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A single DNA-binding transcription factor is sufficient for activation from a distant enhancer and/or from a promoter position

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Typical cell type-specific or inducible mammalian genes are under the control of one or more remote enhancers which transmit their effect to the promoter region located at the initiation site of transcription. Both enhancers and promoters are composed of multiple binding sites for transcription factors. To study the requirements for promoter and enhancer function, we have used a reporter gene that is completely dependent on a single DNA-binding transcription factor *in vivo*. This factor is a truncated, hormone-independent form of the glucocorticoid receptor which interacts strongly with a palindromic binding site. After transfection into HeLa cells, transcription of a reporter gene with one, two or four copies of the binding site upstream of the TATA box is enhanced <10, at least 100 and >1000-fold respectively, in the presence of the receptor. Even when the TATA box is deleted, the four upstream binding sites confer receptor-dependent transcription, though from scattered initiation sites. When four copies of the palindromic binding site are placed downstream of the transcription unit, they form a very strong receptor-dependent enhancer. This enhancer can activate comparably well promoters containing binding sites for either glucocorticoid receptor, Sp1 factor, or octamer factor. Our data show that a single defined DNA-binding factor can mediate both promoter and enhancer activity, and that it can co-operate functionally both with itself and with seemingly unrelated transcription factors.
Key words: DNA–protein binding/enhancer effect/glucocorticoid receptor/transactivator/transcription factor

Introduction

Gene expression in mammalian cells is often controlled by remote enhancers that transmit their effect to the promoter sequences close to the site of RNA initiation. Multiple binding sites for transcription factors are found in promoter as well as in enhancer DNA sequences (for reviews see Serfling *et al.*, 1985a; Maniatis *et al.*, 1987; Ptashne, 1988; Johnson and McKnight, 1989; Mitchell and Tjian, 1989). It is possible to construct very strong enhancers or promoters using multiple copies of the same transcription factor binding site (Ondek *et al.*, 1987; Schirm *et al.*, 1987; Pierce *et al.*, 1988; Westin and Schaffner, 1988). However, these studies were not done with defined, cloned transcription factors. In fact, many specific DNA sequence motifs can be recognized by two or more factors within a mammalian cell (Baldwin and Sharp, 1988; Chodosh *et al.*, 1988; Curran and Franza, 1988; Santoro *et al.*, 1988; Harada *et al.*, 1989; Schreiber *et al.*, 1989 and references therein). Thus it is desirable to use a system involving a cloned transactivator gene and the corresponding responsive reporter gene. It has been shown that a cloned yeast GAL4 factor can function synergistically in mammalian cells with a β-globin promoter and an SV40 enhancer in upstream or downstream positions (Webster *et al.*, 1988; see also Kakidani and Ptashne, 1988). However, the question of whether a single factor is able to activate transcription via its DNA recognition site present both in a promoter and in a remote enhancer position has not yet been addressed. Glucocorticoid receptor is the first *bona fide* mammalian transcription factor whose cDNA has been cloned (Miesfeld *et al.*, 1984) and shown to be active when expressed in mammalian cells (Hollenberg *et al.*, 1985; Miesfeld *et al.*, 1986; reviewed in Yamamoto, 1985; Beato, 1989). For the present study, we have used a truncated glucocorticoid receptor (amino acids 3–556) without the hormone-binding domain (Figure 1), together with a palindromic binding site. Because there is no apparent interference from wild-type glucocorticoid receptor or other

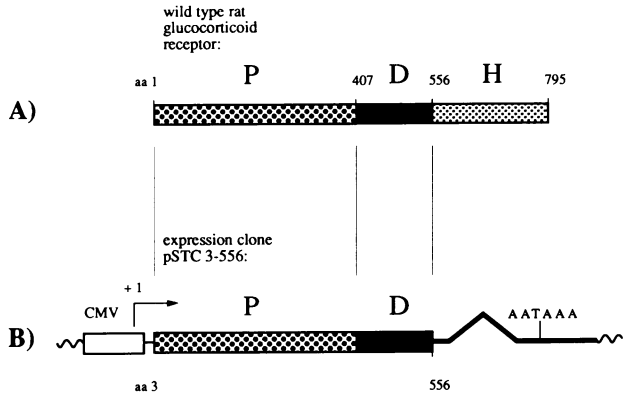
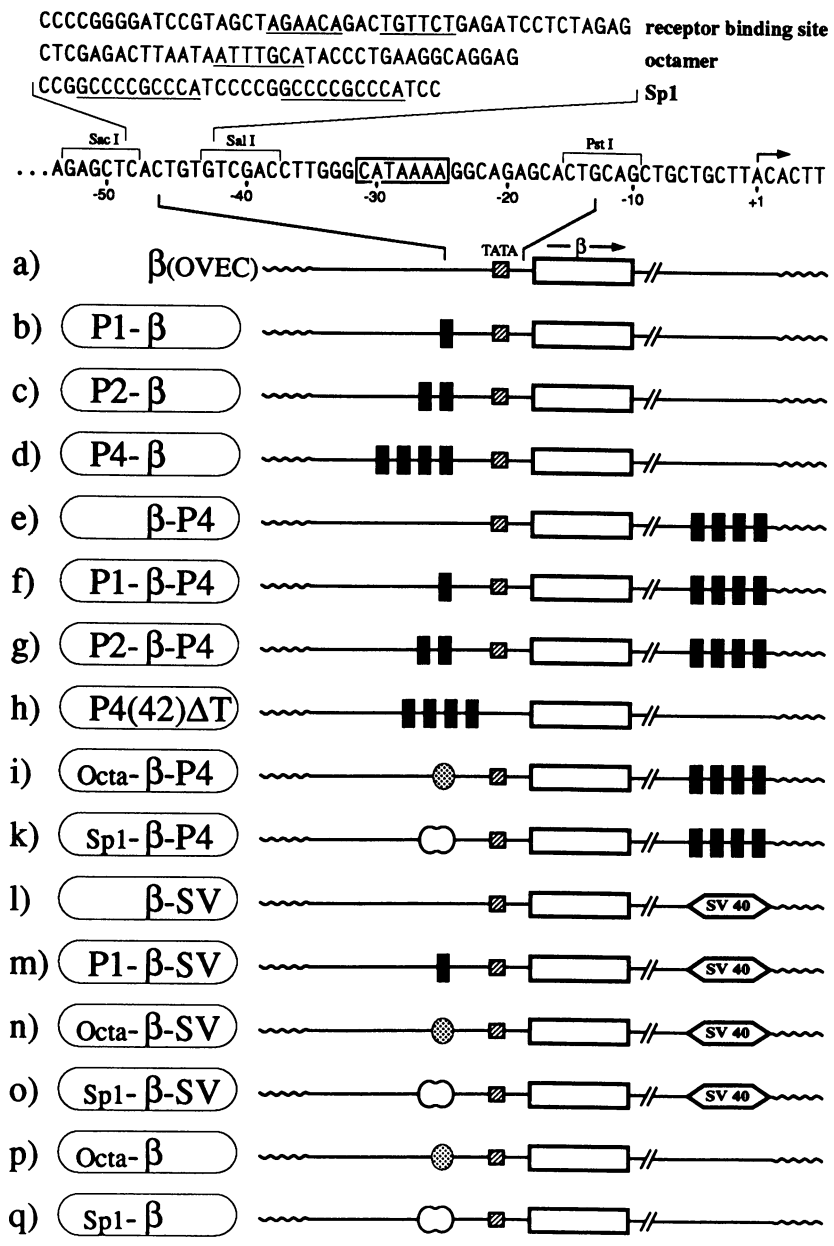


Fig. 1. Structure of the expression vector encoding the rat glucocorticoid receptor. (**A**) The wild-type receptor protein is subdivided in to three major domains which can be operationally defined as follows: residues 1–407 encompass the 'P' (potentiator) domain which harbours a strong activation function. 'D' designates the region of the receptor encoding the DNA-binding domain which is contained within amino acids 440–500 (filled box; Godowski *et al.*, 1987; Hollenberg *et al.*, 1987; Severne *et al.*, 1988). The carboxy-terminal domain, designated 'H' (amino acids 556–795) is the hormone-binding domain (for review see Beato, 1989). (**B**) A truncated version of the wild-type receptor was used in the work presented here. The expression vector pSTC 3-556 (Severne *et al.*, 1988) produces a carboxy-deleted receptor which is constitutively active, even in the absence of hormone (Godowski *et al.*, 1987). Symbols: empty box, cytomegalovirus (CMV) promoter/enhancer (−522/+72, Boshart *et al.*, 1985); thin line, herpes simplex virus thymidine kinase leader, including the initiation codon AUG and two additional amino acids (+55/+104, Rusconi and Yamamoto, 1986); thick line, rabbit β-globin splice/polyadenylation signal (Pääbo *et al.*, 1983).



	TRANSCRIPTION ACTIVITY:		
	- Receptor	+ Receptor	Prom.* + Enh.* / Prom.*
a) β(OVEC)	< 1	< 1	-
b) P1-β	< 1	< 10	-
c) P2-β	< 1	> 100	-
d) P4-β	< 1	> 1000	-
e) β-P4	< 1	30	> 30
f) P1-β-P4	< 1	300	> 30
g) P2-β-P4	< 1	> 300	3
h) P4(42)ΔT	< 1	200	-
i) Octa-β-P4	< 1	15	> 15
k) Sp1-β-P4	10	> 200	> 20
l) β-SV	< 1	< 1	-
m) P1-β-SV	< 1	200	20
n) Octa-β-SV	> 15	> 15	> 15
o) Sp1-β-SV	200	200	20
p) Octa-β	< 1	< 1	-
q) Sp1-β	10	10	-

Fig. 2. Reporter gene constructions. The parent plasmid is shown in **line a**, which depicts the OVEC reporter plasmid (Westin *et al.*, 1987) consisting of a modified rabbit β-globin coding sequence including the initiation (+1) site and the globin TATA box (pos. −31), with the globin promoter region from −37 to −425 replaced by a synthetic linker containing a *Sac*I and a *Sal*I site. Additional elements were cloned immediately upstream of the TATA box and/or 1.8 kb downstream in the region between the *Eco*RI and *Xba*I site, as described below and in Materials and methods. The *Sac*I−*Sal*I region is termed the (proximal) promoter position, whereas the *Eco*RI site at +1800 is defined as the (remote) enhancer position. Symbols: wavy line, plasmid sequences; straight line, globin flanking sequences (upstream from −450 to −1250, downstream from +1700 to +3300); black box, one oligonucleotide containing the hyphenated 15 bp palindromic glucocorticoid responsive element (GRE, Severne *et al.*, 1988, modified from Jantzen *et al.*, 1987; Wieland *et al.*, 1988) (for sequence, see line **a**) in the promoter and also in the enhancer position; hatched box, globin TATA box. The DNA sequence including the TATA box and the +1 initiation site is shown for *OVEC* (**a**) and is the same for all other reporter plasmids except *P4(42)ΔT* (**h**). β-arrow, transcription direction; white box, rabbit β-globin coding sequence. The different reporter plasmids were obtained by insertion of one (*P1/β*, line **b**), two (*P2/β*, line **c**) or four (*P4/β*, line **d**) palindromic receptor sites at the promoter position. To study enhancer effects, the tetrameric GRE cluster (P4) was inserted at position +1800 to yield the plasmids *β/P4*, *P1/β/P4*, *P2/β/P4*, *Octa/β/P4* and *Sp1/β/P4* depicted in (**e**), (**f**), (**g**), (**i**) and (**k**). *P4(42)ΔT* (see **line h**) is a TATA box deleted mutant derived from *P4/β*, in which the number in parentheses indicates the distance in bp between the centre of the proximal palindromic receptor-binding site and the +1 initiation site (for sequence, see Materials and methods). For the remaining constructions (**lines l−q**) the same principle of presentation is used. Symbols: stippled oval, octamer binding sites; white double oval, Sp1 binding site; white hexagon, 195 bp SV40 enhancer fragment (Banerji *et al.*, 1981) inserted at +1800. The first two columns on the right-hand side of Figure 2 indicate the transcript level in the absence of functional receptor (−Receptor) or presence of active receptor (+Receptor). (Note that the 200-fold stimulation in **line h** is an integration of the staggered initiations to account for the overall transcription level of *P4(42)ΔT*.) The rightmost column displays the ratio of transcription level of reporter genes in which both the promoter and the enhancer were activated by receptor (Prom.*+Enh.*) compared to reporter genes with the promoter only (Prom.*). [For example, in **line f** co-transfection of *P1/β/P4* with functional receptor resulted in a 300-fold stimulation which *P1/β* was stimulated only <10-fold by the receptor; 300 divided by <10 yields a ratio of >30 (Prom*+Enh.* divided by Prom.*)].

trans-acting factors, there is an almost undetectable background level of transcription (detectable only on long exposure), with >1000-fold stimulation in the presence of the truncated receptor (Severne *et al.*, 1988). Thus the truncated receptor and its palindromic binding site constitute an ideal system for the study of transcription *in vivo*. Our data show that a single defined DNA-binding factor can mediate both promoter and enhancer activity, and that it can co-operate functionally both with itself and with seemingly unrelated transcription factors.

Results

The glucocorticoid receptor strongly stimulates transcription via upstream binding sites, even in the absence of a TATA box

For our studies we have chosen a series of constructions based on the reporter gene OVEC (Westin *et al.*, 1987), which consists essentially of a β-globin coding sequence with convenient cloning sites in promoter (upstream) and enhancer (downstream) positions, as indicated in Figure 2(a). We have used a reporter gene with β-globin coding sequences, because the high stability of these transcripts facilitates quantitation of gene expression.

The constructs tested contained the following inserted sequences (Figure 2a–q): (i) one, two or four copies of the palindromic receptor binding site located immediately upstream of the TATA box; (ii) four copies of the palindrome placed very close upstream of the transcription initiation site, whereby the TATA box is deleted; (iii) four copies of the palindrome in a remote downstream position, in conjunction with either no palindrome, or one or two palindrome sites upstream of the TATA box; and (iv) Sp1 or octamer sites upstream of the TATA box giving constitutive promoter activity, and with either no downstream elements, four palindromic receptor binding sites downstream of the reporter gene, or an SV40 enhancer at this remote position. The constructs with variable numbers of receptor sites were designed to study possible synergism in transcriptional activation. The reporter plasmids with a constitutive promoter (Sp1 or octamer sites) or a constitutive enhancer (from SV40) were designed to reveal whether or not there is preferential synergism between receptor binding sites in promoter and enhancer positions (for sequences of the binding sites, see Figure 2a and Materials and methods). Finally, the constructs with a promoter consisting of solely the TATA box served as negative controls.

The principle of the assay was to co-transfect into HeLa cells the reporter gene together with the transactivator cDNA

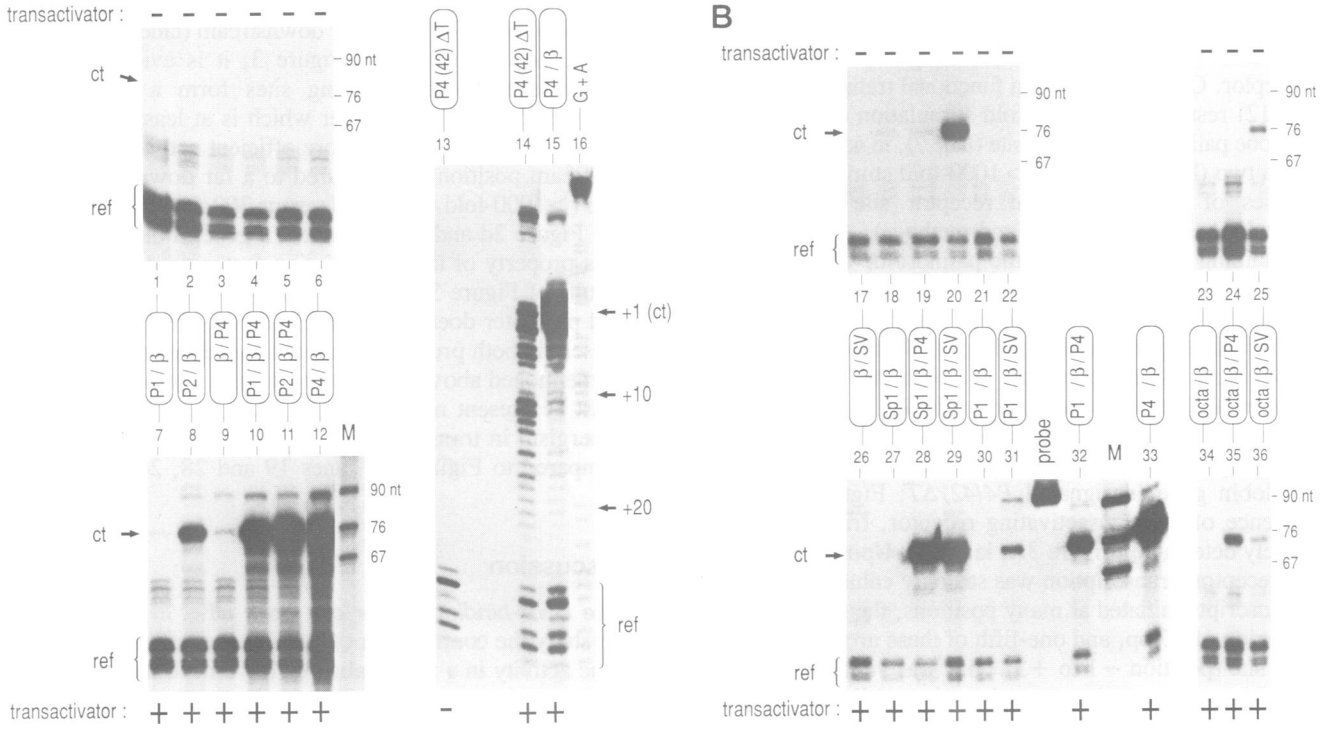


Fig. 3. S1-nuclease mapping of RNA from transiently transfected HeLa cells. The cells were transfected with the reporter genes shown in Figure 2 along with the reference plasmid and either no functional receptor (−) or with the transactivating receptor (+). The panels are displayed such that the corresponding lanes (− and + transactivator) are shown underneath each other (i.e. lanes 1 and 7 refer to the same reporter gene, *P1/β*, tested in the absence or presence respectively of functional receptor). The autoradiograms showing lanes 1–12 are 7 day exposures to reveal weak bands. All other lanes are overnight exposures. Panels in (**A**) show the response to receptor of variable numbers of receptor binding sites in different positions. Lane 16 shows a purine sequencing reaction of the probe (Maxam and Gilbert, 1980; Westin *et al.*, 1987). The lanes in (**B**) demonstrate the versatility of transcriptional stimulation of four palindromic receptor-binding sites in an enhancer position with seemingly unrelated promoter elements such as Sp1- or octamer-binding sites. Symbols: ct, correctly initiated β-globin transcripts; ref, reference gene transcripts from OVEC REF; M, size marker DNA (*Hpa*II-cleaved pBR322); nt, nucleotides; probe, undigested β-globin probe; +1(ct), +10, +20 at the right side of lane 16, initiation of transcripts compared with correctly (+1) initiated RNA. Quantities of transfected DNA and amounts of RNA subjected to S1-nuclease mapping are indicated in Materials and methods. Above the major reference gene signal we observed additional bands from the reference gene expression which became visible upon long exposures of the films only. Partial S1-nuclease digestion or readthrough transcripts generated bands around the 90 nt marker signal.

gene. The latter was driven by the strong enhancer/promoter of the human cytomegalovirus. As a defined transactivating factor we have used a truncated glucocorticoid receptor (amino acids 3−556) that lacks the hormone-binding domain and is constitutively active (Godowski *et al.*, 1987; Hollenberg *et al.*, 1987; Severne *et al.*, 1988) (see Figure 1B and Materials and methods for further details). Two days after transfection, RNA was extracted and the reporter gene transcripts were quantified by S1 nuclease mapping. In mock transfections without the transactivator gene, the same plasmid vector containing a non-functional cDNA insert was used. The transcription stimulation data obtained with all of the constructions are summarized in the three rightmost columns of Figure 2, and the autoradiograms are shown in Figure 3. To improve the presentation of the data, the corresponding lanes of the autoradiograms without and with functional transactivator are shown underneath each other (Figure 3).

In Figure 3(A), lanes 1−6 demonstrate that for all the constructions tested, the transcription level of the reporter genes in the absence of transactivator was almost undetectable. Upon co-transfection of transactivator gene, however, there was a dramatic and stepwise increase in transcriptional efficiency when one, two, or four copies of the palindromic receptor binding site were inserted upstream of the TATA box. These results are consistent with the experiments of Strähle *et al.* (1988), which were, in contrast to our work, conducted with endogenous glucocorticoid receptor. Co-expression of a functional transactivator (lanes 7−12) resulted in a <10-fold stimulation of transcription for one palindrome binding site (lane 7), in at least a 100-fold with two (lane 8) and in a >1000-fold stimulation for four copies of the palindromic receptor site (lane 12; for calculations, see Materials and methods). A reporter gene containing eight copies of the palindrome upstream of the TATA box did not enhance transcription any further, perhaps because other factors become limiting in the transfected cell under our experimental conditions (data not shown).

We also tested a construct in which the TATA box was deleted and the four palindromic binding sites were located such that the centre of the most proximal palindrome was 42 bp upstream of the +1 transcription initiation site in the β-globin gene (designated *P4(42)ΔT*; Figure 2h). In the absence of the transactivating receptor, transcription was barely detectable (Figure 3A, lane 13). Upon co-expression of receptor, transcription was strongly enhanced (lane 14). Transcripts initiated at many positions, the majority within a region of 25 bp, and one-fifth of these around the normal cap site (position −1 to +3). This was verified by running the S1-nuclease resistant products in parallel with a purine sequencing reaction (Maxam and Gilbert, 1980; Figure 3A, lane 16), allowing identification of the various start sites. The overall level of transcription was at least 20% of that seen with the corresponding reporter gene containing the TATA box (lane 15; M.D.Schatt and S.Rusconi, in preparation).

Transactivation by the same factor from a remote enhancer

Four copies of the same palindromic receptor binding site were also inserted 1800 bp downstream of the reporter gene, to test for a genuine enhancer effect, i.e. to see whether transcription could also be stimulated from a position far downstream of the gene. With a promoter consisting of the globin TATA box only, the four palindromic copies in an enhancer position were unable to induce a high level of globin transcription (Figure 3A, lane 9). This is in agreement with previous findings that in mammalian cells an enhancer cannot induce substantial transcription if the promoter contains only a TATA box and no upstream factor binding site (Kuhl *et al.*, 1987; Lin *et al.*, 1988; Westin and Schaffner, 1988; M.Thali, M.Pettersson, S.Rusconi and W.Schaffner, unpublished). The situation is dramatically improved, however, when one or two copies of the receptor binding site are placed in the promoter position (Figure 2f and g). A 300-fold stimulation of transcription in the first case and a >300-fold stimulation in the second case are observed (Figure 3A, lanes 10 and 11). This establishes that in order to respond well to an enhancer, a promoter should consist not only of a TATA box but also of at least one binding site for an upstream transcription factor. In Figure 3(B), we show that this is not a peculiarity of the receptor binding sites, because a promoter that responded strongly to the downstream receptor sites could also be generated from a TATA box and nearby binding sites for Sp1 factor (for reviews see Dynan and Tjian, 1985; Kadonaga *et al.*, 1986) or octamer factor (Wirth *et al.*, 1987; Müller *et al.*, 1988). In mock transfections with non-functional receptor, strong transcription was only detected when the constructions contained an SV40 enhancer downstream (lanes 20 and 25). From the data shown in Figure 3, it is evident that the downstream receptor-binding sites form a very strong receptor-dependent enhancer which is at least as active as the SV40 enhancer. The more efficient activation from an upstream position as compared to a far downstream position (>1000-fold with *P4/β* versus 300-fold with *P1/β/P4*, see Figure 2d and f) may reflect a general distance effect, or a property of the transcription factor used. Finally, the results of Figure 3 show that synergism between enhancer and promoter does not require receptor-binding sites to be present in both promoter and enhancer positions. However, as mentioned above, at least one transactivator-binding site must be present next to the TATA box to obtain a strong synergism in transcriptional activation (Figure 3A, lane 9 compared to Figure 3B, lanes 19 and 28, 24 and 35).

Discussion

One DNA-binding factor can do it all

To study the contribution of a single transcription factor to gene activity in a mammalian cell, we have used a cloned, truncated glucocorticoid receptor gene (amino acids 3−556). Removal of the hormone-binding domain results in constitutive activity even in the absence of glucocorticoid hormone. Reporter genes containing several palindrome-binding sites are transcribed at a very low background level in transfected HeLa cells, and are stimulated >1000-fold by co-transfection of the transactivator gene encoding the truncated receptor. When four copies of the palindromic binding site were placed far downstream of the β-globin transcription unit, they formed a very strong receptor dependent enhancer. However, significant activation of transcription was only observed when the promoter contained at least one palindrome site upstream of the TATA box. This could have meant that enhancer and promoter must be linked

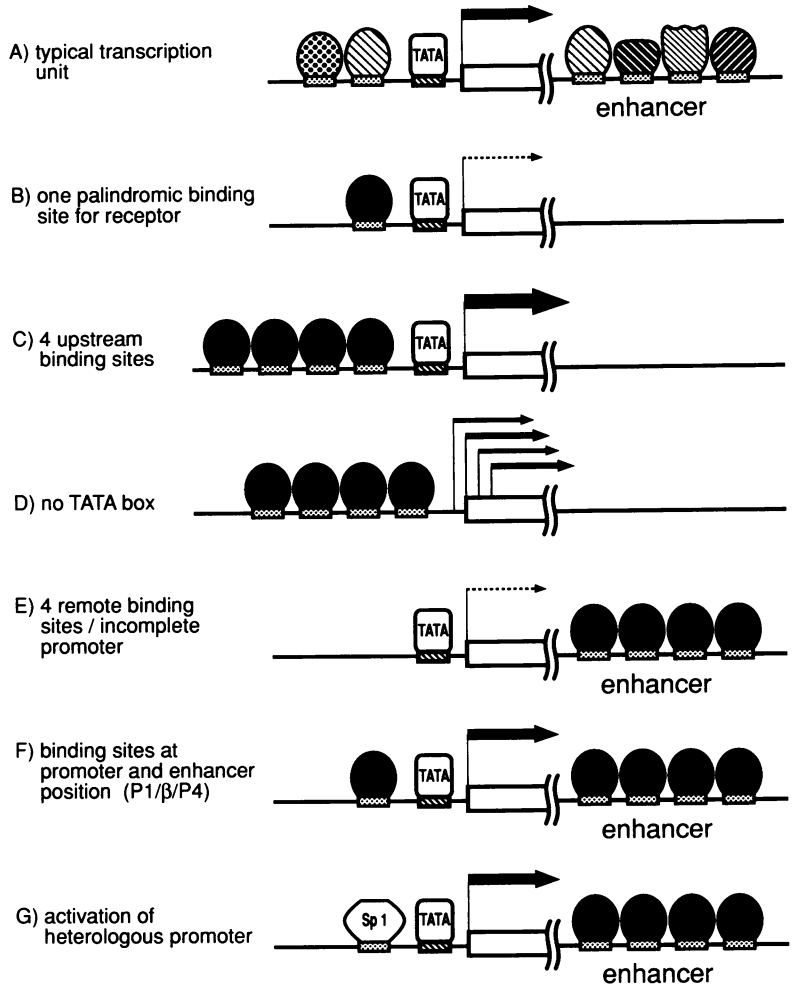


Fig. 4. Schematic representation of various transcription units and their activity. Diagram (**A**) indicates that in a typical mammalian transcription unit, a number of different DNA-binding transcription factors are involved (for reviews see Serfling *et al.*, 1985a; Maniatis *et al.*, 1987; Ptashne, 1988; Johnson and McKnight, 1989; Mitchell and Tjian, 1989). Diagrams (**B**)–(**G**) illustrate the major observations from the present paper. The thickness of the arrows reflects the level of transcription.

via the same factor, in this case the glucocorticoid receptor. Alternatively, it seemed possible that at least one factor binding site upstream of the TATA box/initiation site is required to mediate a strong enhancer effect. From our subsequent experiments we know that the latter is true: the receptor-dependent enhancer can readily interact with other upstream binding factors such as Sp1 or octamer factors that are quite distinct from glucocorticoid receptor. Therefore, we postulate that an ideal, strongly expressed mammalian transcription unit should contain a TATA box/initiation region, at least one factor-binding site upstream of the TATA box, and multiple factor-binding sites in a remote position. A summary of the major conclusions in our paper is presented in Figure 4. Interestingly, strong enhancement mediated by a single defined enhancer/upstream factor is also possible in the absence of a TATA box. In this case, a fraction of transcripts from the TATA-less promoter starts at the correct initiation site, while other initiation sites are scattered around this region. Nevertheless, the total level of transcripts amounts to at least 20% of that seen with the corresponding construction containing a TATA box. From these findings we conclude that a single type of DNA-bound factor is sufficient to induce very strong transcription. This of course does not exclude the requirement for further proteins interacting with the DNA-bound factor (see also above).

An indirect association between enhancer and promoter?

The truncated glucocorticoid receptor used in this study seems to be an ideal DNA-binding transcription factor. It can stimulate transcription from a promoter position upstream of the TATA box, and also via multiple binding sites in a remote enhancer position. Although other transcription factors, for example Sp1, function preferentially in an immediate upstream position, rather than a remote position (Serfling *et al.*, 1985b), our data show that the enhancer effect can in principle be mediated by a single, defined transactivating factor.

What is the mechanism of this remote activation? One possibility would be that RNA polymerase enters at the enhancer site from where it slides along the DNA until it reaches a promoter (scanning model). However, it is now considered most likely that enhancer and promoter associate directly or indirectly with each other to facilitate initiation of transcription. As a consequence of such an interaction, the intervening DNA could be looped out. Looping phenomena are best documented in prokaryotes, including

gene repression by co-operative repressor binding over a distance, site-specific recombination, and DNA replication (reviewed in Ptashne, 1986; Gellert and Nash, 1987; Schleif, 1988; Wang and Giaever, 1988; Gralla, 1989). Activation, rather than repression, of transcription by such a looping mechanism is less well documented. In mammalian cells, perhaps the best evidence compatible with looping, rather than scanning, comes from transcription studies *in vitro*, where we have recently shown that the effect of an enhancer can be transmitted to a promoter even when enhancer and promoter are non-covalently linked (Müller-Storm *et al.*, 1989).

Are our present findings compatible with a loop formation due to co-operative binding of glucocorticoid receptor to enhancer and promoter sites? Our receptor-dependent enhancer activates comparably well promoters containing a binding site(s) for either glucocorticoid receptor, Sp1 factor or octamer factor. This shows that one defined enhancer/upstream factor can functionally co-operate from a distance both with itself and with seemingly unrelated transcription factors. Further studies in our laboratory have extended this concept of a promiscuous interaction. A total of 27 different enhancer/promoter combinations were tested in transfection assays without co-transfection of any transcription factor gene. In every case there was a synergism between enhancer and promoter, i.e. no marked preference was seen for any particular combination (M.Kermekchiev and W.Schaffner, in preparation). This suggests that the promiscuity of enhancer/promoter interactions as observed in our system also applies to a situation where transcription factors are expressed at a physiological level. Thus it seems unlikely, though not impossible, that glucocorticoid receptor directly interacts, with similar efficiency, with Sp1, octamer factors or itself. The flexibility rather suggests that the interaction between receptor-binding sites in an enhancer position and the target promoter is mediated by some adaptor molecule(s), such as RNA polymerase II itself or an accessory factor(s). Along this line of thought, an enhancer could form a stable complex with the promoter via the *trans*-acting factors by looping out of the intervening DNA sequence, and this complex could be recognized as such by RNA polymerase. As an alternative to stable looping, polymerase could bind to the enhancer and from there find the promoter via transient looping. It is also conceivable that the enhancer binds polymerase and thereby increases its local concentration, which in turn would result in a higher availability of polymerase at the promoter via diffusion.

Materials and methods

Cell culture, DNA transfection and RNA analysis

HeLa cells were grown in DMEM (GIBCO) supplemented with 2.5% fetal calf serum (Boehringer), 2.5% new born calf serum (GIBCO), 100 U/ml penicillin and 100 U/ml streptomycin. The cells were transfected by calcium phosphate co-precipitation (de Villiers and Schaffner, 1983). HeLa cells were co-transfected with 10 μg of the globin reporter gene plasmid, 2 μg of OVEC-REF (a reference plasmid, Westin *et al.*, 1987) and 1 μg of the transactivator vector for the experiments shown in lanes 1–12 and 17–36 of Figure 3. For the co-transfection experiments displayed in lanes 13–15, the same amounts of DNA as described above have been used, but only 0.5 μg OVEC-REF DNA was added to the cells. Isolation of β-globin RNA, and S1-nuclease protection assay were performed as described (Westin *et al.*, 1987). In every experiment 20 μg of RNA were used for S1 mapping. The nuclease-resistant products were separated on 10% polyacrylamide/8 M urea gels.

Plasmids

Expression vector for the truncated rat glucocorticoid receptor. The transactivator plasmid pSTC 3-556 (Severne *et al.*, 1988; Wieland *et al.*, 1988) is shown in Figure 1 and consists of a constitutively transcribed vector driven by a cytomegalovirus (CMV) promoter/enhancer (−522/+72, Boshart *et al.*, 1985). A herpes simplex virus thymidine kinase leader, including the initiation codon AUG and two additional amino acids, allows correct translation of the truncated rat glucocorticoid receptor cDNA. Deletion of the few N-terminal amino acids does not significantly affect the activity of the protein (our unpublished observations). Vector pSTC 3-556 encodes a receptor in which the C-terminal hormone-binding domain is deleted (Godowski *et al.*, 1987). The transactivator gene also contains an in-frame stop codon followed by the 3′ region of the rabbit β-globin gene, including the poly(A) site. For mock transfection with a non-functional transactivator we used a mutated version of pSTC 3-556 which cannot bind DNA (pSTC 3-556 Cys482 → Ser, described in Severne *et al.*, 1988).

Reporter plasmids. The various reporter plasmids were obtained by insertion of one (*P1/β*), two (*P2/β*) or four copies (*P4/β*) of an oligonucleotide containing a 15 bp hyphenated palindromic receptor-binding site immediately upstream of the rabbit β-globin gene TATA box into the *Sac*I−*Sal*I site of OVEC (Westin *et al.*, 1987; Figure 2a–d). The sequence of the repeat is: 5′-*GGATCC*GTAGCTAGAACAGACTGTTCTG*GATCT*-3′ (GRE, Severne *et al.*, 1988, modified from Jantzen *et al.*, 1987; Wieland *et al.*, 1988) (*Bam*HI and *Bgl*II sites in italics; the underlined sequences indicate the palindromic glucocorticoid responsive consensus element). Multimerization of the palindromic receptor-binding sites was conveniently carried out by ligation of the *Bam*HI and *Bgl*II ends, which results in a 28 bp distance between the centres of the adjacent palindromes. In some plasmids, a 700 bp *Eco*RI−*Xba*I segment at position +1.8 kb of the β-globin gene was substituted by the corresponding part of the polylinker of the plasmid pSP64 (Promega). To study the enhancer effect, four copies of the palindromic receptor binding site were inserted into the polylinker at position +1.8 kb to yield plasmids *β/P4*, *P1/β/P4* and *P2/β/P4*. *P4(42)ΔT* is a TATA box-deleted mutant, generated from *P4/β*. The *Sal*I−*Pst*I digestion product of *P4/β* was T4 DNA polymerase repaired and ligated. The *Xba*I−*Pst*I region was then substituted with a fragment of the polylinker region of pSP64. *P4(42)ΔT* still contains the four palindromic receptor binding sites, but no TATA box; the number in parentheses indicates the distance in bp between the centre of the proximal palindrome and the +1 initiation site. The sequence of this region is:

5′- . . . AGAACAGACTGTTCTGAGATCCTCTAGAGTCGAC CTGCAGCTGCTGCTTA . . . -3′, in which the dotted underlined sequence identifies the position of the missing TATA box, and the last nucleotide corresponds to the transcription initiation site. Oligonucleotides containing either one octamer binding site (based on the octamer binding site described by Seidman *et al.*, 1979; see Figure 2a, sequence at top) or one palindromic receptor site was inserted into the *Sac*I−*Sal*I site of either *β/P4* or another OVEC plasmid with a 195 bp SV40 enhancer fragment (Banerji *et al.*, 1981) in the *Eco*RI site (+1800). For construction of *Sp1/β/P4* and *Sp1/β/SV40*, a 32 bp blunt-ended oligonucleotide containing two Sp1 binding sites (see Figure 2a, sequence at top) from the herpes virus IE-3 gene promoter (Jones and Tjian, 1985; Höller *et al.*, 1988) was cloned into the blunt-ended *Sal*I site of an OVEC vector with or without either four palindromic receptor-binding sites or the SV40 enhancer at position +1800. Note that *P4/β* is identical to *P4/OVEC* of Severne *et al.* (1988); the new terminology has been introduced for reasons of clarity. All DNA constructions were performed according to standard procedures (Maniatis *et al.*, 1982) and according to the NIH guidelines for recombinant DNA research.

Calculations

For every reporter gene construction, the expression level was corrected for the reference signal (Westin *et al.*, 1987). For the reporter genes that showed an almost undetectable basal level, the stimulation was deduced by considering the sensitivity of the X-ray film (in our case, at least 1 c.p.m./band visible in a 7 day exposure, S.Wieland, personal communication). Everything below this level is referred to as <1 (Figure 2). Stimulation of *P4/β* by the transactivator generated bands ranging from 1000 to 3000 c.p.m., thus we have taken these values as >1000-fold stimulation. The stimulation induced by the truncated receptor was then calculated by comparison of the corrected reporter gene transcription in the presence or absence of the receptor. The relative strength of the enhancers used in our experiments is indicated in the rightmost column of Figure 2. Scintillation counting of the gels and densitometrical analysis of the autoradiograms provided the data for these calculations. Every reporter gene was tested in at least two different transfections of which the arithmetical mean value has been taken for the presentation of the final data.

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