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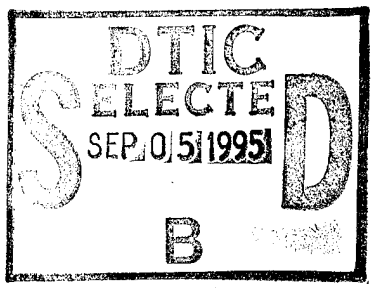

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13. ABSTRACT *(Maximum 200 words)*

We are using two-dimensional gels in combination with genetic and biochemical techniques to identify G1-phase substrates of cyclin-Cdk complexes. We have optimized protocols for labelling proteins with ^{32}P prior to two-dimensional gel electrophoresis. Yeast strains have been constructed bearing six different cyclins expressed from a *GAL* promoter, to allow inducible phosphorylation of the desired substrates. A commercial antibody has been identified and obtained which may specifically immmunoprecipitate phosphorylated forms of the desired substrates. Putative substrates have been examined *in vitro*. Together, these accomplishments provide a good platform from which to identify real, in vivo substrates in the near future.

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Bruce Fuchs June 23, 1995

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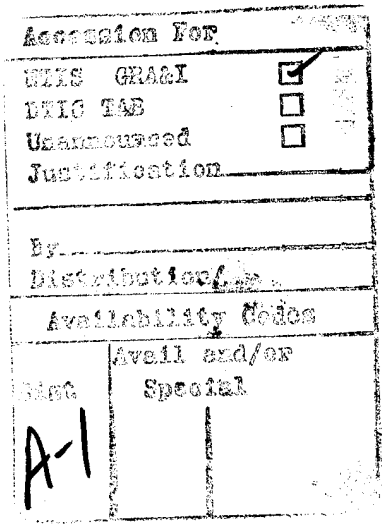

5. Introduction.

There is general agreement that in all eukaryotes, phosphorylation by various cyclin-Cdk complexes controls and orchestrates key cell cycle events. These events include commitment in G1 phase, initiation of DNA synthesis in S phase, and spindle formation and elongation in mitosis. However, despite knowing a great deal about the cyclin-Cdk complexes themselves, and despite years of investigation by many laboratories, we know only about half a dozen substrates of the cyclin-Cdk kinases, and none of these explain the control of critical cell cycle events. In particular, we do not know what substrates have to be phosphorylated for commitment to occur (although in mammalian cells, Rb is almost certainly one of the substrates).

The purpose of the present work is to develop methods for identifying substrates of the cyclin-Cdk complexes. In particular, we are interested in G1 substrates. To begin, experiments will be done in the yeast *S. cerevisiae*, and then the project will expand into mammalian cells.

There are two main approaches. The first approach uses two-dimensional gels to examine phosphoproteins. Various cyclins are expressed from a *GAL* promoter, and cells with the over-expressed cyclins are labelled with ^{32}P. The pattern of spots on a 2D gel is then compared between cells expressing and not expressing the cyclin. Extra spots in the cyclin-expressing cells may be substrates.

The second approach is to develop antibodies against phosphoserine followed by proline, and phosphothreonine followed by proline. Such antibodies would recognize proteins phosphorylated by Cdk complexes. Thus, such proteins could be immunoprecipitated and sequenced. This could also be combined with the 2D gels as an enrichment step.

6. Body of the Report.

The work has gone somewhat slowly, because it took some time to find a well-qualified post-doctoral fellow. It was not until January that the post-doctoral fellow arrived. Nevertheless, some significant progress has been made.

A. Visualization of G1 Cdc28 substrates on 2D gels.

i. Optimize labeling, extraction, and gel conditions. Quite a number of experiments of this kind have been done. The 2D gels are quite limited in the amount of protein that can be loaded. We have modified previous labelling/extraction procedures so that we now label fewer cells with the same amount of ^{32}P. Thus, we actually load more ^{32}P on the gels, which increases sensitivity markedly. The reduced protein load also increases resolution. Gel conditions were also examined. We found that our original conditions were essentially optimal for pH 4-8 and pH 3-10 gels. However, non-equilibrium (NEQ) gels were poor. The non-equilibrium gels are now much improved, but still not as good as the equilibrium gels. The non-equilibrium gels are very important, because even the "pH 3-10" gels actually focus proteins only up to about pH 7.6. The non-equilibrium gels are essential to look at more basic proteins. Experiments with the non-equilibrium gels are continuing.

We examined ^{33}P as a label instead of ^{32}P. Although it had many advantages in terms of ease of handling and resolution, its specific activity was of course much lower (because of the longer half-life), and this reduces the sensitivity so much that it is not a practical alternative to ^{32}P for these experiments.

In summary, the labeling, extraction, and gel systems are now about as good

as we can make them for proteins of iso-electric points 7.6 and lower. Some improvement is still required for more basic proteins.

ii. Construct strains with different inducible cyclins. This has been nearly completed. We have *GAL-CLN1*, *GAL-CLN2*, *GAL-CLN3*, *GAL-PCL2* (Pcl2, also known as OrfD, is a cyclin of uncertain biological role which normally activates the Pho85 Cdk), *GAL-CLB5* and *GAL-CLB2*. *GAL-CLB3* is under construction. This gives us essentially a complete set of relevant cyclins.

iii. Compare the substrates phosphorylated by the different induced cyclins. We have made only modest progress on this part of the project. To make a comprehensive comparison, we need good non-equilibrium gels so that we can see the proteins with a high isoelectric point. Since we have not had really satisfying gels, we have not put our main effort into these comparisons. However, some preliminary experiments have been done. It appears (on pH 4-8 and 3-10 gels) that *GAL-CLN1*, *GAL-CLN2*, and *GAL-CLN3* induce phosphorylation of the same spots (three strings of spots). However, *GAL-CLB2* does not induce phosphorylation of any of these spots. At first sight, this is very encouraging, because it argues that these spots are cyclin specific. This may be true. However, the *GAL-CLB2* does not induce phosphorylation of any spots we can see, although we know that Clb2-associated histone H1 kinase activity is dramatically induced. The *GAL-CLB2* seems to make the cells rather sick, and inhibit incorporation of ^{32}P. Thus, the failure to see induced phosphorylation may not be meaningful.

In the near future, we will try *GAL-PCL1*, which will be especially interesting, since on the one hand it is expressed in G1, and so in some sense is a "G1 cyclin", but on the other hand its Cdk is Pho80, not Cdc28.

B. Develop antibodies against phosphoSer-Pro and phosphoThr-Pro.

The phosphorylated peptide antigens are in the process of being synthesized.

In the meantime, it has become apparent that a pre-existing monoclonal antibody called MPM2 may be exactly the kind of reagent we are trying to make (Westendorf et al. 1994). MPM2 was discovered many years ago (Davis et al. 1983) as a monoclonal antibody detecting antigens which appear specifically during mitosis. It then became clear that MPM2 was detecting certain phosphoproteins, and it is now known that the MPM2 epitope includes phosphoSer-Pro (^{P}SP) or phosphoThr-Pro (^{P}TP). Since these are the key residues in a consensus Cdk phosphorylation site (S/T-P-X-K/R), phosphorylated Cdk substrates are amongst MPM2-reactive proteins. Recent work of Westendorf et al. suggests that some very good Cdk sites (e.g., L^{P}TPLK) are excellent MPM2 antigens, while other good sites (e.g., K^{P}TPVK) are poor MPM2 antigens. Thus, MPM2 may react with some substrates but not others. This will probably be true of any other antibody, as well.

Some other MPM2 epitopes are not cdc2 sites, because they do not have the basic residue in the fourth position. Instead, they are probably MAP kinase sites. Nevertheless, if the only requirement for recognition by MPM2 is phospho-Ser-Pro or phosphoThr-Pro, then MPM2 will recognize phosphorylated cdc2/Cdc28 sites as well as MAP kinase sites.

MPM2 has just become commercially available from UBI, and we have recently received our first vial. We look forward to using MPM2 in combination with the *GAL-CLN* ^{32}P-labelling experiment. That is, we will induce a *CLN* or *CLB* cyclin, label with ^{32}P, then immunoprecipitate with MPM2. The control will be uninduced cells. We will then run the immunoprecipitate on 1D and 2D gels, and look for ^{32}P-labelled spots specific to cyclin induction. If such spots are found, we will try to obtain enough for microsequencing, or for identification by other methods as described in the original grant proposal.

In addition, we have prepared some proteins known to be substrates for Cdc28-cyclin complexes (e.g., histone H1, Mcm3, Sic1), and are asking whether MPM2 will recognize these proteins after (but not before) phosphorylation.

C. Other work.

Two possible G1 substrates of Cdc28 are Sic1 and Mcm3. We have obtained purified Sic1 and Mcm3, and have phosphorylated them in vitro. Both are excellent substrates. Using 2D gels, we have assayed the number of phosphates added (each phosphate gives one extra spot in the isoelectric focusing dimension). Sic1 can gain up to 12 phosphates (!), whereas Mcm3 seems to get 4 or 5. We have not yet characterized Sic1 *in vivo*, but Mcm3 *in vivo* also seems to get 4 or 5 phosphates.

Personnel paid from the grant were B. Futcher (P.I.), D. Marshak (Co-P.I.), G. Sherlock (postdoc), J.P. Liu (lab technician) and E. Araya (part time lab aide).

7. Conclusions.

Except for the late arrival of the post-doctoral fellow, and consequently a delay in some of the research, the project is proceeding more-or-less as planned. The MPM2 antibody may be a valuable alternative to the anti-^{P}SP or ^{P}TP antibodies originally proposed.

8. References:

1. Davis, F. Tsao, T., Fowler, S. and Rao, P. (1983) PNAS 80, 2926-2930.

2. Westendorf, J.M., Rao, P.N., and Gerace, L. (1994) PNAS 91, 714-718.