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Activation of Yeast Polymerase II Transcription by Herpesvirus VP16 and GAL4 Derivatives In Vitro

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Fusion proteins known to activate transcription in vivo were tested for the ability to stimulate transcription in vitro in a recently developed *Saccharomyces cerevisiae* RNA polymerase II transcription system. One fusion protein, whose activation domain was derived from the herpesvirus transcriptional activator VP16, gave more than 100-fold stimulation in the in vitro system. The order of effects of the various proteins was the same for transcription in vitro and in vivo, suggesting that the natural mechanism of activation is preserved in vitro.

Herpesvirus VP16 (or Vmw65) activates expression of immediate-early genes in virally infected cells (21). VP16 appears to be composed of two domains (27), one for interaction with cellular proteins which in turn bind to DNA sequences upstream of immediate-early genes (5, 21, 28) and a second domain which is required for transcriptional activation (27). The functional role of the second domain has been confirmed by fusion to the DNA-binding region of *Saccharomyces cerevisiae* GAL4 protein. When expressed in HeLa or CHO cells, the fusion protein stimulated transcription from promoters with GAL4-binding sites (22). The degree of stimulation was an order of magnitude greater than that obtained with the glucocorticoid receptor and 2 orders of magnitude greater than that obtained with wild-type GAL4 protein. Here we report the effects of the fusion protein GAL4(1-147)-VP16 and other activators (7, 15, 18) on transcription in a yeast nuclear extract. The results are contrasted with those obtained in an extract from HeLa cells.

MATERIALS AND METHODS

Plasmids. pCZGAL is a member of the pCZ family of plasmids (16), with a GAL4-binding oligonucleotide (1) inserted between the *Bam*HI and *Eco*RI sites of the polylinker located about 50 base pairs (bp) upstream of the major *CYC1* TATA box. pCZ3GAL, containing three binding sites for GAL4 (1, 8), was constructed by insertion of the following oligonucleotide (3GAL) between the *Bam*HI and *Eco*RI sites of the polylinker in pCZ:

5′-GATCCGGGTGACAGCCCTCCGACGGGTGACAGCCCTCCGACGGGTGACAGCCCTCCG
GCCCACTGTCGGGAGGCTGCCCACTGTCGGGAGGCTGCCCACTGTCGGGAGGCTTAA-5′

GAL4-binding sites are underlined. pCZ6GAL is derived from pCZ3GAL by tandem duplication of the oligonucleotide. Plasmids pCTGAL and pCT3GAL, containing GAL4-binding sites 150 bp upstream of the TATA box, were made by insertion of the single and triple GAL4-binding site oligonucleotides, respectively, between the *Bam*HI and *Eco*RI sites of the polylinker in pCT (3). pCT6GAL was derived from pCT3GAL by tandem duplication of the oligonucleotide. pJL2 was constructed by ligation of the large *Xba*I-*Xho*I fragment of pTGH21 (15), the *Xba*I-*Hin*dIII fragment of pGEM3 (Promega Biotec) containing the polylinker, and the *Xho*I-*Hin*dIII fragment of pMA540 (22) encoding the carboxy-terminal portion of GAL4(1-147)-VP16. Plasmid pTMC6 was constructed by ligation of the large *Xho*I-*Xba*I fragment of pTGH21, with the *Xba*I end filled, and the *Xho*I-*Sal*I fragment of pB17 (18) encoding the carboxy terminus of GAL4(1-147)-B17, with the *Sal*I end filled.

Purification of fusion proteins. Purification procedures for GAL4(1-147) and GAL4(1-147)-AH have been described previously (15). Purification of GAL4(1-147)-SH was identical to the purification of GAL4(1-147)-AH. GAL4(1-147)-VP16 and GAL4(1-147)-B17 were expressed under control of the tac promoter by plasmids pJL2 and pTMC6, respectively, in *Escherichia coli*. Cultures were grown at 37°C to an A_{600} of 0.7, and expression of the fusion proteins was induced with isopropylthiogalactoside (to 1 mM) for 2.5 h. Cells were harvested, washed in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5)–0.2 M NaCl, suspended in buffer A (20 mM HEPES [pH 7.5], 20 mM 2-mercaptoethanol, 10 μm zinc acetate, 2 μg each of leupeptin, pepstatin A, and tolylsulfonyl phenylalanyl chloromethyl ketone per ml, 20 μg of benzamidine per ml, and 200 μg of phenylmethylsulfonyl fluoride per ml) containing 0.2 M NaCl, and lysed by sonication at 5 to 10°C. All subsequent operations were performed at 0 to 4°C. A crude extract was derived by centrifugation of the lysate for 20 min at 10,000 × *g*. GAL4(1-147)-VP16 was precipitated from a crude extract with polyethlyleneimine (PEI) at 0.25% (wt/vol), and the pellet was washed with buffer A containing 0.4 M NaCl. GAL4(1-147)-VP16 was eluted from the PEI pellet with buffer A containing 0.75 M NaCl and precipitated by adding solid ammonium sulfate to 35% saturation. The ammonium sulfate pellet was suspended in buffer A to a conductivity equal to that of buffer A with 0.1 M NaCl, loaded onto a DEAE column (DE-52; Whatman, Inc.) equilibrated in buffer A with 0.1 M NaCl, and eluted with a gradient of NaCl from 0.1 to 0.4 M. Peak fractions were loaded onto a heparin-Sepharose CL-6B (Pharmacia) column equilibrated in buffer A with 0.2 M NaCl and eluted with 0.6

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M NaCl in buffer A. GAL4(1-147)-VP16 was judged to be 95% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after this step. For GAL4(1-147)-B17 purification, PEI was added to the crude extract to a final concentration of 0.15% (wt/vol) and the precipitate was removed by centrifugation. GAL4(1-147)-B17 remaining in the supernatant was precipitated with ammonium sulfate to 40% saturation. The ammonium sulfate pellet was suspended in buffer A to a final conductivity equal to that of buffer A with 0.2 M NaCl, loaded onto a heparin-Sepharose column equilibrated in buffer A with 0.2 M NaCl, and eluted with 0.6 M NaCl in buffer A. The eluate was diluted to 0.05 M NaCl with buffer A, loaded onto a QAE-Sephadex column (Pharmacia) equilibrated in 0.05 M NaCl, and eluted with a linear gradient of NaCl from 0.05 to 1.0 M in buffer A. The peak fractions were adjusted to 1.5 M NaCl in buffer A, loaded onto a phenyl-Sepharose (Pharmacia) column equilibrated in buffer A with 1.5 M NaCl, and eluted with 0.5 M NaCl in buffer A. GAL4(1-147)-B17 was judged to be 95% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after this step. Milligram amounts of each of the fusion proteins were produced per liter of culture.

Transcription reactions. Transcription was performed with 0.1 or 0.3 μg of template as previously described (3, 16, 17), except that the final concentrations of HEPES and glycerol were 10 mM and 10%, respectively. Purified GAL4 derivatives were incubated with template for 15 min at room temperature before the addition of nuclear extract. After this addition, reactions were allowed to proceed at 20°C for 1 h, and products were recovered as previously described (3, 16, 17), except there were two phenol-chloroform-isoamyl alcohol (25:24:1) extractions in the presence of 1% sodium dodecyl sulfate. The RNA probe for mapping the reaction products was made by transcription of pSPCTV (17) with SP6 polymerase and [α-^{32}P]GTP (650 Ci/mmol; ICN Pharmaceuticals Inc.). Autoradiograms were scanned and integrated with an Ultroscan densitometer (LKB Instruments, Inc.), and the amount of transcription was quantitated by comparison with autoradiograms of standards.

Gel electrophoresis mobility shift assays. *Pvu*II-*Hin*dIII fragments from pCZ3GAL and pCZ6GAL, containing the GAL4-binding sites, were labeled at the *Hin*dIII end with the large fragment of DNA polymerase I (Pharmacia) and [α-^{32}P]dATP (3,000 Ci/mmol; ICN). Binding reactions contained approximately 10^4 cpm of probe fragment, 1 μg of poly(dI-dC), 5 μg of bovine serum albumin in buffer (25 mM HEPES [pH 7.6], 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% Nonidet P-40, 10% glycerol (1), with the amounts of GAL4(1-147)-AH and competitor 3GAL oligonucleotide indicated. Reaction experiments were incubated for 15 min at room temperature and electrophoresed at 100 V for 2.5 h through 4% polyacrylamide gels (acrylamide-bis [80:1], 0.01% Nonidet P-40) in 0.089 M Tris–0.089 M boric acid–0.002 M EDTA (pH 8.3) with circulation of the buffer. Gels were dried and autoradiographed.

RESULTS

Activation of transcription in vitro by GAL4 fusion proteins. Upstream activation sequences placed in front of the yeast *CYC1* promoter have been shown to potentiate transcription by polymerase II in a yeast extract. Upstream activation sequences that bind the multifunctional yeast activator GRF1 (2) (also known as TUF or RAP1 [12, 25]) gave about a sixfold stimulation of transcription, whereas a thymidine-rich upstream activation sequence that was responsible for constitutive expression of the yeast *DED1* gene gave as much as a 30-fold stimulation (3, 16). In the present study, templates for transcription in vitro were designed with one, three, or six copies of a GAL4-binding sequence placed about 50 bp upstream of the major TATA element of the *CYC1* promoter. Proteins consisting of amino acids 1 to 147 of GAL4 protein (representing the DNA-binding domain) fused to various activation regions, were expressed in *E. coli*, purified, and added to transcription reactions. Maximal stimulation of transcription occurred with amounts of activator sufficient to occupy all of the binding sites in the template (confirmed by filter-binding assays; data not shown), and only maximal levels of stimulation are reported here. The GAL4(1-147)-VP16 fusion protein gave a 49-fold stimulation of transcription from 0.1 μg of the template with a single GAL4-binding site (Fig. 1, Table 1). Transcription was due entirely to polymerase II, as shown by inhibition by alpha-amanitin at 10 μg/ml (Fig. 1). Transcription in the presence of GAL4(1-147)-VP16 and with the other GAL4 derivatives described below was initiated at the same sites in the *CYC1* promoter as that found for the unstimulated

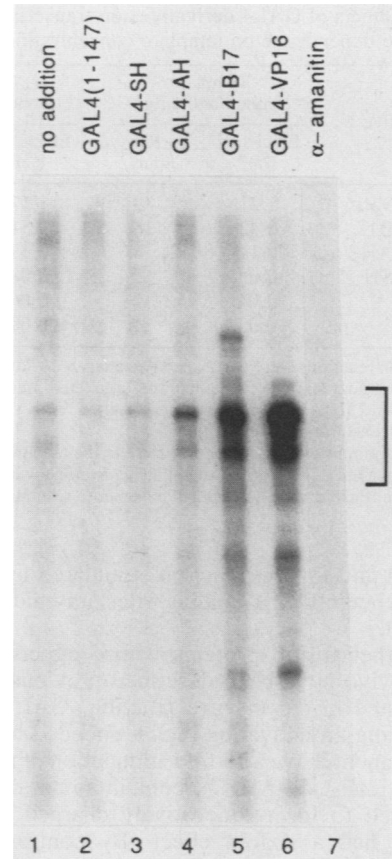

FIG. 1. Effects of GAL4 derivatives on yeast polymerase II transcription in vitro. Template pCZGAL, with a single GAL4-binding site upstream of the *CYC1* promoter, was transcribed in a nuclear extract. The major transcript, about 250 residues in length, and additional minor transcripts are indicated by the bracket beside the autoradiogram. GAL4 derivatives included in the reaction mixtures were GAL4(1-147) (lane 2), GAL4(1-147)-SH (lane 3), GAL4(1-147)-AH (lane 4), GAL4(1-147)-B17 (lane 5), and GAL4(1-147)-VP16 (lane 6). The products of a reaction with no added GAL4 derivative (lane 1) and of a reaction with template pCZ6GAL (see Table 2), GAL4(1-147)-VP16 protein, and alpha-amanitin at 10 μg/ml (lane 7) are shown for comparison.

TABLE 1. Effects of GAL4 derivatives on transcription in vitro and dependence on template concentration[a]

GAL4 derivative	Template concn of 0.1 μg		Template concn of 0.3 μg	
	Efficiency (%)[b]	Rel. trans.[c]	Efficiency (%)	Rel. trans.
GAL4(1-147)-VP16	1.31	49	1.17	14
GAL4(1-147)-B17	0.42	16	0.51	6
GAL4(1-147)-AH	0.11	4	0.18	2
GAL4(1-147)-SH	0.05	2	0.08	1
GAL4(1-147)	0.04	1	0.06	1
No addition	0.03	1	0.09	1

[a] Transcription reactions were performed and analyzed with 0.1 or 0.3 μg of pCZGAL. Autoradiograms were scanned and bracketed areas (Fig. 1) were integrated with an LKB Ultroscan XL densitometer.
[b] Number of transcripts per template.
[c] Rel. trans., Amount of transcription relative to the amount in the reaction mixture with no GAL4 derivative added. The average variation between values of relative transcription determined in separate reactions was less than 20%.

reaction (17) and for the reaction stimulated by other activation sequences (GRF1-binding sites, thymidine-rich element) (3, 16).

Several other fusion proteins whose effects upon transcription in vivo have been described previously (6, 7, 15, 18) were tested in the in vitro reaction. GAL4(1-147)-B17 (18), containing an activating region encoded by an *E. coli* genomic fragment, gave a 16-fold stimulation of transcription in vitro. GAL4(1-147)-AH (7), containing an activating domain designed to form a negatively charged amphipathic alpha helix, had a sixfold effect. By contrast, GAL4(1-147)-SH (7), with the residues of GAL4(1-147)-AH rearranged so an amphipathic helix could not be formed, stimulated transcription less than twofold and GAL4(1-147) (15), and DNA-binding domain of GAL4 alone, did not stimulate transcription (Table 1).

Dependence of activation on number of binding sites and on distance of binding sites from the TATA element. Templates for transcription that contained three and six GAL4-binding sites bound up to three and five equivalents, respectively, of the fusion protein GAL4(1-147)-AH in gel electrophoresis mobility shift assays (Fig. 2). Stimulation of transcription was increased two- to fourfold by incorporation of multiple GAL4-binding sites in the template (Table 2). For GAL4(1-147)-VP16 and six sites in the template, the stimulation was 110-fold, resulting in about 0.04 transcripts per template. The stimulatory effect decreased when the GAL4-binding sites were moved from 50 to 150 bp upstream of the TATA element, but the dependence on number of binding sites in the template was about the same as that for the templates with binding sites closer to the TATA element (Table 2). Similarly, activation by GAL4(1-147)-B17 increased with increasing number of binding sites and decreased when the GAL4-binding sites were moved further upstream of the TATA box (Table 2).

DISCUSSION

The mechanism by which activator proteins exert their effects on transcription will be most easily studied if large stimulatory effects can be observed in vitro. Such effects for mammalian extracts have been reported, and some aspects of the activation mechanism have emerged (4, 9–11, 13, 15, 23). The stimulatory effect presented here for a yeast nuclear extract is comparatively large and has the advantage that it is mediated by an activator available in milligram amounts.

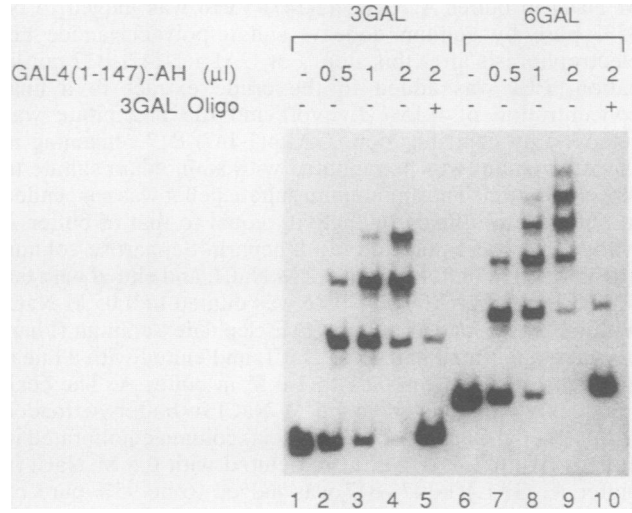

FIG. 2. Gel electrophoresis mobility shift assay showing the binding of multiple equivalents of GAL4(1-147)-AH to pCZ3GAL (lanes 1 to 5) and pCZ6GAL (lanes 6 to 10). Binding reaction experiments were performed with 0.5 (lanes 2 and 7), 1 (lanes 3 and 8), and 2 (lanes 4 and 5 and 9 and 10) μl of GAL4(-1-147)-AH (2.4 μg/ml). The reactions analyzed in lanes 5 and 10 contained 20 ng of 3GAL oligonucleotide. The probe with no additions was analyzed in lanes 1 and 6.

The order of effectiveness of the various activator proteins in the yeast system in vitro parallels the behavior of the proteins in both yeast and HeLa cells. GAL4(1-147)-B17 stimulated transcription three times more strongly in yeast than did GAL4(1-147)-AH, whereas GAL4(1-147)-SH and GAL4(1-147) had no effect (6, 7, 18). Other experiments show that GAL4(1-147)-VP16 is a stronger activator than GAL4(1-147)-B17 in yeast (A. Meikljohn and M. Ptashne, unpublished data). In HeLa cells, GAL4(1-147)-VP16 was 2 orders of magnitude more effective than GAL4(1-147)-B17, whereas GAL4(1-147) gave no stimulation (22).

When two of the activators were tested in HeLa nuclear extracts at similar protein and DNA concentrations, however, the relative stimulatory effects were not correlated with activation in vivo. Specifically, as previously reported, GAL4(1-147) activated transcription almost as well as did GAL4(1-147)-AH, an effect ascribed to a "cryptic" activat-

TABLE 2. Effect of number of GAL4-binding sites and distance from promoter on stimulation of transcription in vitro[a]

No. of sites	Distance (bp)	GAL4(1-147)-VP16		GAL4(1-147)-B17	
		Efficiency (%)[b]	Rel. trans.[c]	Efficiency (%)	Rel. trans.
1	50	1.48	42	0.70	20
3	50	2.84	82	2.12	61
6	50	3.81	110	2.66	77
1	150	0.56	16	ND	ND
3	150	1.01	29	ND	ND
6	150	1.20	35	0.70	20

[a] Reactions were performed and analyzed with 0.1 μg of template. For distances of about 50 and 150 bp between GAL4-binding sites and major TATA element, templates of the pCZ and pCT families, respectively, were used. Templates with three GAL4-binding sites were pCZ3GAL and pCT3GAL, templates with six binding sites were pCZ6GAL and pCT6GAL.
[b] Number of transcripts per template.
[c] See Table 1, footnote *c*.

ing region found between residues 75 and 147 (15). The greater fidelity of the yeast extract with regard to the order of effects of these two activators may reflect a mechanistic difference between the two transcription systems.

A growing number of gene activator proteins and their derivatives have been shown to be functionally interchangeable between yeast and mammalian cells (14, 19, 20, 24, 26). The present findings extend these observations by demonstrating transcriptional effects of some of the activators in vitro; the correlation of the effects with those seen in vivo points to faithful reproduction of the natural mechanism in vitro. It should be possible to elucidate this mechanism through genetic analysis and further studies of transcription in vitro.

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