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Research Article

Nuclear translocation of Fos is stimulated by interaction with Jun through the leucine zipper

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Abstract. Jun and Fos, b-ZIP transcription factors, form a heterodimer and bind to DNA enhancer elements, thereby regulating the expression of target genes. The present study was undertaken to investigate the molecular mechanism underlying nuclear translocation of the Jun/Fos complex. For this purpose, normal rat kidney cells were microinjected with a DNA expression vector containing wild-type or mutant c- or v-*jun* together with c- or v-*fos*, followed by detection of the subcellular localization of Jun or Fos by immunofluorescence staining. The nuclear accumulation of Fos was markedly enhanced by the presence of wild-type Jun, but not by Jun mutants lacking nuclear targeting or zipper dimerization functions, implying that Jun and Fos mutually interact via their leucine zippers and translocate from the cytoplasm to the nucleus using the markedly stronger nuclear localization signal of Jun.

Key words. b-ZIP; Jun; Fos; NLS; nuclear import.

Eukaryotic cells are compartmentalized by various membranous organelles that allow proteins to accumulate at their functional locations by specific sorting mechanisms. The nucleus is separated from the cytoplasm by a double membrane system known as the nuclear envelope. Nuclear proteins are translocated from their site of synthesis in the cytoplasm to the nucleus through nuclear pores, present in components of the nuclear envelope (for a review see [1]). Although small molecules pass freely through the nuclear pores by passive diffusion, molecules as large as M_r 40 kDa or more require machinery for active nuclear transport: this includes shuttling proteins such as importin α/β, components of the Ran-guanosine 5′-triphosphate (GTP)/guanosine 5′-diphosphate (GDP) cycle and a nuclear localization signal (NLS) in the protein for being targeted to the nucleus (for a recent review see [2]).

NLSs are short sequences consisting mainly of basic amino acids in either 1 or 2 clusters, known as mono- or bipartite NLSs (for review see [3]). The monopartite NLS has a stretch of 3 to 5 basic amino acid residues, typified by the SV40 T-antigen NLS (Pro-Lys-Lys-Lys-Arg-Lys-Val) [4]. The bipartite NLS consists of 2 basic amino acids at the amino-terminal side, an intervening spacer of 10 amino acids and a stretch of 3 or 4 basic amino acids at the carboxyl-terminal side, typified by the nucleoplasmin NLS [5]. Up to now, functional mono- or bipartite NLSs have been identified in many nuclear proteins including transcription factors.

Jun and Fos are b-ZIP transcription factors that regulate the expression of target genes possessing an AP-1 site (for a review see [6]). A b-ZIP protein contains a

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basic region for DNA binding and a leucine zipper, an amphipathic α-helix in which 4 to 6 leucine residues are positioned at intervals of 7 amino acids, for formation of homo- or heterodimer complexes. Such a structure is primarily identified in transcription factor C/EBP [7]. Jun was originally found as the product (v-Jun) of the oncogene v-*jun* carried by the avian retrovirus ASV17 [8, 9]. The v-Jun of M_r 65 kDa exhibits a cell cycle-dependent nuclear accumulation that is negatively regulated by phosphorylation of the serine residue at amino acid position 248 adjacent to the NLS in chicken embryo fibroblasts [10, 11]. In contrast, the c-*jun* product (c-Jun) of M_r 36 kDa accumulates in the nucleus, in a cell cycle-independent manner [10]. Both v- and c-Jun possess the same NLS in the basic region, consisting of the sequence Arg^{249}-Lys-Arg-Lys-Leu, and interact strongly with other b-ZIP proteins, including c-Fos, CREB and c-Maf, through their leucine zippers resulting in the formation of dimer complexes that regulate gene expression [9, 12–19]. Although there are many reports on DNA binding of dimeric b-ZIP proteins [9, 12–21], the detailed mechanism of the complex formation and their nuclear translocation is not fully understood.

In this study, we examined the possible role of interactions between b-ZIP proteins in nuclear translocation by comicroinjection of plasmid DNA encoding Jun and Fos into living cells. We found that Fos is most efficiently translocated from the cytoplasm to the nucleus by interaction with c- or v-Jun through their leucine zippers, and mainly by the use of the NLS of Jun.

Materials and methods

Expression vectors of *jun* and *fos*. The complementary DNAs (cDNAs) of v-*jun* (VJ0) and chicken c-*jun* (CJ3) were gifts from Dr. P. K. Vogt (Scripps Research Institute, La Jolla, CA, USA). After digestion with *Cla*I, the entire cDNA of v- or c-*jun* was blunt-end ligated into the *Eco*RV site of a mammalian expression vector pcDNA1 (Invitrogen, San Diego, CA, USA) (then designated pcDNA1-v-*jun* or pcDNA1-c-*jun*, respectively). An NLS-deficient mutant of v-*jun* (pcDNA1-v-*jun*-mtNLS) was constructed by replacing the functional NLS (Arg-Lys-Arg-Lys-Leu) with a defective sequence (Arg-Asn-Asn-Lys-Leu), using a commercial site-directed mutagenesis (Pharmacia Biotech, Tokyo, Japan) with the oligonucleotide, 5′-ccaaaagccggaataataagttg-gaaagg-3′ (mutations are underlined). The original leucine zipper-deficient mutant of v-*jun* (L3P) was a gift from Dr. M. Nishizawa (Kyoto University, Kyoto, Japan) [21]. A fragment including the defective leucine zipper of L3P was transferred to the corresponding site of the pcDNA1-v-*jun* (pcDNA1-v-*jun*-L3P). A double mutant of v-*jun* defective in both the NLS and leucine zipper (pcDNA1-v-*jun*-mtNLS-L3P) was then constructed from v-*jun*-L3P by site-directed mutagenesis as mentioned above. A mouse c-*fos* expression vector, SVE-c-*fos* [22], was obtained from Dr. S. Hirai (Yokohama City University, Yokohama, Japan). The avian retroviral v-*fos*- and mutant v-*fos*-containing vectors, pEF-v-*fos* and pEF-v-*fos*-L3P were gifts from Dr. K. Kataoka (University of Tokyo, Tokyo, Japan) [21]. Structures of the wild-type and mutant Jun and Fos proteins used in this study are schematically shown in figure 1.

Microinjection of DNA. Normal rat kidney (NRK) cells were grown on glass coverslips (11 × 22 mm) in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal calf serum at 37 °C. Microinjection was performed using an automatic Eppendorf microinjector 5242 and micromanipulator 5171 attached to a Nikon inverted phase-contrast microscope. The plasmid DNA at 0.1 μg/μl in Dulbecco's phosphate-buffered saline (PBS) was injected into the nuclei of about 100 cells within 10 min at room temperature. The conditions were set to inject approximately

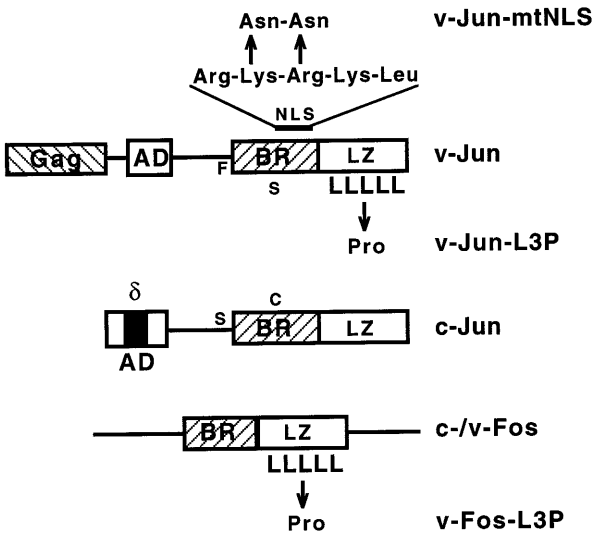

Figure 1. Schematic structures of Jun and Fos mutants used in the present study. The wild-type v-Jun consists of Gag (p19 + Δp10), the activation domain (AD) lacking the δ-region of 27 amino acids, the basic region (BR), the leucine zipper (LZ) and two sporadic mutations, serine (S) to phenylalanine (F) at position 222 and cysteine (C) to serine (S) at 248, from the chicken c-Jun. The NLS-deficient mutant, v-Jun-mtNLS, contains two asparagine residues in place of Lys^{250}-Arg^{251} of the NLS in the basic region. In the leucine zipper-deficient mutant v-Jun-L3P, the third leucine of the leucine zipper is substituted by a proline. The NLS and zipper double mutant is v-Jun-mtNLS-L3P. The c-Jun contains the δ-region in the activation domain. The c-Fos contains a basic region and a leucine zipper. The v-Fos used in this study does not contain the Gag portion [21].

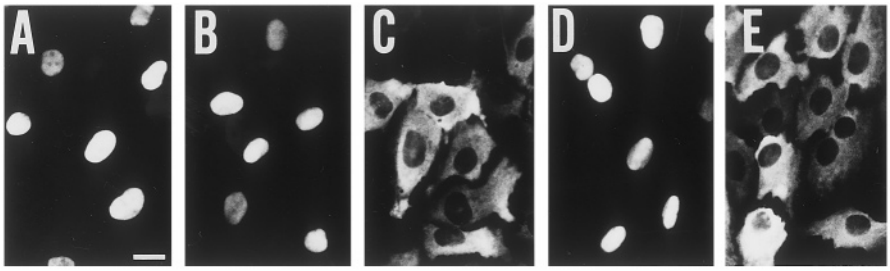

Figure 2. Nuclear accumulation of c-Jun and v-Jun mutants. Immunofluorescence was performed using anti-Jun antibody 2 h after microinjection of c-*jun* (*A*), v-*jun* (*B*), v-*jun*-mtNLS (*C*), v-*jun*-L3P (*D*), or v-*jun*-mtNLS-L3P (*E*). Bar, 20 μm.

1000 copies (50 fl) of the plasmid DNA into the nucleus. After microinjection, the cells were incubated for 2 h at 37 °C in the above medium supplemented with 100 nM staurosporine, which inhibits phosphorylation of the serine residue at 248 of v-Jun, resulting in cell cycle-independent nuclear translocation [11]. The cells were fixed with 4% formaldehyde in PBS at room temperature for 1 h.

Immunofluorescence staining. Rabbit anti-Jun antibody was a gift from Dr. P. K. Vogt. Mouse monoclonal anti-Fos antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. All procedures were performed at room temperature. The fixed cells were washed twice with PBS for 10 min, permeabilized in methanol for 10 min, and treated with 3% goat serum in PBS for 30 min to block nonspecific binding. The cells were incubated with the antibody to Jun or Fos at 10 μg/ml in PBS for 1 h, washed again in PBS, and incubated with anti-rabbit immunoglobulin G (IgG) conjugated to fluorescein isothiocyanate (Zymed, San Francisco, CA, USA) or to Alexa 488 (Molecular Probes, Inc., Eugene, OR, USA), or anti-mouse IgG antibody conjugated to Cy3 (Jackson ImmunoResearch Lab., West Grove, PA, USA) at 20 μg/ml in PBS for 1 h. The cells were washed 3 times with PBS and once with distilled water and mounted on glass slides in a solution of 20% PBS and 80% glycerol. Immunofluorescence was detected at a magnification of 400 × under the fluorescence microscope, BX50 (Olympus, Tokyo, Japan) or the confocal laser scanning microscope, μRadiance AG2 (Bio-Rad, Hertfordshire, UK).

Results and discussion

Rapid gene expression by microinjection. In the present study, a large copy number of plasmid DNA (approximately 1000 copies per cell) was microinjected into the nuclei of NRK cells. The products of the injected genes were rapidly expressed within 2 h under the control of the cytomegalovirus, SV40, and elongation factor 1α promoters in the pcDNA1, SVE and pEF vectors, respectively. The injected cells were subjected to immunofluorescence staining. Under our conditions, the endogenous c-Jun and c-Fos of the NRK cells were not detected by immunofluorescence.

Nuclear accumulation of Jun and its mutants. As shown in figure 2A and B, the specific immunofluorescence signals of v-Jun and c-Jun, respectively, were detected within 2 h after microinjection of the plasmid DNA into the cells. The signals of Jun were found exclusively in the nucleus, whereas no or weak signals were found in the cytoplasm, indicating complete nuclear translocation of both v- and c-Jun. When the plasmid DNAs were transfected via the calcium-phosphate method, similar nuclear accumulation of these products was observed after 24 h (data not shown).

The NLS-deficient v-Jun (v-Jun-mtNLS) failed to localize in the nucleus, and remained in the cytoplasm (figs 2C and 3A). However, the product of v-*jun*-L3P (v-Jun-L3P), a leucine zipper-deficient v-Jun, accumulated almost exclusively in the nucleus (fig. 2D). The double mutant of v-Jun defective in both the NLS and leucine zipper (v-Jun-mtNLS-L3P) was only detected in the cytoplasm (figs 2E and 3D). These mutants were not detected in other subcellular organelles, such as the mitochondria or the nucleoli. These data indicate that the NLS of Jun is required for its nuclear entry, which is consistent with previous reports [11, 23].

Stimulation of nuclear entry of Fos by Jun. Figure 3 illustrates the subcellular distribution of c-Fos. Immunofluorescence signals from c-Fos were detectable under a confocal laser scanning microscope. When c-*fos* was microinjected alone, the c-Fos was detected only weakly in the nucleus, the majority of the signal being detected in the cytoplasm (fig. 3A). Comicroinjection with c-*jun* (fig. 3B, H) or v-*jun* (fig. 3C, I) stimulated the nuclear accumulation of c-Fos in parallel with the nuclear accumulation of c- or v-Jun. On the other hand, the NLS-

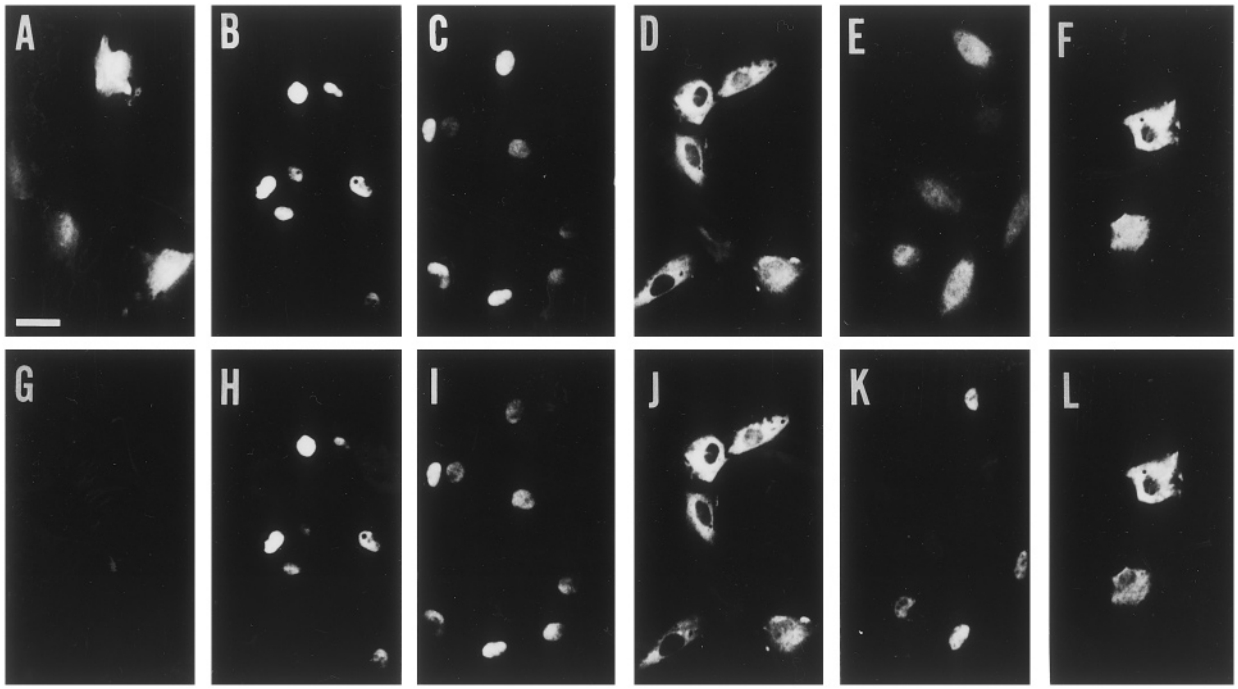

Figure 3. Simultaneous expression of *jun* and *fos*. Localization of c-Fos (*A–F*) and Jun mutants (*G–L*) when c-*fos* was microinjected alone (*A*, *G*) or together with c-*jun* (*B*, *H*), v-*jun* (*C*, *I*), v-*jun*-mtNLS (*D*, *J*), v-*jun*-L3P (*E*, *K*) or v-*jun*-mtNLS-L3P (*F*, *L*). Double-stained images were sectioned optically at 1.0 μm by confocal laser scanning microscopy. Bar, 40 μm.

deficient v-*jun*-mtNLS (fig. 3D, J), the leucine zipper-deficient v-*jun*-L3P (fig. 3E, K) or the double mutant v-*jun*-mtNLS-L3P (fig. 3F, L) did not stimulate but partly inhibited the nuclear accumulation of c-Fos.

Figure 4 shows nuclear translocation of v-Fos and its leucine zipper-deficient mutant (v-*fos*-L3P). When v-*fos* was injected alone, the product v-Fos was detected mainly in the cytoplasm, and only weakly in the nucleus (fig. 4A), as shown for c-Fos (fig. 3A). The zipper-deficient v-Fos-L3P was only detected in the cytoplasm (fig. 4B). Comicroinjection of c-*jun* stimulated nuclear accumulation of the wild-type v-Fos (fig. 4C) but not of the zipper-deficient mutant (fig. 4D). These results indicate that c- or v-Fos is efficiently translocated into the nucleus by interaction with Jun through their leucine zippers and by the use of the NLS of Jun.

Nuclear targeting of Fos is still not fully understood. Roux et al. [24] and Vriz et al. [25] reported that nuclear translocation of c-Fos is regulated by extracellular mitogens and depends on cell-growth conditions. Tratner and Verma [26] reported that human c-Fos contains a bipartite NLS in the basic region, but a mutant lacking the NLS is still localized in the nucleus, suggesting the presence of multiple NLSs in c-Fos as found in the B-*myb* gene product [27] and the p53 tumor suppressor protein [28]. However, rapid transient expression in our study indicated relatively little nuclear translocation of mouse c-Fos and avian retroviral v-Fos. Further studies are needed to understand the functional NLSs in the Fos molecule.

Glover and Harrison [29] reported the crystallographic structure of a heterodimer formed between the b-ZIP regions of Jun and Fos: their leucine zippers interact and form an asymmetric coiled-coil structure, whereas their basic regions interact with DNA. The coiled-coil dimerization of Jun/Fos or C/EBPs in the absence of DNA indicates strong interaction with nanomolar dissociation constants [30, 31]. Recently, Williams et al. reported that C/EBPβ lacking the NLS is localized in the nucleus when coexpressed with C/EBPα [32]. Taken together, this indicates that b-ZIP proteins may form a dimer complex in the cytoplasm before entry into the nucleus.

The present study demonstrated that when a b-ZIP protein encounters an appropriate partner in the cytoplasm, the coupled molecules may enter the nucleus as a complex rather than as a single molecule. A combination of NLSs with varying functions may regulate nuclear entry of b-ZIP complexes and subsequently gene expression.

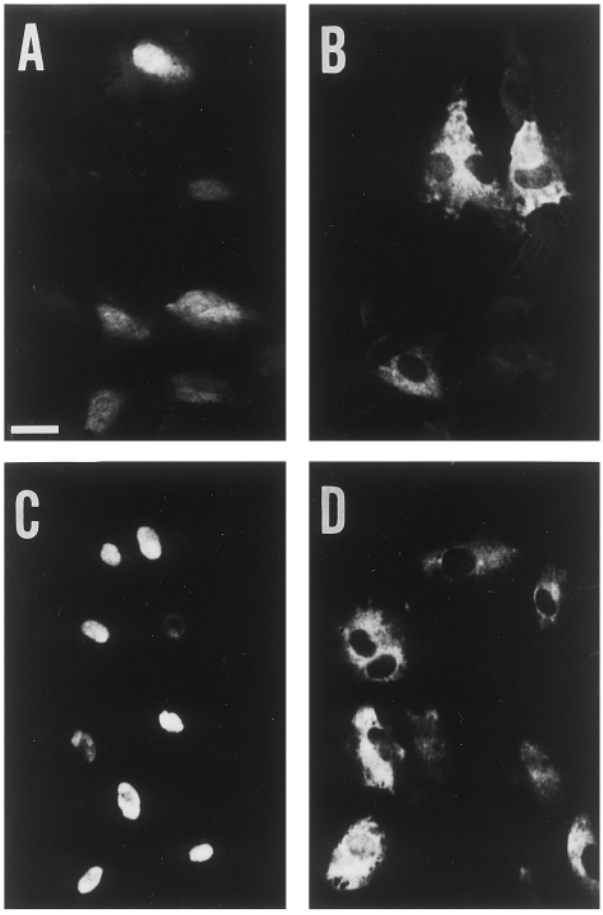

Figure 4. Stimulation of nuclear entry of v-Fos (*A*, *C*), but not of v-Fos-L3P (*B*, *D*) when v-*fos* or v-*fos*-L3P was microinjected alone (*A*, *B*) or together with c-*jun* (*C*, *D*). Images were sectioned optically at 1.0 μm by confocal laser scanning microscopy. Bar, 30 μm.

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