In Vitro Transfer RNA Methylation in Paired Neoplastic and Nonneoplastic Cell Cultures

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A great deal of insight and valuable information has been derived from in vivo tumor systems, suggesting that aberrant methylation may be a primary biochemical lesion resulting in cancer. In these systems, comparisons of RNA methylation capacity are made between tumor tissue extracts and extracts of adjacent normal tissue, regenerating liver, or embryonic tissue. In making such comparisons, it is difficult to control certain factors such as rates of cell division, differences in state of differentiation, and more subtle parameters involving the physiological state and response of the host. This paper describes an in vitro system in which these several factors are more amenable to control and presents results that support and extend the aberrant methylation hypothesis.

Briefly, this in vitro system consists of established paired cell lines where a malignant line is derived from the nonmalignant line (for details, see Ref. 6). A culture is initiated from a minced mouse embryo pool in NCTC 135 supplemented with 10% fetal calf serum. After 60 to 90 days, a line is split off, and the serum supplement is switched to 10% gelding horse serum. This serum switch results in malignant conversion of the line in an additional 120 to 180 days in culture (5). After malignant conversion, the line is switched back to 10% fetal calf serum supplement in order to eliminate any difference due to the serum. The nonmalignant line is continued in culture so that the time in vitro is the same for the paired cultures. Some pertinent points regarding this system are: (a) the growth rates of the paired lines are essentially the same; (b) the cells are grown as monolayers and both lines pile up somewhat but to the same small extent; (c) both lines are fibroblast-like, so the state of differentiation should be equivalent; (d) cancer is monitored by injection into irradiated isologous hosts.

The evidence which suggests that there is a qualitative difference between the RNA methylase enzyme complements of the paired malignant and nonmalignant cell lines is shown in Chart 1. The rate of incorporation for the nonneoplastic cell extract plateaus at about 3 mg/ml of protein, while the rate of the neoplastic cell extract continues to increase rapidly with increasing protein concentration. This indicates that the neoplastic cell extracts have a greater capacity to methylate tRNA than the nonneoplastic cell extracts. At the lower protein concentrations, where enzyme activity is measured, the activities of the 2 extracts are not greatly different.

Since most comparisons reported in the literature (for a recent review, see Ref. 4) show large differences in activity as well as capacity, it was of interest to see whether the activity of the neoplastic cell extracts was low as compared to tumor tissue or whether the activity of the nonneoplastic cell extracts was high compared to normal tissue. In order to determine these activities, extracts from normal tissue produced by injection of cells from the malignant line into irradiated, isologous mice were compared with the extracts from the nonmalignant cell line. These results are shown in Chart 2. It is clear that the methylase activities are essentially the same, while the capacities are dramatically different. The conclusion here is that the nonneoplastic cell methylase activities are high as compared to the reported normal tissues. A plausible explanation for this is that the growth rate of the nonneoplastic cell line is rapid compared to the usual normal tissue growth rates which have been tested.

An alternative to the conclusion that there is a qualitative difference in the RNA methylase complements of the malignant and nonmalignant lines is that there may be a difference in nuclease activity. It is conceivable that the small amounts of nuclease could either create new sites for methylation or destroy existing sites, resulting in apparent enhanced or diminished capacity. Also, the presence of an enzyme that cleaves S-adenosylmethionine or the possibility that something other than the tRNA is the rate-limiting factor could result in an artifactual plateau. These trivial explanations have been experimentally considered, and it was concluded that they are not responsible for the differences in methylase capacity (6).

If these cell-free extracts actually reflect a qualitative difference expressed in the intact cell, it should be possible in principle to isolate a methylated product in the malignant cells which is absent in the nonmalignant cells. In order to look for such a difference, the paired cell lines were grown in methylated methionine, the cells were mixed together, the nucleic acids were extracted and chromatographed on a methylated albumin-Kieselguhr column, and the tRNA peak was pooled and precipitated (7). This purified tRNA was then chromatographed in a reversed phase chromatography system (9), and the radioactivity profiles were compared (Chart 3). The radioactivity profiles indicate that the methyl group distribution is quite similar in the malignant and nonmalignant tRNA's. However, one small reproducible difference to be seen in the early part of the elution profile is readily seen in the plot of $^3$H:$^3$C in the lower inset. The dip in the ratio around Tube 90 indicates that in this area of the profile there is relatively more methylation of the malignant cell tRNA compared to the nonmalignant cell tRNA. The $^3$C:$^3$H plot in the upper inset is an identical experiment in which the methyl-labeled methionine isotopes were reversed; again the...
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forward by Srinivasan and Borek (8) in 1964. Further, these experiments broaden the base support for the hypothesis by extending the original observations to an experimentally more flexible in vitro system. The small differences observed in the intact cell methylation experiments are attributed to more adequate control, and in retrospect this should be expected since the fundamental difference between a malignant and nonmalignant cell is sure to be subtle. The obvious immediate goals in this system are: (a) to see whether a positive correlation exists between the time of malignant conversion and the appearance of this difference, along with other methods of conversion such as by chemical carcinogens and viruses; and (b) to establish the precise nature of these differences.

The emphasis of this symposium is on tRNA methylation, its function, and its relationship to cellular differentiation and cancer. This is as it must be since this is the area in which techniques are available for exploration and much activity is going on as a result. However, other nucleic acids are methylated, and as is evident in Chart 5, the greatest apparent differences in methylation between malignant and nonmalignant cells do not show up in the tRNA in this system (see Ref. 6 for details of this experiment). The first \( A_{260} \) peak is tRNA with the S S RNA shoulder. The 2nd peak is DNA and the material following is large-molecular-weight RNA, mostly rRNA. From \( \text{H}^3 : \text{C}^14 \) plotted in the inset, broad differences appear to be present in the large-molecular-weight RNA, indicating chromatographic heterogeneity, but the most striking differences are evident in the change in ratio through the DNA peak, indicating comparatively large differences.

Chart 1. Comparison of tRNA methylase capacity of a nonneoplastic cell extract with its paired neoplastic cell extract. The rate of incorporation plotted on the ordinate is the slope of the line through 5 points in separate experiments at each protein concentration plotted on the abscissa. A control experiment in which the tRNA was omitted was subtracted from each point. The incubation solution contained 300 \( \mu \)g Escherichia coli B tRNA, 15 \( \mu \)g S-adenosylmethionine-methyl-\( ^3 \)H, and protein, in a total volume of 0.5 ml.

Ratio demonstrates greater relative methylation of the malignant cell tRNA at Tube 90. Five experiments to date, including a 2nd neoplastic line also derived from this nonneoplastic line, assure the reproducibility of this difference.

In an attempt to determine whether the difference found by reversed phase chromatography could be correlated with a difference in the methylated base composition, the tRNA mixture was hydrolyzed in alkali and chromatographed on a Dowex 1-formate column (3). These results are shown in Chart 4. The only reproducible difference in the radioactivity profile is seen in Peak B just following \( A_{260} \) Peak 2, which contains 2'-AMP (for a more detailed discussion of these experiments, see Ref. 7). Consistent with the reversed phase profiles of tRNA, there is relatively greater methylation of the malignant cell nucleotides compared to the nonmalignant cell nucleotides in Peak B, and it appears from the skewed nature of the \( ^3 \text{H} \) at that point that at least 2 components are contributed by the malignant cell tRNA. This difference is also seen in alkaline hydrolysis of the 2nd malignant cell line as well as in reversed label experiments.

The results to date in this in vitro system are consistent with the aberrant methylation hypothesis of neoplasia put forward by Srinivasan and Borek (8) in 1964. Further, these experiments broaden the base support for the hypothesis by extending the original observations to an experimentally more flexible in vitro system. The small differences observed in the intact cell methylation experiments are attributed to more adequate control, and in retrospect this should be expected since the fundamental difference between a malignant and nonmalignant cell is sure to be subtle. The obvious immediate goals in this system are: (a) to see whether a positive correlation exists between the time of malignant conversion and the appearance of this difference, along with other methods of conversion such as by chemical carcinogens and viruses; and (b) to establish the precise nature of these differences.

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Chart 2. Comparison of tRNA methylase capacity of a nonneoplastic cell extract with an in vivo cultured tumor extract (see text). The coordinates are described in Chart 1. Each incubation contained 150 \( \mu \)g E. coli B tRNA, 15 \( \mu \)g S-adenosylmethionine-methyl-\( ^3 \)H, and protein, in a total volume of 0.5 ml.
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Chart 3. Reversed phase chromatography of tRNA from neoplastic and nonneoplastic cell lines labeled with $^{14}$C and $^3$H, respectively. The $^3$H:$^{14}$C plot was constructed from the isotope ratios calculated for each tube counted. The $^{14}$C:$^3$H plot above was constructed from an identical experiment except that the isotope labels were reversed.

Chart 4. Chromatography of the products of alkaline hydrolysis of tRNA. A Dowex 1-formate (200 to 400 mesh) column (1.0 x 45 cm) was used. After initial water wash (400 ml), 0.1 M formic acid (2000 ml) and then 1.0 M formic acid (2500 ml) were applied as indicated at a rate of 90 ml/hr. Nonneoplastic cell nucleotides contain $^{14}$C, and neoplastic cell nucleotides contain $^3$H.

Although DNA is itself methylated, we have been unable to demonstrate any difference in the DNA of the 2 cell lines. However, chromatography and CsCl equilibrium banding experiments indicate that the heterogeneity is largely due to methylated RNA which is associated with the DNA. These experiments (R. Gantt, F. Montes de Oca, and V. J. Evans, manuscript in preparation) add another dimension to the relationship of aberrant methylation and cancer by suggesting that other methylated nucleic acids as well as tRNA may be linked with neoplasia. For example, it is quite plausible that the fundamental biochemical events leading to cancer could occur in the methylation of tRNA which may be intimately involved in gene control; this RNA could be activator RNA (2), chromosomal RNA (1), or an as yet unspecified RNA.

Borek's original formulation (8) of the aberrant methylation hypothesis did not focus on tRNA to the exclusion of other nucleic acids, although tRNA seemed to be the most likely candidate at the time.

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REFERENCES


Discussion

Dr. Bernfield: I am really somewhat puzzled when I see, and I think you have attempted to direct yourself to this question, that is really why I am asking. We see major differences in vitro assays, but when tRNA’s are looked at, homologous tRNA’s within the cell, we see only very minor differences.

I am wondering whether anyone has really looked other than doing a mixing experiment to see if there is an inhibitor, whether or not there are differences in things like S-adenosylhomocysteine hydrolase which has been shown to be an inhibitor at least in bacterial methylation.

Have people looked at other facets of methylating systems which might relate to these major differences we see in vitro?

Dr. Gantt: I don’t know of very many.

Dr. Bernfield: The example of S-adenosylhomocysteine hydrolase, has anyone looked at that?

Dr. Turkington: We have measured that and found no significant differences as a function of cell differentiation or neoplasia.

Dr. Cantoni: To pursue this line of thinking, isn’t it time that somebody, instead of repeating the same experiment in different tumors and finding small differences, that one purifies the enzymes. You may find that there are no differences in the purified enzymes.

Dr. Gantt: I would purify enzymes tomorrow or yesterday if we had the material with which to do it.

Dr. Gallo: That still doesn’t get to the problem of differentiation. The biochemist might purify an enzyme to death and not have the biological system described or controlled.

Dr. Cohen: If you had a good antiserum against the purified enzyme, you could assay for the amount of protein and the original without having to purify.

Dr. Borek: These are beautiful experiments that our biochemist colleagues are suggesting, but unfortunately the enzymes are very unstable.

Moreover, the enzymes in vivo seem to be closely associated, as you will see later on this afternoon, with inhibitors.

Staehelin actually did what Dr. Cantoni suggests. As he was purifying the adenine methylase, it became more active. Its capacity increased but, of course, what was happening was that he was eliminating the inhibitor.

To be sure, there must be a very large difference between methylation in vivo and in vitro.

I would like to call your attention to Dubert’s finding. It is not well known. He found that extracts of myeloma will methylate a population of heterologous tRNA’s to a small extent, but then he found that all of this methylation is in the methionyl-tRNA.

We may be potentiating our results with in vitro assays, and also we may be diluting the methyl groups when we are working with populations of tRNA.

Dr. Magee: Could I ask two questions? This is probably well known, but what are the criteria of malignant transformation in these cells?

Dr. Gantt: Injection into the isologous animals.

Dr. Magee: And the second question was, have you any idea yet which comes first, the increased methylase activity or the transformation or the other way around? Is it a property of the transformed cells?

Dr. Gantt: What you are suggesting is something that we still haven’t done and probably should, and that is to correlate it with time of conversion.
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