Note

5-Bromo-2'-deoxyuridine Efficiently Suppresses Division Potential of the Yeast Saccharomyces cerevisiae

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We established a thymidine-auxotrophic strain of the yeast Saccharomyces cerevisiae to examine biological effects of thymidine analogues. 5-Bromo-2'-deoxyuridine efficiently suppressed the division potential of yeast showing morphology similar to senescent cells.

Key words: yeast; thymidine auxotroph; 5-bromo-2'-deoxyuridine; growth; senescence

5-Bromo-2'-deoxyuridine (BrdU) is an analogue of thymidine and known to modulate various biological functions when incorporated into genomic DNA in mammalian cells. BrdU induces or inhibits cellular differentiation in various types of cultured mammalian cells1 under the conditions where it only slightly inhibits cell growth. BrdU is also known to cause teratogenesis and accelerate aging when administered to embryos of mammals.2-3 Consistently, BrdU is shown to induce or suppress expression of a certain type of tissue-specific genes.4 To date, many efforts have been made to understand the molecular basis for the action of BrdU, but it is yet to be identified.

Recently, we have found that BrdU induces a senescence-like phenomenon in cultured mammalian cells regardless of cell types or species.5 Furthermore, we have shown that the genes up-regulated by BrdU in HeLa cells are also up-regulated in senescent human fibroblasts.6 To identify a molecular target for BrdU, we planned to undertake a genetic approach using the yeast S. cerevisiae because this system has proved to be very useful in identifying responsible genes in various biological phenomena. In the majority of organisms, BrdU is phosphorylated by thymidine kinase (TK) and normally incorporated into cellular DNA. Unfortunately, however, yeast has no functional TK gene and this has long hampered biochemical analysis of cellular DNA with labeled thymidine. Therefore, we first constructed a plasmid containing the herpes simplex virus TK gene driven by the yeast ADHI-promoter, and introduced it into the yeast NNY11 strain (MATa lys2-801 ura3-52 trp1 leu2 his3)7 to isolate a clone that expresses TK. Herpes TK was chosen because it can efficiently phosphorylate various thymidine analogues with its low substrate specificity.8 When thymidine analogues are tested for their biological effects, an endogenous supply of dTMP obscures the effects. In fact, almost all of the BrdU-resistant mutants isolated from mammalian cells are deficient in TK activity and thus unable to use BrdU (unpublished results). Therefore, we have concluded that, although there are several reports on strains that can use exogenous thymidine/BrdU in S. cerevisiae9,10 it is desirable to use a thymidine-auxotrophic strain to isolate BrdU-resistant mutants not involved in the nucleotide metabolic pathways. Thymidylate synthase is the only de novo source of dTMP in eukaryotes. We thus disrupted the yeast thymidylate synthase gene (CDC21/TMP1) by targeted homologous recombination, and isolated a disruptant named Thy- . As expected, this mutant was absolutely auxotrophic for thymidine, but it required unusually high concentrations of thymidine (1-3 mm) for normal growth (Fig. 1A), compared with thymidine auxotrophs in the bacterium E. coli and mammalian cells in which 10 μM is sufficient for their growth. The latter phenotype of yeast suggests that yeast has lost a functional transport system for thymidine with concomitant loss of TK.

To confirm the role of the introduced TK gene, we also established a yeast thymidine auxotroph, Thy- (GAL), that expresses TK driven by the yeast GAL1-promoter. As the GAL1-promoter is activated by galactose in the medium, the cells grew normally on a plate containing galactose and thymidine, but no growth was observed on a plate containing glucose and thymidine (Fig. 1B). Thus, expression of ex-

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Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; TK, thymidine kinase

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Fig. 1. Properties of Thy' Cells.
A. Growth of Thy' cells on agar medium containing thymidine.
Yeast NNY11 cells (WT) were transfected with a YEplasmid containing the herpes TK gene driven by the ADHI-promoter and the URA3 gene to isolate a TK-expressing strain (TK'). TK' cells were transfected with a targeting construct to disrupt the CDC21/TMP gene to yield the Thy' strain. The construct replaces an ORF of the gene by the yeast Leu2 gene. The construct was amplified by PCR with the following primers using pACT2 (clontech) as a template: 5'-atcaacgaggaggtcataacagacgacgtgtagaggt-3' and 5'-atcagacggtcataacagacgacgtgtagaggt-3' in which italic regions are homologous to the CDC21/TMP1 sequence.

An overnight culture of Thy' cells was diluted to approximately 10⁶ cells/ml. Then serial 10-fold dilutions were spotted on YPD medium containing the indicated concentrations of thymidine and incubated for 4 days.

B. Growth of Thy' (GAL) cells on agar medium containing thymidine and either glucose or galactose.
The cells were established as described in the text with a plasmid containing the TK gene driven by the GAL1-promoter instead of the ADHI-promoter. The cells (10⁶ cells/ml) were diluted as described above and spotted on synthetic medium containing glucose or galactose supplemented with 3 mM thymidine.

C. Immunochromatographic analysis of BrdU incorporated into genomic DNA of Thy' cells.
DNA samples were prepared from the cells cultured for 24 hours in medium containing BrdU and thymidine as indicated. The samples were digested with EcoRV, run on agarose gel, and stained with ethidium bromide (left). DNA was then transferred onto a nitrocellulose filter and probed with mouse anti-BrdU antibody (Sigma). The signal was detected on an X-ray film using ECL chemiluminescence detection system (Amersham-Pharmacia) (right).

ogenous TK is essential for the growth of yeast Thy' cells.
We investigated whether BrdU was normally incorporated into cellular DNA in the Thy' cells. The cells were cultured in medium containing BrdU and thymidine at various ratios, and the DNA was analyzed by Southern blot analysis using anti-BrdU antibody. The cells were found to incorporate BrdU into the DNA dose-dependently (Fig. 1C). Then we investigated the effects of BrdU on growth. In medium containing BrdU, growth was efficiently suppressed (Fig. 2A). In medium containing 0.9 mM BrdU and 2.1 mM thymidine, colony-forming ability was greatly diminished (Fig. 2B) and the colonies formed were very small (not shown). The cells cultured in the presence of BrdU became enlarged (Fig. 2C). This cell shape is similar to those of senescent human cells and yeast old mother cells.5

In these experiments, we kept the sum of thymidine and BrdU to 3 mM. To exclude the possibility that a low concentration of thymidine, but not BrdU added, caused the above phenomenon, we investigated the growth on medium containing 1.5 mM thymidine plus various concentrations of BrdU (0-1.5 mM). The Thy' cells grew normally in the presence of 1.5 mM thymidine (Fig. 1A). In this assay, the parental NNY11 cells transfected with an empty vector, NNY11 cells expressing herpes-TK, and the Thy' cells were examined. The former two types of cells grew normally on every plate supplemented with or without BrdU, but the Thy' cells did not grow on plates containing 1-1.5 mM BrdU (Fig. 2D). These experiments clearly indicate that the addition of BrdU, but not a subtraction in the amount of thymidine, affected the division potential in yeast. It is also suggested that the expression of TK alone is not enough for efficient incorporation of thymidine/BrdU into cellular DNA.

Mammalian cells in senescence are metabolically active although unable to divide. Thus, we investigated whether the yeast cells that have incorporated BrdU are alive or dead. Phloxine B is known to stain dead cells pink but not living cells in YPD medium.12 We cultured the Thy' cells in the presence of phloxine B for 24 hours, and examined the cells under a microscope. More than 75% of the cells were not stained and thus judged to be alive although they had lost division potential. (Fig. 3) Therefore, yeast and human cells seem to be similar when cultured in the presence of BrdU.
BrdU is a unique compound in that it efficiently modulates expression of particular genes in mammalian cells. We hypothesize that chromatin structure is involved in the altered expression of the genes. Here we established a thymidine-auxotrophic strain from \textit{S. cerevisiae} and showed that this strain was found to be useful to study the biological roles of BrdU. Recently, we have isolated BrdU-resistant mutants from this strain (not shown). The conventional, powerful genetic approaches in yeast are applicable to these mutants, and will soon lead us to identify their responsible genes. Identification of these genes will facilitate the understanding of the molecular mechanism not only for the old mystery of why BrdU preferentially modulates cellular differentiation but also our new finding that it universally in-

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**Fig. 2.** Effects of BrdU on Growth of Thy− Cells.

**A.** Growth curves of Thy− cells cultured in medium containing thymidine and BrdU.

The cells were cultured in liquid YPD medium containing 3 mM thymidine (0% BrdU) (○), 2.1 mM thymidine and 0.9 mM BrdU (30%) (●), or 1.5 mM thymidine and 1.5 mM BrdU (50%) (▲).

**B.** Colony formation of Thy− cells on agar medium containing thymidine and BrdU.

The cells were plated on YPD medium containing thymidine and BrdU as indicated and colonies formed were counted.

**C.** Morphology of Thy− cells cultured in medium containing thymidine and BrdU.

The cells were cultured in liquid YPD medium containing 3 mM thymidine, 1.5 mM thymidine, or 1.5 mM thymidine and 1.5 mM BrdU for 24 hours and examined under a microscope.

**D.** Growth of the wild-type (WT), TK-expressing (TK+), and Thy− cells on agar medium containing thymidine and BrdU.

The strains used were described in Fig. 1A. WT cells used contain an empty vector. Cells were diluted as described in Fig. 1A and spotted on SD (Ura−) plates containing 1.5 mM thymidine plus various concentration of BrdU as indicated.
Bromodeoxyuridine induces a senescence-like phenomenon in mammalian cells.

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