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A Potent GAL4 Derivative Activates Transcription at a Distance in Vitro

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Transcription of a typical eukaryotic gene by RNA polymerase II is activated by proteins bound to sites found near the beginning of the gene as well as to sites, called enhancers, located a great distance from the gene. According to one view, the primary difference between an activator that can work at a large distance and one that cannot is that the former bears a particularly strong activating region; the stronger the activating region, the more readily the activator interacts with its target bound near the transcriptional start site, with the intervening DNA looping out to accommodate the reaction. One alternative view is that the effect of proteins bound to enhancers might require some special aspect of cellular or chromosome structure. Consistent with the first view, an activator bearing an unusually potent activating region can stimulate transcription of a mammalian gene in a HeLa nuclear extract when bound as far as 1.3 kilobase pairs upstream or 320 base pairs downstream of the transcriptional start site.

GAL4 IS A TRANSCRIPTIONAL ACTIVATOR found in the yeast *Saccharomyces cerevisiae* (*1*). The 881–amino acid protein binds as dimers to 17-bp dyad sites to activate transcription of a nearby gene (*2*). GAL4 will also activate transcription of a gene in many other eukaryotic cells, including mammalian tissue culture cells (*3*), when its binding sites are placed in the vicinity of the target gene. A fragment comprising the first 147 amino acids of GAL4 [GAL4(1–147)] binds DNA but fails to activate transcription in vivo because, according to our current picture, it lacks an activating region (*4*). We used two fusion

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Table 1. Summary of activation on the different templates. Different exposures of the autoradiographs in Figs. 1 and 2 were scanned with a densitometer and quantified. AH refers to GAL4(1–147)+AH and VP16 indicates GAL4(1–147)+VP16. The stimulations listed are all relative to the level of transcription seen with the TATA box alone in the absence of activator: this amount was assigned a value of 1. Addition of a single TATA-proximal ATF site generally gives a twofold stimulation under our reaction conditions. The experiments were repeated on average three times per template with similar results. The ratio of the transcription signal in the presence of GAL4(1–147)+VP16 to that in the presence of GAL4(1–147)+AH is indicated. The size of the insertions between the GAL4 binding sites and the TATA box are indicated as are the actual distances from the edge of the GAL4 sites to the first T of the *E4* TATA; the sites in pG_5E4T already begin 23 bp from the first T of the *E4* TATA box and the ATF site contributes an additional 25 bp.

Template	Insert (bp)	Distance of GAL4 sites from TATA (bp)	ATF site	Activator	Fold stimulation	Fold stimulation VP16/AH
				–	1	
pG_5E4T	0	23	–	AH	50	4.4
				VP16	216	
				–	1	
$pG_5I54E4T$	54	77	–	AH	15	14.7
				VP16	212	
				–	1	
$pG_5I201E4T$	201	224	–	AH	2	10
				VP16	20	
				–	2	
$pG_5I54AE4T$	54	102	+	AH	50	6.6
				VP16	328	
				–	2	
$pG_5I201AE4T$	201	249	+	AH	7	13.1
				VP16	92	
				–	2	
$pG_5I1300AE4T$	1270	1318	+	AH	2	>100*
				VP16	12	
				–	2	
$pAE4TI320G_5$	320	320	+	AH	4	12.5
				VP16	50	

*We are unable to measure activation by GAL4(1–147)+AH on this template and therefore the ratio cannot be determined accurately, but it is probably very high.

derivatives of GAL4(1–147) that bear, respectively, a short peptide designed to form a negatively charged amphipathic helix [GAL4 (1–147)+AH] (*5*) and a 78–amino acid fragment from the Herpes virus protein VP16 [GAL4(1–147)+VP16] (*6*); the latter fusion protein is particularly potent and activates transcription in mammalian cells from sites positioned greater than 2 kb from the gene, whereas the former works more weakly and only from sites close to the gene (*7*).

GAL4(1–147)+AH and GAL4(1–147)+VP16, synthesized in and purified from *Escherichia coli*, were incubated in a HeLa cell nuclear extract with templates bearing five 17-bp GAL4 binding sites positioned various distances from the TATA box of the *E4* gene of adenovirus. Transcription was measured by primer extension of the product RNAs. Some of the templates contained a site for the mammalian transcription factor ATF located adjacent to the TATA box; ATF, which is present in the nuclear extracts, functions synergistically with GAL4 derivatives in vitro (*8*).

Figure 1 describes the effects of the two GAL4 derivatives on transcription when the five GAL4 binding sites were located close to the promoter (*9*). At all positions, GAL4(1–147)+VP16 activated transcription better than GAL4(1–147)+AH. A similar result was obtained when the GAL4(1–147)+VP16 and weaker activators were assayed in either yeast or mammalian tissue culture cells (7). At both positions tested, ATF acted synergistically with GAL4 derivatives to increase transcription (Fig. 1).

The effect of the activators bound to sites located 1.3 kb upstream and 320 bp downstream of an *E4* TATA box is described in Fig. 2 (*10*). The templates also had an ATF site near TATA (Fig. 2B). The GAL4(1–147)+VP16 protein activated transcription efficiently in both cases (Fig. 2A), although stimulation from the downstream position was more efficient than from the far upstream site (Table 1). When the sites were placed downstream, transcription was activated from both the normal *E4* start sites as well as from a point located further upstream (Fig. 2A). It is likely that this latter start site originates near a cryptic TATA box outside the polylinker into which the gene was cloned; this region is rich in A and T residue, and several sequences resemble a natural TATA box. In both cases transcriptional activation was dependent on the GAL4 binding sites; templates lacking these sites were not stimulated measurably by either activator (Fig. 2A). The ATF site significantly increased activation by GAL4 (1–147)+VP16 from the upstream site, but was not absolutely required (*11*).

Our results are summarized in Table 1. In each case, activation falls off with distance, the effect being more dramatic with the weaker activator. We interpret this to mean that transcription is saturated, or nearly so, with the stronger activator when the binding sites are close to the TATA box (within 74 bp). In agreement with this, increasing the number of sites from five to nine had no effect on activation by GAL4(1–147)+VP16 but resulted in a fourfold larger activation by GAL4(1–147)+AH (*11*).

We expect, based on the results of others (*12*), that our HeLa extracts (*13*) do not readily form chromatin structures with add-

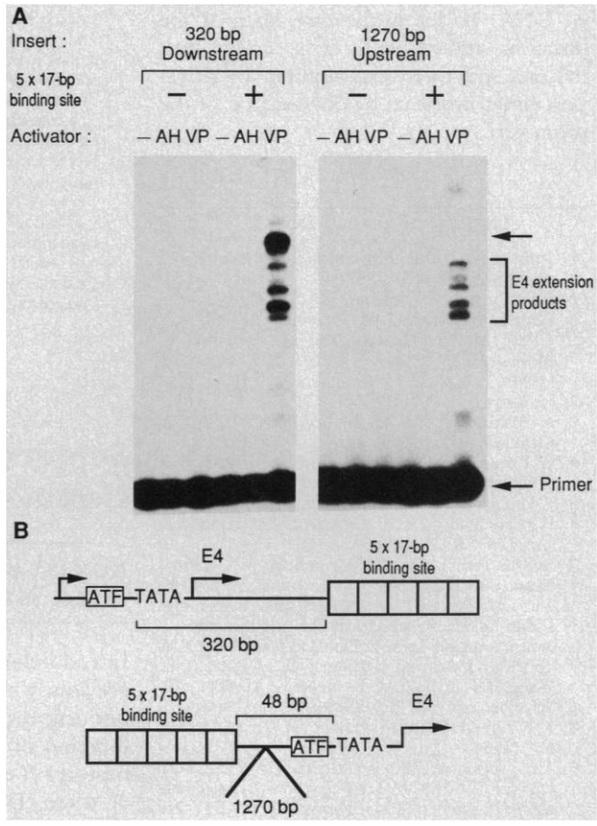

Fig. 2. Transcriptional activation from GAL4 binding sites located downstream and far upstream of the start site. (**A**) The reaction mixtures contained the templates shown in (B), nuclear extract, and the indicated GAL4 derivatives. Templates containing the GAL4 binding sites (+) were compared with templates lacking the sites (−), but containing the spacer DNA. Transcription reactions and product analysis were as described in Fig. 1. The primer and *E4* extension products are indicated. The unlabeled arrow points to a major extension product that appears to result from initiation downstream of a cryptic TATA box in the pGEM3 vector DNA. (**B**) The artificial enhancer was cloned 320 bp downstream (pAE4TI320G_5) or approximately 1.3 kb upstream (pG_5I1300AE4T) from the *E4* TATA box (*10*).

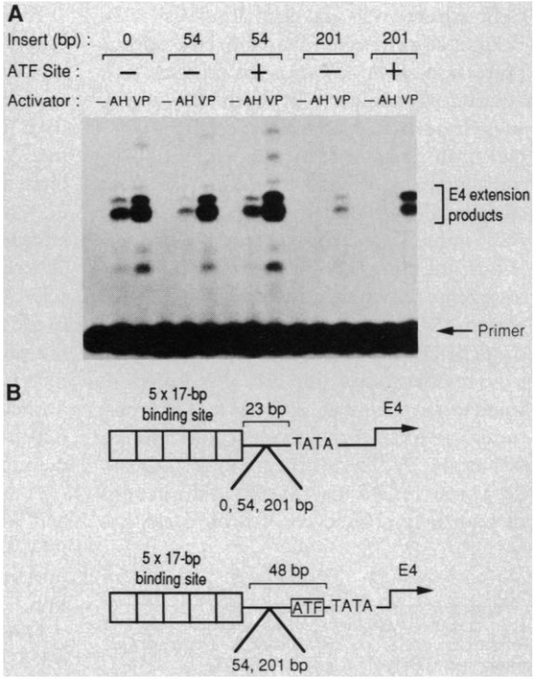

Fig. 1. Transcriptional activation from GAL4 binding sites located close to the promoter. (**A**) The templates shown in (B) were transcribed in HeLa cell nuclear extract (*13*) in the absence (−) or presence (+) of GAL4(1–147)+AH, designated AH, or GAL4(1–147)+VP16, designated VP (*14*). The reaction products were measured by primer extension and fractionated on a polyacrylamide-urea gel. An autoradiograph of the gel is shown. The primer and *E4* extension products are indicated. The multiple products are the result of transcription initiating at different points and are similar to those seen in vivo (*15*). (**B**) The templates contained five tandem 17-bp GAL4 binding sites (indicated by open boxes) positioned either immediately adjacent to the TATA box (23 bp upstream; pG_5E4T) or with 54 (pG_5I54E4T) or 201 (pG_5I201E4T) additional base pairs inserted between the GAL4 binding sites and TATA. Some templates contained the natural *E4* ATF site located between −38 and −63 bp from the major transcription start site (pG_5I54AE4T and pG_5I201AE4T) (*9*).

ed DNA. If this is the case, then at the distances studied here, higher order chromosome structure is not required for activation either upstream or downstream of the promoter.

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9. The plasmid pΔ-38 contained the *E4* TATA box, start site, and 250 bp of coding sequence cloned into the Sma I site of pGEM3. The plasmid pG_5E4T was constructed by inserting five tandem GAL4 binding sites (G_5) into the Hinc II site of pΔ-38, 23 bp upstream from the *E4* TATA box (E4T). The plasmids $pG_5I54E4T$ and $pG_5I201E4T$ were constructed by inserting 54 (I54)– and 201 (I201)–bp Sau 3A fragments from the λ repressor and TFIIIA genes, respectively, into the Bam HI site of pG_5E4T. pΔ-63 contained the natural ATF site immediately adjacent to the *E4* TATA box, and all other sequences were identical to pΔ-38. The Hind III–Bam HI fragment of pG_5E4T, containing the GAL4 binding sites, was first transferred into pΔ-63 to produce pG_5AE4T, and then insertions of 54 and 201 bp were made as described above to generate the plasmids $pG_5I54AE4T$ and $pG_5I201AE4T$.
10. A 1139-bp Pvu II–Bgl I fragment from the adenovirus major late gene was inserted into the Bam HI site of $pG_5I160AE4T$ (*8*) to create $pG_5I1300AE4T$; alternatively, the Hind III–Bam HI fragment (containing the GAL4 binding sites) was excised and the Pvu II–Bgl I fragment was inserted to create pI1300AE4T. The Hind III–Bam HI fragment of pG5E4T was cloned into the Eco RI site of pΔ-63 to generate $pAE4T1320G_5$.
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12. Other workers have failed to observe chromatin formation at ratios of DNA to HeLa nuclear extract protein similar (with a factor of 2) to those used in our experiments [J. L. Workman, S. M. Abmayr, W. A. Cromlish, R. G. Roeder, *Cell* **55**, 211 (1988)].
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14. The 40-μl in vitro transcription mixtures contained 20 m*M* Hepes (*p*H 7.6), 60 m*M* KCl, 7.5 m*M* $MgCl_2$, 12.5% glycerol, 0.6 m*M* dithiothreitol, 0.5 m*M* nucleoside triphosphates, 25 μl (150 μg) of HeLa nuclear extract (*13*), 0.025 n*M* specific template, 200 ng of pGEM3 (Promega) carrier DNA, and either no protein or template-saturating amounts of GAL4(1–147)+AH or GAL4(1–147)+VP16 that had been purified as described previously (*8*) [D. Chasen, J. Leatherwood, M. Carey, M. Ptashne, R. Kornberg, *Mol. Cell. Biol.* **9**, 4746 (1989)]. After 1 hour at 30°C, the reactions were terminated, and the reaction products were purified and analyzed by primer extension [J. Lillie, M. Green, M. R. Green, *Cell* **46**, 1043 (1986)]. The hybridized primers were fractionated on 18-cm long, 1.5-mm thick, 10% polyacrylamide-urea gels. The gels were autoradiographed by exposure to Kodak XAR-5 film, and the autoradiographs were scanned with a Helena densitometer; dilutions of the primer were used to generate a standard curve. The transcription conditions differ from our previous experiments in an important way: the template DNA concentration is 100-fold lower than previously used. We found the amount of GAL4(1–147)+VP16 necessary to fill the binding sites at our standard DNA template concentration of 2.5 n*M* led to an inhibition of transcription, a phenomenon that may be related to "squelching" [G. Gill and M. Ptashne, *Nature* **334**, 721 (1988)]. We therefore lowered the DNA template concentration to 0.025 n*M* and found that the correspondingly lower amount of GAL4(1–147)+VP16 needed to fill the binding sites did not inhibit transcription. These new conditions more accurately mimic the effects observed in vivo with the two GAL4 derivatives.
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Suppression of Tumorigenicity of Human Prostate Carcinoma Cells by Replacing a Mutated *RB* Gene

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Introduction of a normal retinoblastoma gene (*RB*) into retinoblastoma cells was previously shown to suppress several aspects of their neoplastic phenotype, including tumorigenicity in nude mice, thereby directly demonstrating a cancer suppression function of *RB*. To explore the possibility of a similar activity in a common adult tumor, *RB* expression was examined in three human prostate carcinoma cell lines. One of these, DU145, contained an abnormally small protein translated from an *RB* messenger RNA transcript that lacked 105 nucleotides encoded by exon 21. To assess the functional consequences of this mutation, normal *RB* expression was restored in DU145 cells by retrovirus-mediated gene transfer. Cells that maintained stable exogenous *RB* expression lost their ability to form tumors in nude mice, although their growth rate in culture was apparently unaltered. These results suggest that *RB* inactivation can play a significant role in the genesis of a common adult neoplasm and that restoration of normal *RB*-encoded protein in tumors could have clinical utility.

THE RETINOBLASTOMA GENE (*RB*) encodes a nuclear phosphoprotein that is constitutively expressed in most cultured cells and normal tissues (*1, 2*). Retinoblastoma cells invariably lack normal expression of the *RB*-encoded protein (RB) because of mutation of both *RB* alleles, suggesting that loss of functional RB protein is an obligate event during retinoblastoma genesis (*3*). Furthermore, replacement of normal RB protein in retinoblastoma cells induces profound phenotypic alterations, including complete loss of their tumorigenic activity in nude mice (*4*). For this reason, *RB* is aptly termed a tumor suppressor gene (*5*). *RB* mutations are not restricted to retinoblastomas but are also found in some osteosarcomas, soft-tissue sarcomas, breast carcinomas, and small-cell carcinomas of lung (*6, 7*). These observations suggest a broad role for *RB* inactivation in the genesis of human tumors, even of those lacking a known association with retinoblastoma. However, direct biological evidence for this role has not been developed.

Prostate carcinoma is the most common cancer in men, and its incidence increases rapidly with age (*8*). Despite an abundance of clinical material, relatively few cell lines have been successfully established from human prostatic carcinomas (*9*). Three such lines, DU145 (*10*), LNCaP (*11*), and PC-3 (*12*), were readily available (*13*) and were screened for evidence of *RB* inactivation. Whereas LNCaP, PC-3, and control CV-1 cells expressed RB phosphoprotein isoforms in the expected size range (110 to 116 kD), the predominant RB form in DU145 cells migrated ahead of the least phosphorylated normal RB form (Fig. 1A). The same abnormal RB protein in DU145 was also detected by protein immunoblot analysis (*14*) with four polyclonal antisera (*7, 15*) and with monoclonal antibody PMG3-245 (*16*). These data suggested that this aberrant protein might result from a subtle *RB* mutation.

Northern (RNA) blot analysis of DU145 mRNA showed a single *RB* mRNA species

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