The EMBO Journal vol.11 no.1 pp.205-213, 1992

A novel complex between the p65 subunit of NF-κB and c-Rel binds to a DNA element involved in the phorbol ester induction of the human urokinase gene

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Communicated by F.Blasi

The NF-κB subunits, p50 and p65, have extensive sequence homology with the c-*rel* proto-oncogene and the *Drosophila* morphogen *dorsal*. It has recently been shown that *in vitro* translated c-Rel can bind to DNA and form a complex with p50. However, the conditions for DNA binding of c-Rel *in vivo* and its DNA sequence specificity have not been established. Here we report the identification a novel heterodimeric complex that binds to a κB-like, phorbol ester (TPA) responsive DNA sequence, 5′-GGGAAAGTAC-3′, in the 5′ flanking region of the human urokinase (uPA) gene. This sequence was shown to bind two protein complexes, LC and UC. LC was indistinguishable from NF-κB as it reacted with antibodies recognizing the p50 subunit of NF-κB, and was shown by UV crosslinking to contain the p50 and p65 subunits of NF-κB. UC, on the other hand, strongly reacted with anti-v-Rel, but not with the anti-p50 antibodies, and was shown by crosslinking to contain 75 kDa and 85 kDa protein–DNA adducts. The 75 kDa and the 85 kDa adducts could be immunoprecipitated only by anti-p65 and anti-c-Rel antibodies, respectively, showing that c-Rel formed a heterodimer with p65. Both protein complexes were present in inactive forms in HeLa cell cytosol, and their nuclear translocation was induced by TPA. DNA binding of UC and LC could, furthermore, be inhibited by IκB-α. These results are the first demonstration that the c-*rel* proto-oncogene product can bind DNA independently of the p50 NF-κB subunit, in a heterodimeric complex with the p65 subunit of NF-κB. In contrast to the κB element of the κ light chain enhancer, the uPA DNA element was shown to bind both c-Rel–p65 and p50–p65 complexes. Therefore, we have termed this novel TPA responsive element RRBE, for Rel-Related proteins Binding Element.

Key words: c-Rel/p65/IκB/NF-κB/urokinase

Introduction

Urokinase-type plasminogen activator (uPA) is a serine protease required in several tissue remodelling processes (Blasi *et al.*, 1991). uPA is involved in embryogenesis as a regulator of extracellular proteolysis and cell migration, in particular trophoblast implantation (Strickland *et al.*, 1976; Sappino *et al.*, 1989). In this context, uPA also has a role in TGF-β maturation and *in vitro* endothelial cell migration (Sato *et al.*, 1990). In cell culture, uPA synthesis is regulated by essentially all hormones and growth factors, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), cAMP, endotoxin and several lymphokines (reviewed in Blasi and Verde, 1990). The 5′-flanking region of the human uPA gene contains an enhancer between positions −2100 and −1870 and a negative regulatory region between positions −1870 and −1570. The enhancer contains PEA3 and AP-1 binding sequences which act as both basal level and inducible enhancer elements. In addition, a cell-type specific silencer is located between positions −660 and −537 (Verde *et al.*, 1988; Rørth *et al.*, 1990; Cannio *et al.*, 1991; Nerlov *et al.*, 1991).

The transcription factor NF-κB was discovered through its interaction with the κB site in the immunoglobulin kappa (Ig-κ) enhancer (Sen and Baltimore, 1986a). In addition, NF-κB also binds to a palindrome in the H-2K^b enhancer (Baldwin and Sharp, 1988; Israël *et al.*, 1989) and to κB elements in several other target genes involved in immediate early events of immune, inflammatory and acute phase responses, as well as to the HIV-1 LTR and other viral enhancers (Brasier *et al.*, 1990; Libermann and Baltimore, 1990; for review see Baeuerle, 1991). NF-κB is present in an active form in the nuclei of mature B cells, and induction of its activity in pre-B cells correlates with transcription of the κ gene (Sen and Baltimore, 1986b). In pre-B cells and in several non-lymphoid cell lines, NF-κB is present in a cytosolic inactive state in complex with the inhibitor protein IκB (Baeuerle and Baltimore, 1988). Upon release from the inhibitor, NF-κB translocates to the nucleus. *In vitro* treatment of inactive NF-κB with protein kinase C phosphorylates IκB and dissociates the complex thereby activating NF-κB (Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990). *In vivo*, NF-κB is activated by a variety of stimulants, such as TPA, LPS, TNFα or IL-1 (for review see Baeuerle, 1991).

NF-κB binds as a heterodimer of a 50 kDa (p50) and a 65 kDa (p65) subunit to DNA (Urban *et al.*, 1991). p65 is required for binding of IκB and modulation of DNA binding specificity (Baeuerle and Baltimore, 1989; Urban and Baeuerle, 1990; Urban *et al.*, 1991). Both human p50 and p65 share sequence homology with proteins of the *rel* oncogene family and the *Drosophila dorsal* gene product (Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Nolan *et al.*, 1991; Ruben *et al.*, 1991). *In vitro*, p50 and v-Rel can form a heterodimer and bind to the κB site in the H-2K^b enhancer (Kieran *et al.*, 1990). It has recently been shown that *in vitro* translated p50 and c-Rel can be co-immunoprecipitated by anti-p50 antibodies (Logeat *et al.*, 1991). Viral Rel proteins form multi-component complexes *in vivo*, as demonstrated by the ability of v-Rel antibodies to co-precipitate v-Rel and p36, p75, p115 and p124 proteins (Kochel *et al.*, 1991). UV

crosslinkings of a κB site of the IL2Rα enhancer with proteins from TPA-stimulated Jurkat cells have revealed the presence of not only p50, but also p75 and p85 subunits. p75 was suggested to be a novel Rel-related component of κB binding proteins, while p85 was suggested to be identical to c-Rel (Ballard *et al.*, 1990; Molitor *et al.*, 1990). c-Rel has been shown to be a sequence specific transactivator that binds to NF-κB sites (Bull *et al.*, 1990; Inoue *et al.*, 1991). However, the *in vivo* subunit composition of DNA binding complexes containing c-Rel has not been established. Several possibilities exist such as DNA binding of c-Rel by itself, or in complex with p50, p65 or other not yet identified factors. In order to understand the functional role of c-Rel or even v-Rel it is essential to clarify this issue.

In this study we show that a sequence between positions −1592 and −1570 in the human uPA gene contains a κB-like element, which can mediate TPA response in HepG2 and HT1080 cells. In contrast to the κB element of the Igκ enhancer, this element binds two protein−DNA complexes, designated UC and LC. One of the complexes (LC) is identical to NF-κB as it contained p50 and p65 subunits. The other complex (UC), however, is composed of c-Rel and p65. Our data show that c-Rel is associated with the p65 subunit of NF-κB thus forming a novel transcription factor complex. We have therefore termed this TPA responsive element from the uPA promoter Rel-Related proteins Binding Element (RRBE), to indicate its ability to bind two complexes composed of distinct Rel-related subunits and to differentiate it from the classic κB site.

Results

TPA responsiveness of the uPA RRBE

A 5′ deletion analysis of the upstream region of the human uPA gene fused to the CAT reporter gene, has revealed the presence of a TPA responsive element between positions −1592 and −1570 (compare −1592uPACAT with −1570 uPACAT, Table IA). This sequence element, termed RRBE, shows homology to the AP-2 and NF-κB sites in the SV40 enhancer and to the NF-κB consensus sequence (Table II). The effect of TPA is not visible in the presence of the upstream sequences −1870/−1592 (see Table IA); the region −1870/−1570 is the previously observed negative regulatory region functional in several cells, including HT1080 (Verde *et al.*, 1988; our unpublished observations). In order to test the enhancer activity of the TPA responsive RRBE element, the sequence between positions −1592 and −1572 was synthesized, trimerized by ligation and cloned upstream of the minimal uPA promoter (see Materials and methods). pCN23 is a construct containing the uPA minimal promoter, from −86 to +29 of the human uPA gene flanking region, fused to the growth hormone reporter gene (Nerlov *et al.*, 1991). As shown in Table 1B, the presence of three tandem copies of RRBE (pCN24) conferred TPA inducibility to the minimal uPA promoter after transfection into HepG2 cells. The minimal promoter itself (pCN23) was not inducible nor was the control thymidine kinase promoter (TKGH). A trimer of a 2 bp substitution mutant (pCN25) (for sequence see Table II) had no ability to convey TPA induction in HepG2 cells. The data obtained with HepG2 cells could be reproduced with HT1080 cells although the level of induction was lower (data not shown). HepG2 cells were chosen because they do not contain detectable levels of AP-2 (Williams *et al.*, 1988), a transcription factor that binds to a sequence very similar to the uPA RRBE (Table II). Therefore, the data presented in Table I show that AP-2 is not required for the TPA responsiveness of the uPA RRBE. As RRBE was homologous to a NF-κB consensus (Table II), we considered κB-like proteins to be involved in the TPA inducibility of RRBE.

Characterization of RRBE−protein complexes

Using footprinting and an electrophoretic mobility shift assay (EMSA), we have observed the binding of proteins to the

Table I. TPA inducibility of uPA−reporter gene constructs

Construct	Fold stimulation	Number of experiments
A		
−1870uPACAT	0.9	5
−1592uPACAT	4.6	6
−1570uPACAT	1.3	4
B		
pTKGH	0.96	3
pCN23	1.24	3
pCN24	2.93	3
pCN25	1.28	3

(**A**) TPA inducibility of uPACAT constructs in human HT1080 cells. TPA induction (2 days) is expressed as the ratio of CAT activity between stimulated and non-stimulated transfected cells. The values represent the average of several determinations with no more than 20% inter-experimental variation. (**B**) TPA induction (24 h) of RRBE−multimer constructions in HepG2 cells. The induction level is expressed as the ratio between hGH level with or without TPA after subtraction of background (mock-transfected cells). For all constructions at least three experiments were performed and two different plasmid preparations used, with variations not above 20%. The symbols for the constructions are explained in the text and in Materials and methods (plasmid construction).

Table II. Comparison of protein binding motifs in oligonucleotides used for EMSA

A

```
                    -1572                    -1592
                      |                        |
RRBE:          5'-GCTGCCTGCTGGGGAAAGTACAAGT

SV40 TC-II     5'-TGGGGAGCCTGGGGACTTTCCACAC
                          AP-2     NF-κB

Ig-κ NF-κB           5'-CAGAGGGGACTTTCCGAGA

                                 G  GAA
NF-κB consensus        5'-GGGARRTTCC
                                  CC
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B

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hMtIIa         5'-GATCGAACTGACCGCCCGCGGCCCGT

mut RRBE       5'-GCTGCCTGCTGcaGAAAGTAC
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(**A**) Human urokinase TPA responsive region (with the RRBE) aligned with SV40 TC-II (−225 to −249) and murine Ig-κ sequences; AP-2 and NF-κB binding sites are shown. Also NF-κB consensus (Zabel *et al.*, 1991) is aligned, rare nucleotides are indicated by small letters. Nucleotides identical to those of the RRBE are shown in bold. The RRBE oligonucleotides used in EMSA covers the region −1572 to −1592. (**B**) The hMtIIa oligo used for identification of AP-2 is from the distal BLE of the human metallothionein IIa promoter. The AP-2 binding sequence is underlined. mutRRBE oligo is shown with the two base substitutions in lowercase.

uPA RRBE sequence with nuclear extracts from untreated HeLa and HT1080 cells. DNase I footprinting showed that the sequence between positions −1592 and −1572 is protected by proteins present in nuclear extracts of HeLa cells (not shown). Using this information we have synthesized an oligonucleotide (Table II) spanning a sequence from positions −1592 to −1572 (RRBE). EMSAs performed with this probe and HT1080 nuclear extracts showed two retarded bands (Figure 1). The upper complex will be referred to as UC and the lower as LC. The binding activities of LC and UC were specific as both complexes could be competed for by RRBE but not by the 2 bp substitution eliminating the conserved stretch of G residues (mutRRBE, Table II). UC and LC were also observed with HeLa cell nuclear extracts and the same pattern of competition was obtained (Figure 1). The SV40 TC-II oligonucleotide (Table II) competed as efficiently as the wild-type RRBE. These results show that at least two complexes present in extracts from HT1080 and HeLa cells can bind to the RRBE.

To substantiate further the previous conclusion that AP-2 has no role in the RRBE mediated TPA response, we tested nuclear extracts by EMSA for the presence of proteins binding to the human metallothionein IIa (hMtIIa) AP-2 sequence (see Table II). As shown in Figure 1, HT1080 cells contained DNA binding activities specific for this sequence. However, unlabelled RRBE and hMtIIa AP-2 oligonucleotides did not cross-compete. Thus, we conclude that the RRBE sequence does not bind the AP-2 protein. This conclusion is further supported by the separation of the activities contributing to UC and LC from the hMtIIa AP-2 binding activity by gel filtration (data not shown).

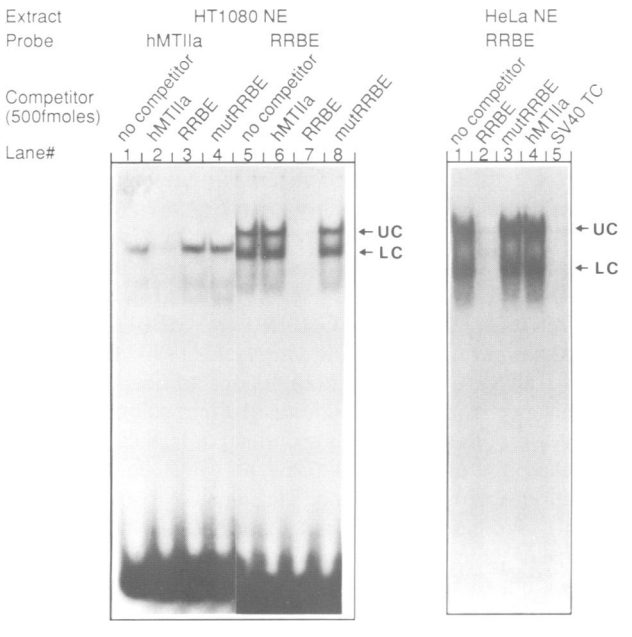


Fig. 1. Electrophoretic mobility shift assay (EMSA) on the TPA responsive region (−1592 to −1570) containing the RRBE of the human uPA promoter. (**Left**) HT 1080 nuclear extract. All lanes: 9 μg protein. Probes and competitor oligonucleotides (200-fold excess) as indicated. Complexes were separated on a 6% polyacrylamide gel. (**Right**) HeLa nuclear extract. All lanes: 24 μg proteins. Competitor oligonucleotides (500 fmol) as indicated. Complexes were separated on a 5% polyacrylamide gel.

The RRBE shows some homology to the Igκ NF-κB site and fulfils a NF-κB consensus sequence (Baeuerle, 1991; Zabel and Baeuerle, 1991; Table II). While the highly conserved half site $GGG^{A}/_{G}N$ is indeed present in RRBE, the second degenerate half site has not yet been found in any other NF-κB binding site studied. We have therefore investigated whether NF-κB-like proteins were involved in forming UC and LC. While in non-lymphoid cells most NF-κB is in an inactive cytoplasmic form, some nuclear NF-κB has been previously observed in HeLa cells (Macchi *et al.*, 1989). However, since cytosolic inactive NF-κB can be activated by detergent treatment (Baeuerle and Baltimore, 1988) we treated HeLa cell cytosol with deoxycholate (DOC) and tested for RRBE binding activity. As shown in Figure 2A, non-treated cytosol has no binding activity towards the oligonucleotide containing the Igκ NF-κB site, nor towards RRBE. Binding activity was, however, obtained with both probes after treatment of cytosol with DOC. While RRBE gave the two band pattern of LC and UC, the Igκ probe gave a single retarded band co-migrating with LC. Furthermore, the cytosolic RRBE binding complexes co-migrated with the nuclear LC and UC. With both probes, complex formation was inhibited by excess unlabelled RRBE and the Igκ sequence. The latter appeared to be a better competitor than uPA RRBE for LC, while RRBE competed better with UC. These results suggested that UC and LC are indeed due to NF-κB or NF-κB-like proteins. To test this idea further, we employed specific antibodies raised against the 105 kDa precursor of the NF-κB p50 subunit (Kieran *et al.*, 1990). As shown in Figure 2B this antibody, but not the control antibody, caused inhibition and displacement of LC and the Igκ−NF-κB complex formed by activated HeLa cell cytosol. UC was not affected by the polyclonal anti-p105 suggesting the absence of the p50 subunit. Thus, the two protein complexes binding the uPA RRBE sequence can be distinguished: LC contains a p50-related protein, but UC does not. LC is indistinguishable in mobility and immunoreactivity from the NF-κB complex formed with the Igκ probe. The latter has been shown to consist of p50 and p65 subunits (Baeuerle and Baltimore, 1989). The demonstration that LC is indeed NF-κB is given below (see Figures 4−6). Our data show that not only LC (NF-κB) but also UC exists as a cytosolic precursor that requires activation to achieve DNA binding activity.

To exclude that the binding specificity was biased by the localization of the κB-like sequence at the 3′ end of the oligonucleotide used as a probe, all of the EMSA reactions have been repeated and confirmed with a 51 nucleotide synthetic DNA probe (data not shown) encompassing the sequence −1605 to −1555 from the human uPA 5′ flanking region (for sequence see Materials and methods).

Both RRBE binding activities are TPA inducible and can be inhibited by IκB-α

We treated HeLa cells with TPA and tested nuclear extracts for binding activity with RRBE and Igκ sequences. As shown in Figure 3, a 30 min treatment with 40 nM TPA induced nuclear translocation of LC (NF-κB) as well as UC forming activities. The TPA-induced LC was specifically displaced by anti-p105 antibodies while UC was not. Similar results have been obtained with HepG2 cells (data not shown).

DOC treatment is known to dissociate NF-κB from a specific protein inhibitor, IκB, resulting in acquisition of

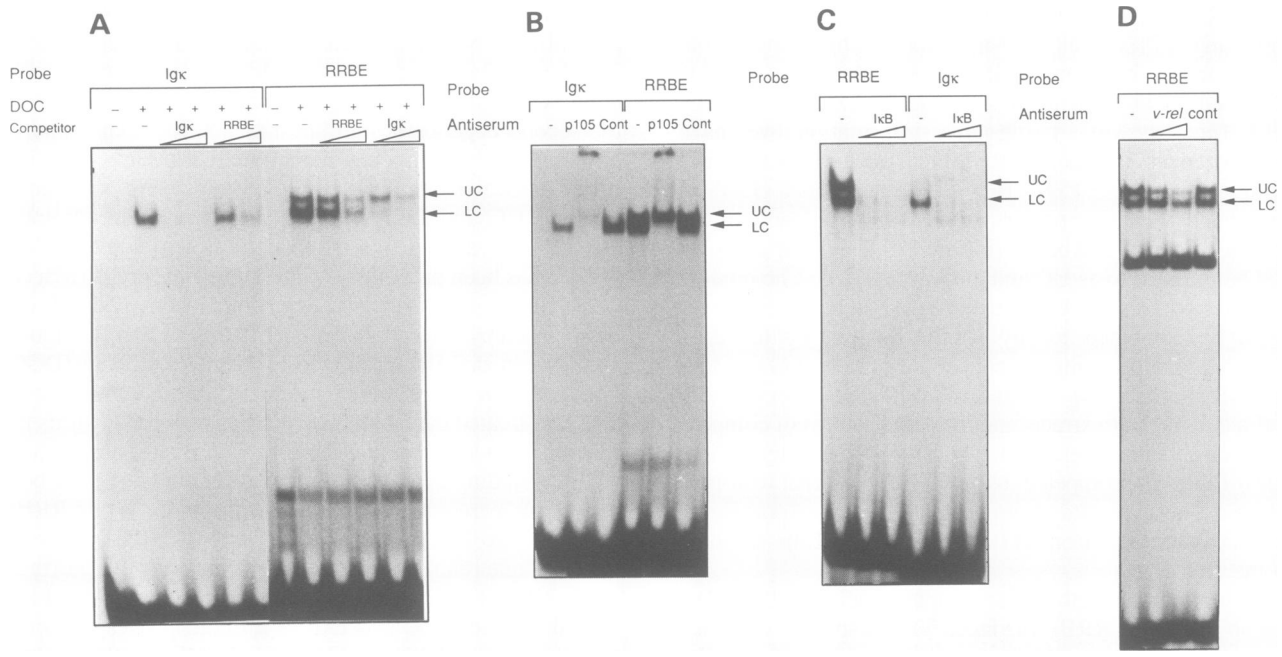


Fig. 2. Identification of NF-$\varkappa$B binding to RRBE. UC and LC (arrows to the right) indicate the migration of upper complex and lower complex respectively. (**A**) *In vitro* activation of DNA binding proteins from HeLa cytosols by deoxycholate (DOC). ± signs signify DOC treatment versus no DOC treatment. Sequences of RRBE and Ig$\varkappa$ probes are shown in Table IIA. In each case 10 μg protein (1 μl) of HeLa cytosols were treated with either 2.5 μl 6.5% DOC or water. Competitor amounts are (from left to right) 25- and 100-fold excess. (**B**) Interaction of LC with a p105 antiserum in EMSA. DOC-activated HeLa cytosols (10 μg protein) were preincubated with 1 μl of antiserum #2 (raised against recombinant p105; Kieran *et al.*, 1990) for 5 min at 0°C before addition of the DNA-containing mixture (total reaction volume 20 μl) and then incubated for 20 min at room temperature (the control is an irrelevant antiserum). (**C**) Interaction of UC and LC with purified I$\varkappa$B-α. 2.5 μl of nuclear extracts (10 μg protein) from TPA-stimulated (30 min) HeLa cells (see also Figure 3) were incubated with 0 (−), 1 or 3 μl of purified I$\varkappa$B-α (Zabel and Baeuerle, 1990), 1 μg poly[d(IC)] and HK2 buffer up to 11 μl for 10 min at room temperature followed by incubation with ^{32}P-labelled probe for 15 min (total volume is 20 μl). (**D**) Interaction of RRBE binding proteins with a v-Rel antiserum. 2.5 μl of nuclear extracts from TPA-stimulated HeLa cells (see Figure 3) were incubated with 0, 1 and 3 μl of v-Rel antiserum (Gilmore and Temin, 1986) or with 3 μl of control antiserum. Extracts and antisera were incubated for 10 min at 0°C before addition of probe, poly[d(IC)], and buffer and were then incubated for 15 min at room temperature. The faster migrating band is a non-specific complex which was not at all affected by the v-Rel antiserum.

DNA binding activity (Baeuerle and Baltimore, 1988). Two forms of I$\varkappa$B have been isolated (Zabel and Baeuerle, 1990; Link *et al.*, 1991) and found to react differently with NF-$\varkappa$B and Rel proteins. I$\varkappa$B-α only inhibited NF-$\varkappa$B by binding to p65, whereas I$\varkappa$B-β inhibited DNA binding of both NF-$\varkappa$B and c-Rel (Kerr *et al.*, 1991). To test further the nature of LC and to investigate its relationship to UC, we tested the effect of the purified alpha form of this inhibitor on the activity of both complexes in TPA induced HeLa nuclear extracts. As shown in Figure 2C, I$\varkappa$B-α inhibited the formation of both LC and UC. Since I$\varkappa$B-α requires the p65 subunit for inhibiting NF-$\varkappa$B activity (Urban and Baeuerle, 1990), this result further substantiates that LC is identical to NF-$\varkappa$B; moreover, it also indicates that UC may contain p65 or a related receptor for I$\varkappa$B-α.

UC but not LC reacts with anti-v-Rel antibodies

The NF-$\varkappa$B element has been shown to bind p50/p55 (p105 products) and p65 (the I$\varkappa$B receptor) subunits. In addition, a synthetic palindromic NF-$\varkappa$B site has also been shown to bind p75 and p85 proteins, the latter corresponding to c-Rel (Ballard *et al.*, 1990; Molitor *et al.*, 1990). In order to test for the presence of the c-Rel protein in the RRBE complexes, we used an antiserum directed against the turkey v-Rel (Gilmore and Temin, 1986). As shown in Figure 2D, v-Rel antibodies strongly inhibited UC formation in nuclear extracts from TPA treated HeLa cells. LC was only slightly affected, possibly due to a cross-reactivity of the serum. The results suggest that UC contains a Rel-homologous protein distinct from NF-$\varkappa$B, possibly c-Rel.

We have tested UC for the presence of factors belonging to a group of highly homologous zinc finger proteins binding the MHC class 2, Ig$\varkappa$ and HIV-1 LTR NF-$\varkappa$B elements [referred to as HIV-EP1, AGIE-BP1 and MBP1/PRDIIBF1 (Maekawa *et al.*, 1989; Baldwin *et al.*, 1990; Ron *et al.*, 1991)], all having highly homologous or identical DNA binding specificity). Upon addition of a monoclonal antibody raised against the DNA binding domain of HIV-EP1 (S.Ishii, personal communication) we saw no effect on UC or LC formation with DOC-activated HeLa cell cytosols (data not shown). We can thus rule out that HIV-EP1-homologous proteins or their proteolytic products are involved in UC and LC binding to RRBE.

In the NF-$\varkappa$B element of the angiotensinogen enhancer, a C-EBP-related protein has been shown to bind and to regulate the function of the NF-$\varkappa$B element (Brasier *et al.*, 1990). We have therefore tested the effect of antibodies directed against synthetic peptides from several isoforms of C-EBP (Landschultz *et al.*, 1988; Birkenmeier *et al.*, 1989; S.McKnight, unpublished). None of these antisera had any effect on UC or LC formation (data not shown).

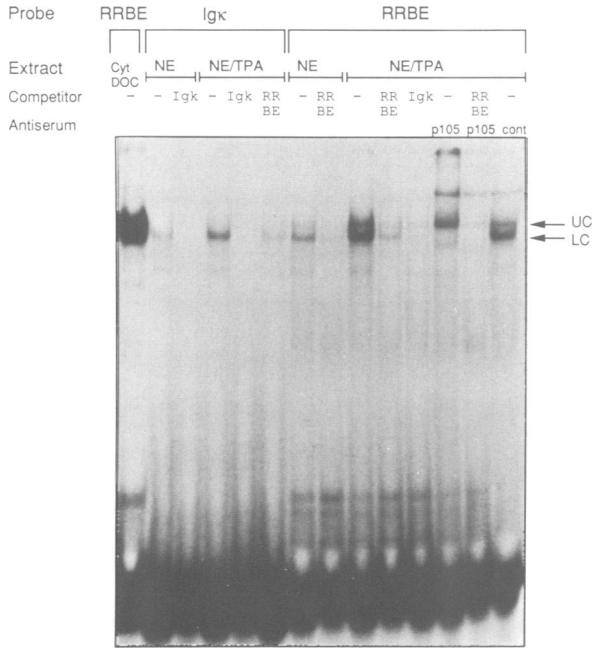


Fig. 3. Stimulation of RRBE binding activity by TPA in HeLa cells. The left lane shows RRBE binding activity of 1 μl of DOC-treated HeLa cytosols for comparison (for details on method see legend to Figure 2A). Binding reactions with nuclear extracts from control (−) or TPA-treated (+) cells contain 8 μg (2 μl) of protein, corresponding to twice as many cells as 1 μl cytosol. Stimulation was carried out with 40 nM of TPA for 30 min at 37°C. In the presence of antiserum, 2 μl of nuclear extracts of TPA-treated cells (+) were preincubated for 10 min at 0°C with 1 μg poly[d(IC)] and 3 μl HK2-N buffer, then 0.5 μl of p150 antiserum #2 (Kieran *et al.*, 1990) or control antiserum was added and incubation was continued for another 5 min at 0°C before addition of probe and competitor/water (final reaction volume is 10 μl). When used, competitors were in 100-fold excess over ^{32}P-labelled probe. It should be noted that the supershift observed with the p105 antiserum was specific since it could be competed for by the unlabelled RRBE oligo.

Identification of the DNA binding subunits within LC and UC

UV crosslinking experiments were performed in order to substantiate our conclusions that UC contained c-Rel and p65, and that LC corresponded to NF-κB and contained p65 and p50. Four different BrdU derivatives of the RRBE oligonucleotide were designed in order to identify the proteins which bind to the individual half sites (Table IIIA). Synthetic BrdU-substituted probes were ^{32}P-labelled at their 5′ end and used in EMSA. The presence of BrdU in the probes had no visible effect on complex formation (data not shown). Crosslinking was performed by UV-irradiating the native acrylamide gel, cutting out the bands corresponding to LC and UC and analysing them by SDS–PAGE. As shown in Figure 4, crosslinking reveals the presence of two major protein adducts in UC of 85 and 75 kDa. LC, on the other hand, forms 75 and 55–60 kDa adducts. The molecular weights have not been corrected for the linked 21 bp oligonucleotide which is likely to decrease the mobility of the proteins in SDS gels.

The nature of the 75 and 85 kDa adducts was investigated by immunoprecipitation with specific anti-p65 and anti-c-Rel antibodies. Crosslinking was performed in the EMSA gel, which was then blotted onto DEAE cellulose paper,

Table III. BrdU substituted probes and summary of crosslinking data

A

Probe no.

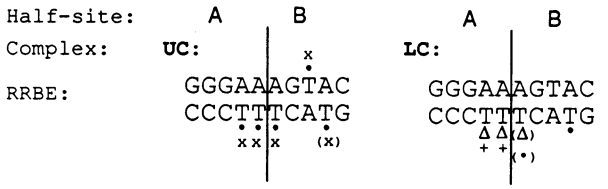
1 5′-GCTGCCTGCTG**GGGAAAGTAC**
CGACGGACGAC**CCCTTTCAXG**

2 .....GGGAAAGTAC
.....CCCXXXCATG

3 .....GGGAAAGTAC
.....CCCTTXCATG

4 .....GGGAAAGXAC
.....CCCTTTCATG

B



Complex	Adducts in kd		Probe	Half-site	Protein
UC	85	x	1,2,3,4	A,B	c-Rel
UC	75	•	1,2,3,4	A,B	p65
LC	75	•	1,2,3	B	p65
LC	60	Δ	2,3	A,(B)	p50
LC	55	+	2	A	p50

(**A**) Oligonucleotides used for crosslinking (X = 5-BrdU) are all identical except for the position of 5-BrdU. (**B**) A (5′-GGGAA) and B (5′-AGTAC) identify the two different half sites of RRBE. Positions where crosslinking between RRBE and protein takes place are summarized from Figure 4 and shown with symbols. Symbols in brackets refer to relatively weak bands in Figure 4. UC and LC refer to the bandshifted complex in which the protein was crosslinked. The factors corresponding to the different adducts are listed to the right.

from which the UC and LC bands were eluted and immunoprecipitated. The procedure included a denaturation step prior to the immunoprecipitation to eliminate co-precipitation of the other subunit–DNA adduct of the heterocomplex. As shown in Figure 5, an anti-p65 antibody could immunoprecipitate the 75 kDa adduct from UC. With anti-c-Rel antibodies, on the other hand, the 85 kDa adduct of UC was immunoprecipitated. Treatment of protein–DNA adducts from LC with anti-c-Rel antibodies did not precipitate any complexes, while anti-p50 antibodies specifically precipitated the 55 kDa adduct. Furthermore, anti-p65 antibodies also immunoprecipitated the 75 kDa adduct of LC (not shown). Our data therefore suggest that the 75 kDa adduct obtained with LC and UC contains the 65 kDa subunit of NF-κB. The immunoprecipitation data also show that the 85 kDa adduct contains c-Rel.

The UV crosslinking data and the results from the immunological characterization are shown summarized in part B of Table III. p50 (55–60 kDa adduct) was specific for LC and detectable with probe 2 and to some extent with probe 3. The 75 kDa adduct of LC (p65) was crosslinked to probe 1 and, to a lesser extent, to probes 2 and 3. Thus p50 was only crosslinked to the conserved half site A and

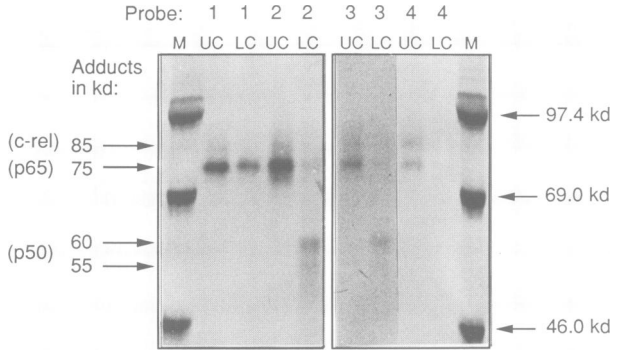


Fig. 4. UV crosslinking of RRBE binding proteins. Probe numbers refer to oligos shown in Table IIIA. EMSA was carried out using probes 1–4, the wet gels were UV-irradiated for 30 min at 306 nm, autoradiographed, and UC and LC cut out and put into the slot of an 8% SDS gel (see Materials and methods for details). The figure shows the autoradiograph of the SDS gel. For these EMSAs, fractionated cytosols were used. A summary of crosslinking of the various proteins to probes 1–4 is shown in Table IIIB.

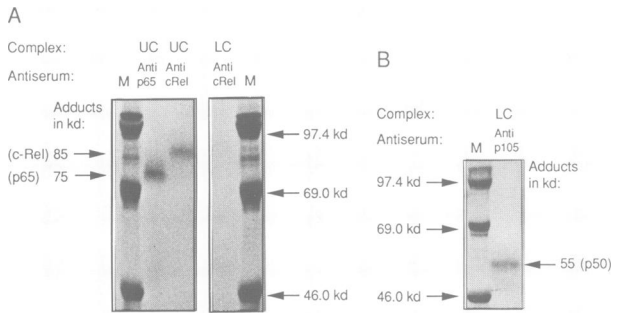


Fig. 5. Immunoprecipitation of UV crosslinked DNA–protein adducts. EMSA was performed with probe 2 (Table IIIA) as described in the legend of Figure 4. Following UV irradiation, the EMSA gel was blotted onto DEAE cellulose paper, the UC and LC bands cut out, eluted and immunoprecipitated (see Materials and methods section). The complex from which the protein–DNA adducts were eluted and the antibodies used for immunoprecipitation are indicated at the top of each lane. M refers to molecular weight markers lane. The numbers to the sides of the gels refer to the migration of molecular weight markers.

p65 to the degenerate half site B, in agreement with previous data (Urban *et al.*, 1991). In UC, however, the situation appears to be different since p65 was crosslinked to all four probes. Also c-Rel (85 kDa adduct), which is specific for UC, was UV crosslinked to probes 2, 3 and 4 and, very weakly, to probe 1. The difference in intensity of the bands of Figure 4 most likely reflects the availability of reactive residues rather than differential affinity. These observations suggest that in UC c-Rel and p65 may have no clear preference for either half site. In conclusion, crosslinking and immunoprecipitation data show that UC is formed by c-Rel and p65, and confirm that LC is a p65/p50 heterodimer.

Discussion

In this study, we provide evidence that the product of the c-*rel* proto-oncogene can complex with the p65 subunit of the NF-$\varkappa$B transcription factor. This evidence is based on the characteristics of a novel protein–DNA complex that binds the $\varkappa$B-like sequence 5′-GGGAAAGTAC-3′ (termed

Complexes formed in EMSA with RRBE probe:	Ig-κ competition:	RRBE competition:	Reactivity with anti-p105 antibodies:	Reactivity with anti-Rel antibodies:	IκB-α inhibition:	UV-cross-linked DNA-protein adducts:	Immunoprecipitation of DNA-protein adducts:
UC:	+	++	-	++	+	85kd 75kd	anti c-Rel anti p65
LC:	++	+	++	(+)	+	75kd 55/60kd	anti p65 anti p50

Fig. 6. Properties and composition of the UC and LC complexes. Summary of the data from Figures 3–5.

RRBE) from the uPA gene regulatory region. The complex is distinct from that of NF-$\varkappa$B as it strongly immunoreacts with Rel antiserum and not with p50 specific antiserum (anti-p105), contains a 85 kDa protein–DNA adduct, migrates more slowly in native gels than NF-$\varkappa$B and has a weaker affinity for the classical NF-$\varkappa$B binding motif 5′-GGGACTTTCC-3′. It is similar to the complex of NF-$\varkappa$B as it contains a 65 kDa subunit indistinguishable, in UV crosslinking and immunoprecipitation experiments, from that of NF-$\varkappa$B, and is inhibited by I$\varkappa$B-α, an inhibitor specific for the p65 subunit of NF-$\varkappa$B (Kerr *et al.*, 1991). The characteristics of the two complexes are qualitatively summarized in Figure 6. From the common and distinct properties of the two complexes, we conclude that nuclei contain two heterodimers, composed of overlapping and distinct protein subunits belonging to the Rel family, which can bind the RRBE sequence. One (LC) is indistinguishable from NF-$\varkappa$B and contains the p50 and p65 subunits. The novel slower migrating complex (UC) shares the p65 subunit with NF-$\varkappa$B but contains instead of the p50 subunit an 85 kDa component. Based on molecular weight and immunoreactivity data we conclude that UC contains c-Rel. And indeed, UC and LC activities co-purified by a procedure including two passages over a RRBE–Sepharose matrix and were shown by silver staining to contain 50, 65 and 77 kDa components; the latter two specifically reacted with anti-p65 and anti-c-Rel antibodies, respectively, in immunoblotting experiments (S.Hansen, in preparation). By analogy to NF-$\varkappa$B (Urban *et al.*, 1991), we expect that UC contains a heterodimer of p65 and c-Rel. Kieran *et al.* (1990) showed that p50 can heterodimerize *in vitro* with v-Rel. It is therefore surprising that there is no complex detectable between c-Rel and p50. It is possible that the additional N-terminal sequences of c-Rel interfere with a heterodimerization of p50 and c-Rel as has recently been proposed for a p65 homodimer (Nolan *et al.*, 1991). As found with FOS/JUN and ATF/CREB proteins (Hai and Curran, 1991), also members of the growing family of Rel-related proteins can apparently mix and form heterodimers of distinct sequence specificity.

Two protein–DNA complexes of different mobility were also observed using the $\varkappa$B motif of the human interleukin-2 receptor α-chain enhancer (Molitor *et al.*, 1990). All complexes showed immunoreactivity with a Rel antiserum, indicating that they all belong to the Rel family of proteins. A novel Rel-related protein of 75 kDa which was present in both complexes was identified by UV crosslinking. In the light of our data, p75 is likely to be the NF-$\varkappa$B p65 subunit. The increased molecular size after UV crosslinking would then be the result of covalently attached DNA. UV crosslinking studies with the purified p65 subunit of NF-$\varkappa$B indeed showed that p65 yields nuclease resistant protein–DNA adducts in the molecular weight range of 70–75 kDa (Urban

et al., 1991). The specific immunoreactivity of the 75 kDa adduct of UC with anti-p65 antiserum confirms that this conclusion is correct.

A recent study has shown that NF-κB contacts binding motifs by a heterodimer of p50 and p65 (Urban *et al.*, 1991). p50 prefers to bind the highly conserved half site with the consensus 5′-GGG$^{A}/_{G}$N-3′, whereas p65 prefers the less conserved second half site. The UV crosslinking data of LC using four probes of the uPA RRBE with photoreactive nucleotides in different sequence positions, are in agreement with this model (Table III). However, in the complex containing c-Rel and p65, the situation is different. The c-Rel subunit could be crosslinked to both half sites, the strongest interaction, however, being with the second less conserved half site (5′-GGGAAAGTAC-3′). Also p65 was found to contact residues within both half sites. Thus p65 and c-Rel were crosslinked to the same residues and to residues on both strands. This suggests that c-Rel–p65 contacts DNA differently from NF-κB. A simple explanation for this observation might be that both p65 and c-Rel can bind to the A or B half site resulting in two different orientations of the complex on the RRBE. The ability of c-Rel to bind also the less conserved right half site makes the RRBE sequence unique and suggests that c-Rel has a DNA binding specificity different from that of p50. RRBE appears to be a κB element able to bind a complex containing one c-Rel subunit. Again, this situation is very similar to that of the Jun/Fos/CREB family of proteins where subtle alterations of the binding motif decide the subunit composition of the heterodimer binding to the respective motif and, ultimately, the responsiveness of the *cis*-acting element. We have termed the uPA κB motif RRBE (Rel-Related proteins Binding Element) to emphasize that it can bind complexes of Rel-related proteins with a composition distinct from that of NF-κB.

At present we cannot tell the physiological significance of c-Rel being present in complex with p65. Trimers of the RRBE function as a TPA inducible enhancer. This effect could be brought about by either the NF-κB (LC) or the c-Rel–p65 (UC) complex, both of which are inducible by TPA and located in inhibited forms in the cytoplasm of cells When both complexes are activated, one can expect that they compete for binding to the RRBE. The functional implications of this competition is at present not known. However, upon activation of c-Rel–p65 (UC) only the RRBE and not the Igκ NF-κB element would mediate a transcriptional response. Thus, the RRBE might be a regulatory element distinct from most of the κB elements described. It is in this context of interest to know the inhibitory components and the precise composition of the inducible cytoplasmic complexes. While in the NF-κB complex, the inhibitor IκB can only bind to p65, the situation in the c-Rel–p65 complex appears more complicated. The inhibition of UC by IκB-α is probably exerted through the p65 subunit, as p65 recognizes this inhibitor (Urban and Baeuerle, 1990); c-Rel, in fact, recognizes the β and not the α form of IκB (Kerr *et al.*, 1991). The inactive, cytoplasmic c-Rel–p65 complex might be expected to contain different combinations of IκB forms: either one IκB-α (binding to p65), or one IκB-β (binding to either subunit) or IκB-α/IκB-β and IκB-β/IκB-β combinations. The presence of two IκB molecules in one complex might not be possible for steric reasons. It is still an open question whether these different IκB forms are inactivated upon different stimuli.

It has been demonstrated that v-Rel can suppress transcriptional activation from κB-like elements (Ballard *et al.*, 1990; Inoue *et al.*, 1991). Here we have shown that c-Rel exists in a DNA binding complex with p65 and not p50. Thus, it might be possible that v-Rel acts as a transcriptional suppressor through the interaction with p65. This possibility becomes even more intriguing as it has been shown that p65 is a strong activator of transcription whereas p50 is a negative regulator (Schmitz and Baeuerle, 1991). One might propose that the transforming properties of v-Rel derive from its substitution of c-Rel, thereby altering p65 activity.

Materials and methods

Antisera

The anti-v-Rel antiserum was kindly donated by Dr H.Temin (Gilmore and Temin, 1986). The anti-c-Rel antiserum, directed against the unique C-terminal 15 amino acids of the human c-Rel protein (Brownell *et al.*, 1989) was kindly donated by Dr N.Rice. The p65 specific anti-peptide antibody was obtained from the synthetic peptide CDTDDRHRIEEKRKRT, corresponding to amino acids 291–305 of human p65 (Ruben *et al.*, 1991). The N-terminal cysteine was used to couple the peptide to maleimidobenzoic acid hydroxysuccinimide ester-activated bovine serum albumin at a molar ratio of 20:1 (Schneider *et al.*, 1983). One rabbit was injected into the thigh muscle and subcutaneously with an amount of conjugate equivalent to 125 μg of peptide in complete Freund's adjuvant for the first injection, and in incomplete Freund's adjuvant for booster injections. Serum obtained after the third boost was passed over epoxy-activated sepharose 4B with coupled peptide (3 ml of resin). After extensive washes with Tris-buffered saline(TBS) pH 8.5 containing 0.2% (v/v) Nonidet P40, the column was washed with 5 vol of 0.1 M glycine–HCl, pH 3.0, followed by elution with 4 M guanidinium hydrochloride. The eluate was extensively dialysed against TBS pH 8.0 and tested by ELISA for specific immunoreactivity. An aliquot of the eluate was subjected to reducing SDS gel electrophoresis followed by Coomassie staining. This revealed the presence of IgG heavy and light chains and gave a rough estimate of the antibody concentration.

Cell lines and cell culture

All cell lines have been described before (Nerlov *et al.*, 1991).

Plasmid constructions

Plasmid pTKGH has been described (Selden *et al.*, 1986). The plasmid pCN23 containing the urokinase promoter (−86 to +30) upstream of the hGH reporter gene has been described elsewhere (Nerlov *et al.*, 1991). Plasmids pCN24 and pCN25 were constructed as follows. The single-stranded oligonucleotides composing RRBE and mutRRBE were phosphorylated and annealed. The annealed oligonucleotides (40 nM final concentration) were ligated into pUC19 cut with *Hin*cII. Plasmids with head to tail trimer inserts were identified by sequencing, inserts were cut out with *Hin*dIII and *Sma*I, and cloned into the unique *Hin*dIII and *Hin*cII sites of pCN23. These plasmids were designated pCN24 (3 × RRBE) and pCN25 (3 × mutRRBE). The human uPACAT constructions have been described (Verde *et al.*, 1988).

Transfections

Transient transfection procedure with HepG2 cells and reporter gene assay are described in Nerlov *et al.* (1991).

Oligonucleotides

Oligonucleotides were synthesized on an Applied Biosystem 381DNA synthesizer. The RRBE oligo corresponding to nucleotides −1592 to −1572 of the human uPA promoter (Verde *et al.*, 1988): 5′-GTACTTTCCCCA-GCAGGCAGC-3′ (blunt ends); mutRRBE sequence: 5′-GTACTTTCTG-CAGGAGC-3′ (blunt ends); the human metallothionein IIa AP-2 binding sequence (hMTIIa oligo): 5′-GATCGAACTGACCGCCCGCGGCCCGT-3 (region −192 to −167; 5′-GATC overhangs) (Imagawa *et al.*, 1987; Williams *et al.*, 1988); the SV40 TC-II containing sequence (SV 40 TC-II oligo): 5′-CTAGGGTGTGGAAAGTCCCCAGGCTCCCCAG-3′ (−250 to −224; 5′-CTAG overhangs) (Mitchell *et al.*, 1987); the Igκ sequence with the NF-κB site: 5′-CAGAGGGGACTTTCCGAGA (Lenardo and Baltimore, 1989). The 51mer oligonucleotides corresponding to

−1605 to −1555 of the human uPA promoter has the following sequence: 5'-GAAAGGCTAACTTGTACTTTCCCCAGCAGGCAGCTG-GCATCCTGAGCCCTC. Oligonucleotides used for UV crosslinking were synthesized by incorporating 5-BrdU (Millipore) in various positions through the X-channel on the DNA synthesizer. The sequence was identical to the RRBE except for the substitutions of dT by 5-BrdU shown in Table IIIA.

Extracts

Nuclear extracts were prepared essentially according to Dignam *et al.* (1983). The following modifications were introduced: (i) the first wash with buffer A was omitted; (ii) the crude nuclear pellet after the 2000 r.p.m. spin was directly suspended in 5 ml of buffer C per 10^9 cells. The NaCl concentration was subsequently adjusted to 0.42 M, using 5 M Nacl; (iii) after stirring on ice, extracts were centrifuged at 80 000 *g* for 60 min; (iv) for footprinting analysis, while still in buffer C, extracts were subjected to a 47% ammonium sulfate precipitation and the precipitate was resuspended in 0.25 times the original volume of buffer D and dialysed against buffer D. For gel retardation and fractionation extracts were dialysed without precipitation. After dialysis, extracts were subjected to another 80 000 *g* centrifugation for 60 min.

Cytosols were prepared as described (Dignam *et al.*, 1983) with the first modification mentioned in the above paragraph. Some preparations were made from cytosols frozen in buffer A (from 4^c A.Miller, Belgium). The S100 supernatant was dialysed against HK1 buffer (25 mM HEPES, pH 7.9, 100 mM KCl, 20% v/v glycerol, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM DTT, 1 mM $Na_2S_2O_5$ and 0.5 mM PMSF) and centrifuged at 80 000 *g* for 60 min.

Footprinting

DNase I footprinting was performed essentially as previously described (Nerlov *et al.*, 1991).

Electrophoretic mobility shift assay (EMSA)

Oligonucleoides were labelled using [γ-^{32}P]ATP and polynucleotide kinase, annealed and gel purified for isolation of double-stranded probes. Specific activity was ~5 × 10^6c.p.m./pmol.

Reactions with nuclear extracts were performed in a 20 μl volume containing 3−10 000 c.p.m. probe, 1 μg poly[d(I-C)] and competitor DNA as indicated. Extracts, in 10 μl HK2 buffer (25 mM HEPES, pH 7.9, 100 mM KCl, 120% v/v glycerol, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT and 0.5 mM PMSF), were incubated with the poly[d(I-C)] at 0°C for 15 min (unless otherwise stated) then added to the probe plus or minus competitor in distilled water. Incubation was continued for 15 min at 20°C after which reaction mixtures were loaded on 5% non-denaturing polyacrylamide gels in 0.5 × TBE (1 × TBE: 89 mM Tris, 89 mM Boric acid, 1 mM EDTA). In some cases HK2-N buffer (HK2 with 0.1% NP40) was used.

In the case of cytosols, 1 μl extract was activated with 2.5 μl 6.5% sodium deoxycholate for 10 min at 0°C before addition to a mixture of probe (10 000 c.p.m.), 3 μg poly[d(IC)], competitor or water and 9 μl HK2 buffer with 2.4% NP40 (total reaction volume: 20 μl). The mixtures were incubated at 20°C for 20 min and complexes were resolved on non-denaturing 5% polyacrylamide gels, as described above.

UV crosslinking

Cytosols prepared from HeLa cells were loaded on a Q-Sepharose HP column (55 ml, Pharmacia) in TK1 buffer (25 mM Tris−Cl pH 7.9, 100 mM EDTA, 1 mM $MgCl_2$, 1 mM DTT, 1 mM $Na_2S_2O_5$, 0.5 mM PMSF). The column was washed with the same buffer and eluted with 500 ml of a 100−600 mM KCl gradient in TK1 buffer. Fractions were screened by EMSA, UC and LC activity was pooled and dialysed against TK1. Half of the pool was passed over a 30 ml mutRRBE−Sepharose column equilibrated in TK1. The mutRRBE column was prepared by coupling 5−50mers of the mutRRBE oligo to CNBr-activated Sepharose 4B (Pharmacia; Kadonaga and Tjian, 1986). The flow through of the mutRRBE column was used in EMSA for UV crosslinking.

Fractionated cytosols (1.5 μl; see above) were activated with 3.5 μl 6.5% DOC for 10 min at 0°C before mixing with 50 000 c.p.m. of BrdU-substituted probe (Table IIIa), 1 μg poly[d(IC)] and buffer as described under EMSA. The wet EMSA gels were UV-irradiated on a trans-illuminator (306 nm) for 30 min at room temperature and autoradiographed at 4°C. The bands corresponding to UC and LC were cut out and equilibrated in 100 mM Tris−Cl pH 6.8, 50 mM DTT and 2% SDS before loading on an 8% SDS−polyacrylamide gel (Laemmli, 1970). Gels were dried and autoradiographed. As markers ^{14}C-labelled protein mixtures (Amersham Int.) were used.

Immunoprecipitation

Immunoprecipitation was performed on UC and LC UV crosslinked *in situ* with probe 2 of Table III. EMSA and UV crosslinking were performed as described.

Following UV-irradiation the gel was blotted to DEAE-cellulose paper (Whatman) and the blot was autoradiographed at 4°C. Parts of the blot corresponding to UC and LC were cut out and the protein−DNA complexes were eluted twice with 100 μl of 20 mM Tris−Cl (pH 8.0), 2 mM EDTA, 1.3 M NaCl, 0.5% SDS, 0.5% Nonidet P40 1 mM DTT and 0.5 mM PMSF. Eluates from five lanes were combined and supplemented with 25 μg BSA and cleared by centrifugation. The resulting supernatant was precipitated with acetone. The pellet was resuspended for 5−10 min at room temperature in 5 μl of 50 mM Tris−Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P40, 0.1% SDS. 100 mM DTT, 0.5 mM PMSF. The sample was then transferred to 0°C and 95 μl of IP-buffer supplemented with 0.5 μg/μl, BSA (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P40, 1 mM DTT, 0.5 mM PMSF) was added and incubated for 10−15 min at 0°C. An appropriate antiserum was added and the mixture was rotated overnight at 4°C. Antigen−antibody complexes were precipitated by 10 μl of protein G−Sepharose (Pharmacia) for 2 h at 4°C. The matrix was washed twice with 150 μl IP-buffer and once with 150 μl 10 mM Tris−Cl (pH 7.5), 0.1% NP40. Complexes were dissolved in 20 μl 100 mM Tris−Cl (pH 6.8), 100 mM DTT and 2% SDS at 65°C for 5 min. The matrix was pelleted by centrifugation and the supernatants were loaded on 8% SDS polyacrylamide gels. Gels were dried and autoradiographed at −70°C.

Acknowledgements

We are grateful to Drs Howard Temin, Alain Israel, Nancy Rice, Steve McKnight and Shunsuke Ishii for their generous gift of antibodies. This work was supported by the Danish Biotechnology Program, Center for Medical Biotechnology, the Danish Cancer Society, the Carlsberg Foundation, the Novo Foundation, the Italian Ministry of Health (AIDS Fund) and the PF Ingegneria Genetica of the Italian Consiglio Nazionale delle Ricerche.

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Received on September 3, 1991; revised on October 18, 1991