able N-H proton of the indole ring. However, the LEFE measurements (*17*) and our own preliminary ^{14}N Q-band ENDOR data indicate that the only nitrogen associated with R· has a low spin density. The complete elimination of nonexchangeable ^{1}H ENDOR signals by deuterating Trp indicates that there is no appreciable spin density on any other moiety bearing a constitutive proton. This result permits interaction of the radical spin with the $Fe^{IV} = O$ moiety of the heme or with a neighboring carboxyl group (Asp^{235}), but apparently precludes substantial delocalization onto other moieties, such as the protoporphyrin ring, the axial His, or the pair of Met residues (230 and 231) that surround Trp^{191}. Of the seven Trp residues in CcP, Trp^{51} and Trp^{191} are by far nearest to the heme active site at 4.1 and 5.1 Å, respectively (*14*). The others lie between 15 and 28 Å from the heme and are poor candidates for the radical center because of *g*-values (*7–10*) and the LEFE results (*17*) strongly suggest that the radical must be near the heme. Moreover, according to the single-crystal EPR study (*10*), Trp^{51} and Trp^{191} are the only Trp residues properly oriented with respect to the heme. Because the $Trp^{51} \rightarrow$ Phe mutation causes only minor perturbations in the ^{1}H ENDOR pattern, the radical site can only be Trp^{191}, not Trp^{51}.

The present study has unambiguously identified the primary site of the ES radical with Trp, most likely Trp^{191}, and thus answers one long-standing question. It does not explain the apparently contradictory results obtained in paramagnetic resonance and CD studies. The search for these explanations represents the next challenge provided by compound ES of CcP.

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26. M. Sivaraja *et al.*, unpublished results.

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Specific Expression of Nuclear Proto-Oncogenes Before Entry into Meiotic Prophase of Spermatogenesis

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The expression of proto-oncogenes representative of several functional categories has been investigated during development of mouse male germ cells. The c-*raf* proto-oncogene and three members of the c-*ras* gene family were expressed in mitotically active stem cells, throughout the prophase of meiosis and to varying extents in post-meiotic cell types. In contrast, the nuclear proto-oncogenes c-*fos*, c-*jun*, and c-*myc* were specifically expressed at high levels in type B spermatogonia. High levels of c-*myc* and c-*jun* RNAs were also detected in spermatocytes early in the prophase of meiosis. The type B spermatogonia represent the last mitotic cell division before entry into meiotic prophase; therefore, these nuclear proto-oncogenes may be involved in altering programs of gene expression at this developmental transition.

A NUMBER OF PROTO-ONCOGENE products fall into five functional categories that appear to constitute elements in signal transduction pathways: growth factors, tyrosine protein kinases, guanosine triphosphate (GTP)–binding proteins, serine and threonine protein kinases, and nuclear proteins. The ability of activated oncogenes to induce abnormal cell growth suggests that their normal cell progenitors, the proto-oncogenes, may function to regulate the growth and differentiation of normal cells. The identification of some proto-oncogenes as known growth factors (*1*), growth factor and hormone receptors (*2*), and transcription factors (*3*) supports the function of these genes as cellular regulatory elements, but the normal physiologic functions of most proto-oncogenes remain unknown. Analysis of the expression and function of these genes within defined normal cell populations provides an approach to identifying genes that may be involved in regulation of cell differentiation, as well as in control of cell growth.

The development of male germ cells in the testis provides a system that is particularly well suited for analysis of gene expression within a cell lineage because cell types can be isolated that represent a spectrum of developmental stages ranging from mitotically proliferating stem cells to mature spermatozoa (*4*). At birth the male germ cells are represented by the primitive type A spermatogonia that differentiate into the renewing stem cell population of type A spermatogonia. In the mouse, type A spermatogonia undergo four mitotic divisions and differen-

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tiate into intermediate and type B spermatogonia. The type B spermatogonia divide once and then enter the prophase of meiosis as preleptotene spermatocytes. Spermatocyte development continues through the leptotene, zygotene, and pachytene stages of meiotic prophase, which is followed by two rapid cell divisions resulting in formation of round spermatids, the earliest haploid postmeiotic cells. Differentiation from the round spermatid to the mature spermatozoa then occurs over a 2-week period.

Stage-specific transcription of a number of genes has been described during spermatogenesis, principally in pachytene spermatocytes and post-meiotic round spermatids (*5*). Specific patterns of transcription of four proto-oncogenes occur in germ cell development. The c-*abl* tyrosine kinase proto-oncogene is transcribed as a 4.7-kb RNA in post-meiotic spermatids, whereas transcripts of higher molecular size are found in a variety of somatic cell types (*6*). The *pim*-1 proto-oncogene, another member of the protein kinase family, is also transcribed in post-meiotic spermatids as an RNA of 2.4 kb in contrast to the 2.8-kb *pim*-1 transcripts detected in somatic tissues (*7*). The c-*mos* proto-oncogene, a member of the serine and threonine kinase family, is expressed most abundantly in male and female germ cells, as a 1.7-kb RNA in round spermatids and as a 1.4-kb RNA in oocytes (*8, 9*). Transcription of the *int*-1 proto-oncogene has been exclusively detected in post-meiotic male germ cells and in the neural tube of the developing mouse embryo (*10*). Because these findings suggest the possible function of several proto-oncogenes in the latter stages of spermatogenesis, we undertook an analysis of the expression of a series of proto-oncogenes representative of different functional classes in each of the spermatogenic cell types that can be isolated in sufficient quantity for biochemical studies.

Spermatogenic cell populations were fractionated to greater than 90% purity by unit gravity sedimentation in bovine serum albumin gradients (*11*). Type A and type B spermatogonia were obtained from testes of 8-day-old mice; preleptotene, leptotene to zygotene, and prepuberal pachytene spermatocytes were obtained from 17- to 18-day-old mice; and adult pachytene spermatocytes, round spermatids, and residual bodies were obtained from adult (70- to 120-day-old) mice. Total cell RNAs isolated from two or three independent preparations of each cell type were analyzed by RNA blot hybridization with specific oncogene probes. Filters were then rehybridized with actin probe to monitor integrity of the RNAs.

We initially examined the transcription of c-*raf* in spermatogenesis because c-*raf* is the only proto-oncogene besides c-*mos* that has been reported to possess serine and threonine protein kinase activity (*12*). A 3.1-kb c-*raf* transcript, which is the same size as in somatic cells (*13*), was detected in both type A and B spermatogonia and throughout meiotic prophase, with some increase in level at the adult pachytene stage (Fig. 1). The c-*raf* transcript was also detected postmeiotically in round spermatids, but was no longer present in residual bodies. Rehybridization of the same filter with actin probe demonstrated similar levels of 2.1-kb tran-

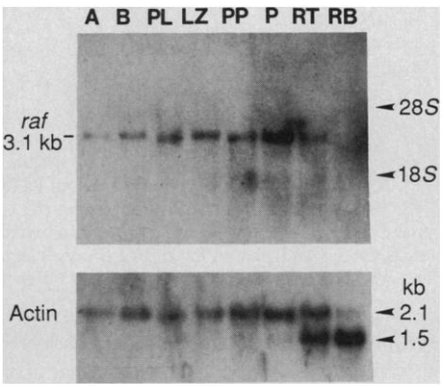

Fig. 1. Expression of c-*raf* in the male germ cell lineage. RNAs of type A spermatogonia (A), type B spermatogonia (B), preleptotene spermatocytes (PL), leptotene to zygotene spermatocytes (LZ), prepuberal pachytene spermatocytes (PP), adult pachytene spermatocytes (P), round spermatids (RT), and residual bodies (RB) were analyzed by RNA blot hybridization. The filter was first hybridized with *raf* probe that was prepared by nick translation of the insert of plasmid p627 (*20*), which contains the complete human c-*raf*-1 cDNA. The filter was subsequently rehybridized with β-actin probe (*21*). Exposure times were 4 days for *raf* and 12 hours for actin. Germ cells were isolated as described (*11*). Type A and B spermatogonia were prepared from testes of 8-day-old CD-1 mice (Charles River Laboratories) (type A: purity, 90 to 93%; contaminants, Leydig cells and type B spermatogonia; type B: purity, 92 to 94%; contaminants, erythrocytes). Prepuberal CD-1 mice (17 to 18 days old) were used for isolation of preleptotene spermatocytes (94 to 96% pure; contaminants, leptotene to zygotene spermatocytes), mixed leptotene to zygotene spermatocytes (88 to 92% pure; contaminants, pachytene spermatocytes), and prepuberal pachytene spermatocytes (88 to 91% pure; contaminants, Leydig cells). Adult CD-1 mice (70 to 120 days old) were used for preparations of pachytene spermatocytes (85 to 93% pure; contaminants, round spermatids), round spermatids (88 to 96% pure; contaminants, residual bodies), and residual bodies (91 to 97% pure; contaminants, condensing and elongating spermatids). RNAs were isolated by the guanidinium isothiocyanate method (*22*), and 10 μg of total cell RNAs were separated by electrophoresis in 1% agarose plus 2.2*M* formaldehyde gels and analyzed by blot hybridization as described (*8*). Probes were prepared by nick translation of gel-purified fragments to specific activities of 1×10^9 to 5×10^9 cpm/μg (*23*).

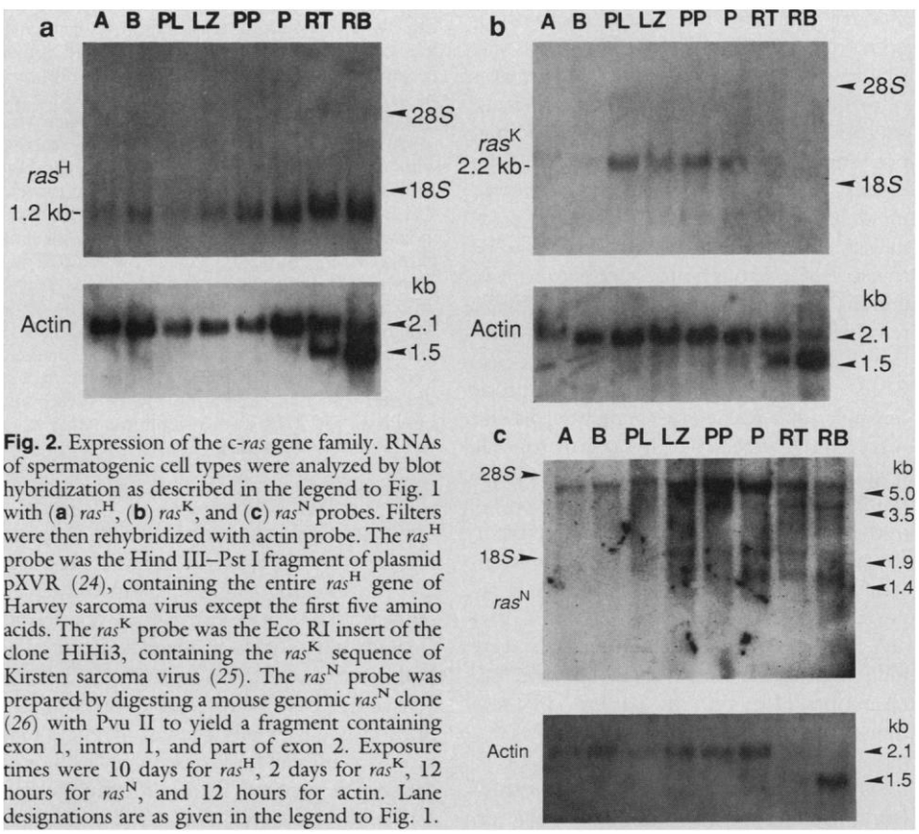

Fig. 2. Expression of the c-*ras* gene family. RNAs of spermatogenic cell types were analyzed by blot hybridization as described in the legend to Fig. 1 with (**a**) ras^H, (**b**) ras^K, and (**c**) ras^N probes. Filters were then rehybridized with actin probe. The ras^H probe was the Hind III–Pst I fragment of plasmid pXVR (*24*), containing the entire ras^H gene of Harvey sarcoma virus except the first five amino acids. The ras^K probe was the Eco RI insert of the clone HiHi3, containing the ras^K sequence of Kirsten sarcoma virus (*25*). The ras^N probe was prepared by digesting a mouse genomic ras^N clone (*26*) with Pvu II to yield a fragment containing exon 1, intron 1, and part of exon 2. Exposure times were 10 days for ras^H, 2 days for ras^K, 12 hours for ras^N, and 12 hours for actin. Lane designations are as given in the legend to Fig. 1.

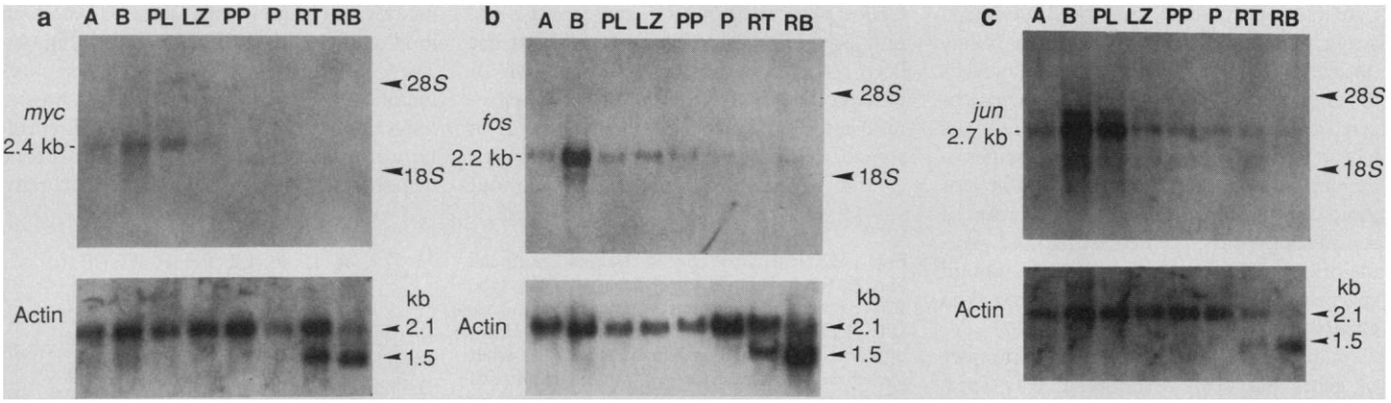

Fig. 3. Nuclear proto-oncogene expression. RNAs were analyzed as in Fig. 1 by hybridization with probes for (**a**) c-*myc*, (**b**) c-*fos*, and (**c**) c-*jun*. The c-*myc* probe was the Xho I fragment of the pMc-myc 54 cDNA clone (*27*), which spans exon 2 and part of exon 3 of mouse c-*myc* cDNA. The *fos* probe was prepared from the 1-kb Pst I fragment of pfos-1 constructed from the FBJ murine osteosarcoma virus (*28*). The *jun* probe was the 900-bp Pst I fragment of pEHJ-2, which contains human c-*jun* cloned in pUC13 (*29*). Exposure times were 6 hours for *fos*, 12 hours for *jun*, 2 days for *myc*, and 12 hours for actin. Lane designations are as given in legend of Fig. 1.

scripts in spermatogonia and spermatocytes, in addition to the specific post-meiotic actin transcript of 1.5 kb (*14*) in round spermatids and residual bodies (Fig. 1). Levels of actin transcripts did not appear to vary significantly at the different stages of spermatogenesis analyzed. These results are consistent with a report of c-*raf* RNA in pachytene spermatocytes (*7*) and indicate that c-*raf*, in contrast to the specific post-meiotic transcript of c-*mos*, is expressed throughout nearly the entire spermatogenic cell lineage.

The c-*ras* gene family consists of three closely related genes (c-ras^H, c-ras^K, and c-ras^N) that encode plasma membrane GTP-binding proteins. The c-ras^H proto-oncogene transcript was detected in mitotically proliferating spermatogonia, in spermatocytes throughout the prophase of meiosis, and post-meiotically in both round spermatids and residual bodies (Fig. 2A). The highest levels of c-ras^H RNA were detected in adult pachytene spermatocytes and post-meiotic cell types. Thus, c-ras^H appears to be transcribed throughout spermatogenesis, and the transcript is maintained through the terminal differentiation of post-meiotic spermatids to mature spermatozoa. The c-ras^K RNA was most visible in preleptone through adult pachytene spermatocytes and was faintly detectable in spermatogonia (Fig. 2B). However c-ras^K RNA was significantly reduced in round spermatids (relative to that seen with c-ras^H) and undetectable in residual bodies, indicating that the transcription or stability of c-ras^K RNA is reduced in post-meiotic cells. The sizes of c-ras^H and c-ras^K RNAs in germ cells were indistinguishable from their somatic cell transcripts (*15*), but the pattern of c-ras^N transcription during spermatogenesis was more complex (Fig. 2C). A 5.0-kb c-ras^N transcript was present in all cell types studied, including residual bodies. However,

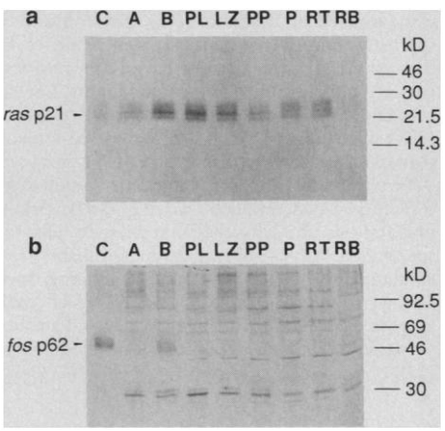

Fig. 4. Expression of c-*ras* and c-*fos* proteins. Extracts of spermatogenic cell types (45 μg of protein per lane) were separated by electrophoresis in (**a**) 15% or (**b**) 10% SDS-polyacrylamide gels and analyzed by immunoblotting either with *ras*-10 monoclonal antibody, which is reactive with all *ras* proteins (*30*) (a), or with a rabbit antibody against bacterially expressed *fos* protein (b). Blots were developed with goat antibody to mouse or to rabbit immunoglobulin G–alkaline phosphatase (Promega). Lane C is *ras* p21 expressed in *Escherichia coli* (*24*) for (a) and c-*fos* p62 expressed in the baculovirus system for (b). Lane designations for spermatogenic cell extracts are as in the legend to Fig. 1. Molecular size markers were phosphorylase B (92.5 kD), bovine serum albumin (69 kD), ovalbumin (46 kD), carbonic anhydrase (30 kD), trypsin inhibitor (21.5 kD), and lysozyme (14.3 kD).

three additional transcripts of 1.4, 1.9, and 3.5 kb were detected in meiotic prophase and post-meiotic stages of germ cell development. The 5.0- and 1.4-kb transcripts coincide with RNAs previously reported in both somatic and germ cells (*7, 15*), but the 1.9- and 3.5-kb transcripts have not been previously described. These differences may be due to the cell populations analyzed or to the specific probes used in different studies. Although the significance of the different sizes of c-ras^N transcripts is unclear, the results indicate that all three c-*ras* proto-oncogenes, like c-*raf*, are transcribed throughout most of spermatogenesis.

In contrast to the continuous expression of c-*ras* and c-*raf*, three nuclear proto-oncogenes, c-*myc*, c-*fos*, and c-*jun*, were specifically expressed at high levels in type B spermatogonia (Fig. 3). In addition, high levels of c-*myc* and c-*jun* RNAs were detected in preleptotene spermatocytes. However, densitometry scanning indicated that the expression of these proto-oncogenes was at least tenfold lower in other spermatogenic cell types. The c-*myc*, c-*fos*, and c-*jun* transcripts in germ cells were the same sizes as in somatic cells (*16*). Studies (*7, 17*) detected c-*myc* and c-*fos* RNAs at low levels in several spermatogenic cell types, but type B spermatogonia were not analyzed.

To determine whether these distinct patterns of cytoplasmic (c-*ras* and c-*raf*) as compared to nuclear (c-*fos*, c-*jun*, and c-*myc*) proto-oncogene transcription correlated with synthesis of the corresponding proteins, we analyzed expression of the c-*ras* and c-*fos* gene products. Consistent with the pattern of RNA accumulation, c-*ras* proteins were detected at all stages of spermatogenesis (Fig. 4A). In contrast, significant c-*fos* protein was visible only in type B spermatogonia (Fig. 4B). The specific transcription of c-*fos* at this stage is therefore correlated with synthesis of c-*fos* protein.

The c-*fos* and c-*jun* proto-oncogenes have recently been identified as components of the transcriptional regulatory factor AP-1 (*3*). The c-*myc* gene product has also been found to function as a transcriptional activator when expressed as a fusion protein in yeast (*18*). Transcription of these nuclear proto-oncogenes in a variety of cell types is increased by stimuli that induce either proliferation or differentiation (*19*), suggesting

that they may act as transcriptional regulatory factors to modulate changes in gene expression. The high level of specific transcription of these proto-oncogenes in type B spermatogonia therefore suggests their potential involvement in mediating changes in gene expression at this stage of germ cell differentiation. Because the type B spermatogonia represents the last mitotic stem cell division before entry into the prophase of meiosis, it is possible that the nuclear proto-oncogenes are involved in altering programs of gene expression at this developmental transition.

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The MHC-Binding and gp120-Binding Functions of CD4 Are Separable

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CD4 is a cell surface glycoprotein that is thought to interact with nonpolymorphic determinants of class II major histocompatibility (MHC) molecules. CD4 is also the receptor for the human immunodeficiency virus (HIV), binding with high affinity to the HIV-1 envelope glycoprotein, gp120. Homolog-scanning mutagenesis was used to identify CD4 regions that are important in class II MHC binding and to determine whether the gp120 and class II MHC binding sites of CD4 are related. Class II MHC binding was abolished by mutations in each of the first three immunoglobulin-like domains of CD4. The gp120 binding could be abolished without affecting class II MHC binding and vice versa, although at least one mutation examined reduced both functions significantly. These findings indicate that, while there may be overlap between the gp120 and class II MHC binding sites of CD4, these sites are distinct and can be separated. Thus it should be possible to design CD4 analogs that can block HIV infectivity but intrinsically lack the ability to affect the normal immune response by binding to class II MHC molecules.

CD4 IS A CELL SURFACE GLYCOPROTEIN found on those T cells that recognize antigen presented by class II MHC molecules (*1*). It is thought that the extracellular region of CD4, which comprises four immunoglobulin-like domains (V_1 to V_4), interacts with nonpolymorphic determinants of class II MHC and that this interaction is important in the efficient recognition of antigen by these cells (*2*). Gene transfer experiments have indicated that CD4 can function as an adhesion molecule that binds to class II MHC molecules on antigen-presenting cells and improves the interaction of the T cell receptor (TcR) with its ligand (*3, 4*). In addition, it has been reported that CD4 may associate with the TcR as part of the antigen recognition process (*5*) and may be directly involved in the resultant signal transduction (*6*). The CD4 molecule serves as the receptor for HIV-1 (*7*), binding with high affinity to the viral envelope glycoprotein, gp120 (*8, 9*). The relationship between the regions of CD4 that are important in its normal function and those involved in HIV-1 binding has been unknown; while several residues within the V_1 domain of CD4 are known to be important in gp120 binding (*10–13*), the residues involved in class II MHC binding have not been mapped.

As soluble forms of CD4 are currently under investigation as AIDS therapeutics (*9, 14–16*), we thought it important to examine whether the regions of CD4 responsible for its different functions are distinct. Although monovalent soluble CD4 analogs have not been found to affect the normal function of CD4-bearing cells (*15, 17*), it is possible that multivalent derivatives [such as CD4 immunoadhesins (*16*)] might do so. We have used homolog-scanning mutagenesis (*18*) to begin to define the MHC-binding regions of CD4 and their relationship to those involved in gp120 binding. We find that the sequences involved in these two functions are not identical, and, although possibly overlapping, can be separated.

Our strategy for generating mutants was based on the observation that, although human and mouse CD4 share a high degree of amino acid sequence identity (*19*), the murine homolog does not bind gp120 (*11*) and does not interact with the human class

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