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The Product of the *mos* Proto-Oncogene as a Candidate "Initiator" for Oocyte Maturation

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The endogenous c-*mos* product, pp39^{mos}, is required for progesterone-induced meiotic maturation in *Xenopus* oocytes. Treatment of oocytes with progesterone induced a rapid increase in pp39^{mos} that preceded both the activation of maturation promoting factor (MPF) and germinal vesicle breakdown (GVBD). Microinjection of synthetic *mos* RNA into oocytes activated MPF and induced GVBD in the absence of progesterone. Thus, the *mos* proto-oncogene product may qualify as a candidate "initiator" protein of MPF and is at least one of the "triggers" for G_2 to M transition.

IN MOST SPECIES, FULLY GROWN OOcytes are naturally arrested at the first meiotic prophase (or late G_2 phase) (*1, 2*). In *Xenopus*, this arrest is released by progesterone and is followed by the appearance of a cytoplasmic activity, MPF, that causes nuclear breakdown or GVBD and chromosome condensation (*3*). MPF was originally described as a cytosolic factor that, when microinjected, could induce G_2-arrested oocytes to enter meiosis in the absence of protein synthesis (*4, 5*). An identical activity was subsequently shown to exist in a variety of maturing oocytes and also in mitotic cells of many species (*6–9*). Recently, *Xenopus* MPF has been purified to near homogeneity and shown to contain two components (*10*). Importantly, one of the components, a 32-kD polypeptide, has been shown to be a *Xenopus* homolog of the fission yeast *cdc2* gene product, p34^{cdc2}, which is a key element controlling entry into mitosis (*11, 12*). MPF is therefore almost certainly a fundamental and universal regulator of M phase or G_2 to M transition in both meiosis and mitosis in eukaryotes (*2, 3*).

Xenopus MPF exists in immature oocytes in an inactive form (pre-MPF) (*2, 13, 14*), and during the first few hours after progesterone treatment, de novo protein synthesis is required for MPF activation (*2, 3*). An "initiator" for MPF activation (*15*) must be synthesized early and in low abundance because a detectable increase in protein synthesis occurs only after MPF is activated, at a time when cycloheximide no longer inhibits GVBD (*2, 3, 13*). Identification of the initiator is essential to understand the molecular mechanisms leading to activation of pre-MPF.

We have shown that *mos* proto-oncogene transcripts are expressed at high levels in adult gonadal tissue (*16–19*) and are specifically associated with germ cells (*20–23*). Synthesis of the endogenous *Xenopus mos* product, pp39^{mos}, occurs during progesterone-induced oocyte maturation and is required for GVBD (*18*). The *mos* product has

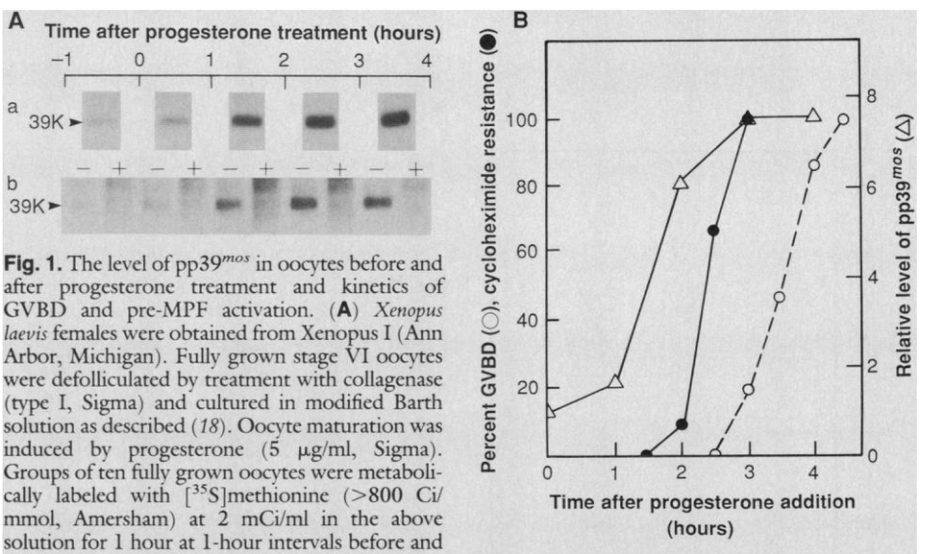

Fig. 1. The level of pp39^{mos} in oocytes before and after progesterone treatment and kinetics of GVBD and pre-MPF activation. (**A**) *Xenopus laevis* females were obtained from Xenopus I (Ann Arbor, Michigan). Fully grown stage VI oocytes were defolliculated by treatment with collagenase (type I, Sigma) and cultured in modified Barth solution as described (*18*). Oocyte maturation was induced by progesterone (5 μg/ml, Sigma). Groups of ten fully grown oocytes were metabolically labeled with [^{35}S]methionine (>800 Ci/mmol, Amersham) at 2 mCi/ml in the above solution for 1 hour at 1-hour intervals before and after progesterone treatment. Procedures for immunoprecipitation and SDS–polyacrylamide gel electrophoresis (PAGE) analyses have been described (*18*). Intracellular pools of free [^{35}S]methionine were estimated from aliquots before immunoprecipitations. Uptake of [^{35}S]methionine was similar up to 3 hours after progesterone treatment, but was 40% lower during the fourth hour, and this sample was normalized accordingly. Oocyte extracts from the equivalent of four oocytes were subjected to immunoprecipitation analysis with either (a) a monoclonal antibody (termed 5S) raised against the *Xenopus* c-*mos* product expressed in *Escherichia coli* (*18*), or (b) a peptide antiserum C232 (*18*) in the presence (+) or absence (−) of the peptide antigen. (**B**) Thirty oocytes of the same batch described above were scored for GVBD every 30 min after progesterone addition (○). Groups of 20 oocytes were also treated with cycloheximide (100 μg/ml) at 30-min intervals after progesterone addition. After 12 hours, oocytes were examined for percent of GVBD, which is expressed as cycloheximide resistance of GVBD (●). Densitometric measurements of autoradiographs as shown in (A) and performed in parallel is shown (△). These data are derived from three independent experiments.

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been shown to function at a later stage during mouse oocyte maturation (*24*). These results suggest that in vertebrates the *mos* proto-oncogene product is required for MPF activity (*18, 24*). Its unique induction during maturation led us to ask how soon after progesterone treatment pp39^{mos} can be detected (Fig. 1A). Low levels of pp39^{mos} were detected in fully grown oocytes during a 1-hour labeling period (*24a*), whereas 1 hour after progesterone addition, the amount increased slightly. By the second hour, the level of pp39^{mos} increased approximately sixfold and approached the high levels observed during both the third and fourth hours (Fig. 1A). In continuous labeling experiments, pp39^{mos} is only detected during progesterone-induced oocyte maturation (*18*). The increase in the level of *mos* product was most likely due to increased translation, but changes in stability have not been ruled out.

In the same group of oocytes, GVBD was first observed between 2.5 and 3 hours after progesterone treatment, and by 3.5 hours, GVBD occurred in 50% ($GVBD_{50}$) of the oocytes (Fig. 1B). We also determined the time period after progesterone treatment when GVBD occurs in the presence of cycloheximide, a time that corresponds to pre-MPF activation (*3, 15*). After progesterone addition, cycloheximide was added to oocytes at half-hour increments, and oocytes were examined 12 hours later for GVBD. During the first 2 hours cycloheximide prevented GVBD, but 65 and 100% of the oocytes matured normally when the inhibitor was addeded at 2.5 and 3 hours, respectively (Fig. 1B). Thus, after 2 hours GVBD becomes protein-synthesis independent. The ratio of this time to the $GVBD_{50}$ (3.5 hours) is ~0.6, and is equivalent to ratios obtained by others for pre-MPF activation (*2, 3*). The significant increase in pp39^{mos} preceded both GVBD and pre-MPF activation and occurred during the second hour after progesterone treatment (~0.3 to 0.4 $GVBD_{50}$).

To test whether the *mos* product can activate maturation in the absence of progesterone, c-*mos*^{xe} RNA was transcribed from pSP64 vector with or without a cap structure. The translation product of this RNA was characterized in vitro before microinjection into oocytes, and two polypeptides (38 and 25 kD) were specifically recognized by C232 antiserum (*25*). The former (p38) was slightly smaller than endogenous pp39^{mos} (*18*), whereas the latter (p25) was presumably derived from initiation at an internal ATG in *mos* RNA (*17*). Oocytes treated with progesterone revealed the endogenous pp39^{mos} band of moderate intensity (Fig. 2A), whereas oocytes microinjected with capped c-*mos*^{xe} RNA showed two *mos* bands (p38^{mos} and pp39^{mos}). The lower band migrates with the p38^{mos} primary translation product detected in reticulocyte lysates (*25*), whereas the upper band most likely corresponds to a phosphorylated form of pp39^{mos} (*18*). The intensity of both bands was about ten times as great as the intensity of endogenous pp39^{mos} in progesterone-treated oocytes. In this system, capped RNA has been shown to be more efficiently translated (*26*), and oocytes injected with uncapped c-mos^{xe} RNA (Fig. 2A) do not show more pp39^{mos} than is detected in fully grown oocytes (Fig. 1A).

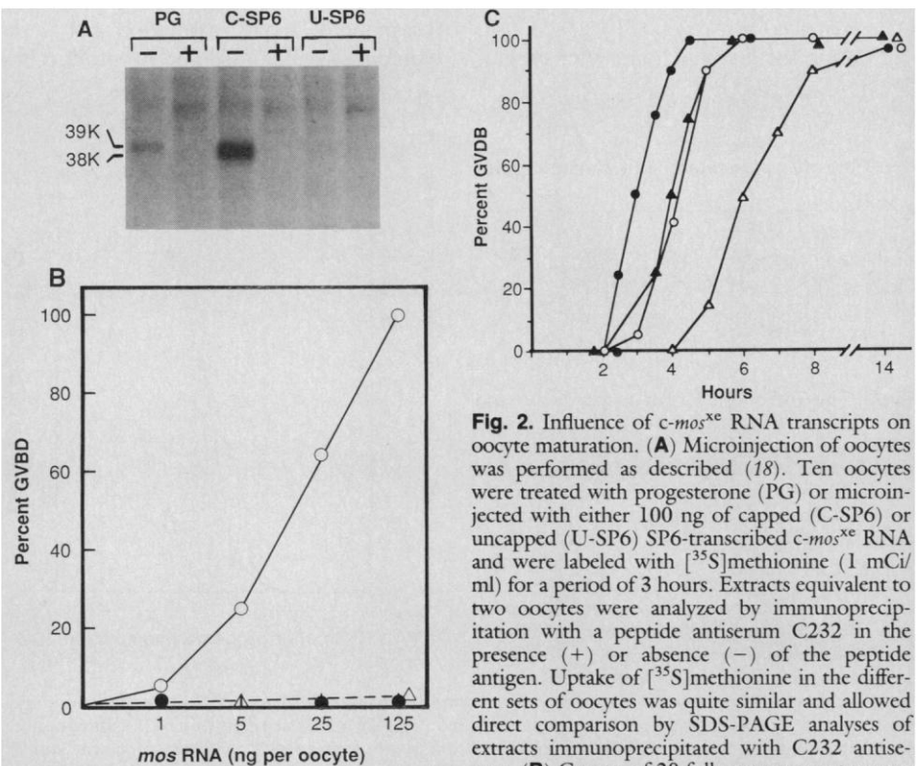

Fig. 2. Influence of c-*mos*^{xe} RNA transcripts on oocyte maturation. (**A**) Microinjection of oocytes was performed as described (*18*). Ten oocytes were treated with progesterone (PG) or microinjected with either 100 ng of capped (C-SP6) or uncapped (U-SP6) SP6-transcribed c-*mos*^{xe} RNA and were labeled with [^{35}S]methionine (1 mCi/ml) for a period of 3 hours. Extracts equivalent to two oocytes were analyzed by immunoprecipitation with a peptide antiserum C232 in the presence (+) or absence (−) of the peptide antigen. Uptake of [^{35}S]methionine in the different sets of oocytes was quite similar and allowed direct comparison by SDS-PAGE analyses of extracts immunoprecipitated with C232 antiserum. (**B**) Groups of 20 fully grown oocytes were microinjected as described above with either capped (○) or uncapped (△) c-*mos*^{xe} RNA at the indicated doses. Capped c-*mos*^{xe} RNA preannealed with an excess (30 ng) of *mos*-specific antisense oligodeoxyribonucleotide G^{-} (*18*) was also microinjected (●). After 12 hours, each oocyte was examined for GVBD both externally and internally (*18*). Under these conditions, 100 to 125 ng of capped c-*mos*^{xe} RNA induced 100% GVBD. (**C**) The time of appearance of GVBD was determined for groups of 20 to 30 oocytes that were: treated with progesterone (○); microinjected with c-*mos*^{xe} RNA (△); simultaneously treated with progesterone and microinjected with c-*mos*^{xe} RNA (▲); and microinjected with c-*mos*^{xe} RNA but treated with progesterone 1 hour later (●) (RNA injections contained 100 ng of capped c-*mos*^{xe} RNA). The data are an average of six independent experiments.

Capped c-*mos*^{xe} RNA induced GVBD in a dose-dependent manner (Fig. 2B). Oocytes microinjected with uncapped c-*mos*^{xe} RNA (even at 125 ng) only yielded 5% GVBD, which was consistent with the lack of pp39^{mos} expression in similarly treated oocytes (Fig. 2A). Moreover, capped c-*mos*^{xe} RNA preannealed with a *mos* antisense of oligodeoxyribonucleotide was also unable to induce GVBD (Fig. 2B). Collectively these results demonstrate that overexpression of pp39^{mos} triggers GVBD in the absence of progesterone.

Oocytes treated with progesterone underwent GVBD between 2 and 5.5 hours after treatment (Fig. 2C), whereas GVBD started at 4 hours and reached nearly 100% at 8 hours in oocytes injected with c-*mos*^{xe} RNA (125 ng per oocyte). Thus, c-*mos*^{xe} RNA induces GVBD, but the kinetics of induction are slower than with progesterone treatment. Ooctyes microinjected with c-*mos*^{xe} RNA and simultaneously treated with progesterone showed no significant acceleration and had similar GVBD kinetics as oocytes treated with progesterone alone (Fig. 2C). These data indicate that there must be additional rate-limiting progesterone-dependent steps involved in maturation. However, if fully grown oocytes are preincubated with c-*mos*^{xe} RNA for 1 hour and then stimulated with progesterone, GVBD is accelerated and the time required for GVBD is reduced ~25% (Fig 2C). Thus preincubation with pp39^{mos} diminishes the requirement for one or more progesterone-dependent steps.

We next tested whether the appearance of MPF activity correlated with the presence of

Table 1. MPF activation in c-*mos*^{xe} RNA or oligodeoxyribonucleotide-injected oocytes. Groups of ten donor oocytes were treated with progesterone, or microinjected with 125 ng of capped c-*mos*^{xe} RNA (C-SP6), or microinjected with mixtures of *mos* sense (S/P) or antisense (AS/P) oligonucleotides. [The mixtures contained as previously described (*18*) 130 ng each of either A^+, B^+, C^+, and D^+ for sense or A^-, B^-, C^-, and D^- for antisense oligodeoxyribonucleotides. After 4 hours of preincubation, the oligodeoxyribonucleotide-containing oocytes were treated with progesterone.] All groups of oocytes were cultured for 6 hours after progesterone treatment or c-*mos*^{xe} RNA microinjection, and crude MPF extracts were prepared (*34*). The variation where observed is given. *n*, number of experiments.

Treatment	Percent GVBD		*n*
	Donor	Recipient	
Progesterone	100	100	2
C-SP6	20 ± 5	35 ± 10	3
S/P	90 ± 8	100	3
AS/P	0	0	3

pp39^{mos}. Oocytes were either treated with progesterone after preinjection of sense or antisense oligodeoxyribonucleotides as before [the latter prevent pp39^{mos} expression and GVBD (*18*)] or injected with capped c-*mos*^{xe} RNA as described in Fig. 2. Six hours after progesterone treatment or c-*mos*^{xe} RNA injection, cytosolic extracts extracts were obtained and tested for the presence of MPF by injection into fully grown cycloheximide-treated oocytes.

Extracts from the control, progesterone-induced oocytes induced 100% GVBD in the recipient oocytes, whereas extracts from c-*mos*^{xe} RNA–injected oocytes induced 35% GVBD (Table 1). The lower value of the latter is consistent with the lower percentage of donor oocytes exhibiting GVBD compared to the progesterone-treated donor oocytes (Table 1). Extracts from oocytes injected with sense oligo-deoxyribonucleotides induced 100% GVBD in the recipient cycloheximide-treated oocytes, whereas those microinjected with *mos* antisense oligodeoxyribonucleotides did not induce GVBD nor did extracts from control uninjected oocytes (Table 1). These results show that pp39^{mos} is required for activating pre-MPF, and overexpression of the product can induce pre-MPF activation in the absence of progesterone. Since the amount of pp39^{mos} increases early after addition of progesterone (Fig. 1A) and the increase precedes activation of pre-MPF, pp39^{mos} has the properties of a putative initiator protein. However, the *mos* product persists throughout maturation (*18, 24*) and appears to be required at a later stage in maturing mouse oocytes (*24*).

Certain gene products, such as the regulatory subunit of adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (*27*), the *ras* protein (*28*), and invertebrate cyclin mRNA (*29*), have been shown to induce GVBD when microinjected into *Xenopus* oocytes in the absence of progesterone. Other products such as *src* protein (*30*) and nerve growth factor receptor mRNA (*31*) can accelerate the rate of progesterone-induced oocyte maturation when exogenously introduced. Thus, in some way, all of these products influence MPF activity. However, thus far only the *mos* product has been shown to function endogenously during progesterone-induced maturation.

We have been interested in determining the normal function of the *mos* proto-oncogene product for it is likely that this will help explain what specific properties are responsible for its activity as an oncogene. Parallels may exist between the induction of oocyte maturation by *mos* overexpression and the expression of the transformed phenotype as a result of constitutive *mos* expression in somatic cells (*24*). We have postulated (*18, 24*) that *mos* oncogene expression in somatic cells may activiate, directly or indirectly, components of MPF prematurely and that transformation and the transformed phenotype may be due to the expression of mitotic phenotypes at nonmitotic stages of the cell cycle (*18*). Similar arguments have been made for the c-*src* product (*32*). MPF activation through overexpression of pp39^{mos} shows some similarity with regulators of *cdc2* in yeast *Schizosaccharomyces pombe* such as *nim1* (*33*). The *nim1* product is a protein kinase that suppresses the function of mitotic inhibitor *wee1* and induces entry into mitosis probably via *cdc2* function (*33*).

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24a. Although a low level of pp39^{mos} in fully grown oocytes (Fig. 1a) was observed in collagenase-treated oocytes, pp39^{mos} was not detectable in manually defolliculated oocytes. The high level of pp39^{mos} in progesterone-treated oocytes was the same for those oocytes that were defolliculated by either method [N. Sagata, unpublished observations].

25. The *Xenopus* c-*mos*^{xe} coding region was inserted into pSP64 [D. A. Melton and P. A. Krieg, *Nucleic Acids Res.* **12**, 7035 (1984)] between the Sac I and Eco RI polylinker sites with a synthetic double-stranded oligodeoxyribonucleotide that corresponds to the first 12 codons of the c-*mos*^{xe} coding sequence (*21*). Into this oligodeoxyribonucleotide (CACCATGGCTTCCCC-AATCCCCGTGGAGCGTTTCCTGCCGC), an initiator ATG codon (underlined) in the optimal sequence context [M. Kozak, *Microbiol. Rev.* **47**, 1 (1983)] was introduced changing the second CCT codon (proline) to a GCT codon (alanine). This was inserted between the Sac I site of pSP64 and the Sac II site (position 34) in the c-*mos*^{xe} coding region (*18*). The 3′ end of the c-*mos*^{xe} sequence (Bal I site 200 base pairs downstream of the *mos* stop codon) (*18*) was ligated to the Eco RI site of pSP64 with an Eco RI linker. The structure of the construct was linearized at the unique Bgl I site in pSP64 and was transcribed in vitro into c-*mos*^{xe} RNA with or without a cap structure according to the method recommended by the supplier (Promega). Uncapped SP6 c-*mos*^{xe} RNA was in vitro translated in a reticulocyte lysate in the presence of [^{35}S]methionine. Uninjected control oocytes or oocytes microinjected with 50 ng of capped SP6 transcribed c-*mos*^{xe} RNA were metabolically labeled with [^{35}S]methionine (1 mCi/ml) for 3 hours. Translation products of both reticulocyte lysates and oocytes were analyzed by immunoprecipitation (as described in Fig. 1 caption) with either C232 or C237 peptide antiserum (*18*) in the presence (+) or absence (−) of the peptide antigen (data not shown).

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34. Crude MPF preparations were obtained from oocytes treated by a modification of the method described by Dabauvalle *et al.* [M. C. Dabauvalle, M. Doree, R. Bravo, E. Karsenti, *Cell* **52**, 525 (1988)]. Briefly, ten oocytes in an Eppendorf tube were homogenized in 20 μl of a buffer containing 80 m*M* β-glycerophosphate, 15 m*M* EGTA, and 10 m*M* $MgCl_2$ (*p*H 7.3) and centrifuged at 14,000*g* for 5 min at 4°C. This supernatant was used for microinjection. To assay for MPF activity, 20 recipient oocytes treated for 1 hour with cycloheximide (100 μg/ml) were injected with 65 nl of the supernatant from each group of donor oocytes. After 12 hours of cultivation in the presence of cycloheximide, recipient oocytes were scored for GVBD.

35. We thank S. Fujiwara for densitometric measurement of the X-ray films and M. Zweig for electroelution of protein used as antigen. We also thank R. Paules, F. Propst, and N. Yew for valuable discussion and P. Hall for manuscript preparation. Research sponsored in part by the National Cancer Institute, Department of Health and Human Services (DHHS), under contract NO1-CO-74101 with Bionetics Research, Inc. The contents of this publication do not necessarily reflect the views or policies of the DHHS, nor does mention of trade names,

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Preferential Heterodimer Formation by Isolated Leucine Zippers from Fos and Jun

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The products of the nuclear oncogenes *fos* and *jun* are known to form heterodimers that bind to DNA and modulate transcription. Both proteins contain a leucine zipper that is important for heterodimer formation. Peptides corresponding to these leucine zippers were synthesized. When mixed, these peptides preferentially form heterodimers over homodimers by at least 1000-fold. Both homodimers and the heterodimer are parallel α helices. The leucine zipper regions from Fos and Jun therefore correspond to autonomous helical dimerization sites that are likely to be short coiled coils, and these regions are sufficient to determine the specificity of interaction between Fos and Jun. The Fos leucine zipper forms a relatively unstable homodimer. Instability of homodimers provides a thermodynamic driving force for preferential heterodimer formation.

FOS AND JUN EACH CONTAIN A LEUcine zipper region in which five leucine residues are spaced periodically at every seventh residue (*1, 2*). The leucine zipper regions of these proteins are known to be important for heterodimer formation (*3–7*), as originally proposed in the leucine zipper model (*2*). Jun can also form a homodimer (*3, 4, 8*), whereas there has been no evidence for dimerization of Fos (*3, 4, 7, 8*).

A synthetic peptide, corresponding to the isolated leucine zipper of the yeast transcriptional activator GCN4, self-associates to form a very stable dimer of parallel α helices, probably as a short, parallel coiled coil (*9*). Both Fos and Jun show sequence homology to GCN4 in regions within and adjacent to the leucine zipper (*10*). We investigated whether the leucine zipper regions of Fos and Jun are sufficient by themselves to mediate specific heterodimer formation.

Peptides corresponding to the leucine zippers (Fos-p1N and Jun-p1N) were synthesized (*11*). In each case, a Cys residue (to permit S–S bond formation) and two Gly residues (for flexibility) were added at the NH_2-terminus (Fig. 1A). Equimolar amounts of the peptides were mixed in a redox buffer (which favors S–S bond formation), and the amounts of S–S homodimers and heterodimer were measured by high-performance liquid chromatography (HPLC).

The results (Fig. 1B) show that only heterodimers are detected. This reaction is at equilibrium since an equimolar mixture of S–S Fos-p1N and Jun-p1N homodimers also rearranges to give only heterodimer (Fig. 1C). In both cases, a homodimer would have been detected if it were present at 1/1000 the concentration of the heterodimer (*12*). Only when oxidation of the peptides is performed in conditions where the peptides are unfolded [6.8*M* guanidine hydrochloride (GuHCl)] are homodimers observed.

Circular dichroism (CD) spectra of all three S–S dimers display minima at 208 and 222 nm (Fig. 2A), indicating that they are predominantly α helical (*13*). All of the S–S dimers are also resistant to thermal denaturation (*14*). Thus the leucine zippers of Fos and Jun, like that of GCN4 (*9*), appear to be helical dimerization sites (*15*).

The orientation of the helices in the peptide dimers was determined by monitoring the stability of the S–S species as a function of peptide concentration. The S–S bond joins the peptides in a parallel manner. If the helix orientation is parallel, then the stability of the S–S dimers should be independent of peptide concentration. If the orientation is antiparallel, S–S dimers should associate and show concentration-dependent stability. [Such higher order association is observed when leucine zippers of GCN4 are forced to be antiparallel (*16*).] The stabilities of the S–S Fos-p1N homodimer, Jun-p1N homodimer, and Fos-p1N–Jun-p1N heterodimer are independent of peptide concentration [(*14*) Fig. 2B], indicating that the helix

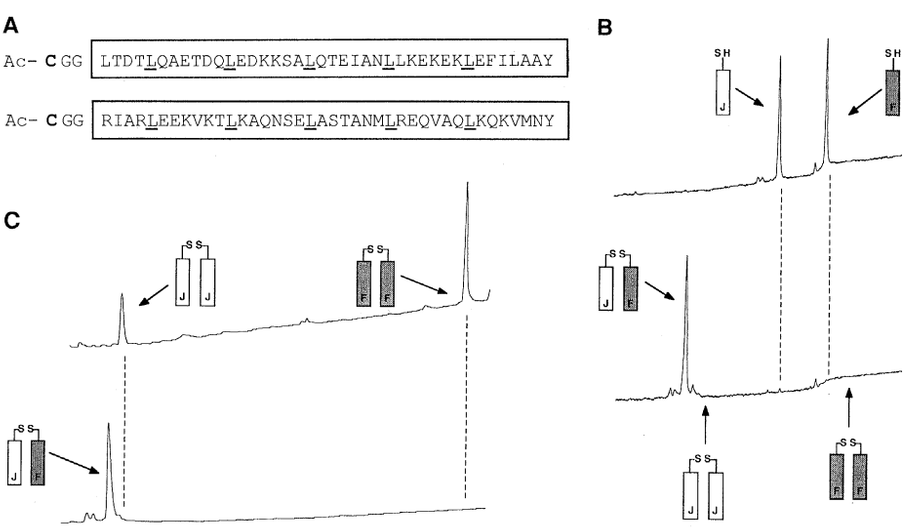

Fig. 1. Preferential heterodimer formation by isolated leucine zipper peptides from Fos and Jun. (**A**) Sequences of the peptides (Fos-p1N, top, and Jun-p1N, bottom) used in the S–S assay (*11*). Residues defining the leucine repeat are underlined. (**B**) HPLC analyses of reduced Fos-p1N + reduced Jun-p1N in 2 m*M* HCl (top) and redox buffer (bottom) (*29*). In redox buffer (where reshuffling of S–S bonds is permitted) only the heterodimer is detected (*12*). The arrows at the bottom indicate the elution positions for the two homodimers. (**C**) HPLC analyses of the S–S Fos-p1N homodimer + Jun-p1N homodimer in 2 m*M* HCl (top) and in redox buffer (bottom) (*29*). Only heterodimer is observed in redox buffer, confirming that the reaction mixtures are at equilibrium. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr.

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