Nucleic Acids Research, 1997, Vol. 25, No. 18 **3621–3628**

The transcriptional activation and repression domains of RFX1, a context-dependent regulator, can mutually neutralize their activities

Yael Katan, Reuven Agami and Yosef Shaul*

Department of Molecular Genetics, The Weizmann Institute of Science, Rehovot 76100, Israel

Received June 2, 1997; Revised and Accepted July 31, 1997

ABSTRACT

EP is a DNA element found in regulatory regions of viral and cellular genes. While being a key functional element in viral enhancers, EP has no intrinsic enhancer activity but can stimulate or silence transcription in a context-dependent manner. The EP element is bound by RFX1, which belongs to a novel, evolutionarily conserved protein family. In an attempt to decipher the mechanism by which EP regulates transcription, the intrinsic transcriptional activity of RFX1 was investigated. A functional dissection of RFX1, by analysis of deletion mutants and chimeric proteins, identified several regions with independent transcriptional activity. An activation domain containing a glutamine-rich region is found in the N-terminal half of RFX1, while a region with repressor activity overlaps the C-terminal dimerization domain. In RFX1 these activities were mutually neutralized, producing a nearly inactive transcription factor. This neutralization effect was reproduced by fusing RFX1 sequences to a heterologous DNA-binding domain. We propose that relief of self-neutralization may allow RFX1 to act as a dual-function regulator via its activation and repression domains, accounting for the context-dependent activity of EP.

INTRODUCTION

The transcription of eukaryotic genes is regulated by promoters and enhancers, each of which is composed of multiple elements. Many such elements possess an intrinsic activity and can thus function independently, when multimerized and linked to a reporter gene. Another class of regulatory elements exert their proper effect only when positioned within their natural DNA contexts. Reported mechanisms of context-dependent regulation include alterations in the structure of DNA or chromatin and promotion of protein–DNA or protein–protein interactions between enhancer-bound transcription factors (1–3). In other cases the transcriptional effect of a specific factor can be reversed by a neighbouring DNA-bound protein (4–6). Several context-dependent regulators were shown to contain both activation and repression domains (7–13), suggesting that the differential activity of such a transcription factor may result from an interplay between its independent functional regions.

A well studied binding site exhibiting a context-dependent activity is the EP (or EF-C) element of the hepatitis B virus (HBV) enhancer (14,15). This inverted repeat element is conserved in hepadnaviruses from other species (15,16), and related sequences are present in several viral enhancers (14,16–19) and regulatory regions of cellular genes (20–26). Mutational analyses have shown these sites to play a key stimulatory role in the HBV and polyomavirus enhancers (15,16,24,27–29) and to be functionally important positive elements in the promoters of the major histocompatibility complex (MHC) class II (20,30,31) and the ribosomal protein rpL30 (22,23) genes. Yet, when taken outside of the HBV enhancer and multimerized, the EP element cannot stimulate transcription significantly and thus possesses no intrinsic enhancer activity (16). Moreover, a multimer of either the HBV or the polyomavirus enhancer EP element, or of a homologous binding site present in intron 1 of the human c-*myc* gene, was shown to function as a transcriptional silencer in several different contexts (32–34, our unpublished data). The observation that the c-*myc* site is a target for mutations in Burkitt's lymphoma raised the suggestion that this element may negatively regulate c-*myc* expression (35,36). A transcriptional inhibitory binding site (NREγ) upstream of the HBV core promoter was recently shown to be an EP-homologous element (37), and the X box, the EP-like site of MHC class II promoters, was also reported to possess an inhibitory activity (38).

The EP element binds a ubiquitous nuclear protein complex (14,15,18). This complex was shown to contain the c-Abl tyrosine kinase (41,42) as well as dimers of the RFX1, RFX2 and RFX3 proteins (24,43,44). The latter are members of a novel family of DNA-binding proteins, highly conserved in evolution (24,43–47). The RFX proteins share several regions of homology, including a DNA-binding domain (DBD) and a dimerization domain (43,44,46). In contrast to the common situation in dimeric transcription factors, the DBD and dimerization domain of the RFX proteins are non-adjacent and functionally independent, so that the dimerization domain is not required for DNA-binding (43,44). RFX1–3, identified in humans and mice, are expressed in a tissue-specific manner, with only RFX1 being ubiquitously expressed (44). This major EP-binding protein was originally cloned on the basis of its affinity for the MHC promoter X box (48) and was shown, by antisense experiments, to play a role in the induction of MHC class II genes by interferon-γ (43,49). Overexpression and introduction of antisense oligonucleotides also demonstrated the involvement of RFX1 in the stimulatory activity of the HBV

*To whom correspondence should be addressed. Tel: +972 8 9342320; Fax: +972 8 9344108; Email: lvshaul@weizmann.weizmann.ac.il

enhancer (24). In addition, the overexpression of RFX1 can activate the HBV core promoter through the upstream NREγ site (37).

The mechanism by which the effect of RFX1 is exerted is presently unknown. The ability of the EP site to both enhance and silence transcription, in a context-dependent manner, prompted us to investigate the intrinsic transcriptional activity of RFX1, which could account for the EP-mediated effects. A functional dissection of RFX1 identified an N-terminal activation domain containing a glutamine-rich region and a C-terminal repressive region overlapping the dimerization domain. These positive and negative effects were mutually neutralized, causing RFX1 to be nearly transcriptionally inactive. Relief of self-neutralization, resulting in a net effect of activation or repression, could allow RFX1 to act as a dual-function regulator, the properties of which may underlie the context-dependent differential activity of EP.

MATERIALS AND METHODS

Plasmid constructions

Expression plasmids of GAL4 derivatives. All GAL4 derivative expression plasmids, presented in Figure 2, are based on the pECE expression vector. GAL4-Fos and GAL4 DBD have been described (50). G4-RFX[1–435] and G4-RFX[529–738] were constructed by digesting GAL4-Fos with *Eco*RI and *Xba*I to remove the c-Fos insert and inserting polymerase chain reaction (PCR)-generated fragments containing bp 93–1399 and 1677–2307 of RFX1 (numbered according to ref. 43), respectively, into these sites. G4-RFX[198–435] and G4-RFX[1–199] were derived from G4-RFX[1–435] by replacing an *Eco*RI–*Kpn*I fragment or a *Kpn*I–*Xba*I fragment, respectively, with the corresponding linker from pGEM-3Z (Promega). G4-RFX[77–435] was constructed by inserting an *Ecl*136II–*Kpn*I RFX1 fragment (bp 326–689) into the *Ecl*136II/*Kpn*I site of G4-RFX[198–435]. To construct RFX[1–435]-G4, a *Hin*dIII(filled-in)–*Kpn*I fragment of G4-RFX[1–435], containing the GAL4 DBD and a portion of RFX1, was replaced by an *Eco*RI(filled-in)–*Kpn*I fragment from pSG5RFX1 (24) containing bp 1–689 of RFX1. Then a PCR-generated fragment containing the GAL4 DBD was inserted into the *Xba*I site at the 3′ end of the RFX1 sequence. To construct G4-RFX[728–979], the RFX1 insert of G4-RFX[198–435] was deleted by digestion with *Ecl*136II and *Xba*I(fill-in), and a *Hin*cII fragment of ~1200 bp extending from bp 2275 of RFX1 was inserted. Constructs G9–G12 were generated by creating in-frame deletions in G4-RFX[728–979]: *Stu*I–*Msc*I (bp 2461–2830) for construct G12, *Pst*I (bp 2390–2531) for construct G11, *Stu*I–*Fsp*I (bp 2461–2692) for construct G9, and *Fsp*I–*Msc*I (bp 2692–2830) for construct G10. To construct G4-RFX[728–913], the c-Fos insert of GAL4-Fos was deleted by digestion with *Eco*RI and *Ecl*136II, and an *Eco*RI–*Msc*I fragment from G4-RFX[728–979], containing bp 2275–2830 of RFX1, was inserted. To construct G4-RFX[914–979], an *Msc*I–*Xba*I fragment (starting at bp 2830 of RFX1) was excised from G4-RFX[728–979], subcloned into the *Sma*I–*Xba*I site of pGEM-3Z to generate pGEM.RFX-Ac, excised by digestion with *Eco*RI and *Xba*I, and inserted into GAL4-Fos digested with *Eco*RI and *Xba*I. To construct G4-RFX[529–979], a *Kpn*I–*Bam*HI fragment from pSG5RFX1 was subcloned into the *Kpn*I–*Bam*HI site of pGEM-3Z, and then a *Sac*I–*Xba*I fragment was excised and inserted into G4-RFX[529–738] digested with *Sac*I and *Xba*I. RFX[Δ436– 528]-G4 was constructed by inserting an *Eco*RI fragment containing the RFX1 sequence of G4-RFX[529–979] into the *Eco*RI site of RFX[1–435]-G4, at the 3′ end of the GAL4 DBD.

Expression plasmids of HA-RFX1 derivatives. All HA-RFX1 derivative expression plasmids, presented in Figure 1, are based on pSG5RFX1, which expresses the RFX1 cDNA under the control of SV2 (24). pSG5.HA-RFX1 (construct 1) was generated by altering bp 91–96 of pSG5RFX1 to an *Nde*I site and inserting an *Eco*RI–*Nde*I fragment encoding the HA epitope in frame into the *Eco*RI–*Nde*I site 5′ of the RFX1 coding sequence. The resulting plasmid expresses the whole RFX1 protein tagged at its N-terminus with HA. HA-RFX1-derived constructs 2, 6, 7 and 8 were constructed by digesting pSG5.HA-RFX1 with *Sac*II, which cuts at bp 2312 and in the 3′-untranslated region (UTR), and inserting *Sac*II fragments from GAL4–RFX1 constructs G12, G9, G10, and G11 (Fig. 2), respectively, into the *Sac*II sites. HA-RFX1-derived construct 5 was generated by creating an in-frame *Nco*I deletion of bp 2267–2666 in pSG5.HA-RFX1. Construct 3 was generated by digesting pSG5.HA-RFX1 with *Sma*I, which cuts at bp 1899 and in the 3′-UTR, and inserting an *Ecl*136II–*Hin*cII fragment from pGEM.RFX-Ac (containing the RFX1 sequence from bp 2830) into the *Sma*I site. Construct 4 was generated by creating an in-frame *Msc*I deletion of bp 1675–2830 in pSG5.HA-RFX1. Construct 9 was generated by deletion of the pSG5.HA-RFX1 sequence between the *Nde*I and *Sac*I (bp 326) sites and insertion of a synthetic *Nde*I–*Sac*I linker. Constructs 10 and 14 were generated by creating in-frame *Pst*I deletions of bp 269–791 in constructs 1 and 3, respectively. Constructs 11 and 15 were generated by replacing a *Kpn*I–*Msc*I (bp 689–1144) fragment of constructs 1 and 3, respectively, with a linker. Constructs 12 and 16 were generated by replacing an *Nhe*I–*Kpn*I fragment of constructs 11 and 15, respectively, with an *Nhe*I–*Kpn*I linker from pGEM-3Z. Constructs 13 and 17 were generated by subcloning a *Sty*I (filled-in) fragment of RFX1 (bp 1338–2267) into the *Hin*cII site of pGEM-3Z, then excising a 530 bp fragment with *Xba*I and *Ecl*136II (bp 1859 of RFX1) and inserting it into constructs 1 and 3, respectively, each digested with *Nhe*I and *Ecl*136II. Construct 18 was generated by inserting the same *Xba*I–*Ecl*136II fragment into pSG5.HA-RFX1 digested with *Nhe*I and *Msc*I (bp 2830), thus altering the reading frame after amino acid 588 and leading to premature termination.

Reporter plasmids. The structure of G5-luciferase has been described (51). To construct E5G5-luciferase, five copies of the HBV enhancer E element oligonucleotide (16) were multimerized and inserted into the *Hin*dIII–*Pst*I site of G5-luciferase. TATA-luciferase was constructed by inserting an oligonucleotide encoding the E1B TATA box (5′-GGGTACCAGATCTTATATAATGAG-CT-3′) upstream of the luciferase gene. EP4-luciferase was constructed by multimerizing four copies of the EP oligonucleotide (16) and inserting them into TATA-luciferase, upstream of the TATA box.

Cell culture, transfection and luciferase and β-galactosidase analyses

Cells were cultured in Dulbecco's modified Eagle minimal essential medium (GIBCO Laboratories) containing 100 U/ml penicillin and 100 μg/ml streptomycin, supplemented with 8% fetal bovine serum. Transfection was performed by the calcium phosphate precipitation method, as previously described (52). At the time of transfection the cells were 30–60% confluent. For luciferase assays, 6 cm plates were transfected with 1–2 μg of a luciferase reporter plasmid, 1 μg of the SV2-β-galactosidase internal control plasmid, and an expression plasmid. The amount of SV2 elements and the total amount of DNA was kept constant in each

experiment by addition of pSV2 and Bluescript (Stratagene), respectively. Each RFX1-derived construct was examined several times and at different concentrations. For preparation of whole-cell extracts, each 6 cm plate was lysed with 50 µl of lysis buffer containing 0.1 M KPi (pH 7.8), 0.5% Triton X-100 and 1 mM DTT. The luciferase assay was performed with a substrate buffer (Promega) and was read in a Turner TD-20e luminometer. β-Galactosidase was assayed as described (53). The normalized luciferase activity of each plate was calculated by dividing the results of the luciferase assay by those of the β-galactosidase assay.

Gel retardation and Western blot analyses

Whole-cell extracts for protein analyses were prepared by lysing each 6 cm plate with 100 µl of buffer A containing 20 mM HEPES–KOH (pH 7.9), 250 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM DTT, and a cocktail of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 10 µg/ml pepstatin A, 1 µg/ml aprotinin, 1 µg/ml leupeptin) and phosphatase inhibitors (2 mM $NaVO_3$, 10 mM NaPi, 10 mM NaPPi, 50 mM NaF). For preparation of nuclear extracts, 6 cm plates were rinsed twice with cold PBS, scraped from the plates, and allowed to swell in 250 µl of swelling buffer (10 mM KCl, 30 mM Tris–HCl pH 7.5, 5 mM magnesium acetate, 5 mM EDTA, 45 mM β-mercaptoethanol) for 10 min on ice. The cells were lysed by adding 10 µl of 20% Nonidet P-40 and vortexing. The lysates were overlaid on an equal volume of swelling buffer containing, in addition to the above constituents, 25% glycerol and 0.1% Nonidet P-40 and centrifuged at 400 *g* for 5 min. The upper (cytoplasmic) fraction was removed, and the nuclear pellet was washed twice with 500 µl of swelling buffer. Nuclei were extracted in 50 µl of high-salt buffer (50).

For Western blot analysis, cellular extracts were subjected to SDS polyacrylamide gel electrophoresis, and the resolved proteins were electroblotted onto nitrocellulose membranes. For analysis of GAL4 derivatives, the blots were incubated with anti-GAL4 rabbit immunoglobulin G (protein G purified), produced in our laboratory, followed by protein A conjugated with horseradish peroxidase (HRP). HA-RFX1 derivatives were analyzed using the anti-HA monoclonal antibody 12CA5 (Pharmingen, San Diego) and goat anti-mouse conjugated with HRP. The immune complexes were detected by the ECL detection system (Amersham).

Gel retardation analysis of HA-RFX1 derivatives was conducted essentially as described (16), with several modifications. The binding reaction was performed for 45 min on ice with 2×10^4 c.p.m. of the EP element oligonucleotide (sequence shown in ref. 42), end labeled by a fill-in reaction, and 5–9 µl of whole-cell extract, and the samples were run on a 5% polyacrylamide gel. Gel retardation for assaying GAL4 derivatives was performed as described (54), by incubating 2×10^4 c.p.m. of the GAL4 binding site oligonucleotide (5′-AATTCAGCGGAGTACTGTCCTCCGAGGAATT-3′), end labeled by a fill-in reaction, with 9 µl of nuclear extract.

RESULTS

RFX1 contains stimulatory and inhibitory transcriptionally active regions

To examine whether RFX1 possesses an intrinsic transcriptional activity and to locate the specific regions involved in this effect, the wild-type hemagglutinin-tagged RFX1 (HA-RFX1) and its deletion mutants were assayed in transient transfections. Constructs expressing the various HA-RFX1 derivatives were cotransfected into HepG2 hepatoma cells together with a reporter plasmid containing four copies of the EP element upstream of the luciferase gene. As a control, cotransfections with a similar luciferase reporter plasmid that lacks these EP sites were performed in parallel. Western (Fig. 1B) and gel retardation (Fig. 1C and data not shown) analyses indicated that the HA-RFX1 derivatives were well-expressed (with only constructs 7 and 8 having significantly lower levels) and bound the EP DNA efficiently (except for construct 4). The identity of the DNA–protein complexes containing exogenous HA-RFX1 proteins was verified by supershift with an anti-HA antibody (Fig. 1C).

The wild-type HA-RFX1 induced only a mild increase in the level of transcription (Fig. 1A, construct 1), as did RFX1 (not shown). However, upon deletion of a large part of the dimerization domain or most of the C-terminal half of the protein, the activation level dramatically increased (Fig. 1A, constructs 2 and 3). This suggests that the wild-type RFX1 possesses an intrinsic stimulatory activity that is counteracted by an inhibitory C-terminal region. A similar stimulatory effect of the C-terminal deletion was observed with other HA-RFX1 derivatives lacking sequences from the N-terminal part of RFX1 (compare constructs 10 and 14, 11 and 15, 12 and 16, 13 and 17), indicating that the inhibitory effect of the C-terminal region does not necessarily require an interaction with the RFX1 N-terminus. Extending the deletion further up to the DBD reduced the activation (construct 4), yet this effect could result from the inefficient DNA-binding of this mutant (Fig. 1C). Partial deletions within the dimerization domain (constructs 5–8) resulted in a small increase or no increase in activation, although the expression of at least some of these constructs (5 and 6) was not substantially lower than that of construct 2 (Fig. 1A and B). Collectively, these results indicate that the dimerization domain, or regions within it, can downregulate the transcriptional activity of RFX1. The inability of the partial deletions to induce full activation suggests that these mutant proteins retain at least part of their inhibitory function, meaning that different regions within the dimerization domain can negatively affect transcription (see below).

A region responsible for the ability of the HA-RFX1 mutants to activate transcription was localized by the examination of deletions within the N-terminal half of RFX1. These deletions were introduced either into the wild-type HA-RFX1 or in combination with an ‘activating’ C-terminal deletion, creating double mutants. The deletion of amino acids (aa) 1–77 (Fig. 1A, construct 9) or 59–232 (compare constructs 1 and 10, 3 and 14) revealed that the first 232 aa of RFX1 are not required for activation. However, a more internal deletion of aa 200–351 reduced activation considerably (compare constructs 1 and 11, 3 and 15). The extension of the N-terminal deletion resulted in an additional small reduction or no reduction in activity (constructs 12, 13, 16 and 17). Therefore, this analysis has localized the region required for maximal activation to aa 233–351 within the N-terminal half of RFX1, which includes the third glutamine-rich region. The above data also suggest that the RFX1-induced activation may be partially inhibited by a region within residues 59–232, since deletion of this region from an ‘activated’ RFX1 mutant (construct 3) resulted in enhanced activity (construct 14).

Although the deletion of the RFX1 N-terminus caused a major reduction in activation, the double deletion mutants 16 and 17 still induced a weak activation. A further extension of the C-terminal deletion produced a 173 aa HA-RFX1 derivative (construct 18) that exhibited the same effect. This weak activation did not result

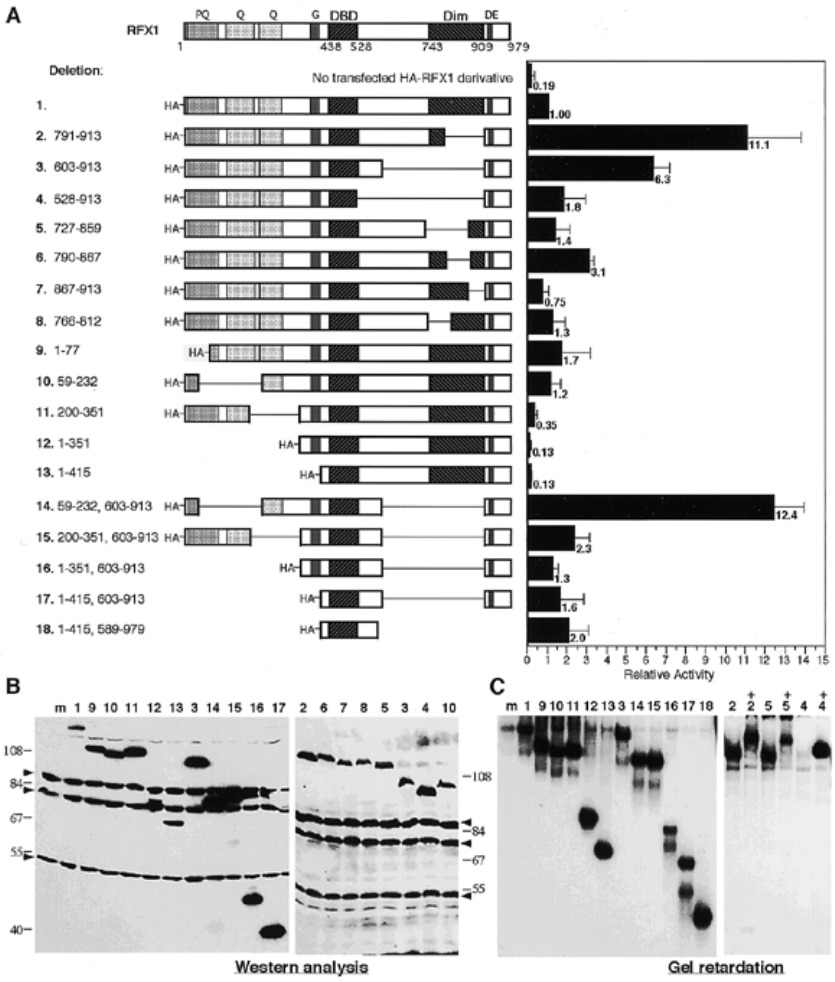

Figure 1. (**A**) Structure and transcriptional activity of HA-RFX1 deletion mutants. The structure of RFX1 is shown schematically at the top (according to ref. 43), including its DNA-binding domain (DBD), dimerization domain (Dim), and the following structural regions: proline- and glutamine-rich (PQ), glutamine-rich (Q), glycine-rich (G) and a highly acidic stretch (DE). Expression plasmids of the hemagglutinin-tagged RFX1 (HA-RFX1) or its deletion mutants lacking the indicated amino acid sequences (3 μg) were cotransfected into HepG2 cells, together with 1 μg of a luciferase reporter plasmid controlled by four copies of the HBV EP element (EP4-luciferase) or a similar control reporter plasmid lacking the EP elements (TATA-luciferase), and 1 μg of the SV_2-β-galactosidase internal control plasmid. For each construct, the ratio between the normalized luciferase activities obtained with EP4-luciferase and TATA-luciferase was calculated, and divided by the ratio obtained with the wild-type HA-RFX1, to yield the relative activity. The results shown are the mean and SD of two independent experiments. (**B** and **C**) Expression of HA-RFX1 deletion mutants. Plasmids expressing the indicated HA-RFX1 derivatives, numbered as above, (4 μg) were transfected into HepSK1 cells. Whole-cell extracts were prepared and subjected to the following analyses. (B) Western analysis was performed using an anti-HA antibody. The bands marked with arrowheads represent endogenous proteins that cross-react with anti-HA. Molecular weight markers (in kDa) are indicated. (C) Gel retardation analysis was performed using the EP element probe. Anti-HA was added to the binding reaction where indicated by +. m, mock transfected.

from the addition of the HA epitope, since an untagged derivative of construct 16 was similarly active (data not shown). These data suggest that the region between aa 416 and 588, located around the DBD, may have a weak activation capacity. Alternatively, the stimulatory effect of this minimal HA-RFX1 derivative may result from the displacement of an endogenous inhibitory EP-binding protein.

The functional regions of RFX1 can activate and repress transcription when fused to a heterologous DNA-binding domain

The above deletion analysis identified specific regions that are required for stimulation or inhibition of transcription in the context of the EP-bound RFX1. To examine whether RFX1 contains independently active regions, which are sufficient for mediating transcriptional regulation when linked to a heterologous DBD, various RFX1 sequences were fused downstream of the DBD of the yeast GAL4 activator (codons 1–147). Constructs expressing these GAL4–RFX1 chimeric proteins were transiently transfected into differentiated HepG2 (Figs 2A and 3A) or undifferentiated HepSK1 (data not shown) hepatoma cells, together with the G5-luciferase reporter plasmid. In both cell types, transcription activation was observed with GAL4 constructs containing sequences from the N-terminal half of RFX1. Constructs G4-RFX[1–435] and G4-RFX[198–435] were also tested in HeLa cells and showed a similar behaviour (Fig. 3B). The minimal construct with significant activation potential, which induced the strongest activation, was G4-RFX[198–435], thus defining a 238 aa region from the N-terminus of RFX1 as sufficient for activation

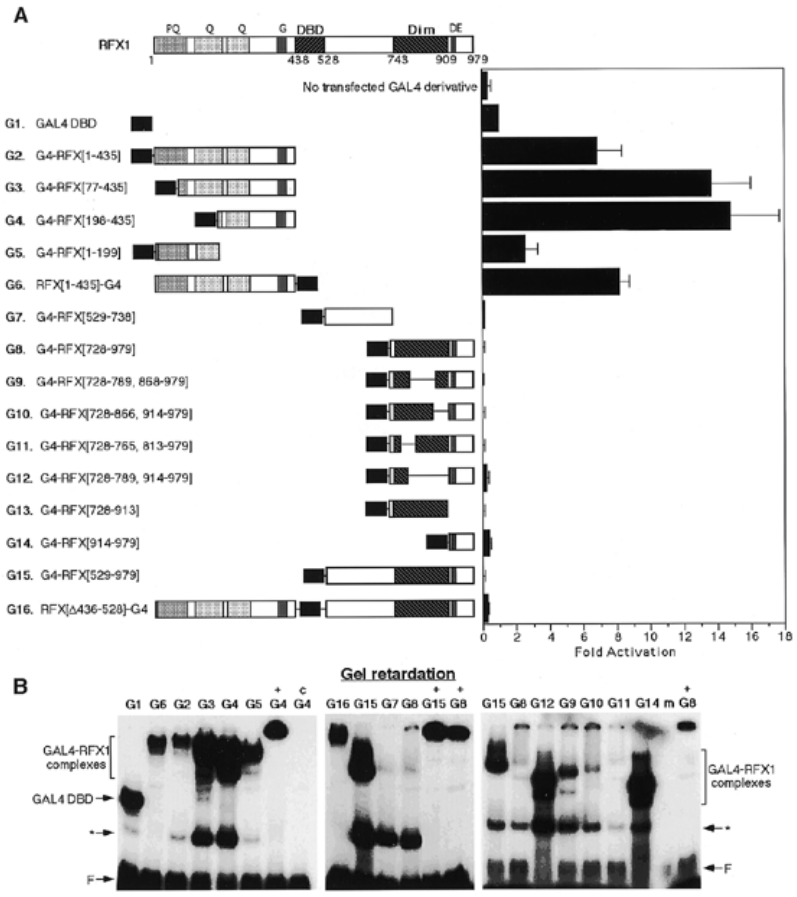

Figure 2. (**A**) Structure and transcriptional activity of GAL4–RFX1 chimeric proteins. Various regions of RFX1 were fused to the GAL4 DBD (aa 1–147). For constructs G2–G15, numbers in brackets indicate the fused RFX1 aa sequences. For construct G16, residues deleted from the wild-type RFX1 and replaced by the GAL4 DBD are indicated. The structure of RFX1 is shown schematically above the fusion constructs (Fig. 1A). A filled box represents the GAL4 DBD. The fusion plasmids (0.75 µg) were cotransfected into HepG2 cells with 1 µg of a luciferase reporter plasmid controlled by five copies of the GAL4 binding site (G5-luciferase) and 1 µg of the SV_2-β-galactosidase internal control plasmid. Normalized luciferase activities relative to activity of the GAL4 DBD alone are presented as fold activation. Each result represents the mean and SD of several independent experiments: three experiments for constructs G6 and G16, five experiments for constructs G2, G4 and G5, and two experiments for the rest. (**B**) Gel retardation analysis of GAL4–RFX1 chimeric proteins. GAL4–RFX1 expression plasmids (3 µg) were transfected into HepSK1 cells. Nuclear extracts were prepared and analyzed by gel retardation, using the GAL4 binding site probe. An anti-GAL4 antibody (+) or an excess of GAL4 competitor oligonucleotide (c) was added where indicated. Constructs are numbered as in (A). The band marked with an asterisk is GAL4-specific, as shown by the anti-GAL4 supershift, and probably represents a degraded complex. m, mock transfected. F, free probe.

when fused to the GAL4 DBD. This part of the protein includes the region identified by the deletion analysis as required for maximal activation (aa 233–351). Varying the amounts of transfected GAL4–RFX1 N-terminal expression constructs produced a dose–response curve typical of transcriptional activators (55), in which the level of activation increases with increasing activator amounts until a maximal level is reached, followed by a decrease in the activation level, termed squelching (Fig. 3). Another N-terminal fusion construct was tested, in which the RFX1 N-terminus was located upstream of the DBD (RFX[1–435]-G4), as is the case in the wild-type RFX1. The activation level obtained with this fusion was similar to that of G4-RFX[1–435] (Fig. 2A), indicating that in this system the activation capacity of the RFX1 N-terminus is not affected by its position relative to the DBD. Thus, the GAL4 and deletion analyses of RFX1 identified an activation domain in the N-terminal part of the protein (aa 233–435), overlapping a glutamine-rich region.

Interestingly, the larger N-terminal construct G4-RFX[1–435] was a less potent activator than the shorter constructs G4-RFX[77–435] and G4-RFX[198–435] lacking the extreme N-terminus of RFX1 (Figs 2A and 3), suggesting a possible inhibitory function for this region. The existence of an inhibitory function within the RFX1 N-terminus (aa 59–232) was also suggested by the deletion analysis (Fig. 1). In Western (data not shown) and gel retardation (Fig. 2B) analyses of whole-cell and nuclear extracts, respectively, GAL4–RFX1 constructs containing the extreme N-terminal region (aa 1–76) exhibited a significantly lower expression and DNA-binding activity than those lacking this region. Taken together, these results suggest that the extreme N-terminus of RFX1 can function as a modulator region, either at the level of transcriptional activation capacity, or at the level of expression, or both.

The transcriptional activity observed with several fusion constructs containing sequences from the C-terminal half of RFX1 suggested that these RFX1 sequences may downregulate transcription (Fig. 2 and data not shown), in agreement with the analysis of RFX1 C-terminally deleted mutants (Fig. 1). In order to substantiate the repressive effect of the RFX1 C-terminal

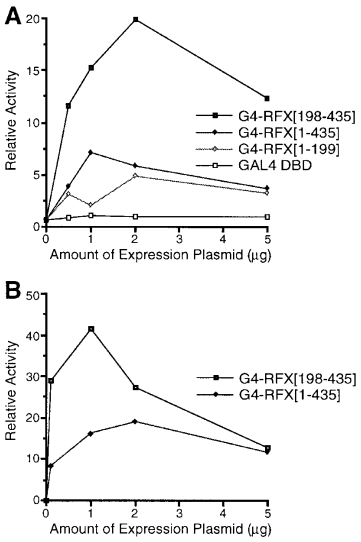

Figure 3. Dose-dependent activation by GAL4–RFX1 chimeric proteins containing sequences from the N-terminal half of RFX1. Increasing amounts of GAL4 derivative expression plasmids (Fig. 2A) were cotransfected into HepG2 (**A**) or HeLa (**B**) cells with 2 μg of the G5-luciferase reporter plasmid and 1 μg of the SV_2-β-galactosidase internal control plasmid. The results shown are normalized luciferase activities relative to the activity obtained with 2 μg of the GAL4 DBD.

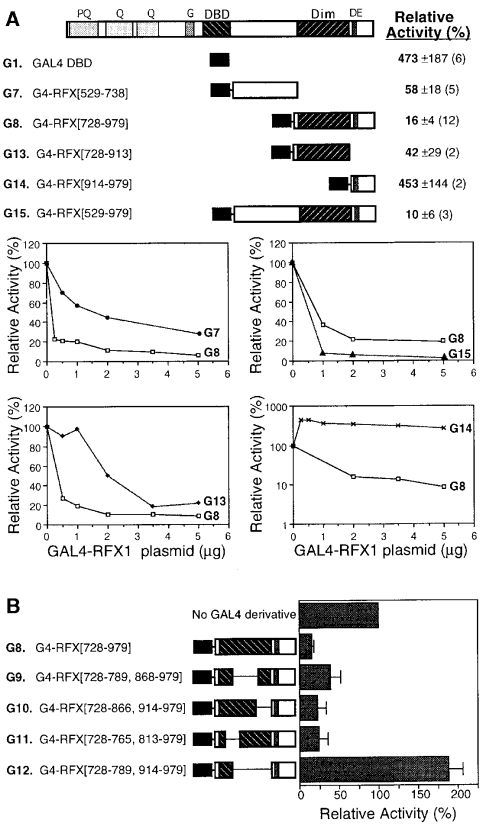

Figure 4. Transcriptional repression by GAL4–RFX1 chimeric proteins containing sequences from the C-terminal half of RFX1. GAL4–RFX1 expression plasmids (numbered as in Fig. 2A) were cotransfected into HepG2 cells, together with 2 μg of a luciferase reporter plasmid containing five copies of the GAL4 binding site and five copies of the E element (E5G5-luciferase), 0.3 μg of a c-Jun expression plasmid (RSVc-Jun), and 1 μg of the SV_2-β-galactosidase internal control plasmid. Normalized luciferase activities are expressed as percent of the 'basal' activity obtained in the absence of a GAL4 derivative. (**A**) The numbers to the right of each construct represent the mean and SD of 2–12 independent experiments (as indicated in brackets), performed with 2 μg of GAL4–RFX1 expression plasmids (except for 5 μg used for construct G13). The results of four representative experiments, using increasing amounts of expression plasmids, are shown below. (**B**) The transfections included 2 μg of a GAL4–RFX1 expression plasmid containing the dimerization domain and acidic region of RFX1, or deleted derivatives of this construct. The results shown are the mean and SD of two independent experiments.

sequences, we used the E5G5-luciferase reporter construct, which includes five copies of the E element [an AP-1-like site present in the HBV enhancer (14)] in addition to five GAL4 binding sites, upstream of the luciferase gene. The cotransfection of a plasmid expressing c-Jun was used to further activate this reporter through the E element. With this activated reporter, as with the basal G5-luciferase reporter, the GAL4 DBD alone induced a slight activation (Fig. 4A). By contrast, G4-RFX[728–979] (containing the dimerization domain and acidic region), G4-RFX[529–738] (containing the 'central region' between the DBD and the dimerization domain), and G4-RFX[529–979] (containing both segments) all reduced the level of transcription relative to that observed in the absence of a GAL4 derivative, in a dose-dependent manner (Fig. 4A). The latter construct was also transfected into HeLa cells and exhibited the same repressive effect (data not shown). When the RFX1 portion of G4-RFX[728–979] was dissected into two GAL4 fusions containing either the dimerization domain or the acidic region, the former, but not the latter, repressed transcription (Fig. 4A). Thus, both the 'central region' and the dimerization domain of RFX1 appear to possess a repressive capacity, while the acidic region shows no significant effect in this assay.

A further dissection of the dimerization domain by deleting various parts of it from G4-RFX[728–979] produced fusion constructs (G9, G10, and G11) that still exhibited a repressive effect on activated transcription (Fig. 4B). Since construct G12, containing the combined deletions of constructs G9 and G10, did not cause this effect, though exhibiting a high DNA-binding activity in a gel retardation assay (Fig. 2B), the repressive activities of fusions G9 and G10 can be attributed to aa 868–913 and 790–866, respectively, within the dimerization domain, consistent with the results of the HA-RFX1 deletion analysis. Thus, three non-overlapping regions within the C-terminal half of RFX1 appear to possess a repressive capacity, two within the dimerization domain and one in the 'central region'.

The dissection of RFX1 into GAL4 chimeras identified an N-terminal stimulatory region as well as C-terminal repressive regions. A fusion construct containing all these regions (RFX[Δ436–528]-G4) was generated by replacing the RFX1 DBD (aa 436–528) with the GAL4 DBD and showed no significant effect on transcription in this system in both HepG2 and HeLa cells (Fig. 2A and data not shown). Since the fusion protein formed an easily detectable DNA–protein complex (Fig. 2B), its lack of activity appears to result from the combined effects of the positively and negatively acting regions of RFX1. The main findings of the GAL4 analysis correlate with those obtained with the RFX1 deletion mutants, identifying independently active stimulatory and repressive regions that are likely to constitute functional regions of the native RFX1 protein, as shown schematically in Figure 5. In both assay systems used, the positive and negative activities of RFX1 were mutually neutralized, so that the wild-type RFX1 and the corresponding fusion protein

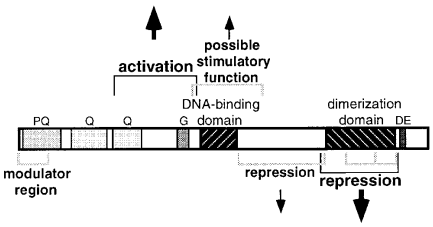

Figure 5. The different functional regions of RFX1, identified by the GAL4 and deletion analyses.

RFX[Δ436–528]-G4 exhibited little effect on transcription, though containing transcriptionally active regions.

DISCUSSION

The conservation of the EP element in viral enhancers and the unusual transcriptional properties of EP-homologous sites suggest that these elements fulfill an important and unique function. While possessing no intrinsic enhancer activity, these regulators of viral and cellular genes can both stimulate and silence transcription (15,16,22,27,29,33,34), depending on the DNA context. In order to gain insight into the EP mechanism of action, we focused on the intrinsic transcriptional properties of the ubiquitous EP-binding protein RFX1. Cotransfection experiments using RFX1 deletion mutants and GAL4–RFX1 chimeras identified several regions with independent transcriptional activity. While the wild-type RFX1 had little effect on the level of transcription in this system, a C-terminal deletion turned this protein into a transcriptional activator. The major RFX1 activation domain was localized to an N-terminal part of the protein (aa 233–435), containing a glutamine-rich region. The C-terminal dimerization domain (aa 791–913) was shown to downregulate the activation capacity of RFX1 and to possess an independent repressive activity and thus appears to be, or overlap, a repression domain. Since two non-overlapping parts of the dimerization domain exhibited a repressive effect, it is likely that the transcriptional-inhibitory activity is not fully dependent on the dimerization function. However, as these RFX1 regions are involved in both dimerization and transcriptional repression, it is possible that their repressive activity is modulated by the formation of intradimeric protein–protein interactions. The GAL4 analysis showed another region, the ‘central region’ (aa 529–738), to possess a repressive activity. Therefore, the overall repression induced by the C-terminal half of RFX1 may be the combined effect of several smaller inhibitory regions. Another inhibitory activity appears to be located at the extreme N-terminal part of the protein, since the deletion aa 59–232 (from an RFX1 derivative) or 1–76 (from a GAL4–RFX1 fusion) resulted in increased activation. The inefficient accumulation of GAL4–RFX1 chimeras containing aa 1–76 raises the possibility that an inhibitory activity in terms of protein expression, such as a degradation signal, is located in this part of RFX1. However, the attribution of the weaker activation of the longer N-terminal fusion constructs to their lower expression is inconsistent with the observation that upon increasing the amount of transfected expression construct beyond a certain level, the activation decreased (apparently due to squelching). Thus, the extreme N-terminus of RFX1 may function as a modulator region by affecting the intrinsic transcriptional activity of this protein, its expression or both. Collectively, these results show RFX1 to possess an intrinsic stimulatory activity, which is counteracted by inhibitory regions of the same protein (Fig. 5).

Several regions of RFX1 are homologous to the corresponding regions of other RFX proteins (44,46). The major transcriptionally active parts of RFX1 include such regions of homology. The N-terminal activation domain of RFX1 and the corresponding regions of RFX2 and RFX3 contain a conserved sequence preceded, and partly overlapped, by a glutamine-rich region. The transcriptionally repressive dimerization domain of RFX1 is homologous to sequences of other RFX family members, from humans and mice (RFX2, 3), *Caenorhabditis elegans*, and *Schizosaccharomyces pombe* (the cell-cycle regulator *sak1*) (44,46). It therefore remains to be determined whether the activities identified in RFX1 are also performed by other members of the RFX family.

The identification of independent activation and repression domains suggests that RFX1 can act as a dual-function regulator via these regions. In a system controlled by the multimerized EP site alone, the positive and negative activities of RFX1 appear to be of similar potency and are thus mutually neutralized. This neutralization can be reproduced in the GAL4 system, where the transcriptionally active regions of RFX1 are tethered to the DNA via a heterologous DBD. Under these conditions the wild-type RFX1 and the corresponding GAL4–RFX1 fusion protein exhibited little or no transcriptional activity. Such an effect may be achieved by one of several possible mechanisms. The activation and repression domains of RFX1 could function by contacting different components of the basal transcription complex, as observed for the transcriptional regulator Kruppel (56). Alternatively, these domains could contact different surfaces of the same transcriptional component, or directly compete with each other for a common binding site within the transcription complex. In these cases, the resulting effect of transcriptional inertness, rather than a dominance of one activity over the other, is likely due to the relative affinities of the different RFX1 domains for their respective targets. Another possibility is the existence of a direct interaction between the activation and repression domains of RFX1. Since these two domains can function independently of each other, the potential to form such an inhibitory interaction with the RFX1 repression domain could not be specific for the RFX1 activation domain but, rather, should be common to all activation domains or a subset of them. Despite this lack of specificity, the particular array of activation and repression domains in RFX1 may be designed to allow the mutual neutralization of their activities and, possibly, the relief of this neutralization under certain conditions.

Although the intact RFX1 did not exhibit substantial transcriptional activity, a mechanism that converts this protein from an inactive state to a state of positive or negative activity may serve to turn on its different functions, in a regulated manner. Dual-function transcription factors were shown to switch their activity by various mechanisms, including the binding of a ligand (57), interaction with a regulatory protein (11,58,59), cooperation with a neighbouring DNA-bound transcription factor (4–6), concentration-dependent homodimerization (10,56), and interaction with a specific DNA sequence (60). One of these mechanisms may enable RFX1 to either activate or repress transcription, depending on the DNA or cellular context. In particular, the enhancer/silencer activity of the EP-homologous RFX1 binding sites may be attributable to the different functional regions of RFX1. In such cases, the self-neutralizing effect of RFX1 would be relieved by functional or physical interactions with other

DNA-bound transcription factors, such as those that bind the HBV and polyomavirus enhancers, resulting in a net effect of activation or repression. A regulatory interaction between DNA-bound factors has been detected in the upstream regulatory region of the human papilomavirus type 18, where the ability of YY1 to activate rather than repress transcription is determined by a switch region (4). Another example is the conversion of Dorsal from an activator to a repressor of the *zen* promoter by its interaction with DNA-bound DSP1 (6).

Previous studies have implicated RFX1 in the activation of specific natural regulatory elements through EP-homologous sites. The data presented here indicate that RFX1 is not a conventional transcription activator; like its binding sites, RFX1 exhibits both stimulatory and inhibitory activities but is nearly inactive on its own, thus appearing to function only in conjunction with other factors. Interestingly, its lack of activity results from a mutual neutralization of the effects exerted by its independent activation and repression domains. Further studies are needed in order to determine how the activities of the different functional regions of RFX1 are integrated to modulate context-dependent transcription, and to uncover regulatory mechanisms controlling this system. The continued investigation of RFX1, at the functional and molecular level, may eventually elucidate the as yet unknown mechanism by which EP cooperates with other binding sites in regulating the expression of viral and cellular genes.

ACKNOWLEDGEMENTS

We thank B. Mach and W. Reith for the gift of pSG5RFX1, I. Haviv for preparation of anti-GAL4, and H. Greif for helpful discussions and construction of reporter plasmids. This work was supported by the Ebner Family Biomedical Research Foundation at the Weizmann Institute of Science in Memory of Alfred and Dolfi Ebner, and by a grant from the Basic Research Foundation administered by the Israel Academy of Sciences and Humanities.

REFERENCES

1 Du, W., Thanos, D. and Maniatis, T. (1993) *Cell*, **74**, 887–898.
2 Tjian, R. and Maniatis, T. (1994) *Cell*, **77**, 5–8.
3 Wolffe, A.P. (1994) *Cell*, **77**, 13–16.
4 Bauknecht, T., Jundt, F., Herr, I., Oehler, T., Delius, H., Shi, Y., Angel, P. and Zur Hausen, H. (1995) *J .Virol.*, **69**, 1–12.
5 Ip, Y.T. (1995) *Curr. Biol.*, **5**, 1–3.
6 Lehming, N., Thanos, D., Brickman, J.M., Ma, J., Maniatis, T. and Ptashne, M. (1994) *Nature*, **371**, 175–179.
7 Bushmeyer, S., Park, K. and Atchison, M.L. (1995) *J. Biol. Chem.*, **270**, 30213–30220.
8 Licht, J.D., Grossel, M.J., Figge, J. and Hansen, U.M. (1990) *Nature*, **346**, 76–79.
9 Sauer, F. and Jackle, H. (1991) *Nature*, **353**, 563–566.
10 Sauer, F. and Jackle, H. (1993) *Nature*, **364**, 454–457.
11 Shi, Y., Seto, E., Chang, L.S. and Shenk, T. (1991) *Cell*, **67**, 377–388.
12 Wang, Z.Y., Qiu, Q.Q. and Deuel, T.F. (1993) *J. Biol. Chem.*, **268**, 9172–9175.
13 Wang, Z.Y., Qiu, Q.Q., Gurrieri, M., Huang, J. and Deuel, T.F. (1995) *Oncogene*, **10**, 1243–1247.
14 Ben-Levy, R., Faktor, O., Berger, I. and Shaul, Y. (1989) *Mol. Cell. Biol.*, **9**, 1804–1809.
15 Ostapchuk, P., Scheirle, G. and Hearing, P. (1989) *Mol. Cell. Biol.*, **9**, 2787–2797.
16 Dikstein, R., Faktor, O., Ben-Levy, R. and Shaul, Y. (1990) *Mol. Cell. Biol.*, **10**, 3682–3689.
17 Garcia, A.D., Ostapchuk, P. and Hearing, P. (1991) *Virology*, **182**, 857–860.
18 Ostapchuk, P., Diffley, J.F., Bruder, J.T., Stillman, B., Levine, A.J. and Hearing, P. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 8550–8554.
19 Zhang, X.-Y., Asiedu, C.K., Supakar, P.C., Khan, R., Ehrlich, K.C. and Ehrlich, M. (1990) *Nucleic Acids Res.*, **18**, 6253–6260.
20 Kouskoff, V., Mantovani, R.M., Candeias, S.M., Dorn, A., Staub, A., Lisowska-Grospierre, B., Griscelli, C., Benoist, C.O. and Mathis, D.J. (1991) *J. Immunol.*, **146**, 3197–3204.
21 Labrie, C., Lee, B.H. and Mathews, M.B. (1995) *Nucleic Acids Res.*, **23**, 3732–3741.
22 Safrany, G. and Perry, R.P. (1993) *Gene*, **132**, 279–283.
23 Safrany, G. and Perry, R.P. (1995) *Eur. J. Biochem.*, **230**, 1066–1072.
24 Siegrist, C.A., Durand, B., Emery, P., David, E., Hearing, P., Mach, B. and Reith, W. (1993) *Mol. Cell. Biol.*, **13**, 6375–6384.
25 Zhang, X.-Y., Jabrane-Ferrat, N., Asiedu, C.K., Samac, S., Peterlin, B.M. and Ehrlich, M. (1993) *Mol. Cell. Biol.*, **13**, 6810–6818.
26 Zhang, X.-Y., Supakar, P.C., Wu, K.Z., Ehrlich, K.C. and Ehrlich, M. (1990) *Cancer Res.*, **50**, 6865–6869.
27 Bolwig, G.M. and Hearing, P. (1991) *J. Virol.*, **65**, 1884–1892.
28 David, E., Garcia, A.D. and Hearing, P. (1995) *J. Biol. Chem.*, **270**, 8353–8360.
29 Garcia, A.D., Ostapchuk, P. and Hearing, P. (1993) *J. Virol.*, **67**, 3940–3950.
30 Tsang, S.Y., Nakanishi, M. and Peterlin, B.M. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 8598–8602.
31 Tsang, S.Y., Nakanishi, M. and Peterlin, B.M. (1990) *Mol. Cell. Biol.*, **10**, 711–719.
32 Blake, M., Niklinski, J. and Zajac-Kaye, M. (1996) *J. Virol.*, **70**, 6060–6066.
33 Reinhold, W., Emens, L., Itkes, A., Blake, M., Ichinose, I. and Zajac-Kaye, M. (1995) *Mol. Cell. Biol.*, **15**, 3041–3048.
34 Weisinger, G., Remmers, E.F., Hearing, P. and Marcu, K.B. (1988) *Oncogene*, **3**, 635–646.
35 Yu, B.W., Ichinose, I., Bonham, M.A. and Zajac-Kaye, M. (1993) *J. Biol. Chem.*, **268**, 19586–19592.
36 Zajac-Kaye, M., Gelmann, E.P. and Levens, D. (1988) *Science*, **240**, 1776–1780.
37 Buckwold, V.E., Chen, M. and Ou, J.-H. (1997) *Virology*, **227**, 515–518.
38 Riley, J.L. and Boss, J.M. (1993) *J. Immunol.*, **151**, 6942–6953.
39 Reith, W., Kobr, M., Emery, P., Durand, B., Siegrist, C.A. and Mach, B. (1994) *J. Biol. Chem.*, **269**, 20020–20025.
40 Reith, W., Siegrist, C.A., Durand, B., Barras, E. and Mach, B. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 554–558.
41 Dikstein, R., Agami, R., Heffetz, D. and Shaul, Y. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 2387–2391.
42 Dikstein, R., Heffetz, D., Ben-Neriah, Y. and Shaul, Y. (1992) *Cell*, **69**, 751–757.
43 Reith, W., Herrero-Sanchez, C., Kobr, M., Silacci, P., Berte, C., Barras, E., Fey, S. and Mach, B. (1990) *Genes Dev.*, **4**, 1528–1540.
44 Reith, W., Ucla, C., Barras, E., Gaud, A., Durand, B., Herrero-Sanchez, C., Kobr, M. and Mach, B. (1994) *Mol. Cell. Biol.*, **14**, 1230–1244.
45 Steimle, V., Durand, B., Barras, E., Zufferey, M., Hadam, M.R., Mach, B. and Reith, W. (1995) *Genes Dev.*, **9**, 1021–1032.
46 Emery, P., Durand, B., Mach, B. and Reith, W. (1996) *Nucleic Acids Res.*, **24**, 803–807.
47 Wu, S.Y. and McLeod, M. (1995) *Mol. Cell. Biol.*, **15**, 1479–1488.
48 Reith, W., Barras, E., Satola, S., Kobr, M., Reinhart, D., Herrero-Sanchez, C. and Mach, B. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 4200–4204.
49 Siegrist, C.A. and Mach, B. (1993) *Eur. J. Immunol.*, **23**, 2903–2908.
50 Haviv, I., Vaizel, D. and Shaul, Y. (1995) *Mol. Cell. Biol.*, **15**, 1079–1085.
51 Deng, T. and Karin, M. (1993) *Genes Dev.*, **7**, 479–490.
52 Honigwachs, J., Faktor, O., Dikstein, R., Shaul, Y. and Laub, O. (1989) *J. Virol.*, **63**, 919–924.
53 Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor University Press, Cold Spring Harbor, NY.
54 Chasman, D.I., Leatherwood, J., Carey, M., Ptashne, M. and Kornberg, R.D. (1989) *Mol. Cell. Biol.*, **9**, 4746–4749.
55 Ptashne, M. (1988) *Nature*, **335**, 683–689.
56 Sauer, F., Fondell, J.D., Ohkuma, Y., Roeder, R.G. and Jackle, H. (1995) *Nature*, **375**, 162–164.
57 Baniahmad, A., Ha, I., Reinberg, D., Tsai, S., Tsai, M.J. and O'Malley, B.W. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 8832–8836.
58 Maheswaran, S., Park, S., Bernard, A., Morris, J.F., Rauscher, F.J.d., Hill, D.E. and Haber, D.A. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 5100–5104.
59 Treitel, M.A. and Carlson, M. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 3132–3136.
60 Kurokawa, R., Soderstrom, M., Horlein, A., Halachmi, S., Brown, M., Rosenfeld, M.G. and Glass, C.K. (1995) *Nature*, **377**, 451–454.