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A Yeast Transcription Assay Defines Distinct Rel and Dorsal DNA Recognition Sequences

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Recent data have demonstrated that vRel, cRel, Dorsal, and NF-κB are members of a larger family of DNA-binding regulatory proteins. Rel proteins interact to form homo- and heterodimers that recognize specific sites on DNA, and it is likely that such protein-protein and protein-DNA interactions contribute to proper regulation of target gene expression by these proteins. Here we describe the use of a yeast transcription activation assay to study binding of three Rel family proteins to their native binding sites. These results show that the vRel and cRel proteins recognize two known NF-κB binding sites; the Dorsal protein does not recognize NF-κB sites, but does recognize related sites upstream of the *Drosophila zerknüllt* gene. Our experiments demonstrate that the members of this protein family recognize similar, but not identical, sites in the promoters of target genes, and we are able to identify a particular nucleotide that is apparently involved in the DNA-protein interaction. We exploit the properties of LexA fusion proteins to study the dimerization and DNA-contacting domains of cRel. Our results suggest that the cRel protein forms homodimers and that dimer formation may be necessary for cRel to bind DNA. Finally, our results show that transcription activation by these proteins is cooperative; such cooperativity may be important for correct temporal and spatial regulation of target gene expression.

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v-*rel* is the transforming oncogene of the avian reticuloendotheliosis virus T (Chen et al., 1981; Stephens et al., 1983; Wilhelmsen et al., 1984). c-*rel,* its cellular homolog, has been isolated from a number of avian and mammalian species (Capobianco et al., 1990; Hannink and Temin, 1989). *dorsal* is a *Drosophila* maternal effect gene that is required for the establishment of dorsal-ventral polarity in the blastoderm (Anderson, 1987; Steward et al., 1984). All three gene products (here called vRel, cRel, and Dorsal) share a region of sequence similarity with the 50- and 65-kd subunits of the NF-κB transcription factor (p50 and p65) (Bours et al., 1990; Ghosh et al., 1990; Kieran et al., 1990; Nolan et al., 1991; Steward, 1987).

Several lines of evidence suggest that this sequence similarity reflects a functional homology. First, the activity of all of these proteins appears to be regulated by their subcellular localization. The NF-κB p50-p65 complex is retained in the cytoplasm by the IκB inhibitor protein; phosphorylation of IκB releases NF-κB and allows it to enter the nucleus and activate transcription of target genes (Baeuerle and Baltimore, 1988). The Dorsal protein is similarly retained in the cytoplasm, probably by the product of the *cactus* gene; shortly after fertilization Dorsal is localized to nuclei in the *Drosophila* blastoderm in a ventral to dorsal gradient (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). The c-*rel* protein is also retained in the cytoplasm in some cell types, although the mechanism of retention is not known (Capobianco et al., 1990; Gilmore and Temin, 1986; Hannink and Temin, 1989).

Second, these proteins all regulate transcription. NF-κB was originally identified as a factor that binds sites in the Igκ enhancer; transfection experiments have demonstrated that NF-κB stimulates (transactivates) the expression of many genes that contain similar sites (reviewed in Lenardo and Baltimore, 1989; Sen and Baltimore, 1986). Dorsal is a maternal effect gene product that is required for proper development of ventral structures in the *Drosophila* embryo, and genetic studies suggest that it functions by activating the expression of zygotic genes such as *twist* and *snail* and by repressing expression of *zerknüllt* (*zen*) (Anderson, 1987; Boulay et al., 1987; Doyle et al., 1989; Rushlow et al., 1987; Steward et al., 1984; Thisse et al., 1987; see below). vRel and cRel activate transcription when bound to DNA by LexA or GAL4 DNA-binding

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domains, and deletions in vRel that affect the integrity of its transcription activation domain (region I; Kamens et al., 1990) abolish its ability to cause oncogenic transformation (Bull et al., 1990; Gelinas and Temin, 1988; Kamens et al., 1990).

The final line of evidence for functional homology among vRel, cRel, Dorsal, and NF-κB is that these proteins all bind specific sites on DNA. Truncated versions of both the p50 and p65 proteins bind to NF-κB sites, and, in vitro, the amino-terminal Rel homology domain is required for this function (Ghosh et al., 1990; Kieran et al., 1990; Nolan et al., 1991). Native Dorsal binds DNA sequences in the promoter of the *zen* gene in vitro (Ip et al., 1991). vRel and cRel bind to sites recognized by NF-κB in vitro, and cRel has recently been shown to bind NF-κB sites in activated T cells (Ballard et al., 1990). The high level of similarity in their amino-terminal domains suggests that site recognition by these proteins may depend on the conserved region; immunoprecipitation studies with vRel, cRel, and p50 NF-κB derivatives show that this region may also be involved in oligomerization (Davis et al., 1990a; Davis et al., 1990b; Kieran et al., 1990; Lim et al., 1990).

We and others have used yeast transcription activation to study DNA recognition and oligomerization by higher eukaryotic proteins (Hanes and Brent, 1989; Hanes and Brent, 1991; Samson et al., 1989), to localize transcription activation domains of LexA-Rel fusion proteins (Bull et al., 1990; Kamens et al., 1990), to isolate binding sites (Wilson et al., 1991), and even to detect interacting proteins (T. Zervos and J. Gyuris, unpublished results; D. Shore, personal communication). Here we describe the use of yeast transcription activation to study DNA recognition by native Rel proteins. Our results show that like the homeodomain proteins, the members of the Rel protein family recognize similar, but distinguishable, sites in the promoters of target genes. Our results also suggest that transcription activation by Rel proteins is cooperative. Finally, we use LexA fusion proteins in a novel way to show that the cRel protein contains regions that function in dimerization and that this dimerization is required for cRel to recognize its native site.

RESULTS AND DISCUSSION

vRel and cRel, But Not Dorsal, Activate Transcription of Target Genes That Contain NF-κB Sites

In a series of experiments native vRel, chicken cRel, and Dorsal were expressed in yeast and assayed for their ability to activate the expression of reporter genes that carried potential binding sites (Table 1). Gene expression by the reporter gene was measured, and the results are expressed as units of β-galactosidase.

Table 1. Activation of gene expression by vRel, cRel, and Dorsal.

Plasmids directing the synthesis of native proteins were introduced into yeast cells that carried a plasmid containing the appropriate reporter construct.

Reporter construct*	β-Galactosidase activity (units)			
	pBC102	vRel	cRel	Dorsal
Igκ-1	<1	<1	4	<1
Igκ-2	2	6	50	3
Igκ-3	2	50	430	2
IL2-4	<1	<1	2	3
Rα-3	<1	9	390	3
ZenF	3	20	130	640
ZenR	4	20	120	740
Zen1-1	<1	<1	2	<1
Zen1-2	<1	2	2	9
Zen1-3	<1	1	<1	50
Zen2-1	<1	<1	<1	2
Zen2-2	2	2	2	30
Zen2-3	<1	<1	2	80
Zen3-1	2	2	2	<1
Zen3-2	<1	3	9	6
Zen3-3	<1	9	20	20
IgκA-2	2	5	9	2
IgκA-3	2	20	160	4
Zen2T-2	<1	2	2	8
Zen2T-3	2	2	2	50

*Described in Table 2 and Materials and Methods.

We tested whether cRel, vRel, and Dorsal recognized an NF-κB site found in the enhancer region of the Ig κ gene (here called an Igκ site). cRel strongly activated expression of *Igκ-GAL1-lacZ* reporter genes that contained three copies of the Ig κ enhancer NF-κB binding site (Table 1) (Sen and Baltimore, 1986). Activation was dependent on the production of Rel protein and on the presence of Igκ sites upstream of the reporter gene (not shown). vRel also activated these *Igκ-GAL1-lacZ* reporter genes, but to a lesser extent, consistent with our previous results that showed that LexA-vRel fusion proteins are weaker activators than LexA-cRel fusion proteins (Kamens et al., 1990). While vRel activates transcription when bound to DNA upstream of target genes in yeast (Kamens et al., 1990, and this study), it has been observed that vRel represses transcription in mammalian cells (Ballard et al., 1990; Richardson and Gilmore, 1991). Two factors may explain this difference: First, vRel is a rather weak activator in yeast, suggesting that its activity may be too low to detect in mammalian assays, and second, as has been suggested, vRel probably represses transcription in mammalian cells indirectly, by blocking other Rel family members not found in yeast from binding to DNA and activating transcription (Ballard et al., 1990; Richardson and Gilmore, 1991).

Activation of transcription by vRel and cRel was apparently cooperative. For example, cRel did not

activate reporter constructs that contained only one copy of the Igκ site; however, it directed the synthesis of 50 units of β-galactosidase when the reporter gene contained two copies of the site, and 430 units when the reporter gene contained three copies of the site (Table 1). Dorsal did not stimulate expression of any of these reporter constructs.

Both vRel and cRel also activated expression of reporter constructs that contained a second type of NF-κB–binding site, from the human interleukin-2 (IL-2) receptor α subunit (Böhnlein et al., 1988), but did not activate expression of reporter constructs that contained copies of a third type of binding site, although NF-κB reportedly binds this site in vitro (a human IL-2 site; Table 1, lines 4 and 5) (Hoyos et al., 1989). The failure to activate transcription of a reporter containing copies of the IL-2 binding site can be attributed to the inability of the Rel proteins to bind this site at the concentration of protein present in the yeast cell. It is possible that this site is not actually bound by NF-κB in higher eukaryotic cells. Consistent with this idea, the IL-2 site differs from the consensus site at position 9.

vRel, cRel, and Dorsal Recognize Sites in the zen *Promoter*

To identify DNA sequences recognized by Dorsal, we subcloned and sequenced a 285–base pair (bp) region of the *zen* promoter that deletion studies had identified as being necessary for Dorsal-mediated repression (Fig. 1) (Doyle et al., 1989). We tested Dorsal's ability to regulate transcription of reporter genes containing this region. Table 1 shows that Dorsal recognized this *zen* fragment; it strongly stimulated transcription of reporter constructs with this promoter region inserted upstream of the reporter gene. Stimulation was independent of orientation. vRel and cRel also activated expression of these *zen-GAL1-lacZ* reporter genes, but less strongly than Dorsal (Table 1, lines 6 and 7) (see below).

Note that although Dorsal apparently represses expression of the *zen* gene in the ventral cells of the embryo, it activates transcription in our system, an effect similar to its proposed effect on the *Drosophila twist* and *snail* genes. As in previous studies (Samson et al., 1989), we account for this difference by suggesting that *Drosophila* contain accessory factors that are required for repression of the *zen* promoter but that are absent in yeast (see Conclusions).

Recently, Ip et al. showed that the Dorsal protein binds to three smaller fragments in the 285-bp ventral repression element of the *zen* promoter (Ip et al., 1991). These three fragments contain 10-bp sites that are similar to the NF-κB binding site consensus sequence GGGR(CAT)TYYCC (Table 2) (Lenardo and Baltimore, 1989). We introduced multiple copies of these 10-bp sites upstream of the *GAL1-lacZ* fusion gene and tested these constructs for recognition by Rel and Dorsal proteins in yeast. Dorsal recognized all three of these *zen* promoter sites (Table 1). Dorsal directed the synthesis of 80 units of β-galactosidase from a target gene containing three copies of site 2 (Table 1, Zen2-3). Activation of constructs containing sites 1 and 3 was less strong (50 and 20 units of β-galactosidase, respectively), in agreement with in vitro data that shows that Dorsal binds less strongly to sites 1 and 3 (Ip et al., 1991). vRel and cRel recognized only site 3. Of the three sites, site 3 most strongly resembles the NF-κB consensus; it differs only at positions 6 and 10. As was the case with the Rel proteins, the increase in Dorsal-dependent activation of reporter genes with increasing numbers of sites was more than additive, showing that activation of transcription by Dorsal was also cooperative.

Dorsal activates transcription more strongly from the intact ventral repression element than it does from three copies of any of the individual sites. It is possible that this effect is due to the fact that the ventral repression element contains additional, cryptic Dorsal-binding sites that are recognized when the adjacent sites are bound. Alternatively, this effect may reflect a requirement for proper spacing between target sites for efficient activation by Dorsal.

Inspection of these sequences suggested that the base pair at position 6 of the NF-κB consensus binding site might be important for the different site prefer-

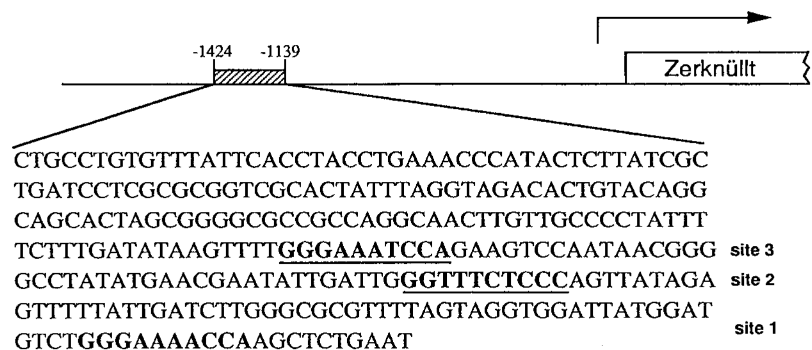

Figure 1. Sequence of the *zerknüllt* promoter region required for Dorsal-mediated repression.

Three proposed protein-binding sites are underlined and designated as site 1, 2, and 3 by the notation to the right of the sequence. This fragment was subcloned into a Bluescript SK+ polylinker, and both strands were sequenced.

Table 2. Reporter genes used for this study.

Reporter constructs contain the indicated number of binding sites and were made as described in Materials and Methods. The 10-bp NF-κB–like sites present in each construct are aligned to allow comparison.

Reporter construct	Number and orientation of inserts	Sequence	Gene
Igκ-1 Igκ-2 Igκ-3	← → → ← ← ←	GGGACTTTCC	Mouse Ig κ light chain
IL2-4	→ → ← →	GGGATTTCAC	Human IL-2
Rα-3	→ ← ←	GGGAATCTCC	Human IL-2Rα
ZenF ZenR	——— → ← → ← → ← ———	285 bp fragment	Drosophila Zerknüllt
Zen1-1 Zen1-2 Zen1-3	← ← ← → ← →	GGGAAAACCA	Zerknüllt (site 1)
Zen2-1 Zen2-2 Zen2-3	← ← ← → ← →	GGGAGAAACC	Zerknüllt (site 2)
Zen3-1 Zen3-2 Zen3-3	← ← ← → ← →	GGGAAATCCA	Zerknüllt (site 3)
NF-κB consensus		C GGGRATYYCC T	

ences of the Rel and Dorsal proteins (Table 2). There is a T at position 6 in the sites recognized by vRel and cRel (and in all known NF-κB sites), but there is an A at this position in the three sites in the *zen* promoter that are recognized by Dorsal. However, when we changed the A to a T at position 6 of the Zen2 site, Dorsal still recognized the site while cRel did not (Table 1, Zen2T-2 and Zen2T-3). Similarly, when we changed T to an A at postion 6 of the Igκ site cRel still recognized this site and Dorsal did not (IgκA-2 and IgκA-3). In both cases changing this base pair led to reduced affinities of the proteins for their sites. These results show that the base pair at position 6 is involved in site recognition by vRel, cRel, and Dorsal, but that the identity of position 6 alone is not responsible for the differences in site preference.

Lesions in the Conserved Region Affect Dimerization

To examine more closely which regions of the Rel proteins contact DNA we introduced a number of small deletions and insertions into the conserved region of the c-*rel* gene (see Materials and Methods). As expected, cRel derivatives containing alterations comparable to those that abolish the ability of vRel to transform (and to bind DNA in vitro; Ballard et al., 1990) did not recognize NF-κB–binding sites (Fig. 2, cRel-D2 and cRel-D2N). Similarly, a deletion comparable to one that does not affect the ability of vRel to transform did recognize NF-κB sites (cRel-D1). We believe that it is unlikely that the inability of derivatives cRel-D2 and cRel-D2N to activate transcription was due to their failure to enter the nucleus; the cRel-D2N derivative contains an insertion of the SV40 nuclear localization sequence, which has been shown to be sufficient to localize these proteins to the nucleus (Gilmore and Temin, 1988).

To confirm that these derivatives were stable in yeast, we constructed expression plasmids that produced comparable cRel derivatives with the LexA DNA-binding domain fused to their amino termini and measured the ability of these fusion proteins to stimulate LexA operator–containing target genes. We expected that such derivatives would activate gene expression strongly, since cRel's strong carboxyl-terminal activation domain (region II; Kamens et al., 1990) was not disturbed by the mutations. Surprisingly, Lex87cRel-D2 and Lex87cRel-D2N did not activate expression of reporter genes containing LexA-binding sites (Fig. 2).

The LexA DNA-binding region used in this study does not contain LexA's carboxyl-terminal dimerization domain. Since LexA fusion proteins apparently

Construct	Target	Units of β-galactosidase
cRel	Igκ-3	580
cRel-D1	Igκ-3	320
cRel-D2	Igκ-3	1
cRel-D2N	Igκ-3	1
Lex87cRel	Lexop-2	720
Lex87cRel-D1	Lexop-2	750
Lex87cRel-D2	Lexop-2	4
Lex87cRel-D2N	Lexop-2	30
Lex202cRel	Lexop-2	930
Lex202cRel-D1	Lexop-2	1080
Lex202cRel-D2	Lexop-2	590
Lex202cRel-D2N	Lexop-2	860

Figure 2. Gene activation by mutant derivatives of cRel.

The indicated deletion and insertion derivatives of cRel, Lex87cRel, and Lex202cRel were introduced into yeast cells containing the appropriate reporter plasmids. The Lex operator reporter construct is on plasmid pJK101, which contains two LexA-binding sites (Kamens et al., 1990). Igκ-3 contains binding sites for native cRel (Table 1). β-galactosidase activity was measured, and the results are expressed as units of β-galactosidase. Background levels of β-galactosidase activity for pJK101 and pIgκ-3 in this experiment were 1 and 2 units, respectively. Grey areas denote the region of sequence similarity between Rel, Dorsal, and NF-κB proteins. Striped areas represent sequences derived from the bacterial LexA protein. Black areas denote the 15–amino acid insertion containing the SV40 nuclear localization sequence (Gilmore and Temin, 1988). D2 and D2N derivatives are from non-transforming vRel mutants and D1 derivatives are from a transforming vRel mutant (Gilmore and Temin, 1986; Kamens et al., 1990). The dimerization and transcription activation functions of the different segments of the fusion proteins are described in the Results and Discussion section.

bind DNA as dimers, we hypothesized that the reason these derivatives of cRel did not activate gene expression was that they did not dimerize and therefore could not bind DNA (Brent and Ptashne, 1985; Little and Mount, 1982; Ruden et al., 1991; Schnarr et al., 1985; Schnarr et al., 1988). To test this hypothesis we used expression plasmids that produced proteins composed of the cRel deletion derivatives fused to full length LexA. These fusion proteins, Lex202cRel-D1, Lex202cRel-D2, and Lex202cRel-D2N, all activated transcription (Fig. 2). This fact suggests that deletion of amino acids 264 to 322 in the conserved domain of cRel disturbs a dimerization motif whose function can be replaced by the LexA carboxyl terminus.

Nolan et al. (1991) have observed that unlike the NF-κB p50 subunit, the full-length product of the NF-κB p65 protein does not efficiently bind DNA. Since p65 apparently contains a DNA-binding domain (truncated versions of p65 do bind efficiently), they suggest that p65 does not bind DNA because it is unable to form homodimers. Since p65 is more homologous to cRel than to NF-κB p50 (73% versus 56% in the Rel homology region), Nolan et al. also suggest that cRel is similarly unable to form homodimers, but might bind DNA only by complexing with another Rel protein, for example, p50. Our results suggest that cRel does homodimerize and that, in our system, cRel is able to bind DNA only when it forms dimers.

CONCLUSIONS

Site Recognition

These experiments have shown that vRel and cRel recognize at least two sites recognized by NF-κB and another site in the *zen* promoter that closely matches the NF-κB consensus binding site. Dorsal does not recognize NF-κB–binding sites, but does recognize sites in the *zen* promoter with sequences that that are somewhat similar to the NF-κB consensus sequence. This suggests that the proteins in the Rel family, like the homeodomain proteins, recognize a related set of binding sites (Hanes and Brent, 1991). Our initial experiments suggested that NF-κB, vRel, and cRel might prefer T at position 6 of the consensus sequence and that Dorsal might prefer A. Our results suggest that this is true, but that this base alone is not sufficient to determine site identity. Additional studies will be

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necessary to show what other bases influence binding specificity, which amino acid residues are responsible for this specificity, and which larger regions of the protein are required for DNA binding.

The yeast transcription assay for DNA binding has some advantages over the in vitro approaches commonly employed. First, DNA binding takes place inside a living cell, at physiological pH and salt concentration, and in the presence of nonspecific DNA (Hanes and Brent, 1989; Hanes and Brent, 1991). Second, binding is typically measured at protein concentrations higher than those used in vitro, but similar to those found in the cells of higher eukaryotes (E. Golemis, unpublished results). Transcription activation also has some disadvantages compared with in vitro assays. First, it measures a complex phenotype that depends on entry of proteins into the nucleus, on protein stability, and often on proper oligomerization; in order to draw meaningful conclusions about DNA specificity, these variables must be carefully controlled. Second, presumably because *S. cerevisiae* lacks modifying proteins found in higher eukaryotes, proteins that are negative regulators in higher eukaryotes are sometimes activators in yeast (Samson et al., 1989; see Results and Discussion). In the future, we anticipate actually taking advantage of these discrepancies to isolate higher eukaryotic proteins responsible for such differences in regulatory activity.

Dimerization

We demonstrate here that two cRel derivatives fused to the DNA-binding domain of LexA (which does not contain a dimerization function) do not bind to LexA sites; however, when these derivatives are fused to the entire LexA protein (which does contain a dimerization function) they bind to LexA sites and activate transcription. Studies of DNA binding by Rel family proteins in gel shift assays have shown that non-transforming derivatives of vRel (i.e., those that have small deletions in the Rel homology domain) do not bind NF-κB sites in vitro (Ballard et al., 1990). We have shown, using a transcription activation assay, that comparable deletion derivatives of cRel do not bind to NF-κB sites in vivo. We then show that these deletion derivatives cannot activate transcription when fused to the LexA DNA binding domain, but do activate transcription when fused to a LexA protein that contains the dimerization region. Our results suggest that Rel proteins must dimerize to recognize their native binding sites and that the lesions in the conserved domain affect dimerization, raising the possibility that lesions in this region may not affect amino acids involved in contacting the DNA. Similar comparisons of transcription activation by Lex87 and Lex202 fusion proteins might help distinguish the DNA-binding and dimerization functions in other proteins.

cRel is expressed primarily in cells of hematopoietic origin, and NF-κB exerts its effect in these same cells, which raises the possibility that these proteins might affect one another's functions (Lenardo et al., 1988; Moore and Bose, 1989). Taken together with previous results, our study suggests at least two ways in which such interactions might occur. First, cRel might form mixed dimers with one or both of the NF-κB subunits, and these heterodimers might have altered regulatory properties (Kieran et al., 1990). Second, cRel and NF-κB might interfere with one another's function by competing for regulatory sites upstream of target genes; such competition for sites between cRel homodimers and NF-κB heterodimers might occur normally in cells where both proteins are expressed and be necessary for the correct regulation of target genes. Since vRel also exerts its effects in lymphoid cells and also recognizes NF-κB sites, transformation by vRel might involve either of these two mechanisms.

Cooperativity

Our results show that there is a more than additive increase in gene activation by vRel, cRel, and Dorsal of reporter genes with increasing numbers of binding sites. This fact suggests that vRel, cRel, and Dorsal exhibit either cooperative DNA binding or cooperative gene activation when bound to DNA ("synergy"; see Carey et al., 1990; Ptashne, 1988). This cooperative gene activation may be important for the function of the Rel family proteins in higher cells. In particular, cooperative activation by Dorsal may well explain how the somewhat shallow longitudinal Dorsal gradient is translated into the observed sharp boundaries of zygotic target gene expression, thus helping to establish the correct embryonic dorsal/ventral polarity.

MATERIALS AND METHODS

Strains, Media, and β-Galactosidase Assays

Bacteriological procedures were performed by standard techniques with *Escherichia coli* strain SCS1 [F- *endA1 hsdR17* ($r_K^- m_K^+$) *supE44 thi-1 λ-recA1 gyrA96 relA1*] (Stratagene). *S. cerevisiae* strain BJ2168 (a *leu2 trp1 ura3-52 pep4-3 prb1-122 prc1-407*) (Zubenko et al., 1980) was used in all experiments involving yeast. Yeast cells were grown in complete minimal medium containing 2% glucose but lacking uracil and tryptophan, and transformations were done using the lithium acetate procedure. β-galactosidase assays of liquid cultures were carried out as previously described (Hanes and Brent, 1989; Harshman et al., 1988; Sherman et al., 1978). Units of β-galactosidase activity are calculated as $[1000(A_{420})]/[OD_{600} \times \text{time} \times \text{volume}]$ where time is measured in minutes and volume, in milliliters. At least three individual yeast transformants were assayed for each sample.

Values greater than 9 units were rounded to the nearest multiple of 10 units. Standard deviations (SDs), expressed as a percentage of the mean values, were as follows: For values less than 1, SDs did not exceed 30% of the mean. For values greater than 1 and less than 10, SDs did not exceed 30% of the mean except in two cases: For cRel on the Zen3-2 target the SD was 43% of the mean, and for Dorsal on the IL2-4 site the SD was 37% of the mean. For values greater than 10 and less than 100, SDs did not exceed 26% of the mean and most were less than 20%. For values greater that 100, SDs did not exceed 20% of the mean.

Construction of Reporter Plasmids

All DNA manipulations were carried out by standard methods (Ausubel et al., 1987). Reporter plasmids all contain a *URA3* gene, a *GAL1-lacZ* fusion gene, and a 2 μm replicator and were constructed by inserting multiple copies of double-stranded synthetic oligonucleotides into the unique XhoI site located 167 base pairs (bp) upstream of the *GAL1-lacZ* reporter gene transcription start site in plasmid pLR1Δ1 (West et al., 1984). Top-strand oligonucleotide sequences were: Igκ, 5′-TCGAGCAGAGGGGACTTTCCGAGAGC-3′; IL-2, 5′-TCGAGAAGAGGGATTTCACCTACAT-3′; Rα, 5′-TCGAGCAGAGGGGAATCTCCCAGAGC-3′; Zen1, 5′-TCGACTGGGAAAACCAA-3′; Zen2, 5′-TCGACTGGGAGAAACCC-3′; Zen3, 5′-TCGATTGGGAAATCCAG-3′; IgκA, 5′-TCGAGCAGAGGGGACATTCCGAGAGC-3′; Zen2T, 5′-TCGACTGGGAGTAACCC-3′. Note that the orientation of the Zen2 oligonucleotide has been reversed in reference to its orientation in the *zen* promoter to make apparent its alignment with the other sequences (Fig. 1). The number and orientation of inserts is as shown in Table 2 or as follows: IgκA-2 contains two inserts, both in the reverse orientation; IgκA-3 contains three inserts, all in the forward orientation; Zen2T-2 contains two inserts, the first in reverse orientation and the second in the forward orientation; Zen2T-3 contains three inserts with the same orientations as the inserts in pZen1-3 (Table 2).

To construct pZenF and pZenR reporter plasmids, a 285-bp fragment that was shown by Doyle et al. to be important for repression of the *zen* gene in ventral regions of the blastoderm (the ventral repression element; Doyle et al., 1989) was isolated from plasmid car20, which contains 600 bp of the *zen* promoter. This blunt-ended XmnI to PvuII fragment was inserted into XhoI-cut, Klenow enzyme–treated pLR1Δ20B, which contains an XhoI site located 128 bp upstream of the *GAL1-lacZ* reporter gene. pZenF has the XmnI-PvuII fragment inserted in pLR1Δ20B in the same orientation in which it is found in the *zen* promoter. In pZenR the orientation is reversed.

pJK101 is a derivative of pLR1Δ20B with LexA-binding sites inserted in the XhoI site (Kamens et al., 1990). All insert constructions were confirmed by sequence analysis.

Construction of Expression Plasmids

Plasmids directing the synthesis of native vRel, cRel, and Dorsal proteins were constructed as follows: pJKvRel, a gift of P. Richardson, was constructed by inserting an XbaI-XbaI fragment containing the entire v-*rel* gene into the EcoRI site of pBC102, which had been treated with Klenow enzyme to generate blunt ends. pBC102 contains a unique EcoRI site flanked by the yeast *ADH1* promoter and terminator, a *TRP1* gene, and *CEN/ARS* sequences to allow single-copy maintenance in yeast cells (Kamens et al., 1990). pJKcRel was constructed by isolating a SmaI-KpnI fragment from p304cRel, treating it with T4 DNA polymerase, and inserting it into EcoRI-cut, Klenow enzyme–treated pBC102. p304cRel contains the full length c-*rel* gene inserted into a plasmid containing a Bluescript pSK+ polylinker (Stratagene). The first ATG of the gene is located just after a SmaI restriction site. Treatment of the SmaI-KpnI fragment with T4 DNA polymerase led to the deletion of two base pairs at the amino-terminus of the fragment, leaving a 5′-CATG-3′ flush end to fuse with the filled-in EcoRI end of pBC102. pJKDsl was constructed by inserting an SspI-SalI fragment from pJD214-Dsl containing the entire *dorsal* gene into the polylinker of pJK304TPI at the SmaI and SalI sites. pJK304TPI is a 2 μm–based plasmid containing a *TRP1* gene and the yeast triosephosphate isomerase (TPI) gene promoter followed by a polylinker (V. MacKay, Zymogenetics, Inc.). The *dorsal* gene was expressed from this very strong promoter on a high copy number plasmid because Dorsal is unstable in yeast and we wished to produce as much of the protein as possible in the cell (Kamens et al., 1990; J. Kamens, unpublished results). vRel, cRel, and Dorsal proteins of the expected sizes were detected by immunoblot analysis of yeast cell extracts with anti-vRel or anti-Dorsal antiserum (not shown).

pLex87cRel was constructed by inserting a SmaI-KpnI fragment carrying the entire c-*rel* gene into pJK1521 that had been digested with SmaI and KpnI. pJK1521 is derived from pBC102 (see above) and contains a DNA fragment that codes for the 87–amino acid LexA DNA-binding domain, followed by restriction sites for SmaI and KpnI (Kamens et al., 1990). pLex202cRel was constructed by inserting a SmaI-SalI fragment from p304cRel into the polylinker of pLex202pl that had been cut with BamHI, treated with Klenow, and then cut with SalI. This treatment generated a fusion gene that codes for a protein containing all 202 amino acids of the LexA protein fused to the entire cRel protein. This fusion gene was subcloned into pJK1521, creating a plasmid identical to pLex87cRel except in the region between the codon for amino acid 87 of LexA and the codon for amino acid 1 of cRel. The sequence in this region, beginning with the last three base pairs of the LexA coding sequence, is 5′-CTGGAATTCCCGGGGATCGGGATG . . .-3′. The LexA-vRel and LexA-cRel fusion proteins did not activate expression of target genes that contained Igκ sites (Kamens et al., 1990).

To construct the deletion derivatives of cRel, ClaI-EspI fragments from vRel derivatives containing the indicated alterations were introduced into the *cRel, Lex87cRel,* and *Lex202cRel* genes in place of the corresponding wild-type ClaI-EspI fragment (Gilmore and Temin, 1986; Kamens et al., 1990). The cRel deletion derivatives correspond to the vRel mutants as follows: D1, dHae/Hinc (deletes amino acids 314-322 of cRel); D2, dStu/Hinc (deletes amino acids 264-322); D2N, TG20 (deletes amino acids 264-322 and has

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an insertion of 15 amino acids containing the SV40 nuclear localization sequence) (Gilmore and Temin, 1986).

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