LA-UR-01-6806
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Title: **Vibrational Spectroscopy of Viable, Paired Tumorigenic and Non-Tumorigenic Cells**

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Submitted to:

Vibrational Spectroscopy of Viable, Paired Tumorigenic and Non-Tumorigenic Cells

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ABSTRACT

Infrared absorption of two pairs of non-tumorigenic and tumorigenic cells suspended in phosphate buffered saline have been measured. Suspensions of cells with several different growth cycle distributions were measured. The effect of both growth cycle and tumorigenity on the infrared absorption spectrum will be presented. For example, changes in absorption in the phosphate absorption region were observed for suspensions with different cell cycle distributions. We will discuss the biochemistry which may cause these changes. We have found that spectra of isolated nuclei allow the DNA spectra to be studied, without the confounding influence of RNA. Therefore, the measurement of isolated nuclei may represent a method of detecting changes in DNA architecture with cell cycle. As part of this exploratory study we have also examined the variation in spectra with cell type and compared epithelial cells with fibroblast cells. Very little change was observed. Similarly we saw very little change in the spectra of tumorigenic and non-tumorigenic cells harvested with similar cell cycle distributions. Changes in the spectra were observed when rapidly growing tumorigenic cells were compared to slowly replicating nontumorigenic cells.

INTRODUCTION

IR and Raman spectroscopic measurements can provide detailed molecular information. Consequently, these spectroscopies have the potential to generate important biochemical information for tissue diagnosis. Some of the medical applications of vibrational spectroscopy currently under investigation include characterization of atherosclerosis (plaque in arteries), and detection of cancer.

METHODS

Biochemical components of cells were obtained and measured in aqueous media. Type I "highly polymerized" calf thymus DNA (Sigma) was dissolved in TE buffer (pH 8.02, 10mM TRIS, 1mM EDTA). Type IV calf liver RNA was also dissolved in TE buffer. L-α-phosphotidylcholine and L-α-phosphotidylethanolamine were dissolved in a buffer consisting of 0.01 M, pH 7.3 TRIS, 0.1 M NaCl and 0.02 M sodium azide. Albumin was measured in a pH 7 phosphate buffer and lysozyme was measured in pH 3.8 sodium citrate buffer.

Four types of cells derived from rat embryo fibroblast (REF) cells were used. M1 and Rat1 cells are immortalized but non-tumorigenic derivatives of REF cells. MR1 cells are a tumorigenic derivative of M1 cells: specifically, these cells were transfected with the point-mutated T24Ha-ras-oncogene. Similarly, Rat1-T1 cells are a tumorigenic derivative of Rat1 cells obtained by a stable transfection with the same mutant h-ras oncogene. Cells were cultured as monolayers in standard tissue culture flasks using Dulbecco's modified eagle's medium containing 4.5 g/l D-glucose, 5% (v/v) fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Two types of epithelial cells were also measured. AT3.1 and AT6.1 are androgen-independent malignant rat prostate carcinoma cells[1] kindly supplied by Dr. Rinker-Schaeffer of the University of Chicago. Cells were cultured as monolayers in α-MEM (Invitrogen) containing 10% (V:V) fortified calf serum (Hyclone Laboratories) and antibiotics (50 μg/ml streptomycin and 50 U/ml penicillin, Invitrogen).

Cell suspensions were obtained from monolayer cultures by treatment for 10 minutes with 0.25% trypsin in a Dulbecco's phosphate buffered saline. The cell suspensions were subsequently centrifuged to obtain a cell pellet; subsequently, phosphate buffered saline (PBS) was added to the cell pellet to obtain a cell suspension with approximately 10^8 cells/ml. The cell cycle distribution of the cells was determined by flow cytometric DNA content analysis.

Nuclei were prepared from cell suspensions using a hypotonic lysis and sucrose gradient procedure. Briefly, a cell suspension in media was centrifuged (1000 rpm x 10 minutes) and the pellet was resuspended in a hypotonic lysis buffer (HLB:10 mM Tris, 5 mM KCl, 1.5 mM MgCl2, pH 7.9) for 10 minutes on ice then passed 12 times through a 22-guage needle to lyse the cells. The nuclei suspension was layered on top of a sucrose cushion (800 mM sucrose in HLB) then centrifuged (5000 rpm x 10 minutes). The nuclei pellet was resuspended in sucrose buffer (250 mM sucrose, 10 mM Tris, 3.3 mM MgCl2, pH 7.9), centrifuged at low speed, and the pellet resuspended in sucrose buffer. This nuclei suspension was then layered on top of another sucrose cushion (350 mM sucrose, 0.5 mM MgCl2) and spun again at high speed. This final nuclei pellet was washed twice with phosphate-buffered saline (PBS) using low-speed centrifugation then resuspended in ice-cold PBS for analysis.

Infrared absorption of cells in PBS and of nuclei in PBS was measured on a Mattson Cygnus-100 FTIR with a DTGS detector. The cell suspension was loaded into a 50 micron thick space between two $BaFl_2$ windows. The absorption was calculated as $-\log(I_c/I_{PBS})$, where I_c is the intensity measured when the sample cell was loaded with cells and I_{PBS} is the intensity measured when the sample cell was loaded with saline. Each intensity measurement consisted of 200 scans at 2 cm^{-1} resolution. For each experiment alternating measurements of cells and PBS were made. Typically two measurements of saline, two measurements of cells, two measurements of saline, etc. For analysis the spectra were scaled to have an absorbance of 0.075 at 1400 cm^{-1}. No baseline correction was performed accept were specifically mentioned in the results section.

RESULTS

Spectra of Biochemical components

In order to attribute changes in the spectra of biological cells to changes in biochemical composition, it is necessary to know the spectral regions where the individual biochemical components of cells absorb. Albumin and lysozyme were measured to obtain an estimate of which absorption bands of cells are primarily due to proteins. The absorption spectra of RNA, DNA and a 50/50 mix of phosphotidylcholine and phosphotidylethanolamine were also measured. Comparison of the cell spectra and component spectra in Figure 1 demonstrates that cell absorption bands at 1400, 1450 and 1548 cm^{-1} are primarily due to proteins, although the 1450 band may have a small contribution from phospholipid. The broad band between 1200 and 1250 cm^{-1} has contributions from phospholipid, protein, and the nucleic acids. The band at 1120 cm^{-1} is due to absorption by RNA, the two peaks at 1087 and 1054 cm^{-1} are due to both RNA and DNA with an underlying phospholipid absorption. The absorption at 971 cm^{-1} is due primarily to DNA with some contribution from both phospholipids and RNA.

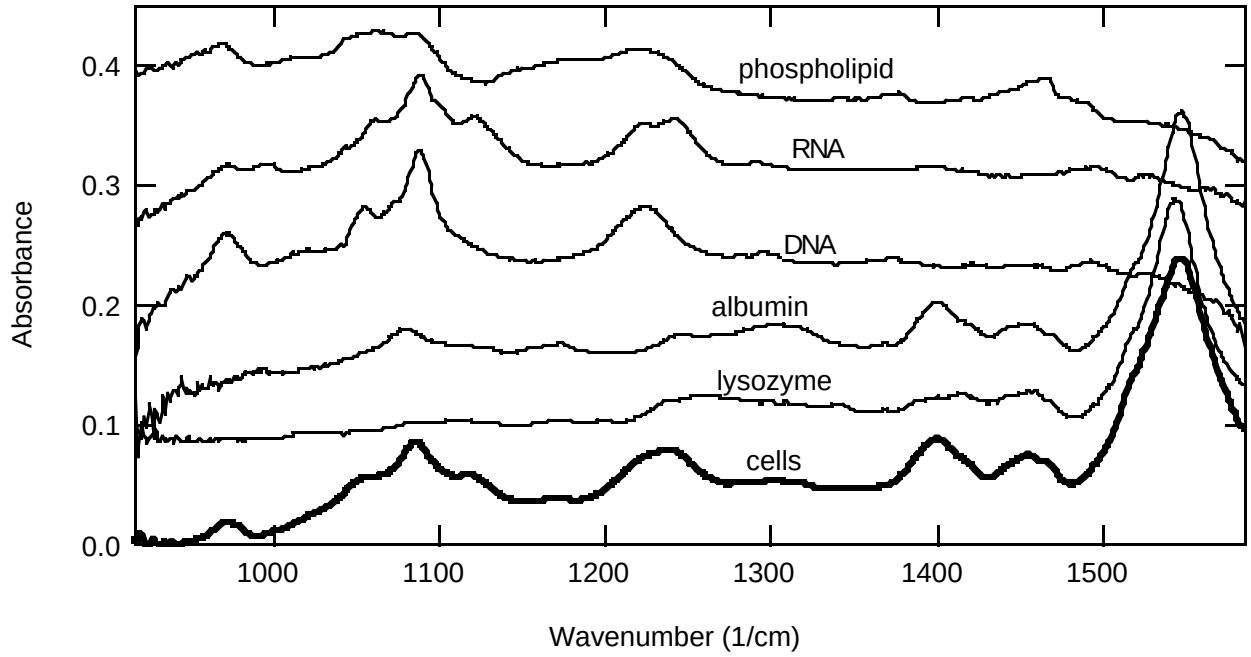

Figure 1. Absorption spectra of several biochemical compounds of expected to be present in biological cells as well as the absorption spectra of some fibroblast cells. Spectra have been offset on the y-axis for clarity.

Effect of growth stage

For each suspension of cells measured the percent of cells in G1, S and G2 was determined. The spectra were then grouped based on the percent of cells in G1 as shown in Table 1. The groups were chosen such measurements were made of at least four cell preparations in each group. The two exception were, M1 cells with less than 50% of the cells in G1 for which we only had one measurement and Rat-T1 cells with more than 75% of the cells in G1 for which we had no measurements. Results for M1, MR1, Rat1 and Rat1-T1 cells are shown in Figure 2a,b,c,and d. The spectral shape from 1000 cm^{-1} to 1150 cm^{-1} changes with the percent of cells in G1. To quantify one of the spectral changes, we fit the spectral region 1051 to 1066 cm^{-1} to a straight line. The slopes of these lines are given in Table 1. The slope decreases as the fraction of the cells in G1 increases. This change probably represents an alteration in nucleic acid content or conformation.

	Slope from 1051 to 1066 cm^{-1}		
	% cells in G1 < 50%	% cells in G1 < 50% & > 75%	% cells in G1 > 75%
Rat1	0.00054	0.00022	-0.00011
Rat1-T1	0.00053	0.00021	No data
M1	0.00040	0.00037	-0.00016
MR1	0.00038	0.00031	-0.00001

Table 1. Slope from 1051 to 1066 cm^{-1} of spectra obtained of cell suspensions with different cell cycle distributions.

In addition to the change in the spectral shape of the phosphate absorption, there is a trend that the absorption due to phosphate relative to the absorption due to protein decreases with increasing G1 content. The ratio of the absorption at 1087 cm^{-1} to the absorption at 1400 cm^{-1} is given in Table 2. With the exception of the M1 cells, this ratio decreases with increasing G1 content. The ratio given for M1 cells with greater than 75% of the cells in G1 may have significant error. This number was

calculated for only one measurement, whereas all of the other ratios were calculated from an average of measurements of multiple cell preparations.

	A(1086.6)/A(1400.2)		
	% cells in G1 < 50%	% cells in G1 < 50% & > 75%	% cells in G1 > 75%
Rat1	1.27	0.97	0.89
Rat1-T1	1.12	1.08	No data
M1	1.09	1.03	1.13
MR1	1.17	1.16	1.12

Table 2. Ratio of the absorption at 1087 cm^{-1} to the absorption at 1400 cm-1 as a function of cell cycle distribution.

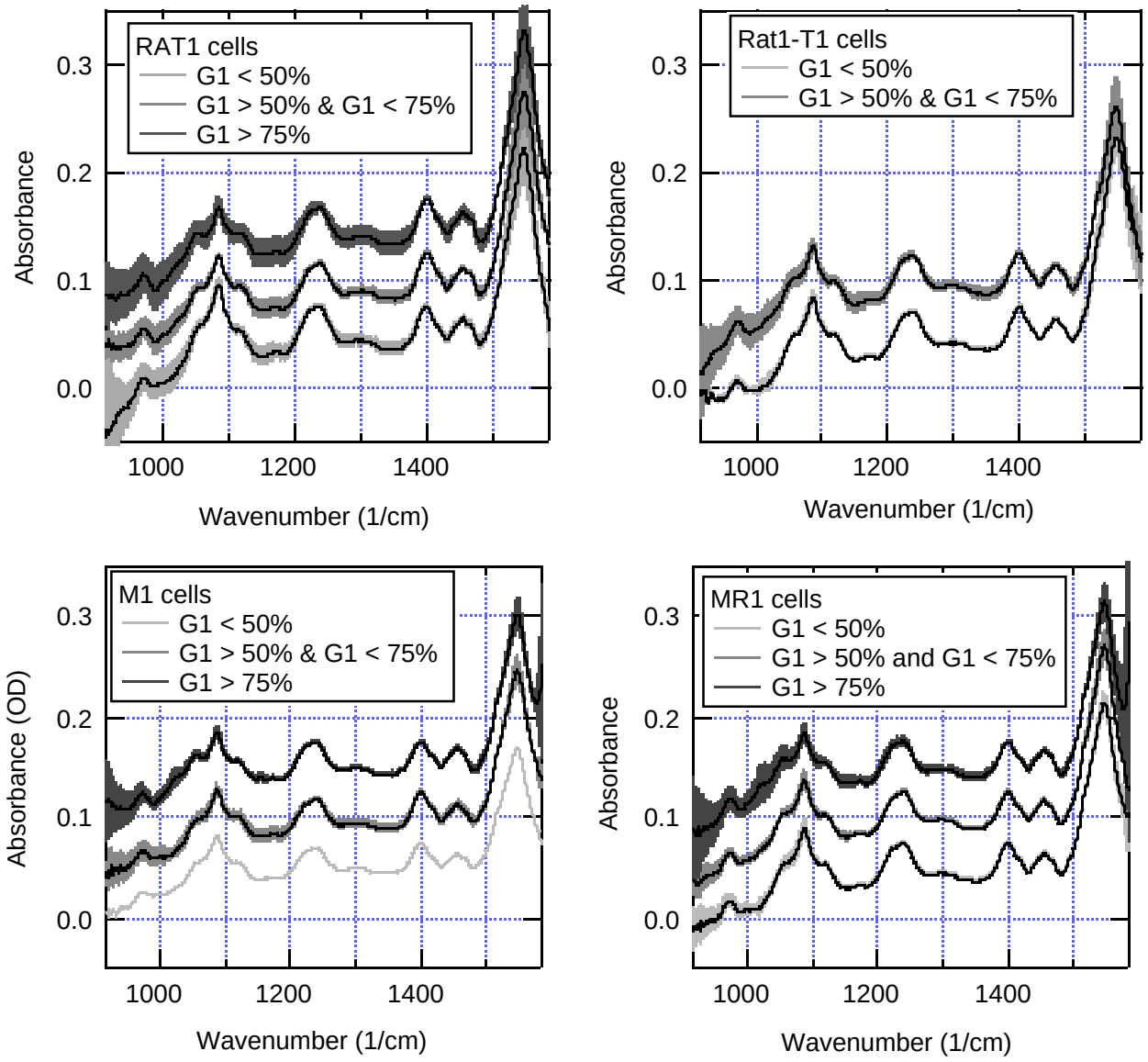

Figure 2. Spectra of tumorigenic and non-tumorigenic cells as a function of the percent of cells in the G1 phase of the cell cycle. Except for M1 cells with the G1 percent less than 50%, multiple preparations of cells were measured for each spectra shown. The broad bands show the standard deviation of the absorbance at each wavenumber while the solid black line is the average of the spectra. No baseline correction was performed. The spectra for cell suspensions with the percent of cells in G1 between 50 and 75% are offset by 0.05 absorbance units. The spectra for cell suspensions with G1 greater than 75% are offset by 0.1 absorbance units.

Difference in tumorigenic and nontumorigenic cells

In precancerous tissue and early stage tumors, cells typically replicate rapidly. Consequently, there are only a small percent of the cells are in the G1 phase of the cell cycle. In normal tissue a high fraction of cells are in G1. Therefore, we have compared spectra of tumorigenic cell suspensions with less than 50% of the cells in G1 to spectra of non-tumorigenic cells with more than 75% of the cells in G1. Figure 3a shows difference spectra for both the M1/MR1 pair and the Rat1/Rat1-T1 pair. The spectral differences are attributable to differences in cell cycle distribution as opposed to intrinsic cellular differences. In Figure 3b the spectral differences between cell suspensions with less than 50% of the cells in G1 and more than 75% of the cells in G1 are shown.

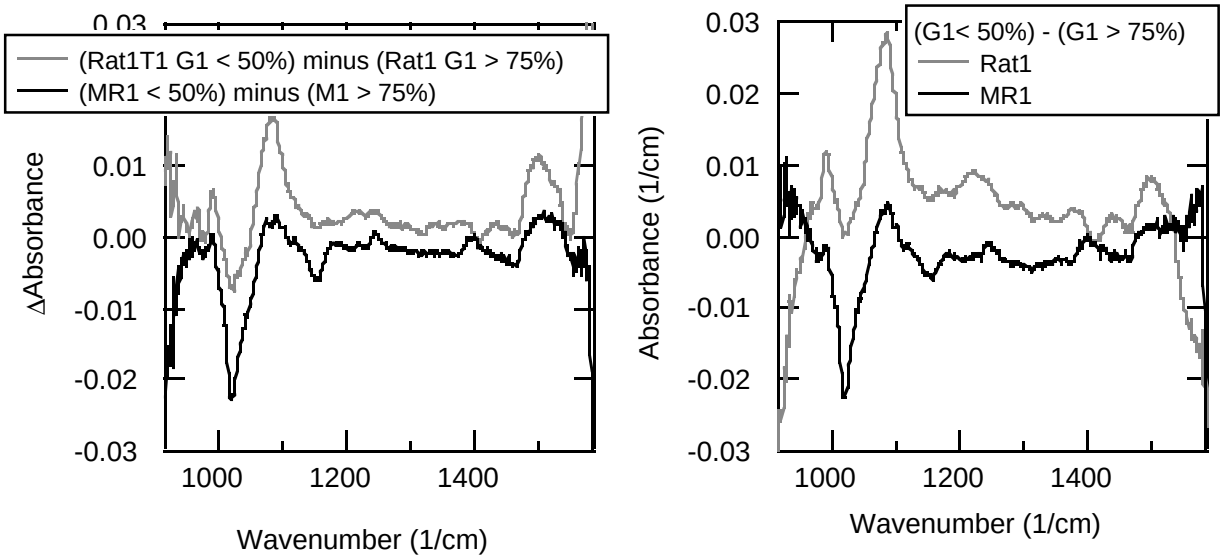

Figure 3. a) Spectral differences between rapidly growing tumorigenic cells and slowly growing non-tumorigenic cells. Before the subtraction of the M1 and MR1 cells spectra was performed, a constant was subtracted from the M1 spectrum and the spectrum was rescaled to have the same absorption at 1400 cm^{-1} as the MR1 spectra. b) Spectral differences between slowly and rapidly growing cells of the same cell type.

Comparison of Epithelial cells and fibroblast cells

Spectra of epithelial and fibroblast cells were compared to determine how the spectra of cells may differ with cell type. Figure 4 shows spectra of Rat1 fibroblast cells and AT3.1 epithelial cells. Both cell suspensions had very similar cell cycle distributions. For the Rat1 cells, 40% were in G1, 39% in S, and 22% in G2. For the AT3.1 cells, 40% were in G1, 38% in S, and 23% in G2. The absorption spectra are similar with peaks in the same locations. There are some small changes in relative peak heights which could indicate an increased protein concentration relative to DNA, RNA and phospholipid for the AT3.1 cells.

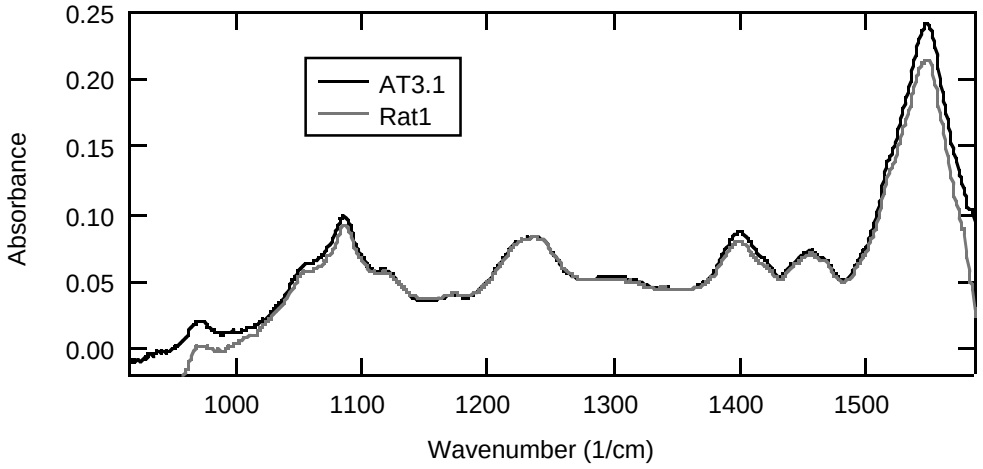

Figure 4. Comparison of the spectra of AT3.1 and Rat1 cells. As described in the text the cell cycle distribution was the same for the two cells suspensions.

Infrared absorption of nuclei

MR1 nuclei were isolated from MR1 cells as described in the methods section. Figure 5 shows a spectra of the nuclei. There are some striking differences between the spectra of the nuclei and the spectra of MR1 cells. The peak at 1120 cm^{-1} due to RNA is gone and the absorption in the phosphate region looks like the absorption of DNA. The band between 1200 and 1250 cm^{-1} is different in shape with the intensity at 1241 cm^{-1} decreasing. This change is also consistent with the nuclei containing much less RNA than the cells.

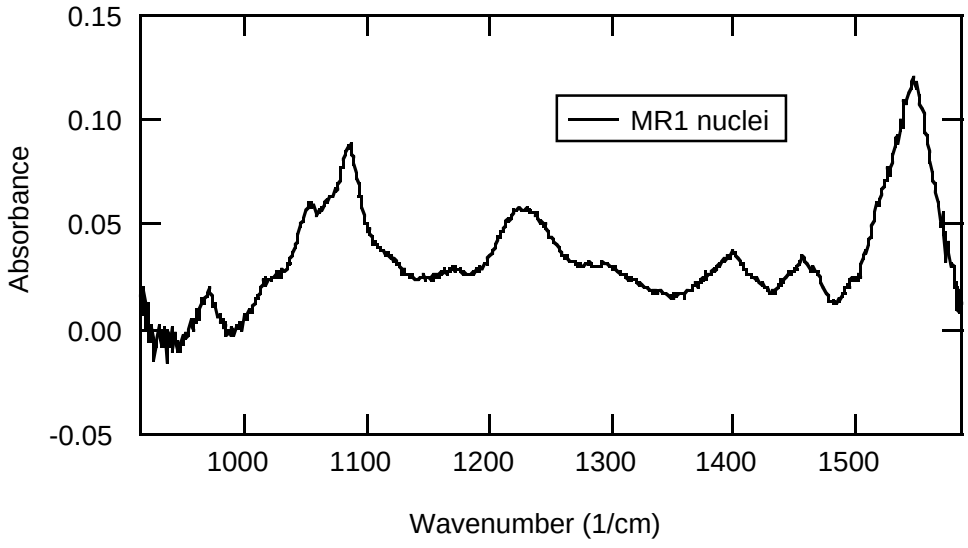

Figure 5. Spectra of nuclei isolated from MR1 cells.

DISCUSSION

Spectral differences have been observed for four types of fibroblast cells as a function of cell cycle. In particular changes were noted as a function of the percent of cells in G1. These spectral changes could also be correlated with a change in S-phase concentration. We found a strong correlation between a decreasing percent of cells in G1 and an increase in the percent of cells in S-phase, shown in Figure 6. The spectral changes occur in regions where RNA and DNA are the major absorbers. We noted two particular changes. 1. There is a change in slope from 1051 to 1066 cm^{-1} when the percentage of cells in G1 changes. 2. There is an increase in phosphate absorption at 1087 cm^{-1} compared to protein absorption at 1400 cm^{-1} with decreasing G1 content. To obtain a better understanding of the biochemistry behind the change in slope we attempted to fit the spectral region from 1035 to 1141 cm^{-1} to a superposition of RNA, DNA and phospholipid absorption plus a linear baseline. We found that the data was not well fit. This results indicates that either our spectra of the components do not accurately represent the *in vivo* spectra, there is an absorbing species we are not accounting for, or there are changes in the conformation of nucleic acids as a function of cell cycle that affects their *in vivo* spectra.

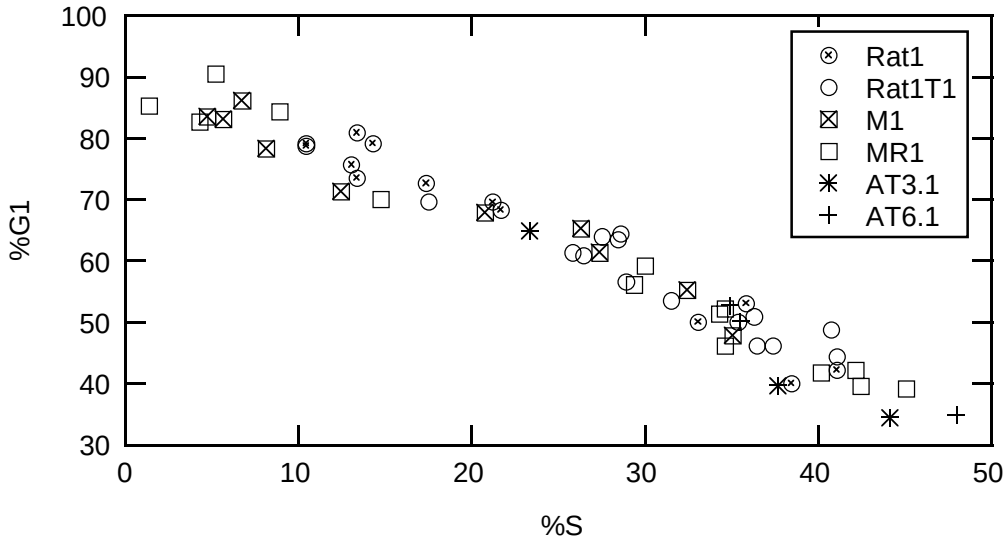

Figure 6. Correlation between the percent of cells in the G1 phase of the cell cycle and the percent of cells in the S phase of the cell cycle.

Changes in the phosphate absorption region as a function of cell cycle have also been observed in myeloid leukemia cells[2]. There are a couple of significant differences in how that data was obtained compared to our measurements. One major procedural difference was that the cells were fixed with ethanol and a single cell layer was measured on a polyethylene membrane rather than measuring a suspension of cells in PBS. This difference may be related to a significant difference in the published spectra of the myeloid leukemia cells in the phosphate absorption region, 1020 – 1150 cm^{-1}. The spectra of the fixed cells did not show the spectral detail seen in our spectra of fibroblast and epithelial cells, although the measurements were obtained at the same resolution. Possibly this difference is caused by the ethanol fixation, although we can not rule out that the spectra of leukemia cells are simply different from that of epithelial and fibroblast cells. The idea that the spectral differences are caused by the dehydration accompanying fixation is supported by the fact that spectra of dried RNA and DNA presented in that paper have less sharp spectral features than the spectra of RNA and DNA we obtained in aqueous media. The other significant difference in the procedure for measuring the myeloid leukemia cells is that the myeloid leukemia cells were separated by centrifugation and spectra were obtained for cells in G1, S, and G2 rather than measuring cell suspensions with varying cell cycle distributions. The authors report that the absorption in the phosphate region decreases when the cells are in G2. This result

is somewhat surprising, because the DNA content is higher in G2. The authors explain their result by saying that "assuming, for simplicity, a spherical nucleosome with 500 nm diameter, its optical density would exceed 50" and concluding that "the nucleosomes of an inactive nucleus will appear as 'black' dots that yield no spectral information from the DNA". While this is a very interesting idea, that the packing causes some of the DNA to be invisible, our measurements indicate that DNA is not packed densely enough for this scenario. DNA in a cell is wrapped around nucleosome core particles, creating a particle with a radii of about 5 nm. This particle contains146 base pairs of DNA[3]. Our calculations show that the concentration of DNA bases is roughly 8 mM in this structure. We have measured a DNA suspension that was roughly 30 mM in DNA bases using a 50 micron spacer. The greatest absorbance was 0.1 OD. Therefore, the absorption of the DNA in a cell can not be great enough to cause an effective shading of some of the DNA.

Finally, regarding the spectral changes with cell cycle we note that we have considered whether these differences could be systematic artifacts, such as a change in the cell size causing the displacement of PBS to be different or the growth media not being washed off. The changes in the spectra are inconsistent with either artifacts due to phosphate buffer or growth media absorption.

One of the goals of this work was to determine whether there are spectroscopic differences in tumorigenic and non-tumorigenic cells. Primarily we are interested in comparing rapidly growing tumorigenic cells to slowly replicating nontumorigenic cells. Differences in the spectra of these cells were noted and were attributed primarily to differences in cell cycle distributions. We also compared the spectra of tumorigenic and nontumorigenic cell suspensions with similar cell cycle distributions. This comparison was somewhat difficult because of a lack of data for Rat1T1 cell suspensions with more than 75% of the cells in G1 and lack of data for M1 cell suspensions with less than 50% of the cells in G1. The most consistent change we saw was a decrease in absorption at 1400 cm^{-1} between tumorigenic and nontumorigenic cells with between 50% and 75% of the cells in G1.

The near lack of a difference between spectra of tumorigenic and non-tumorigenic cell suspensions with similar cell cycle distributions is consistent with the small spectral changes seen between epithelial and fibroblast cells. The small spectral differences seen at 1400 and 1548 cm^{-1} may be due to a relative change in protein concentration. The changes seen in Figure 4 in the phosphate region are most likely baseline errors in the measurements.

Finally, we found that the nuclei of a cell has a very different absorption spectra from the cell itself. There is virtually no absorption due to RNA and the absorption in the phosphate region resembles that of DNA. By measuring the spectra of nuclei isolated from cell suspensions with different cell cycle distributions we hope to be able to understand the spectroscopic differences noted in Tables 1 and 2 as a function of cell cycle.

ACKNOWLEDGEMENTS

This work was funded by NIH CA89255. We would also like to acknowledge Tamara Johnson for measurements of growth media and phospholipid.

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