C-Jun phosphorylation (Ser-63) in the testis of the lizard, *Podarcis s. sicula*

P Chieffi[1,2], A Picascia[2], R Stanzione[2] and **D Tramontano[3]**

[1]Dipartimento di Fisiologia Umana e Funzioni Biologiche Integrate 'F Bottazzi' II Università di Napoli, Naples, Italy

[2]Dipartimento di Biologia e Patologia Cellulare e Molecolare 'L Califano', Università di Napoli 'Federico II', Naples, Italy

[3]Dipartimento di Medicina Sperimentale e Clinica 'Gaetano Salvatore', Università di Catanzaro 'Magna Graecia', Catanzaro, Italy

(Requests for offprints should be addressed to P Chieffi, Dipartimento di Biologia e Patologia Cellulare e Molecolare, via Pansini, 5, 80131 Napoli, Italy; Email: chieffi@unina.it)

Abstract

Proto-oncogenes play an important role in the regulation of cellular growth and differentiation. C-Jun activity has been studied in the testis of a non mammalian vertebrate, the lizard *Podarcis s. sicula*, during two different periods: winter stasis and the breeding season. C-Jun protein was localized by immunocytochemistry in the cytoplasm of the spermatogonia (SPG) and stage I and II spermatocytes (SPC) during the winter stasis (from December until March), while the protein was present in the nuclei of the same cells during the active spermatogenic period (April/May). The different localization of c-Jun has been confirmed by Western blot and immunoprecipitation analysis. In addition, when Jun is present in the nuclear compartment, it is phosphorylated on Ser-63 and is complexed with Fos protein.

These data suggest that the nuclear localization of the Jun protein in the SPG and stage I and II SPC, with strong phosphorylation on Ser-63 during the breeding period, could be the signal of increasing transcriptional activity in the lizard testis.

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Introduction

Spermatogenesis is a hormonally regulated and unique developmental process whereby diploid stem cells differentiate through an ordered sequence of steps into haploid spermatozoa, which are highly specialized in structure and function. This process can be divided into mitotic, meiotic, and postmeiotic phases that are synchronized and are an ideal model for studying the control of cellular growth and differentiation. The molecular mechanisms that regulate and coordinate the expression of genes throughout spermatogenesis in vertebrates are not completely understood.

The expression of many different proto-oncogenes during male germ cell development has been investigated (Iawaoki *et al.* 1993, Schultz *et al.* 1995). mRNAs for c-Raf, c-Ras H, c-Ras N, and c-Ras K have been detected throughout the spermatogenic cell cycle (Wolfes *et al.* 1989). The c-Myc, c-Fos, and c-Jun (Wolfes *et al.* 1989) proto-oncogenes are expressed primarily during the premeiotic phase of mouse spermatogenesis. More specifically, high levels of c-Jun transcripts are found in isolated populations of type B spermatogonia and lower c-Jun mRNA levels are found in germ cells from earlier and later stages of the spermatogenic cycle (Wolfes *et al.* 1989).

Activator protein 1 (AP-1) is a sequence-specific transcription factor composed of either homo- or heterodimers among members within the Jun family (c-Jun, JunB, and JunD) or among proteins of the Jun and Fos (c-Fos, FosB, Fra1, and Fra2) families (for review see Angel & Karin 1991). Among them, c-Jun is the major component of the AP-1 complex (Bohmann *et al.* 1987, Angel *et al.* 1988) and c-Fos is its best known partner (Karin 1995, Karin *et al.* 1997). C-Jun was originally isolated as the cellular homolog of v-Jun, the oncogene in avian sarcoma virus 17 (Maki *et al.* 1987). C-Jun transcriptional activity is enhanced by amino-terminal phosphorylation on Ser-63/73 (Pulverer *et al.* 1991, Smeal *et al.* 1991). This inducible phosphorylation is mediated by members of the Jun amino-terminal kinase (JNK, also known as stress-activated protein kinase, SAPK) subfamily (Hibi *et al.* 1993, Dérijard *et al.* 1994, Kyriakis *et al.* 1994, Minden *et al.* 1994).

Seasonal breeders, in which morphological and physiological changes in the testis are a normal part of an annual reproductive cycle, may be a suitable system to provide insight into the putative role played by hormonally activated proto-oncogenes during spermatogenesis. In the fox, *Vulpes vulpes* (Cohen *et al.* 1993), frog, *Rana esculenta* (Chieffi *et al.* 1995), and lizard, *Podarcis s. sicula* (Chieffi

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et al. 1997) proto-oncogene activity is differentially expressed during the different phases of the annual testicular cycle.

Therefore, c-Jun activity, and in particular its phosphorylation on Ser-63, was determined in the testis of the lizard, *Podarcis s. sicula* during two different periods of the reproductive cycle: winter stasis and the breeding season.

Materials and Methods

Animals

Intact male lizards (*Podarcis s. sicula*) were collected in the vicinity of Naples during two different periods (n=20/month): winter stasis (from December until March) and the breeding season (April and May). The lizards were killed after anesthesia with MS222 (Sigma Chemical Co., St Louis, MO, USA) and the testes were immediately removed and stored at − 80 °C until processed for protein preparation or quickly prepared for histological examination.

Protein extract preparation

Frozen tissues were homogenized directly in cold TEDG lysis buffer containing 50 mM Tris–HCl pH 7·5, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonylfluoride (PMSF), 10% glycerol, 1% Triton-X-100) (1:2 w:v), and protease inhibitors (1 mM PMSF, 1 μM aprotinin, 0·5 mM sodium orthovanadate, 20 mM sodium pyrophosphosphate; Sigma Chemical Co.). Large particulate material, including the cell nuclei was removed by centrifugation at 800 × ***g*** for 40 min and retained for the preparation of nuclear protein extracts. The supernatant fraction was carefully removed and centrifuged at 100 000 × ***g*** for 40 min at 4 °C to obtain cytosolic extracts. The crude nuclear pellet was subjected to a washing step to remove residual soluble material and was then resuspended in TKM buffer (5 mM Tris–HCl pH 7·5, 5 mM $MgCl_2$, 2·5 mM KCl, 250 mM sucrose) in the presence of protease inhibitors, filtered through a sterile gauze, and the sucrose concentration was increased to 1·6 M. Samples were stratified on 2·2 M sucrose and centrifuged at 60 000 × ***g*** for 1 h at 4 °C. The residual pellets were resuspended in a small volume of TKM buffer and sonicated. Protein concentrations of cytoplasmic and nuclear extracts were estimated by BioRad (Melville, NY, USA) assay.

Antibodies

Antibodies were purchased from the following sources: anti-c-Jun (# sc-1694), anti-JNK1 (# sc-474-G), c-Jun (79) (cat # sc-4113) cognate peptide from Santa Cruz Biotechnology, Santa Cruz, CA, USA; phospho c-Jun Ser-63 (# 9261L) from New England Biolab, Beverly, MA, USA; anti c-Fos (OA-11–822), c-Fos (OP-11–3210) cognate peptide from Cambridge Research Biochemicals, Northwich, Cheshire, UK.

Immunocytochemistry

Lizard testes, rapidly removed and fixed in Bouin's fluid, were dehydrated in ethanol, cleared in xylene, and embedded in paraffin wax. Tissue sections (5 μm) were processed by the peroxidase-antiperoxidase technique (PAP). Ten sections/animal/month were examined. The sections were treated for 20 min with H_2O_2 to block endogenous peroxidase. Incubations were performed at 4 °C in a moist chamber for 16 h with the primary antisera. These were diluted 1:250 to 1:500 in 0·1 M phosphate-buffered saline (PBS) at pH 7·4 containing 1% swine serum. After washing in PBS, the sections were incubated for 1 h with rabbit PAP complex (Dakopatts). The antigens were visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co.) and 0·3% H_2O_2 in PBS solution. To check the specificities of the immunoreactions, controls omitting the primary antibodies or using the primary antiserum preabsorbed with an excess (10^{-6} M) of the cognate peptide were used.

Immunoprecipitation and Western blot

For immunoprecipitations 50 μl protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden) were incubated with 5 μl rabbit anti-c-Jun or anti-JNK1 antibodies for 1 h at 4 °C, and subsequently incubated with equal amounts of protein (500 μg) for 2 h at 4 °C. Immunoprecipitates were washed four times with HTNG (20 mM Hepes, 150 mM NaCl, 0·1% Triton-X-100, 5% glycerol) and boiled in Laemmli's buffer (prepared according to Laemmli 1970) for 5 min before electrophoresis. Immunoprecipitates were subjected to SDS-PAGE (10% polyacrylamide gel) under reducing conditions. After electrophoresis, proteins were transferred to nitrocellulose membranes (Immobilon; Millipore Corporation, Bedford, MA, USA); complete transfer was assessed using prestained protein standards (BioRad).

For Western blots, proteins (40 μg/lane) were separated using 0·1% SDS/10% polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes (Immobilon; Millipore Corporation) and complete transfer was assessed as previously described. The membranes were blocked with 5% BSA (in Tris-buffered saline: 10 mM Tris–HCl pH 7·6, 150 mM NaCl) and then incubated for 1 h at room temperature with the primary antibody against: (1) c-Jun (diluted 1:4000), (2) phospho

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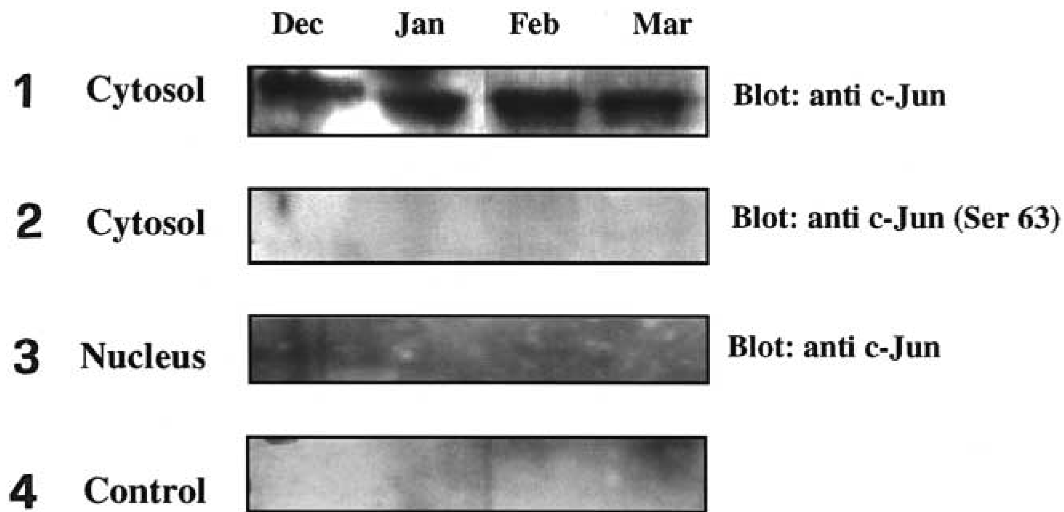

Figure 1 Western blot detection of c-Jun protein in the cytosolic and nuclear testicular extracts of *Podarcis s. sicula* during the winter stasis (December to March). Proteins (40 μg/lane/month) were resolved by SDS-PAGE, transferred to nitrocellulose membranes and then incubated with antibody raised against c-Jun protein (1, 3) and c-Jun Ser-63 protein (2), and with the same antibody preabsorbed with an excess amount (10^{-6} M) of the antigen (4). A specific band of about 39 kDa was observed in the cytosol by comparison with comigrating size markers. The blots are representative of four separate assays.

c-Jun Ser-63 (diluted 1:4000), (3) c-Fos (diluted 1:5000), (4) JNK1 (diluted 1:2000) or with the primary antibodies preabsorbed with 10^{-6} M of the antigen overnight at 4 °C on an orbital shaker. The membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (1:2000) for 45 min at room temperature and the reactions were detected with an enhanced chemiluminesence system (ECL; Amersham Life Science, Little Chalfont, Bucks, UK).

Kinase assay

Frozen tissues were lysed at 4 °C in a buffer containing 25 mM Hepes pH 7·5, 0·3 M NaCl, 1·5 mM $MgCl_2$, 0·2 mM EDTA, 0·5 mM dithiothreitol, 1% Triton-X-100, 0·5% sodium deoxycholate, 0·1% SDS, 20 mM β-glycerophosphate, 1 mM PMSF, and 20 μg/ml leupeptin. The epitope-tagged JNK was immunoprecipitated from the cleared lysates (500 μg) by incubation with the specific antibody anti-JNK1 (as previously described) for 1 h at 4 °C. Immunocomplexes were recovered with the aid of Gamma-Bind Sepharose beads (Pharmacia) and washed three times with PBS containing 1% NP-40 and 2 mM sodium vanadate, once with 100 mM Tris (pH 7·5), 0·5 M LiCl, and once with kinase reaction buffer (12·5 mM MOPS pH 7·5, 12·5 mM β-glycerophosphate, 7·5 mM $MgCl_2$, 0·5 mM EGTA, 0·5 mM sodium fluoride, 0·5 sodium vanadate). The JNK activity present in the immunoprecipitates was determined by resuspension in 30 μl kinase reaction buffer containing 1 μCi [γ-^{32}P]ATP per reaction and 20 μM unlabeled ATP, using 1 μg GST-c-Jun (79) fusion protein as a substrate, as previously described (Coso *et al.* 1995). After 20 min at 30 °C, reactions were terminated by the addition of 10 μl 5 x Laemmli's buffer. Samples were heated at 95 °C for 5 min and analyzed by SDS electrophoresis on 12% polyacrylamide gels. Autoradiography was performed with the aid of an intensifying screen. Parallel anti-JNK1 immunoprecipitates were processed for Western blot analysis using a JNK-specific antiserum.

Results

Expression of c-Jun proteins during the seasonal cycle

The antisera used in the present study fulfil the criteria of specificity for investigating the distribution of immunoreactive materials. In particular, immunoadsorption tests revealed that the labeling was totally blocked by preincubation with 10^{-6} M of the cognate peptide, but was not affected by the same concentrations of non-related oncoproteins.

Western blot analysis was used to examine c-Jun protein expression in lizard testes, testing cytosolic and nuclear protein extracts. In cytoplasmic preparations c-Jun proteins are present during the winter stasis (from December until March) and show as a specific band of about 39 kDa without phosphorylation in Ser-63 (Fig. 1). In nuclear preparations Jun proteins are present during the spermatogenic wave (April/May) showing as a single specific

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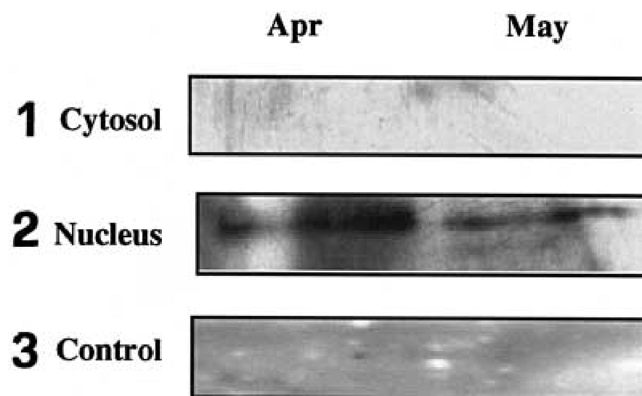

Figure 2 Western blot detection of c-Jun-Ser-63 protein in the cytosolic and nuclear testicular extracts (1, 2) of *Podarcis s. sicula* during the breeding season (April/May). Membranes were prepared and probed as described in Fig. 1. A specific band of about 39 kDa, with a strong phosphorylation on Ser-63, was observed in the nucleus by comparison with comigrating size markers. (3) Control blot represents the same membrane incubated with the same antibody preabsorbed with an excess amount (10^{-6} M) of the antigen. The blots are representative of four separate assays.

band of about 39 kDa (Fig. 2) with strong phosphorylation in Ser-63.

Immunocytochemistry was performed on serial sections and confirmed the different localizations; in fact, in the germinal compartment there was strong Jun immunoreactivity in the cytoplasm of spermatogonia (SPG) and stage I and II spermatocytes (SPC) during the winter stasis (Fig. 3*a*), whereas it was detected in the nuclei of the same cells during the spermatogenic wave (Fig. 3*b*).

Immunostaining of germ cells was not homogeneous. Indeed, several negative SPG were seen. A positive reaction was evident in Sertoli cells adjacent to the SPG, particularly during April (Fig. 3b).

C-Jun proteins bind with Fos-related proteins

Immunoprecipitation experiments supported the different localization of c-Jun proteins in the two different periods. C-Jun proteins were present in cytoplasmic preparations during the winter stasis (December/January) (Fig. 4*a*), whereas they were detected in the nuclear preparations during the spermatogenic wave (April/May), with strong phosphorylation on Ser-63 (Fig. 4*b*). When c-Jun proteins are present in the nuclei they bind with a 60 kDa Fos-related protein (Fig. 4*b*). The nuclear localization of Fos-related proteins in SPG, and stage I and II SPC was confirmed by immunocytochemistry on serial sections (Fig. 5*a*).

Positive Sertoli cells adjacent to some SPG were also detected during the breeding period when there is active spermatogenesis (Fig. 5*a*).

C-Jun-Ser-63 phosphorylation

JNK1 proteins were immunoprecipitated to assay for their activity to phosphorylate the Ser-63/73 on c-Jun proteins. A kinase assay was then utilized to test for the ability of JNK1 to phosphorylate the GST-c-Jun (79) fusion protein in the two different periods (winter stasis, breeding season) analyzed in this study. This experiment shows there to be strong activity in the breeding period, in contrast with only very low activity during the winter stasis (Fig. 6).

Discussion

The activity of Jun proteins in the testis of the lizard, *Podarcis s. sicula*, is associated with seasonal changes and correlates with steroidogenesis and spermatogenic activity (Andò *et al.* 1992). This lizard presents an annual spermatogenic cycle with two periods of spermatogenic activity (spring and autumn); only during the spring does mating occur and there is complete development of secondary sexual characters. The autumn spermatogenic wave is abortive and animals display neither spermiation nor androgen-dependent characteristics. Jun protein is present in the SPG and in stage I and II SPC of *P. s. sicula*. The immunoreactivity is stored in the cytoplasm, forming a ring at the edge of the nucleus during the winter stasis (from December to March) and is available in the nuclei of SPG, and stage I and II SPC during the breeding period (April/May), as observed by immunocytochemistry, Western blot, and immunoprecipitation experiments. Although the presence of c-Jun immunoreactivity both in Western blot and in immunoprecipitation of the whole

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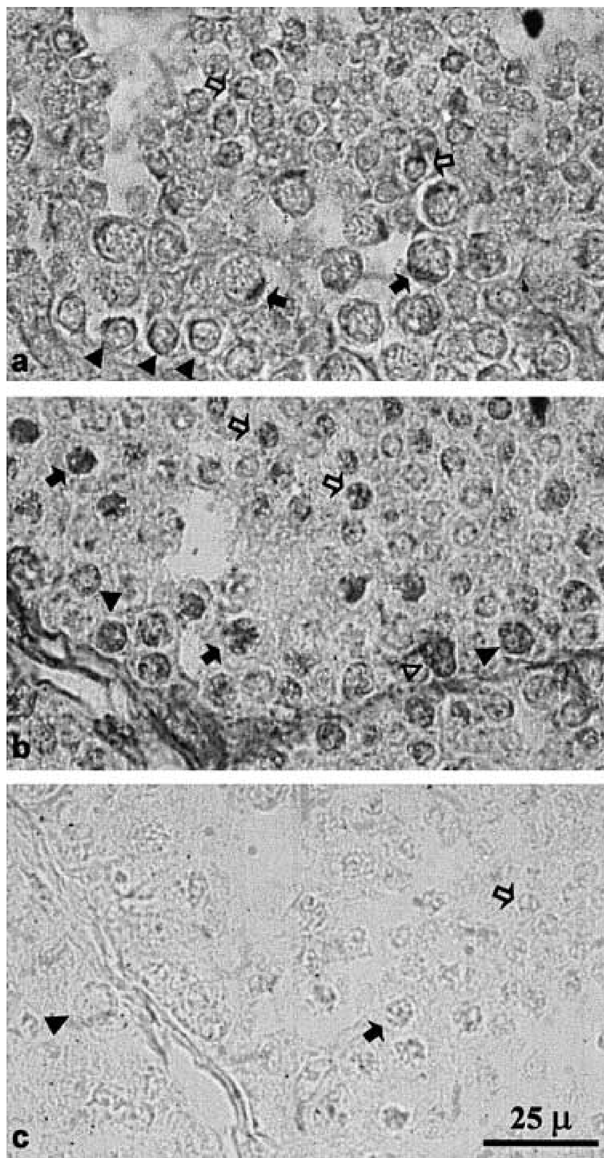

Figure 3 Immunocytochemistry for c-Jun protein in the testis of *Podarcis s. sicula* showing positive reaction in (a) cytoplasm of SPG (solid triangles), and stage I (solid arrows) and II (open arrows) SPC during the winter stasis (from December until March), and in (b) nuclei of SPG (solid triangles), and stage I (solid arrows) and II (open arrows) SPC during the spermatogenic wave (April and May); positive Sertoli cells nuclei (open triangle) can be seen adjacent to SPG (solid triangles). (c) Control section incubated with excess amount (10^{-6} M) of Jun. Symbols are the same as those used to show positive reactions. Bar=25 µ.

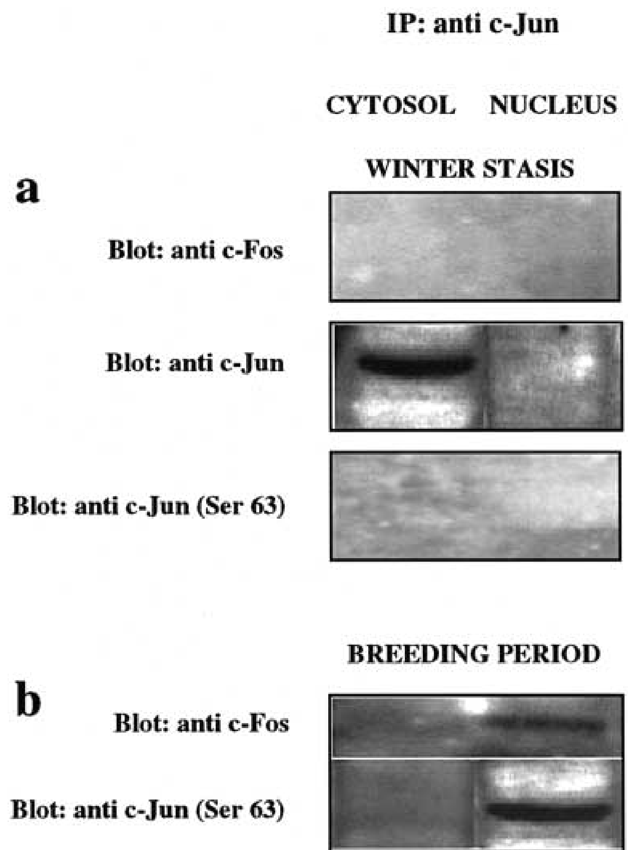

Figure 4 Western blot detection of c-Jun and c-Fos-related proteins after immunoprecipitation (IP) of the cytosolic and nuclear testicular extracts of *Podarcis s. sicula* in the two different periods. (a) During the winter stasis (December and January were considered) a specific band of 39 kDa was immunoprecipitated in the cytosolic testicular extracts. (b) During the breeding period (April and May were considered) a specific band of 39 kDa, with a strong phosphorylation on Ser-63, was immunoprecipitated in the nuclear testicular extracts and c-Fos-related proteins (60 kDa) were coimmunoprecipitated. The blots are representative of four separate assays.

testis could suggest that c-Jun might also derive from somatic cells, immunocytochemistry fails to show any cytoplasmic localization in somatic cells in either the present or a previous study (Chieffi *et al.* 1997). It is relevant to note that steroid hormones regulate c-myc, c-Fos and c-Jun expression in reproductive tissue in rats (Schuchard *et al.* 1993), birds (Subramaniam *et al.* 1993), and sea stars (Marsh & Walker 1995). In this respect, it should be noted that in the plasma of *P. s. sicula* the peak of estradiol correlates well with the new annual spermatogenic wave (Andò *et al.* 1992). However, the possibility does exist that in some months these proteins are not efficently translocated to the nucleus and are therefore present but not functional, or that these cells accumulate a stock of c-Jun proteins, without phosphorylation, which can be post-translationally activated upon mitogenic stimulation in order to play a role in cell division. This supports a role for Jun proteins in an epithelium with proliferative capability. Because germinal surface cells are hormonally responsive, an influence of the hormonal climate on the expression of the Jun proteins is expected.

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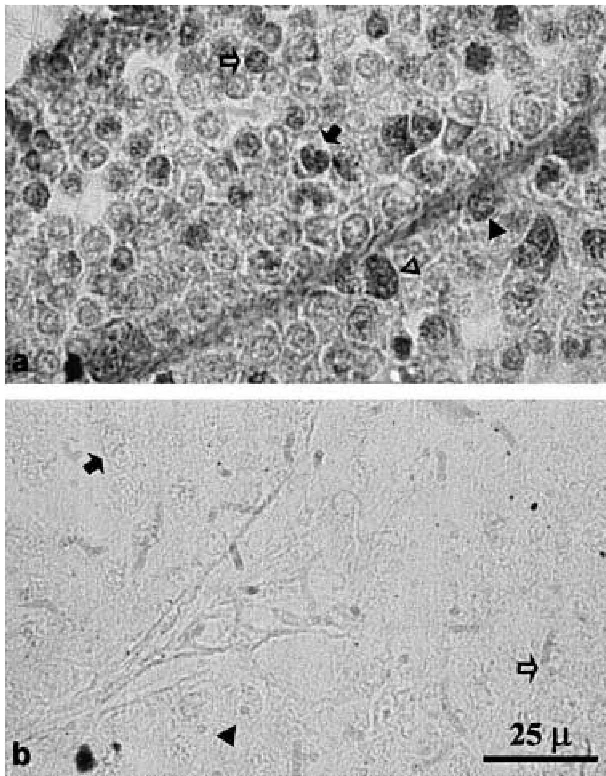

Figure 5 Immunocytochemistry for c-Fos protein in the testis of *Podarcis s. sicula* showing positive reaction in (a) the nuclei of SPG (solid triangles) and the nuclei of stage I (solid arrows) and II (open arrows) SPC during the spermatogenic wave (April and May); positive Sertoli cells nuclei (open triangles) can be seen adjacent to SPG (solid triangles). (b) Control section incubated with an excess amount (10^{-6} M) of Fos. Symbols are the same as those used to show positive reactions. Bar=25 µ.

The c-Jun proto-oncogene belongs to a family of genes that encodes proteins that contribute to dimeric complexes which bind to the AP-1 consensus DNA (Lee *et al.* 1987). While the Jun proteins can form homodimers which bind to the DNA (Halozonetis *et al.* 1988, Nakabeppu *et al.* 1988, Rauscher *et al.* 1988), the Fos family proteins cannot form homodimers and therefore do not bind to DNA or influence transcription in the absence of Jun protein. In this way, the localization of Jun proteins, with strong phosphorylation on Ser-63, and Fos proteins in the nuclei of SPG and stage I and II SPC during the breeding period could be the signal to increase the transcriptional activity of Fos/Jun heterodimers and Jun/Jun homodimers during the spermatogenic wave (spring).

In *Vulpes vulpes* a cytoplasmic localization for the AP-1 complex in SPG and SPC has been observed (Cohen *et al.* 1993). In *Xenopus laevis,* Myc accumulates as an abundant protein during oogenesis, and fertilization triggers its translocation into the nuclei (Guesse *et al.* 1989). In *Rana esculenta*, the oncoproteins Myc, Fos, and Jun appear in the cytoplasm in SPG nuclei at the onset of and during the annual period characterized by the new spermatogenic wave (Chieffi *et al.* 1995).

The cytoplasmic localization of classical 'nuclear oncoproteins', clearly seen in *R. esculenta*, is supported by the present data as well as by recent reports in human ovarian cancer and normal ovarian epithelium (Neyns *et al.* 1996), and in breast cancer (Li *et al.* 1996).

The activity of AP-1 components are modulated through their phosphorylation. So far, this form of post-translational control has been demonstrated for c-Jun, c-Fos, and ATF2 (activated transcription factor 2), but it is likely that other Jun and Fos proteins are similarly regulated. In the case of c-Jun, phosphorylation at Ser-63 and Ser-73, located within its transactivation domain, potentiates its ability to activate transcription as either a homodimer (Pulverer *et al.* 1991, Smeal *et al.* 1991) or a heterodimer with c-Fos (Deng & Karin 1994). These residues, which do not affect DNA binding activities, are phosphorylated by members of the mitogen-activating protein kinase (MAPK) family, the Jun kinases or JNKs (Hibi *et al.* 1993, Dérijard *et al.* 1994), which are the only protein kinases found to efficiently phosphorylate the N-terminal site of c-Jun.

It is relevant to note that the c-Jun (Ser-63) phosphorylation in the nuclei of SPG, and stage I and II SPC during the breeding period in the lizard testis is concomitant with the strong JNK1 activity, as shown in the present kinase assay.

Fos and Jun have also been found in Sertoli cells with the immunoreactivity of the oncoproteins predominantly present in cells adjacent to negative SPG. The immunoreactivity was strongest during the breeding period, supporting the idea that proto-oncogene activity is an integral part of a network of local interacting factors controlling Sertoli and germ cell communication (Guraya 1995) during spermatogenesis.

In conclusion, in the lizard model, which is characterized by a discontinuous progression of spermatogenesis, we have demonstrated the presence of c-Jun activity in the testis and suggest that it may be involved in mechanisms related to active spermatogenesis. This seasonally dependent system may represent an important model with which to study the various steps of spermatogenic progression, since each step of this cascade of events can be analyzed separately at different times. In species in which the regulation of spermatogenesis is continuous, only pharmacological stimulation can be used to modulate the seminiferous epithelium, whereas in a seasonal system the physiological progression permits the investigation of each step separately.

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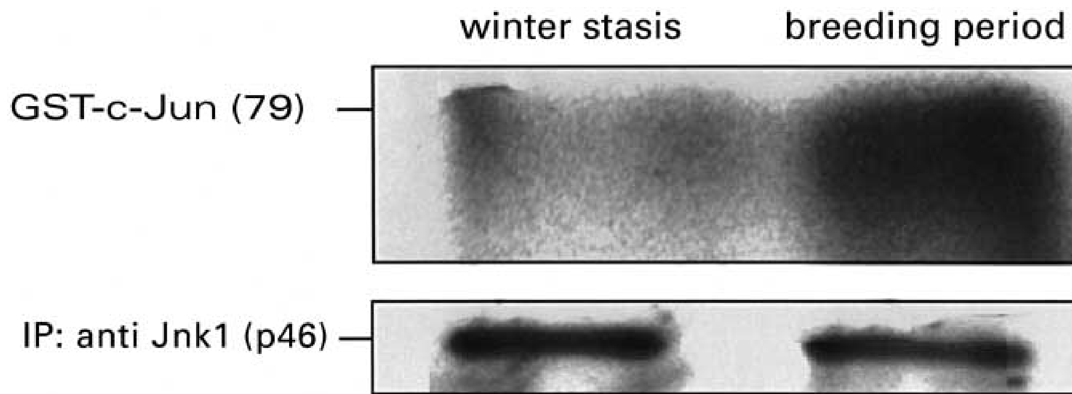

Figure 6 JNK1 kinase activity during the winter and during the breeding period. JNK1 kinase was immunoprecipitated from whole extracts obtained from each period, and equal amounts of protein (bottom panel) were added to GST-c-Jun in the presence of [γ-^{32}P]ATP, as described in Materials and Methods. The samples were then subjected to SDS-PAGE and autoradiography. The blots are representative of four separate assays. IP, immunoprecipitation.

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