The Schaechter–Bentzon–Maaløe experiment and the analysis of cell cycle events in eukaryotic cells

Stephen Cooper

The Schaechter–Bentzon–Maaløe (SBM) experiment, performed more than 40 years ago, provides an important lesson for the analysis of the eukaryotic cell cycle. Before this experiment, temperature shifts had been used to synchronize bacteria and determine the pattern of DNA synthesis during the bacterial division cycle. These experiments indicated that DNA replication occurred during a fraction of the division cycle with gaps before and after DNA synthesis, a pattern similar to the eukaryotic division cycle. The SBM experiment studied DNA replication during the division cycle by labeling an unperturbed culture with a short pulse of tritiated thymidine. All cells were found to be labeled, indicating that unperturbed cells synthesize DNA throughout the division cycle. Thus, the SBM experiment was a control experiment demonstrating that artifacts can be introduced by synchronization methods. The idea of an control experiment under unperturbed conditions is proposed for the analysis of data on cell-cycle-specific gene expression in yeast and mammalian cells.

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Stephen Cooper
Dept of Microbiology, University of Michigan School of Medicine, Ann Arbor, MI 48109-0620, USA.
e-mail: cooper@umich.edu

Can an experiment that was carried out more than 40 years ago have meaning for contemporary high-tech analysis such as microarray analysis of eukaryotic gene expression? It is argued here that a lesson from a simple experiment performed in Copenhagen years ago to analyze DNA synthesis during the bacterial division cycle should be regarded as a model control experiment for analyzing eukaryotic gene expression during the division of eukaryotic cells.

Microarray analysis has recently been used to propose that many genes in *Saccharomyces cerevisiae* are expressed in a cell-cycle-specific manner. In one set of experiments [1], cells were synchronized in one of three ways (α-factor arrest, temperature arrest of a temperature-sensitive mutant or elutriation synchronization), the mRNA was extracted at various times during the cell cycle, and the expression patterns of various genes were determined using a two-color microarray protocol. Using a Fourier-fitting algorithm, it was proposed that several genes were expressed in a cell-cycle-specific manner [1]. A separate approach used two temperature-sensitive mutants to synchronize *S. cerevisiae* [2], and RNA expression during the division cycle was analyzed using Affymetrix chips. In both experiments, it was proposed that numerous genes were expressed in a cell-cycle-specific manner. Additionally, a similar analysis of >7000 genes in mammalian (human) cells indicated that 387 genes are expressed in a cell-cycle-specific manner [3]. These mammalian cells were 'synchronized' using a double-thymidine-block method and gene expression was determined using an Affymetrix microarray.

The question remains, however, whether the observed periodicities are truly representative of the normal cell cycle in unperturbed cells. It has been argued that the batch synchronization methods (α-factor arrest, temperature-sensitive inhibition or nocodazole) used to analyze the *S. cerevisiae* cell cycle do not actually synchronize cells but merely align cells with particular cellular properties [4]. More importantly, it is generally accepted that such methods of inhibition can lead to the introduction of artifacts or anomalous periodicities into the experimental material that are not present in unperturbed cells [5].

'...batch synchronization methods...can lead to the introduction of artifacts or anomalous periodicities into the experimental material that are not present in unperturbed cells.'

An experiment performed in 1959 by Schaechter, Bentzon and Maaløe [6] presented one approach to eliminating problems arising from artifacts introduced by the synchronization procedure.

DNA synthesis during the bacterial division cycle

More than 40 years ago, Lark and Maaløe performed a series of experiments on the timing of DNA synthesis during the bacterial division cycle. Bacterial cells were 'synchronized' by temperature shifts [7]. Subsequent measurements of DNA synthesis during the division cycle of these temperature-entrained cells indicated that synthesis occurred during the middle of the division cycle, with a gap at either side [8]. This result was similar to the proposed pattern of DNA replication during the eukaryotic cell cycle [9,10], which has a central S phase between two gap periods, G1 and G2. If the bacterial result was correct, there was a clear parallel between the patterns of DNA replication in bacteria and eukaryotes.

Although the unifying aspect of this proposed pattern was certainly welcome, it was accompanied

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by the ever-present problem of artifacts being introduced by temperature shifts. A control experiment was thus performed, and it is to this experiment that we now turn.

The Schaechter–Bentzon–Maaløe experiment

The experiment performed by Schaechter, Bentzon and Maaløe was extremely simple [6]. Exponentially growing bacteria were pulse-labeled for a short time with tritiated thymidine, the cells were fixed and the amount of radioactivity in individual cells was determined by autoradiography. If the results using synchronized cells were correct, then only a proportion of the cells would be expected to be labeled as cells that were in the G1 and G2 phases during the labeling period would be unlabeled. The result obtained showed that all cells incorporated thymidine; no unlabeled cells were observed that could not be eliminated by statistical considerations. It was therefore concluded that all cells were synthesizing DNA at all times during the division cycle.

The importance of the Schaechter–Bentzon–Maaløe (SBM) experiment becomes apparent when we consider that this experiment was a control experiment to test the proposal that DNA synthesis in bacteria is confined to the central period of the division cycle, which is flanked by two gap periods. Because the unperturbed control experiment was free of artifacts owing to synchronization, it was concluded not only that DNA synthesis occurred throughout the cell cycle, but also that temperature shifts could alter bacterial physiology and give incorrect results.

To give the SBM experiment its proper due, it must be realized that the original bacterial results were important and exciting because they related bacteria to eukaryotes and unified the patterns of DNA synthesis. There was no strong imperative to critique the results from synchronization studies as there was a strong theoretical basis for believing that they were satisfactory. The brilliance of the SBM group in rechecking the bacterial model for artifacts thus shines through in this classic paper. Subsequent determinations of the pattern of DNA replication during the bacterial division cycle indicated that DNA synthesis obeyed rules that eliminated gaps in synthesis [11–13]. It was now clear that at the growth rates studied by Schaechter, Bentzon and Maaløe there would be no gap in DNA synthesis.

As an aside, it must be emphasized that I am not suggesting here that DNA synthesis is continuous in eukaryotic cells. The evidence for the existence of periods devoid of DNA synthesis (G1 and G2/M phases) is clear and abundant. However, it is interesting to note that the original discovery of the G1 and G2 phases was not the result of a synchrony experiment but a non-synchrony experiment: the frequency of labeled mitoses (FLM) method [9,10].

Two further points should be taken from the SBM experiment. First, one can study the cell cycle without synchronizing cells. If the SBM experiment had been carried out first, the times during the division cycle when DNA synthesis occurred would not have been measured. No gaps would have been postulated and therefore there would have been no need to test when during the division cycle these gaps appeared. An understanding of at least one aspect of synthesis would have been obtained by analyzing unsynchronized cells. Second, the methods that are used to synchronize cells (in this case, temperature-shift entrainment) should be viewed with caution until it is demonstrated that not only are the cells synchronized but also that no artifacts are introduced by the synchronization procedure. It has also been proposed that no batch treatment, such as when temperature shifts are used, can ever synchronize cells [4,5].

Analysis of gene expression during the eukaryotic cell cycle

Let us now apply the lessons of the SBM experiment to the problem of the timing of gene expression during the division cycle of human and yeast cells.

One of the most striking results obtained using *S. cerevisiae* is that there is continuous variation in the timing of gene expression during division [1]. This implies that the cell has a timing mechanism to turn on gene expression for different genes at different points during the division cycle. The methods used to analyze the cell cycle, however, are subject to question. If it is true that no batch method of synchronization can actually synchronize cells [4], then the use of α-factor arrest and temperature-sensitive arrest to align cells at a particular point in the division cycle should be avoided.

The arguments previously presented that batch treatments cannot, in theory, synchronize cells [4,5,14] do not directly apply to the elution results. In this regard, it is interesting to note that when a single value describing cell-cycle-specific synthesis in yeast was calculated, the elutriation data were not included. The timing of expression was calculated only from the α-factor, CDC15 and CDC28 synchronization results. The reason that the elutriation data were not included was that 'it was not possible to calculate a [value] that maximized the value of more than a handful of the known genes' [1]. It is interesting that in a paper devoted to analyzing the extant yeast cell cycle [15], the classification of genes according to function was different from the original [1] classification. The explanation given [15] was:

> 'This may be due to the poor quality of the elutriation expression data, as synchronization by elutriation was not very effective in this experiment. For the α-factor-synchronized cell cycle expression there is much better agreement between the two classifications.'

One possible explanation for the different classification results, and the lack of conformity of the

elutriation data with the α-factor, CDC15 and CDC28 results, is that the elutriation data are closer to the expression pattern in unperturbed cells. It is possible that in the unperturbed cells there is little variation in gene expression during the division cycle. Conversely, the α-factor-synchronized cell cycle data give stronger periodicities that lead to a more robust classification scheme. If this were the case, one would expect reproducible results for the α-factor experiments but less reproducible results for the elutriation data. However, it is important to note that this reproducibility and strength of periodicity should not be taken as an indication that a particular gene is expressed in a cell-cycle-specific manner during the normal, unperturbed cell cycle.

To put this more clearly, the results from the α-factor synchronization might lead to more defined and reproducible analyses than the data from the elutriation experiment. Such reproducibility could be useful for gene expression analysis. However, utility is not a proper criterion for accepting data that might be tainted by perturbations or artifacts. It is possible that the weaker cyclicities in the elutriation data are closer to the natural situation in growing cells. For this reason, one should be wary of extending the batch synchronization methods (whether by α-factor or temperature-sensitive arrest) to determine the pattern of gene expression during the division cycle. A similar critique can be applied to the double-thymidine block used to synchronize human cells [3].

This viewpoint is a minority position. Numerous papers have used batch methods to synchronize cells, whether by starvation for growth requirements (e.g. low serum to put cells into a state of G1-phase arrest) or chemical inhibition (e.g. lovastatin or double-thymidine block). How can one resolve the question of whether or not these methods introduce artifacts? It is proposed here that the SBM approach should be applied. What does this mean? It means that if one wants to confirm whether or not cell-cycle-specific expression patterns are truly present in growing cells and are independent of synchronization methods, then one should test the gene expression results in an unperturbed experimental situation.

Analysis of the cell cycle without synchronization

There are many ways to test the pattern of gene expression during the division cycle without synchronization. Here, I will suggest two methods to illustrate possible approaches. This list should not be taken as exhaustive or as the final list as, with technical improvements, other methods are sure to be developed that will test the proposition that one or another gene is expressed in a cell-cycle-specific pattern.

One method that would be strictly analogous to the SBM experiment would be to look at fluorescent *in situ* hybridization (FISH) to assess whether all cells express a particular mRNA. Thus, if it were postulated that a particular mRNA was expressed only during a particular phase of the division cycle and was absent in the other phases, this would be observed as some cells being fluorescent and others not being fluorescent.

The microscopic FISH method is probably not quantitative enough to determine quantitative differences during the division cycle. However, one can imagine that the same methods could be carried out using flow-cytometry to measure the fluorescence per cell. Linking this with measurements of DNA content or light-scattering measurements of cell size could lead to a method to test whether different phases of the cell cycle have less or more of a particular transcript. Of course, one would have to have controls with other mRNAs that are known to be expressed throughout the cell cycle to ensure this FISH method does not produce artifacts. If the default, non-cell-cycle-specific pattern of expression is exponential and continuous synthesis during the division cycle, one would expect the mRNA content to vary by a factor of approximately two within the population of exponentially growing cells.

It is also possible to analyze the cell cycle chemically without synchronization. For example, the original description of the pattern of DNA replication during the division cycle by Howard and Pelc was determined using the FLM method. In this approach, the DNA of exponentially growing cells is labeled with a short pulse of a radioactive precursor and then the label in cells undergoing mitosis is determined by autoradiography. At first, no mitoses are labeled (as these cells were in G2 when the label was added), then labeled mitoses are observed (cells labeled during S phase), followed by a period without labeled mitoses (reflecting the cells in G1 phase at the time of labeling).

The SBM experiment is also a non-synchrony approach to cell cycle analysis although the question it asked was quite limited. When pulse labeling was combined with measurements of cell length it was possible to determine the pattern of protein synthesis during the division cycle without synchronization [16], a result subsequently confirmed by more accurate methods [17].

Another method to analyze gene expression during the cell cycle without perturbation involves elutriation. The following experiment is proposed. Yeast cells or human cells should be grown exponentially at low density to prevent any problems with overgrowth leading to some cells ceasing growth. The growing cells should be fixed chemically to prevent synthesis or breakdown of mRNA. The fixed cells can then be separated out on a gradient according to size. This size distribution will also reflect the age distribution, as larger cells are found later in the cell cycle, and the mRNA can then be extracted and assayed. To be precise, although there is a correlation between cell size and age, separation by size will blur the age distribution. But we are not looking for precise timing in this experiment. In fact, if a peak in activity occurred at some specific cell size, it would not be possible to identify precisely what time during the division cycle

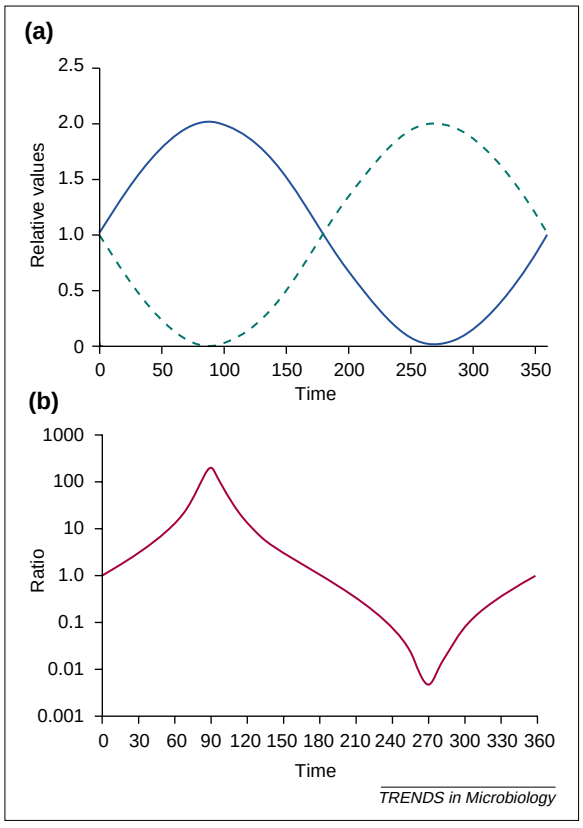

Fig. 1. Analysis of gene expression during the cell cycle without synchronization. The proposed confirmation of microarray results without synchronization is illustrated for two genes that are expressed out of phase during the division cycle (a). If the ratio of these genes is analyzed it is clear that the ratio gives an even larger difference than the absolute measurements of any one gene (b). The abscissa in both panels are relative values for position in a gradient that separates cells according to relative size.

that particular synthetic activity occurred. All that could be stated is that there was a particular change in gene expression during the division cycle.

To give the proposal of cell-cycle-specific gene expression during the division cycle its best chance to succeed, one should look at two genes that are proposed to be expressed completely out of phase based on the microarray experiment. That is, take two genes where a peak in the expression of one occurs when there is a dip or valley in the expression of the other and vice versa. Two gene expression patterns that exhibit this inverse relationship are shown in Fig. 1a. Then let us assay both genes across the size spectrum using a traditional method such as northern blotting and determine the ratio of gene expression for the two genes. As shown in Fig. 1b, the ratio of the two genes should be more obvious than the absolute values of any individual gene relative to the entire complement of mRNA molecules.

The question raised is whether the genes in different phases are really cycle specific when looked at in an unperturbed situation. In a sense, this is a replay of the SBM experiment where a cell cycle result obtained using a problematic method was tested using a non-perturbing approach to cell cycle analysis.

Rb phosphorylation during G1 phase

One of the most well known G1-phase events is the specific phosphorylation of the retinoblastoma protein (Rb). I will not review the data for this proposal as I have done so elsewhere [18]. However, I will point out that this classic example of a G1-phase event has an alternative explanation. It appears that much of the data supporting G1-phase Rb phosphorylation is based on a problem with how the cells are grown and analyzed. Not only is it possible to grow cells where all cells have only phosphorylated Rb – which can be criticized as being related to those specific cells – but also an analysis of NIH 3T3 cells grown to different densities leads to an explanation of why it appears as though Rb was phosphorylated specifically during G1 [18,19].

The ultimate test: reproducibility and standardization

The ultimate demonstration and convincing proof that a gene is synthesized in a meaningful way during the cell cycle is that this pattern of gene synthesis is studied in more than one laboratory and can be analyzed in different laboratories in a reproducible manner. I have previously proposed that the ultimate analysis of gene expression requires a standard system allowing researchers in different laboratories to reproduce work from other laboratories [20]. Similarly, if a G1-phase gene-expression system truly exists, then it must be reproducible and quantifiable in more than one laboratory and in more than one experiment.

Controls and the burden of proof

It is sometimes difficult to do control experiments. One has a result, the results look satisfying, so it is difficult to be self-critical and to test the experimental results by running controls to eliminate possible artifacts. Part of the brilliance of the SBM experiment is that it was a control experiment that questioned a result that not only appeared consistent with eukaryotic data but could also be understood and explained in its own right. There was no reason to question the original results showing mid-cycle DNA replication with gaps at either end of the cell cycle. Who should do the controls? I propose that those who perform synchronization experiments should take on the burden of proof that an experiment is free of artifacts introduced by the methods of synchronization.

The lessons of Schaechter, Bentzon and Maaløe must be reinforced again and again to ensure that our understanding of the eukaryotic cell cycle is not the result of perturbations introduced by experimental conditions.

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References

1 Spellman, P.T. *et al.* (1998) Comprehensive identification of cell cycle-regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization. *Mol. Biol. Cell* 9, 3273–3297
2 Cho, R.J. *et al.* (1998) A genome-wide transcriptional analysis of the mitotic cell cycle. *Mol. Cell* 2, 65–73
3 Cho, R.J. *et al.* (2001) Transcriptional regulation and function during the human cell cycle. *Nat. Genet.* 27, 48–54
4 Cooper, S. (1998) Mammalian cells are not synchronized in G1-phase by starvation or inhibition: considerations of the fundamental concept of G1-phase synchronization. *Cell Prolif.* 31, 9–16
5 Cooper, S. (1991) *Bacterial Growth and Division*, Academic Press
6 Schaechter, M. *et al.* (1959) Synthesis of deoxyribonucleic acid during the division cycle of bacteria. *Nature* 183, 1207–1208
7 Lark, K.G. and Maaløe, O. (1954) Induction of cellular and nuclear division in *Salmonella typhimurium* by means of temperature shifts.

Biochim. Biophys. Acta Mol. Cell Res. 15, 346–354
8 Lark, K.G. and Maaløe, O. (1956) Nucleic acid synthesis and the division cycle of *Salmonella typhimurium*. *Biochim. Biophys. Acta Mol. Cell Res.* 21, 448–457
9 Howard, A. and Pelc, S. (1951) Nuclear incorporation of P^{32} as demonstrated by autoradiographs. *Exp. Cell Res.* 2, 178–187
10 Howard, A. and Pelc, S. (1951) Synthesis of nucleoprotein in bean root cells. *Nature* 167, 599–600
11 Cooper, S. and Helmstetter, C.E. (1968) Chromosome replication and the division cycle of *Escherichia coli* B/r. *J. Mol. Biol.* 31, 519–540
12 Helmstetter, C.E. *et al.* (1968) The bacterial life sequence. *Cold Spring Harbor Symp. Quant. Biol.* 33, 809–822
13 Helmstetter, C.E. (1967) Rate of DNA synthesis during the division cycle of *E. coli* B/r. *J. Mol. Biol.* 24, 417–427
14 Cooper, S. (2000) The continuum model and G1-control of the mammalian division cycle. *Prog. Cell Cycle Res.* 4, 27–39
15 Alter, O. *et al.* (2000) Singular value decomposition for genome-wide expression data processing and modeling. *Proc. Natl. Acad. Sci. U. S. A.* 97, 10101–10106
16 Ecker, R.E. and Kokaisl, G. (1969) Synthesis of protein, ribonucleic acid, and ribosomes by individual bacterial cells in balanced growth. *J. Bacteriol.* 98, 1219–1226
17 Cooper, S. (1988) Leucine uptake and protein synthesis are exponential during the division cycle of *Escherichia coli* B/r. *J. Bacteriol.* 170, 436–438
18 Cooper, S. and Shayman, J.A. (2001) Revisiting retinoblastoma protein phosphorylation during the mammalian cell cycle. *Cell. Mol. Life Sci.* 58, 580–595
19 Cooper, S. *et al.* (1999) Phosphorylation-dephosphorylation of retinoblastoma protein is not necessary for passage through the mammalian division cycle. *IUBMB Life* 1, 27–39
20 Cooper, S. (2000) Toward a standard system for the mammalian cell cycle. *ASM News* 66, 71–75

Anti-fungal therapy at the HAART of viral therapy

Carol A. Munro and Bernhard Hube

HIV-positive patients receiving combination therapy (highly active anti-retroviral treatment, HAART) suffer significantly fewer oral infections with the opportunistic fungal pathogen *Candida albicans* than non-HAART-treated patients. One component of HAART is an inhibitor of the HIV proteinase, the enzyme required for correct processing of retroviral precursor proteins. It would appear that HIV proteinase inhibitors also have a direct effect on one of the key virulence factors of *C. albicans*, the secreted aspartic proteinases (Saps). This suggests that the reduction in *C. albicans* infections in HIV-positive patients might not be solely the result of improved immunological status but could also be caused by the HAART treatment directly inhibiting *Candida* proteinases.

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Carol A. Munro*
Dept of Molecular and Cell Biology, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, UK AB25 2ZD.
*e-mail: c.a.munro@abdn.ac.uk

Bernhard Hube
Robert Koch-Institut, NG4, Nordufer 20, D-13353 Berlin, Germany.

Patients infected with HIV are susceptible to a large panel of opportunistic microbial infections. The treatment of HIV-positive patients with combination therapies including a cocktail of reverse transcriptase (RT) and proteinase inhibitors has proved successful in delaying the onset of AIDS. Administration of highly active anti-retroviral treatment (HAART) results in a significant improvement in the immune status of HIV-positive patients, which is reflected in an increase in the number of $CD4^+$ T cells. These patients also tend to have a greatly reduced incidence of oral infection with the fungus *Candida albicans* than non-HAART-treated patients. It is not yet clear whether this is just a reflection of the recovery of the $CD4^+$ T cell count in HAART-treated patients or whether this treatment regimen has direct effects on opportunistic fungi such as *C. albicans*. Here, we summarize evidence that the HIV proteinase inhibitors used in combination therapies have at least one direct effect on *C. albicans*: inhibition of known *C. albicans* virulence factors, the fungal aspartic proteinases.

'...the anti-proteinase drugs in HAART regimens have a direct inhibitory effect on *Candida* aspartic proteinases and hence candidosis.'

The incidence of disease (candidosis) caused by *C. albicans* has increased over the past two decades mainly owing to a rise in the number of immunocompromised individuals. Candidosis can range from mild superficial infections of the mucosal membrane, commonly called thrush, to severe life-threatening infections. Superficial oropharyngeal infections are common in patients infected with HIV and could affect as many as 90% of HIV-positive individuals [1]. It has been reported that HIV-positive patients receiving dual- or triple-therapy HAART regimens, which include an HIV proteinase inhibitor, lose *C. albicans* progressively from the oral mucosa and suffer significantly fewer oropharyngeal infections than patients not receiving HAART [2,3]. In these reports, the patients responded positively to HAART treatment and the loss of oral candidosis was accompanied by an increase in the $CD4^+$ T cell count. However, in 1998, Hoegel *et al.* published a case of a patient receiving HAART who recovered from a *Candida* infection but maintained a low $CD4^+$ T cell count [4], hinting that perhaps HAART might have a direct effect on *C. albicans*. Soon after this, Cauda *et al.* [5] documented that, in a prospective case-controlled study of 93 subjects, $CD4^+$ T cell counts and the recovery of specific anti-*Candida* immunity could not account for the early clinical benefit of HAART on recurrent oral candidosis. Subsequently, several lines of evidence have indicated that the anti-proteinase drugs in HAART regimens have a direct inhibitory effect on *Candida* aspartic proteinases and hence candidosis.

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