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Subject: Interim Report on Cell Cycle dependent expression of proto-oncogenes (2-89-0036)

At the time the project "Cell cycle dependent expression of proto-oncogenes p53 and c-myc in hematologic malignancies" was proposed, both in vitro transfection experiments and in vivo analysis seemed to indicate that, in some cases, levels of the p53 protein correlated with the proliferative potential of the cells. We thus proposed to analyze the levels of p53 by several techniques, first in cultured cells and then in human hematologic malignancies. During the last one year, a major part of the first phase of the project was completed:

A) IMMUNOPRECIPITATION-IMMUNOBLOT

A transformed lymphocyte cell line (RAJI), as well as SV40 transformed MKSA cells were used throughout this study. Total cell protein was extracted with NP40 detergent and mixed with either specific monoclonal antibody against p53 (421) or control antibody. The immunocomplexes were then precipitated by adding protein A from Staphylococcus aureus. After several washings, the samples were applied to a denaturing polyacrylamide gel and the proteins separated according to their size. After transfer to a nitrocellulose membrane, specific bands were detected by probing with ^{125}I-labeled antibody against p53. The size of the resulting bands, present only with specific antibody, indicate that we identified p53 in these cell lines.

B) METABOLIC LABELING

RAJI cells were incubated in methionine-free medium with S-methionine, proteins extracted and immunoprecipitated as before. After washing, the labeled proteins were separated by denaturing polyacrylamide gel and autoradiographed. Although many labeled proteins were visualized, a band of about 53 kDa was present only when specific anti-p53 antibody was used.

C) EFFECT OF STORING CONDITIONS

The effect of storage conditions on both the stability of p53 and the ability of RAJI cells to incorporate 35S-methionine was investigated. According to our results, preformed p53 is stable to incubation of the cells for 2 hours at either 4°C or room temperature. The ability to synthesize p53, measured by the amount of S-labeled p53, was not impaired after 3 h at RT or 4°C. The minimal amount of cells required to identify p53 by immunoprecipitation-immunobloting was 5 million whereas by metabolic labeling, this protein was visible with 1 million cells.

D) IMMUNOFLUORESCENCE

We tried to develop an immunofluorescence method that would specifically stain the p53 protein in transformed lymphocytes. Briefly, RAJI cells were cyto-spun into glass slides and fixed with 50% normal goat serum and the primary antibody (421 anti-p53 or control antibody) was added. After washing PBS with 0.2% Tween 20, the secondary antibody was added. Different secondary antibodies (affinity purified IgG or Fab2 fragments of goat anti-mouse antibodies) labeled with either fluorescein or biotin-streptavidin-fluorescein, and absorbed with lymphocyte or liver extracts were all tried. Specific staining of intranuclear proteins was observed only in MKSA cells (grown in cover slips) but not in cyto-spun RAJI cells due to high background staining (probably from non-specific adsorption of the secondary antibody).

In summary, we were able to measure p53 levels in cultured cells by immunoprecipitation and gel-electrophoresis using fairly large amounts of cells. In order to use clinical material, we would need a much more sensitive assay, ideally measuring p53 levels on a cell by cell basis. Flow-cytometry seems adequate for this purpose but we were unable to specifically stain RAJI cells with fluorescent antibodies due to high background problems.

Moreover, recent studies reexamined the in vitro experiments and showed that in many transfection assays where p53 was shown to promote cell growth and transformation, a mutated p53 gene was used. In vivo studies showed deletion and mutation of p53 genes in naturally occurring tumors. This has led to the current belief that p53 is not a proto-oncogene as initially thought but a tumor suppressor gene. Thus, any evaluation of the role of p53 in neoplastic transformation should take into account the possibly different role of mutated vs. normal p53 gene. Simple detection of steady state levels of p53, even at different phases of the cell cycle, as done by Flow Cytometry, may not be of significant informative value in the absence of sequencing data. Sequencing or mutation detection of the p53 genes is technically difficult, would out of scope of the present study.

FUTURE DIRECTIONS

After establishing the basic techniques for the study of proto-oncogenes in the first part of the project we will like to embark on the second part viz. the study of in vitro model of neoplastic transformation. This model system is composed by:

- a non-transformed liver epithelial cell line (C61)
- a non-transformed C61 clone (PF3) that was transfected with the proto-oncogene c-fos
- a transformed clone (PF3/4) derived from PF3 cells by selecting for growth in soft agar medium.

Important characteristics of this system are:

- the y-glutamyl-transpeptidase (GGT) gene is silent in non-transformed cells but always active in transformed clones, and is thus an easily measurable marker of neoplastic transformation.
- the expression of the endogenous c-fos gene is inducible by serum in non-transformed cells but it can't be induced in transformed cells.

We think that the correlation between GGT expression and c-fos non-inducibility with transformation can yield very important clues about the basic mechanisms of transformation by analyzing the regulation of those 2 genes. An important part of this studies would involve a cell by cell analysis of GGT and c-fos expression by immunocytochemistry and flow-cytometry.

We have conserved most of the grant money, which we believe can now be usefully applied to our model system. However the present study requires extension of the time frame to one more year. We shall appreciate your consideration for the extension.

Sincerely,

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AS:pf

Enclosure

Figure 1

Immunoprecipitation - Western Blot with pAB 421

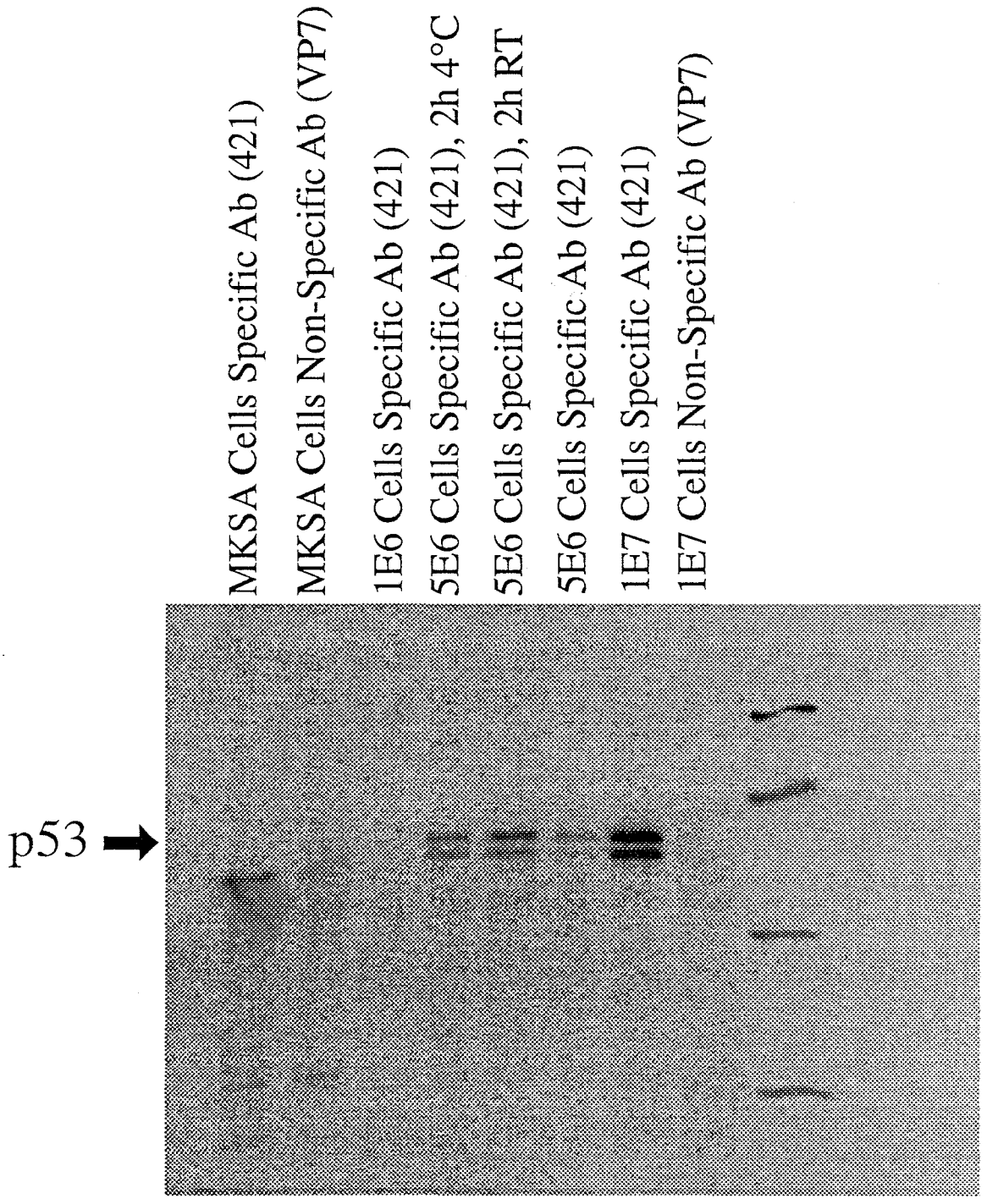
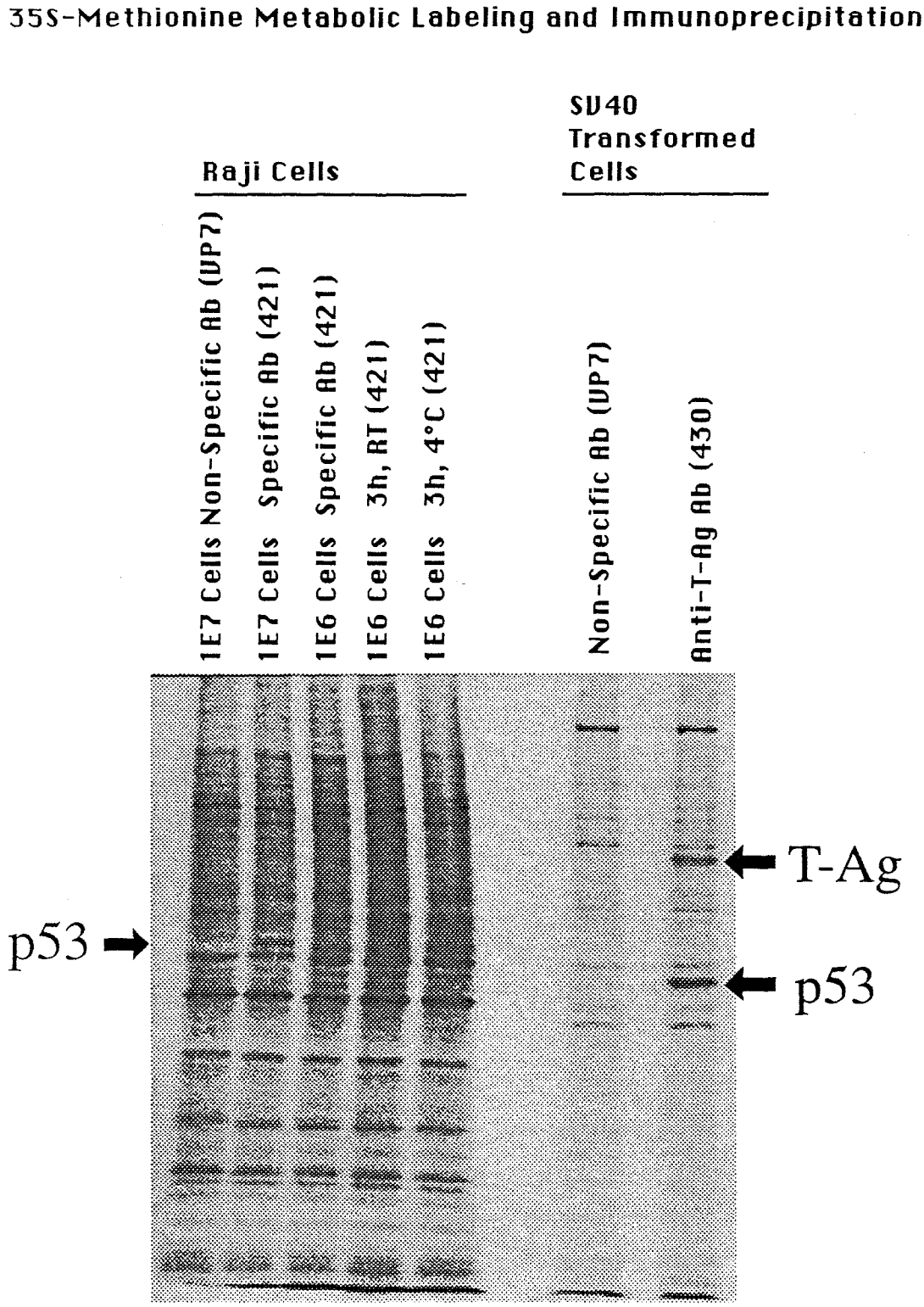


Figure 2


35S-Methionine Metabolic Labeling and Immunoprecipitation
Raji Cells
SV40 Transformed Cells
1E7 Cells Non-Specific Ab (UP7)
1E7 Cells Specific Ab (421)
1E6 Cells Specific Ab (421)
1E6 Cells 3h, RT (421)
1E6 Cells 3h, 4°C (421)
Non-Specific Ab (UP7)
Anti-T-Ag Ab (430)
p53
T-Ag
p53