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13. ABSTRACT (Maximum 200 words) Telomerase is an enzyme needed to maintain telomeres, and is therefore needed for the indefinite growth of cancer cells. An anti-telomerase drug might therefore be a good anti-tumor agent. To help in finding such drugs, two-hybrid and three-hybrid screens have been done in an attempt to identify new components of the telomerase complex. In addition, telomerase activity has been reconstituted in vitro. Reconstituted telomerase will eventually be useful for drug screening.				
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FOREWORD

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Bruce Fentz Aug 11, 1999
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Introduction.

Chromosome ends are capped by structures called telomeres. Since DNA polymerases synthesize DNA only in the 5' to 3' direction, they cannot completely replicate the ends of a linear molecule (Watson 1972; Olovnikov 1973). The problem is solved by an enzyme called "telomerase", which adds repetitive DNA (TTAGGG, in mammals) to chromosome ends to balance the loss of sequences at replication (Greider and Blackburn 1985; Greider and Blackburn 1987). Normal somatic cells lack telomerase activity, and so the telomeres of somatic cells get shorter and shorter with every cell doubling (Harley et al. 1990). Somatic cells have a limited replicative capacity (Hayflick 1961), and the lack of telomerase seems to be the reason for this, since expression of telomerase in otherwise normal fibroblasts allows them to double indefinitely, escaping the Hayflick limit (Bodnar et al. 1998).

Immortal cells must have a method of maintaining telomeres, and indeed it has been found that immortalized cell lines and all or almost all tumor cells have telomerase (e.g., Kim et al. 1994; Langford et al. 1995; Hiyama et al. 1996; Sommerfeld et al. 1996). Thus, it seems that switching on telomerase is an essential step in tumorigenesis. Telomerase may be the perfect target for anti-tumor drugs--normal somatic cells neither have it nor need it, while tumor cells do have it and do need it. The long term objective of this proposal is to enable the development of anti-telomerase drugs for cancer therapy.

However, since telomerase is a non-abundant and poorly characterized enzyme, our short term objectives have been to identify new components of the telomerase complex, and to produce telomerase activity *in vitro* for biochemical characterization. Candidates for novel telomerase components have been found, and are undergoing further testing. We have made small amounts of telomerase activity *in vitro*, and will soon produce telomerase in larger quantities.

Body of the Report.

There are three known components of human telomerase, hTR (the telomerase RNA) (Feng et al. 1995), hTERT (the reverse transcriptase) (Lingner et al. 1997; Meyerson et al. 1997; Nakamura et al. 1997; Harrington et al. 1997), and a protein called either TR1 or TLP1 (Harrington et al. 1997; Nakayama et al. 1997). hTR and hTERT are necessary and sufficient for *in vitro* activity; the function of TR1/TLP1 is not known. However, it seemed likely that a function as complex as that fulfilled by telomerase might require additional components, and therefore we used several methods to find additional proteins that might be part of the telomerase complex.

Aim 1. Identification of novel telomerase components using a 3-hybrid screen.

In Aim 1, which was largely completed during the first year, we used the three-hybrid screen (SenGupta et al. 1996) to find novel proteins interacting with hTR, the telomerase RNA. We found 36 different proteins in this screen (described and listed last year). Many of these appeared to be uninteresting, and may have been relatively non-specific. There were nine proteins about which absolutely nothing is known, and these are still difficult to evaluate. In addition, there were 5 proteins that appeared to be relatively interesting. These were: (1) hax-1, the protein we found most frequently; (2) DEK, a potential oncogene with slight homology to the Tetrahymena telomerase

component p95; (3) poly (ADP-ribose) polymerase (PARP), a protein with a BRCA1 domain, and involved in DNA repair; (4) KIAA0078, the human homolog of *S. pombe* Rad21, which is a cohesin; and (5) c-myc, an oncogene, and a transcription factor known to help control expression of telomerase. Our plan with these proteins was to immunoprecipitate them, and see if telomerase activity or the telomerase RNA co-precipitated.

We have had difficulty obtaining antibodies which successfully immunoprecipitate these proteins with acceptably low non-specific precipitation of other material. To begin to solve this problem, we tagged hax-1 with three tandem copies of the HA epitope, expressed the tagged hax-1 in human cells, and immunoprecipitated the hax-1 protein with monoclonal antibody from the 12CA5 cell line against HA. We labeled proteins with ³⁵S, and in this way showed that our immunoprecipitations were successful, clean, and specific. Unfortunately, we could not detect any telomerase activity or telomerase RNA above background in these immunoprecipitates. Therefore we conclude that hax-1 is probably not (tightly) associated with telomerase *in vitro*.

We now have an antibody which will probably allow us to immunoprecipitate PARP, and this will be the next protein to be tested.

We also tested the expression of many of the three-hybrid positives in various transformed or primary cell lines. We did not see a convincing correlation between expression and telomerase activity for any of the novel candidates tested. c-myc was not tested, since it was already clear from previous work that there would likely be a positive correlation.

Aim 2.

The original Aim 2 was to clone the reverse transcriptase subunit by an expression strategy. However, the telomerase subunit from yeast and *Euplotes* was found by other labs using other methods, and the human subunit subsequently appeared in databases as a result of cDNA sequencing projects Harrington et al. 1997; Lingner et al. 1997; Meyerson et al. 1997; Nakamura et al. 1997). Therefore we discontinued Aim 2, and substituted Aims 3 and 4.

Aim 3. Identification of novel telomerase components using a two-hybrid screen.

The two-hybrid screen is a method of finding proteins interacting with a given protein (Fields and Song 1989). Since the human reverse transcriptase subunit of telomerase, hTRT, had just been found, the two-hybrid screen seemed a natural and high-priority method to find novel telomerase components. This screen occupied most of our effort in the last year.

The hTRT gene was amplified by PCR from plasmid pCI-Nco-hEST2-HA (a gift of Dr. R. Weinberg's lab) and subcloned into each of two two-hybrid vectors, pGBD-C1 (TRP1 marker), and pGBDU-C1 (URA3 marker). These two vectors were then transformed into yeast strain PJ69-4a (James et al. 1996), which has three reporter genes for positive two-hybrid interactions: ADE2 (extremely stringent), HIS3 (moderately stringent), and lacZ (low stringency). Because the subcloned hTRT was tagged with HA, we were able to show using Westerns and the 12CA5 antibody that the human enzyme was indeed expressed in the yeast transformants.

Strain pJ69-4a bearing pGBD-C1 (TRP1) – hTRT was transformed with a HeLa

cDNA library (Hannon et al. 1993) in pGAD-GH (LEU2 marker). A total of 7×10^6 transformants were obtained. In addition, train pJ69-4a bearing pGBDU-C1 (URA3) – hTRT was transformed with the HeLa cDNA library and a total of 3.6×10^6 transformants were obtained. Summing the two, just over 10^7 transformants were examined.

Interactors were selected on –his plates (either –his –leu –trp or –his –leu –ura). A total of 62 colonies appeared within 3 days, and an additional 449 appeared by 7 days. These were tested for their expression of *ADE2* (i.e., their ability to grow on –ade plates) and their expression of B-galactosidase. 20 were Ade+, and 10 expressed B-galactosidase, but there was no overlap between the two groups (i.e., none of the 20 Ade+ colonies also expressed B-galactosidase).

All the Ade+ colonies were tested for dependence of the Ade+ phenotype on the hTRT plasmid (*TRP1* or *URA3*). The 20 clones were grown non-selectively, and replica-plated to –trp or to –ura medium, as appropriate. For all 20 clones, we were able to obtain Leu+ Trp- or Leu+ Ura- segregants. Unfortunately, all 20 were still Ade+, showing that the Ade+ phenotype did not require the hTRT plasmid, and so was not due to any interaction of any protein with hTRT.

Similarly the B-galactosidase phenotype was not dependent on the hTRT plasmid, and in fact the B-galactosidase phenotype was difficult to reproduce. Difficulties with the B-galactosidase phenotype have also been encountered by other investigators using this strain.

Finally, we checked all of the remaining colonies of the 62 that appeared within 3 days of transformation for dependence of the His+ phenotype on the hTRT plasmid. The clones were grown non-selectively, and replica-plated to –trp or to –ura medium, as appropriate. We obtained Leu+ Trp- or Leu+ Ura- segregants. Unfortunately, all of these were still His+, showing that the His+ phenotype did not require the hTRT plasmid, and so was not due to any interaction of any protein with hTRT. In summary, there is no evidence that we have obtained any genuine hTRT-interacting proteins.

The three-hybrid screen (SenGupta et al. 1996) had yielded putative telomerase RNA interactors, and these interactors were obtained from a two-hybrid activation domain library. A protein interacting with the telomerase RNA might also interact with the reverse transcriptase, and this was easy to test since we already had hTRT in a two-hybrid DNA binding construct. Therefore, we tested some of the positives from the three-hybrid screen against hTRT for a two-hybrid (i.e., protein-protein) interaction. So far, we have tested clones #70 (FRG1), #339 (DEK), #20 (NF90), #266 (hax-1), #60 (hax-1), #62 (cyclin G), #693, #315, #124, and #331. None of these gave a His+ phenotype, so there was no evidence for a two-hybrid interaction.

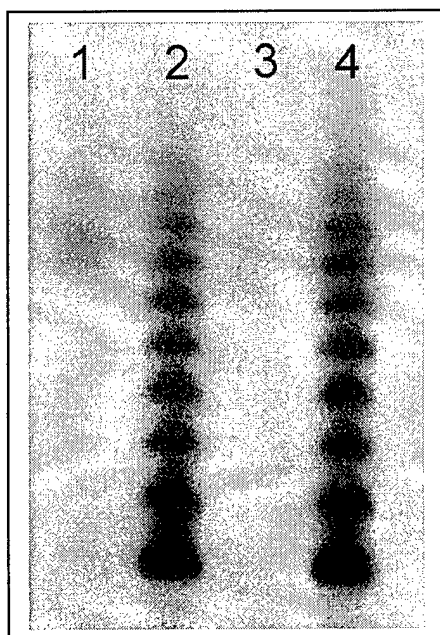
All these experiments were done with an HA-tagged hTRT clone obtained from Dr. R. Weinberg. We had used the HA-tagged form so that we could use Western analysis to demonstrate that the protein was actually being expressed in yeast before we did a large-scale two-hybrid screen. Recently, Dr. Weinberg informed us that this HA-tagged hTRT does not behave exactly like wild-type, untagged hTRT. Both the tagged and the untagged forms give telomerase activity *in vitro*. However, unlike wild-type hTRT, the tagged hTRT fails to maintain telomeres *in vivo* in human cells. It is possible

that this defect of the HA-tagged hTRT also prevents some wild-type two-hybrid interaction.

Aim 4. Reconstitution of telomerase activity *in vitro*.

We plan to reconstitute telomerase activity on a large scale. However, as a preliminary experiment, we have reconstituted telomerase activity on a small scale using *in vitro* transcription/translation. The telomerase RNA and the reverse transcriptase (hTRT) were both cloned behind a T7 polymerase promoter. These were then transcribed and translated *in vitro* using the TNT T7 coupled reticulocyte lysate system from Promega Corporation. After transcription/translation, telomerase activity was assayed using the TRAP assay. Results are shown in Fig. 1. Reconstitution of telomerase activity *in vitro* has also been accomplished by other workers (Beattie et al. 1998; Weinrich et al. 1997)

FIGURE 1. Reconstitution of Telomerase activity *in vitro*.



A TRAP assay on *in vitro* transcribed/translated hTR and hTRT. The ladder of bands in lanes 2 and 4 shows a positive result. Lane 1, negative control, no hTR or hTRT. Lane 2, 1 μ l of the hTR/hTRT mixture. Lane 3, 2 μ l of the hTR/hTRT mixture. Lane 4, 5 μ l of the hTR/hTRT mixture. It is not known why a negative result was obtained in Lane 3.

Research Accomplishments:

- Tested hax-1 for association with telomerase
- Screened over 10^7 HeLa cDNA clones for interaction with the human telomerase reverse transcriptase
- Screened 10 positive clones from the three-hybrid screen for a two-hybrid interaction with human telomerase reverse transcriptase

- Reconstituted human telomerase *in vitro*.

Conclusions:

The work this year focused on the two-hybrid screen. Unfortunately no genuine interactors were found. This may truly mean that there is no protein that interacts strongly with hTERT; or it may mean that such proteins are extremely non-abundant and so difficult to find; or it could mean that the version of hTERT used in our screen was defective for the hypothetical interaction. We confirmed that hTR and hTERT are sufficient to reconstitute telomerase activity *in vitro*.

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