jected to acute anoxia indicates the need for caution in the use of these drugs in hypoxic human newborns.

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## Stimulated DNA Synthesis in Frog Nuclei by Cytoplasmic Extracts of Temperature-Sensitive Mammalian Cells

Abstract. *Cytoplasmic extracts of proliferating cells stimulate DNA synthesis in isolated nuclei of* Xenopus laevis *liver. When tested by the same assay, cytoplasmic extracts of resting cells are completely inactive. When cytoplasmic extracts are prepared from cell cycle–specific temperature-sensitive mutants arrested in the $G_1$ phase of the cell cycle by the nonpermissive temperature, they also fail to stimulate DNA synthesis in frog nuclei. The results indicate that, to stimulate DNA synthesis in isolated frog nuclei, essentially all information of $G_1$ cells must be present.*

Jazwinski *et al.* (*1*) reported that DNA synthesis in nuclei isolated from frog liver or spleen can be directly stimulated by the addition to the incubation mixture of cytoplasmic extracts prepared from proliferating cells. Electron microscopic analysis of the DNA molecules from the reaction mixture strongly indicated that the cytoplasmic extracts stimulated initiation of DNA replication in the chromatin of the normally resting frog nuclei. A comparison of the stimulatory activity in extracts from various mammalian and avian sources showed that the activity was present only in proliferating cells. In contrast, cytoplasmic extracts from normally resting tissues and cells had no stimulatory activity on DNA synthesis in isolated frog nuclei. In subsequent experiments, Jazwinski and Edelman (*2*) used the same cell-free system, consisting of isolated frog nuclei, to investigate the stimulatory effect of cytoplasmic extracts from temperature-sensitive (ts) mutants of *Saccharomyces cerevisiae*. The ts mutants used by Jazwinski and Edelman (*2*) were mutants of the cell division cycle isolated and described by Hartwell and collaborators (*3*). All of these mutants were deficient in events of the dependent pathway leading to the initiation of DNA synthesis in the yeast cell cycle. When the yeast cells were incubated for one generation at the nonpermissive temperature, 36°C, their extracts showed very low or no stimulatory activity on DNA synthesis in frog liver nuclei. On the other hand, cytoplasmic extracts from yeasts grown at the permissive temperature had stimulatory activity.

We have extended these studies to ts mutants of the mammalian cell cycle and, specifically, to ts mutants in the $G_1$ phase of the cell cycle. These are operationally defined as mutants that, at the nonpermissive temperature, are arrested in the $G_1$ phase of the cell cycle. Three such mutants have been studied in our laboratory: (i) AF8 cells, originally derived from BHK cells by Basilico and coworkers (*4*). The execution point of the ts defect in these cells has been located by shift-up experiments at 8.6 hours from the beginning of the S phase (*5*); (ii) K12 cells, a ts mutant from Chinese hamster cells originally isolated by Roscoe *et al.* (*6*) and characterized by Smith and Wigglesworth (*7*). This mutant is also arrested in $G_1$ at the nonpermissive temperature, and the execution point has been located by Ashihara *et al.* (*5*) at 1.6 hours before the S phase; and (iii) ts13 cells, another $G_1$ phase ts mutant from BHK cells originally isolated and partially characterized by Talavera and Basilico (*8*). Its execution point has been located (*9*) at 3.2 hours before the S phase.

In these experiments we asked a simple question, namely, Is the activity in the cytoplasmic extracts of proliferating cells that is responsible for the stimulation of DNA synthesis in frog nuclei present when the cells are prevented from reaching S by exposure to the nonpermissive temperature? The advantages over the previous experiments with yeast cells (*2*) are essentially two: (i) the work is extended to mammalian cells and (ii) more important, by appropriate manipulations the cells can be arrested in $G_1$ without their having to be exposed to the nonpermissive temperature for a whole generation.

Temperature-sensitive mutants were made quiescent by serum deprivation (36 to 48 hours in 0.5 percent serum) and were then stimulated by fresh medium plus 10 percent serum at either the permissive or the nonpermissive temperature. Under these conditions, at the permissive temperature, 85 to 90 percent of the cells enter DNA synthesis with median times of entry into S of 24, 11, and 24.5 hours for AF8, K12, and ts13, respectively. At the nonpermissive temperature, less than 5 percent of the cells are labeled by continuous exposure to [$^3$H]thymidine (*5, 10*). Cytoplasmic extracts were prepared as described by Jazwinski *et al.* (*1*) from quiescent cells,


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Table 1. Characteristics of the cell lines used for the preparation of cytoplasmic extracts.

| Cell line | Parent cell line | Temperature (°C) | | Execution point* (hours) | Ability to reactivate chick nuclei at† | |
|---|---|---|---|---|---|---|
| | | Permissive | Nonpermissive | | Permissive temperature | Nonpermissive temperature |
| tsAF8 | BHK | 34° | 40.6° | 8.6 | + | − |
| tsK12 | CHL | 34° | 40.6° | 1.6 | + | − |
| ts13 | BHK | 34° | 39.5° | 3.2 | + | − |
| BHK | (Syrian hamster) | 37° | (40.6°)‡ | | + | + |
| HeLa | (Human) | 34° | (40.6°)‡ | | + | + |

*The execution point of a ts function is defined as the point at which shift-up to the nonpermissive temperature no longer prevents the entry of cells into S (*5*). †Reactivation of chick nuclei after fusion in heterokaryons is defined here as induction of DNA synthesis in chick nuclei (*9, 10*). ‡Both BHK and HeLa cells grow well at 40.6°C.

cells stimulated by 10 percent serum at the permissive temperature, and cells stimulated by 10 percent serum at the nonpermissive temperature. The extracts were then studied for their ability to stimulate directly DNA synthesis in nuclei isolated from frog liver. The method we used for the assay of DNA synthesis in frog liver nuclei was that described by Jazwinski *et al.* (*1*). We also used liver nuclei from *Xenopus laevis*, prepared as described (*1, 2*).

We used as controls both cytoplasmic extracts from exponentially growing HeLa cells and cytoplasmic extracts from BHK cells stimulated to proliferate at both 37° and 41°C (BHK cells are the parent cell line of both AF8 and ts13 cells). The characteristics of the cell lines used are summarized in Table 1.

Table 2 gives the results on the ability of cytoplasmic extracts from the different cell lines to stimulate directly DNA synthesis in isolated frog nuclei. In these experiments, the amounts of cytoplasmic extracts were standardized at 300 $\mu$g of protein per assay, and each experiment was repeated at least three times. The results indicate that exponentially growing HeLa cells, and BHK cells stimulated at either 37° or 41°C have, in their cytoplasmic extracts, sufficient activity to stimulate DNA synthesis. The K12, AF8, and ts13 cells stimulated at the permissive temperature are also capable of stimulating DNA synthesis in the isolated frog nuclei system. However, if these cells are not stimulated (for instance when the cytoplasmic extracts are prepared from cells in 0.5 percent serum), or when the cells are stimulated at the nonpermissive temperature, the cytoplasmic extracts are incapable of stimulating DNA synthesis in frog nuclei. This is especially interesting considering that some of these mutants are blocked in $G_1$ at points that are rather close to the beginning of the S phase.

Jazwinski *et al.* (*1*) stated that mouse lymphocytes stimulated by concanavalin A and arrested prior to the S phase by hydroxyurea, were still capable of initiating DNA synthesis in frog nuclei. However, in the present experiments cytoplasmic extracts from cells blocked in $G_1$ are not capable of stimulating DNA synthesis in frog nuclei although the K12 cells are blocked at a point that is only 1.6 hours from the beginning of the S phase. The temperature itself is not responsible for the failure to stimulate DNA synthesis in frog nuclei since extracts from BHK cells, stimulated at 41°C, are still active. Furthermore, since the cells were stimulated at the nonpermissive temperature for only 16 to 24 hours, the results cannot be attributed to cell death. In all these ts mutants, cell death does not begin for at least 30 hours, after the cells are shifted up to the nonpermissive temperature (*4, 7, 9*) and, especially in AF8, it proceeds very slowly. These results therefore seem to indicate that for DNA synthesis to be stimulated in isolated frog nuclei by the assay of Jazwinski *et al.* (*1, 2*), essentially all information, or almost all the information, of $G_1$ cells must be present. This would be in agreement with recent results by Tsutsui *et al.* (*10*) and Lipsich *et al.* (*11*) who found that reactivation of DNA synthesis in chick nuclei in either heterokaryons or "cybrids" was cell cycle–dependent. Only cells or cytoplasts from S phase cells had all the necessary information to reactivate DNA synthesis in chick nuclei after fusion.

It seems, therefore, that the initiation of DNA synthesis in frog nuclei in the isolated system of Jazwinski *et al.*, and the reactivation of chick nuclei after fusion, measure essentially the same amount of cell cycle information (compare Tables 1 and 2). Both systems could be useful in an analysis of the biochemical mechanisms that regulate cell flow in the $G_1$ phase of the cell cycle.

Table 2. Induction of DNA synthesis in frog nuclei by cytoplasmic extracts. Cells were serum-starved (0.5 percent) for 2 days at permissive temperature followed by serum stimulation (10 percent) for 24 hours at either permissive or nonpermissive temperature. Cytoplasmic extracts and frog liver nuclei were prepared, and the assays were carried out, as described by Jazwinski *et al.* (*1*). Each assay contained about 300 $\mu$g of protein and $10^6$ frog nuclei. The results are expressed in picomoles of [$^3$H]thymidine triphosphate incorporated into DNA, after subtraction of background counts (300 count/minute). In some repeated experiments K12 cells were stimulated for only 16 hours. The HeLa cytoplasmic extracts were always prepared from exponentially growing cells.

| Cell line | Permissive temperature and 10 percent serum | Nonpermissive temperature and 10 percent serum | Permissive temperature and 0.5 percent serum |
|---|---|---|---|
| AF8 | 30.0 | 7.5 | 2.9 |
| K12 | 30.1 | 8.7 | 7.5 |
| ts13 | 18.4 | 7.4 | 3.0 |
| HeLa | 53.4 | | |
| BHK, 37°C | 25.0 | | |
| BHK, 40°C | 17.3 | | |
| CE from frog liver | 3.2 | | |

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