Evidence for Positive and Negative Regulatory Elements in the 5'-Flanking Sequence of the Mouse Sparc (osteonectin) Gene*

(Received for publication, November 28, 1988)

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We have investigated the role of 5'-flanking DNA sequences in regulating the expression of the murine Sparc (osteonectin) gene in parietal endoderm cells and in F9 embryonal carcinoma cells induced to differentiate into parietal endoderm with retinoic acid and cyclic AMP. Varying lengths of flanking sequences extending up to 3.0 kilobase pairs 5' of the transcription initiation site were linked to the bacterial chloramphenicol transacetylase gene in the Bluescript M13 vector. The constructs were tested in transient assays, using a β-galactosidase plasmid as a transfection control. Sequences between 78 and 169 base pairs upstream of the cap site are the minimum required for cell-type specific promoter activity; this region is dominated by two oligonucleotide/oligopyrimidine stretches or "GAGA" boxes and is highly conserved between the mouse and bovine genes. Addition of the sequence between -169 and -449, which includes part or all of a third GAGA box, results in increased parietal endoderm-specific transcription, up to a maximum of 6.3-fold higher than in undifferentiated F9 cells. Further addition of sequences between -449 and -638 markedly reduces promoter activity in both cell types but parietal endoderm-specific activity is restored in constructs containing 2.2 and 3.0 kilobase pairs of flanking DNA. In addition, we have identified sequences related to the consensus sequence for steroid response elements, one of which is able to confer progesterone-enhanced transcription when tested with a heterologous promoter in steroid responsive cells. These results suggest that negative and positive elements normally interact to regulate the temporal and tissue-specific patterns of Sparc gene transcription seen in vivo.

Sparc is a Mr = 43,000 phosphorylated Ca2+-binding glycoprotein originally identified as a major secreted product of mouse embryo parietal endoderm cells and F9 teratocarcinoma cells treated with retinoic acid and cAMP (Mason et al., 1986a and 1986b; McVey et al., 1988). More recent studies from a number of laboratories have shown that Sparc is identical to three other proteins, osteonectin, originally isolated from fetal bone (Termine et al., 1981; Young et al., 1986; Bolander et al., 1988; Findlay et al., 1988), a 43,000-endothelial cell "culture shock" protein (Sage et al., 1984), and BM40, isolated from the mouse EHS tumor matrix (Mann et al., 1987). In addition, Northern blot analysis and in situ hybridization studies have shown that the murine and human Sparc genes are differentially expressed in a wide variety of adult and embryonic cells (Holland et al., 1987; Nemura et al., 1988; Swaroop et al., 1988). In particular, high levels of Sparc RNA are seen in tissues actively engaged in the synthesis and remodeling of extracellular matrices rich in either basal lamina components (e.g., mouse embryo parietal endoderm, maternal deciduall, and regions of angiogenesis) or type I collagen (e.g., membrane and endochondral bone, odontoblasts, and tendon). The frequent association of high levels of Sparc expression with matrix remodeling has led to the speculation that the protein may play a role in some Ca2+-dependent processes associated with the assembly, processing, or turnover of extracellular matrix molecules (Mason et al., 1986b; Engel et al., 1987). Finally, the Sparc gene is down-regulated in cells transformed with SV40, abl, and src (Mason et al., 1986b).

The complex temporal and tissue-specific pattern of Sparc gene expression observed in vivo is presumably mediated by the interaction between regulatory proteins and specific DNA sequences within or adjacent to the Sparc gene. Additional elements may confer responsiveness to hormones and growth factors in vivo and modulate the increase in Sparc gene transcription that occurs in F9 cells treated with retinoic acid and cyclic AMP (Mason et al., 1986a, 1986b). There is now good evidence that retinoic acid is a natural morphogen in vivo (Thaller et al., 1987, Tickle et al., 1982) and a number of DNA binding proteins of the steroid and thyroid hormone receptor family have been identified which bind retinoic acid with different affinities (Giguere et al., 1987; Petkovich et al., 1987; Brand et al., 1988; Benbrook et al., 1988). It is formally possible that the Sparc gene contains a retinoic acid-responsive element analogous to the steroid and thyroid hormone response elements already characterized in other genes (for review, see Evans et al., 1988). However, an increase in the level of Sparc RNA is not seen in F9 cells until about 48 h after addition of retinoic acid and cAMP (Mason et al., 1986b), suggesting that Sparc gene transcription is modulated by downstream elements of a regulatory cascade, rather than by the retinoic acid receptor directly.

In order to identify factors regulating Sparc expression, we have analyzed DNA sequences extending up to 5 kb 5' of the...
cap site for their ability to direct parietal endoderm specific expression of a bacterial chloramphenicol transactase (CAT) reporter gene. These experiments define a basal promoter between the cap site and −78 and two regions which specifically increase transcription 3.2–8.0-fold in parietal endoderm compared with undifferentiated F9 cells. These regions are separated by a third which, by contrast, removes cell-type specificity and decreases the level of Sparc gene transcription in both F9 cells and parietal endoderm. Evidence is also presented for at least one steroid response element which may modulate Sparc transcription in vivo by progesterone. We conclude that the temporal and tissue-specific pattern of Sparc gene expression observed during development is the result of interactions between factors binding to positive and negative regulatory elements.

EXPERIMENTAL PROCEDURES

Oligonucleotide Plasmid Constructions—Chemically synthesized oligonucleotides were cloned into the XbaI site of pBLCAT2, which contains the thyminode kinase promoter from herpes simplex virus linked to the CAT gene (Lucow and Schutz, 1987). For example, the 16-bp sequence from −230 to −246 (Fig. 5) was synthesized as two complementary oligonucleotides (5'-ctagaTGTCTCATCTGTTCTGt-3'), (5'-ctgagtTCTGTTCTGTTCTG-3') (lower case denotes nucleotides added to generate XbaI linkers). These were mixed in equal quantities, phosphorylated, and heated to 65° C for 30 min. After cooling, the annealed oligonucleotides were ligated with XbaI-digested pBLCAT2. Recombinants were confirmed by sequencing.

Sparc-CAT Constructions—A UC18CAT, containing the CAT gene as a 1.6-kb EcoRI-BamHI fragment of pSV2-CAT cloned into the XbaI-BamHI site of pUC18 was kindly provided by Dr. Roger Watson, was digested with SacI and religated to generate a construct containing the β-galactosidase gene transferred into the BamHI site of Bluescript plasmid double digested with ApaI and PstI to remove the Sparc 5'-flanking sequence and relax the PstI-PstI fragment covering the region −1950 to +11, and a PstI-PstI fragment covering −2100 to +11 were cloned into the ApaI-PstI sites of pGEM-1 and then transferred as an ApaI-PstI fragment into the HindIII-PstI site of plasmid pUC18. Finally, a BamHI fragment containing the Sparc 5'-flanking sequence and CAT gene was transferred into the BamHI site of Bluescript M13\* to generate the plasmid Bluescript 638 Sparc CAT (pSP638CAT).

To generate a unidirectional deletion series, pSP638CAT was double-digested with Apol and ClaI and incubated with EcoRI and Mung-bean nucleases, using conditions recommended by Stratagene. The positions of the deletions were confirmed by sequencing. To generate a negative control for the CAT transient assays, pBP573CAT was digested with PstI to remove the Sparc 5'-flanking sequence and relax the PstI-PstI fragment covering the region −1950 to +11, and a PstI-PstI fragment covering −2200 to +11 were cloned into the ApaI-PstI sites of pGEM-1 and then transferred as an ApaI-PstI fragment into the HindIII-PstI site of pUC18. Then, a 3.0-kb SacI fragment was then transferred to the SacI site of pBP573CAT to generate a construct covering −3000 to +1 of the Sparc gene linked to the CAT gene (pSP57300CAT).

For RNA protection assays a 445-bp Poul-Feull fragment from pSP573CAT containing 272 bp Sparc 5'-flanking sequence, 11 bp of Sparc exon 1 and 122 bp of the CAT gene was subcloned into the HindIII site of pGEM-1. This plasmid is designated p2p.

Sequencing—The NcoI-PstI fragment from −638 to +71 was sequenced, by use of a combination of deoxy chain termination and chemical sequencing methods. As a result of this sequencing the construct in pUC18 designated pSP638-CAT in McVey et al., 1988 is the same as pSP638CAT in this paper.

Cell Culture and Transfections—The murine parietal endoderm cell line, PYS, and undifferentiated F9 teratocarcinoma cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. F9 cells were transfected as described above, with a 1-min gal locus shock and fresh DMEM usually containing retinoic acid, dibutyryl cAMP, and isotbutylylxythane added. 40 h post-transfection cells were washed with phosphate-buffered saline, harvested with a rubber policeman, and cell lysates prepared for β-galactosidase assays.

For studying steroid response elements, the human mammary epithelial cell line ZR-75-1 (Ham et al., 1988) was cultered in DMEM with 3% charcoal-treated fetal bovine serum and 10⁻⁶ M estradiol, which is required for growth. 24 h prior to transfection, cells were seeded at a density of 5 × 10⁵ cells/60-mm dish, and the medium was changed 2 h before the calcium phosphate-DNA coprecipitates were added. 10 µg of DNA was used for transfection/plate, and calcium phosphate precipitates were prepared as described (Gorman et al., 1982). The precipitates were removed by washing with DMEM after 6 h, and fresh medium containing hormones was added to the cells which were incubated 40 h later. As a positive control transfected cells were cotransfected with the construct CA-124/-125 containing a 15-bp mutant steroid response element from the MMTV LTR in the vector pHBLCAT2 (Ham et al., 1988).

CAT Assay—Nonchromatographic CAT assays were carried essentially as described (Sleigh et al., 1986). Collected cells were washed with 10 ml of 10 mM NaCl, 50 mM Tris-HCl, pH 7.8, lysed in 0.1 ml of 250 mM Tris-HCI, pH 7.8, 0.5% Nonidet P-40 at room temperature for 15 min followed by incubation at 65° C for 10 min. After centrifugation for 5 min at 4° C, supernatants were collected and used as cell extracts. 10 µl of cell extract were mixed with 50 µl of 250 mM Tris-HCI, pH 7.8, 20 µl of 8 mM chloramphenicol, and 20 µl of 0.5 mM acetyl-CoA containing 0.05 µCi of [14C]acetyl-CoA (Amersham International) and then incubated at 37° C for 1 h. Reaction products were extracted twice with 100 µl of ethylacetate and radioactivity in the pooled extracts was counted by liquid scintillation. CAT activity was normalized by β-galactosidase activity (cpm/β-gal activity) for experiments with F9 and PYS cells or protein concentration (cpm/µg protein) for ZR-75-1 cells. Protein assays were performed using the Bio-Rad kit. Each experiment was repeated more than three times with duplicate dishes. In some experiments, CAT assays were carried out as described by Gorman et al., 1982, using chromatography to separate reaction products. Similar results were obtained with both chromatographic and nonchromatographic methods.

β-Galactosidase Assay—30 µl of cell lysate was mixed with first 30 µl of solution A (10 mM KCl, 1 mM MgCl₂, 10 mM Tris-HCl, pH 7.6, 5 mM β-mercaptoethanol, 0.5 mM ATP, 0.2% sodium azide, 0.1 mM phenylmethylsulfonyl fluoride, 10 µM dithiothreitol, 10 µM 3-(N-morpholino)propanesulfonic acid) and then 20 µl of solution B (150 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 25 mM sodium fluoride, 10 mM β-mercaptoethanol, 1 mg/ml 4-methylumbelliferone-β-d-galactoside). The final reaction mixture was incubated at 37° C for 90 min, and 20 µl were then added to 2 ml of 1 mM NaOH and the fluorescence read immediately, using an excitation wavelength of 365 nm and emission wavelength of 440 nm. A standard calibration curve was made using purified β-galactosidase, and activity was measured in the linear range.

RNA Protection Assay—A 445-bp single-stranded RNA probe was made by T7 RNA polymerase using HindIII linearized plasmids. The RNA was transfected into undifferentiated F9 cells as described (Krunlauf et al., 1987) and poly(A) RNA isolated by oligo(dT) cellulose column chromatography (Mamia et al., 1982). 2 µg of poly(A) RNA was hybridized with 2 × 10⁶ cpm (440 pg) probe in 80% formamide, 40 mM PIPES, pH 6.7, 400 mM NaCl, 1 mM EDTA at 48° C for 16 h and then digested with a mixture of 20 µg/ml RNase A and 10 µg/ml RNase T1 at 28° C for 2 h. The protected fragment was analyzed on an 8% acrylamide, 7 M urea gel followed by autoradiography, using 32P end-labeled MspI-digested pBR322 as a size marker.

REFERENCES

Organization of the 5'-Flanking Region of the Murine Sparc Gene—Fig. 1 shows a map of the cosm id pcosSP13 containing the 5'-flanking region of the Sparc gene linked to the CAT gene (McVey et al., 1988). Se-
Regional regulatory elements in mouse Sparc gene.

**FIG. 1.** Map of mouse Sparc 5'-flanking DNA. The cosmid pcosSp13 (McVey et al., 1988) contains the first 75-bp exon of the mouse Sparc gene (boxed). A detailed map of the SacI-PstI fragment from -3.0 kb to +11 is also shown, with restriction sites used to generate the constructs Bsp3000CAT, Bsp2200CAT, Bsp1600CAT, Bsp638CAT, and Bsp38CAT.

**FIG. 2.** Parietal endoderm-specific expression of the Sparc promoter and evidence for negative regulatory element(s). Relative CAT activity was measured in extracts of PYS cells (hatched bars), undifferentiated F9 cells (filled bars), and F9 cells differentiated into parietal endoderm by 4-day treatment with retinoic acid and cAMP (open bars), 40 h after transfection with Sparc-CAT constructs containing varying amounts of 5'-flanking sequence. CAT activity was measured as conversion of [14C]acetyl-CoA to [14C]-acetylated chloramphenicol (Sleigh et al., 1986). Results are plotted as cpm/β-galactosidase activity and are the average of four experiments, each with duplicate dishes. Values above the hatched bars give relative CAT activity in extracts of PYS cells compared with undifferentiated F9 cells.

**FIG. 3.** Transient expression of Sparc-CAT constructs with progressive deletions of 5'-flanking DNA. CAT activity was measured in extracts of PYS cells (hatched bars) and undifferentiated F9 cells (filled bars) following transfection with a deletion series containing various amounts of Sparc 5'-flanking sequence linked to the CAT gene. Relative CAT activity (cpm/β-galactosidase activity) was measured as described in Fig. 2, and results are the average of three experiments, each with duplicate dishes. Values above the hatched bars are relative activity in PYS cells compared with undifferentiated F9 cells.
transcription and abolition of cell-type specificity. This inhibition appears to consist of two components: first, loss of parietal endoderm specificity with the addition of sequences between -449 and -476, followed by suppression of transcriptional activity in both cell types as the amount of flanking sequence is extended.

Initiation of Sparc-CAT Transcripts at the Correct Cap Site—RNase protection experiments were performed to determine whether RNA transcripts from the Sparc-CAT constructs in Bluescript M13 were initiating at the same cap site as in the endogenous Sparc gene (McVey et al., 1988). A 32P-labeled 445-bp single-stranded RNA probe was prepared from the plasmid pp2, as shown in Fig. 4B. In RNase protection experiments this probe gave a fragment of the size predicted for transcription initiation at the correct cap site (173 bp) (A). Moreover, the amount of protected fragment varied as expected from the level of CAT activity observed with the different constructs in differentiated compared with undifferentiated F9 cells (Fig. 2). Finally, no fragment was observed in cells transfected with the plasmid Bsp+11CAT which contains only the first 11 bp of (untranslated) exon 1.

Sequence of 638 bp of 5'-Flanking DNA—The nucleotide sequence of a Ncol-Pst fragment covering the region -638 to +11 of the mouse Sparc gene is shown in Fig. 5. The absence of “TATA” and “CAAT” motifs has already been noted, together with certain features of the sequence to -130 bp (McVey et al., 1988). Inspection of the sequence shown in Fig. 5 reveals a number of additional features. Most striking is the presence in the positive strand of two stretches of polypurine

Fig. 4. Correct transcripational initiation in Sparc-CAT constructs transfected into F9 cells. A, poly(A) RNA was isolated from undifferentiated (-) and differentiated (+) F9 cells 40 h after transfection with various Sparc-CAT constructs (see Fig. 2 for associated CAT activity) and 2 μg used in RNase protection assays as described under “Experimental Procedures,” with a 32P-labeled 445-bp probe generated as shown in B. Protected fragments were analyzed on an 8% acrylamide, 7 M urea gel followed by autoradiography. The position of the 32P-labeled MspI fragments of pBR322 is shown on the right. The discrepancy between the expected size of the protected fragment (173 bp) and the size of the adjacent marker fragment (180 bp) is due to the larger size of ribonucleotides compared with deoxyribonucleotides. The apparent larger size of the protected fragment with Bsp449CAT is due to a “smile” in the gel in this experiment, and was not consistently seen. B, strategy for generating 445-bp probe for RNase protection.

sequence between -83 and -135 (interrupted by four Cs) and -148 and -176 (interrupted by one C). Because of the presence of repeats of the sequence GGA, AGA, AGGA, etc. we have termed these regions “GAGA” boxes 1 and 2, respectively. A third region, from -348 and -396, is composed almost entirely of purines or pyrimidines in one strand and identified as GAGA boxes 1-3. The positions of deletion constructs used for the transfections in Figs. 2 and 3 are shown, together with the position of three oligonucleotides tested for steroid responsiveness with a heterologous promoter (underlined).
Evidence for steroid regulatory elements in mouse Sparc gene

The experiments described in Table 1A and B, progestin produced a 3.7-7.1-fold induction of CAT activity in cells transfected with pBLCAT2 containing oligo B. In a third experiment a value of 2.3-fold was obtained but in a fourth experiment no difference was observed. Table 1C shows that progestin induction of CAT activity was also observed in cells transiently transfected with constructs BSp356, 296, and 272CAT, all of which contain the sequence of oligonucleotide B, but not with BSp214CAT, in which the sequence is deleted.

Comparison of Mouse and Bovine 5'-Flanking Regions—The sequence of 1 kb 5' to the cap site of the bovine osteonectin gene has recently been determined (Young et al., 1989). Fig. 6 shows a comparison of the bovine and mouse sequences. There is extensive homology between the two flanking regions from +1 to about -300 of the mouse sequence. This region includes two polypurine/polypyrimidine stretches which we have termed here “GAGA” boxes 1 and 2. Between -300 and -500 the regions of sequence identity are much shorter and separated by gaps. The sequence termed GAGA box 3 in the mouse gene does not appear in the bovine gene. Between about -500 and -638 the homology between the mouse and bovine genes is again high.

DISCUSSION

Cell-type Specific Promoter Activity Associated with Polypurine/Polypyrimidine Sequences—The experiments described here have identified sequences within the 5'-flanking region of the mouse Sparc gene regulating its complex temporal and tissue-specific pattern of expression during development. In particular, we are interested in determining those regions responsible for high levels of expression in embryonic parietal endoderm cells and the increase in transcription seen when F9 teratocarcinoma cells differentiate into parietal endoderm in response to retinoic acid and CAMP. It is clear from the results shown in both Figs. 2 and 3 that sequences up to -78 constitute a minimal promoter element, while addition of nucleotides -78 to -169 are required to confer parietal endoderm specificity on this promoter. A striking feature of the sequence from -78 to -169 is the presence of two stretches of 27 and 53 nucleotides which consist almost entirely of purines in one strand. We have termed these polypurine sequences -78 to -169 of the mouse Sparc gene as box 1 in the mouse gene. Box 1 is inserted in a supercoiled recombinant plasmid. The mouse Sparc sequence contains an inverted repeat of 9 bp centered at nucleotide -98 within GAGA box 1. A similar inverted repeat has been observed in the promoter region of the mouse osterectin gene in chicken a2(I) collagen gene where it was suggested that it forms a S1 nucleosome-sensitive single-stranded loop. These models, however, are based on mirror repeat sequences which may be disrupted by short inserts. Although the particular conditions of the model systems (e.g. mirror repeats) do not pertain in the Sparc or osteonectin promoter regions, Young et al. (1989) have demonstrated an S1 nucleosome-sensitive site toward the 3' end of one of the bovine osteonectin GAGA boxes (box 1 in Fig. 6) when this sequence is inserted in a supercoiled recombinant plasmid. The mouse Sparc sequence contains an inverted repeat of 9 bp centered on nucleotide -98 within GAGA box 1.

Evidence for steroid regulatory elements in mouse Sparc gene

Table I

<table>
<thead>
<tr>
<th>DNA transfected</th>
<th>Induction factor</th>
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<tbody>
<tr>
<td></td>
<td>No addition</td>
<td>+10^-7 M R5029</td>
<td>+10^-7 M dexamethasone</td>
</tr>
<tr>
<td>A. pBLCAT2</td>
<td>1.0</td>
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<td>0.99</td>
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<tr>
<td>pBLCAT2 + oligo(A)</td>
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<td>0.38</td>
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<tr>
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<td>3.73</td>
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<tr>
<td>pBLCAT2 + oligo(C)</td>
<td>1.0</td>
<td>1.43</td>
<td>1.00</td>
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<td>CA-124/-123</td>
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<td>B. pBLCAT2</td>
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<tr>
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The cap site in parietal endoderm cells to which regulatory elements, the experiments described here also suggest that negative elements are present in upstream region of the mouse Sparc gene. Thus, a significant inhibition of parietal endoderm-specific promoter activity is seen when sequences between −449 and −465 are added to BSp449CAT (Figs. 2 and 3). Further inhibition of transcription in both parietal endoderm and undifferentiated F9 cells is seen when constructs are extended to include up to −638 bp of 5′-flanking sequence. Sequences inhibiting cell-type specificity of promoters have been reported in a number of mammalian genes, e.g. Bouvagnet et al., 1987; Borras et al., 1988; Larsen et al., 1986; Muglia and Rothman-Denes, 1986; Goodbourn and Maniatis, 1988; Sassone-Corsi and Verma, 1987; Smith et al., 1988, but little is known at present about the proteins that may bind to these regions and the way in which they confer negative effects on transcriptional initiation.

In the experiments described here maximal parietal endoderm-specific expression is seen with the CAT construct containing 3 kb of Sparc 5′-flanking sequence, which shows approximately 8-fold higher activity in differentiated F9 cells and PYS cells, compared with undifferentiated F9 (Fig. 2). The corresponding level of endogenous Sparc mRNA in 5'-differentiated F9 cells and PYS cells is approximately 20-fold higher than in undifferentiated F9 cells (Mason et al., 1986a, 1986b). Although it has been clearly shown that the increase in mRNA level in differentiated F9 cells is accompanied by an increase in Sparc gene transcription (Mason et al., 1986b), quantitative data is not yet available. Assuming no change in Sparc mRNA stability during differentiation the data presented here suggest that additional elements outside the 3-kb 5′-flanking sequence are required for full parietal endoderm-specific Sparc gene expression. Further studies must therefore include an assessment of the contribution to developmental regulation made by sequences within the gene, for example the first exon and intron, which are conserved in the mouse and bovine genes, and the identification of nuclelease sensitive sites flanking the Sparc gene in vivo. It will also be important to see if constructs such as BSp638CAT and BSp56CAT show the same differences in expression in other cell types producing high levels of Sparc protein, e.g. osteoblasts, decidual cells, and endothelial cells.

Acknowledgments—We thank Drs. Karen Lyons, Yasuhisa Matsui, Jeffrey Hok, Lynn Matrisian, and Roland Stein for helpful and critical comments on the manuscript and Dr. Marian Young for communicating her manuscript before publication. Special thanks are also due to our colleague, Dr. Victor Fet for mapping pcosSP13. We acknowledge the skill and patience of Janet McDonald in preparing the manuscript.

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