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Regulation of DNA replication *in vitro* by the transcriptional activation domain of GAL4-VP16

(chromatin/DNA-binding protein/chimeric transcription factor)

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ABSTRACT **Studies of DNA viruses have provided evidence that eukaryotic transcriptional activator proteins can enhance the efficiency of DNA replication as well as transcription. The mechanism of this effect was studied *in vitro* using the chimeric transcription factor GAL4-VP16 and a DNA template containing GAL4 binding sites adjacent to the simian virus 40 origin of DNA replication. The binding of GAL4-VP16 prevented the repression of DNA replication which otherwise occurred when the template was assembled into chromatin. Relief of repression by GAL4-VP16 required both its DNA-binding and transcriptional activation domains but did not require RNA synthesis. The results are consistent with a general model in which transcriptional activators stimulate eukaryotic DNA replication by modifying the outcome of the competition between initiation factors and histones for occupancy of the origin.**

Both DNA replication and transcription involve the initiation of polynucleotide synthesis at specific sites in chromatin. Studies of animal virus model systems have provided evidence that the two processes may be regulated by similar mechanisms. In particular, it has been demonstrated in several systems that sequence elements known to activate transcription at cellular promoters can also stimulate initiation of DNA replication at viral origins of DNA replication (1, 2). This phenomenon was first observed in the case of polyomavirus, but similar observations were subsequently reported for simian virus 40 (SV40), adenovirus, Epstein–Barr virus, and bovine papillomavirus. Recent evidence suggests a role of transcriptional activators in cellular DNA replication as well (3). A number of possible explanations for the stimulatory effects of transcriptional regulatory elements have been suggested, but the underlying molecular mechanisms remain unclear.

SV40 has proven to be a useful model for studying the mechanisms of mammalian DNA replication *in vivo* and *in vitro* (1, 4, 5). The viral genome replicates in the nucleus of infected cells, where it is complexed with histones to form a minichromosome that is similar in structure to cellular chromatin. SV40 DNA replication requires a single virus-encoded protein, the large tumor (T) antigen, but is otherwise dependent upon cellular replication proteins. The replication reaction has recently been reconstituted *in vitro* with T antigen and seven highly purified cellular proteins (6, 7). The minimal SV40 origin of DNA replication consists of a 65-base-pair (bp) DNA segment that contains binding sites for the SV40 T antigen. Studies from a number of laboratories have demonstrated that the transcriptional regulatory elements immediately adjacent to the minimal origin dramatically stimulate DNA replication *in vivo* (8–12). These elements include a series of G+C-rich repeats which bind the transcriptional factor Sp1 and the SV40 enhancers which bind several different cellular transcriptional factors. The presence of either element is sufficient to increase replication efficiency by at least 10-fold when positioned within 100 bp of the minimal origin (8–12). Moreover, the binding sites for other cellular transcriptional activators (e.g., NF-I/CTF) can also stimulate SV40 DNA replication when substituted for the naturally occurring elements (13).

In previous studies we have made use of the cell-free SV40 DNA replication system to analyze the effects of the transcriptional activator NF-I/CTF on the replication of templates containing the NF-I/CTF recognition site adjacent to the minimal SV40 origin of DNA replication (13). The activator protein had little effect on replication efficiency in the standard cell-free system in which the template was introduced as naked DNA. In contrast, large effects on replication efficiency *in vitro* were observed when chromatin templates were used. In the absence of NF-I/CTF (or the NF-I/CTF recognition site) preassembly of the template into chromatin strongly repressed DNA replication. However, the repression was relieved if purified NF-I/CTF protein was present during chromatin assembly (13). These observations suggested that transcriptional activators might stimulate DNA synthesis by enhancing the ability of replication initiation factors to compete with histones for interaction with the origin of DNA replication.

Genetic studies have demonstrated that eukaryotic transcriptional activators are composed of at least two distinct functional domains (14–16). One of these domains (the DNA-binding domain) interacts with specific DNA sequence elements, and the other domain (the activation domain) is thought to interact with components of the transcriptional apparatus. To further analyze the biochemical mechanism responsible for the stimulation of DNA replication by transcriptional activators, we have tested whether DNA binding alone is sufficient to prevent repression of DNA replication by chromatin assembly or whether an activation domain is also required. For this purpose we used derivatives of the well-characterized yeast transcriptional activator GAL4 (17–20). The DNA binding and dimerization domains of GAL4 are located at the N terminus of the protein. A highly potent hybrid transcriptional activator has been constructed by fusing the DNA-binding domain of GAL4 (residues 1–147) to the 78-amino acid acidic activation domain of the herpes virus trans-activator protein, VP16 (19). The chimeric protein, GAL4-VP16, binds as a dimer to the GAL4 recognition site and is capable of stimulating transcription in extracts from both mammalian and yeast cells (19–21). To test the ability of this protein to stimulate DNA replication, we constructed a

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Abbreviations: SV40, simian virus 40; T antigen, large tumor antigen.

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DNA template (pUC.HNO/GAL4) which contains five tandem copies of the GAL4 recognition sequence inserted immediately adjacent to the SV40 origin of DNA replication (Fig. 1*a*). Using this template and the chromatin replication system described above, we compared the stimulatory activity of the hybrid protein GAL4-VP16 with that of GAL4/DB, a protein which contains only the GAL4 DNA-binding and dimerization domains (residues 1–94) but lacks any activation domain. We observed that the binding of GAL4-VP16 prevented the repression of DNA replication which occurred when the template was assembled into chromatin. However, binding of GAL4/DB did not. Therefore, relief of repression by GAL4-VP16 required an intact transcriptional activation domain. The results suggest that transcriptional activators can facilitate DNA replication by modifying the outcome of the competition between initiation factors and histones for occupancy of the origin.

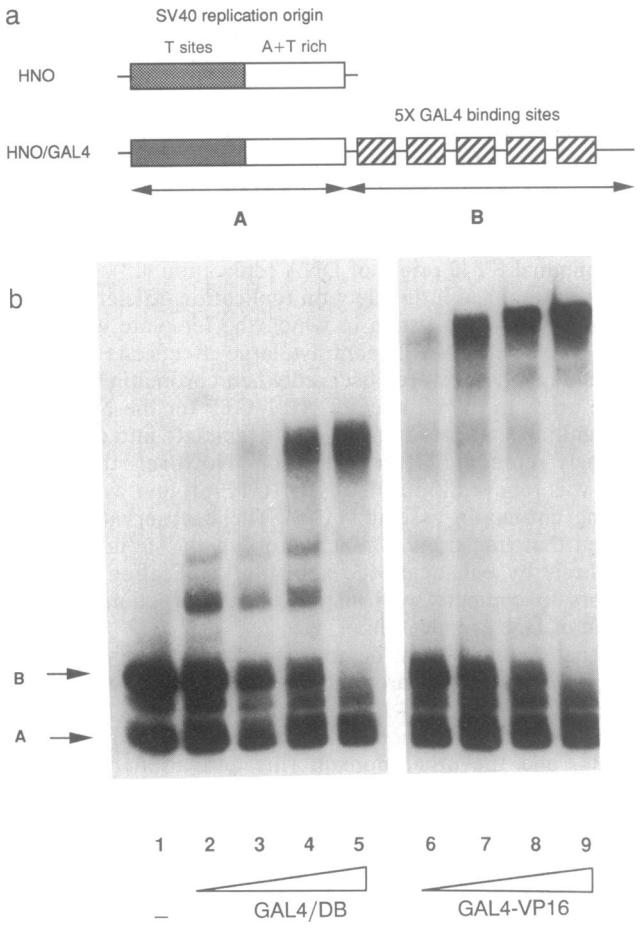

FIG. 1. Binding of GAL4 derivatives to DNA. (*a*) Templates for DNA replication. Plasmids pUC.HNO and pKP.HNO contain the identical SV40 origin of DNA replication, including T-antigen binding sites (T sites) and an A+T-rich region, and support efficient DNA replication in the cell-free system for replicating SV40 DNA (13). Plasmid pUC.HNO/GAL4 contains the SV40 origin plus five tandem GAL4 binding sites. Plasmid pHNO.GAL4 was used as a template for the polymerase chain reaction (PCR) to generate two radioactive DNA probes (A and B) for the following binding studies. (*b*) Binding of GAL4-VP16 and GAL4/DB proteins to DNA. The two DNA fragments (13 fmol of each) were incubated simultaneously with various quantities of either GAL4-VP16 (lanes 2–5) or GAL4/DB (lanes 6–9) protein. The amounts of GAL4 monomer proteins added were as follows: lanes 2 and 6, 16 fmol; lanes 3 and 7, 32.5 fmol; lanes 4 and 8, 65 fmol; lanes 5 and 9, 130 fmol. Reaction mixtures containing unbound DNA and DNA–protein complexes were loaded onto a 5% acrylamide gel and electrophoresed in 0.5× TBE buffer. Positions of the unbound nonspecific (A) and specific (B) probes are indicated by arrows.

MATERIALS AND METHODS

Templates for DNA Replication. The plasmids pUC.HNO and pKP.HNO contain the identical SV40 origin of DNA replication including T-antigen binding sites (T sites) and an A+T-rich region and support efficient DNA replication in the cell-free system for replicating SV40 DNA (13). The plasmid pKP.HNO, which is smaller, is used as an internal control in the replication assay. The plasmid pUC.HNO/GAL4 contains the SV40 origin plus five tandem GAL4 binding sites (Fig. 1*a*). This construct was obtained by excising the *Xba* I–*Pst* I fragment containing the GAL4 binding sites from the plasmid $pG_5E1b.CAT$ (21) and inserting it into the corresponding sites of pUC.HNO, adjacent to the A+T-rich region of the SV40 origin.

Preparation of Protein Extracts for Chromatin Assembly and SV40 DNA Replication. Oocyte extracts capable of *in vitro* nucleosome assembly were prepared from *Xenopus laevis*. Isolated mature oocytes were homogenized, and extracts were prepared in EB buffer [10% (vol/vol) glycerol/20 mM Hepes, pH 7.5/5 mM KOAc/1.5 mM $Mg(OAc)_2$/0.5 mM EGTA/1 mM dithiothreitol] as described (13). Cytoplasmic extracts required for supporting SV40 replication *in vitro* were prepared from HeLa S3 cells as described (23).

Purification of GAL4 Derivatives and SV40 T Antigen. GAL4-VP16 and GAL4/DB (containing the first 94 residues of GAL4) were overexpressed and purified from *Escherichia coli* (22). The two proteins were about 60% pure as judged by SDS/PAGE and were stored in buffer Z (20% glycerol/25 mM Hepes, pH 7.5/50 mM NaCl/0.01% Nonidet P-40/20 mM 2-mercaptoethanol/10 μM $ZnCl_2$/0.1% bovine serum albumin). SV40 T antigen was purified from HeLa cells infected with the recombinant adenovirus vector R284 (23). Protein concentrations were determined by the method of Bradford (24), with bovine serum albumin as a standard.

Binding of GAL4 Derivatives. To generate radioactive DNA probes for binding studies, the plasmid pUC.HNO/GAL4 was used as a template for the polymerase chain reaction (PCR) with two primers flanking the SV40 origin on one side and the GAL4 binding sites on the other. The radioactive PCR product was digested with *Nco* I to generate a control fragment A (100 bp), containing the SV40 origin, and a test fragment B (150 bp), containing the five tandem GAL4 binding sites (see Fig. 1*a*). The 6-μl binding reaction mixture contained 13 fmol of each labeled DNA fragment (45 ng of total DNA per reaction), 0.1 M disodium creatine phosphate, 2 mM Hepes (pH 7.5), and 2 μl of either GAL4-VP16, GAL4/DB, or buffer Z. After incubation at 25°C for 15 min, 6 μl of EB buffer was added (EB buffer is used for extraction of *Xenopus* oocytes). The final composition of the binding reaction was the same as that in the chromatin assembly/replication assay (see below). Reaction mixtures were loaded onto a 5% acrylamide gel and electrophoresed in 0.5× TBE buffer (45 mM Tris/45 mM boric acid/1 mM EDTA) at 10 V/cm for 2 hr. Gels were dried and exposed to Kodak XAR-5 film with an intensifying screen at −80°C.

***In Vitro* Replication of Assembled Templates.** Twenty-five nanograms of the test template (pUC.HNO/GAL4, 13 fmol), containing the SV40 origin plus adjacent GAL4 binding sites, and 20 ng of the control template (pKP.HNO, 13 fmol), containing the SV40 origin alone, were incubated with a GAL4 derivative or the buffer Z in 6-μl binding reaction mixtures as described above. After addition of 6 μl of the oocyte extract, the mixtures were incubated at 37°C for up to 3 hr to allow chromatin assembly. Then the components required for SV40 DNA replication (T antigen, HeLa cytosol, etc.) were added to generate a standard 25-μl replication reaction mixture (23). All replication reactions were allowed to proceed for 1 hr at 37°C in the presence of [α-^{32}P]dCTP. The reaction products were deproteinized and subjected to

electrophoresis through a 1% agarose gel containing ethidium bromide (0.5 μg/ml). Under these conditions the various topoisomers of the test and control DNAs were compressed into single bands. Gels were dried and exposed to Kodak XAR-5 film with an intensifying screen at −80°C.

RESULTS

Specific Binding of GAL4 Derivatives on DNA. To verify that both GAL4-VP16 and GAL4/DB bound specifically to the GAL4 recognition site under the conditions used for chromatin assembly, gel retardation assays were carried out. Each protein was incubated with a mixture of two radioactive DNA fragments: fragment B, containing five tandem copies of the GAL4 recognition site, and as an internal control, fragment A, containing only the SV40 replication origin (Fig. 1*a*). Both GAL-VP16 and GAL/DB bound specifically to fragment B, forming a series of slower migrating bands containing increasing numbers of GAL4 dimers (from one to five; Fig. 1*b*). At the highest protein level studied (≈20 nM monomer protein/2 nM DNA), >90% of fragment B was shifted to the slowest mobility form, whereas the mobility of the nonspecific fragment A was virtually unaffected. At intermediate protein concentrations the pattern of slow migrating forms was similar for both proteins. This result is consistent with previous reports that the affinities of GAL4-VP16 and GAL4/DB proteins for the GAL4 recognition site are similar (21, 22, 25).

Effect of GAL4-VP16 on the Replication of Assembled Templates. The ability of GAL4-VP16 protein to activate the replication of chromatin templates was tested in a coupled chromatin assembly/DNA replication system (13). The plasmid pUC.HNO/GAL4, containing five tandem GAL4 recognition sites adjacent to the SV40 origin, and pKP.HNO (13), a smaller, control plasmid containing only the SV40 replication origin, were mixed with GAL4-VP16 or buffer and then incubated for various periods of time in the chromatin assembly system derived from *Xenopus* oocytes. The components required for SV40 DNA replication were subsequently added, and DNA synthesis was allowed to proceed for a fixed period of time. The products of the replication reaction were fractionated by agarose gel electrophoresis (Fig. 2). The following observations were made. (*i*) In the absence of chromatin assembly (Fig. 2*a*, lanes 1 and 2), GAL4-VP16 protein had no effect on the replication of either the plasmid containing the GAL4 recognition sites or the plasmid lacking such sites. (*ii*) In the absence of GAL4-VP16 protein (Fig. 2*a*, lanes 4, 7, and 10), assembly of the plasmid DNA templates into chromatin strongly repressed subsequent DNA replication, consistent with previous results (13). The extent of repression was similar for the two plasmids, since the molar ratio of the two product DNAs was independent of incubation time and roughly equal to that of the input DNA templates. (*iii*) In the presence of GAL4-VP16 protein (Fig. 2*a*, lanes 5, 8, and 11), the replication of the test template, containing the GAL4 recognition sites adjacent to the origin, was inhibited only slightly by chromatin assembly,

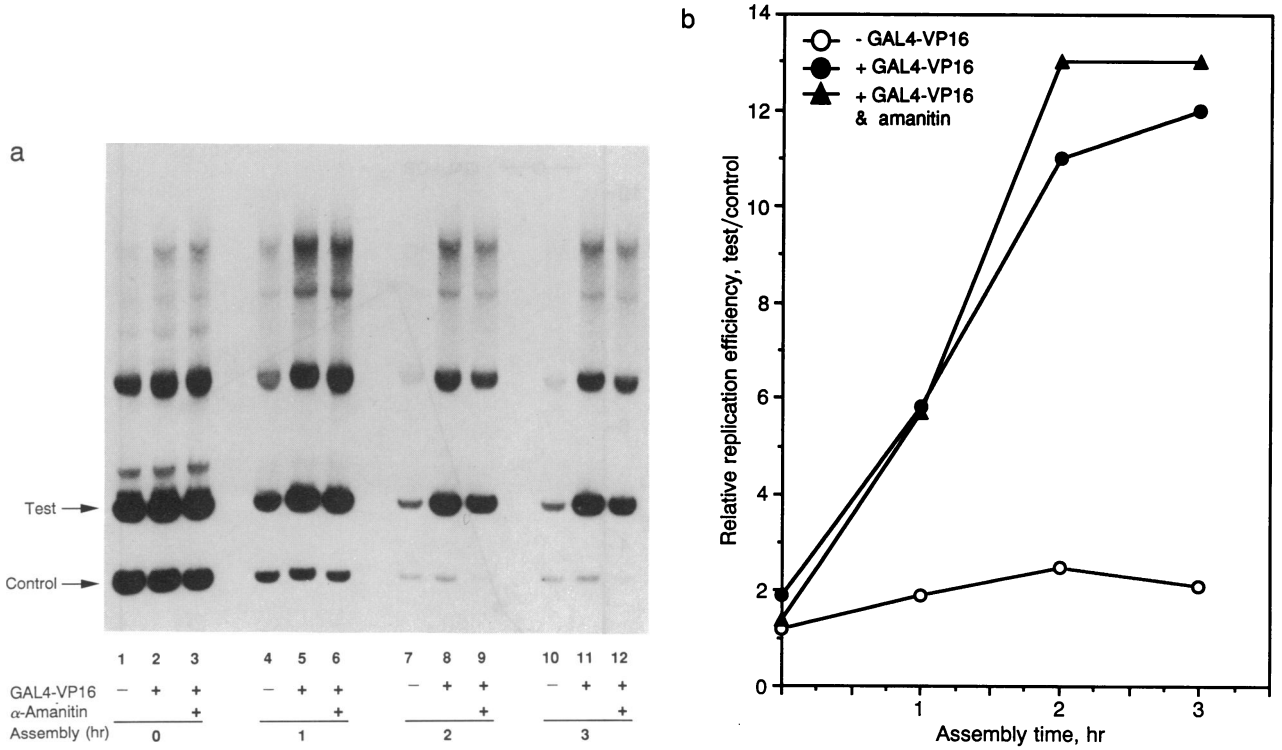

FIG. 2. *In vitro* replication of SV40 minichromosomes assembled in the presence or absence of GAL4-VP16. (*a*) Gel electrophoresis of replication products. The test template (pUC.HNO/GAL4, containing the SV40 origin plus adjacent GAL4 binding sites) and the control template (pKP.HNO, containing the SV40 origin alone) were incubated with 65 fmol (monomer) of GAL4-VP16 or buffer Z. Oocyte extract was then added, and the reaction mixtures were incubated for 0, 1, 2, or 3 hr to allow chromatin assembly. Where indicated the assembly was carried out in the presence of α-amanitin (Boehringer Mannheim) at 10 μg/ml. After chromatin assembly, the components required for SV40 DNA replication were added and replication reactions were allowed to proceed for 1 hr in the presence of [α-^{32}P]dCTP. The reaction products were deproteinized and subjected to electrophoresis through a 1% agarose gel containing ethidium bromide. Locations of complete replication products (unit-length circular DNA molecules) are indicated by arrows. Radioactive species with mobilities less than those of the monomer plasmids represent DNA dimers and various replication intermediates. (*b*) Quantitation of DNA replication. Incorporation of [α-^{32}P]dCMP into unit-length circular DNA products was determined by excising the appropriate bands from the dried gel after autoradiography and assaying radioactivity in a scintillation counter. Data are plotted as the ratio of the incorporation into the test plasmid to the incorporation into the control plasmid.

while the replication of the control template, which lacked GAL4 sites, continued to be strongly repressed. (*iv*) The extent of DNA replication in the presence of GAL4-VP16 was not reduced significantly by addition of α-amanitin at a concentration sufficient to inhibit RNA synthesis by RNA polymerase II or III (26). Thus, GAL4-VP16 protein was able to substantially prevent the repression of DNA replication by chromatin assembly, but the relief of repression was not dependent upon transcription from the DNA template (see Fig. 2*b* for quantitation). Furthermore, the relief of repression by GAL4-VP16 was not due to global inhibition of chromatin assembly, since the average number of nucleosomes assembled onto the DNA was roughly the same in the presence or absence of GAL4-VP16 at this concentration (L.C. and T.J.K., unpublished data).

The DNA-Binding Domain of GAL4 Is Not Sufficient for the Stimulation. The ability of GAL4/DB protein, which contains only the DNA-binding domain of GAL4, to relieve repression due to chromatin assembly was assessed in the same system (Fig. 3). In contrast to the GAL4-VP16 protein, which stimulated the replication of pUC.HNO/GAL4 chromatin about 10-fold, GAL4/DB protein was unable to stimulate DNA replication more than 2-fold despite the fact that it binds to the GAL4 recognition site with equal affinity under these conditions (see above; refs. 22 and 25). At the highest concentration of GAL4-VP16 or GAL4/DB (130 fmol), a small increase in the replication of both templates was observed (Fig. 3*a*, lanes 4 and 7). This was correlated with an inhibitory effect on chromatin assembly (unpublished data). Thus, simple occupancy of a DNA site adjacent to the origin by a site-specific DNA-binding protein is not sufficient to activate DNA replication. This conclusion is consistent with the results of experiments in which we examined the ability of *E. coli lac* repressor protein to activate replication of a chromatin template containing the *lac* operator adjacent to the SV40 origin. Although the repressor bound to the operator site under the conditions used for chromatin assembly, little effect on DNA replication was observed (L.C. and T.J.K., unpublished data).

DISCUSSION

In this study, we have found that the activation domain of GAL4-VP16 is required for efficient replication of chromatin templates *in vitro* since the DNA-binding domain is not sufficient. The data are consistent with a recent *in vivo* study of polyomavirus DNA replication (27). The replication of the polyomavirus genome resembles that of SV40 in many ways, including the requirement for transcriptional elements adjacent to the origin of DNA replication (1, 2). When the natural polyoma transcriptional elements were replaced by GAL4 recognition sites, the replication of the viral genome in mouse cells was significantly enhanced by transfection of a plasmid that expressed a functional transcriptional activator protein binding to the GAL4 sites. Transfection of a plasmid expressing the GAL4 DNA-binding domain alone did not activate

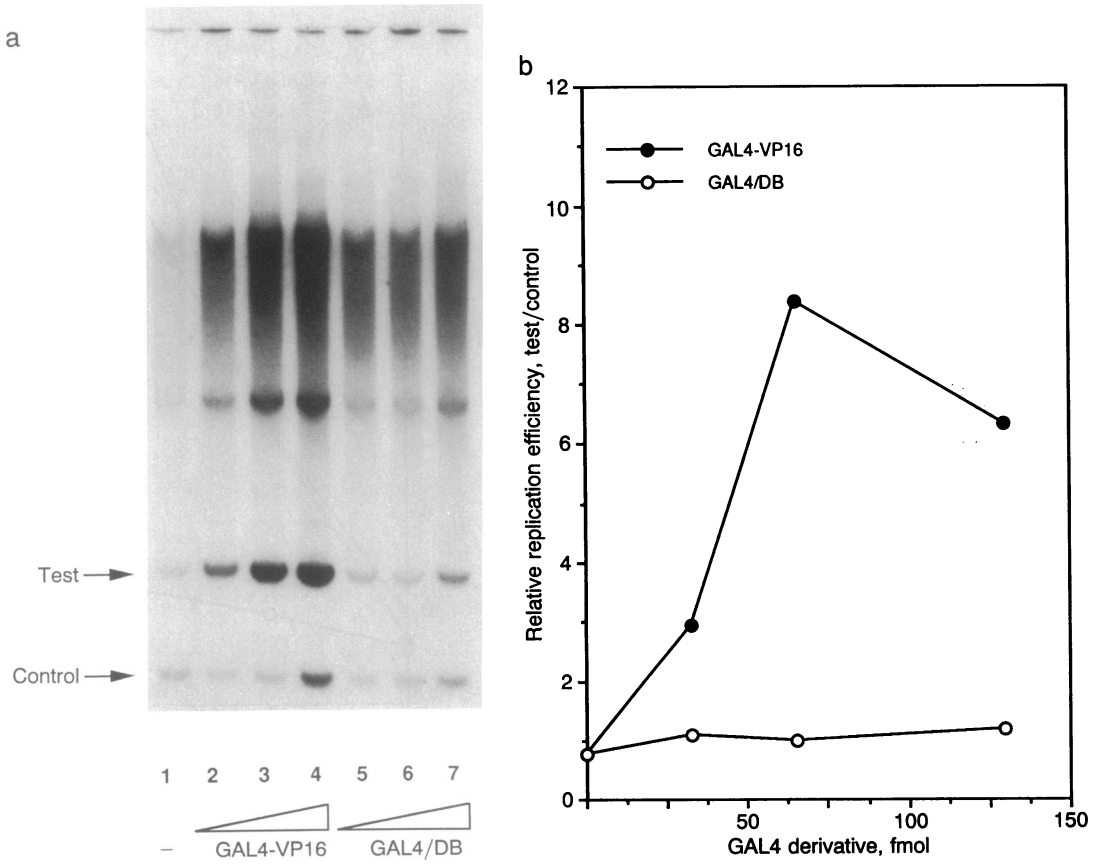

FIG. 3. The DNA-binding and dimerization domains of GAL4 are not sufficient for stimulating the replication of SV40 minichromosomes. Test (pUC.HNO/GAL4) and control (pKP.HNO) plasmids were preincubated in the chromatin assembly system in the presence or absence of GAL4 derivatives for 3 hr as described in the legend of Fig. 2. The resulting minichromosomes were then assayed for their ability to support DNA replication *in vitro*. (*a*) Gel electrophoresis of replication products. Lanes 1–4, titration of GAL4-VP16 (0, 33, 65, and 130 fmol, respectively); lanes 5–7, titration of GAL4/DB (33, 65, and 130 fmol, respectively). (*b*) Quantitation of DNA replication efficiency. As in Fig. 2, incorporation of [α-^{32}P]dCMP into unit-length circular DNA products was determined by excising the appropriate bands from the dried gel after autoradiography and assaying radioactivity in a scintillation counter. Data are plotted as the ratio of the incorporation as a function of the amount of GAL4-VP16 (●) or GAL4/DB (○).

DNA replication *in vivo* (27). Although the mechanism responsible for the stimulatory effect of GAL4 activator protein *in vivo* is not yet clear, the data presented here suggest that it may be due to prevention of repression by histones.

Many previous studies have demonstrated that transcription is strongly repressed by assembly of the DNA template into chromatin (see review articles in ref. 28). Both the nucleosomal core histones and histone H1 have been implicated in repression. Recent studies have shown that GAL4-VP16 can alleviate repression of transcription by nucleosome assembly if the active promoter is adjacent to multiple GAL4 recognition sites (22, 25). The GAL4/DB protein, which lacks an activation domain, is insufficient to preserve promoter function during chromatin assembly even though it binds to the template with a stability comparable to that of GAL4-VP16. In these transcription experiments GAL4-VP16 moderately stimulated transcription in the absence of nucleosome assembly. However, assembly of the template into chromatin increased the dependence of transcription on the VP16 activation domain 10-fold. Thus, the 78-residue acidic activation domain of GAL4-VP16 is required for activation of both transcription and DNA replication.

Our data are consistent with a model in which histones compete with replication initiation factors (T antigen, etc.) for occupancy of the SV40 origin of DNA replication. In this model, activator proteins such as GAL4-VP16 perturb this competition in favor of the replication initiation factors. A similar idea has been proposed to explain the effect of GAL4-VP16 on transcription of chromatin templates (22). There are two general ways that such activator proteins could influence the outcome of the competition between histones and initiation factors: (*i*) they could interfere with chromatin assembly in the vicinity of the origin or (*ii*) they could facilitate binding of the initiation factors to the origin. For example, the acidic activation domain could destabilize nearby nucleosomes by direct interaction or promote the formation of a transcription preinitiation complex that sterically blocks nucleosome formation in the origin region. Alternatively, the activation domain could make specific protein–protein contacts with replication initiation factors, thus bringing them into proximity to the origin. All of these possibilities have in common a requirement for the activation domain of the activator protein but do not require RNA synthesis. Whatever mechanism proves correct, the ability of transcriptional activators to facilitate the binding of initiation factors to the replication origin may prove to be an important mechanism for the regulation of DNA replication in eukaryotic cells.

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