A general protocol for evaluating the specific effects of DNA replication inhibitors

Victor Levenson and Joyce L. Hamlin*

University of Virginia School of Medicine, Department of Biochemistry, Charlottesville, VA 22908, USA

Received June 3, 1993; Revised and Accepted July 21, 1993

ABSTRACT

Inhibitors of DNA replication in mammalian cells are of great interest because of their potential use in chemotherapy and in cell synchronizing protocols in the laboratory. We have used a combination of isotopic labelling protocols and a two-dimensional gel replicon mapping procedure to determine the specific effects of five different replication inhibitors in cultured cells. Utilizing this protocol, we show that hydroxyurea, aphidicolin, and cytosine arabinoside, three known chain elongation inhibitors, are rather ineffective at preventing fork progression even at relatively high concentrations. In contrast, two related compounds that have been suggested to be G₁/S inhibitors (mimosine and cyclopyrox olamine [CPX]) actually appear to inhibit initiation at origins. One of these agents (CPX) appears also to inhibit replication in yeast, opening the possibility that the gene encoding the target (initiator?) protein can first be identified in yeast by genetic approaches and can then be used to isolate the mammalian homologue.

INTRODUCTION

In bacteria, inhibitors of DNA synthesis have been used primarily to dissect out various components of the replication machinery (see Ref. 1 for review). In animal cells, emphasis has been placed on identifying replication inhibitors that can be used either to arrest actively dividing cells in chemotherapeutic protocols or to devise synchronizing regimens for cultured cells. Among the most widely used drugs in these applications are hydroxyurea, methotrexate, 5'-fluorodeoxyuridine, 1-beta-D-furanosylarabino-cytosine (ara-C), and aphidicolin.

Most of these agents have an extensive history as chemotherapeutic agents (e.g., 2–4), each has been used in cell synchronizing regimens (e.g., 5–9), and their mechanisms of action are well understood. Hydroxyurea, methotrexate, and 5'-fluorodeoxyuridine [which inhibit ribonucleotide reductase (10), dihydrofolate reductase (11), and thymidylate synthetase (8), respectively] slow replication by lowering deoxyribonucleotide pool levels, while ara-C has been shown to be a leaky chain terminator (reviewed in 12). Aphidicolin, which is the newest of these drugs, inhibits DNA replication by interacting with at

least three of the known mammalian DNA polymerases (13). In recent years, aphidicolin has become the agent of choice for synchronizing cultured cells (e.g., 14–16).

While the action of each of these agents is somewhat different, all five block chain elongation. There is no *a priori* reason to believe that any of these drugs inhibits initiation at origins. This distinction could prove critical in designing effective chemotherapeutic applications and, as we will demonstrate below, plays a major role in the efficacy of synchronizing regimens performed in the laboratory. [In this paper we will use the term 'initiation' to refer to the initial helix destabilizing event that must occur at origins prior to the actual priming of nascent daughter strands.]

For example, a common procedure for synchronizing cultured cells at the beginning of the S period is to arrest cells in G₀ by nutritional or serum deprivation, followed by release into complete medium containing an inhibitor such as hydroxyurea or aphidicolin. After a time long enough to allow the slowest cells to traverse the G₁ phase and collect near the G₁/S boundary, the drug is removed and the population enters the S phase in a parasynchronous wave (e.g., 5,14).

While neither hydroxyurea or aphidicolin is expected to prevent initiation at early-firing origins under these circumstances, it was anticipated that these agents would arrest replication forks very close to their respective origins during the block. Indeed, for assessing the approximate time of replication of defined sequences within the S period, this synchronizing procedure has proven to be more than adequate (e.g., 14,17,18).

However, in recent two-dimensional (2-D) gel electrophoretic studies on replication intermediates in the amplified dihydrofolate reductase (DHFR) locus of a methotrexate-resistant CHO cell line (CHOC 400), we found that aphidicolin is a surprisingly leaky inhibitor of chain elongation when utilized in this protocol: many origins of replication fire in its presence and the corresponding replication forks move significant distances away from the origin (19). Several years ago, Walters *et al.* suggested that hydroxyurea might be similarly leaky (20).

More recently, we initiated studies on the plant amino acid, mimosine, which was reported to be a late G₁ inhibitor (21), in hopes that it could be used in this regimen to collect cells prior to initiation at origins in early S (22). Our eventual goal is to identify *trans*-acting protein factors that regulate initiation at

* To whom correspondence should be addressed

origins of replication. In the course of investigating the action of this drug on CHO 400 cells, we developed a comprehensive protocol for discriminating between inhibitors of initiation and elongation, and also for assessing the efficacy of any given inhibitor. In the present study, we compare the effects of five different agents on DNA replication in cultured Chinese hamster ovary cells. Our results suggest that two of these agents (mimosine and a related compound, cyclopyrox olamine [23]) specifically inhibit initiation of replication. Since the latter agent also appears to inhibit replication in yeast, it may be possible to use yeast genetics to eventually clone the gene encoding a mammalian initiation protein.

MATERIALS AND METHODS

Cell culture and synchronization

The methotrexate-resistant Chinese hamster cell line, CHO 400, was propagated as described previously (24). Experiments were performed on asynchronous or synchronous populations growing either in multiwell dishes (uptake studies) or in 15 cm tissue culture dishes (for 2-D gels). In all experiments, cell cycle distribution was determined by fluorescence-activated cell sorter (FACS) analysis as previously described (22), performed either at the University of Virginia FACS Facility or at SmithKline Beecham's Flow and Adherent Cytometry Laboratory. For synchronized populations, cultures were deprived of isoleucine for 45 hr, which induces G_0 arrest (25), and were then returned to complete medium to allow G_1 traverse; thirty min later, one of five different inhibitors (see text) was added to each culture; 14 hr after release from G_0 (by which time all cells have

accumulated near the G_1/S boundary), the drugs were removed by washing twice with prewarmed drug-free medium and the cells were returned to drug-free medium, allowing entry into S.

Stock solutions of drugs were as follows: 60 mM aphidicolin (Sigma) in DMSO; 1 M hydroxyurea (Sigma) in water; 0.1 M ara-C (Sigma) in DMSO; 50 mM mimosine (Aldrich) in culture medium; 0.1 M CPX (Sigma) in DMSO. Stock solutions were divided into small aliquots and stored at -20°C .

Pulse-labelling protocols

In the uptake studies in Fig. 1&2, cells were pulsed for 15 min with 5 $\mu\text{Ci/ml}$ ^3H -thymidine and incorporation into TCA-precipitable material was determined as previously described (22). Data was processed with the Sigmaplot 4.1 program (Jandel Corp.). Each point represents the mean of duplicate samples and bars indicate the standard error.

Two-dimensional (2-D) gel analysis (26)

Nuclear matrices were prepared by a modification (27) of the method of Mirkovitch *et al.* (28) and DNA loops were removed from the matrix by digestion with EcoRI. The DNA remaining with the matrix represents only 4–5% of the total DNA but contains >95% of the replication intermediates (29). The matrix fraction was subjected to chromatography on benzoylated naphthoylated DEAE-cellulose (Sigma)(30) and the material eluting with caffeine (containing the replication intermediates) was subjected to 2-D neutral/neutral gel electrophoretic analysis (26) as described in a previous publication (27). Each gel in Fig. 6–8 was loaded with DNA from $\sim 8 \times 10^7$ cells (four 15 cm plates).

Figure 1. Determining effective drug concentrations on asynchronous cultures. CHO 400 cells were plated at $\sim 2 \times 10^5$ per 2.4 cm well; 36 hr later, the medium was refreshed to insure that the cells were still in log growth phase. Twelve hr later (time 0), the medium was replaced with prewarmed medium containing the indicated concentration of each drug. ^3H -thymidine incorporation during a 15 min pulse was determined at hourly intervals thereafter and samples were processed as described in Materials and Methods.

Figure 2. Efficacy of drugs as synchronizing agents. Panel A. CHOC 400 cells were released from a G₀ block into complete medium. Thirty min later the medium was replaced with complete medium with no additions ('rel' in the figure) or the indicated concentrations of each drug; 13.5 hr later, ³H-thymidine was added for 15 min in the presence of drug and samples were harvested and analyzed as described above. Panel B. CHOC 400 cells growing in multiwell dishes were synchronized at the G₁/S boundary as above. The drugs were removed and cultures were washed with and returned to prewarmed medium. Progress through the subsequent S period was determined by monitoring ³H-thymidine uptake during a 15 min pulse at 90 min intervals thereafter. The first point was taken immediately after drug removal.

Figure 3. Effect of mimosine on cell cycle progression. Cells were plated at 2.5×10^6 cells per 60 mm dish and 24 hr later, drug-free medium was replaced with fresh medium containing each of the drugs and incubation was continued for 16 hr. Samples were taken from each culture and processed for FACS analysis.

approximately 2 hr prior to S (21,23). This conclusion was based on one- and two-dimensional fluorescence-activated cell sorter (FACS) analysis of log-phase populations of human HL60 and LAZ463 cells treated for 16 hr with these agents.

However, when we used the FACS to analyze changes in cell cycle distribution upon addition of mimosine or CPX to asynchronous CHO 400 cells, we were not able to arrest cells exclusively in the G_1 compartment. Typical results are illustrated in Fig. 3, in which log phase cells were incubated with each of the five inhibitors for 16 hr, a time long enough to allow the majority of CHO 400 cells to traverse a complete cell cycle (average cycle time is ~ 20 hr).

The only obvious difference between *any* of the drug-treated samples and the drug-free control is a diminution in the number of cells in the G_2 channel. This is the result expected if each of these agents is a replication inhibitor: any cell in the S period at the time of drug addition will be arrested in S, while cells in G_2 , M, and G_1 will continue in the cycle until they reach the G_1/S boundary (note that most of the cells remaining in the G_2 peak represent G_1 phase tetraploids; 22).

Drug effects on a single replicon

To determine the effect of each drug at the level of a single replicon, we analyzed replication intermediates in the amplified DHFR domain of CHO 400 cells by a two-dimensional gel electrophoretic technique (26). This method takes advantage of the different hydrodynamic properties of fragments containing either single replication forks, replication bubbles, or termination structures to identify fragments containing origins of replication.

The patterns traced in a 2-D gel by each of the possible replication intermediates are shown in Fig. 4, where the non-replicating fragment is detected as a large $1n$ spot lying on the diagonal of linear fragments (indicated by the dotted line). Single fork arcs (panel A), bubble arcs (panels B and C), and X-shaped

Figure 4. Principle of the 2-D gel electrophoretic replicon mapping procedure. Patterns that would be obtained on a 2-D gel when a fragment containing either single forks (A), centered bubbles (B), off-center bubbles (C), or two convergent forks (D) is analyzed with an appropriate probe. A restriction digest containing replicating DNA is separated in the first dimension on the basis of size, which, for any given fragment, will range from $1n$ to $2n$. In the second dimension, the fragments are then separated on the basis of both size and shape. Symbols: a—diagonal of non-replicated fragments from the entire genome; $1n$ —the non-replicated fragment homologous to the probe; b—single fork arc; c—bubble arc; d, e, and f—arcs resulting from termination structures.

Figure 5. Map of the 240 kb dihydrofolate reductase amplicon in CHO 400 cells. A map of a single 240 kb repeating unit (amplicon) is presented, showing the positions of the DHFR and 2BE2121 genes flanking the 50 kb initiation locus. The intergenic region is expanded below, showing the positions of relevant fragments (discussed in text).

Figure 6. Two-dimensional gel analysis of replicative intermediates from synchronized cells. CHO 400 cells were arrested in G_0 by isoleucine starvation and were returned to complete medium containing the indicated drug for 14 hr. Time-zero samples were taken and the remainder of the plates were returned to complete medium. At the indicated times, additional samples were harvested. DNA was isolated and digests were separated on a 2-D gel as described in Materials and Methods, and the resulting transfers were probed with a fragment specific for a 6.2 kb EcoRI fragment from the DHFR initiation locus (fragment F'; Fig. 5). Note that the multiple dots on the diagonal of linear results from the presence of some partially digested products in each sample.

termination structures (panel D) are displaced above the diagonal of linears. A restriction fragment of interest is analyzed by separating a genomic DNA digest on a 2-D gel, transferring the digest to a membrane, and hybridizing with a probe specific for that fragment.

A map of the 240 kb DHFR amplicon in CHO 400 cells is presented in Fig. 5 and shows the position of the replication initiation locus between two convergently transcribed genes. We have shown that, *in vivo*, nascent chains can initiate at any of a large number of positions scattered throughout this 50 kb region, with the result that any restriction fragment within the initiation locus is sometimes replicated passively by forks emanating from outside of the fragment and sometimes by bidirectional bubbles that initiate within the fragment itself (31,19). This phenomenon

Figure 7. Replication intermediates in the DHFR gene in cells blocked with inhibitors. The transfers containing the time-zero samples shown in Fig. 6 were stripped and reprobbed with a 300 bp fragment that is specific for the 3' end of the DHFR gene (fragment H in Fig. 5).

results in a composite pattern composed of a bubble arc and a single fork arc (see below).

In the experiments described below, we analyzed the pattern of replication intermediates in a 6.2 kb EcoRI fragment (fragment F', Fig. 5) from this locus under three different circumstances: 1) in cells collected near the G_1/S boundary with each of the five agents; 2) after cells were released from the G_1/S block and allowed to enter S; and 3) at intervals after adding the drugs to cells growing asynchronously.

In the experiment summarized in Fig. 6, replicate plates of CHO 400 cells were arrested in G_0 by isoleucine deprivation and were then released into complete medium; 30 min later, one of the indicated drugs was added. Fourteen hr later, when all cells had traversed the G_1 period (9), a time-zero sample was taken for each drug, and the remainder of the plates were released into drug-free medium. Additional samples were harvested at the indicated times.

In the time-zero samples from cells treated with each of the three chain elongation inhibitors (aphidicolin, hydroxyurea, and ara-C), a composite pattern of complete bubble arcs and single fork arcs was observed (Fig. 6) (the bubble arcs are extremely faint in the reproductions, but are visible on the original films). Note that, although a very faint fork arc can be observed in isoleucine-starved cells prior to the 14 hr drug treatment, it constitutes less than 10% of the signal seen in the time-zero samples treated with either aphidicolin, hydroxyurea, or ara-C. Thus, none of these agents inhibited the formation of initiation bubbles or prevented replication forks from moving away from origins of replication (as evidenced by the complete single fork arcs). In fact, when the same transfers were stripped and reprobbed for a fragment lying upstream in the DHFR gene (fragment H in Fig. 5), single fork arcs were also observed (Fig. 7), although the intensity was considerably lower than in fragment F' (compare to the 1n spot in each case).

Therefore, in the presence of a relatively high level of either aphidicolin, hydroxyurea, or ara-C, some forks can travel 10 kb or more from their respective start sites in the initiation locus. In contrast, in the samples blocked either with mimosine or with CPX, no replication intermediates were observed at time-zero either in fragment F' (Fig. 6) or in the 3' end of the DHFR gene

Figure 8. The effect of inhibitors on replication fork movement in the DHFR initiation locus in log cells. CHO 400 cells in exponential growth were treated with each drug for either 2 or 8 hr and replicative intermediates were isolated and separated as in Fig. 6. The resulting transfers were probed with a 1 kb fragment specific for fragment F' in the DHFR initiation locus. Note again the presence of partial digestion products, resulting in a series of $>1n$ dots on the diagonal of linears.

(Fig. 7). These two agents must therefore be highly effective inhibitors of initiation, elongation, or both. [Note that in Fig. 6–8, the restriction digests that gave rise to the replication intermediates in each sample did not go to completion, resulting in partial digestion products. These appear as spots along the diagonal of linears, and, in some cases, also appear as shadow arcs (e.g., as in Fig. 6&8). Note in particular that the fork arc in the sample treated with ara-C is somewhat obscured by the $1n$ spot of a partial digestion product but is nevertheless present.]

After the drugs were removed, the kinetics of recovery from each inhibitor were also quite different (Fig. 6). Twenty min after release from aphidicolin, the bubble and fork arcs are already quite intense, but the pattern is qualitatively similar to that seen at time-zero. This result again suggests that aphidicolin slows replication forks but does not prevent cells from entering the S period and initiating at origins. The intensity of the bubble arcs appears to be maximal ~40 min after release from aphidicolin and the bubbles disappear by 80 min. After removal of hydroxyurea or ara-C, the peak of initiation (bubble arcs) in this locus occurs ~60 min after drug removal (note that the hydroxyurea samples are somewhat underexposed in this experiment).

When cells are released from a mimosine block, there is a 30–40 min lag before cells enter the S period, in agreement with earlier studies (19,22). Since this lag is decreased when lower drug levels are used to block the cells near the G_1/S boundary (data not shown), the lag is likely due to an inability to completely remove the drug by washing. Recovery from CPX is quite rapid relative to mimosine, even though the number of cells that recover and, therefore, the number of replication intermediates detected on gels, is markedly reduced (Fig. 6; also see Fig. 2B).

Both mimosine and CPX inhibit initiation

Of the five drugs that we have examined here, mimosine and CPX are the most effective at preventing DNA replication when administered to cells released from a G_0 block (Fig. 6). This finding could simply mean that these two drugs are very effective chain elongation inhibitors. However, when added to asynchronous cultures (Fig. 1), both drugs display the slow-stop phenotype that characterizes initiation mutants in bacteria (32). While the latter phenomenon could be explained by an inordinately slow equilibration of these agents inside the cell, an examination of replication intermediates by 2-D gel electrophoresis suggests that, in fact, mimosine and CPX probably inhibit initiation at cellular origins.

CHO 400 cells in exponential growth were treated with each of the five DNA replication inhibitors and cells were harvested 2 hr and 8 hr later. Replication intermediates in the region of fragment F' were then analyzed by 2-D gel electrophoresis.

As expected, the 2 and 8 hr drug-free controls ('log' in Fig. 8) both show a complete single fork arc and a very faint bubble arc (more obvious in the original films). [Note that only 10–20% of the 1,000 DHFR amplicons in this cell line actually sustain an active initiation event, with the remainder of amplicons being replicated passively by forks from neighboring amplicons; thus, the bubble-to-fork arc ratio in log cells is much lower than in cells synchronized in early S, when only those that are actively initiating are detected (see Discussion in Ref.19,31)].

After either 2 or 8 hr, the patterns for samples treated with either aphidicolin or ara-C appear to be unchanged relative to the drug-free log control. These inhibitors must therefore either arrest replication forks altogether, or allow a slow flux of forks through this fragment. In combination with the results of the uptake studies in Fig. 1 and the 2-D gel studies in Fig. 6, the latter interpretation seems more likely.

The samples treated with mimosine and CPX are very similar to one another and display a different phenotype than the other three inhibitors: at 2 hr, there is a relative diminution of small forks that is manifested as a discontinuity along the fork arc just below the peak; by 8 hr, the entire fork arc has disappeared except for a narrow zone just below the peak (Fig. 8). Thus, it appears that mimosine and CPX allow replication forks that were in transit to continue, but prevent any new initiations from occurring). The accumulation of forks near the center of the fragment further suggests that some sequence and/or protein transiently slows fork progression in this region.

The samples treated with hydroxyurea differ from all of the other drugs: even by 2 hr, the single fork arc has virtually disappeared except for a small amount of material near the peak; after 8 hr in hydroxyurea, the pattern does not change significantly (note that the intermediates accumulating on the single fork arc in the presence of hydroxyurea are slightly larger than those accumulating in mimosine or CPX).

DISCUSSION

Our initial interest in developing the drug screening protocol described in this paper was to allow the identification of compounds that could be used to synchronize cultured cells at various cell cycle positions—in particular, at the G_1/S boundary

or at various stages of DNA replication. However, it could equally well be used to screen agents for potential applications to chemotherapy. By combining several standard approaches, this protocol retains the strengths of each but obviates many of the weaknesses inherent in any single methodology. For screening large numbers of unknown compounds, the entire protocol is somewhat involved. However, there are certain relatively simple elements that can be extracted that give immediate clues as to a drug's action.

For example, short-term ^3H -thymidine labelling studies on log populations treated with various inhibitors should be able to distinguish inhibitors that arrest a step in G_1 from those that affect DNA replication: the former agent should require a full cell cycle before ^3H -thymidine uptake is reduced to zero, since some cells will have to traverse the entire cycle before they come to the pre-replicative drug-sensitive step; a replication inhibitor, on the other hand, will completely inhibit uptake within, at most, a few hours (e.g., as in Fig. 1).

Provided that rate studies are performed (which are more sensitive to kinetic differences than are cumulative uptake measurements), thymidine uptake data also suggest whether replication inhibitors act on the initiation or elongation phases (Fig. 1). This is so because mammalian cells exhibit the same 'slow-stop' phenotype in the presence of an initiation inhibitor as do bacterial cells that carry temperature-sensitive initiation mutations when subjected to the non-permissive temperature (32). A caveat, however, is that a particular drug might take an inordinately long time to equilibrate in cells, also resulting in a slow-stop phenotype. Hence, 2-D gel studies on a defined replicon would be required in subsequent studies to distinguish between inefficient uptake and effects on initiation.

We have also observed that cells released from a G_0 block into an initiation inhibitor for various time intervals (10–18 hr) exhibit the same low initial rate of ^3H -thymidine uptake when the agent is subsequently removed (as with mimosine and CPX in Fig. 2B; 22). In contrast, when the agent inhibits elongation, the initial rate *increases* with the duration of the block, as we have shown previously for aphidicolin (22) and hydroxyurea (J.L. Hamlin, unpublished observations).

The latter observations could be explained if some drugs are more effective chain elongation inhibitors than others. In addition, some drugs (e.g., ara-C and aphidicolin) may selectively inhibit chromosomal replication without affecting mitochondrial DNA synthesis, which might be higher at later times in the cell cycle. While this could account for the refractile uptake detected at the highest levels of certain drugs (Fig. 1&2A), the most likely explanation is that elongation inhibitors allow more and more origins to fire as the incubation time increases. Thus, when the drug is removed, a larger number of replication forks are assembled and ready to go, accounting for an increasing initial rate as drug exposure time increases.

In our experience, fluorescence-activated cell sorter analysis of cell populations is a relatively insensitive monitor of drug action that is fraught with difficulties. Obvious problems relate to the inability to distinguish cells in early G_1 , late G_1 , and early S based solely on DNA content. There are additional problems related to the lengthy time intervals required to see a quantitative drug effect on cell cycle position. When we performed FACS analyses on supposedly log populations of CHO 400 cells that had been treated with mimosine for 24 or 48 hr (1 or 2 cell cycles), we found that most cells were in the G_1 cell compartment (unpublished observations). We therefore concluded

that mimosine was a late G_1 inhibitor in CHO cells, as originally reported by Lalande and coworkers for human cells (21, 23). However, when companion drug-free cultures were analyzed 24 and 48 hr later, they also had come to rest in the G_1 compartment, probably because of nutritional deprivation.

In fact, we found it rather difficult to ensure even that the starting population was totally asynchronous, since, like many other laboratories, we allow our cells to come to stationary phase in G_0 over the weekend, and these stocks are then used to seed plates for experiments on Monday. Several days of culture with adequate feeding are then required to render the population fully cycling and asynchronous. When we tested the activities of mimosine and CPX on asynchronous CHO, murine, and monkey cells prepared in this way, it became apparent that these agents were inhibiting some aspect of replication *per se*, and not the G_1/S transition (22; and data not shown).

The novel application in the screening protocol described in this paper is the use of a 2-D gel electrophoretic procedure to examine drug effects at the level of a single replicon. *A priori*, the method allows one to focus exclusively on nuclear replication, and to distinguish between effects on initiation versus elongation. In the present study, this method dramatically confirms the suggestion from uptake studies that mimosine and CPX prevent initiation at origins when administrated to cells released from a G_0 block (Fig. 6). Furthermore, in combination with uptake data in log cells (Fig. 1), the 2-D gel data argue convincingly that mimosine and CPX allow replication forks to progress to their destinations (i.e., these agents don't appear to arrest chain elongation *per se*), but they do not allow new forks to be set up (i.e., they prevent new initiations from occurring) (Fig. 8). We believe that this is the only method currently available for rapidly distinguishing between these two potential effects in mammalian cells (DNA fiber autoradiography can do so to some extent, but only after 3–4 month exposures) (33).

A most important finding in our study is that CPX, though relatively toxic to CHO 400 cells, appears to specifically inhibit initiation. While its cytotoxicity indicates that CPX would not be a useful drug for synchronizing mammalian cells, this drug may still prove extremely useful in studies designed to identify the initiation protein that is apparently the target of CPX (and/or mimosine). CPX is a relatively broad-spectrum anti-fungal agent (34) and, in preliminary experiments, we have found that CPX arrests *Saccharomyces cerevisiae* primarily in the G_1 /early S compartment (R.J. Kalejta and J.L. Hamlin, unpublished observations). If it can be shown that CPX also inhibits initiation at origins of replication in yeast using the 2-D gel methodology described above, it may be possible to utilize the powerful genetic systems in this organism to clone the gene encoding the target and to find its homologue in mammalian genomes.

It is important to point out that we would have eliminated CPX from our study because of its cytotoxicity (as deduced from uptake studies) had we not had the additional information from 2-D gel studies that the agent inhibits initiation selectively. Thus, we argue that the relatively comprehensive drug screening protocol that we outline here can give valuable information that would otherwise not be available.

ACKNOWLEDGEMENTS

We would like to thank Carlton White for his excellent and dedicated technical assistance and Chris Eichmann for his help with FACS analysis. We particularly extend our thanks to Pieter

Dijkwel for his advice throughout this project. This work was supported by grants from the NIH to J.L.H. (RO1GM26108 and RO1CA52559).

REFERENCES

1. Kornberg, A., and Baker, T.A. (1992) *DNA Replication* (2nd ed.). W.H. Freeman & Co. San Francisco.
2. Yarbrow, J.W. (1992) *Seminars Oncol.* 19 (Supp. 9), 1–10.
3. Chen, A.P., and Grem, J.L. (1992) *Current Opinion Oncol.* 4, 1089–1098.
4. Welch, A.D. (1965) *Ann. NY Acad. Sci.* 123, 19–40.
5. Tobey, R.A., Crissman, H.A. (1972) *Exp. Cell Res.* 75, 460–464.
6. Hamlin, J.L., and Pardee, A.B. (1976) *Exp. Cell Res.* 100, 265–275.
7. Pedrali-Noy, G., Spadari, S., Miller-Faures, A., Miller, A.O.A., Kruppa, J., and Koch, G. (1980) *Nucl. Acids Res.*, 8, 377–387.
8. Rueckert, R.R., and Mueller, G.C. (1960) *Cancer Res.* 20, 1584–1591.
9. Heintz, N.H., and Hamlin, J.L. (1983) *Biochemistry* 22, 3552–3556.
10. Skoog, L., and Nordenskjold, B. (1971) *Eur. J. Biochem.* 19, 81–89.
11. Werkheiser, W.C. (1961) *J. Biol. Chem.* 236, 888–893.
12. Cozzarelli, N.R. (1977) *Ann. Rev. Biochem.* 46, 641–648.
13. Wang, T.S.-F. (1991) *Ann. Rev. Biochem.* 60, 513–552.
14. Heintz, N.H., and Hamlin, J.L. (1982) *Proc. Natl. Acad. Sci. USA* 79, 4083–4087.
15. Kaufman, G., Zannis-Hadjopoulos, M., and Martin, R.G. (1985) *Mol. Cell. Biol.* 5, 721–727.
16. Burhans, W.C., Vassilev, L.T., Caddle, M.S., Heintz, N.H., and DePamphilis, M.L. (1990) *Cell* 62, 955–965.
17. Goldman, M.A., Holmquist, G.P., Gray, L.A., Caston, L.A., and Nag, A. (1984). Replication timing of genes and middle repetitive sequences. *Science* 224, 686–692.
18. Taljanidisz, J., Popowski, J., and Sarkar, N. (1989) *Mol. Cell. Biol.* 9, 2881–1889.
19. Dijkwel, P.A., and Hamlin, J.L. (1992) *Mol. Cell. Biol.* 12, 3715–3722.
20. Walters, R.A., Tobey, R.A., and Hildebrand, C.E. (1976) *Biochem. Biophys. Res Commun.* 69, 212–217.
21. Lalande, M. (1990) *Exp. Cell Res.* 186, 332–339.
22. Mosca, P.J., Dijkwel, P.A., and Hamlin, J.L. (1992) *Mol. Cell. Biol.* 12, 4375–4383.
23. Hoffman, B.D., Hanauske-Abel, H.M., A., Flint, and Lalande, M. (1991) *Cytometry* 12, 26–32.
24. Milbrandt, J.D., Heintz, N.H., White, W.C., Rothman, S.M., and Hamlin, J.L. (1982) *Proc. Natl. Acad. Sci. USA*, 78, 6043–6047.
25. Ley, K.D., and Tobey, R.A. (1970) *J. Cell Biol.* 47, 453–459.
26. Brewer, B.J., and Fangman, W.L. (1987) *Cell* 51, 463–471.
27. Dijkwel, P.A., Vaughn, J.P., and Hamlin, J.L. (1991) *Mol. Cell. Biol.* 11, 3850–3859.
28. Mirkovitch, J., Mireault, M.E., Laemmli, U.K. (1984) *Cell*, 39, 223–232.
29. Vaughn, J.P., Dijkwel, P.A., Mullenders, L.N.F., and Hamlin, J.L. (1990) *Nucl. Acids Res.* 18, 1965–1969.
30. Levine, A.J., Kang, H.S., and Billheimer, F.E. (1970) *J. Mol. Biol.* 50, 549–565.
31. Vaughn, J.P., Dijkwel, P.A., and Hamlin, J.L. (1990) *Cell* 61, 1075–1087.
32. Wechsler, J.A., and Gross, J.D. (1971) *Mol. Gen. Genet.* 133, 273–284.
33. Huberman, J.A., and Riggs, A.D. (1968) *J. Mol. Biol.* 32, 327–341.
34. Jue, S.G., Dawson, G.W., and Brogden, R.N. (1985) *Drugs* 29, 330–341.