20% glycerol, 500 mM KCl, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) by repeated freeze-thaw cycles, as described previously (15). Whole cell lysates were normalized for total protein concentration. 4 μg of total protein extract were incubated in 20 μl of extraction buffer for 10 min at room temperature with a 32P end-labeled double-stranded DNA probe containing the 17-base pair GAL binding site (5′-GATC-CGGGTTCGGAGTACTGTCCTCGACTGC-3′). Protein-DNA complexes were separated from free probe on native 4% polyacrylamide gels as described in Ref. 20 and revealed by phosphorimaging (Molecular Dynamics).

RESULTS AND DISCUSSION

To evaluate the role of transcription factors in the activation of SV40 replication, COS7 cells were transfected with a replication reporter plasmid containing five GAL binding sites adjacent to the SV40 ori core, pSGALori, along with chimeric cDNA expression vectors for GAL fusion proteins. These encode the DNA binding and dimerization domain of the yeast GAL4, either alone (amino acids 1–147; GAL DBD) or fused to the coding sequences of the transcriptional activation domains of CTF/NF-1 (amino acids 399–499; GAL Pro) or Sp1 (amino acids 132–243, GAL Sp1) (Fig. 1A). A plasmid with the full origin of replication, including both auxiliary sequences, was used as an internal reference plasmid for transfection and replication (Fig. 1A, pUCori). SV40 DNA replication was assayed by the addition of [3H]thymidine to the growth medium and by the extraction of radiolabeled replicated DNA. To ascertain that the recovered radiolabeled plasmids were true replication products, they were digested with DpnI, which only cleaves methylated DNA that has not been replicated. As an additional control, a digestion with MboI was performed (Fig. 1B) because MboI is dam methylation sensitive and cuts only non-methylated DNA. The recovered plasmids are cleaved by MboI, but not by DpnI, and are therefore replication products.

The presence of the five GAL4 binding sites had no influence per se on the basal replication activity of the modified replication origins. No difference in replication activity was detected between the two replication reporter plasmids pSGALori and pCori, a plasmid in which the aux-2 sequence is deleted (data not shown). The proline-rich transcriptional activation domain of CTF/NF-1 fused to GAL (Fig. 1B, GAL Pro) efficiently activated replication of pSGALori. The expression of the DNA binding domain of GAL4 (GAL DBD) had a weak but significant activation effect on SV40 replication in the presence of GAL4 binding sites, consistent with previously published results (21, 22). In the absence of binding sites, GAL DBD and
other GAL4 fusions had no effect on replication (data not shown).

**H3-interacting Transcriptional Activation Domains Activate Replication**—It has been proposed that Sp1 and NF-I bind histone H3 and reorganize chromatin. Oct2, but not VP16, was also found to interact with histone H3 in two-hybrid assays (11). Thus, the activation domains of the latter transcription factors were tested for their capacity to activate replication using the same experimental approach as that used for NF-I. The activation by GAL NF-I was fixed as the reference level of replication and is used in all comparisons below. Whereas GAL Oct2 and GAL Sp1 stimulated replication, they did so to a lesser extent than GAL NF-I. Activation by these transcription factors was not due to the mere presence of a transcriptional activation domain because expression of a GAL fusion containing the VP16 activation domain or a portion thereof did not result in detectable replication (Fig. 2A; data not shown). Activation of replication by GAL Sp1 was expected because the aux-2 sequence contains Sp1 binding sites (23), and replacement of the aux-2 sequence with artificial Sp1 binding sites has been reported to activate SV40 replication in CV1 cells (24). Activation by GAL Oct2 is also consistent with the previous finding that Oct binding sites, which are found in the enhancer sequence of the SV40 promoter, can substitute for aux-2 when adjacent to the A/T-rich element of the ori core (4, 25). NF-I and Oct2 may therefore substitute for Sp1 in replication activation by using the same basic mechanism. However, the activation domain of VP16 did not activate replication in our assay in COS7 cells, indicating that the presence of a potent transcriptional activation domain is not sufficient for replication activation. This suggests that it is not transcriptional activity per se that activates SV40 replication and that another activity is required. Thus, there is a direct correlation between the potential of the activation domains to activate SV40 replication and the ability of the activation domains to interact with histone H3.

To test for the interaction of these fusion proteins in COS7 cells, two-hybrid transcription experiments were performed in parallel to the replication assays. COST7 cells were transfected with the CAT reporter construct, expression vectors for the indicated GAL fusion proteins, and expression vectors for the VP16 activation domain, either alone (VP) or fused to mouse histone H3.3 (VP-H3; Fig. 2B). An interaction between a GAL fusion protein and H3 in VP-H3 recruits the full-length VP16 transcriptional activation domain to the promoter, thus leading to higher levels of transcription. When assayed for transcriptional activity with VP16 alone, all GAL4 fusions activated transcription, and the most potent of all was GAL VP16, as seen previously (11). In the two-hybrid assay, the proline-rich activation domain of NF-I interacts efficiently with histone H3 in COS7, as do the activation domains of Sp1 and Oct2. However, no interaction between the acidic transcriptional activation domain of VP16 and the VP16-H3 fusion could be detected.

Control experiments were performed to rule out the possibility that different activation levels may actually be due to different expression levels of the activator proteins (Fig. 2C). Gel mobility shift assays demonstrated that all the fusion proteins were expressed at comparable levels and that the presence of H3-VP16 had no effect on the expression levels of the GAL4 fusion proteins. We find a close correlation between the H3 interaction and replication activation. The three transcription factors that interacted with histone H3 also significantly activated SV40 replication, whereas both VP16 and GAL DBD did not significantly activate replication and did not interact with histone H3 in the two-hybrid assay. Together, these data suggest that the presence of a strong transcriptional activation domain, as seen in GAL VP16, is not sufficient for replication activation and that the other domains induce SV40 replication by interacting with H3.

**Activation of Replication by Two NF-I Subdomains**—Six deletion mutants of the proline-rich domain of NF-I were used to determine more precisely which of its subdomains activate the
SV40 origin of replication. The C-terminal deletion mutants GAL(399–472) and GAL(399–438) activated replication but did so less efficiently than the full-length activation domain. This indicates that part of the replication activity maps to the C terminus of the NF-I activation domain. Further deletion of the N-terminal portions of the NF-I transcriptional activation domain, as seen in GAL(438–499), resulted in the loss of all activation potential. Thus, a second part of the replication activation potential of the proline-rich domain resides in the N-terminal portion.

**Fig. 3. Two subregions of the NF-I proline-rich domain mediate replication and interaction with histone H3 in COS7 cells.**

A, COS7 cells transfected with the indicated deletion mutants of the CTF/NF-I transcriptional activation domain fused to the GAL4 DNA binding domain, and DNA replication was assayed as described in the Fig. 2 legend. Replication activity was normalized to that of the internal standard plasmid, pUCori, and the value obtained for cells expressing the Gal-NF-I and VP16-histone H3 fusions was set as 100%. B, the C-terminal part of the proline-rich domain of CTF/NF-I mediates interaction with histone H3 in COS7 cells. COS7 cells were transfected as described in the Fig. 2 legend with the deletion mutants of the transcriptional activation domain indicated. CAT activity was assessed 36 h after transfection. C, bandshift analysis of GAL4 fusion of NF-I deletion mutants. The experiment was performed as described in the legend to Fig. 2C.
H3-interacting Domains Activate SV40 DNA Replication

Activation of replication is abolished by a point mutant that markedly affects transcription. A. COS7 cells were transfected with the replication reporter plasmid, p5GALori, the internal standard plasmid, pUCori, and expression vectors for GAL-NF-I fusion proteins. The tyrosine at position 497 was changed to either aspartic acid (GAL 399–499 Y497D) or phenylalanine (GAL 399–499 Y497F). Radiolabeling, cell harvesting, and quantification of the replicated plasmids were performed as described in the legend to Fig. 2. B. COS7 cells were transfected with G5BCAT, CMV/gal, the vector coding for either VP-H3 or VP alone, and expression vectors for GAL-NF-I bearing point mutations in the C-terminal portion of the transcriptional activation domain, as specified in the figure. CAT activity was determined 36 h after transfection and is expressed relative to the normalized CAT activity obtained for cells expressing wild type GAL(399–499), which was set as 100. C. Bandshift analysis of GAL4 fusions of NF-I point mutants. The experiment was performed as described in the legend to Fig. 2C.

(Fig. 3A). The loss of activation potential of GAL(438–499) and GAL(399–472) compared with GAL(399–438) and GAL(486–499) is most likely due to the presence of a central inhibitory subdomain (amino acids 438–472). These results indicate that both the C- and N-terminal portions of the NF-I transcriptional activation domain are required for full activation of the replication origin. In contrast, the central portion of the proline-rich domain has no detectable effect on replication activity.

The same deletion mutants were used to map the region of GAL(399–499) that interacts with histone H3 in the two-hybrid assay. The assays for H3 interaction and replication activation were performed in the same transfections to allow a direct comparison of the results. The C-terminal deletion mutants GAL(399–472) and GAL(399–438) show strongly reduced transcriptional activation, 30- and 20-fold lower than that of GAL(399–499) (Fig. 3B). However, their interaction with H3 decreased but remained detectable, in keeping with their transcriptional activity. Conversely, the N-terminal deletion mutant GAL(438–499) showed both reduced transcriptional activity and H3 binding activity. As seen before, the expression of GAL4 fusions was not affected by the coexpression of the histone fusion constructs (Fig. 3C). These data indicate that two NF-I domains interact with H3 and mediate replication activation. One domain maps to the 13 C-terminal amino acids and is similar to the histone H3-interacting domain first identified in mouse fibroblastic cells (11). Moreover, GAL(472–499) and GAL(486–499) only modestly activate transcription on their own, but they show high levels of activity when assayed for interaction with the VP-histone H3 fusion protein and replication activation. In addition, we identified another portion of the proline-rich domain that also shows interaction with histone H3. This second histone H3-interacting domain activated SV40 replication to approximately the same extent as the C terminus, yet it did not significantly activate transcription on its own. Deletion of both the C- and N-terminal domains in mutant GAL(438–472) was required to inhibit both interaction with histone H3 and replication activation.

Overall, the data indicate a dual role for C- and N-terminal extremities of the NF-I transcriptional activation domain in histone H3 interaction and replication activation and imply that interaction with the histone, rather than mere transcriptional activation, confers SV40 ori activation by NF-I.

A NF-I Point Mutant Inhibits both H3 Interaction and DNA Replication—The C-terminal portion of the transcriptional activation domain is clearly one determinant of H3 interaction and replication activation. To further evaluate the role of this domain, we tested three GAL NF-I fusions bearing single point mutations at the C terminus.

GAL(399–499, Y491D) (tyrosine 491 changed to aspartate) and GAL(399–499, Y497F) (tyrosine 497 changed to phenylalanine) efficiently activated both replication and transcription, and interaction with VP16-H3 did not significantly differ from wild type GAL(399–499) (Fig. 4). In contrast, the protein bearing a mutation of tyrosine to aspartate at position 497, GAL(399–499, Y497D), abolished the activation of p5GALori replication. Transcriptional activation and histone interaction were also similarly reduced with this mutation (Fig. 4B). Further deletion of the N-terminal H3 interaction domain in the Y497D mutant abolished H3 interaction and replication activation, as expected (data not shown). Overall, these results provide evidence that the proline-rich domain of NF-I activates SV40 replication and further implicate the interaction with histone H3 in SV40 replication activation.

Conclusions—Previous studies have indicated that NF-I can
remodel chromatin in vitro, whereas Sp1 has been suggested to maintain the SV40 origin nucleosome-free (14, 26). These findings, together with the association of chromatin remodeling and transcriptional activation at many promoters (27–29), may thus point to chromatin remodeling as a common mechanism for the activation of both transcription and replication. In both cases, chromatin structure alteration by the action, direct or indirect, of transcription factors would be an early step in activation. It is interesting to note that the H3-binding domain of NF-I is capable of restructuring the chromatin of target promoters and concomitantly prevents heterochromatic silencing in yeast. Therefore, the binding of histone H3 by NF-I may be the key step by which this transcription factor overcomes chromatin repression on both promoters and replication origins.

Acknowledgments—We are grateful to J. Sogo for pSVori Mariacarmen and to M. Zahn and L. Hunt for critical reading of the manuscript.

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