REFERENCES AND NOTES

1. J. A. Habeshaw and A. Dalgleish, *J. Acquired Immune Defic. Syndrome* **2**, 457 (1989).
2. G. M. Shearer, *N. Engl. J. Med.* **308**, 223 (1983).
3. J. L. Ziegler and D. P. Stites, *Clin. Immunol. Immunopathol.* **41**, 305 (1986).
4. J. M. Andrieu *et al.*, *AIDS Res.* **2**, 163 (1986).
5. R. B. Forsyth and G. W. Hoffmann, *J. Immunol.* **145**, 215 (1990).
6. D.-W. Hsu *et al.*, *Int. Immunol.* **1**, 197 (1989).
7. G. W. Hoffmann *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3060 (1991).
8. G. W. Hoffmann *et al.*, *J. Immunol.* **137**, 61 (1986).
9. D. C. Diamond *et al.*, *ibid.* **141**, 3715 (1989); K. J. Weinhold *et al.*, *ibid.* **142**, 3091 (1989); Y. Thomas *et al.*, *Immunol. Rev.* **74**, 113 (1983); Q. J. Sattentau, *Science* **234**, 1120 (1986); L. K. Clayton *et al.*, Nature **339**, 548 (1989); D. Lamarre *et al.*, *Science* **245**, 743 (1989).
10. One question that might be raised is whether there is overlap between the CD4 binding site on class II MHC and the T cell receptor binding site on class II MHC. The experiments that localize the interaction of the T cell receptor with class II MHC exclusively to the neighborhood of the cleft on the MHC molecules are the same ones that are concerned with the interaction of T cell receptors with peptides in the clefts. We have questioned the conventional absolute interpretation of such data [G. W. Hoffmann and M. D. Grant, *Lect. Notes Biomath.* **83**, 386 (1989)]. The data typically come from experiments in which the T cells have been primed to an antigen and therefore reflect the properties of a part of the repertoire that are biased by the priming process. Different antigen fragments survive for different lengths of time. The fragments that are broken down most slowly are presumably stimulatory for the longest time, and fragments that happen to fit into a cleft of the MHC molecules will be protected from proteolytic degradation. One might expect those fragments to persist in stimulating a subset of T cells that then become the dominant population specific for a particular antigen + MHC. Therefore, the specificity repertoire of primed T cells is a reflection of the selection process for antigen fragments, and it may be a mistake to extrapolate from that repertoire to the naive T cell repertoire. Naive helper T cells may include clones that interact with various exposed parts of MHC class II, including the CD4 binding site, and not just those residues near the cleft.
11. H. Golding *et al.*, *J. Exp. Med.* **167**, 914 (1988); H. Golding *et al.*, *J. Clin. Inves.* **83**, 1430 (1989).
12. J. A. T. Young, *Nature*, **333**, 215 (1988).
13. M. Vega *et al.*, *ibid.* **345**, 26 (1990).
14. G. W. Hoffmann, in *The Semiotics of Cellular Communication in the Immune System*, E. E. Sercarz *et al.*, Eds. (Springer-Verlag, Berlin, pp. 257–271; G. W. Hoffmann *et al.*, in *Theoretical Immunology*, A. S. Perelson, Ed. (Addison-Wesley, Redwood City, CA, 1988), pp. 291–319.
15. T. A. Kion, unpublished results.
16. B. R. Kaye, *Ann. Intern. Med.* **111**, 158 (1989).
17. ELISA plates coated with 20 ng per well of recombinant gp120 or recombinant p24 (MicroGeneSys, West Haven, CT) and blocked with 5% casein were incubated with sera diluted 1 in 100 with phosphate-buffered saline (PBS)–Tween 20 plus 1% casein. The bound antibodies were detected with biotinylated–goat anti-mouse IgG (Bethesda Research Laboratories–Gibco, Burlington, Canada) and avidin conjugated to alkaline phosphatase (Calbiochem). The plates were incubated with the substrate for 3 hours (Fig. 1, A and B) or 1 hour (Fig. 1, C and D). The numbers of mice for Fig. 1, A and B (independent samples), were five B6 (normal), five B6 (immunized), six CBA (normal), and six CBA (immunized); and for Fig. 1, C and D, the numbers were ten for each MRL group and eight for each CBA group.
18. The ELISA was performed as described in (*17*), except that the sera were diluted 1 in 200 (final concentration) and were added together with the inhibitor. The results given are the mean inhibition ±SEM for five each of the mouse strains MRL-*lpr/lpr* (age 4 months) and MRL-+/+ (age 12 months).
19. Hyperimmune sera preparation and inhibition of cytotoxicity were as in (*8*). Sera from 2-month-old MRL-*lpr/lpr* mice were absorbed with normal BALB/c and CBA Ig (3 mg/ml) or rabbit antibodies to mouse Ig (12 mg/ml) (Cedarlane Laboratories) coupled to Sepharose 4B (Sigma). Pooled sera from groups of 8 to 30 mice per group were used. Sera (200 μl) were absorbed against 200 μl of absorbant for 1 hour at room temperature. In the absence of inhibitory sera, BALB/c anti-CBA killed 65% of CBA targets, B10.D2 anti-B10.BR killed 79% of B10.BR targets, and CBA anti-BALB/c killed 65% of BALB/c targets.
20. ELISA plates that had been coated with sera at the dilution indicated and blocked with 5% casein were incubated with biotinylated, affinity-purified monoclonal antibody (100 ng per well) diluted in PBS–Tween 20 plus 1% casein. Bound antibody was detected with avidin conjugated to alkaline phosphatase. Anti-I-A^k (clone 10-3.6.2), anti-H-$2K^kD^k$ (clone 16-1-2N), anti-I-A^b (clone AF6.120.1.2), and anti-H-$2K^bD^b$ (clone 12-2-2S) were obtained from the American Type Culture Collection. Anti-β-2-6-linked-fructosan (clone UPC 10) was purchased from Sigma. Anti-$V_{\beta 8}$ (clone F23.1) was a gift from H.-S. Teh, University of British Columbia, Vancouver, BC. Pooled sera from 15 mice (MRL-*lpr/lpr*) and 10 mice (MRL-+/+) were used in this experiment. Similar results were obtained in a separate single-point experiment in which we tested sera from individual mice with 8 to 10 mice per group. The anti-I-A^k and anti-H-$2K^kD^k$ monoclonal antibodies do not react significantly with normal CBA sera (*15*).
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Specific DNA Binding by c-Myb: Evidence for a Double Helix-Turn-Helix–Related Motif

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The c-Myb protein is a sequence-specific DNA binding protein that activates transcription in hematopoietic cells. Three imperfect repeats (R_1, R_2, and R_3) that contain regularly spaced tryptophan residues form the DNA binding domain of c-Myb. A fragment of c-Myb that contained the R_2 and R_3 regions bound specifically to a DNA sequence recognized by c-Myb plus ten additional base pairs at the 3′ end of the element. The R_2R_3 fragment was predicted to contain two consecutive helix-turn-helix (HTH) motifs with unconventional turns. Mutagenesis of amino acids in R_2R_3 at positions that correspond to DNA-contacting amino acids in other HTH-containing proteins abolished specific DNA binding without affecting nonspecific DNA interactions.

THE C-MYB NUCLEAR ONCOPROTEIN is a transcriptional activator whose expression is linked to the differentiation state of hematopoietic cells (*1, 2*). The c-Myb protein functions in expression of *mim-1*, c-*myc*, *cdc2*, and the gene that encodes DNA polymerase α (*3*). It also activates transcription from the human immunodeficiency virus–1 long terminal repeat (*4*). Oncogenic activation of c-*myb* can occur when truncated versions of c-Myb are expressed that give rise to versions that lack either an NH_2-terminal phosphorylation site that regulates specific DNA binding (*5*) or a COOH-terminal trans-repressor domain (*1*). In addition, point mutations in the DNA binding domain can impose alternative differentiation phenotypes on transformed myeloid cells (*6*). The DNA binding domain is located near the NH_2-terminus and is composed of three highly conserved, imperfect 51- or 52-residue repeats (designated R_1, R_2, and R_3); only R_2 and R_3 are required for sequence-specific DNA binding (*7, 8*). Each repeat contains three regularly spaced tryptophans that are important for maintaining an active DNA binding structure (*9, 10*). In order to examine the minimal DNA binding domain, we engineered a 312-bp region of chicken c-*myb* (*11*) that encoded the R_2R_3 domain by the polymerase chain reaction (PCR) for expression in *Escherichia coli* (*12*). The R_2R_3 recombinant polypeptide was purified to near homogeneity (*13*) for use in the studies.

We used the electrophoretic mobility shift assay (*14*) to monitor DNA binding to an oligonucleotide that contained two Myb recognition elements (2xMRE-probe). Two complexes (C1 and C2) were observed (Fig. 1A) in a proportion that was dependent on the protein-to-DNA ratio. When increasing amounts of protein were added, complex C1 was formed first, followed by C2, which was the predominant complex at high protein-to-DNA ratios. Competition with specific and nonspecific oligonucleotides showed that both complexes are specific (*15*). These results demonstrate that R_2R_3 is sufficient

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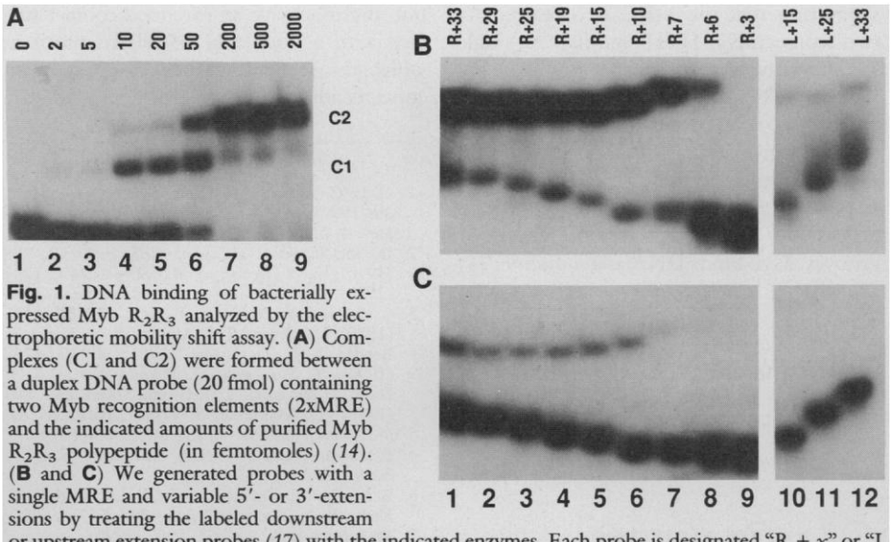

Fig. 1. DNA binding of bacterially expressed Myb R_2R_3 analyzed by the electrophoretic mobility shift assay. (**A**) Complexes (C1 and C2) were formed between a duplex DNA probe (20 fmol) containing two Myb recognition elements (2xMRE) and the indicated amounts of purified Myb R_2R_3 polypeptide (in femtomoles) (*14*). (**B** and **C**) We generated probes with a single MRE and variable 5′- or 3′-extensions by treating the labeled downstream or upstream extension probes (*17*) with the indicated enzymes. Each probe is designated "R + *x*" or "L + *x*" where *x* gives the size of the right (R, downstream) or left (L, upstream) extension (in base pairs) beyond the MRE hexamer: R + 33, no treatment (lane 1); R + 29, Bam HI + Klenow (lane 2); R + 25, Ava I + Klenow (lane 3); R + 19, Rsa I (lane 4); R + 15, Hind III + Klenow (lane 5); R + 10, Xba I + Klenow (lane 6); R + 7, Bam HI + Klenow (lane 7); R + 6, Xba I + mung bean nuclease (lane 8); R + 3, Bam HI + mung bean nuclease (lane 9), L + 15, Hind III + Klenow (lane 10); L + 25, Ava I + Klenow (lane 11); and L + 33, no treatment (lane 12). Complex formation was analyzed with 12.5-fmol of probe and 200-fmol of pure R_2R_3 polypeptide either in the absence (B) or in the presence (C) of a large excess (1 μg) of nonspecific polyd(I·C) competitor.

for specific DNA binding to the MRE consensus sequence (*16*). However, the C1 and C2 complexes had different half-lives ($t_{1/2}$); C2 ($t_{1/2}$ < 5 min) was less stable than C1 ($t_{1/2}$ ≅ 1 hour) (*15*). Complex C2 may contain two molecules of R_2R_3 bound in a less stable complex.

Using a smaller probe (18 bp) with a single MRE (*5*), we observed a single, specific complex that migrated in a position similar to C1 but had a short half-life (*15*). To assess the contribution of sequences flanking the core consensus sequence to the stability of the complex, we designed two probes with a single MRE that contained an extension of about three helical turns in either the 5′ or 3′ direction and a series of restriction sites that allowed the length of the probe to be varied (*17*). The binding of R_2R_3 to this series of probes was analyzed by the electrophoretic mobility shift assay. In the absence of competitor, complex formation required a 3′ extension of at least 6 to 7 bp (Fig. 1B). Under the same conditions, complex formation with probes that had variable 5′ extensions was substantially reduced. In the presence of excess nonspecific competitor, no complexes were observed with probes containing 5′ extensions; however, a 3′ extension of 10 bp or more allowed complex formation (Fig. 1C). This suggests that R_2R_3 is asymmetrically positioned relative to the consensus core sequence and that nonspecific interactions on one side of the recognition element within approximately one turn of a helix are important for stabilizing the protein-DNA complex. However, although downstream extensions increased the half-lives of the complexes, the sequences of the 3′ extensions also influenced complex stabilities (*15*).

Each of the $R_1R_2R_3$ repeats was predicted to contain three α helices, and it was hypothesized that the second and third helices were similar to the helix-turn-helix (HTH) motif found in bacterial transcriptional repressors and eukaryotic homeodomain-containing proteins (*18*). It was suggested that one of the R_2R_3 HTH-like domains participates in sequence-specific binding to DNA, whereas the other interacts nonspecifically with DNA (*10, 19*). To refine such a model so that it could be tested by mutagenesis, we reanalyzed the R_2 and R_3 sequences (*20*) using two structure prediction methods (*21*). When a direct alignment based on the highly conserved glycine at HTH position 9 was used (4–amino acid turn), the similarity between HTH domains and R_2R_3 was not significant. However, by extending the turn in R_3 by one amino acid and the turn in R_2 by two amino acids, we obtained significant similarity between an HTH motif and R_2R_3 (*21*) (Fig. 2A).

In order to predict which amino acids in the putative recognition helices of R_2 and R_3 participate in sequence-specific recognition of DNA, we aligned the amino acid sequences of R_2 and R_3 with those of transcriptional repressors and homoeotic proteins in which amino acids that are directly involved in base pair contacts have been identified (recognition positions) (*22, 23*). Arginine, glutamine, and asparagine residues (amino acids frequently found in direct hydrogen-bond interaction with base pairs in DNA) (*22*) thus identified in both R_2 and R_3 were mutagenized to alanine. As controls, glutamines and arginines outside of the putative HTH domains of R_2 and R_3 were similarly mutagenized (Fig. 2A) (*24*).

Soluble bacterial extracts that contained mutated R_2R_3 were analyzed for specific DNA binding to the 2xMRE probe at two protein concentrations (Fig. 2B) (*25*). The three mutants outside the putative recognition helices (Q14A and R15A in R_2 and R66A in R_3) (Fig. 2, A and B) bound the probe like the wild-type protein. All eight mutations in the putative recognition helices had decreased specific DNA binding. Three of these (N52A in R_2 and R103A and R104A in R_3) retained the ability to bind the MRE motif at higher protein concentrations. The other five (Q42A, R46A, and N49A in R_2, and N96A and N99A in R_3) completely eliminated sequence-specific interaction. These five are all in recognition positions (Fig. 2A). Thus, the amino acids in R_2R_3 predicted to participate in DNA binding by alignment with other HTH-containing proteins were found to be the most important for sequence-specific recognition of the MRE.

To confirm the conclusion that both HTH motifs contribute to sequence-specific DNA binding, we investigated the effect of the mutations on nonspecific DNA binding with nonspecific DNA coupled to magnetic Dynabeads (*26*). At high DNA concentrations, the R_2R_3 polypeptides bound the DNA-containing beads and were rapidly extracted from the solution by a magnet. This approach allowed isolation of complexes with short half-lives or low affinity and avoided potential problems caused by denaturation of the proteins that occurs in Southwestern (DNA-protein) blot experiments. When tested in this manner, all mutants except R46A bound nonspecific DNA as well as did wild-type R_2R_3 (Fig. 2C). The R46A mutant bound to the nonspecific DNA with reduced affinity. We therefore conclude that regions involved in sequence-specific DNA recognition are present in both R_2 and R_3. Therefore, our data do not show that specific interactions can be attributed to one repeat and nonspecific interactions to the other.

Our mutational analysis supports the hypothesis that the DNA binding motif of the c-*myb* proto-oncogene is related to the HTH motif. The extra amino acids in the

turn may allow for a different angle between the two helices, possibly placing the repeated tryptophans in configurations that allow them to interact (*10*). We have observed a hyperchromicity effect with denaturation of purified R_2R_3, consistent with a direct stacking interaction between the tryptophans (*15*). Our data show that sequence-specific interactions occur through the putative recognition helices of both R_2 and R_3, suggesting that the MRE is contacted by two consecutive HTH motifs. A single straight α helix, such as that found in the classical HTH repressors, is only able to contact 4 to 6 bp because of the curvature of the major groove (*27*). A larger recognition surface is created in these proteins by formation of dimers that allow two recognition helices to contact neighboring major grooves. Myb binds DNA as a monomer (*8*) but might achieve an extended contact surface with a double HTH-related motif in which the recognition helix of each motif contacts adjacent major grooves.

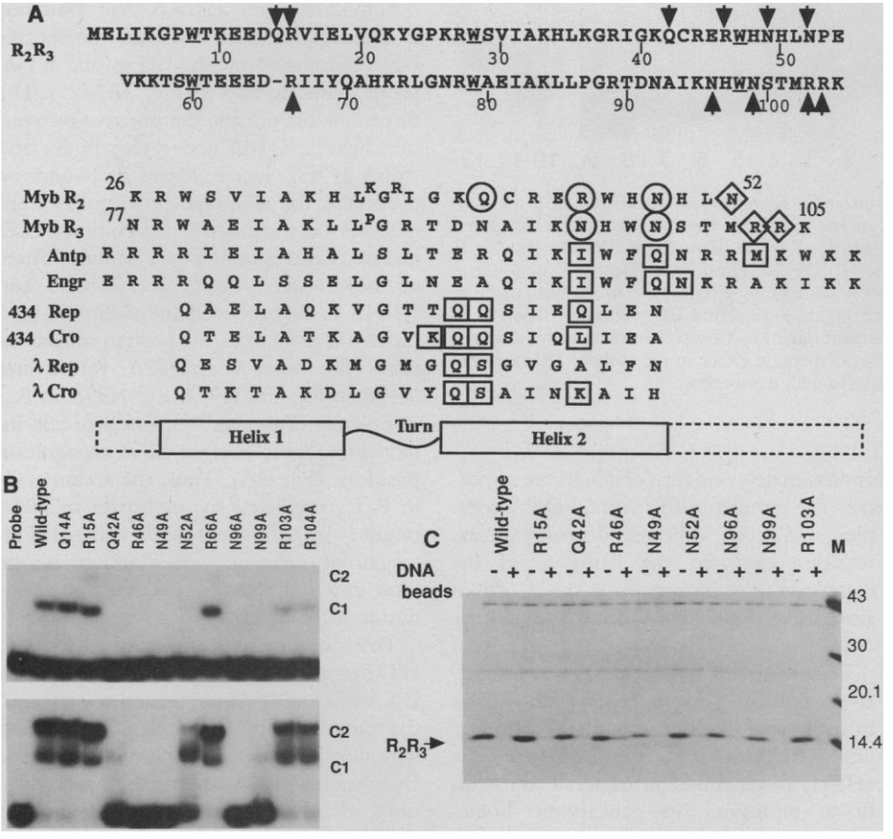

Fig. 2. Specific and nonspecific DNA binding of mutated Myb R_2R_3. (**A**) (Top) Sequence of recombinant chicken Myb R_2R_3 aligned by the tryptophans in R_2 and R_3. The NH_2-terminal methionine introduced during the PCR engineering is not removed in the expressed protein (*15*). Positions that were later mutated are indicated by arrows. Repeated tryptophans are underlined. (Bottom) Alignment of Myb R_2 and R_3 with HTH polypeptides for which structures of protein-DNA complexes have been reported (*22, 23*). Antp, Antennapedia; Engr, Engrailed; Rep, repressor. Myb R_2 is aligned with a 6–amino acid turn; R_3 with a 5–amino acid turn. To fit in these larger turns in the alignment, we superscripted some amino acids in the turn region of R_2 and R_3. Amino acids in helix 2 that are directly involved in base pair contacts are marked as boxes. The DNA contact positions given for the two Cro proteins are from low-resolution structures (*28*) and are thus probable positions. Positions in Myb R_2 and R_3 that strongly affect specific DNA binding when mutated are circled. Positions with a moderate effect on DNA binding are marked as diamonds. (**B**) Bacterial extracts that contained similar concentrations of recombinant Myb R_2R_3 polypeptides were analyzed for specific DNA binding with the 2xMRE probe (*14*) in the presence of polyd(I·C) (1 μg). In the upper gel, approximately equimolar amounts of R_2R_3 protein and DNA were used (giving mainly C1) whereas the protein-to-DNA ratios in the lower gel were 100-fold higher (giving predominantly C2). The mutants analyzed are indicated above each lane. The first letter indicates the wild-type amino acid, the number indicates the position of the mutated amino acid according to the numbering given in (A), and the second letter indicates the amino acid after mutation. Lane 1 shows free probe. (**C**) We monitored nonspecific DNA binding by adsorbing the bacterial extracts to magnetic DNA-affinity beads without MRE sequences and then analyzing the unbound polypeptides (+) by SDS–polyacrylamide gel electrophoresis in parallel with nontreated extracts (−). The mutants analyzed are indicated above each lane. The arrow indicates the R_2R_3-overexpressed polypeptide. Molecular size markers are shown at the right in kilodaltons. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

REFERENCES AND NOTES

1. G. L. C. Shen-Ong, *Biochim. Biophys. Acta* **1032**, 39 (1990); B. Lüscher and R. N. Eisenman, *Genes Dev.* **4**, 2235 (1990).
2. D. McClinton *et al.*, *Mol. Cell. Biol.* **10**, 705 (1990); B. L. Weber *et al.*, *Science* **249**, 1291 (1990).
3. S. A. Ness, Å. Marknell, T. Graf, *Cell* **59**, 1115 (1989); J. L. Evans, T. L. Moore, W. M. Kuehl, T. Bender, J. P. Ting, *Mol. Cell. Biol.* **10**, 5747 (1990); Y. Furukawa, H. Piwnica-Worms, T. J. Ernst, Y. Kanakura, J. D. Griffin, *Science* **250**, 805 (1990); D. Venturelli, S. Travali, B. Calabretta, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5963 (1990).
4. P. Dasgupta *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8090 (1990).
5. B. Lüscher, E. Christenson, D. W. Litchfield, E. G. Krebs, R. N. Eisenman, *Nature* **344**, 517 (1990).
6. M. Introna *et al.*, *Cell* **63**, 1287 (1990).
7. H. Sakura *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5758 (1989).
8. K. M. Howe *et al.*, *EMBO J.* **9**, 161 (1990).
9. P. Saikumar, R. Murali, E. P. Reddy, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8452 (1990).
10. C. Kanei-Ishii *et al.*, *J. Biol. Chem.* **265**, 19990 (1990).
11. D. Rosson and E. P. Reddy, *Nature* **319**, 604 (1986).
12. The R_2R_3 region of a subclone that harbored the chicken c-*myb* gene (*11*) was amplified by PCR with the following oligonucleotides (*myb* sequences are underlined): 5′-TTTACATATGGAACTTATCAAAGGTCCATGG-3′ and 5′-GGGGAGGCCTGGATCCTTACTACTTCCGGCGCATGGTGGAATTCC-3′. The amplified product was digested with Nde I and Bam HI and inserted into the pET3a vector [F. W. Studier, A. H. Rosenberg, J. J. Dunn, J. W. Dubendorff, *Methods Enzymol.* **185**, 60 (1990)]. The resulting plasmid was designated pMX3 and was expressed in *E. coli* strain BL21(DE3)LysS. After induction with isopropyl-1-thio-β-D-galactoside (IPTG), the recombinant bacteria accumulated about 20% of their total protein content as the soluble form of the expected 12-kD polypeptide.
13. A purification procedure consisting of two chromatographic steps resulted in homogeneous protein (99.5% pure) with a yield of about 3 to 4 mg pure protein per liter of culture (based on total amino acid analysis) (*15*).
14. DNA binding was monitored by the electrophoretic mobility shift assay [M. G. Fried, *Electrophoresis* **10**, 366 (1989)]. The oligonucleotide 2xMRE probe (*8*) used was as follows (MRE in italic):
    5′-CTAGAAAAT*TAACTG*CTAT*TAACTG*TCAATT-3′
    3′-TTTTA*ATTGAC*GATA*ATTGAC*AGTTAAGATC-5′
    We end-labeled our probes with α-^{32}P–labeled deoxycytidine triphosphate and then added an excess of unlabeled deoxyribonucleotides to produce duplex probes (with a specific activity of 14, 000 dpm/fmol). Protein-DNA complexes were formed at 25°C in 20 μl of binding buffer (20 mM tris-HCl; 0.1 mM EDTA; 10% glycerol; 50 mM NaCl; 0.1 mM dithiothreitol; pH 8) that contained labeled probe (20 fmol) and the indicated amounts of protein. Where indicated, polyd(I·C) (1 μg) was used as a nonspecific competitor. After complexes were formed, the samples were loaded onto a 7% polyacrylamide gel, in 0.5× TBE buffer (45 mM tris-boric acid and 1 mM EDTA) and resolved by electrophoresis at 10 V/cm at 4°C, and then the gel was analyzed by autoradiography.
15. O. S. Gabrielsen and A. Sentenac, unpublished observations.
16. Directional deletion analysis of c-Myb and v-Myb have identified the second and third repeat as necessary for DNA binding (*7–9*). The smallest Myb polypeptide that binds DNA and was expressed in *E. coli* was mouse $R_1R_2R_3$ fused at the NH_2-terminus to 25 amino acids derived from vector sequences (*10*).
17. Four oligonucleotides with convenient restriction

sites were annealed as follows (MRE in italic):

3′ extension:

5′-CTAGAA*TAACGG*AA-3′

3′-TT*ATTGCC*TTCTAGATCTTCGAACCATGGGCCCTAGGCGG-5′

5′ extension:

5′-GGCGGATCCCGGGTACCAAGCTTCTAGATCTAA*TAACGG*AA-3′

3′-TT*ATTGCC*TTGATC-5′

We labeled both duplexes by a Klenow fill-in reaction with [α-^{32}P] deoxycytidine triphosphate, followed with an excess of unlabeled deoxyribonucleotides to produce duplex probes. We made shortened probes by subsequent digestion with one of the indicated restriction enzymes and then used either a fill-in reaction or treatment with mung bean nuclease to produce blunt-ended probes.

18. J. Frampton *et al.*, *Nature* **342**, 134 (1989).
19. T. Oehler, H. Arnold, H. Biedenkapp, K. H. Klempnauer, *Nucleic Acids Res.* **18**, 1703 (1990).
20. The real sequences, not the $R_1R_2R_3$ consensus average used by Frampton *et al.* (*18*), were used because the differences between R_2 and R_3 are conserved and probably of importance.
21. The two structure prediction methods used were found in R. G. Brennan and B. W. Matthews, *J. Biol. Chem.* **264**, 1903 (1989); I. B. Dodd and J. B. Egan, *Nucleic Acids Res.* **18**, 5019 (1990). The significant scores for the two methods, respectively, were as follows: R_2, 0.80 and 2.86 (for a 6–amino acid turn); and R_3, 0.79 and 4.16 (for a 5–amino acid turn).
22. S. C. Harrison and A. K. Aggarwal, *Annu. Rev. Biochem.* **59**, 933 (1990); G. Otting *et al.*, *EMBO J.* **9**, 3085 (1990); T. A. Steitz, *Q. Rev. Biophys.* **23**, 205 (1990).
23. C. R. Kissinger, B. Liu, E. Martin-Blanco, T. B. Kornberg, C. O. Pabo, *Cell* **63**, 579 (1990).
24. Site-directed in vitro mutagenesis was performed as described [T. A. Kunkel, J. D. Roberts, R. A. Zakour, *Methods Enzymol.* **154**, 367 (1987)]. We subcloned the expression casette from pMX3 (Eco RV–Bgl II fragment) into Pvu II–digested Bluescript SKII$^+$ vector (Stratagene) to produce uracil-containing single-stranded DNA that we annealed to the mutagenic oligonucleotides. We used plasmids from positive clones, identified by DNA sequencing, to transform the expression strain BL21(DE3)LysS directly. Similar amounts of protein were produced after IPTG induction for all mutants.
25. The same mutants have been tested for DNA binding with the 6-bp and 33-bp 3′-extended probes, and all mutants gave results comparable to those in Fig. 2B.
26. Nonspecific DNA binding was monitored by adsorption to magnetic DNA-affinity beads made as described [O. S. Gabrielsen, E. Hornes, L. Korsnes, A. Ruet, T. B. Øyen, *Nucleic Acids Res.* 17, 6253 (1989)]. A 260-bp DNA fragment that harbored the yeast *tRNA^{Tyr} Sup 4-o* gene, which contains no MRE sites, was immobilized on streptavidin-coated magnetic Dynabeads (Dynal As, Oslo, Norway) (with 7 μg of DNA per milligram of beads). No specific complexes were formed with R_2R_3 and the same DNA fragment in mobility-shift assays. Bacterial extracts (3 μg of total protein) were incubated for 10 min at 25°C with magnetic DNA-affinity beads (2 mg) in 25 μl of buffer (20 mM tris-HCl; 1 mM EDTA; 10% glycerol; 100 mM NaCl; 1 mM dithiothreitol; and 0.05% Triton X-100; pH 8). Nonadsorbed proteins were recovered after magnetic separation and analyzed by SDS–polyacrylamide gel electrophoresis in parallel with nontreated extracts.
27. C. O. Pabo, *Annu. Rev. Biochem.* **53**, 293 (1984).
28. R. G. Brennan, S. L. Roderick, Y. Takeda, B. W. Matthews, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8165 (1990); C. Wolberger, Y. Dong, M. Ptashne, S. C. Harrison, *Nature* **335**, 789 (1988).

29. We thank E. Quemeneur and C. Doira for oligonucleotide synthesis and DNA sequencing, C. Carle for amino acid sequencing and composition analysis, J. Huet for the gift of DNA Dynabeads, and B. Perbal, who encouraged us to initiate this study and for providing the chicken c-*myb* gene.

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Ca^{2+}-Induced Ca^{2+} Release in Sea Urchin Egg Homogenates: Modulation by Cyclic ADP-Ribose

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Calcium-induced calcium release (CICR) may function widely in calcium-mediated cell signaling, but has been most thoroughly characterized in muscle cells. In a homogenate of sea urchin eggs, which display transients in the intracellular free calcium concentration ($[Ca^{2+}]_i$) during fertilization and anaphase, addition of Ca^{2+} triggered CICR. Ca^{2+} release was also induced by the CICR modulators ryanodine and caffeine. Responses to both Ca^{2+} and CICR modulators (but not Ca^{2+} release mediated by inositol 1,4,5-trisphosphate) were inhibited by procaine and ruthenium red, inhibitors of CICR. Intact eggs also displayed transients of $[Ca^{2+}]_i$ when microinjected with ryanodine. Cyclic ADP-ribose, a metabolite with potent Ca^{2+}-releasing properties, appears to act by way of the CICR mechanism and may thus be an endogenous modulator of CICR. A CICR mechanism is present in these nonmuscle cells as is assumed in various models of intracellular Ca^{2+} wave propagation.

EXTRACELLULAR SIGNALS INDUCE OSCILLATIONS of $[Ca^{2+}]_i$ or propagated waves of intracellular Ca^{2+} release in various nonmuscle cell types. The classic example of such complex spatiotemporal behavior of $[Ca^{2+}]_i$ during cell signaling is observed during fertilization of the egg. In sea urchin, starfish, fish, and frog eggs, fertilization elicits a single propagated wave of increased $[Ca^{2+}]_i$ starting at the site of sperm entry and sweeping in a regenerative fashion across the egg at about 10 μm/s (*1*). Hamster eggs display both Ca^{2+} waves and a series of periodic transients of $[Ca^{2+}]_i$ after fertilization (*2*). The $[Ca^{2+}]_i$ transient or transients at fertilization regulate the metabolic and developmental activation of the egg (*3*). Other nonmuscle cell types also display oscillatory or propagated $[Ca^{2+}]_i$ transients in response to calcium mobilizing hormones or Ca^{2+} itself (*4, 5*). Calcium-induced Ca^{2+} release (CICR), whereby an increase in the concentration of extravesicular free Ca^{2+} triggers Ca^{2+} release from intracellular stores, has been characterized in muscle fibers and sarcoplasmic reticulum (SR) vesicles (*6, 7*). CICR is mediated by the ryanodine receptor (*8, 9*) and may function in producing both Ca^{2+} waves and oscillations (*10, 11*) by acting in concert with inositol 1,4,5-trisphosphate (IP_3)–mediated Ca^{2+} release. Pharmacological agents that modulate CICR include the stimulators caffeine and ryanodine and the inhibitors procaine and ruthenium red (*6, 12*), but no endogenous modulating compounds (other than adenosine nucleotides) have yet been reported. We now report direct evidence for and characterization of a CICR mechanism (distinct from the IP_3-mediated release mechanism) in sea urchin egg homogenates. Also, we present evidence suggesting that CICR occurs in the intact cell. Further, cyclic adenosine diphosphate (ADP)–ribose (cADPR), which is a metabolite of nicotinamide adenine dinucleotide (NAD^+) and is present in homogenates of urchin eggs (*13*) and various mammalian cells (*14*), was found to modulate the urchin egg CICR mechanism. Like IP_3, cADPR triggers calcium mobilization and egg activation in urchin eggs (*15*).

Homogenates of sea urchin eggs supplemented with an adenosine triphosphate (ATP)–regenerating system, mitochondrial inhibitors, and the calcium-reporting dye fura 2, sequester added Ca^{2+} into vesicular stores in an ATP-dependent manner and release Ca^{2+} in response to nanomolar concentrations of either cADPR or $InsP_3$ (Fig. 1) (*15*). Such homogenates display desensi-

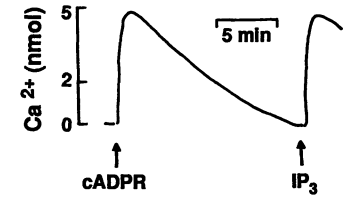

Fig. 1. Ca^{2+} release induced by cADPR and IP_3 in egg homogenates measured fluorometrically with fura 2. Addition of cADPR (20 nM, final concentration) elicited a large rapid Ca^{2+} release, which was then resequestered. IP_3 (100 nM) subsequently triggered a similar release, which was also resequestered. The absolute amount of Ca^{2+} released is indicated on the ordinate. Breaks in the record occurred during additions to the cuvette. Abbreviations: cADPR, cyclic ADP-ribose; IP_3, inositol 1,4,5-trisphosphate. See (*30*) for methods.

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