Comparison of DNA Replication and Repair Enzymology Using Permeabilized Baby Hamster Kidney Cells*

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The roles of DNA polymerases in replication and repair were examined in lysolecithin-permeabilized baby hamster kidney cells. Lysolecithin treatment yields cell preparations that are capable of normal replicative DNA synthesis at in vivo rates, when supplied with deoxyribonucleoside triphosphates (dNTPs) (Miller et al. (1978) Biochemistry 17, 1073–1080; Miller et al. (1979) Exp. Cell Res., 120, 421–425). The K\textsuperscript{m} of dNTPs for replication is 50 μM. Evidence presented here shows that permeabilized G\textsubscript{0} cells carry out DNA repair synthesis after exposure to bleomycin. The K\textsuperscript{m} of dNTPs for repair is 160 μM.

The sensitivity of replication and repair in permeabilized cells to known inhibitors of DNA polymerases α and β in cell-free extracts was investigated. Replication synthesis was strongly inhibited by cytosine arabinoside triphosphate, high KCl, and N-ethylmaleimide, as is DNA polymerase α in extracts. Repair synthesis was relatively insensitive to these agents, as is DNA polymerase β activity in extracts. Thus replication and repair synthesis can be clearly distinguished by these inhibitors.

These differences in inhibition, K\textsuperscript{m}, and cell cycle occurrence indicate that DNA polymerase α is the major replication polymerase, whereas DNA polymerase β is the major repair polymerase.

Ribonucleoside diphosphates serve well as precursors for DNA replication and repair in lysolecithin-permeabilized cells, indicating that ribonucleotide reductase is highly active. Hydroxyurea inhibits replication and ribonucleotide reductase. But hydroxyurea does not inhibit repair, except partially at very low concentrations of substrates. This result suggests that noninhibition of repair by hydroxyurea has a quantitative basis—the very low requirement of dNTPs for repair.

Animal cells contain at least three DNA polymerases: α, β, and γ. The α and β enzymes have been highly purified, and the γ enzyme has been partially purified (1). The mitochondrial DNA polymerase is probably a form of the γ enzyme (2).

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The abbreviations used are: lysolecithin, L-a-lysophosphatidylcholine; BHK, baby hamster kidney cells; CU, counts per minute; HU, hydroxyurea; Ara-C, cytosine arabinoside triphosphate; dNTPs, deoxyribonucleoside triphosphates; rNDPs, ribonucleoside diphosphates; dNMPs, deoxyribonucleoside monophosphates.
Solution A was added to the cells for 2 min at 0°C, at a concentration in detail previously (10, 11). A typical concentration was 150 µg/ml; >98% of the cells stained with trypan blue following this treatment. DNA Synthesis in Permeable Cells—The maximum rate of DNA synthesis measured by [3H]TTP incorporation was obtained by incubating each dish of permeable cells in 1 ml of Solution A containing 10 mM phosphoenolpyruvate, 1.25 mM ATP, 0.12 mM CTP, UTP, and GTP, and 0.25 mM [3H]TTP (0.1 Ci/mmol), dATP, dCTP, and dGTP, at 37°C. At the indicated times, the DNA assay mixture was aspirated from replicate plates. Each dish was washed once with 3 to 4 ml of phosphate-buffered saline and the cells were inactivated with 5% trichloroacetic acid containing 0.2 M sodium pyrophosphate for 15 min at 4°C. The precipitates were washed five times with cold 5% trichloroacetic acid, then dissolved in 1.0 ml of 0.1 N NaOH for 30 min at 37°C, neutralized with 0.1 ml of 1.0 N HCl, and the entire sample was counted in a liquid scintillation counter with 10 ml of Biofluor.

RESULTS

Replication of DNA in Permeable Cells—We have previously shown that DNA synthesis in exponential cultures of lysosome-permeabilized cells proceeds in a normal replicative fashion; i.e., it is semiconservative, discontinuous, cycle dependent, and occurs at in vivo rates (10, 11). This in situ system was further utilized to examine the DNA polymerase activities in BHK cells. The Kₚ of dNTPs was determined by varying the concentration of all four dNTPs simultaneously over a wide range and measuring incorporation of [3H]TTP into DNA of permeabilized exponential cells. Incorporation from 30 s to 20 min of incubation using 0.25 mM dNTPs showed that extrapolating to zero time yields very close to zero incorporation and that incorporation is linear (10). The Kₚ for dNTPs was calculated from 15-min incubations. From the specific activity of the assay mix, we obtained the picomoles of dNMPs incorporated into DNA/unit of time. A Lineweaver-Burk plot of a typical experiment is presented in Fig. 1. The Kₚ of dNTPs for replication synthesis in BHK cells was 0.05 mM (±0.02 mM), and Vₘₐₓ was 220 (±40) pmol of dNMPs/min/10⁷ cells. Similar values were obtained using permeabilized Chinese hamster ovary cells grown in suspension (10) and with BHK cells made permeable using a high NaCl concentration (13).

We next examined the sensitivity of DNA replication to inhibitors that affect mammalian DNA polymerases in extracts differently. The cells were permeabilized and then incubated in the DNA synthesis assay solution containing different concentrations of KCl or N-ethylmaleimide (1). Fig. 2A shows that replication activity is inhibited >80% at concentrations of KCl above 0.3 M. NaCl at 0.08 M inhibited replication by 50% (not shown). Fig. 2B shows that N-ethylmaleimide concentrations above 0.3 mM inhibit DNA replication by >90%. We have previously reported that 20 µM cytosine arabinoside triphosphate inhibited DNA synthesis by 90%, as did 1.0 mM hydroxyurea when rNTPs were supplied as precursors to permeable cells (10, 13). The preferential inhibition of DNA polymerase α by Ara-CTP is well documented (see ref. 14 for a review).

DNA Repair in Permeable Cells—To measure repair synthesis, BHK cells were first arrested in G₀ by placing them in medium containing 0.2% calf serum for 48 h. This greatly reduced replication synthesis which otherwise would obscure the much lower repair activity. The low serum medium was replaced with medium containing 10% calf serum for 1 to 2 h prior to permeabilization to ensure that the cells were as physiologically healthy as possible. Flow microfluorimetry showed virtually none of these cells in S phase. The undamaged cells incorporated very little [3H]TTP—approximately 0.1% of the replication rate (Fig. 3).

DNA was damaged by exposing these cultures to x-rays or UV light prior to permeabilization. Other cultures were permeabilized and incubated in the DNA synthesis assay solution containing bleomycin. Bleomycin is a basic glycopeptide that binds to DNA and causes nicking and strand scission. DNA repair follows any of these treatments (see ref. 1b and 16 for reviews).

Permeable BHK cells were shown to carry out repair synthesis after x-ray treatment. BHK cells arrested in G₀ were
et al. (17). Double-stranded (or native) DNA will not bind to
ylated DEAE-cellulose columns using the method of Scudiero
DNA is found in double stranded DNA and elutes with 1
and Cooper (18). Following this procedure, we found that 85%
identical to the BUdR density-labeling procedure of Hanawalt
of the increase in radioactivity after bleomycin treatment was
single-stranded and remains bound to the column during a 1
M NaCl, whereas [3H]TTP incorporated into repaired DNA was
exposed to x-rays 30 min prior to permeabilization with lyso-
lecithin. There was a 1.5- to 2-fold increase in [3H]TTP
incorporation relative to unirradiated permeable GO cells (Fig.
3B).
Irradiation with UV (1200 µW/cm² for up to 5 min) did not
increase [3H]TTP uptake appreciably.
Bleomycin produced an increase in [3H]TTP incorporation,
which was dose-dependent up to 10 µg/ml (Fig. 3A). Maximum
repair after bleomycin treatment (Fig. 3) occurred at about
1% of the replication rate in an exponential culture (10), about
a 10-fold increase in [3H]TTP incorporation relative to unir-
radiated permeable GO cells. Synthesis was linear for at least
15 min. In contrast to replication, omitting GTP, CTP, and
UTP from the DNA synthesis assay mixture did not affect
the rate or amount of DNA synthesis observed after bleomy-
cin treatment, so these rNTPs were omitted from subsequent
DNA repair experiments.
The DNA synthesized after bleomycin treatment of Chinese
hamster ovary cells was analyzed on benzoylated naphto-
ylated DEAE-cellulose columns using the method of Scudiero
et al. (17). Double-stranded (or native) DNA will not bind to
the column and washes through with 1 mM NaCl. Single-
stranded DNA will bind tightly to the column and does not
elute with 1 mM NaCl. [3H]TTP incorporated into repaired
dNA is found in double stranded DNA and elutes with 1 mM
NaCl, whereas [3H]TTP incorporated into replicating DNA is
single-stranded and remains bound to the column during a 1
M NaCl wash. This method has been shown to yield results
identical to the BUdR density-labeling procedure of Hanawalt
and Cooper (18). Following this procedure, we found that 85%
of the increase in radioactivity after bleomycin treatment was
eluted with 1 mM NaCl, indicating it is in repaired DNA.

To estimate an overall K_m for all four dNTPs for the
bleomycin-induced synthesis, we used the data from Fig. 3A
and from several similar experiments. The calculation was
based on six different concentration pairs from three experi-
ments, and applying the following derivation of the Michaelis-
Menton equation:
\[
\frac{v_1}{v_2} = \frac{S_1/(K_m + S_1)}{S_2/(K_m + S_2)}
\]
where the subscripts denote to which member of the concentra-
tion pair the reaction velocity (v) and substrate concentra-
tion (S) refer. The K_m value obtained for all four dNTPs was
0.170 mM ± 0.05 mM. This is substantially higher than the
0.05 mM K_m found in replication synthesis (Fig. 1).
To further distinguish this unscheduled DNA synthesis
from replication activity, the sensitivity of bleomycin-induced
synthesis to KCl and N-ethylmaleimide was examined. Bleo-
mycin-induced DNA synthesis was much less sensitive than
replication to both high KCl (Fig. 2A) and N-ethylmaleimide
(Fig. 2B). Furthermore, 20 µM Ara-CTP, a concentration
which inhibits replication in permeable cells by 90% (10, 13),
reduced bleomycin-induced synthesis only 20%. Eliminating
ATP and phosphoenolpyruvate from the assay mixture in-
hibited bleomycin-induced synthesis by 10 to 30% in different
experiments; the absence of these energy sources reduced
replication synthesis by >80% (10, 13).
The rNDPs can support repair synthesis in permeable cells.
Using ADP, GDP, and CDP with [3H]TTP as the DNA
synthesis precursor consistently gave 80 to 90% as much
incorporation relative to unirradiated permeable Go cells (Fig.
10).

DISCUSSION
It is well documented that DNA replication and repair in
animal cells are very different processes (19, 20), and our data
are consistent with these observations. A major purpose of
the experiments reported here was to assign functions to the DNA

TABLE I
Effect of HU on DNA repair

| Concentration | Rate of repair | Rate of repair |
|---------------|----------------|----------------|
|               | - HU           | + HU           |
| dNTPs or rNDPs | dNTPs rNDPs | dNTPs rNDPs |
| µM            | rNMPs/min/10⁶ cells | rNMPs/min/10⁶ cells |
| 50            | 1.60 1.45 0.90 | 1.68 1.82 0.91 |
| 10            | 0.65 0.55 0.85 | 0.62 0.53 0.85 |
| 5             | 0.45 0.40 0.89 | 0.46 0.40 0.87 |
| 1             | 0.20 0.16 0.80 | 0.18 0.12 0.67 |
| 0.5           | 0.13 0.11 0.85 | 0.14 0.09 0.64 |

Fig. 3. Unscheduled DNA synthesis in permeable BHK cells. BHK cells were plated in 60-mm culture dishes at 5 x 10⁶ cells/dish. After 24 h the cells were put in Dulbecco’s medium containing 0.2% calf serum for an additional 48 to 60 h. A, GO cells were permeabilized and exposed to the DNA synthesis solution containing bleomycin at the indicated doses. ○, 0.05 mM dNTPs; □, 0.01 mM dNTPs. B, GO cells were exposed to the indicated dose of x-rays 30 min prior to permeabilization. The DNA synthesis solution contained 0.05 mM dNTPs.

All incubations were for 20 min at 37°C. 20 µCi/ml [3H]TTP was used in each experiment.

BHK cells were arrested in G0 and permeabilized as described under “Experimental Procedures.” They were incubated with 20 µg/ml of bleomycin and with the indicated concentrations of either dNTPs or rNDPs plus dTTP (20 µCi of [³H]TTP/ml). After 20 min the reaction was stopped, and the cells were processed for scintillation counting as described under “Experimental Procedures.” The amount of incorporation by permeabilized G0 cells not incubated with bleo-
mycin was subtracted from each point. The concentration of HU was 5 mM.

To further distinguish this unscheduled DNA synthesis from replication activity, the sensitivity of bleomycin-induced synthesis to KCl and N-ethylmaleimide was examined. Bleo-
mycin-induced DNA synthesis was much less sensitive than replication to both high KCl (Fig. 2A) and N-ethylmaleimide (Fig. 2B). Furthermore, 20 µM Ara-CTP, a concentration which inhibits replication in permeable cells by 90% (10, 13), reduced bleomycin-induced synthesis only 20%. Eliminating ATP and phosphoenolpyruvate from the assay mixture in-
hibited bleomycin-induced synthesis by 10 to 30% in different
experiments; the absence of these energy sources reduced
replication synthesis by >80% (10, 13).
polymersases $\alpha$ and $\beta$. Many properties of these enzymes have been well studied in cell extracts, and their activities with respect to cell cycle phase have been examined using synchronized intact cells (see Ref. 1). To bridge the gap between enzymes and their roles in intact cells, we have used lysolecithin-permeabilized BHK cells. This subcellular yet highly physiological cell preparation carries out normal, replicative DNA synthesis (10, 11) as well as repair synthesis (Fig. 3).

Bleomycin-induced [$^3$H]TTP incorporation was shown to be predominantly (>80%) repair by the following criteria, which distinguish it from replication: 1) It is unscheduled synthesis; i.e. it occurs in non-S phase cells (Fig. 3). 2) [$^3$H]TTP incorporation reached a plateau at higher bleomycin concentrations; saturation at high levels of DNA damage is characteristic of repair (17, 21). 3) It is not dependent on ATP. 4) It elutes from BND-cellulose columns in the manner expected of repaired DNA.

A small amount (10 to 20%) of the bleomycin-induced [$^3$H]TTP incorporation does not elute in the 1 M NaCl repair fraction. This could be due to a background of replication carried out by the few cells not arrested in $G_0$ or to the induction of a small amount of replication synthesis by bleomycin in $G_0$ cells. The presence of a small amount of replication synthesis is consistent with the 10 to 30% inhibition of bleomycin-induced [$^3$H]TTP incorporation produced by N-ethylmaleimide, KCl, and Ara-CTP. We consider the remaining incorporation to be repair synthesis.

Table II compares the characteristics of both types of DNA synthesis to the properties of DNA polymerases $\alpha$ and $\beta$, as studied in extracts by other investigators. Each of the differences between replication and repair is characteristically different between DNA polymerases $\alpha$ and $\beta$, respectively. DNA replication involves many proteins and enzymes besides a polymerase. Although it is possible that one or more of these molecules is sensitive to salt, N-ethylmaleimide, or Ara-CTP, it is unlikely that another component of the replication (or repair) process would have the same sensitivity to all three inhibitors as DNA polymerase $\alpha$ (or, if so, that none of the components of the repair system have these sensitivities). These data provide strong evidence that the $\alpha$ enzyme is the polymerase for replication synthesis, whereas the $\beta$ enzyme is the polymerase for repair synthesis. The simplest explanation for the apparently rate-limiting role of these polymerases is that they catalyze elongation, the polymerization of the bulk of the DNA precursors. An alternative explanation that we cannot rule out is that these polymerases are involved in an initiation or priming step which limits the subsequent rate.

The primary action of HU is to inhibit ribonucleotide reductase (13), although HU also has been reported to inhibit a gap-filling step of SV40 DNA synthesis (22). HU is frequently used to inhibit replication preferentially while repair is measured (23). Insensitivity of repair to HU is generally attributed to the far smaller requirement for dNTPs, as compared to replication, so that preexisting pools of dNTPs might be adequate. Another possibility that we test here is that inhibition of ribonucleotide reductase by HU might be so incomplete under the usual conditions that enough dNTPs would be produced to permit repair. If this is so, HU should inhibit DNA synthesis most strongly when the reductase catalyzes the reaction at a low rate, e.g. at low substrate (rNDP) concentrations.

In contrast to strong inhibition by 1 mM HU of DNA replication in permeable cells, even when high concentrations (500 $\mu$M) of rNDPs were supplied (10, 13), 5 mM HU did not inhibit repair synthesis until the concentrations of rNDPs were below 5 $\mu$M (Table I). Even at 0.5 $\mu$M rNDPs, inhibition was only about 30%. At first glance, the higher $K_m$ of repair would seem to make it more sensitive to HU than replication. However, the repair of DNA is a low rate multistep process in which the high activity of ribonucleotide reductase appears to be far from rate-limiting. If so, then a nearly complete inhibition of reductase activity would be required before any possible effect on repair could be observed. Even under conditions of low substrate and high HU concentrations, no definitive evidence was found for the inhibition of repair by HU.

Use of permeable cells to study enzyme kinetics assumes that 1) exogenous substrate has free access to the enzyme, and 2) there are no intracellular pools of substrates available to the enzyme. In these experiments, cells were treated with high doses of lysolecithin, resulting in "highly permeable" cell preparations (11). This should reduce intracellular pools (e.g. of dNTPs) and also ensure maximum accessibility of exogenous compounds to the enzyme of interest. Even so, complete elimination of enzyme or intracellular pool compartmentalization is not certain. For instance, the lack of dependence of bleomycin-induced repair on ATP is characteristic of "short-patch" repair observed in prokaryotes and eukaryotes (21). We cannot rule out the possibility that permeable cells still contain a protected pool of ATP, or that salvage pathways are still active and are supplying ATP for repair synthesis.

The results presented here have examined enzymological aspects of DNA replication and repair in lysolecithin-permeabilized BHK cells. Since a wide variety of animal cells can be rapidly and easily permeabilized (11, 13), the technique should be useful for examining many cellular processes, especially for relating results obtained with enzymes and cell extracts to phenomena observed in vitro.

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| TABLE II Comparison of DNA replication and repair in permeable cells with DNA polymerase $\alpha$ and $\beta$ |  |
|---|---|---|---|---|
| Per cent inhibition by | Overall $K_m$ for dNTPs | Overall $V_{max}$ | Activity in $G_0$ |
| | $\mu$M | pmol of dNMP/ min/10$^6$ cells |  |
| Replication | 90 | 90 | 90 | 80 | 50 | 220 | No |
| DNA polymerase $\alpha$ | 95 | 90 | 90 | 10-50 | 170 | 2 | Yes |
| Repair | <25 | 10 | 20 | 30 | 0 | 10-190 | Yes |
| DNA polymerase $\beta$ | 10 | 10 | 10-20 | 0 | 10-190 | Yes |
REFERENCES

1. Weissbach, A. (1977) *Annu. Rev. Biochem.* **46**, 25-47
2. Bolden, A., Noy, G. P., and Weissbach, A. (1977) *J. Biol. Chem.* **252**, 3361-3366
3. Kornberg, A. (1974) *DNA Synthesis*. W. H. Freeman, San Francisco
4. Chang, L. M. S., and Dollum, F. J. (1973) *J. Biol. Chem.* **248**, 3398-3404
5. Baril, E. F., Jenkins, M. D., Brown, O. E., Lasslo, J., and Morris, H. P. (1973) *Cancer Res.* **33**, 1187-1193
6. Roodman, G. D., Hutton, J. J., and Bollum, F. J. (1975) *Exp. Cell Res.* **91**, 269-278
7. Chiu, J.-F., Craddock, C., Morris, H. P., and Hnilica, L. S. (1974) *Cancer Biochem. Biophys.* **1**, 13-21
8. Craig, R. K., Costello, P. A., and Keir, H. M. (1975) *Biochem. J.* **145**, 233-240
9. Bertazzoni, A., Stefanini, M., Pedrali-Noy, G., Giuolotto, E., Nuzzo, F., Falaschi, A., and Spadari, S. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73**, 785-789
10. Miller, M. R., Castellot, J. J., Jr., and Pardee, A. B. (1978) *Biochemistry* **17**, 1073-1080
11. Miller, M. R., Castellot, J. J., Jr., and Pardee, A. B. (1979) *Exp. Cell Res.* **120**, 421-425
12. Schneider, E. L., Stanbridge, E. J., and Epstein, C. J. (1974) *Exp. Cell Res.* **84**, 311-318
13. Castellot, J. J., Jr., Miller, M. R., and Pardee, A. B. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 351-355
14. Cozzarelli, N. R. (1977) *Annu. Rev. Biochem.* **46**, 641-668
15. Umezawa, H. (1973) *Biomedicine* **8**, 459-475
16. Chabner, B. A., Myers, C. E., Coleman, C. N., and Johns, D. G. (1975) *N. Engl. J. Med.* **292**, 1107-1112
17. Scudiero, D., Henderson, E., Norin, A., and Strauss, B. (1975) *Mutation Res.* **29**, 473-487
18. Hanawalt, P., and Cooper, P. (1971) *Methods Enzymol.* **21**, 221-229
19. Hanawalt, P., and Setlow, R. (1976) *Molecular Mechanisms for the Repair of DNA*. Plenum, New York
20. Strauss, B., Scudiero, D., and Henderson, E. (1976) in *Molecular Mechanisms for the Repair of DNA* (Hanawalt, P., and Setlow, R., eds.) Plenum, New York
21. Regan, J. D., and Setlow, R. B. (1973) in *Chemical Mutagens: Principles and Methods for their Detection* (Hollaender, A., ed) Vol. 3, Plenum, New York
22. Laipis, P. J., and Levine, A. J. (1973) *Virology* **56**, 580-584
23. Brands, W. N., Flamm, W. G., and Bernheim, M. J. (1972) *Chem.-Biol. Interact.* **6**, 327-339
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