reduced with the addition of formamide or urea. However, the SI5'3' precursor cleaved to an extent comparable with the SA1 RNA. Therefore, stable base-pairing in stem I was required for efficient self-cleavage activity.

A similar approach was taken for stem II. A SII5' mutation was made with a U17A and a C18G change (Fig. 1). This had only a moderate effect on the extent of cleavage in the absence of denaturants, but in the presence of formamide or urea the extent of cleavage was reduced (Fig. 3*c*). A SII3' mutation was made with a G82C and an A83U change. Again, a decrease in the amount of cleavage was observed which was exaggerated by addition of denaturants to the reactions. The SII5'3' variant restored the potential for base-pairing in stem II and resulted in similar levels of cleavage to that of the SA1-2 precursor. Cleavage of the SII5'3' precursor was not reduced by the addition of denaturants. Therefore, an intact and stable stem II facilitates self-cleavage in the presence of denaturants. This is consistent with the finding that sequences forming the 3' side of that stem are required only in formamide (Fig. 4). We propose that, under favourable conditions, the self-cleaving structure can form in the absence of stem II, but stem II can stabilize the self-cleaving structure. Stem II may therefore contribute to the phenomenon of denaturant-enhanced self-cleavage of certain transcripts[5] by stabilizing the self-cleaving structure under conditions where competing inactive structures are disrupted. □

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A mediator required for activation of RNA polymerase II transcription *in vitro*

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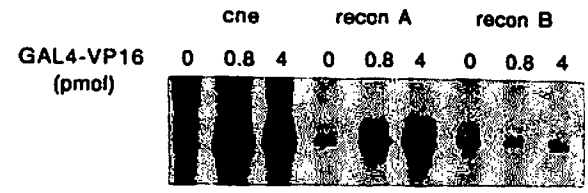
ACTIVATOR proteins bind to enhancer DNA elements and stimulate the initiation of transcription. It has been proposed[1–3] that activators contact general initiation factors at a promoter, and evidence for such direct interaction has been obtained[4–10]. Studies of transcription *in vitro*, however, have suggested that activators might function through an intermediary molecule(s) distinct from the general factors. In the first of these studies[11,12], we exploited the finding that one activator could inhibit transcription stimulated by a second activator (activator interference or 'squelching')[13–15]. This inhibition, which is attributed to competition between the activators for a common target factor, could not be relieved by addition of a large excess of general initiation factors, suggesting that the target for which activators compete is distinct from these factors. Similar conclusions came from the observation that TFIID's expressed from cloned genes[16–18] fail to replace partially purified 'natural' TFIID fractions in supporting activation, evidently because they lacked some component present in the impure fractions. While these lines of evidence for a novel 'mediator' of activation were negative, we also showed that a partially purified fraction from yeast would reverse activator interference[12]. This positive effect of a presumptive mediator provided an assay for its activity, but its role in activation was still only inferred. We now present direct evidence for a mediator which is required for stimulation of transcription *in vitro* by the activators GAL4–VP16 and GCN4, but which has no effect on transcription in the absence of activator protein.

For a compelling demonstration of a mediator between activator proteins and the transcription apparatus, the mediator must be resolved from all components of the transcription apparatus and shown to be required for activation. Efforts to achieve this using a mammalian transcription system have been unsuccessful[17], possibly because of interactions between the mediator and general initiation factors. We now find that mediator activity can be distinguished from that of general initiation factors in a yeast transcription system. Yeast extracts can be resolved into five fractions, designated a, b, c, d and e, all of which are required in addition to RNA polymerase II for initiation at a yeast or mammalian promoter[19]. Fraction d can be replaced by cloned, bacterially expressed yeast TFIID (yTFIID). Both the starting extract and the system reconstituted from the five fractions (Fig. 1, cne and recon A, respectively) are responsive to activator proteins. For example, the activator GAL4-VP16, consisting of the DNA-binding domain of yeast GAL4 protein fused to the acidic activation domain of human herpes VP16 (ref. 13), activates transcription 5.3-fold in the extract and 5.7-fold in the reconstituted system (Fig. 1). Further purification of fraction a eliminated the response of the reconstituted system to GAL4-VP16 (Fig. 1, recon B), but did not affect 'basal' transcription in the absence of GAL4-VP16. This suggests that a component required for response to GAL4-VP16 was removed during the purification of fraction a.

The response of the reconstituted system to GAL4-VP16 could be restored by the addition of a DEAE-Sephacel fraction shown previously[12] to relieve activator interference or 'squelching' by GAL4-VP16 (Fig. 2*a*). The effect was proportional to the amount of the DEAE fraction added within the range tested (Fig. 2*b*). In contrast, even at the highest levels tested, the DEAE fraction did not appreciably stimulate basal transcription (Fig. 2*b*), indicating that the fraction does not contain a general stimulatory factor whose effect is unrelated to the presence of activator. This also argues that the DEAE fraction does not merely supply a general initiation factor present in less than saturating amounts in the reconstituted system. Moreover, adding as much as ten times more of fractions a, b, e, yTFIID and RNA polymerase II to the reconstituted reaction had no effect on the level of transcription in the presence of GAL4-VP16, but without the DEAE fraction (Fig. 2*c*). This shows that all fractions were originally present in saturating amounts in the reconstituted system, and that none contains highly inhibitory material. We conclude that the DEAE fraction supplies a component(s) required for activation by GAL4-VP16 which we refer to as a mediator of activation.

The requirement for a mediator fraction for stimulated transcription in the reconstituted system provides a straightforward assay for mediator activity. Using this assay, the mediator has been enriched from the starting yeast extract about 1,000-fold (Fig. 3, left panel). An amount of purified fraction sufficient for activation contained barely detectable activities of general initiation factors or RNA polymerase (*6*, *11*, *0*, *0*, 8 and 3% of the amounts of factors a, b, c, yTFIID, e and RNA polymerase II, respectively, required for optimal transcription). As the mediator could be purified in a straightforward manner and the activity did not split in the course of extensive enrichment, it presumably

FIG. 1 Loss of response to the activator GAL4-VP16 during fractionation of a yeast RNA polymerase II transcription system. Transcription reactions contained 100 ng of pGAL4CG⁻, which has a GAL4-binding site upstream of the yeast *CYC1* promoter fused to a G-less sequence[22], GAL4-VP16 (ref. 23) in the amounts indicated, and 75 μg of crude yeast nuclear extract[12,24] (cne) or the following set of proteins (recon): fractions b (0.02 μg) and e (6 μg) from whole-cell extract and fraction c (3 μg) from nuclear extract, prepared as described[19] with modifications (see below), bacterially expressed yeast TFIID (ref. 25) (yTFIID, 0.4 μg), and pure yeast RNA polymerase II (ref. 26) (0.04 μg). Recon A contained fraction a (6 μg) prepared from whole-cell extract[19]; recon B contained Bio-Rex flow-through (1.5 μg) and 600 mM potassium acetate Bio-Rex eluate (1 μg) derived from fraction a (see below). Specifically initiated transcripts, processed as described[22], formed the major bands in each lane. Radioactivity in specific transcripts (determined with an AMBIS Radioanalytic Imaging System) obtained in the presence of GAL4-VP16 divided by that obtained in its absence (fold stimulation) was: for reaction with cne, 5.3 (0.8 pmol GAL4-VP16) and 3.5 (4 pmol); for recon A, 3.9 (0.8 pmol) and 5.7 (4 pmol); for recon B, 1.0 (0.8 pmol) and 1.0 (4 pmol). Details of fractions a, b and e preparations were as follows. Whole-cell extract was applied to DEAE-cellulose and eluted with 120 and 350 mM ammonium sulphate in buffer A as described[19]. The 350 mM eluate was dialysed against buffer A to the conductivity of buffer A containing 100 mM potassium acetate and applied to a Bio-Rex 70 column equilibrated in the same buffer. The column was washed in the same buffer and eluted with buffer A containing 375 and 600 mM potassium acetate.

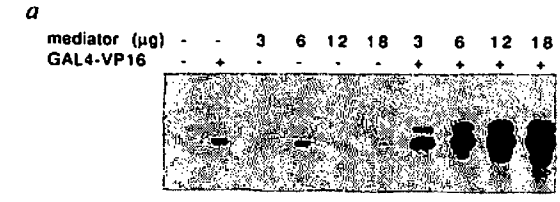


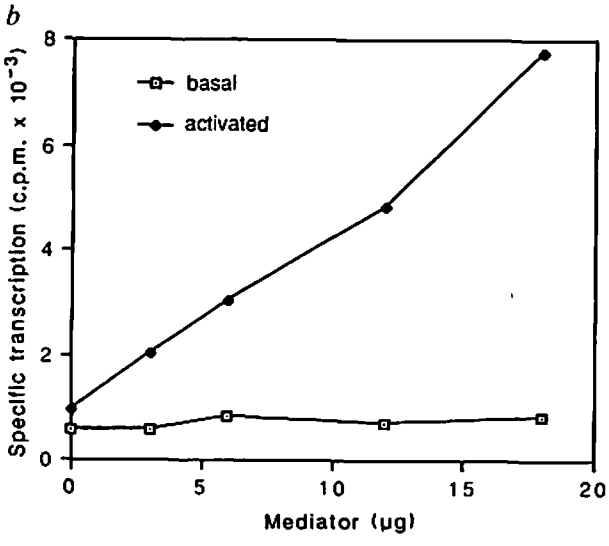
The flow-through and wash and the 600 mM eluate supplied fraction a activity in recon B. For fraction b, the 120 mM ammonium sulphate eluate from DEAE-cellulose was adjusted to 48% of saturation with ammonium sulphate. The precipitate was suspended in buffer A and passed through a Sephacryl S300 column. Fractions containing activity were pooled and dialysed against buffer A to the conductivity equivalent of buffer A containing 70 mM KCl and applied to a phophocellulose (P11) column (Whatman) equilibrated in the same buffer. The column was developed with a gradient of 70–700 mM KCl in buffer A, and fraction b activity eluted between 300 and 400 mM KCl. For fraction e, the 120 mM ammonium sulphate eluate from DEAE-cellulose was adjusted to 70% of saturation with ammonium sulphate. The precipitate was suspended in buffer A, dialysed to the conductivity of buffer A containing 100 mM potassium acetate, and applied to a P11 column equilibrated in the same buffer. The column was washed with buffer A containing 300 mM potassium acetate, and fraction e activity was eluted with buffer A containing 700 mM potassium acetate.

comprises one or a small number of components. The possibility that mediator activity resides in a dissociable subunit of one of the general initiation factors or even in a modified form of one of these factors is not excluded.

Two observations indicate that the mediator is a protein, or at least contains an essential protein component. The activity is heat-labile, with a sharply defined transition centred at about 42 °C, and it is abolished by treatment with proteinase K (data not shown).

As the mediator fraction described here is from yeast, and the activation domain of GAL4-VP16 is of human viral origin, it can be asked whether their interaction is physiologically significant. A yeast activator whose binding site is a thymidine-rich sequence seems to require the same mediator as GAL4-VP16, as its action is subject to interference by GAL4-VP16 (ref. 12). The thymidine-rich-binding activator has not been isolated, however, and little is known about it. GCN4 is a better characterized yeast activator which, like VP16, possesses an acidic activation domain and stimulates transcription in a yeast nuclear extract[20]. As with GAL4-VP16, stimulation by GCN4 is lost when the extract is fractionated, and restored when the mediator fraction is added back (data not shown).





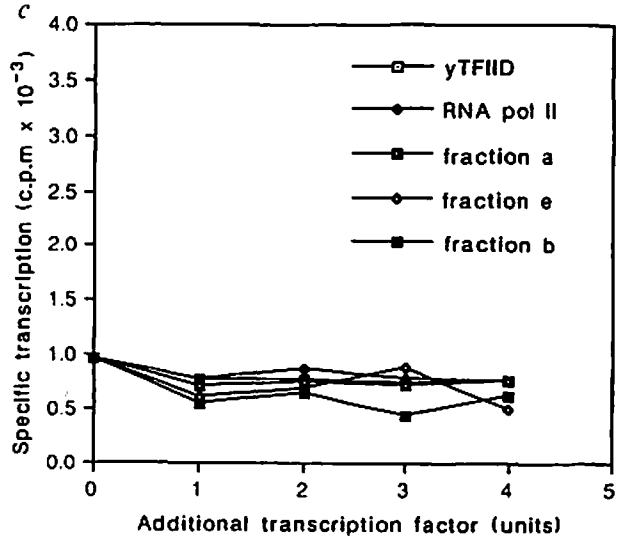


FIG. 2 Restoration of GAL4-VP16 effect on transcription by the addition of mediator. *a*, Transcription reactions as described for recon B in Fig. 1 contained 0.8 pmol of GAL4-VP16 (+) or not (−) and mediator fraction in the amounts indicated. Mediator fraction was the DEAE eluate shown previously to be active in the relief of interference or 'squelching' by GAL4-VP16 (ref. 12). *b*, Radioactivity in specific transcripts in *a* was determined as in Fig. 1 for reactions that contained GAL4-VP16 (activated) or not (basal). *c*, To recon B transcription reactions containing 0.8 pmol of GAL4-VP16 were added extra quantities of transcription factors in multiples of an arbitrary unit of protein defined as follows: fraction a (600 mM Bio-Rex eluate, 1.4 μg), b (0.6 μg), e (3 μg), yTFIID (0.4 μg), RNA polymerase II (0.04 μg).

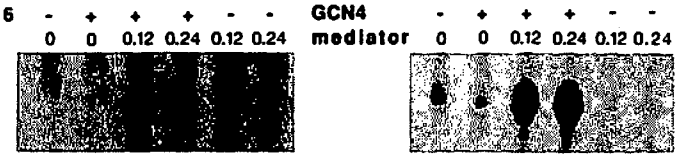


FIG. 3 Partially purified mediator supports activation by both GAL4-VP16 and GCN4. Transcription reactions as described for recon B in Fig. 1 contained (left panel) 100 ng pGAL4CG$^-$ and 0.8 pmol GAL4-VP16 or (right panel) 100 ng pS(GCN4)^{2}CG$^-$ (this template DNA contains two copies of a consensus GCN4-binding sequence[27] upstream of the yeast *CYC1* promoter fused to a G-less cassette in pSP73 (Promega)) and 0.64 pmol homogeneous GCN4. Amounts of mediator fraction added to the various reactions are indicated in μg. The mediator fraction was derived from yeast whole-cell extract by conventional chromatography on cation and anion exchangers[19], and HPLC on a Bio-Gel SP-5-PW column, as follows. The Bio-Gel SP column was loaded with 25 mg protein in 0.1 M potassium acetate and developed with a linear gradient of 0.2–0.6 M potassium acetate in buffer A. The increase in specific activity of the mediator was estimated to be 10-fold, 12-fold, and 9-fold for the three chromatographic steps, giving an overall enrichment of 1,080-fold from the starting extract.

Although the mediator fraction used in these experiments was the relatively crude DEAE fraction described above (Fig. 2, and ref. 12), two observations suggest that GAL4-VP16 and GCN4 use the same mediator. First, mediator activity enriched some 1,000-fold on the basis of activation by GAL4-VP16 also supports activation by GCN4 (Fig. 3, right panel). Second, the two activators interfere with each other (Fig. 4) when increasing amounts of GCN4 (or GAL4-VP16) are added to reactions containing a constant level of GAL4-VP16 (or GCN4) and a template with a single GAL4-binding site (or a template with a pair of GCN4-binding sites). The amount of GCN4 required to cause 50% inhibition of transcription dependent on GAL4-VP16 was ~20-fold greater than the amount of GAL4-VP16 required in the reciprocal experiment, perhaps reflecting a lower affinity of GCN4 for the mediator.

The evidence that a single mediator supports the effects of both GAL4-VP16 and GCN4 raises the possibility that this mediator is required for transcriptional enhancement by all acidic activators. It has been suggested that different mediators are needed by other types of activation domain, such as glutamine- or proline-rich domains[17]. Further studies should reveal whether this is the case, and whether or not the classification of activation domains on the basis of amino-acid composition is appropriate. These studies may be facilitated by the recent development of assays for mediators of activation by GAL4-VP16 (P. Chambon, personal communication) and by USF, Sp1, and NFκB (R. Roeder, personal communication) in the HeLa transcription system.

We have not demonstrated a direct interaction between the activation domains of GAL4-VP16 and GCN4 and the mediator, and even if these interactions occur, there may be additional direct contacts between activation domains and general initiation factors or RNA polymerase. For example, the data presented here are not incompatible with evidence for direct activator-TFIID interaction[4-9].

If mediator is the target of an activation domain, what molecule does the mediator act on to influence transcription? The amino-terminal domains of human and *Drosophila* TFIIDs seem to be required for activation by Sp1 but not for basal level transcription in the HeLa transcription system[18]. Similarly, the amino-terminal domain of yeast TFIID is not required for basal transcription in the HeLa system[21]. But a truncated form of yeast TFIID lacking the amino-terminal domain supports activated transcription in the yeast system *in vitro* (R.J.K., P.M.F., D. I. Chasman and R.D.K., manuscript in preparation). The amino-terminal domain of yeast TFIID is thus ruled out as a target, leaving the conserved core of TFIID or other general factors as possible molecules with which the mediator interacts to influence transcription. □

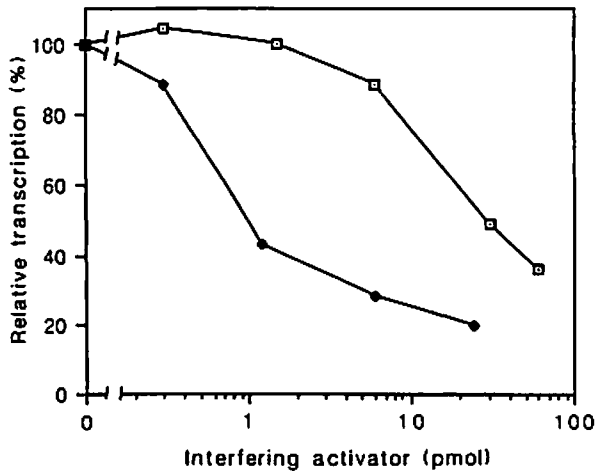


FIG. 4 Activator interference between GAL4-VP16 and GCN4. Transcription reactions with pS(GCN4)^{2}CG$^-$ (100 ng) contained an amount of GCN4 protein that caused maximal activation (1.5 pmol), the amounts of GAL4-VP16 (interfering activator) indicated, GAL4-binding oligonucleotide[23] (250 ng, 24 pmol), and cne (64 μg) (■). Reactions with pGAL4CG$^-$ (100 ng) contained an amount of GAL4-VP16 that caused maximal activation (1.2 pmol), the amount of GCN4 (interfering activator) indicated, GCN4-binding oligonucleotide[27] (250 ng, 24 pmol), and cne (64 μg) (□). Radioactivity in specific transcripts, determined as for Fig. 1, is expressed as a percentage of that obtained in the absence of interfering activator.

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