for a large fraction of the catalytic advantage in this reaction (*15*), consistent with the classic notion of transition-state complementarity of Haldane (*16*) and Pauling (*17*).

This behavior is in contrast to that of an antibody-catalyzed transesterification reaction recently reported by Lerner and co-workers (6) (in which the antibody was raised to a negatively charged phosphonate monoester rather than a neutral phosphonate diester). This antibody-catalyzed reaction, which has a similar rate of acceleration and which also showed high specificity for the acyl acceptor versus water, proceeds through a ping-pong mechanism involving a covalent antibody · substrate complex. Thus, the tremendous diversity of the immune system has provided two mechanistic alternatives for similar acyl transfer reactions, in much the same way that enzymes have evolved with similar catalytic properties but different mechanisms (such as the acid, serine, and Zn^{2+} proteases). Further characterization of these catalytic antibodies should provide additional insight into the essential requirements for efficient acyl transfer catalysts as well as optimal hapten structures for generating such catalysts.

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15. From transition-state theory (*9, 23*)

$$\frac{K_{Ab}^{\neq}}{K_N^{\neq}} = \frac{K_A K_B}{K_T} = \frac{k_{Ab}}{k_N}$$

as defined in the scheme:

$$\begin{array}{ccc} & \overset{K_N^{\neq}}{} & \\ Ab+A+B & \rightleftarrows & [AB]^{\neq}+Ab \rightarrow P+Ab \\ \Updownarrow K_A K_B & & \Updownarrow K_T \\ & \overset{K_{Ab}^{\neq}}{} & \\ Ab{\cdot}A{\cdot}B & \rightleftarrows & [Ab{\cdot}A{\cdot}B]^{\neq} \rightarrow P{\cdot}Ab \end{array}$$

Substituting $K_m(3)$, $K_m(7)$, and $K_d(2)$ for K_A, K_B, and K_T, respectively, affords $K_A K_B / K_T \geq 834$ M, which can be compared with $k_{Ab}/k_N = 2.7 \times 10^4$ M [where $K_d(2)$ corresponds to the phenyl rather than the cyanomethyl leaving group and is therefore probably an upper limit].
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18. Hapten **1** was prepared as follows: β-alanine benzyl ester was treated with phosgene to produce the isocyanate. This was combined with thymidine, and the 5′-acylated product was isolated by silica-gel chromatography (3% methanol–methylene chloride). Phenethyl alcohol was treated with phosgene to yield the chloroformate, which was quenched in aqueous ammonia. The resulting carbamate was combined with acetaldehyde and triphenylphosphite in acetic acid and heated to 90°C for 1 hour. The racemic diphenyl phosphonate was purified by recrystallization from methanol and hydrolyzed to the monolithium salt with 0.95 equivalent of lithium hydroxide in *N,N*-dimethyl formamide–H_2O. Reaction with thionyl chloride produced the phosphonyl chloride, which was combined with the thymidine derivative and one equivalent of triethylamine. The product was purified by silica-gel chromatography (3% methanol–methylene chloride), and the benzyl ester was cleaved by hydrogenolysis with 10% palladium hydroxide on carbon as a catalyst to yield hapten **1**. Carrier protein conjugates were prepared from the *N*-hydroxysuccinimide ester of hapten **1**. Epitope densities of 11 (KLH) and 12 (BSA) were determined by ultraviolet (UV) absorption (270 nm); protein concentrations were determined by the method of Lowry *et al.* (*19*). Allyl amine was coupled to hapten **1** with dicyclohexylcarbodiimide (DCC) and subsequently reduced with tritium gas to yield radiolabeled derivative **2**. Substrate **3** was prepared by treatment of thymidine with ethyl isocyanate in pyridine. The 5′-acylated product was isolated by silica-gel chromatography (5% methanol–methylene chloride). L-Alanine and D-alanine were treated with phenethyl chloroformate to yield the parent acids of compounds **4** through **11**. We prepared compounds **4**, **5**, **6**, **9**, **10**, and **11** by coupling the appropriate parent acid with the appropriate alcohol, using DCC and 4-dimethylaminopyridine. Esters **7** and **8** were prepared from the L and D parent acids by reaction with excess chloroacetonitrile. Compounds **4** through **11** were purified by silica-gel chromatography (**4** through **9**: 50% ethyl acetate–hexane; **10** and **11**: 5% methanol–methylene chloride). All compounds were characterized by nuclear magnetic resonance spectroscopy and mass spectroscopy.
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8 January 1992; accepted 2 March 1992

Identification of a Naturally Occurring Transforming Variant of the p65 Subunit of NF-κB

Ramaswamy Narayanan, John F. Klement, Steven M. Ruben, Kimberly A. Higgins, Craig A. Rosen*

Transcription factor NF-κB comprises two proteins, p50 and p65, that have sequence similarity to the v-*rel* oncogene. In primary hematopoietic cell populations an alternatively spliced form of NF-κB p65 mRNA was observed that encoded a protein designated p65Δ. Expression of the p65Δ cDNA in Rat-1 fibroblasts resulted in focus formation, anchorage-independent growth in soft agar, and tumor formation in athymic nude mice, effects not obtained with expression of p65 or a p65Δ mutant that contains a disruption within the transcriptional activation domain. Thus, p65Δ, which associated weakly and interfered with DNA binding by p65, may sequester an essential limiting regulatory factor or factors required for NF-κB function.

The NF-κB transcription factor complex contains the proteins p65 and p50 and participates in the induction of numerous cellular and viral genes (*1*). Both proteins are members of the *rel* family of proteins (*2, 3*). The v-*rel* oncogene causes lymphoid cell tumors in young birds (*4*). The ability either to activate or repress transcription is a feature shared by each of the known *rel*-related family members (*5*). Proteins in the *rel* family interact with DNA after dimerization through regions within the *rel* conserved domain (*2, 3, 6*).

Amino acids 222 to 231 in human NF-κB p65 are required for association of p65 with p50 and for DNA binding (6). We identified an alternatively spliced form of p65 mRNA designated p65Δ, which lacks nucleotides (nt) that encode amino acid residues 222 to 231. The prevalence of p65Δ mRNA in certain cell lineages was

R. Narayanan and K. A. Higgins, Department of Molecular Genetics, Hoffmann–La Roche, Inc., Nutley, NJ 07110.
J. F. Klement, S. M. Ruben, C. A. Rosen, Department of Gene Regulation, Roche Institute of Molecular Biology, 340 Kingsland Street, Nutley, NJ 07110.

*To whom correspondence should be addressed.

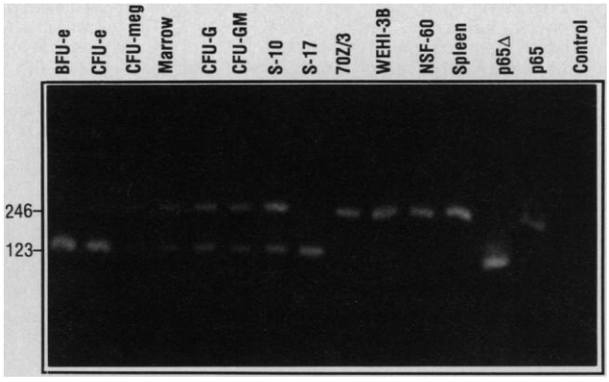

Fig. 1. PCR analysis of p65 and p65Δ expression in RNA from erythroid colonies (BFU-e, CFU-e, and CFU-meg), murine primary bone marrow, myeloid colonies (CFU-G and CFU-GM), lymphoid colonies (S-10 and S-17, pre-B), lymphoid cell lines (70Z/3, WEHI-3B, and NSF-60) (*16*), and normal mouse spleen cells. Samples were subjected to electrophoresis on 3% agarose gels. Molecular size markers are indicated at the left in base pairs. PCR products corresponding to p65 and p65Δ contain 150 and 180 nt, respectively. Control, PCR reaction without cDNA addition.

suggestive of a potential physiologic function. To explore this possibility further, we examined the relative abundance of p65Δ mRNA in primary hematopoietic progenitor cell populations derived from murine primary bone marrow (*7–9*). Myeloid, erythroid, and lymphoid colonies were established, and the presence of p65Δ mRNA was identified by polymerase chain reaction (PCR) and with primers that discriminate between p65 and p65Δ (*10*). The relative amount of p65Δ and p65 transcripts was variable with respect to the individual cell types and stage of differentiation (Fig. 1). The overabundance of p65Δ transcripts in the S17, pre-B, and erythroid colonies, to the near exclusion of p65, suggests that recognition of the alternate splice acceptor used to generate p65Δ is a regulated event.

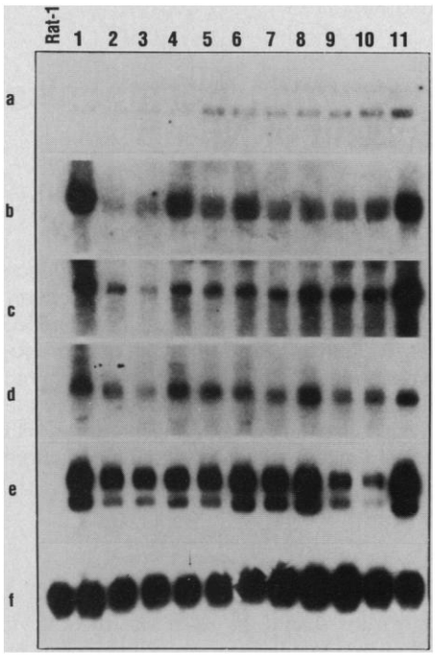

Fig. 2. Expression profile of p65Δ and immediate-early genes in the Rat-1–derived foci. Total RNA (15 μg) from 11 independent p65Δ foci and Rat-1 cells was isolated with RNAzol B (Cinna Biotex, Houston, Texas) and analyzed for expression of (a) p65, (b) *jun*-B, (c) c-*fos*, (d) *egr*-1, (e) c-*jun*, and (f) glyceraldehyde 3-phosphate dehydrogenase (GADPH) by Northern blot analysis (*17*). For detection of human p65Δ expression in Rat-1 cells, we hybridized blots at high stringency (65°C) to avoid cross-hybridization with endogenous Rat p65.

To begin to explore the physiological function of p65Δ, we examined its ability to elicit transformation. The similarity between p65 and v-*rel* provided a precedent for this type of analysis (*2, 3*). Rat-1 cells (*11*) were chosen for the transformation assay because they have a low background of spontaneous foci and do not express a detectable amount of p65Δ RNA. Cells were transfected with an expression vector that contained either human p65Δ or p65 cDNA under control of the cytomegalovirus (CMV) immediate-early promoter (6). After 30 days, foci were evident in monolayers transfected with p65Δ (Table 1). In contrast, expression of wild-type p65 did not result in focus formation (Table 1). Because anchorage-independent growth is a reliable indicator for transformation, 11 of the foci were picked, expanded, and plated in soft agar. Each of the 11 clones formed colonies in soft agar (Table 1), which confirmed their transformed phenotype. The altered growth rate of these foci was also shown by the two- to threefold increase in their saturation density as well as by a decrease in doubling time (Table 1). The morphology of the foci-derived cell lines was, however, indistinguishable from that of the parental Rat-1 cells. Integrated sequences and expression of exogenous p65Δ cDNA were detected in these foci by PCR with primers that distinguish endogenous p65 and p65Δ (*10*). The amount of p65Δ expression was further examined by Northern (RNA) blot analysis (Fig. 2). Consistent with the PCR results, each focus exhibited substantial p65Δ mRNA.

We also examined the amounts of mRNAs encoding various oncogenes whose expression often correlates with altered cell growth. Several members of the immediate-early gene family, including c-*fos*, c-*jun*, *jun*-B, and *egr*-1, showed marked elevation in each of the 11 p65Δ-induced foci (Fig. 2). Expression of another oncogene product, c-*myc*, was not elevated in these foci (*12*). The tumorigenic properties of the p65Δ-expressing Rat-1 cells were examined by subcutaneous injection of these cells into athymic nude mice (Table 1). Within 4 weeks, tumors were evident in mice with the transformed p65Δ Rat-1 clones. No tumors were found in mice injected with Rat-1 cells or cells expressing wild-type p65.

Antibodies (Abs) that distinguish p65Δ from p65 are not yet available. Therefore, an expression vector that contained p65Δ cDNA with an epitope tag of nine amino acids corresponding to an antigenic epitope in the influenza hemaglutinin protein (HA) (6) was constructed (pCMVINHAp65Δ). This epitope is recognized by a mouse monoclonal antibody to HA (anti-HA) (*13*). Rat-1 cells expressing HAp65Δ

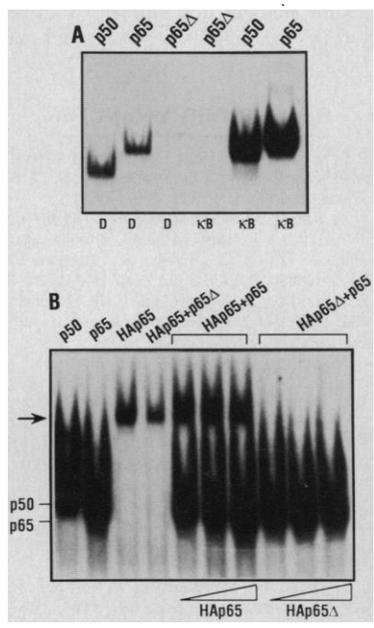

Fig. 3. The inability of p65Δ to associate with DNA. (**A**) Purified *Escherichia coli* p50 (amino acids 1 through 377) (*6*), p65 (1 through 309) (*6*), and p65Δ (1 through 299) (*6*) were incubated with a ^{32}P-labeled κB DNA probe (κB) or a degenerate DNA probe that contained 18 randomly substituted nucleotides (D) in an electrophoretic mobility-shift assay (*18*). (**B**) Electrophoretic mobility-shift assays were performed as in (A), except that the binding reactions contained a mixture of purified p65 or p65Δ with purified HAp65 or HAp65Δ. All reactions were incubated in the presence of anti-HA (*13*). The DNA bound to complexes that contained HA-tagged protein appeared as a supershifted complex in the gel (arrow). Triangles depict addition of an increasing amount of purified HAp65 or HAp65Δ protein.

Table 1. Biological characterization of Rat-1 fibroblasts transfected with p65Δ and p65. Doubling times were determined from the linear portion of growth curves. Saturation density was measured as the maximum number of cells obtained after 4 × 10^3 cells per centimeter squared were incubated in growth medium that was changed every 3 days. For anchorage-independence assays, cell suspensions (1 × 10^3 cells per milliliter) were plated in agar (0.33%) in growth media, and visible macroscopic colonies were scored at day 18. For the nude mice tumorigenicity assay, 1 × 10^6 cells were injected subcutaneously into BALB/c nude mice. Tumors were evaluated after 6 weeks. Rat-1 cells were transfected by the calcium phosphate precipitation method (*21*) with 5 μg of plasmid DNA encoding p65, p65Δ, or p65Δ-ΔL442 (*6*). Foci were scored at 4 weeks, subcloned by dilution, and propagated for further analysis. The p65Δ Neo^R transfectants were obtained by cotransfection of pCMVINHAp65Δ (10 μg) with plasmid RSVneo (1 μg) and 48 hours later were plated in media containing G418 (800 μg/ml). Resistant colonies were picked at day 14 and expanded for further analysis. Parental Rat-1 cells are provided for comparison. ND, not done.

Transfection	Number of foci*				Anchorage-independent growth (%)†	Saturation density (10^6/cm^2)†	Doubling time (hours)†	Tumorigenicity†
	1	2	3	4				
p65Δ	14	12	13	18	3.5 ± 0.2 (n = 2)	2.9 ± 0.3 (n = 2)	18–19	12/13
p65	0	0	0	0	ND	ND	ND	0/6
p65Δ-ΔL442	0	0	0	ND	ND	ND	ND	ND
ras	43	39	36	41	6.4 ± 0.5 (n = 8)	7.6 ± 0.4 (n = 2)	13	3/3
Rat-1	0	0	0	0	<0.1 ± 0.02 (n = 4)	1.02 ± 0.1 (n = 2)	24	0/6

*Number of foci obtained per microgram of DNA per 10^6 cells, in four separate experiments. †Four independent foci of p65Δ were analyzed, including one $G418^R$ colony.

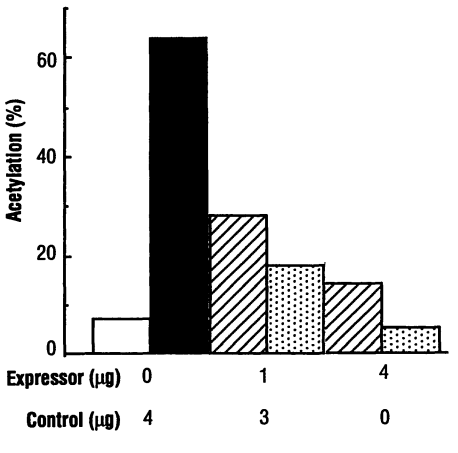

Fig. 4. Expression of p65Δ suppresses NF-κB function. Jurkat T cells were transfected with either a CAT reporter plasmid driven by a synthetic sequence containing four copies of the κB motif upstream of an SV40 promoter (*19*) alone, or with CMV-p65Δ or CMV-p65 amino acids (1 through 309) and stimulated with phorbol 12-myristate 13-acetate (PMA) (5 ng/ml) 30 hours after transfection. Cells were harvested at 40 hours after transfection for CAT assays. In each transfection, we kept the amount of CMV promoter constant to avoid results reflecting competition for transcription factors. Plasmid CMV-p65 (1 through 309) encodes the DNA binding domain of p65 [lacks amino acids 310 through 551 which encompass the activation domain (*6, 20*)] and thus suppresses NF-κB function through competition for κB binding sites. Open bar, cells transfected with the HκB4 CAT reporter alone; solid bar, with PMA activation; hatched bars, with PMA activation plus CMV-p65Δ; dotted bars, CMV-p65 (1 through 309). A typical result is shown. Three independent transfections were performed with absolute values differing by no more than 20%.

mRNA produced HAp65Δ protein [as shown by immunoprecipitation (*14*)], demonstrated anchorage-independent growth in soft agar, and formed tumors in nude mice (Table 1), consistent with the transforming capability of p65Δ.

The p65Δ protein is unable to associate with p50 or interact with DNA that contains an NF-κB binding site (6). However, it remained possible that p65Δ interacts with a DNA motif other than the canonical κB element. To examine this possibility, we prepared a degenerate oligonucleotide randomly substituted for a length of 18 nt. Purified p65 bound to labeled random oligonucleotides in an electrophoretic mobility-shift assay (Fig. 3A). However, no association of the DNA with p65Δ was evident. The ability of p65-p65Δ complexes to associate with κB DNA was also examined. Purified HAp65 and HAp65Δ were renatured individually with p65, and the resulting complexes were assayed for DNA binding in the presence of anti-HA with the electrophoretic mobility-shift assay (Fig. 3B). Although addition of the anti-HA caused a supershift of HAp65-p65 bound to κB DNA, no supershift was evident in binding reactions that contained heterodimers of HAp65Δ and p65.

Our study demonstrates that a naturally occurring variant of p65, p65Δ, can elicit changes in cell growth. Although results obtained with an in vitro transformation assay must always be viewed with caution, we propose that p65Δ functions similarly in its natural environment. The apparent overabundance of p65Δ transcripts in primary as opposed to continuous cell lines may provide insight to p65Δ function. The amount of p65Δ transcripts was highest in cells before terminal differentiation; therefore, p65Δ may permit unrestricted cell expansion, consistent with the finding that its unregulated expression can elicit transformation. We speculate that p65Δ, which cannot associate with κB DNA but contains a functional activation domain (6), may affect cell growth by disrupting NF-κB function. Two models, neither mutually exclusive, are consistent with this hypothesis. In the first model, p65Δ associates with p65, albeit weakly, and prevents DNA binding. In the second model, p65Δ may contain a domain that interacts with and sequesters an essential limiting regulatory factor required for NF-κB function. In support of the latter, a p65Δ protein that contained a mutation within the putative activation domain (6) failed to transform Rat-1 cells (mutant p65Δ-ΔL442) (6) (Table 1). Furthermore, the ability of p65Δ expression to suppress endogenous NF-κB activity after mitogen stimulation of Jurkat T lymphocytes, as demonstrated by the reduction in activity of a transfected κB choline acetyltransferase (CAT) reporter plasmid, provides direct evidence for the ability of p65Δ to interfere with NF-κB function (Fig. 4).

Our findings suggest that NF-κB p65 may regulate a set of genes whose expression prevents uncontrolled growth. This possibility is consistent with studies that show v-*rel* also inhibits NF-κB activity (*15*); how similar the functions of p65Δ and v-*rel* are depends on their molecular targets. Although we have shown that p65Δ can form inactive heterodimers with p65, p65Δ may also associate with a different *rel*-related protein, which may elicit transformation and altered cell growth properties.

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10. Total RNA (1 μg) from cell lines including mouse myeloid leukemia (NSF-60 and WEHI-3B), pre-B (70Z/3), normal mouse spleen, and primary mouse bone marrow, as well as total RNA from individual progenitor myeloid colonies (CFU-G and CFU-GM), erythroid colonies (*9*) (BFU-e, CFU-e, and CFU-meg), and lymphoid pre-B colonies (*8*) (S-10 and S-17) was reverse-transcribed with random hexamers (*9*), and one-tenth of the reaction mixture was used for PCR amplification with primers 5′-GCGGCCAAGCTTCAAGATCTGCCGAGTT-3′ (sense) and 5′-CGCTGCTCTAGAACACAATGGCCACTTGCCG-3′ (antisense) spanning the exons surrounding the p65 deletion. Amplification cycles were 94°C for 1 min, 58°C for 2 min, and 72°C for 3 min for 45 cycles on a thermocycler (Perkin-Elmer). One-fifth of the reaction was analyzed by agarose (3%) gel electrophoresis and visualized by staining with ethidium bromide.
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16. The S-10 and S-17 lymphoid colonies were obtained by the addition of stromal cell conditioned medium to Whitlock-Witte cultures (*7*). Factors from the S-17 cell line potentiate the growth of colonies in which cells progress up to various stages of development. Some of the cells in S-10 pre-B colonies express surface immunoglobulin M (*8*).
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18. DNA binding reactions were carried out as described (*6*) with the addition of dithiothreitol (1 mM) and NP-40 (0.1%) to the binding buffer [10 mM Hepes (pH 7.9), 50 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, and 20% glycerol]. A bacterial protein (~20 ng) and a ^{32}P-labeled oligonucleotide probe (1.5 ng; 500,000 cpm) were used in the binding reactions. Nondenaturing gels (4%) were subjected to electrophoresis at 4°C in tris-borate buffer (0.5 × TBE buffer) for 2 hours. The κB DNA probe used in the binding reactions contained the sequence 5′-GGATCCTCAACAGAGGGGACTTTCCAGGCCA-3′, which corresponds to the κB motif present in the immunoglobulin light chain enhancer. The sequence of the degenerate primer was 5′-TGCCTAGAGGATCCGTGAC(X)$_{18}$CGGAATTCCTTAAGAAGCTTCCGAGCG-3′, where X equals A, G, C, or T. For labeling the degenerate oligonucleotide, a primer complementary to the 3′ sequence (5′-CGCTCGGAAGCTTGAATTC-3′) was annealed to the random oligonucleotide. The primer was then extended with DNA polymerase (Klenow fragment) and 40 μCi of each ^{32}P-labeled deoxynucleotide triphosphate (dNTP), and after extension of the primer, we added 250 μM unlabeled dNTPs to ensure complete synthesis. For (B), we renatured together purified proteins by first diluting each with 6 M guanidine-HCl (1:10) and then mixing the two denatured proteins in the ratio of 1:1, 1:5, and 1:20 before dialysis (*6*).
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19 November 1991; accepted 27 January 1992

Calcium-Regulated Phosphorylation Within the Leucine Zipper of C/EBPβ

Michael Wegner, Zhaodan Cao, Michael G. Rosenfeld*

Alterations in intracellular calcium levels activate several signal transduction pathways resulting in distinct patterns of gene expression. Here, a pathway for calcium-mediated signals is demonstrated that involves C/EBPβ, a member of the bZip family of transcription factors. In pituitary cells C/EBPβ was phosphorylated in response to increased intracellular calcium concentrations as a consequence of the activation of a calcium-calmodulin–dependent protein kinase. Phosphorylation of serine at position 276 within the leucine zipper of C/EBPβ appeared to confer calcium-regulated transcriptional stimulation of a promoter that contained binding sites for C/EBPβ.

Many regulatory molecules that function by binding to plasma membrane receptors cause changes in the intracellular Ca^{2+} concentration (*1*). This second messenger can modulate the expression of target genes by effecting changes in the phosphorylation status of specific transcription factors. Many Ca^{2+}-mediated changes in gene expression have been attributed to the phosphorylation of transcription factors by protein kinase C (*2*). However, fluctuations in intracellular Ca^{2+} concentrations can also activate Ca^{2+}-calmodulin–dependent kinases (*3*, *4*), the most studied of which is the multifunctional Ca^{2+}-calmodulin–dependent protein kinase II (CaMKII) (*5*) isozymes, which are expressed in most tissues (*6*).

In transient transfections of a pituitary cell line (G/C), the DNA sequence motif 5′-AAATGTAGTCTTATGCAATACACTTGTAGTCTTGCAACA-3′ rendered a reporter gene responsive to a constitutively active mutant of the brain-specific α-subunit of CaMKII (*4*). Critical positions within this CaMKII responsive element (CaMRE) coincide with binding sites for the nuclear factor C/EBP (*7*) (Fig. 1A). We hypothesized that CaMKII might exert its stimulation either directly or indirectly through a member of the C/EBP family of transcription factors (*8*). Members of this family belong to the bZip class of transcription factors. These factors contain a basic DNA-binding region adjacent to a leucine zipper dimerization domain and can form homodimers or heterodimers with other bZip proteins (*9*–*12*).

Electrophoretic mobility shift experiments with a ^{32}P-labeled CaMRE and G/C nuclear extract yielded four complexes (C1 to C4) (*4*) (Fig. 1B), all of which displayed the same methylation interference pattern (*13*) (Fig. 1A). This pattern was also identical to the one obtained with bacterially produced recombinant C/EBPα. Antisera to known members (α, β, and δ) of the C/EBP family (*9*, *10*) were used to establish the identity of the CaMRE binding activity in G/C nuclear extracts (Fig. 1B). Only the antiserum to C/EBPβ reacted specifically with the complexes formed between G/C nuclear extract and the CaMRE. Whereas low concentrations of antiserum caused the formation of new complexes with lower mobility, higher concentrations of the C/EBPβ-specific antiserum resulted largely in the elimination of the G/C cell–specific complexes (Fig. 1C). The observation that complexes C1 to C4 were equally affected by the C/EBPβ-specific antiserum suggests that C/EBPβ is present in all of these complexes and, as the major CaMRE binding protein in G/C cells, is a potential target for CaMKII.

To assess the ability of C/EBPβ to be phosphorylated, we performed immunoprecipitations on nuclear extracts prepared from G/C cells labeled in vivo with [^{32}P]orthophosphate (*14*) (Fig. 2A). The amount of phosphorylated C/EBPβ was 2.5-fold greater in untreated G/C cells than in cells treated with the CaMKII inhibitor KN-62. KN-62 competitively inhibits the binding of calmodulin to CaMKII by interacting with the calmodulin binding site and thus inactivates the enzyme (*15*). The amount of phosphorylation of C/EBPβ was

M. Wegner and M. G. Rosenfeld, Eukaryotic Regulatory Biology Program and Howard Hughes Medical Institute, Cellular and Molecular Medicine Building, University of California, San Diego, La Jolla, CA 92093–0648.
Z. Cao, Department of Embryology, Howard Hughes Research Laboratories, Carnegie Institution of Washington, Baltimore, MD 21210.
*To whom correspondence should be addressed.