First, the lipids in free bilayers may be expected to be able to undergo elastic and other deformations even more easily than our supported bilayers. Second, although we "depleted" our bilayers to expose hydrophobic groups, in the case of vesicles and biomembranes such exposed areas would arise from certain stresses, that is, from inhomogeneous ionic or osmotic stresses, or local packing stresses induced by integral membrane proteins. Indeed, both lipid vesicles and biological membranes may fuse by going through similar stages as depicted in Fig. 3 (*1, 10*). Such a molecular mechanism is very simple and does not involve any complex intermediate nonbilayer or inverted micellar structures.

Recent studies have increasingly implicated the hydrophobic interaction in the adhesion and fusion of membranes (*23–26*) through mechanisms not unlike the ones we have found for supported bilayers, namely, through membrane conformational changes involving the exposure of hydrophobic "domains" (*24*), "pockets" (*25*), or "segments" (*26*). Our force measurements are not inconsistent with these observations on membrane adhesion and fusion, and—more generally—shed new light on the forces and mechanisms that govern such interactions.

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17. The surfactants and lipids were of the highest purity available (>99%) from Sigma (St. Louis, Missouri) or Avanti (Birmingham, Alabama) and were used without further purification. The water was distilled, filtered in a Labconco purification unit, and then redistilled. The NaBr from Mallinckrodt (Paris, Kentucky) was analytical grade. Abbreviations for lipids: CTAB, hexadecyltrimethylammonium bromide; DGDG, plant digalactosyldiglyceride lipids; MGDG, plant monogalactosyldiglyceride lipids; DLPC, L-α-dilaurylphosphatidylcholine; DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; egg-PC, egg-yolk phosphatidylcholine; DPPE, L-α-dipalmitoylphosphatidylethanolamine.
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Cognate DNA Binding Specificity Retained After Leucine Zipper Exchange Between GCN4 and C/EBP

PETER AGRE,* PETER F. JOHNSON,† STEVEN L. MCKNIGHT

Both C/EBP and GCN4 are sequence-specific DNA binding proteins that control gene expression. Recent evidence implicates C/EBP as a transcriptional regulator of genes involved in lipid and carbohydrate metabolism. The C/EBP protein binds avidly to the dyad symmetric sequence 5′-ATTGCGCAAT-3′; GCN4 regulates the transcription of genes that control amino acid biosynthesis in yeast, and binds avidly to the dyad symmetric sequence 5′-ATGA(G/C)TCAT-3′. Both C/EBP and GCN4 bind DNA via the same structural motif. This motif has been predicted to be bipartite, consisting of a dimerization interface termed the "leucine zipper" and a DNA contact surface termed the "basic region." Specificity of DNA binding has been predicted to be imparted by the basic region. As a test of this hypothesis, recombinant proteins were created wherein the basic regions and leucine zippers of GCN4 and C/EBP were reciprocally exchanged. In both of the recombinant polypeptides, DNA binding specificity is shown to track with the basic region.

A NEWLY RECOGNIZED CLASS OF SEQUENCE-SPECIFIC DNA binding proteins has been described (*1*) that includes biochemically defined DNA binding proteins such as C/EBP and AP1 (*2*), genetically defined regulatory proteins such as GCN4 (*3*), and transforming proteins encoded by proto-oncogenes such as *fos* and *jun* (*4*). These proteins have been proposed to interact with DNA through a common, bipartite DNA binding motif (*5*). One component of the motif is a dimerization interface termed the leucine zipper. The zipper is thought to form as a result of the intermolecular association of two amphipathic α helices. Helix association is believed to result, at least in part, from hydrophobic interactions generated by aliphatic amino acids in a manner analogous to the coiled-coil intertwining of filamentous, structural proteins (*6*). As a result of dimerization, highly basic polypeptide regions located on

Howard Hughes Research Laboratories, Department of Embryology, Carnegie Institution of Washington, 115 West University Parkway, Baltimore, MD 21210.

*On sabbatical leave from the Department of Medicine, Hematology Division, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205.

†Present address: BRI, Basic Research Program, National Cancer Institute, Frederick Cancer Research Facility, P.O. Box B, Building 539, Frederick, MD 21701.

the immediate NH_2-terminal side of the zipper are predicted to become closely juxtaposed. According to this model, the cleft formed between appropriately juxtaposed basic regions constitutes the DNA contact surface. We term these bZIP proteins in order to distinguish them from other transcriptional regulatory proteins that use leucine zippers in the context of other DNA contact surfaces.

A number of experiments have been conducted to test the validity of the aforementioned model. Amino acid substitutions and deletions have been introduced into the leucine repeat segment of several bZIP proteins. Changes in amino acid sequence that disrupt helix amphipathy interfere with DNA binding. These include substitutions of leucines by residues less suitable for hydrophobic packing (*7–11*), introduction of helix-disrupting residues such as proline (*8*), and deletions that result in an interruption of helical periodicity (*9*). It has been assumed, and in certain cases established (*11*), that the failure of these zipper-defective mutants to bind DNA is concordant with their inability to associate as dimers.

Several bZIP proteins have also been altered by site-specific mutagenesis in regions corresponding to the putative DNA contact surface. Two variant forms of C/EBP that bear small clusters of basic to uncharged substitutions lack sequence-specific DNA binding activity despite retaining the capacity to form dimers (*11*). Similar results have emerged from studies on the Fos and Jun polypeptides (*8–10*). Perhaps the most compelling evidence favoring the importance of the basic region has emerged from classical genetic studies. Inactive forms of a regulatory gene from *Neurospora crassa* that encodes a bZIP protein have been sequenced and shown to encode polypeptides that substitute single amino acids within the basic region (*12*).

Although molecular genetic studies such as those outlined in the preceding paragraphs are consistent with the bipartite structural model hypothesized for bZIP proteins, they do not provide unambiguous evidence that DNA binding specificity resides in the basic region. In order to perform a more rigorous test of this hypothesis, we have prepared and expressed a set of chimeric proteins that contain the basic region of one protein fused to the leucine zipper of another protein (Fig. 1A).

The two proteins chosen for study, C/EBP and GCN4, have the canonical properties of bZIP proteins (Fig. 1B), yet recognize different sites on DNA. C/EBP binds to a variety of different sites whose consensus appears to be a 10-bp sequence—5′-ATTGCGCAAT-3′—consisting of two directly abutted dyad half sites (*13, 14*); GCN4 binds to the 9-bp sequence—5′-ATGA^G_CTCAT-3′—consisting of two dyad half sites that overlap at a central G · C base pair (*15*). In order to construct chimeric proteins, Xho I restriction sites were introduced into the genes that encode C/EBP and GCN4 at positions between the basic region and leucine zipper. The hexanucleotide sequence that specifies cleavage by Xho I, 5′-CTCGAG-3′, encodes leucine–glutamic acid. This same set of amino acids occurs starting at the NH_2-terminal leucine of the GCN4 zipper (Fig. 2). The gene encoding GCN4 was mutagenized with a synthetic oligonucleotide (*16*), allowing production of a polypeptide termed G1. Although the nucleotide sequence of the gene encoding G1 was changed so as to introduce the Xho I restriction site, the protein sequence of

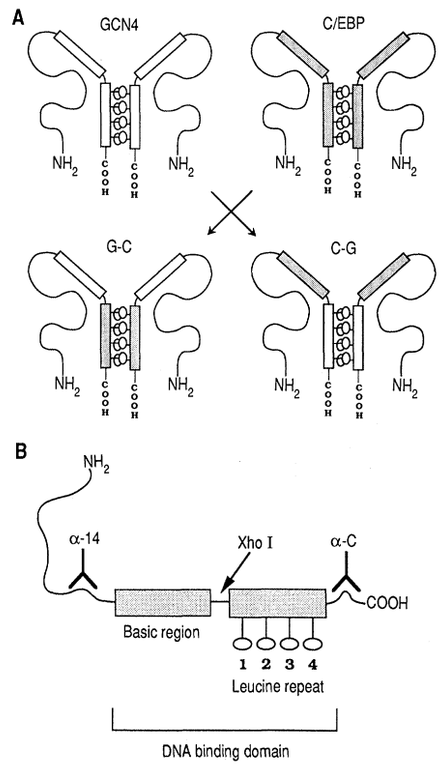

Fig. 1. (**A**) Scheme used to reciprocally exchange leucine zippers of GCN4 and C/EBP. The upper portion shows parental, dimeric forms of GCN4 (open rectangles) and C/EBP (stippled rectangles). Chimeric polypeptides shown in the lower portion were prepared by reciprocal exchange of basic regions and leucine repeats, resulting in G-C (basic region of GCN4 fused to leucine zipper of C/EBP) and C-G (basic region of C/EBP fused to leucine zipper of GCN4). (**B**) Anatomy of a monomeric chain of C/EBP. The DNA binding domain of C/EBP consists of a basic region (about 20 amino acids) linked to a leucine repeat (about 30 amino acids). Reciprocal exchange with corresponding segments of GCN4 was made possible by insertion of an Xho I restriction enzyme recognition site at one of two positions (Fig. 2). Expression of parental C/EBP and chimeric derivatives was monitored with antibodies specific to either of two epitopes. One epitope was located within a sequence of 14 amino acids located on the NH_2-terminal side of the basic region (α-14). The other epitope was located within a 15–amino acid sequence located at the COOH-terminus of C/EBP (α-COOH).

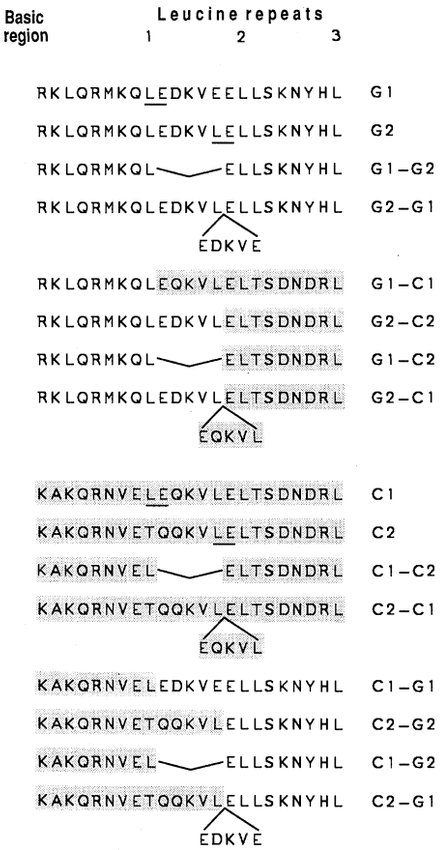

Fig. 2. Locations of reciprocal exchange between leucine zippers of GCN4 and C/EBP. Amino acid sequences of portions of GCN4, C/EBP, and chimeric fusions are shown using the single amino acid code (*22*). Top four sequences show a segment of the GCN4 polypeptide sequence starting within the basic region (arginine residue number 235 out of the 271 residue polypeptide) and progressing approximately halfway through the leucine repeat (terminating with leucine residue number 257). Positions of Xho I linkers are indicated by underlined leucine–glutamic acid (*LE*). Recombination of G1 basic region with G2 leucine repeat leads to deletion of five residues (G1G2), and recombination of G2 basic region with G1 leucine repeat leads to insertion of five residues (G2G1). Third set of sequences (stippled) show a segment of the C/EBP polypeptide sequence starting within the basic region (lysine residue number 302 out of the 359 residue polypeptide) and progressing approximately halfway through the leucine repeat (terminating with leucine residue number 324). Second and fourth sets of sequences show chimeric polypeptides consisting of GCN4 basic region fused to the C/EBP leucine repeat (second set), and C/EBP basic region fused to the GCN4 leucine repeat (fourth set). In all cases, C/EBP sequences are designated by stippling. In addition to appropriately matched chimeric polypeptides (G1C1, G2C2, C1G1, and C2G2), four illegitimately matched chimeras were also prepared (G1C2, G2C1, C1G2, and C2G1).

GCN4 remained unchanged. The analogous position of C/EBP, according to interpretations outlined by Vinson *et al.* (*14*), contains the sequence Thr-Glu. The DNA sequence encoding these amino acids was changed to specify cleavage by Xho I, leading to a gene that encodes an altered form of C/EBP termed C1 (Fig. 2).

Not knowing whether this first site of reciprocal exchange would be suitable, we also introduced Xho I restriction sites into the two genes at positions five amino acids closer to their respective COOH-termini (Fig. 2). In this second case, mutagenesis led to a form of GCN4, termed G2, that bore a single amino acid substitution (glutamic acid to leucine). In contrast, C/EBP already contained the sequence leucine–glutamic acid at this position. Therefore, introduction of the Xho I site led to expression of a polypeptide bearing the native C/EBP amino acid sequence (termed C2). These four polypeptides, G1, G2, C1, and C2, were expressed in bacterial cells and tested by deoxyribonuclease I (DNase I) footprinting for sequence-specific interaction with DNA. As is shown below, all four proteins bound DNA selectively. Three of the four proteins bound to their cognate sites on DNA as avidly and specifically as "native" protein expressed from unaltered GCN4 and C/EBP genes. However, the apparent affinity of the C1 protein was reduced by 70 percent relative to native C/EBP as measured by titrating C1 and native C/EBP. The molecular basis for the reduced affinity of the C1 variant of C/EBP has not been resolved.

Reciprocal exchanges were made by recombining the C/EBP and GCN4 genes at the newly introduced Xho I sites. Recombinants containing the basic region of C/EBP fused to the zipper of GCN4 were termed C1G1 and C2G2. Likewise, recombinants containing the basic region of GCN4 fused to the zipper of C/EBP were termed G1C1 and G2C2 (Fig. 2). Having introduced the Xho I site at two locations in each gene (separated by five amino acids), we also prepared derivatives of the parental proteins that either inserted or deleted five amino acids in the region separating the basic region from the zipper. These variants of GCN4 were termed G1G2 (deletion of five amino acids) and G2G1 (insertion of five amino acids). The corresponding derivatives of C/EBP (C1C2 and C2C1) were also prepared (Fig. 2). Finally, improperly matched recombinants were prepared by fusing the basic region of GCN4 to the inappropriate site on the C/EBP zipper (G1C2 and G2C1), and the basic region of C/EBP to the inappropriate site on the GCN4 zipper (C1G2 and C2G1).

Parental and recombinant proteins were expressed in bacterial cells, solubilized (*17*), and fractionated on three polyacrylamide electrophoresis gels. One gel was stained with Coomassie brilliant blue, and the other two were prepared for immunoblot analysis with two C/EBP-specific antibodies. One antibody, termed α-14, was prepared against a 14–amino acid epitope located immediately "NH_2-terminal" from the C/EBP basic region. The other antibody, termed α-COOH, was prepared against an epitope corresponding to the COOH-terminal 15 amino acids of C/EBP (Fig. 1B). The α-14 antibody allowed detection of parental and recombinant polypeptides that contained the basic region of C/EBP, whereas the α-COOH antibody allowed detection of polypeptides that contained the zipper of C/EBP. Coupled with Coomassie staining, the immunoblots (Fig. 3) verified the presence of the appropriate polypeptides in each bacterial extract. These analytical studies showed that the inappropriately matched recombinant proteins, which either inserted or deleted five amino acids (Fig. 2), migrated with the expected, altered mobilities.

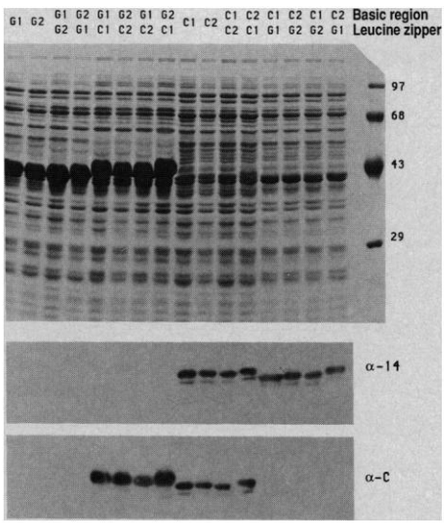

Fig. 3. SDS gel electrophoresis of parental and recombinant forms of GCN4 and C/EBP. Soluble extracts prepared from bacterial cells (*17*) were applied to three electrophoresis gels. Protein nomenclature identifying individual gel lanes (top) is as designated in Fig. 2. (**Top**) One gel was stained with Coomassie brilliant blue. Protein bands on right represent molecular markers. The other two gels were subjected to immunoblot analysis with antibodies to two epitopes of C/EBP (Fig. 1B). One antibody, α-14, recognized an epitope located on the NH_2-terminal side of the C/EBP basic region (**center**). This antibody reacted with polypeptides carrying the basic region of C/EBP, but failed to react with polypeptides carrying the basic region of GCN4. The other antibody, termed α-COOH, recognized an epitope located on the COOH-terminal side of the C/EBP leucine zipper (**bottom**). This antibody reacted with polypeptides carrying the leucine zipper of C/EBP, but failed to react with polypeptides carrying the leucine zipper of GCN4. Parental and recombinant polypeptides exhibited electrophoretic mobilities in the range of 40 kD.

Having found that parental and recombinant polypeptides could be produced in a soluble form, we then tested each protein for sequence-specific interaction with DNA. The binding sites used in DNase I footprinting assays were synthesized as complementary oligonucleotides and inserted into the same vector DNA molecule (*18*). The nucleotide sequence chosen for the C/EBP binding site was 5′-ATTGCGCAAT-3′, and that for the GCN4 binding site was 5′-ATGAGTCAT-3′. As shown in the upper panel of Fig. 4, the GCN4 binding site was protected by four protein samples (G1, G2, G1C1, and G2C2). The lower panel of Fig. 4 shows that the C/EBP binding was also footprinted by four protein samples (C1, C2, C1G1, and C2G2). These assays also provide evidence of weak interaction between the C/EBP binding site and the four proteins that bound avidly to the canonical GCN4 binding site (G1, G2, G1C1, and G2C2). All eight proteins that initiated translation at the NH_2-terminus of GCN4 were expressed more effectively in bacteria than those that initiated at the NH_2-terminus of C/EBP (Fig. 3). This disparity in protein concentration probably accounts for part of the perceived promiscuity of GCN4 binding.

Three general conclusions can be drawn from the observations presented in Fig. 4. First, DNA binding specificity is conferred by amino acid sequences located upstream from the leucine zipper. G1C1 and G2C2 bound to DNA in a manner indistinguishable from G1 (which consisted of an entirely native GCN4 amino acid sequence) or G2 (which contained a single amino acid substitution). Likewise, C1G1 and C2G2 bound DNA in a manner indistinguishable from C1 (which contained a single amino acid substitution) or C2 (which consisted of an entirely native C/EBP amino acid sequence). Other than the zipper, the only aspect of C/EBP necessary for DNA binding is the basic region (*11, 19*). Thus, we conclude that the DNA binding specificity of a bZIP protein is determined by its basic region (*20*). However, we emphasize that DNA binding is heavily dependent on dimerization; mutations in the C/EBP leucine zipper that fully disrupt dimerization also lead to complete inactivation of DNA binding (*11*).

Second, the fact that exchange of leucine zippers between C/EBP and GCN4 yields functional DNA binding proteins demonstrates the autonomous nature of the leucine zipper. That is, its capacity to mediate dimer formation is not substantially influenced by its polypeptide environment. This "autonomy" argues against the notion that the leu-

cine zipper relies on tertiary interactions with other polypeptide determinants in order to adopt its appropriately folded configuration.

The third observation that emerged from our studies is that all "illegitimate" recombinants of the parental proteins (G1G2, G2G1, C1C2, and C2C1) failed to bind DNA (Fig. 4). Likewise, all four of the illegitimate chimeric recombinants (G1C2, G2C1, C1G2, and C2G1) failed to bind DNA. Since it was possible to document the presence of these proteins in soluble bacterial extracts (Fig. 3), we do not believe that their inactivity can be attributed simply to a failure to be expressed in a stable form. Moreover, cross-linking assays conducted on C1C2 and C2C1 show that both proteins are fully competent in dimer formation (*21*). It would thus appear that proteins bearing small deletions or insertions between the basic region and leucine zipper are not completely distorted in three-dimensional structure. We instead believe that their failure to bind DNA reflects the disruption of a register or phasing between the basic region and leucine zipper that is conserved in all proteins of this class. The nature of this register is described by Vinson *et al.* (*14*).

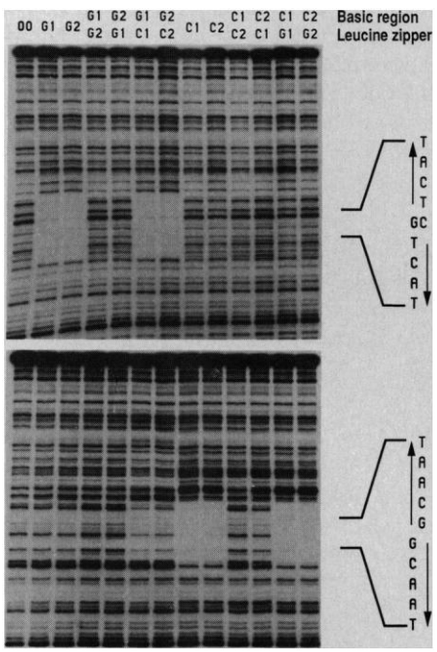

Fig. 4. DNase I footprint assays of parental and recombinant forms of GCN4 and C/EBP. Soluble extracts prepared from bacterial cells (*17*) were tested in footprint assays with two DNA substrates (*18*). One substrate contained a high affinity binding site for GCN4 (**top**). The other substrate contained a high-affinity binding site for C/EBP (**bottom**). Left lane (00) of each set of assays contained substrate DNA that had been exposed to DNase I in the absence of added protein. Remaining lanes contained DNA that had been exposed to parental and recombinant proteins as indicated above each gel lane. Positions and sequences of GCN4 and C/EBP binding sites are indicated on the right.

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16. Recombinant DNA copies of the genes encoding GCN4 and C/EBP were cloned into pEMBL-19 and pEMBL-18, respectively (constructed by S. Lazarowitz), and transfected into *Escherichia coli* strain JM101. Cultures (20 ml) of T broth were superinfected with 20 μl of the VCS-M13 "helper" bacteriophage, allowing production and subsequent purification of single-stranded DNA. Single-copy Xho I restriction sites were introduced at two locations in each gene (Fig. 2) by oligonucleotide-directed mutagenesis [M. J. Zoller and M. Smith, *Methods Enzymol.* **100**, 469 (1983)]. Mutagenic oligonucleotides of the following sequences:
5′-AACCTTGTCCTCGAGTTGTTTCATTC-3′ (GCN4 1)
5′-CGAAAGCAACTCGAGAACCTTGTC-3′ (GCN4 2)
5′-CACCTTCTGCTCGAGCTCCACGTTG-3′ (C/EBP 1)
5′-ACTGGTCAACTCGAGCACCTTCTG-3′ (C/EBP 2)
were annealed with single-stranded GCN4 or C/EBP cDNA. The annealed oligonucleotide was extended with the Klenow fragment of *E. coli* DNA polymerase (Boehringer Mannheim), ligated, and transformed into *E. coli* strain JM101. The entire transformation mix was grown overnight in a 5-ml liquid culture of T broth containing ampicillin (50 μg/ml). Double-stranded plasmid DNA was purified, cleaved with Xho I restriction endonuclease, and subjected to electrophoresis on a 1% agarose gel. Plasmid DNA corresponding to the linear form was excised from the agarose gel, recovered, ligated, and transformed a second time into *E. coli* strain JM101. Since both parental plasmids lacked Xho I restriction sites, this procedure allowed selection of derivatives that had been mutagenized by the synthetic oligonucleotide. After undergoing transformation a second time, cells were spread onto L broth plates containing ampicillin. Individual clones were picked and grown in liquid culture, allowing preparation of plasmid DNA. Recombinant derivatives (Fig. 2) were prepared by conventional techniques (T. Maniatis, E. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982), and commercially obtained restriction endonucleases (Boehringer) and DNA ligase (New England Biolabs). All recombinants were subjected to enzymatic sequencing (Sequenase, U.S. Biochemical) through the region of recombination and mutagenesis.
17. Parental and recombinant forms of GCN4 and C/EBP were synthesized in bacteria with the use of a bacteriophage T7 expression system [F. W. Studier and B. A. Moffatt, *J. Mol. Biol.* **189**, 113 (1986)]. Both GCN4 and C/EBP were engineered to allow translation starting at their natural AUG initiation codons. Translation initiation was enhanced by use of a termination-initiation coupler designed by S. Eisenberg [see note 16 in (*11*)]. After IPTG induction, cells were harvested by centrifugation, resuspended in phosphate-buffered saline (PBS) containing 5*M* urea, 1 m*M* benzamidine, 1 m*M* EDTA, and 0.2% Triton X-100. Resuspended cells were frozen at −80°C, quick-thawed at 37°C, and sonicated. The lysates were passed over a DEAE-cellulose column equilibrated with PBS containing 5*M* urea, 1 m*M* benzamidine, and 1 m*M* EDTA. The flowthrough fraction was dialyzed against PBS. In the SDS–gel electrophoresis analysis 15 μl of extract were used in each gel lane. DNase I footprint assays were performed using 0.3 μl of extract for proteins that initiated translation at the NH_2-terminus of GCN4 and 1.0 μl of extract for proteins that initiated at the amino terminus of C/EBP.
18. Oligonucleotides corresponding to high-affinity C/EBP and GCN4 binding sites were synthesized. The C/EBP oligonucleotide consisted of the sequence 5′-GATCAAGCTGCAGATTGCGCAATCTGCAGCTT-3′, which, upon annealing, generated a symmetric dyad with directly abutted half sites. The GCN4 oligonucleotides, 5′-GATCCATCCATGACTCATCCT-3′ and 5′-GATCCATCCATGAGTCATCCT-3′, generated a symmetric dyad with half sites separated by a central G · C base pair. Each of the annealed oligonucleotides contained 4-bp protrusions of the sequence 5′-GATC-3′, allowing insertion into a Bam HI restriction endonuclease cleavage site that had been introduced into the herpes simplex virus (HSV) thymidine kinase gene (*tk*). The Bam HI site was located between 6 and 16 bp upstream from the *tk* mRNA start site, in a derivative of the *tk* promoter termed linker scanning mutant −16/−6 [S. L. McKnight and R. C. Kingsbury, *Science* **217**, 316 (1982)]. Probe DNA molecules were prepared for DNase I footprinting by digesting the *tk* DNA at a Bgl II restriction site located 56 bp downstream from the mRNA start site, end-labeling with [γ^{32}P]ATP, then digesting at an Eco RI site located 80 bp upstream from the mRNA start site. DNase I footprinting reactions were as described (*11*).
19. J. Shuman and S. L. McKnight, unpublished observations.
20. The DNA binding assays (Fig. 4) were done with protein samples that were contaminated to varying extents by bacterial proteins (see Fig. 3). Accurate measurements of the specific activity of each parental and chimeric polypeptide were therefore not obtained. However, when protein input levels were reduced by sequential titration, the relative patterns of binding shown in Fig. 4 persisted.
21. P. Agre and S. L. McKnight, unpublished observations.
22. Amino acid sequence of GCN4 was determined by A. Hinnebusch, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6442 (1984), and G. Thireos, M. D. Penn, H. Greer, *ibid.*, p. 5096. Amino acid sequence of C/EBP was determined by W. H. Landschulz, P. F. Johnson, E. Y. Adashi, B. J. Graves, S. L. McKnight, *Genes Dev.* **2**, 786 (1988). Abbreviations for amino acid residues are 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.

23. We thank R. Kingsbury for technical assistance, A. Hinnebusch for providing a recombinant DNA clone of the yeast gene encoding GCN4, C. Vinson for construction of plasmid vectors containing C/EBP and GCN4 binding sites, K. LaMarco for editorial comments, W. Landschulz for helpful discussion, and C. Norman for clerical assistance. Supported by grants from NIH, American Heart Association, Johns Hopkins University Hematology Division (P.A.), Howard Hughes Medical Research Institute (S.L.M.), and Carnegie Institution of Washington (S.L.M.).

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Assembly of the Native Heterodimer of *Rana esculenta* Tropomyosin by Chain Exchange

SHERWIN S. LEHRER, YUDE QIAN,* SØREN HVIDT

***Rana esculenta* tropomyosin assembles in vivo into a coiled-coil α helix from two different subunits, α and β, which are present in about equal concentrations. Although the native composition is αβ, a mixture of equal amounts of αα and ββ is produced by refolding dissociated α and β at low temperature in vitro. Refolding kinetics showed that αα formed first and was relatively stable with regard to chain exchange below approximately 20°C. Equilibration of the homodimer mixture at 30° and 34°C for long times, however, resulted in the formation of the native αβ molecule by chain exchange. Biosynthesis of αβ from separate α and β genes is, therefore, favored thermodynamically over the formation of homodimers, and biological factors need not be invoked to explain the preferred native αβ composition.**

TROPOMYOSIN PURIFIED FROM SEVeral sources (skeletal, cardiac, and smooth muscle) is composed of two kinds of subunits, α and β, which differ slightly in amino acid sequence (*1*). In some muscles, α and β are present in about equal amounts (*2*). Assembly of the two subunits in such muscles after chain biosynthesis, or in vitro after chain dissociation, can in principle produce a mixture of equal numbers of homodimers (αα and ββ), all heterodimer (αβ), or a mixture of the three species. Attempts to determine the native composition and to find out if it is controlled by thermodynamic and kinetic parameters or by biological factors have been made for tropomyosin from rabbit skeletal muscle (*3–7*) and fowl gizzard smooth muscle (*8–12*).

Tropomyosin is a coiled-coil α helix, which dissociates into subunits (*13, 14*) in parallel with a major cooperative helix-coil transition (*13, 15, 16*). Thus, the dissociation and association processes can be studied by directly monitoring changes in helical content with circular dichroism (CD) methods. We have purified tropomyosin from *Rana esculenta* muscle, in which α and β are present in about equal amounts, and found that the native composition is predominantly αβ. The native αβ molecule and the two reconstituted homodimers, αα and ββ, had different thermal stabilities characterized by different helix thermal unfolding profiles. This allowed us to determine the relative amounts of the three species refolded in vitro from separated α and β chains.

The thermal unfolding profile of the α helix of αα (*17*) showed one main transition at 50°C and that of ββ showed a broad transition centered at 30°C, both of which were reversible on slow cooling (Fig. 1A). In contrast, the unfolding profile of αβ showed two cooperative transitions with midpoints at 36° and 50°C (Fig. 1A, curve 1) and was not reversible on refolding by cooling, as made evident by the missing 36°C transition (Fig. 1A, curve 2). A second unfolding run of the unfolded and refolded αβ sample gave data that coincided with the refolding profile (Fig. 1A, curve 3); this profile consisted of a broad transition between 20° and 40°C and a sharp transition at 50°C, suggesting that the curve originated from contributions from ββ and αα, respectively. The agreement between a curve calculated by taking equal contributions of the unfolded and refolded αβ (Fig. 1A, curve 3) showed that the heating and cooling processes resulted in a conversion of the αβ heterodimer to equal amounts of homodimers.

Samples of the three different dimers were dissociated separately in 5*M* guanidinium hydrochloride, and dimers were allowed to re-form by dialyzing the samples at 5°C. The thermal unfolding profiles of the homodimers refolded from guanidinium hydrochloride (Fig. 1B) were essentially the same as those obtained for untreated homodimers (Fig. 1A). The unfolding profile of the

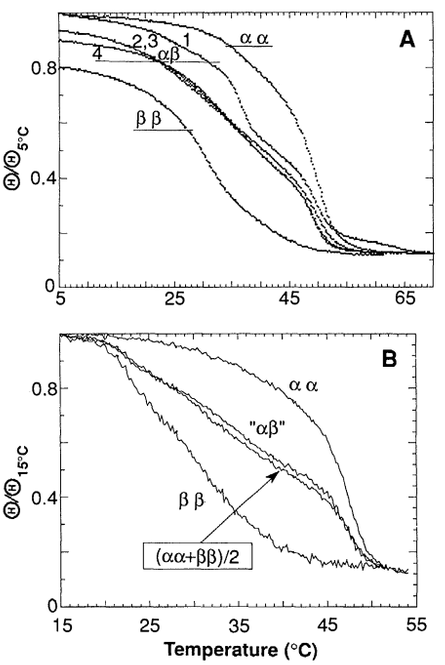

Fig. 1. Helix thermal unfolding and refolding profiles of the three species of *R. esculenta* tropomyosin. The αβ tropomyosin refolds as a 1:1 mixture of αα and ββ. (**A**) Temperature dependence of ellipticity (Θ) at 222 nm normalized to the value for αα at 5°C. The αα and ββ profiles were reversible whereas that of αβ was not for these cooling rates. The αβ profiles are as follows: 1, on heating; 2, on cooling; 3, on reheating; and 4, calculated profile where Θ = 1/2 ($\Theta_{\beta\beta}$ + $\Theta_{\alpha\alpha}$) at each temperature. Initial value of helicity for ββ was lower than that of αα and αβ. (**B**) Temperature dependence of ellipticity at 222 nm normalized to the αα value at 15°C for αα, ββ, and "αβ" refolded from 5*M* guanidinium hydrochloride by dialysis at 5°C. The (αα + ββ)/2 curve was calculated by taking equal contributions from the αα and ββ profiles. Ellipticity was measured on tropomyosin solutions (0.5 mg/ml) in a 1-mm cell (A), or on solutions (0.05 mg/ml) in a 1-cm cell (B), in buffer [0.5*M* NaCl, 20 m*M* sodium phosphate (*p*H 7.0), 1 m*M* EDTA, and 1 m*M* dithiothreitol] with an Aviv 60DS CD spectropolarimeter and a Hewlett-Packard 8910A temperature controller. Data were collected in steps of 0.2°C after 0.3-min dwell times with 10-s averaging, which gave a 0.4° per minute heating or cooling rate. The αβ and αα tropomyosin were purified from frog back and leg muscle by isoelectric precipitation, ammonium sulfate fractionation, and hydroxyapatite chromatography (*4*) at 0° to 5°C and were found to contain 86 and 96% αβ from leg and back muscle, respectively, and a corresponding small amount of αα (14 and 4%). The ββ dimer and also αα when needed in greater quantities were prepared by ion-exchange chromatography on urea-separated chains of αβ (*24*), and the chains were refolded by dialysis.

S. S. Lehrer and Y. Qian, Department of Muscle Research, Boston Biomedical Research Institute, 20 Staniford Street, Boston MA 02114, and Department of Neurology, Harvard Medical School, Boston, MA 02115.
S. Hvidt, Risø National Laboratory and Roskilde University, DK 4000, Roskilde, Denmark.

*Present address: Chemistry Department, Hangzhou University, Hangzhou, People's Republic of China.