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Different activation domains stimulate transcription from remote ('enhancer') and proximal ('promoter') positions

Katja Seipel, Oleg Georgiev and Walter Schaffner[1]

Institut für Molekularbiologie II der Universität Zürich, CH-8057 Zürich, Switzerland
[1]Corresponding author

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We reported previously that the lymphocyte-derived octamer transcription factor 2A (Oct-2A or OTF-2A) activated both natural immunoglobulin promoters and synthetic promoters which contain the 'octamer' site, but was unable by itself to stimulate transcription from a remote enhancer position. Here we examine a larger set of transcription factors with respect to their proximal versus remote activation. Since a transcription factor may contain more than one activation domain, we have chosen to study the potential of individual activation domains in the context of fusion proteins that contain the DNA binding domain of GAL4. We have identified at least two distinct functional classes of transcriptional activation domains. 'Proximal' activation domains, exemplified by glutamine-rich domains of Oct-1, Oct-2A and Sp1, stimulate transcription only from a position close to the TATA box, usually in response to a remote enhancer. 'General' activation domains, derived from VP16, GAL4, p65 (NF-κB), TFE3, ITF-1 and ITF-2, can activate transcription from remote as well as proximal positions. These domains contain many acidic amino acids and/or other features such as clusters of serine and threonine. The proline-rich activation domains of AP-2 and CTF/NF1 may represent a third class with considerable promoter activity and low but significant enhancer activity. Furthermore, activation domains of both the acidic and glutamine-rich types seem to have a modular structure, since duplicated subdomains can substitute for the entire domain.

Key words: activation domains/enhancer/GAL4-fusion proteins/promoter/transactivation

Introduction

Enhancers stimulate transcription of nearby genes in an orientation-independent manner and act relatively independently of their distance from the initiation site (Banerji *et al.*, 1981; Moreau *et al.*, 1981). The SV40 enhancer, often considered a paradigmatic enhancer, has a modular structure and contains multiple binding sites for different nuclear protein factors, as does the immunoglobulin heavy chain gene enhancer, the first identified cellular and cell-type specific enhancer (for reviews see Müller *et al.*, 1988a and Staudt and Lenardo, 1991). The modular organization of factor binding sites in enhancers has been most convincingly demonstrated by multimerization experiments. When a short enhancer-derived sequence motif containing factor binding site(s) was ligated in multiple copies, a synthetic enhancer could be generated that exhibited high transcriptional activity (Müller *et al.*, 1988a). Since a modular structure had also been demonstrated for promoters (Cochran and Weissmann, 1984) and since we were able to translocate enhancer sequences from promoter proximal positions to remote positions (Serfling *et al.*, 1985a,b; Schirm *et al.*, 1987), we adopted the working hypothesis that factors binding upstream of the TATA box and factors binding in a remote enhancer position acted via the same fundamental mechanism.

Here we show that this simple hypothesis needs to be revised: not all binding sites for transcription factors will, in multiple copies, generate an enhancer, even though the same sites work well at a (proximal) promoter position. Thus we considered the existence of two functional modes of action, proximal versus distal, mediated by different classes of activation domains. To test this concept, we made a series of fusion protein constructions, all based on the GAL4 DNA binding domain (amino acid residues 1-93) fused to activation domains from well characterized transcription factors, with the assumption that these activation domains are independent functional units (Brent and Ptashne, 1985). We tested glutamine-rich domains from Oct-1, Oct-2A and Sp1, proline-rich domains from CTF/NF1 and AP-2, negatively charged domains of VP16, GAL4, p65 (NF-κB) and TFE3 and serine-/threonine-rich domains of ITF-1 and ITF-2. All of these chimeric activators stimulated transcription when allowed to bind next to the TATA box. By contrast, only some of them were able to stimulate transcription from a remote position. The typical domains for promoter activation were found to be glutamine-rich, indicating that they are functionally distinct from the remote activation domains, which include domains with negatively charged residues. Therefore, our studies introduce evidence for differential function of promoters and enhancers.

Results

Oct-1, Oct-2 and Sp1 by themselves fail to enhance transcription from remote positions

Our previous experiments showed that multiple copies of a 50 bp fragment from the immunoglobulin heavy chain (IgH) enhancer can activate the β-globin promoter from a remote position in B cells (Gerster *et al.*, 1987). It appeared from mutagenesis experiments that the essential component of this 50 bp fragment was the octamer sequence ATGCAAAT, and that other components (*in vivo* footprint site E4 and flanking regions of the octamer) were required for full activity of the fragment in B cells (Gerster *et al.*, 1987, and data not shown). From this we concluded that a single binding site for an octamer factor, when multimerized, might be sufficient to create an enhancer. However, all attempts to create such an enhancer with shorter

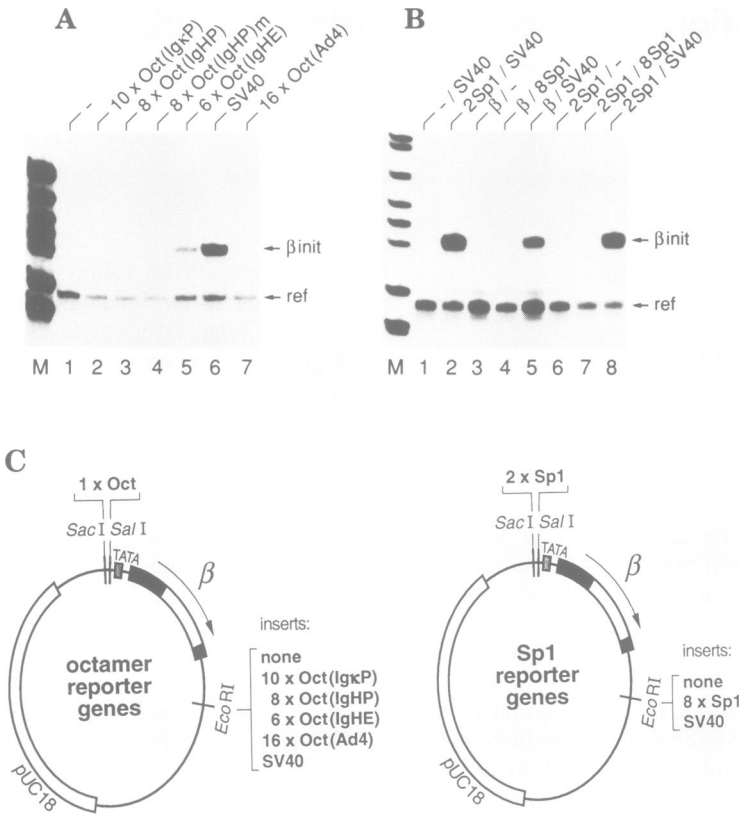

Fig. 1. Oct-1, Oct-2 and Sp1 fail to activate transcription from remote positions. (**A**) RNase protection analysis of a B cell transfection experiment with reporter genes containing the β-globin promoter and various octamer enhancers (see Materials and methods for the sequences): no enhancer (lane 1), 10×Oct(IgκP) (lane 2), 8×Oct(IgHP) (lane 3), 8×Oct(IgHP)$_m$ (lane 4), 6×Oct(IgHE) (lane 5), SV40 (lane 6), 16×Oct(Ad4) (lane 7). The only enhancers active in B cells are the SV40 enhancer and the derivative of the IgH enhancer containing six copies of the 50 bp fragment. β-init indicates the position of the correctly initiated RNA from the reporter genes and ref indicates the position of the reference signal from OVEC-REF. Lane M, size marker (*Hpa*II-cleaved pBR322). (**B**) RNase protection analysis of a HeLa cell transfection with the following reporter genes: −/SV40 (lane 1), 2 Sp1/SV40 (lane 2), β-globin/− (lane 3), β-globin/8 Sp1 (lane 4), β-globin/SV40 (lane 5), 2 Sp1/− (lane 6), 2 Sp1/8 Sp1 (lane 7), 2 Sp1/SV40 (lane 8). '−' indicates the absence of an upstream promoter or downstream enhancer element. (**C**) Schematic drawing of the reporter genes based on plasmid OVEC-1 (Westin *et al.*, 1987). OVEC-1 is derived from β1E (Gerster *et al.*, 1987) with a deletion of the β-globin promoter upstream sequences between positions −425 and −37. A *Sac*I and a *Sal*I site have been introduced immediately upstream of the β-globin gene TATA box. An octamer sequence or two Sp1 sites have been inserted between the *Sac*I and *Sal*I sites. Not depicted are the reporter genes derived from β1E which contain a complete β-globin promoter. Different inserts have been introduced into the *Eco*RI site of either β1E or OVEC-1 downstream of the coding region (see Materials and methods for sequences). Figure 1A shows the results obtained with the octamer enhancer reporter genes derived from β1E. The same results were obtained with the octamer enhancer reporter genes derived from OVEC-1 with the octamer promoter insert as depicted here (data not shown). Figure 1B shows results with the Sp1 reporter genes.

segments containing the octamer site failed, although these same DNA segments were very active in promoter proximal positions upstream of the TATA box of a reporter gene (Kemler *et al.*, 1991, and data not shown). We tested the activity of reporter constructs (Figure 1C) containing multiple copies of various octamer sequences in remote enhancer positions in the context of either the β-globin promoter (Figure 1A) or an octamer-containing promoter (data not shown). These octamer sites included ones derived from an immunoglobulin kappa promoter (IgκP) (Seidman *et al.*, 1979), a heavy chain promoter (IgHP) (Ballard and Bothwell, 1986), the origin of replication of adenovirus 4 (Ad4) (Prujin *et al.*, 1987) and a short segment of the heavy chain enhancer (IgHE) (Gerster *et al.*, 1987). All of these octamer sequences were inactive in HeLa cells, while in B cells the only active constructs were derivatives of the IgH enhancer encompassing more than just the octamer sequence (Figure 1A). We previously demonstrated that Oct-2 could not act from a remote position in HeLa cells (Müller-Immerglück *et al.*, 1990). We conclude that neither Oct-2 nor Oct-1 can activate transcription from remote positions, even in the presence of a proximal octamer site, and that an additional factor is needed for the B cell-specific activity of the 50 bp IgH enhancer fragment. We then tested other transcription factors to see if some of them were also unable to activate from an enhancer position.

The ubiquitous transcription factor Sp1 has often been considered to be a proximal promoter factor, since functional binding sites are usually found within a few hundred base pairs of the transcriptional start site (Dynan and Tjian, 1985). We examined the remote activation potential of Sp1, using reporter genes (Figure 1C) containing Sp1 binding sites from the HSV IE-3 promoter (Jones and Tjian, 1985) in proximal and/or remote positions in HeLa cells. Endogenous Sp1 factor activated transcription from a promoter but not from an enhancer position, even when Sp1 binding sites were present both in proximal and remote positions (Figure 1B). Interestingly, the cell-type specific Oct-2 and the ubiquitous proteins Oct-1 and Sp1 share a common feature, namely transcriptional activation domains relatively rich in glutamine residues (Courey and Tjian, 1988; Müller-Immerglück *et al.*, 1990). These domains lack the conspicuous excess of

Table I. Summary of transactivation results

GAL4 fusion constructs	Prevalent amino acids	Net charge	Activation	
			Proximal[a,c]	Remote[b,c]
GAL4(1−93)			(+/−)	−
GAL4(1−93)−Oct1(175−269)	Q	+1	++	−
GAL4(1−93)−Oct2(99−161)	Q	+2	++	−
GAL4(1−93)−Oct2(143−160)$_1$	Q	0	+	−
GAL4(1−93)−Oct2(143−160)$_2$	Q	0	++	−
GAL4(1−93)−Sp1(132−243)	Q	0	++	−
GAL4(1−93)−Sp1(340−485)	Q	+1	++	−
GAL4(1−93)−[Q]$_{43}$	Q	0	(+/−)	−
GAL4(1−93)−AP-2(31−76)	P>S+T	0	+	(+/−)
GAL4(1−93)−CTF(399−499)	P>S+T	+3	+	(+/−)
GAL4(1−93)−[P]$_{43}$	P	0	−	−
GAL4(1−93)−GAL4(753−881)	S+T>D+E	−8	++	++
GAL4(1−93)−VP16(413−490)	D+E	−18	++	++
GAL4(1−93)−VP16(n)(413−454)	D+E	−11	++	++
GAL4(1−93)−VP16(c)(454−490)	D+E	−7	+	+
GAL4(1−93)−VP16(437−447)$_1$	D+E	−5	+	(+/−)
GAL4(1−93)−VP16(437−447)$_2$	D+E	−10	++	++
GAL4(1−93)−p65(286−518)	P>S+T>D+E	0	++	++
GAL4(1−93)−p65(520−550)	S>D+E	−5	++	++
GAL4(1−93)−ITF1(1−427)	S+T>P	+8	++	+
GAL4(1−93)−ITF2(2−451)	S+T>P	+16	++	++
GAL4(1−93)−TFE3(2−216)	S+T>D+E	−6	++	++
GAL4(1−93)−Nuc(170−277)	D+E	−57	−	−
GAL4(1−93)−cbl2(352−469)	D+E	−14	−	−
GAL4(1−93)−[D]$_{120}$	D	−120	−	−
GAL4(1−93)−[E]$_{120}$	E	−120	−	−

[a]Transcriptional activation on the reporter genes 1GAL/SV40, 2GAL/8MRE and 2GAL/SV40.
[b]Transcriptional activation on the reporter genes β-globin/5GAL, 1SP1/5GAL, 2SP1/5GAL and 1GAL/5GAL.
[c]++ indicates strong, + weak, (+/−) very weak and − indicates no transcriptional activation by the corresponding activation domain.

Table II. Amino acid sequences of the activation domains

1.	**GAL4**	(1- 93)	Lgt
2.	**-Oct1**	(175-269)	LgtpaaasidpDLQQLQQLQQQNLNLQQFVLVHPTTNLQPAQFIISQTPQGQQGLLQAQNLQTQLPQQSQANLLQSQPSITLTSQPATPTRTIAATPIQTLPQSQSv
3.	**-Oct2**	(99-161)	LgtpaaastpLAGDIQQLLQLQQLVLVPGHHLQPPAQFLLPQAQQSQPGLLPTPNLFQLPQQTQGALLTSQPRv
4.	**-Oct2**	(143-160)$_1$	LgtrsNLFQLPQQTQGALLTSQPgstsss
5.	**-Oct2**	(143-160)$_2$	LgtrsNLFQLPQQTQGALLTSQPgsNLFQLPQQTQGALLTSQPgstsss
6.	**-Sp1**	(132-243)	LgtpaaastpNRTVSGGQYVVAAAPNLQNQQVLTGLPGVMPNIQYQVIPQFQTVDGQQLQFAATGAQVQQDGSGQIQIIPGANQQIITNRGSGGNIIAAMPNLLQQAVPLQGLANNVLSGQTgss
7.	**-Sp1**	(340-485)	LgtpaaastrSSGTNSQGQTPQRVSGLQGSDALNIQQNQTSGGSLQAGQQKEGEQNQQTQQQQILIQPQLVQGGQALQALQAAPLSGQTFTTQAISQETLQNLQLQAVPNSGPIIIRTPTVGPNG QVSWQTLQLQNLQVQNPQAQTITLAPMQGVSLgv
8.	**-Q43**		LgtpaaastptattpQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQ
9.	**-AP2**	(31- 76)	LgtpaaasidpLGTVGQSPYTSAPPLSHTPNADFQPPYFPPPYQPIYPQSQDPYSHVgss
10.	**-CTF**	(399-499)	LgtpaaastqfpgiDLVSLACDPASQQPGPLNGSGQLKMPSHCLSAQMLAPPPPGLPRLALPPATKPATTSEGGATSPTSPSTSPPDTSPANRSFVGLGPRDPAGIYQAQSWYLG
11.	**-P43**		LgtpaaastPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPPrl
12.	**-GAL4**	(753-881)	LgtpaaastpacstmaLQSLVPLTPSALFGGANFNQSGNIADSSLSFTFTNSSNGPNLITTQTNSQALSQPIASSNVHDNFMNNEITASKIDDGNNSKPLSPGWTDQTAYNAFGITTGMFNTTTM DDVYNYLFDDEDTPPNPGKKElargsl
13.	**-VP16**	(413-490)	LgtpaastqfpgiwAPPTDVSLGDELHLDGEDVAMAHADALDDFDLDMLGDGDSPGPGFTPHDSAPYGALDMADFEFEQMFTDALGIDEYGG
14.	**-VP16**	(413-454)	LgtpaastqfpgiwAPPTDVSLGDELHLDGEDVAMAHADALDDFDLDMLGDGDSPGL
15.	**-VP16**	(454-490)	LGPGFTPHDSAPYGALDMADFEFEQMFTDALGIDEYGG
16.	**-VP16**	(437-447)$_1$	LgtrsDALDDFDLDMLgstsss
17.	**-VP16**	(437-447)$_2$	LgtrsDALDDFDLDMLgsDALDDFDLDMLgstsss
18.	**-p65**	(286-518)	LgtpaaastqFQYLPDTDDRHRIEEKRKRTYETFKSIMKKSPFSGPTDPRPPPRRIAVPSRSSASVPKPAPQPYPFTSSLSTINYDEFPTMVFPSGRSARPRLGPAPPQVLPQAPAPAPAPAMVS ALAQAPAPVPVLAPGPPQAVAPPAPKPTQAGEGTLSEALLQLQFDDEDLGALLGNSTDPAVFTDLASVDNSEFQQLLNQGIPVAPHTTEPMLMEYPEAITRLVTGAQRPPDPAPAPLG
19.	**-p65**	(520-550)	LgtpaaastPGLPNGLLSGDEDFSSIADMDFSALLSQISS
20.	**-ITF1**	(1-427)	LgtpaaastptvspEFGGGECLAWCGPSAVHRCADVGLGMVSARTAPGKSGERGAYASFGRDAGVGGLTQAGFLSGELALNSPGPLSPSGMKGTSQYYPSYSGSSPPPAADGSLDTQPKKVRKVP PGLPSSVYPPSSGEDYGRDATAYPSAKTPSSTYPAPFYVLPLPPGSGPVGSSGSSSTFGGLHQHERMGYQLHGAEVNGGLPSASSFSSAPGATYGGVSSHTPPVSGADSLLGSRGTTAGSSGDAL GKALASIYSPDHSSNNFSSSPSTPVGSPQGLAGTSQWPRAGAPGALSPSYDGGLHGLQSKIEDHLDEAIHVLRSHAVGTAGDMHTLLPGHGALASGFTGPMSLGGRHAGLVGGSHPEDGLAGSTS LMHNHAALPSQPGTLPDLSRPPDSYSGLGRAGATAA
21.	**-ITF2**	(2-451)	LgtapcscvdqFGGVEDRSSSGSWGNGGHPSPSRNYGDGTPYDHMTSRDLGSHDNLSPPFVNSRIQSKTERGSYSSYGRESNLQGCHQQSLLGGDMDMGNPGTLSPTKPGSQYYQYSSNNPRRRP LHSSAMEVQTKKVRKVPPGLPSSVYAPSASTADYNRDSPGYPSSKPATSTFPSSFFMQDGHHSSDPWSSSSGMNQPGYAGMLGNSSHIPQSSSYCSLHPHERLSYPSHSSADINSSLPPMSTFHR SGTNHYSTSSCTPPANGTDSIMANRGSGAAGSSQTGDALGKALASIYSSDHTNNSFSSNPSTPVGSPPSLSAGTAVWSRNGGQASSSPNYEGPLHSLQSRIEDRLERLDDAIHVLRNHAVGPSTA MPGGHGDMHGIIGPSHNGAMGGLGSGYGTGLLSANRHSLMVGTHREDGVALRGSHSLLPNQVPVQLPVQSATSPDLNPPQDPYRGgl
22.	**-TFE3**	(2-216)	LgtpaaastqFGRASQALTPPPGKASAQPLPAPEAAHTTGPTGSAPNSPMALLTIGSSSEKEIDDVIDEIISLESSYNDEMLSYLPGGTTGLQLPSTLPVSGNLLDVYSSQGVATPAITVSNSCP AELPNIKREI
23.	**-Nuc**	(170-277)	LgtpaaastqfrIEPAAMKAAAAAPASEDEDDEDDEDDEDDDDDEEDDSEEEAMETTPAKGKKAAKVVPVKAKVAEDEDEEEDDEDEDDDDDEDDEDDDDEDDEEEEEEEEEEPVKEAPgl
24.	**-cbl2**	(352-469)	LgtpaaastLCEPTPQDHIKVTQEQYELYCEMGSTFQLCKICAENDKDVKIEPCGHLMCTSCLTSWQESEGQGCPFCRCEIKGTEPIVVDPFDPRGSGSLLRQGAEGAPSPNYDDDDDERADDTL FMgss
25.	**-D~120**		LalqlrrpDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDgv
26.	**-E~120**		LgtplrrpEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEgv

All the indicated sequences were tested for transcriptional activation as fusion to the GAL4 DNA-binding domain. The sequences start with the leucine residue 93 of GAL4. Amino acids encoded by artificial linkers are shown in small letters. Peptides used as minimal activation domains derived from Oct-2 (lane 3) and VP16 (lane 13) are underlined. They were used for the constructions in lanes 4, 5 and 16, 17, respectively. The region of highest homology to the Oct-2 activation domain is positioned at residues 223-241 of the Oct-1 activation domain (lane 2).

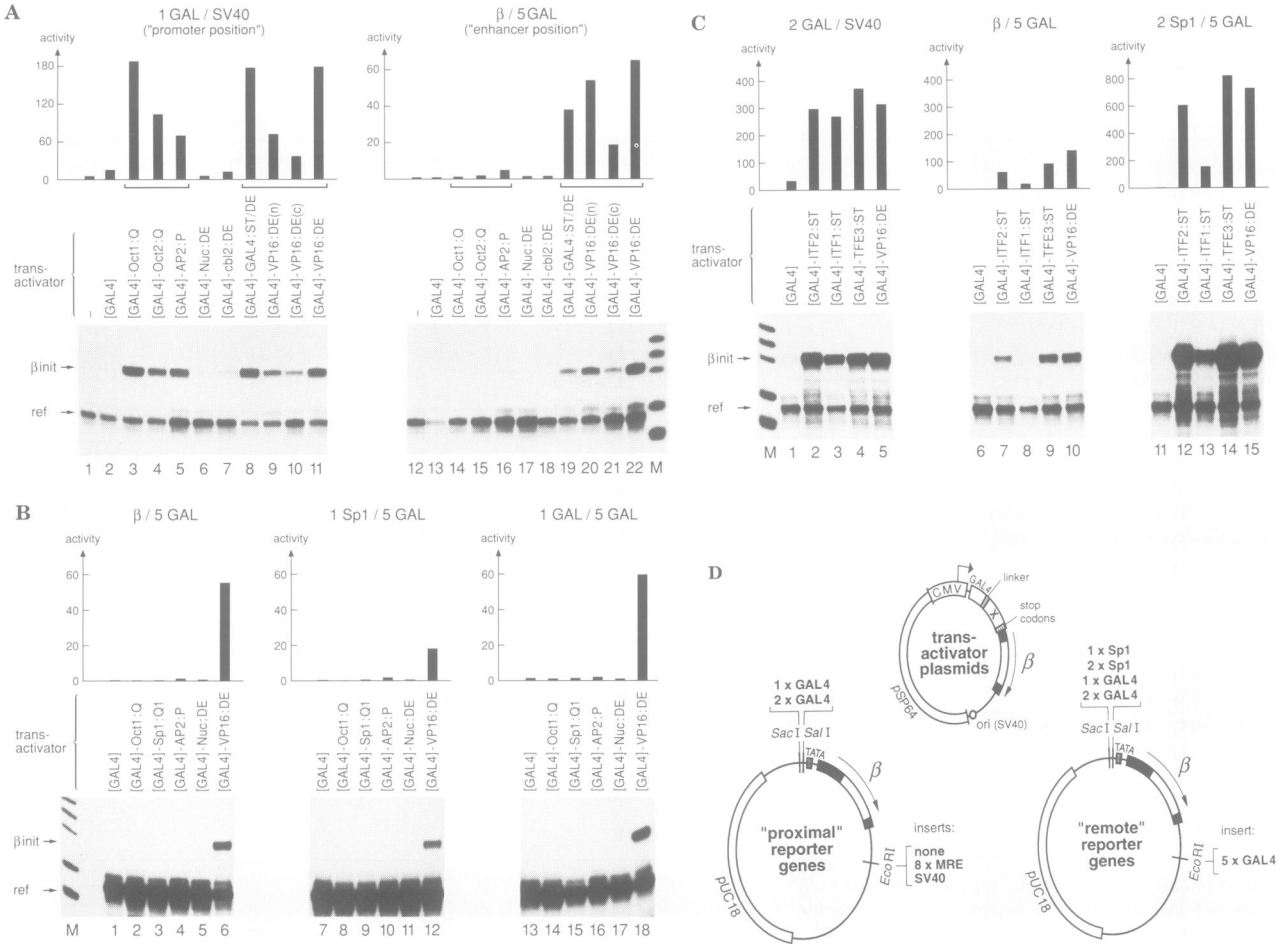

Fig. 2. The glutamine-rich activation domains from several known transcription factors fail to activate transcription from remote positions. RNase protection analysis of representative HeLa cell transfection experiments. Quantification of the signals is shown above the autoradiographs. The 'activity' numbers are arbitrary units of the signals from the reporter genes (β-init) relative to the signal from the reference gene (ref). Variability of transfection efficiency leads to the differences in 'activity' (compare panel A lane 22 with panel B lane 6, panel C lane 10 and Figure 3 lane 19). **(A)** RNase protection analysis of a HeLa cell transfection with the reporter genes 1 GAL/SV40 and β-globin/5 GAL and the indicated GAL4 fusions as transactivators (see Table II for the sequences). **(B)** RNase protection analysis of a HeLa cell transfection with the reporter genes β-globin/5 GAL, 1 Sp1/5 GAL, 1 GAL/5 GAL and the indicated GAL4 fusions as transactivators (see Table II for the sequences). **(C)** RNase protection analysis of a HeLa cell transfection with the reporter genes 2 GAL/SV40, β-globin/5 GAL, 2 Sp1/5 GAL and the indicated GAL4-fusions as transactivators (see Table II for the sequences). **(D)** Schematic drawing of the transactivator and the reporter plasmids. The depicted reporter genes are derived from OVEC-1 (see Figure 1C). Not depicted are the reporter genes derived from β1E which contain a complete β-globin promoter. All possible combinations of proximal and remote elements were tested as reporter genes. All transactivator plasmids (GAL4)-X were based on the plasmid pSCTEV-GAL4(1−93), which is designated (GAL4). The linker consists of an oligonucleotide with restriction sites in three frames. DNA fragments coding for transcriptional activation domains and other protein domains were fused to (GAL4) and are designated as (X). For the amino acid sequences see Table II.

negative charges present in some transcription factors like GCN4 (Hope and Struhl, 1986), GAL4 (Ma and Ptashne, 1987), VP16 (Triezenberg *et al.*, 1988), TFE3 (Beckmann *et al.*, 1990) and NF-κB (Schmitz and Baeuerle, 1992). Other types of activators that we have tested previously and found to be active from remote positions include multiple binding sites of the metal-regulated enhancer element (MRE) (Westin and Schaffner, 1988), the glucocorticoid receptor element (GRE) (Schatt *et al.*, 1990), NF-κB (Pettersson *et al.*, 1990; see also Pierce *et al.*, 1988) and the papilloma virus transactivator E2 (our unpublished results; see also Forsberg and Westin, 1991). Therefore, we postulated that there were two modes of activation, proximal and remote, and that these represent functions of two different types of activation domain.

The activation domains from several known transcription factors can be divided into at least two functional groups

In order to study individual protein domains for transcriptional activation, we created a series of fusion constructions based on the DNA binding domain of the yeast factor GAL4 fused to different activation domains from known transcription factors (Tables I and II): the glutamine-rich regions of Oct-1, Oct-2 and Sp1, the proline-rich regions of AP-2, CTF/NF1 and p65 (NF-κB), the serine- and threonine-rich domains of ITF-1 and ITF-2 and the negatively charged regions from VP16, GAL4, p65 (NF-κB) and TFE3. ITF-1, ITF-2 and TFE3 are helix−loop−helix factors that bind to the IgH enhancer. We also tested fusion proteins representing protein domains from

non-transcription factors, namely the highly acidic regions of the product of the *cbl-2* oncogene and of nucleolin.

For good promoter activity, at least one copy of an upstream factor binding site is needed in addition to the TATA box and initiator region. Increasing the number of factor binding sites in the enhancer to two, four and eight increases the promoter activity without qualitatively changing the results (Gerster *et al.*, 1987; Schatt *et al.*, 1990; M.Pettersson and W.Schaffner, unpublished). Therefore we used reporter plasmids with one or two factor binding sites next to the TATA box and multiple binding sites in a remote position.

The reporter genes were of two types (Figure 2D). The 'proximal GAL4' reporter genes contained one or two GAL4 binding sites next to the TATA box, and either no enhancer, an SV40 enhancer or eight metal-regulatory elements (MREs) downstream of the reporter gene. The 'remote GAL4' reporter genes contained five GAL4 binding sites downstream of the reporter gene and, as promoter, the β-globin TATA box in combination with one of the following proximal upstream elements: the rabbit β-globin upstream region (~2 kb; Dierks *et al.*, 1983), one or two Sp1 sites, one or two GAL4 sites, or an octamer site. With the exception of the glutamine-rich activation domains, all activation domains were able to activate transcription from proximal and remote positions, although to different extents (Figure 2, panels A, B and C, data summarized in Table I). The glutamine-rich domains activate transcription only from a proximal position. The acidic clusters of the product of the *cbl-2* oncogene and of nucleolin and most of the homopolymers failed to activate the GAL4 reporter genes. The effects were not specific for a given promoter or enhancer, since different reporter gene constructions responded similarly (Table I, Figure 2 panels A, B and C, and data not shown). All the fusion proteins were able to bind to the GAL4 DNA binding site and were expressed at similar levels, as judged by gel retardation analysis with nuclear extracts from COS cells transfected with the same quantity of plasmid DNA as in the HeLa cell transfections. Only the acidic domain of GAL4 and the acidic homopolymers shifted less of the probe than the others (data not shown). From these experiments we conclude that there exist at least two classes of transcriptional activating domains (Table I): one exemplified by the glutamine-rich activation domains of Oct-1, Oct-2 and Sp1, the second by the negatively charged domains of VP16, GAL4, p65 (NF-κB) and TFE3 and the serine-/threonine-rich domains of ITF-1 and ITF-2 (which would also be negatively charged if phosphorylated). The proline-rich domains of AP-2 and CTF/NF1 may belong to a third class, since they have considerable activity from proximal positions and also weak, but significant, activity from remote positions.

The activation domains of Oct-2 and VP16 can be reduced to short peptides and still retain full transcriptional activation potential

In order to characterize further the functions of the different classes of activation domains, we attempted to design minimal functional units of activation domains. In a first approach we tested homopolymeric stretches of the prevalent amino acids of different activation domains, namely poly-glutamate, poly-aspartate, poly-glutamine and poly-proline stretches. Only one of the homopolymers, namely the poly-glutamine sequence, when fused to the GAL4 DNA binding domain, mediated some transcriptional activation from a proximal position (Table I and data not shown). The next approach was to take subdomains of defined activation domains. The subdomain of the Oct-2 factor was taken from the most conserved region between the glutamine-rich regions of Oct-1 (Sturm *et al.*, 1988) and Oct-2 (Müller *et al.*, 1988b) (Table II). This segment of 18 amino acid residues, which was originally tested in the laboratory of W.Herr (unpublished data), functioned weakly, but, if dimerized to two tandem copies, it functioned almost as well as the complete Oct-2 activation domain (Figure 3). The 11 amino acid residue subdomain of VP16 contains the important Phe442 and may have a helical structure (Cress and Triezenberg, 1991). This segment was weakly active as a single copy, but if dimerized, it was as active as the entire 80 amino acid VP16 activation domain (Figure 3), indicating that transcriptional activation domains have a modular structure.

Discussion

Different mechanisms for different classes of activation domains

Transcriptional activation domains have been characterized by a number of criteria: general amino acid content (Mitchell and Tjian, 1989), requirement for coactivators (Tanese *et al.*, 1991; Berger *et al.*, 1992), activity in histone antirepression assays (Croston *et al.*, 1991; Laybourn and Kadonaga, 1991; Workman *et al.*, 1991), and *in vivo* interference or 'squelching' (Tasset *et al.*, 1990). This study introduces evidence for differential function of promoters and enhancers. Activation domains are classified according to their ability to stimulate transcription from remote and/or proximal positions (Table I). The activation domains we tested fall into at least two groups: one, represented by the glutamine-rich domains, can activate only from a position close to the TATA box ('proximal' activation), usually in response to a remote enhancer, whereas another, which includes the acidic domains, can activate from remote positions as well as from proximal positions ('general' activation). These two functional classes reflect qualitative differences rather than merely quantitative ones, i.e. 'strong' versus 'weak' activation. For example, the small acidic VP16 fragments, which are less effective than the glutamine-rich domains in a proximal position, nevertheless function well from a distance, unlike the glutamine-rich domains (Figure 2A). From these findings one might wonder whether the glutamine-rich domains even qualify as bona fide activators. They can, however, activate transcription in cell-free systems without the need for additional enhancer components. We have tested, in collaboration with Danny Reinberg and his colleagues, the effect of Oct-2 factor in an *in vitro* transcription system with individually purified components. Oct-2 clearly stimulates transcription in the absence of nucleosomes (Arnosti *et al.*, 1993). A simple explanation would be that proximally active domains interact directly with the basal transcription machinery, while a major function of domains with long-range potential is to reorganize the chromatin for efficient transcription *in vivo*.

It is also possible that the two classes of activation domains

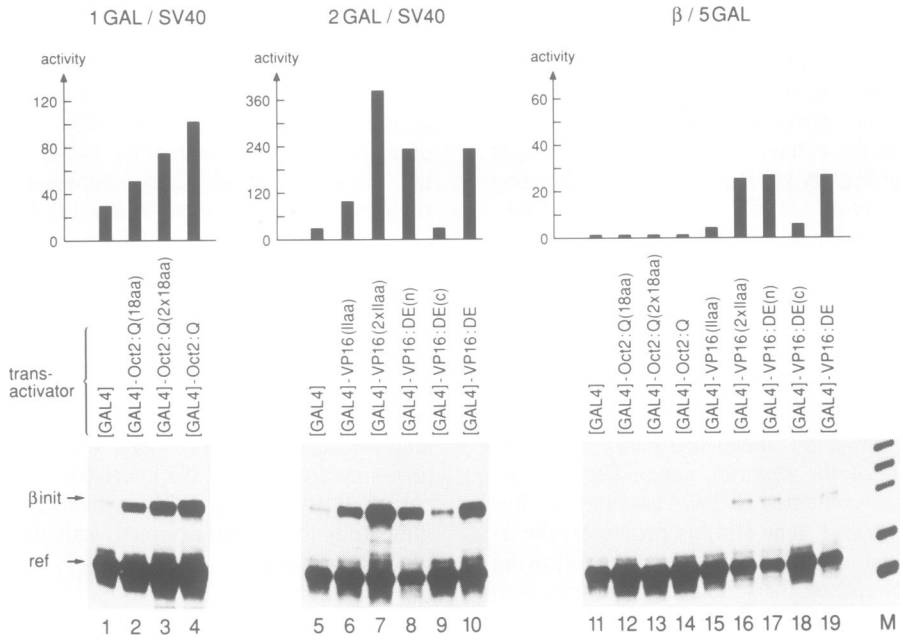

Fig. 3. The activation domains of Oct-2 and VP16 can be reduced to short peptides and still retain full transcriptional activation potential if dimerized. The 18 amino acid segment is the most conserved region in the glutamine-rich activation domains of Oct-1 and Oct-2 and was previously found to be active by W.Herr (personal communication),. The 11 amino acid VP16 segment includes the critical Phe442 (Cress and Triezenberg, 1991). RNase protection analysis of a representative HeLa cell transfection with the reporter genes 1 GAL/SV40, 2 GAL/SV40 and β-globin/5 GAL and the indicated GAL4 fusions as transactivators (see Figure 2D and Table II for reporter and transactivator plasmids).

interact with distinct proteins in the basal transcription complex. Furthermore, the target proteins in the basal complex might be differentially accessible: while the target for proximal activation domains would be accessible only from positions close to the TATA box, the target for the general activation domains would be accessible from any position, presumably by looping out of the intervening DNA. If general and proximal activators have distinct pathways of activation, then one would expect to see synergistic activation when both elements are combined. Such a synergism is found with glutamine-rich domains of Oct-1, Oct-2 and Sp1. These 'proximal' activation domains are weak activators in the absence of an enhancer, but respond strongly to a remote enhancer. In contrast, acidic activation domains can be very active by themselves, with no need for an extra enhancer (our unpublished data). Thus the glutamine-rich activation domains, which have not been found in yeast so far, may be of particular importance in higher eukaryotes, where one gene can be independently controlled by several remote enhancers. We propose that the 'proximal' activation domains have evolved to allow for efficient channelling of an enhancer effect to the responsive promoter.

Can Oct-2 and Sp1 still contribute to enhancer function?

Even though domains for proximal and remote activation might in principle be present within one and the same transcription factor, this seems not to be the case for Oct-1, Oct-2 and Sp1, which in our experiments do not activate from an enhancer position (Figure 1A and Müller-Immerglück *et al.*, 1990). However, in a specific context both Oct-2 and Sp1 may contribute to enhancer function. A 50 bp segment of the IgH enhancer harboring an octamer site was found to be a strong B cell-specific enhancer, and the activity of this enhancer depended on the integrity of the octamer site (Gerster *et al.*, 1987). However, in further studies, we found that enhancer activity was also lost by mutations upstream and downstream of the octamer site (unpublished results), suggesting a requirement for further factors on the 50 bp fragment. The most likely explanation is that Oct-2 binds to the IgH enhancer and facilitates binding of one or more additional factors with long-range activation potential. Consistent with this suggestion, Annweiler *et al.* (1992) found that Oct-2 could contribute to IgH enhancer function in B cells, even when the protein lacked its glutamine-rich activation domain, which is essential for proximal activation. In this instance, the C-terminal domain was required for efficient enhancer function. They concluded that a B cell-restricted cofactor was interacting with Oct-2, which is consistent with the idea of an 'anchoring' role for Oct-2.

As observed with octamer promoters, promoters containing Sp1 sites were very active in response to a remote SV40 enhancer, but endogenous Sp1 factor was not active from a remote position in our constructions (Figure 1B). We note that these results are at variance with findings of Courey *et al.* (1989), who reported long-range activation by Sp1 factor that was highly expressed in *Drosophila* Schneider cells. There are several possible explanations for this difference. For example, in *Drosophila* cells, there may be some quantitative effect due to Sp1 overexpression, or a cofactor with long range activity may be fortuitously recruited by Sp1. Our results with the GAL4 fusion proteins were consistent with the inactivity of endogenous Sp1 from distal positions. As with the glutamine-rich activation domains of Oct factors, the glutamine-rich activation domains of Sp1 fused to GAL4 yielded factors that were active from

a promoter position in response to a remote SV40 enhancer but inactive from the enhancer position (Table I, Figure 2B and data not shown). There was one exception: the chimeric factor containing residues 132–243 of Sp1 (GAL4-Sp1:Q1) very weakly activated the 2Sp1/5GAL reporter gene (data not shown). However, it could not activate the 2Sp1/– or the 2GAL/5GAL reporter gene, which indicates that the glutamine-rich activation domain of Sp1 functionally interacted, if at all, only with the complete Sp1 factor bound in the promoter position. Furthermore, the stimulation by GAL4-Sp1:Q1 from remote positions required very high intracellular factor concentrations, consistent with the findings of Courey *et al.* (1989).

Modular structure of activation domains

Comparison of activation domains reveals a striking variety in primary amino acid sequence, but also a clustering of certain amino acids like glutamine, serine/threonine or a high density of negative charges (Table II; Mitchell and Tjian, 1989). The latter has been found to be a characteristic of activation domains of two yeast transcription factors (Hope and Struhl, 1986; Ma and Ptashne, 1987). It has been postulated that a negatively charged amphipathic helix is a common structural theme, and such a synthetic construction has indeed been found to be active (Giniger and Ptashne, 1987). However, while it has been calculated that the C-terminal activation domain of NF-κB assumes a helical structure, this is not true for most activation domains. Cress and Triezenberg (1991) found in their analysis of the acidic VP16 activation domain that helix-breaking mutations did not necessarily impair function. Unexpectedly, some mutations that removed acidic amino acids also retained activity, while a phenylalanine to alanine mutation in the N-terminal half of the VP16 domain abrogated activity. Our studies show that the C-terminal portion of the VP16 domain also works as an activation domain, demonstrating that a region outside of the critical phenylalanine (Phe442) has activity on its own. Nevertheless, the importance of the region encompassing the phenylalanine is emphasized by our studies where a segment of 11 amino acids including Phe 442 shows strong activity when dimerized (Figure 3). The fact that such subfragments of the VP16 domain have independent activity reveals a modular structure for this activation domain. Glutamine-rich activation domains also seem to have a modular structure, since one or two copies of an 18 amino acid segment of Oct-2 activate transcription (Figure 3). The implications of modular structure for gene regulation have been reviewed recently (Frankel and Kim, 1991). The single modules of an activation domain could have different functions, for example they could interact with different adaptors. Alternatively, in a more straightforward manner, they could reinforce interaction with a single class of target(s) whose concentration is limiting (see also Pettersson and Schaffner, 1990).

Materials and methods

Construction of plasmids

The constructs containing different octamer sequences are based on the plasmids β1E (Gerster *et al.*, 1987) or OCTA(1) (Müller-Immerglück *et al.*, 1990). They contain either the SV40 enhancer or multimerized copies of double stranded oligonucleotides cloned in the downstream *Eco*RI site:

1 x Oct (IgHE): 5'-CTGAGCAAAACACCACCTGGGTAATTTGCATTTCTAAAATAAGTCGA
CGTTTTGTGGTGGACCCATTAAACGTAAAGATTTTATTCAGCTGACT-5'

1 x Oct (IgHE)m: 5'-CTGAGCAAAACACACCCGGGGTAATTTGCATTATGCAAATAAGTCGA
CGTTTTGTGTGGGCCCCATTAAACGTAATACGTTTATTCAGCTGACT-5'

1 x Oct (IgHP): 5'-TCGAGTCCTCATGAATATGCAAATCATTGG
CAGGAGTACTTATACGTTTAGTAACCAGCT-5'

1 x Oct (IgHP)m: 5'-TCGAGTGCACGTTAATCTGAACATCAATTGG
CACGTGCAATTAGACTTGTAGTTAACCAGCT-5'

5 x Oct (IgkP): 5'-CGAGTAATAATTTGCATACCCTTAATAATTTGCATACCCTTAATAATTTGCAT-
TCGAGCTCATTATTAAACGTATGGGAATTATTAAACGTATGGGAATTATTAAACGTA-

-ACCCTTAATAATTTGCATACCCTTAATAATTTGCATG
-TGGGAATTATTAAACGTATGGGAATTATTAAACGTACAGCT-5'

4 x Oct (Ad4): 5'-TCGAGAATATGCAAATAATATGCAAATAATATGCAAATAATATGCAAATG
CTTATACGTTTATTATACGTTTATTATACGTTTATTATACGTTTACAGCT-5'

The oligonucleotide containing two Sp1 sites was cloned as a single copy into the blunt ended *Sal*I site in front of the TATA box of OVEC-1 (Westin *et al.*, 1987; Westin and Schaffner, 1988), or as multimerized copies into the blunt-ended downstream *Eco*RI site of β1E:

2 x Sp1: 5'-CCGGGCCCCGCCCATCCCCGGGCCCCGCCCATTC
GGCCGGGGCGGGTAGGGGCCGGGGCGGGTAAG-5'

The constructs containing GAL4 binding sites are based on the plasmids β1E (Gerster *et al*, 1987) or OVEC-1 (Westin *et al.*, 1987). They contain either no enhancer, the SV40 enhancer, or eight copies of an oligonucleotide containing an MRE (Westin and Schaffner, 1988) in the downstream *Eco*RI site:

1 x MRE: 5'-GAGCTCTGCACTCCGCCC
AGACGTGAGGCGGGCTCG-5'

The constructs bearing GAL4 binding sites in promoter position were prepared by inserting a synthetic oligonucleotide containing two consensus GAL4 binding sites, or two mutant binding sites, into the *Sac*I and *Sal*I sites of the OVEC-1 and the OVECS plasmid which is a derivative of the OVEC-1 plasmid (Westin *et al.*, 1987) containing the SV40 enhancer in the *Eco*RI site downstream of the coding region:

2 x GAL4: 5'-CGAGCGGAGGACTGTCCTCCGGAGCTCCGGAGGACTGTCCTCCG
TCGAGCTCGCCTCCTGACAGGAGGCCTCGAGGCCTCCTGACAGGAGGCAGCT-5'

2 x GAL4M: 5'-CGAGGGGAGTACTGTCCTCCCGAGCTCGGGAGTACTGTCCTCCC
TCGAGCTCCCCTCATGACAGGAGGGCTCGAGCCCTCATGACAGGAGGGAGCT-5'

The construct bearing five GAL4 binding sites in enhancer position was prepared by inserting a *Pst*I–*Xba*I fragment from plasmid pG5E4 (Carey *et al.*, 1990) into the *Eco*RI site of plasmid β1E (Gerster *et al.*, 1987).

The expression plasmids were all based on the plasmid pSCTEV, a derivative of plasmid pSCT (Rusconi *et al.*, 1990), containing the three stop codons from pEV3S (Matthias *et al.*, 1989). The expression plasmids all contain the DNA binding domain of GAL4 (amino acid residues 1–93) from plasmid pSG424 (Sadowski and Ptashne, 1989) and a polylinker with the restriction sites *Kpn*I, *Pst*I, *Sal*I, *Sma*I and *Xba*I in three frames. The following activation domains were cloned into the *Sma*I site: from Oct-1, residues 175–269 (*Xho*II–HindII fragment of human cDNA) (Sturm *et al.*, 1988); from Oct-2, residues 99–161 [*Kpn*I–*Sma*I fragment of plasmid p0(99–479) (Müller-Immerglück *et al.*, 1990)]; from Sp1, residues 132–234 (*Hin*f1 fragment from plasmid pPacSp1) and 340–485 (*Sau*3AI–*Sty*I fragment from plasmid pPacSp1-516C) (Courey and Tjian, 1988); from AP-2, residues 31–76 (*Pvu*II–*Hin*dII fragment of human cDNA) (Williams *et al.*, 1988); from CTF/NF1, residues 399–499 (*Eco*RI fragment from plasmid pGAL-CTF1(399–499) (Martinez *et al.*, 1991); from VP16, residues 413–453 (*Sma*I fragment from pSGVP), residues 454–490 (*Sma*I–*Kpn*I fragment from pSGVP) and residues 413–490 (*Eco*RI–*Kpn*I fragment from pSGVP) (Sadowski *et al.*, 1988); from GAL4, residues 753–881 (*Hin*dIII–*Bam*HI fragment from pSCTGalX556) (Rusconi *et al.*, 1990); from ITF-1, residues 1–427 (*Hin*dII fragment of GAL4-E2.5); from ITF-2, residues 2–452 (*Eco*RI–*Sma*I fragment of GAL4-E2.2) (Henthorn *et al.*, 1990); from TFE3, residues 2–126 (*Eco*RI fragment of GAL4-λ3Δ2) (Beckmann *et al.*, 1990); from NF-κB p65, residues 286–518 (*Xho*I–*Hin*dIII fragment of GAL4ΔNΔ*Sma*) and residues 520–550 (*Xho*I–*Hin*dIII fragment of GAL4Δ*Sma*) (Schmitz and Baeuerle, 1992). Other domains fused to the GAL4 DNA binding domain were: from nucleolin, residues 170–277 (*Eco*RI–*Bst*N1 fragment of human cDNA

clone ASA4) (A.Seiler, unpublished; Srivastava *et al.*, 1989); from cbl-2, residues 352–469 (*Bsr*I–*Bsp*HI fragment of human cDNA clone) (W.Langdon, unpublished; Blake,T., Shapiro,M., Moise,M.,III, Langdon,W., EMBL data library accession number X57110). Additional sequences fused to the GAL4 DNA binding domain were multimerized oligonucleotides coding for homopolymeric stretches of 43 glutamine (Q), 34 proline (P), or ~120 aspartic (D) or glutamic (E) acid residues.

Transfections and RNA analysis

B cells (X63) were transfected by the DEAE–dextran procedure (Gerster *et al.*, 1987) with 5 μg reporter plasmid and 1 μg reference plasmid or 4 μg reporter plasmid, 2 μg transactivator plasmid and 1 μg reference plasmid. HeLa cells and COS cells were transfected by the calcium phosphate coprecipitation procedure (Westin *et al.*, 1987) with 10 μg reporter plasmid, 5 μg transactivator plasmid, 1 μg reference plasmid and 5 μg sonicated salmon sperm DNA as carrier. The reference plasmid used in all transfections was OVEC-REF (Westin *et al.*, 1987). Zinc inductions were done as described by Westin and Schaffner (1988). After incubation of the cells for ~40 h, cytoplasmic RNA was extracted according to Schreiber *et al.* (1989). 20 μg of RNA from HeLa or COS cells and 50 μg of RNA from X63 cells were used for hybridization to a radioactive complementary strand RNA probe (covering positions −37 to +179 of the vector), generated by SP6 RNA polymerase. Hybridization was performed at 37°C overnight. Hybridization products were digested with RNase A (6.5 μg/ml) and RNase T1 (10 U/ml) at 37°C for 60 min and separated on a 6% polyacrylamide/7 M urea gel. For quantification, radioactive bands were cut out of the gel and measured by scintillation counting. The signals derived from the reference transcripts were used to normalize for variability in the transfection efficiency.

Preparation of nuclear extracts and bandshift experiments

Nuclear extracts from COS cells transfected with the same quantity of plasmid DNA as in the HeLa cell transfections were prepared as described by Schreiber *et al.* (1989). For bandshift experiments, an end-labelled 42 bp double stranded DNA probe containing a perfect palindromic consensus GAL4 binding site was used:

5'- TCGACGAGCTCGGGTCGGAGGACTGTCCTCCGACTGCTCGAG-3'
3'- GCTCGAGCCCAGCCTCCTGACAGGAGGCTGACGAGCTCAGCT-5'

Bandshift conditions were as described in Carey *et al.* (1989). 10 fmol of end-labelled probe were incubated with 10 μg of nuclear extract. To confirm the specificity of binding, 2 pmol of specific competitor containing two wildtype or two mutated GAL4 binding sites (see above) were added to the reaction.

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