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1993 PROGRESS REPORT

"Regulation of Cell Division in Higher Plants" Grant No. DE-FG02-90ER20008

Two manuscripts-in-preparation are attached. These are briefly summarized below, followed by a description of more recent work.

Prewett ME, HS Feiler H and TW Jacobs (1993) Developmental patterns of *cdc2*, cyclin and histone H1 kinase activity in pea (*in preparation*)

A version of this manuscript was included in last year's report. On the advice of journal editors, we have delayed final submission of this manuscript until further data were available, which we now have in hand. In this manuscript, we describe a developmental survey of pea *cdc2* and cyclin transcript levels as well as a parallel characterization of the levels of histone H1 kinase activity. In order to accurately quantify pea *cdc2* transcript levels, we used the PCR-generated partial cDNA clone in RNase protection experiments. We surveyed a variety of tissues and found that levels of *cdc2* mRNA per microgram total RNA closely reflect the mitotic state of the tissues: etiolated stem and root tips have more, roots and stems have less, leaves and tendrils hardly any at all, and seeds, none detectable. It is likely that, were we to dissect out germinating embryos from imbibed seeds, that we might see a signal above the massive null background of the cotyledon. This finding presents an interesting cellular/developmental challenge: What is the mechanism by which *cdc2* transcription is turned off during seed (esp. cotyledon) maturation, since one can assume that it is probably on when cotyledonary cells are dividing during seed maturation?

We used PCR to obtain a partial pea cyclin clone. We have since obtained and fully sequenced a full-length clone of the PCR fragment used in these experiments, and this clone's sequence will be reported in the revised version of this manuscript. Once again, we found that mitotic tissues display more cyclin transcript, on a per microgram total RNA basis, than their less-mitotic counterparts.

We used precipitation with p13-Sepharose as a means of affinity purifying the pea "MPF" complex from a variety of tissues. This method has become the industry standard for p34^{cdc2} kinase assays and is based on the natural affinity of p13, the fission yeast cell cycle regulator, for the p34-cyclin complex. When this matrix was used to precipitate histone H1 kinase activity from a variety of pea tissues, a result consistent with that described above was obtained: Activity was concentrated in mitotic tissues, when activity was expressed on a per microgram protein basis.

In order to refine this analysis and eliminate developmental differences from one tissue to the next, we selected the 10 cm pea root tip as a developmental gradient along which to investigate pea MPF activity and its components. Five successive 2 mm segments from the pea root tip are collected and pooled from a population of pea roots. The meristem proper is located in the second segment (2-4 mm from the tip). We were surprised to find that the levels of *cdc2* transcript were relatively constant along the root gradient, on a per cell basis, whereas the pea cyclin message was much more abundant, on a per cell basis in the meristematic zone of the root tip. The cyclin profile was closely paralleled by the p13-precipitable histone H1 kinase activity along the root tip gradient. These results suggest that, within the pea root tip developmental profile, cyclin and p34^{cdc2} kinase activity reflect -- and possible control -- mitotic activity of cells, whereas the level of *cdc2* transcription is relatively unregulated on a per cell basis. This may reflect the potential for pea root cells to re-enter the proliferative state upon stimulation by *Rhizobium* or other developmental programs such as lateral root emergence or cambial growth. The persistence of *cdc2* message and protein suggests

that plant cells have an intermediate state in terms of proliferative potential. That is, cells can lack cyclin message (and presumably protein) but retain substantial amounts of p34 protein and *cdc2* message. It will be interesting to examine more closely the levels, phosphorylation and quaternary states of *cdc2* proteins in a variety of developmental and physiological contexts in plant tissues.

Feiler HS and TW Jacobs (1993) Developmental patterns of *cdc2*, cyclin and histone H1 kinase activity in pea (*in preparation*)

In this manuscript, we describe the use of p13-Sepharose affinity chromatography for examining the abundance, polymorphism and associated histone H1 kinase activity of p34^{*cdc2*} along the cell cycle pathway in pea root apical meristem cells. Using 5-aminouracil (5-AU) as a DNA synthesis blocker and inducer of mitotic synchrony, we observed a set of Coomassie Blue stainable proteins that cycle in abundance, with peaks at S- and M-phases. When blots of such proteins were probed with an anti-p34^{*cdc2*} antibody, the abundance of the crossreactive material was not seen to fluctuate over the 12 hour course of the cell cycle, suggesting that p34^{*cdc2*} protein levels do not dictate passage through stages of the cell division cycle.

Histone H1 kinase (H1K) assays of p13-Sepharose precipitates prepared at hourly intervals following release from the 5-AU block revealed a strong peak of activity in S-phase and a lesser one at M. This was unexpected, since the best characterized and phylogenetically conserved H1K activity peak occurs at G2/M, where it catalyzes entry into mitosis. The finding of a strong peak at S is doubly intriguing, since the complex that expresses this activity in vertebrate cells also contains such components as the p107 relative of the retinoblastoma tumor suppressor protein and the E2F transcription factor, neither of which have been identified in higher plants to date. Thus, the intriguing question is opened as to what other components comprise the complex, in pea, that gives this high S-phase H1K activity in p13-precipitates.

Interestingly, H1K assays of the supernatants from these staged p13-Sepharose precipitations revealed a constitutive H1K activity as well as phosphorylation of what appears to be a single set of proteins at both S- and M-phases. This finding suggests that not only do peas employ a p13-Sepharose precipitable protein kinase as part of the regulatory circuitry governing S- and M-phase passage, but other S- and M-phase specific phosphorylations also play a role in control of the cell cycle in the pea root meristem. The observed phosphorylations could result from a periodic protein kinase activation, a periodic phosphatase activation or the periodic availability or degradation of the substrates visualized on these films. What makes this observation especially intriguing is the fact that it appears to be *the same set of proteins* that are phosphorylated at S and at G2/M in these assays. Passage through these the stages of the cell cycle is expected to be dependent upon rather different checkpoint controls. Therefore, posttranslational modification of the same proteins at each passage is difficult to rationalize. Clearly, determination of the identities of these proteins will be of great interest, as will identification of the enzymes that catalyze their phosphorylation.

Recent Work

During the final year of this 3-year funding cycle, work on the project has been focused almost exclusively on obtaining and characterizing cDNA clones encoding cyclin-dependent kinases (CDKs, including *cdc2* and its many cousins) and cyclins from pea. All of our work up to this time has relied on small PCR-generated cDNA clones of 2 putative pea CDKs (~550 bp each) and a putative pea mitotic cyclin (~200 bp), as well as anti-CDK antibodies of poor affinity and questionable specificity. Therefore, it has become a high priority for us to generate clones, probes and immunological tools in our own system.

Postdoctoral fellow Houqi Chen prepared and sent Stratagene Corporation a batch of pea root

tip RNA, from which the company constructed and sold us a lambda ZAPII cDNA library. Dr. Chen, with the help of various other members of our group, screened this library, using our PCR clones as probes. About half a million plaques were screened with each probe. As of this writing, we have four putative CDKs (*CdkPs1,2,3,&4*) and five putative cyclins (*CycPs1,2,3,4,&5*), the DNA sequences of which have been determined to varying degrees of completeness.

Cyclin-Dependent Kinases. *CdkPs1* appears to be a full-length clone of the original PCR product obtained by Heidi Feiler and reported in our 1990 PNAS paper. The open reading frame of the clone encodes a polypeptide of 297 amino acids and predicted molecular mass of 34,087 Da, in perfect agreement with that predicted for an authentic *cdc2* homologue. The protein carries the hallmark "GEGTYG", "WYRAPE", and "PSTAIRES" motifs, characteristic of all authenticated *cdc2* homologues reported to date. Authentication of this clone is being pursued by Julie Poole (a senior this year, who also did the sequencing), by assaying its ability to rescue temperature-sensitive *cdc28* mutants of budding yeast, supplied to us by Steve Reed (Scripps).

Ms. Poole has also taken some steps toward providing us with the protein product of *CdkPs1*. She has cloned all but the amino terminal 20% of the protein into one of New England Biolabs's pMAL expression vectors, from which it will be produced as a fusion protein to the maltose binding protein. This should aid in its purification. We intend to use this fusion protein as a "hook" to try to fish out the protein kinase that phosphorylates -- and thus activates -- the product of *CdkPs1* at Thr-161. This is the consensus activation site of all true *cdc2* homologues. Ms. Poole's construct was designed to lack the other presumed phosphorylation site at Tyr-15. She has also determined that the carboxy terminus of the protein encoded by *CdkPs1* carries enough unique sequence information that an antibody raised against it should be very specific for this particular CDK. She has therefore cloned the carboxy-terminal 90 amino acids of *CdkPs1* into pATH10, another *E. coli* expression vector. This expression system produces proteins fused to the *trpE* gene of *E. coli*, largely in inclusion bodies that can be recovered from cell lysates and used as effective antigens in rabbits.

Betty Prewett (M.S. student) is working on completing the sequencing of *CdkPs2, 3, and 4*. *CdkPs4* appears to be a truncated version of the clone reported by Feiler and Jacobs in 1991 as "*cdc2*-like". When combined with sequence data from the PCR clone obtained by Heidi Feiler (with which *CdkPs4* overlaps significantly and with perfect identity), this clone would appear to be somewhat removed from the mainstream of CDK sequences. A highly modified "PSTAIRES" is present (in the form of "PCTAIRES") and other hallmark motifs are only partially identifiable. It will be interesting to investigate the cell cycle regulation of expression of this protein, and any cyclin associations it may have. But these experiments may have to wait for a full-length version of the gene to be recovered.

Preliminary sequence data on *CdkPs2* and *CdkPs3* suggest that the former is very closely related to *CdkPs1* and is therefore likely to be an authentic CDK, whereas the latter is of the same class as *CdkPs4*, and therefore has a probable, but unknown role in cell cycle control. Complete sequence data on all of these clones should be available by summer's end, 1993.

Cyclins. Dr. Chen also labeled Heidi Feiler's short PCR clone of a putative mitotic cyclin from pea and probed the pea root tip cDNA library. Of the five putative cyclin clones recovered, complete sequence information is only available for one, *CycPs1*, as of this writing. This clone represents a full-length version of Dr. Feiler's PCR product, that used by Betty Prewett in the developmental studies described above. Its open reading frame encodes a polypeptide of 51,950 kDa and has a cyclin "destruction box" 35 amino acids from the N-terminus, with the sequence RAALHDIGN, wherein only the H diverges from the absolutely conserved positions in the consensus sequence for animal cyclin destruction boxes of RAALGVISN. This suggests that the pea protein may be regulated by ubiquitin-mediated proteolysis at anaphase, as is the case with its animal counterpart. But this remains to be demonstrated biochemically, of course. This protein would appear to fall, roughly, into the "B" class of cyclins, and is strikingly similar to the soybean

cyclin published by Hata's group in 1991.

CycPs2 has been sequenced on only one strand in its entirety and appears to encode polypeptide of 51,162 kDa. It has a putative "destruction box" starting at amino acid 20 (a little early for the animal consensus) of RAGLTDTVNT, violating the strictly conserved residues of the animal consensus with its G and T. Again, the consequences of these departures from the animal consensus destruction box can only be discerned with biochemical analysis. This cyclin would appear to be an "A" type, and is very similar to the carrot cyclin published by Hata's group.

CycPs3 appears to encode another "A" type cyclin and some data suggest that its regulation may be interesting. This clone was only picked up once in the library screen, and it is *very* difficult to detect its message in pea root tip RNA. This is in contrast to *CycPs1* and *CycPs2*, the mRNAs of which are relatively abundant in the pea root tip message pools, and were picked up several times in our library screen. However, it should be noted that the *CycPs3* clone also appears to contain a cloning artifact that has resulted in the repetition of some of the clone's 5' sequence information, making the clone somewhat longer than a full-length cyclin clone could possibly be. Tina Roush (postdoc) is currently working on finishing off the sequencing of this clone and sorting out its somewhat problematic 5' end.

Finally, *CycPs4* and *CycPs5* appear to be extremely closely related clones. In the course of restriction mapping them, Christine Doss (summer undergraduate student) has noted a restriction fragment length polymorphism between them, in the middle of what is expected to be protein coding sequence. Houqi Chen kept these clones separate in his original screening because he also detected a size difference in some of his initial digests. However, we have found essentially identical sequences at the 5' and 3' ends of these clones, where different genes would be expected to diverge significantly. Therefore, Ms. Doss is concentrating her sequencing efforts on just one of these clones (*CycPs4*) at present, but has subclones of the other clone covering the region in which the polymorphism resides. Further sequencing will be required to differentiate these clones, and to determine what class of cyclins their translation products represent.

Once the DNA sequences of this collection of putative pea cyclins and CDKs are completed, we will proceed to test their function by complementing appropriate yeast strains, examine the abundance of their cognate mRNAs over the cell cycle, and begin to raise antibodies which will specifically recognize and immunoprecipitate their cognate proteins from appropriate pea cell extracts.

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