Direct activation and anti-repression functions of GAL4–VP16 use distinct molecular mechanisms

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In order to determine whether the molecular mechanisms used for direct activation by GAL4–VP16 are the same as those used for anti-repression, we have employed monoclonal antibodies specific for the VP16 activation domain. In the absence of added repressors, GAL4–VP16 was able to stimulate transcription from a template containing GAL4-binding sites, and the antibodies raised against the VP16 activation domain failed to inhibit this direct activation. GAL4–VP16 also was able to prevent histone H1-mediated repression by a mechanism that was strongly dependent on the presence of specific GAL4-binding elements in the promoter. However, in contrast to the assays conducted in the absence of repressors, the antibodies were strong inhibitors of GAL4–VP16-activated transcription in the presence of histone H1. Thus the binding of the antibodies distinguished between the direct activation and anti-repression

functions of GAL4–VP16, indicating that these functions operate through distinct molecular mechanisms. The anti-repression-specific mechanism that is inhibitable by the antibodies acted at an early stage of preinitiation complex formation. Deletions of individual subdomains of the VP16 activation domain demonstrated that there was not a discrete subdomain responsible for the anti-repression function of GAL4–VP16. Thus, the inhibitory effect of the antibodies appeared to be due to the location of the epitope within the activator protein rather than to some inherent biochemical property of that region of the protein that is required specifically for anti-repression. The inhibitory effect of the antibodies also ruled out the possibility that steric exclusion of repressor proteins from the promoter was the sole means of anti-repression by the transcriptional activator.

INTRODUCTION

The transcription of genes in eukaryotic cells by RNA polymerase II is controlled through a balance of repression and activation. During the activation of genes *in vivo*, nucleosomes and histone H1 are removed or structurally altered, leaving key sites of the promoter and enhancer regions accessible to regulatory proteins [1–9]. Experiments performed *in vitro* have demonstrated that nucleosomes and H1 are repressors of transcription and that transcriptional activator proteins are able to overcome this repression in a pre-emptive manner [10,11]. The chimaeric transcription factor, GAL4–VP16, is a potent activator of transcription, both *in vivo* and *in vitro* [12–17]. This property of activation can manifest itself in two ways: as direct activation (or 'true activation' [18]), in which transcription is stimulated in the absence of repressors; and as anti-repression, in which transcription is protected from the inhibitory effects of repressors [18–21]. The repressors may be chromatin or non-chromatin proteins [18,19,22–26].

The molecular mechanisms by which transcriptional activators can prevent repression by chromatin constituents, such as histones, are poorly understood at this stage. In particular, it is not clear whether an activator anti-represses by the same mechanisms as those by which it directly activates transcription. Both paths of activation require cofactors, in addition to the activator and the general factors required for basal levels of transcription [21,27–29]. A simple model can be invoked that explains both paths of activation in terms of a common mech-

anism: that of the activator protein facilitating transcription factor (TF) IID binding to the promoter [10,20,30,31]. In the absence of repressors, this facilitation results in active TFIID complexes being better able to compete with other TATA-binding protein (TBP)-containing complexes for occupancy of the TATA-box region of the promoter. In the presence of repressors, it also enables TFIID to compete more efficiently with the repressors for binding to the TATA box. In both modes of activation, the bound TFIID then acts as a nucleus for preinitiation complex formation by binding the other general transcription factors.

In the present study, we have employed monoclonal antibodies (mAbs) to determine that the VP16 activation domain uses molecular interactions for anti-repression that are distinguishable from those used for direct activation. At the same time, the experiments demonstrate that steric hindrance of repressor–DNA binding by sheer molecular bulk of the activator is not sufficient to explain anti-repression.

EXPERIMENTAL

Transcription assays were performed at 25 °C, in a volume of 20–100 µl of the following buffer: 20 mM Tris/HCl, 140 mM KCl, 10 mM NaCl, 2.5 mM MgCl₂, 1 mM spermidine, 0.1 mM EDTA, 0.5 mM dithiothreitol, pH 8.0. The dithiothreitol was added to components from a freshly prepared stock solution just prior to the commencement of each assay. The higher-than-usual ionic strength was chosen to approximate physiological

Abbreviations used: TF, transcription factor; TBP, TATA-binding protein; mAb, monoclonal antibody; HepUG, heparin–Ultragel; H1, histone H1.

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conditions. mAbs, when used, were preincubated for 30 min at a concentration of 1 mg/ml with GAL4-VP16 (5 µg/ml) in one fifth of the final assay volume. General transcription factors [heparin-Ultrogel (HepUG)(0.6) fraction, unless stated otherwise] and H1 (2 µg/ml in final volume) or other repressors were then added, and preinitiation complex formation was begun by the addition of the DNA template (5 µg/ml in final volume). The template was 17₅·TATA-CAT6, unless otherwise stated. After 30 min, nucleoside triphosphates were added to a final concentration of 0.5 mM each, and transcription was allowed to proceed for 60 min. The RNA transcripts were detected by quantitative primer extension analysis [32].

The plasmid transcriptional templates were based on TATA-CAT6. TATA-CAT6 was constructed by inserting the *NcoI*-*SalI* fragment of TATA-CAT [13], containing the adenovirus major late promoter, into the corresponding sites of pBLCAT6 [33]. 17₅·ERE·TATA-CAT6 and 17₅·TATA-CAT6 were constructed similarly, except that synthetic oligonucleotides containing one (CTAGCGGAGGACTGTCCTCCGTGCCGAGGTCACTGTGACC) or two GAL4-binding elements (CTAGCGGAGGACTGTCCTCCGTGCCGAGGACTGTCCTCCG), respectively, had been inserted between the *XbaI* and *BglII* sites. In order to make 17₅·TATA-CAT6, the *BglII*-*BglII* fragment of 17M5pAL7 [34], containing five tandem GAL4-binding elements, was inserted into the *BglII* site of TATA-CAT6. The expression plasmids for mutant GAL4-VP16 proteins were derived from the GAL4-VP16/pET3c plasmid [34], as follows. GAL4-VP16(F442A)/pET3c was constructed by excising the *SstI*-*KpnI* fragment of GAL4-VP16/pET3c and replacing it with the corresponding fragment of a GAL4-VP16 coding region in which the phenylalanine at residue 442 had been changed to an alanine by site-directed mutagenesis, yielding plasmid GAL4-VP16(F442A)/pET3c. The *SstI*-*KpnI* fragment, including the coding region for the carboxy end of the VP16 activation domain, was subcloned into the corresponding sites of pBluescript IISK⁺ (Stratagene), giving VP16(SK)/BIISK⁺. VP16(SK;F442A)/BIISK⁺ was created by subcloning the *SstI*-*KpnI* fragment of GAL4-VP16(F442A)/pET3c, which contains the F442A mutation, into pBluescript IISK⁺. The coding region for VP16 amino acids 456–490 was removed by excising the *AvaI*-*KpnI* fragments of VP16(SK)/BIISK⁺ and VP16(SK;F442A)/BIISK⁺ and inserting synthetic oligonucleotides of sequence CCGGGGCCGTAGTAATGAGCTAGCGGTAC, giving plasmids VP16(SK;Δ456–490)/BIISK⁺ and VP16(SK;Δ456–490;F442A)/BIISK⁺ respectively. The coding region for VP16 amino acids 435–452 was removed by excising the *SphI*-*XmaI* fragment of VP16(SK)/BIISK⁺, filling in with T4 DNA polymerase and self-ligating, creating plasmid VP16(SK;Δ435–452)/BIISK⁺. Finally, the *SstI*-*KpnI* fragments of plasmids VP16(SK;Δ456–490)/BIISK⁺, VP16(SK;Δ456–490;F442A)/BIISK⁺ and VP16(SK;Δ435–452)/BIISK⁺ were subcloned back into the corresponding restriction sites in GAL4-VP16/pET3c, giving plasmids GAL4-VP16(Δ456–490)/pET3c, GAL4-VP16(Δ456–490;F442A)/pET3c and GAL4-VP16(Δ435–452)/pET3c respectively.

GAL4-VP16 wild-type and mutant proteins were expressed in *Escherichia coli* as described previously [34]. They were purified chromatographically, as follows. Cell lysates were applied by gravity feed to a 5 ml column of DEAE-Sephacel (Pharmacia) equilibrated in 20 mM Tris/HCl/1 mM EDTA/0.5 mM dithiothreitol/8.7% glycerol (pH 8.0) (column buffer), containing 0.1 M NaCl. The column was washed with column buffer containing 0.1 M NaCl and eluted sequentially with column buffer containing 0.2 M, 0.4 M and 1 M NaCl. The GAL4-VP16 proteins eluted in both the 0.2 M and 0.4 M NaCl fractions,

which were used for further purification on a 1 ml sequence-specific DNA-affinity column composed of concatemeric 17-mer GAL4-binding elements linked to cyanogen bromide-activated Sepharose (Pharmacia) [35]. The purified proteins were > 90% pure, as determined by gel electrophoretograms and Western blots stained with specific antibodies.

Crude nuclear extracts were prepared from HeLa cells as described previously [36], up to the point of (NH₄)₂SO₄ precipitation. The HepUG(0.6) fraction used routinely for transcription assays was obtained by precipitating the nuclear extract with 2 M (NH₄)₂SO₄, redissolving the precipitate and fractionating it on a column of HepUG (IBF) as described previously [37], except that the column and precipitate were equilibrated in buffer containing 0.24 M KCl. H1 was purified from the chromatin pellets derived from HeLa cell nuclear extracts either by using CM-Sephadex (Pharmacia) [38], or by extraction with 0.5 M NaCl followed by precipitation of the DNA and bound nucleosomes with 10% poly(ethylene glycol) 6000. Both methods gave equivalent results. mAbs were purified from mouse ascites fluid by precipitation with caprylic acid and (NH₄)₂SO₄ [39]. mAb B10, specific for the N-terminal activation domain of the human oestrogen receptor [40], was used as a control antibody.

RESULTS

In order to investigate the relationship between direct activation and anti-repression, we have employed monoclonal antibodies directed against the VP16 activation domain [41] in transcription assays performed *in vitro*. The ability of GAL4-VP16 to activate transcription in the presence of mAbs was dependent upon the source of general transcription factors that were used. When a crude nuclear extract was used as a source of general transcription factors, the basal level of transcription was very low but was stimulated strongly by GAL4-VP16 (Figure 1a, lanes 7 and 8). The mAb, 5GV7, which binds specifically to VP16 amino acids 441–452, was inhibitory to GAL4-VP16-stimulated transcription (Figure 1a, compare lanes 8 and 12), in agreement with assays performed previously using a whole-cell extract [41]. However, when the general transcription factors were supplied by the HepUG(0.6) fraction, a partially purified preparation that supports both basal and activated transcription [34,37], two differences became apparent in the transcription assays. Firstly, the basal level of transcription was considerably higher than when nuclear extract was used (Figure 1a, compare lanes 1 and 7). Secondly, 5GV7 was no longer inhibitory to stimulation by GAL4-VP16 (Figure 1a, compare lanes 2 and 6). In fact, the transcriptional activity that was stimulated by GAL4-VP16 was often slightly super-stimulated by the mAbs, perhaps by stabilization of dimers. A control mAb had no inhibitory effect on either basal or activated transcription, whereas the effects of mAbs 2GV4 and 5GV2, which bind to the same epitope as 5GV7, were the same as those of 5GV7 (Figure 2c and results not shown).

This parallel between a decreased level of basal transcription and an increased susceptibility of GAL4-VP16 to inhibition by the mAbs suggested that repressor proteins in the crude nuclear extract that were separated from general transcription factors during preparation of the HepUG(0.6) fraction may determine both phenomena. Repressors that were present in the crude nuclear extract that were not present in the HepUG(0.6) fraction were identified by a complementation procedure. It has been reported that the supernatants of (NH₄)₂SO₄ precipitations of nuclear extracts contain high levels of repressor activity [18]. In agreement with that report, the addition of the (NH₄)₂SO₄ supernatant to the HepUG(0.6) fraction repressed basal tran-

Figure 1 Monoclonal antibodies inhibit anti-repression, but not direct activation, by GAL4-VP16

(a) Primer extension analysis of *in vitro* transcripts, showing the resistance of GAL4-VP16 to inhibition by mAbs in HepUG(0.6). GAL4-VP16 was preincubated with buffer only (—), a control mAb (Con) or a mAb directed against the VP16 activation domain (5GV7). The mixture was then added to transcription assay reactions containing partially purified general transcription factors [HepUG(0.6)] or a crude nuclear extract, and the specific RNA transcripts were analysed by primer extension as described in the Experimental section. (b) Transcription assay demonstrating the repression of basal transcription by nuclear extract fractions. Fractions of nuclear extract obtained by $(\text{NH}_4)_2\text{SO}_4$ precipitation and heparin-affinity chromatography were dialysed against assay buffer and added to general transcription factors [the HepUG(0.6) fraction, derived from 7 μl of crude nuclear extract], which were then assayed for transcriptional activity. Five quantities of each fraction were tested, derived from 40 μl (lanes 1, 6 and 11), 20 μl (lanes 2, 7 and 12), 10 μl (lanes 3, 8 and 13), 5 μl (lanes 4, 9 and 14) or 2.5 μl (lanes 5, 10 and 15) of crude nuclear extract. A control reaction contained HepUG(0.6) only (lane 16). Abbreviation used: sn, supernatant. (c) Dependence of anti-repression by GAL4-VP16 on the number of specific elements in the promoter. Transcriptional templates containing 0, 1, 2 or 5 GAL4-binding elements were incubated with HepUG(0.6) containing H1 (200 ng per 500 ng of DNA template) and/or GAL4-VP16, as indicated. The plasmids were TATA-CAT6 (0 elements), 17·ERE-TATA-CAT6 (1 element), 17₂·TATA-CAT6 (2 elements) and 17₅·TATA-CAT6 (5 elements). (d) mAb 5GV7 inhibits anti-repression by GAL4-VP16 in a dose-dependent manner. GAL4-VP16 was preincubated with various amounts of mAb 5GV7 before its inclusion in the transcription assay. The mAb can be seen to reduce the level of transcription in the presence of H1 (lanes 9–12), but not in its absence (lanes 3–6). (e) H1 represses transcription in a dose-dependent manner. Various amounts of H1 were added to the general transcription factors and 500 ng of 17₅·TATA-CAT6 in the absence (lanes 1–12) or presence (lanes 13–24) of GAL4-VP16. The preincubation of GAL4-VP16 with mAb 5GV7 (lanes 6–12 and 19–24) can be seen to reduce its effectiveness as an anti-repressor.

scription in a dose-dependent manner, whereas the 0.24 M KCl and 2.0 M KCl eluants from the heparin-affinity column did not (Figure 1b). GAL4-VP16 was able to overcome this repression.

Histone H1 is the major repressor in the $(\text{NH}_4)_2\text{SO}_4$ supernatant fraction, being present in the nucleus at levels that exceed the available DNA binding sites [18]. In order to test the hypothesis that H1 in the crude nuclear extract could be responsible for both the decreased level of basal transcription and the increased susceptibility of GAL4-VP16 to inhibition by the mAbs, purified H1 was included during preinitiation complex formation in transcription assays utilizing HepUG(0.6) as the source of general transcription factors. GAL4-VP16 was able to activate transcription in the absence of H1, a low level of activation being observed even from a template containing no GAL4-specific binding sites (Figure 1c, lanes 5–8). The addition of H1 to transcriptional templates had two effects. As expected from previous studies [18], H1 repressed transcription in the absence of GAL4-VP16 (Figure 1c, compare lanes 1–4 with 9–12). H1 also made the activation by GAL4-VP16 more dependent on the presence of GAL4-specific binding sites, there being no activation from the template lacking these sites (lane 13)

and a steeply increasing level of activation as the number of responsive elements increased (compare lanes 13–16 with 9–12). Phosphorescent imaging demonstrated that the degree of activation of transcription by GAL4-VP16 on templates containing 0, 1, 2 and 5 elements changed from 1.3-, 3.5-, 2.3- and 6.2-fold, respectively, in the absence of H1 (lanes 5–8) to 0.9-, 7.2-, 21- and 85-fold, respectively, in the presence of H1 (lanes 13–16). Repression by deposition of nucleosomes on the templates had a similar effect on activation (results not shown).

The inhibition of basal transcription by H1 was dose dependent (Figure 1e, lanes 1–6). As little as 100 ng of H1 per 500 ng of DNA template caused significant inhibition, while 200 ng per 500 ng of DNA abolished almost all basal transcription. In contrast, when GAL4-VP16 was present during preinitiation complex formation, H1 caused no noticeable inhibition when present at up to 200 ng per 500 ng of DNA, and significant levels of transcripts were detected even when 500 ng of H1 per 500 ng template were used (Figure 1e, lanes 13–18). However, when mAb 5GV7 was present during preinitiation complex formation, GAL4-VP16 could no longer overcome the H1-mediated repression. This inhibitory effect of the mAb was noticeable at low

Figure 2 GAL4-VP16 mutants lacking either subdomain of the VP16 activation domain retain anti-repression activity

(a) Comparison of the VP16 activation domain sequences of the wild-type and four mutant forms of GAL4-VP16. Also shown is the corresponding sequence of the VP16 gene isolated from a different strain of herpes simplex virus (Strain 2); vertical bars represent identity between the two strains, whereas colons represent conservative substitutions. The regions corresponding to the epitope recognized by the inhibitory mAbs and to subdomains 1 and 2 are indicated by lines below the sequences. Amino acid substitutions are in bold typeface. Internal deletions are represented by hyphens. Sequences are from [61]. (b) Western blot analysis of wild-type and mutant forms of GAL4-VP16 proteins. Molar equivalents of purified forms of GAL4-VP16 were subjected to SDS/PAGE and electroblotted onto nitrocellulose. Replicate blots were analysed for reactivity with mAb 2GV3 (lanes 1–5), which binds to the GAL4 DNA-binding domain, and mAb 2GV4 (lanes 6–10), which binds to subdomain 1 in the VP16 activation domain. (c) Transcription assay demonstrating the retention of anti-repression by mutants of GAL4-VP16. Equivalent amounts of wild-type or mutant forms of GAL4-VP16, or buffer only (None), were preincubated with a control mAb (Con) or one of the three inhibitory mAbs (2GV4, 5GV2 and 5GV7), as indicated. H1, HepUG(0.6) and 17_s·TATA-CAT6 transcriptional template (500 ng) were added, and the transcription assay was continued as in Figure 1. In control reactions, GAL4-VP16 was omitted (lane 2) and both GAL4-VP16 and H1 were omitted (lane 1).

concentrations of H1 (Figure 1e, compare lanes 20 and 21 with 14 and 15 respectively), but more pronounced at higher concentrations: when 300–500 ng of H1 were used per 500 ng of DNA, significant levels of transcription were retained in the presence of GAL4-VP16 (Figure 1e, lanes 16–18), but were abolished when mAb 5GV7 was included (Figure 1e, lanes 22–24). mAb 5GV7 did not inhibit basal transcription (compare lanes 1 and 7 in Figure 1e; see also Figure 1a), or GAL4-VP16-mediated direct activation in the absence of added repressor (Figure 1e, compare lanes 13 and 19). This inhibitory effect of the mAb in the presence of H1 was dose-dependent (Figure 1d) and was shared by mAbs 2GV4 and 5GV2 (Figure 2c). Thus purified H1 was able to restore the susceptibility of GAL4-VP16 to inhibition by the mAbs.

These results have clear implications for the relationship between direct activation and anti-repression by GAL4-VP16.

In HepUG(0.6) there were very low levels of repressors, as evident from the considerable levels of basal transcription. Thus, under those conditions, GAL4-VP16 was stimulating transcription primarily by direct activation. However, when repressors were present, either in crude nuclear extract or by the addition of purified H1 to the HepUG(0.6) fraction, GAL4-VP16 was stimulating transcription by a mechanism that relied on anti-repression. Taken together, the results above show that the mAbs had no effect on the direct activation of transcription by GAL4-VP16, but were able to inhibit its anti-repression function. Thus the molecular mechanisms that are used by GAL4-VP16 for direct activation are at least partially distinct from those used for anti-repression, and the mAbs can be used to distinguish those two mechanisms.

This ability of the mAbs to distinguish between the direct activation and anti-repression functions of GAL4-VP16

suggested that they prevent the VP16 activation domain from interacting with a target molecule that is not required for direct activation but is required for its anti-repression effect. One explanation for the distinct effects of the mAbs on direct activation and anti-repression is that the VP16 activation domain is composed of two distinct sub-domains: one subdomain that includes the epitope recognized by the mAbs and is responsible for anti-repression, and a second subdomain that is responsible for direct activation but not anti-repression. Comparison of the amino acid sequences of VP16 genes isolated from two different strains of herpes simplex virus reveals two highly conserved regions within the activation domain (amino acids 435–453 and 465–492; subdomains 1 and 2 respectively) separated and flanked by less conserved amino acids (Figure 2a). Highly conserved amino acids 416–431 were not considered to be part of an activation subdomain, as they are expendable for transcriptional activation [42]. The epitope to which the inhibitory mAbs bind is in subdomain 1 and includes the phenylalanine at VP16 residue 442, an amino acid reported to be extremely sensitive to mutations [43]. We postulated that subdomain 1 is responsible for the anti-repression effect by taking part in a molecular interaction that is specific to anti-repression and is inhibitable by the mAbs, whereas subdomain 2 contributes to direct activation, but not to anti-repression.

We tested this hypothesis by analysing four GAL4–VP16 mutants that had deletions of each subdomain and/or a substitution within subdomain 1 (Figure 2a): Δ 435–452, in which subdomain 1 was deleted; Δ 456–490, in which subdomain 2 was deleted; F442A, in which phenylalanine 442 was replaced with an alanine in a full-length activation domain; and Δ 456–490;F442A, in which subdomain 2 was deleted and phenylalanine 442 was replaced with an alanine.

The mutant proteins were purified from *E. coli*, and then tested for reactivity with mAbs in Western blots and for direct activation and anti-repression in transcription assays. All five proteins were recognized in Western blots by mAb 2GV3 (Figure 2b), which binds specifically to the GAL4 DNA-binding domain [41]. The mutant Δ 435–452, which lacks the epitope recognized by the mAbs that inhibit anti-repression, did not react with inhibitory mAb 2GV4 in Western blots, whereas the other mutants did react with this mAb, albeit to varying extents (Figure 2b, lanes 6–10). The Δ 456–490 mutant, in which the C-terminus of the VP16 activation domain was truncated close to the epitope, showed somewhat reduced reactivity with mAb 2GV4 when compared with the wild type. The introduction of the F442A point mutation, which lies within the epitope in the wild-type and Δ 456–490 proteins, caused a marked reduction in their affinity for mAb 2GV4, although they could still be detected in a Western blot (Figure 2b, compare lane 6 with lane 7, and lane 9 with lane 10). The two other inhibitory mAbs, 5GV2 and 5GV7, had staining specificities in Western blots that were identical to that of 2GV4, although the bands in each case were less strong than with 2GV4 (results not shown).

In order to determine whether the introduction of mutations had destroyed the direct activation and anti-repression activities of GAL4–VP16, the mutant proteins were added to transcription assays. Although the addition of H1 repressed basal transcription (Figure 2c, compare lanes 1 and 2), wild-type GAL4–VP16 was able to overcome this repression (lane 3), consistent with previous experiments. However, deletion of subdomain 1 (Δ 435–452; lane 13) did not diminish this anti-repression effect greatly, demonstrating that its presence in GAL4–VP16 was not required for anti-repression. Similarly, the mutant deleted for subdomain 2 (Δ 456–490) retained its anti-repression activity (lane 18). The introduction of the F442A mutation did not ablate anti-re-

pression, in the context of either the full-length GAL4–VP16 or the Δ 456–490 truncation (compare lanes 3 and 18 with lanes 8 and 23 respectively). Like the wild type, the four mutants were capable of direct activation of transcription in the absence of H1 and were not inhibited from doing so by mAbs (results not shown). Thus all four mutants retained the ability to activate transcription through both pathways: anti-repression and direct activation.

The anti-repression activity of the mutants remained sensitive to inhibition by the mAbs for those that retained strong immunoreactivity. The inhibitory effect of the mAbs was diminished in the cases of 5GV2 and 5GV7, but was retained by the higher-affinity mAb, 2GV4, which was able to inhibit anti-repression by wild type, F442A and Δ 456–490 (Figure 2c). Mutants Δ 456–490;F442A and Δ 435–452, which reacted with the mAbs poorly and not at all, respectively (Figure 2b), were refractory to the mAbs (Figure 2c). From the ability of the mutant lacking the epitope (Δ 435–452) to anti-repress, we can conclude that anti-repression cannot be assigned to one particular region of the VP16 activation domain, and that the ability of the mAbs to distinguish anti-repression from direct activation must rely on the location of the epitope in the activation domain, rather than on some inherent biochemical activity of the region encompassing that epitope.

DISCUSSION

The results of this study show that the molecular mechanisms used by GAL4–VP16 for anti-repression are distinct from those used for direct activation. The ability of specific mAbs to inhibit anti-repression, but not direct activation, allowed us to discriminate between these two modes of activation of transcription. Some aspects of the nature of the molecular event that is specifically inhibited by the mAbs can be deduced from the results of this and a previous study.

One hypothesis that could have explained the specificity of the mAbs for inhibiting anti-repression was that separate sub-domains of the VP16 activation domain are responsible for the separate functions of anti-repression and direct activation. The existence of two subdomains was postulated previously [44], although no functions were assigned to them. Despite early results that were consistent with this hypothesis, further experiments discounted it, since the elimination of the putative anti-repression region (subdomain 1) by deletional mutagenesis did not eliminate the ability of GAL4–VP16 to anti-repress. Thus, it is the location of the epitope in the VP16 activation domain that seems to be important to the inhibitory effect of the mAbs, since replacing subdomain 1 at that position with subdomain 2 yielded a protein (Δ 435–452) that retained its ability to anti-repress, but was no longer susceptible to inhibition by the mAbs (Figure 2c). By binding to that region of the wild-type GAL4–VP16 that is proximal to the DNA-binding domain, the mAbs were able to interfere with an interaction between the VP16 activation domain and some other transcription factor that is required for the anti-repression effect. The identity of the factor with which GAL4–VP16 interacts is not known, but it could be a factor that has been identified previously as interactive with the VP16 activation domain, such as TFIID and TFIIB [17,45–47], or perhaps even H1 itself.

Our finding that direct activation and anti-repression by GAL4–VP16 are mediated by non-identical molecular mechanisms is fundamental to the view of how transcriptional activators work. The ability of activators such as GAL4–VP16 to facilitate the recruitment of TFIID and TFIIB to the promoter, forming a template-committed complex, is believed to account

for the direct activation of transcription that occurs in the absence of repressors. The TFIID that is recruited must be present as an appropriate complex of proteins: TBP alone is not sufficient [15,16,27,28,34,48–51]. By selectively recruiting active TFIID complexes to the TATA box of the RNA polymerase II-transcribed gene, thereby excluding inactive TBP-containing complexes such as RNA polymerase I and III transcription factors [31,52] and TFIID complexes specific for other activators [53], there is a greater chance of an RNA polymerase II preinitiation complex forming. This TFIID complex that has been recruited to the promoter by GAL4–VP16 is able, in turn, to recruit other essential RNA polymerase II general factors [41,49]. It has been shown that TFIID is required for anti-repression by GAL4–VP16, also, and a simple model based on competitive binding could explain the anti-repression effect: GAL4–VP16, in concert with cofactors [21,28,29], facilitates the binding of TFIID to the TATA box; as a result, the TBP is better able to compete with repressors for occupancy of that region of the promoter. However, the ability of the mAbs to distinguish between the direct activation and anti-repression functions of GAL4–VP16 shows that the molecular mechanisms required for the two phenomena are not identical, and some additional complexity must be introduced for the model to be consistent with this observation and with the existence of the GAGA factor, an activator that apparently lacks a direct activation function [18]. Two alternatives are possible. Firstly, the competition model may hold true, and the anti-repression effect of GAL4–VP16 occurs solely by enhancing the recruitment of active TFIID, thereby enabling it to out-compete H1 for occupancy of the TATA box. In this case, the mechanism for the enhanced recruitment of TFIID to the promoter in the presence of H1 must include a mAb-sensitive step that is not required in the absence of that repressor; thus, at least two distinct pathways would have to exist through which GAL4–VP16 facilitates TFIID binding to the promoter. In the second alternative, the anti-repression effect may require some molecular mechanism in addition to recruitment of active TFIID for keeping the promoter accessible to the transcription machinery in the presence of repressors, and it is this additional mechanism that is inhibited by the mAbs.

We can deduce some characteristics of the mAb-inhibitable molecular event that is specific to anti-repression by re-examining the results of previous studies in the light of the results of the present study. In a previous study, the mAbs were used in kinetic experiments to block the action of GAL4–VP16 at discrete times during preinitiation complex formation, in order to determine the requirements for stimulation of transcription by GAL4–VP16 [41]. That study determined that GAL4–VP16 acts on preformed template-committed complexes to increase the number of preinitiation complexes formed, without affecting the rate at which they form. From the present study, it is clear that the stimulation being blocked by mAbs in the previous study was not direct activation, but was anti-repression. Thus, the molecular event that is specific to anti-repression is completed within the first 10–20 minutes of preinitiation complex assembly, since it becomes resistant to inhibition by mAb 2GV4 at that time (see Figures 4c and 5b in [41]). In contrast, complete stimulation of transcription by GAL4–VP16 requires at least 30 minutes (see Figure 1b in [41]; results not shown). Also, when a whole-cell extract is fractionated chromatographically, a fraction containing active TFIID is essential for anti-repression, since its omission during preinitiation complex formation leaves activation vulnerable to inhibition by mAb 5GV2 (see Figure 6d in [41]). Finally, the DNA transcriptional template must be present during the incubation of GAL4–VP16 with general factors in

order for transcription to become mAb-resistant and, by inference, for the anti-repression-specific molecular event to occur. This observation, combined with the other results of the previous study and those of the present study, is consistent with the idea that the mAb-inhibitable molecular event that is specific to anti-repression involves some structural modification of the promoter DNA that renders it refractory to the binding of repressors. In this respect, it may be relevant that some transcriptional coactivators have been identified as helicases [54–57].

Another conclusion that may be drawn from the data in this study is that occlusion of repressors from the promoter by steric hindrance is not sufficient to account for the pre-emptive anti-repression effect of GAL4–VP16, since the addition of extra molecular bulk, in the form of bound antibodies, not only failed to increase the anti-repression activity of GAL4–VP16, but actually inhibited it. Although the GAL4 DNA-binding domain may contribute to anti-repression by destabilizing nucleosome–DNA contacts [58], previous experiments have shown that the GAL4 DNA-binding domain alone was unable to anti-repress nucleosomes, whereas GAL4–VP16 is able to do so [20]. From this observation, it was interpreted that an activation domain was required for anti-repression. However, another valid interpretation of those previous experiments is that transcriptional activators bound to promoters are able to prevent repressors from binding to the promoter through steric hindrance. According to this interpretation, the GAL4 DNA-binding domain was not large enough to offer sufficient steric hindrance to inhibit the binding of repressors to the promoter, and the addition of an activation domain enabled anti-repression simply by adding molecular bulk. We aimed to test this hypothesis by comparing the anti-repression activity of GAL4–VP16 with that of a GAL4 DNA-binding domain fused to a protein domain that is the same size and general shape as the VP16 activation domain, but which lacked transcriptional stimulation activity. The F442A mutation of the VP16 activation domain was chosen as the non-stimulatory protein domain, since it had been reported previously, by the group of Triezenberg, to lack stimulatory activity in transfection experiments in the context of a VP16 protein truncated at residue 456 [43]. In agreement with subsequent experiments by that group [44], the F442A mutation had little effect on direct activation in the context of the full-length activation domain. However, this mutation was also functionally silent in the context of the truncated form of GAL4–VP16 (Δ 456–490;F442A), unlike the previous results. This discrepancy may be explained by the effect of the F442A mutation being dependent on the use of the entire VP16 protein, rather than GAL4–VP16, or being specific for an event that occurs in transfection experiments that cannot be reproduced in transcription assays performed *in vitro*. Although the F442A mutants were inappropriate for the purpose of comparing anti-repression by GAL4–VP16 with that of a like-sized, functionally impaired protein, the experiments with the mAbs allowed us to rule out the possibility that molecular size alone determines the anti-repression effect of activators. If the anti-repression effect of derivatives of the GAL4 DNA-binding domain had been related directly to the sheer bulk of the protein, then we would have expected the added bulk of the mAbs bound to the activation domain to have increased the anti-repression effect of GAL4–VP16, contrary to the decrease that was observed. Thus by ruling out steric hindrance as the means of anti-repression by GAL4–VP16, this study reinforces the hypothesis that a transcriptional activation domain is required for efficient anti-repression to occur.

A pre-emptive mechanism for anti-repressing H1 may be important to the control of transcription in at least two distinct situations *in vivo*. Firstly, as recognised by others [10,59], the

presence of a pre-emptive anti-repressor may enable transcription following a round of DNA replication, during which chromatin structures are disrupted, by preventing the deposition of chromatin repressors on the target promoter during the chromatin reassembly that follows replication. Such a mechanism may account for enhanced transcription of late response genes that require DNA replication to respond to a hormonal or other stimulus. Secondly, the presence of levels of H1 in the nucleus that exceed available DNA binding sites [18], resulting in a pool of H1 that is free in the nucleoplasm, suggests a role for controlling transcription through anti-repression in the absence of DNA replication. During activation of transcription *in vivo*, nucleosomes are disrupted, causing the promoter DNA to become accessible to binding by transcriptional activators and general transcription factors [9,60]. The availability of a pool of free H1 in the nucleoplasm ensures that these transcription factors will be in competition with H1 for binding to the DNA during nucleosome disruption, similar to the scenario in the transcription assays used here. Thus, a mechanism for pre-emptive anti-repression may be critical to both immediate-early and late responses to stimuli for the enhanced transcription of genes.

We thank K. Bavik for excellent technical assistance; I. Davidson for use of the oligonucleotide affinity column; the Egly laboratory and J. Wu for samples of whole-cell extracts and helpful discussions; C. Brou for the plasmids GAL4-VP16(F442A)/pSG5, GAL4-VP16/pET3c and 17M5pAL7; L. Tora for the TATA-CAT plasmids; the cell culture laboratory for HeLa cells; Y. Lutz and the hybridoma laboratory for ascites fluid; and S. Vicaire for assistance with DNA sequencing. JGL was supported by a research associateship and fellowships from CNRS, INSERM, Université Louis Pasteur and the Fondation Pour La Recherche Médicale.

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