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DOWN-REGULATION OF c-*myc* GENE EXPRESSION WITH INDUCTION OF HIGH MOLECULAR WEIGHT DNA FRAGMENTS BY FLUORODEOXYURIDINE

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Abstract—5-Fluoro-2′-deoxyuridine (FdUrd), a potent inhibitor of thymidylate synthase, induces extensive bulk DNA damage at drug concentrations that produce significant *in vitro* growth inhibition of human ileocecal carcinoma (HCT-8) cells. Constant- and pulsed-field gel electrophoresis (CFGE and PFGE), to detect size distribution of DNA double-strand breaks and repair kinetics, in parallel with northern and western blot analyses, to quantitate c-*myc* gene and protein expression, were utilized to analyze drug effects. At 24-hr post *in vitro* drug treatment, when maximum bulk DNA damage was detected, FdUrd produced a broad range of high molecular weight DNA fragments, clustering between 0.1 and 5.7 megabases in size, and resulted in a decrease in the level of c-*myc* transcripts and protein with no significant effect on the level of v-*myc* and H-*ras*. These effects preceded the observed cellular growth inhibition. Addition of the reduced folate leucovorin potentiated the effects induced by FdUrd, indicating that thymidylate synthase inhibition is an important initial step in drug effect followed by DNA fragmentation and suppression of c-*myc* expression. Changes in the integrity of the genetic materials and regulatory genes occurred prior to the observed cell growth inhibition by FdUrd, suggesting that these molecular alterations by FdUrd may be associated with subsequent FdUrd-induced cell growth inhibition.

Key words: fluorodeoxyuridine; thymidylate synthase; DNA fragmentation; c-*myc* gene expression

FdUrd§ a TS inhibitor, is a clinically active agent against a variety of human solid tumors. Sustained inhibition of TS by FdUrd is an important determinant of response [1–4]. Additionally, this agent produces a high level of DNA SSBs and DSBs in a concentration- and time-dependent manner, reaching a maximum level by 16–24 hr post-treatment [5–8]. These effects were reversible with dThyd and potentiated by [6RS]LV.

c-*myc* Oncogene expression is linked to changes in the control of cell proliferation [9–11]. In recent years, several investigators have demonstrated elevated expression of c-*myc* mRNA in a wide variety of tumors including colon carcinoma [12–14]. The findings that DNA-damaging agents can suppress the expression of specific oncogenes [15–17] would suggest that these agents are capable of inducing changes in oncogene expression in association with the induction of DNA strand breakage.

In this report, the effect of FdUrd on induction of high molecular weight DNA fragments and suppression of specific oncogene expression was investigated. We initiated the present study to evaluate the relationship between DNA fragmentation, suppression of oncogene expression, and cell growth inhibition.

MATERIALS AND METHODS

Drugs and chemicals. FdUrd, [6RS]LV and dThyd were purchased from the Sigma Chemical Co. (St. Louis, MO). Proteinase K was obtained from Bethesda Research Laboratories (Gaithersburg, MD) and [^{14}C]dThyd (sp. act. 56 mCi/mmol) from Moravek Biochemical, Inc. (Brea, CA).

Cell culture. Human ileocecal adenocarcinoma cells (HCT-8, ATCC CCL 244) were obtained from the American Type Culture Collection (Rockville, MD) and maintained as monolayers in RPMI 1640 medium supplemented with 10% dialyzed horse serum (GIBCO, Grand Island, NY) and 1 mM sodium pyruvate. All treatments were performed on exponentially growing cell cultures.

Growth inhibition assessment by the SRB assay. Cell growth inhibition was determined by the SRB

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§ Abbreviations: FdUrd, 5-fluoro-2′-deoxyuridine; TS, thymidylate synthase; dThyd, thymidine; [6RS]LV, [6*RS*]-5-formyltetrahydrofolate, leucovorin; SRB, sulforhodamine B; HBSS, Hanks' balanced salt solution; PFGE, pulsed-field gel electrophoresis; CFGE, constant-field gel electrophoresis; Mb megabase; and SSBs and DSBs, single- and double-strand breaks.

assay, as described previously [18, 19]. Exponentially growing cells were seeded into 96-well plates, treated for 2 hr, and then incubated for 8, 24, 32, 48, 72, 96 and 120 hr in FdUrd-free medium with or without [6RS]LV. The cells were fixed with 10% trichloroacetic acid and stained for 10 min with SRB solution (0.4 g/100 mL in 1% acetic acid). SRB was removed by rinsing five times with 1% acetic acid and the bound dye solubilized in 10 mM Tris base. Absorbance was read at 570 nm using an automated Bio Kinetics reader (model EL 340, Bio-Tek Instrument, Winooski, VT).

Preparation of DNA plugs. Cells were embedded in agarose plugs for PFGE and CFGE analyses [20, 21]. Approximately 1.0×10^7 cells were washed twice with HBSS and resuspended in 0.2 mL of HBSS. An equal volume of 2.0% low melting point agarose was added. Agarose plugs were digested in 20 vol. of lysis buffer, containing 0.5 M EDTA, pH 8.0, 10 mM Tris, 1% Sarkosyl and 1 mg/mL of proteinase K, for 24 hr and then were incubated for 1 hr in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) containing 0.2 mg RNase A/mL. Each agarose plug contained approximately 1×10^6 cells.

Constant- and pulsed-field gel electrophoresis. Agarose gels were prepared in 0.5 × BRL pulsed-field TBE buffer (75 mM Tris, 25 mM boric acid and 0.1 mM EDTA, pH 8.9). Agarose plugs containing purified DNA were inserted into the wells and sealed with a small amount of liquid agarose. Electrophoresis was carried out using a Hex-A-Field horizontal gel electrophoresis apparatus (GIBCO-BRL, Grand Island, NY), containing a hexagonal array of electrodes having a reorientation angle of 120. Electrophoresis was performed in TBE buffer at 14°. After electrophoresis, the gels were stained with ethidium bromide (5 μg/mL in water) for 10 min and then destained in clean water for 10 min, and photographs of the gels were taken on a trans-illuminator. Megabase I and IV (GIBCO-BRL) were used as DNA markers.

CFGE agarose gels were prepared using 1 × TAE buffer (1.6 M Tris, 0.8 M sodium acetate, 40 mM EDTA, pH 7.2), and electrophoresis was performed in TAE buffer at room temperature. For quantitative analysis of DNA fragmentation, cells were prelabeled with [^{14}C]dThyd for 24 hr and, following CFGE, each band was cut and the quantity of ^{14}C-labeled DNA was determined by scintillation counting.

Northern hybridization. Cellular RNA extracted with guanidine was centrifuged over a cesium chloride gradient [22, 23]. RNA was resolved on 1.2% agarose-formaldehyde gel. Ethidium bromide staining of the agarose gels was used to monitor RNA integrity, uniform RNA loading, and transfer onto the Hybond-N membranes by capillary blotting. Prehybridization was carried out at 45° for 2 hr in 50% formamide (deionized), 6× SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7) 10% dextran sulfate, 1% SDS and 100 μg/mL of sheared, heat-denatured salmon testes DNA. Following prehybridization, ^{32}P-labeled probes for c-*myc*, v-*myc*, H-*ras* or β-actin were added at a final concentration of 10^6 to 10^7 cpm/mL. Hybridization was performed at 45° overnight. Following the high stringency washes, the membranes were exposed to Kodak film at −70° using Dupont Cronex lighting plus intensifying screens. Film density was quantitated with a Biomed Instruments Soft Laser Scanning Densitometer. Ethidium bromide staining of the gel was used to quantitate the amounts of RNA loaded in each lane.

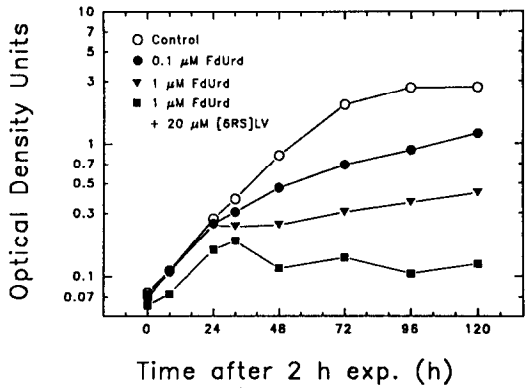

Fig. 1. Effects of FdUrd on HCT-8 cell proliferation. Cells were treated with 0.1 and 1 μM FdUrd with or without 20 μM [6RS]LV for 2 hr, washed and grown in drug-free medium in the presence or absence of [6RS]LV. Inhibition of cell growth was determined at 0, 8, 24, 32, 48, 72, 96 and 120 hr post-drug treatment. Symbols represent averages of three separate experiments, each with 8 wells of cultures.

Western blotting. HCT-8 cells (5×10^6) were washed twice with HBSS, and the cell pellet was digested with RIPA buffer containing 50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 0.5% SDS, 0.5% sodium deoxycholate, 0.5% Nonidet P40, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 0.4 TIU aprotinin. Cell lysates were homogenized on ice, transferred to a microfuge tube, and pelleted for 10 min at 4°. The supernatant was divided into aliquots, and the protein content was determined by the Bio-Rad DC protein assay. Fifty micrograms of extract was loaded onto an SDS–PAGE system according to the method of Laemmli [24]. After electrophoresis, the proteins were transferred to nitrocellulose membrane (Bio–Rad, Hercules, CA) and blocked with 5% nonfat milk in PBS at 37° for 2 hr. The first antibody was added and incubated for 1 hr at room temperature. The second antibody was then incubated with the blot, and the proteins were detected by chemiluminescence as described by the manufacturer (Amersham Life Science, Amersham, U.K.).

RESULTS

Cell growth inhibition. FdUrd inhibited HCT-8 cell growth in a concentration-dependent manner (Fig. 1). Treatment with 0.1 and 1.0 μM FdUrd slowed growth, observed beginning 32 hr after drug treatment. At 24 hr post-drug treatment, more than 90% of cells remained viable as determined by the trypan blue exclusion assay. The data in Fig. 1 also demonstrate that [6RS]LV potentiated the FdUrd-induced growth inhibition by approximately 2-fold.

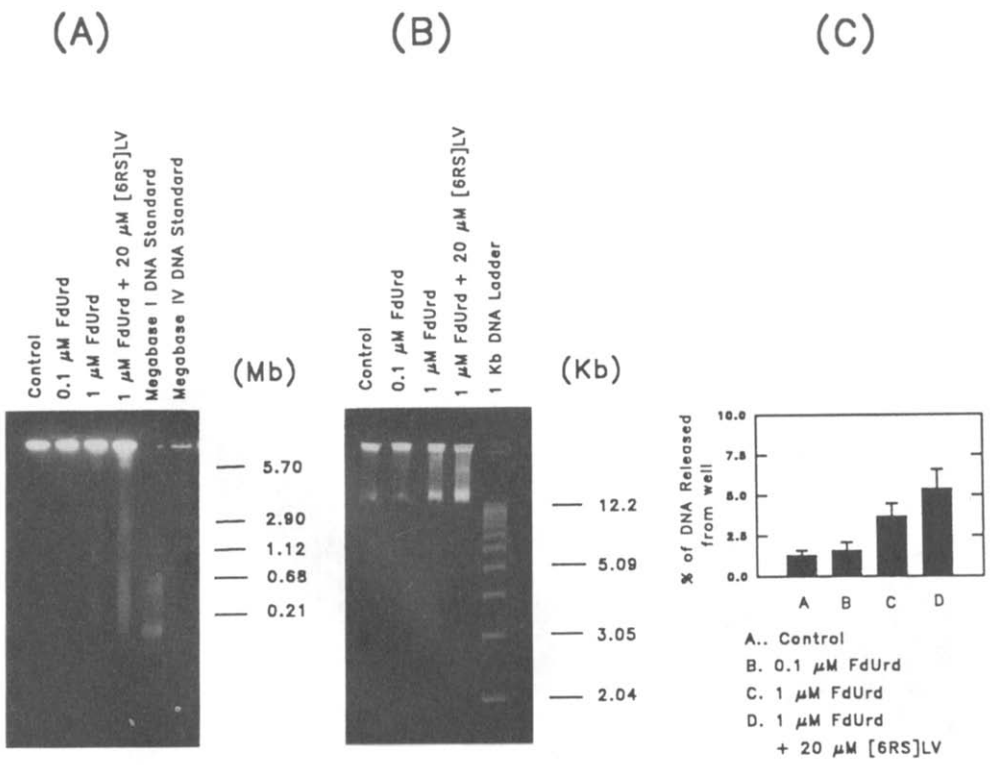

Fig. 2. Quantitation of high molecular weight DNA fragments induced by various concentrations of FdUrd with or without [6RS]LV using pulsed- (A) and constant-(B) field gel electrophoresis. Quantitation of percentages of DNA released from the wells is shown in (C). Agarose plugs containing DNA in HCT-8 cells were processed as described in the text. (A) Measurement of DNA DSBs was carried out by PFGE at 14° in 0.8% agarose. Megabase I DNA standards were used for sizing linear double-stranded DNA from 0.2 to 1.9 Mb and megabase IV DNA standards were used for sizing from 1.03 to 3.30 Mb. (B) Measurement of DNA DSBs by CFGE was performed for 48 hr (voltage: 1.7 V/cm) at room temperature. (C) Quantitation of the percent of DNA released from the wells was determined by scintillation counting. Following CFGE, each band was cut, and the quantity of ^{14}C-labeled DNA was determined. The data represent an average ±SD of three experiments.

Analysis of DNA fragmentation as a function of drug concentration. The data in Fig. 2 demonstrate that the level of DNA damage induced by FdUrd was concentration dependent, determined by both PFGE (Fig. 2A) and CFGE (Fig. 2B). A broad range of high molecular weight DNA fragments, ranging in size from 0.1 to 5.7 Mb, was detected (Fig. 2A). Although DNA size distribution patterns were similar for each treatment condition, the intensity of DNA damage increased with increased drug concentration or by addition of [6RS]LV (Fig. 2B and C). The fraction of ^{14}C-labeled DNA released from the wells was 1.6 ± 0.5% and 3.7 ± 0.8% of total DNA for cells treated with 0.1 and 1 μM FdUrd, respectively, as compared with 1.3 ± 0.3% for controls. The formation of FdUrd-induced DNA DSBs was enhanced by [6RS]LV treatment (from 3.7 ± 0.8 to 5.4 ± 1.2%) (Fig. 2C).

Kinetics of DNA damage induced by FdUrd. The time-dependent effect of DNA damage induced by a cytotoxic concentration of FdUrd (1 μM, IC_{90}) was investigated, and the results are shown in Fig. 3. A maximum level of DNA DSBs was observed by 24 hr post-drug treatment and maintained up to 72 hr. The broad range of high molecular weight DNA fragments was first observed at 16 hr after drug treatment. The data in Fig. 3A indicate that the distribution of DNA damage was similar in size at all time points after drug treatment. Quantitation of the data from Fig. 3B demonstrates the maximum DNA damage and repair (Fig. 3C).

Oncogene expression by northern blot analysis. The effect of FdUrd on the expression of c-*myc* RNA in HCT-8 cells was investigated, and the results are shown in Fig. 4. Two concentrations of FdUrd were used, 0.1 and 1.0 μM. FdUrd treatment suppressed c-*myc* transcription in a concentration-dependent manner, so that the level of c-*myc* transcripts was decreased to approximately 30% of control in HCT-8 cells exposed to 1 μM FdUrd. The addition of [6RS]LV potentiated the FdUrd-induced suppression of c-*myc* expression. FdUrd treatment had no significant effect on the steady-state levels of v-*myc* and H-*ras* RNA transcripts (Fig. 5).

c-myc Protein expression by western blotting. The effect of FdUrd alone and in combination with

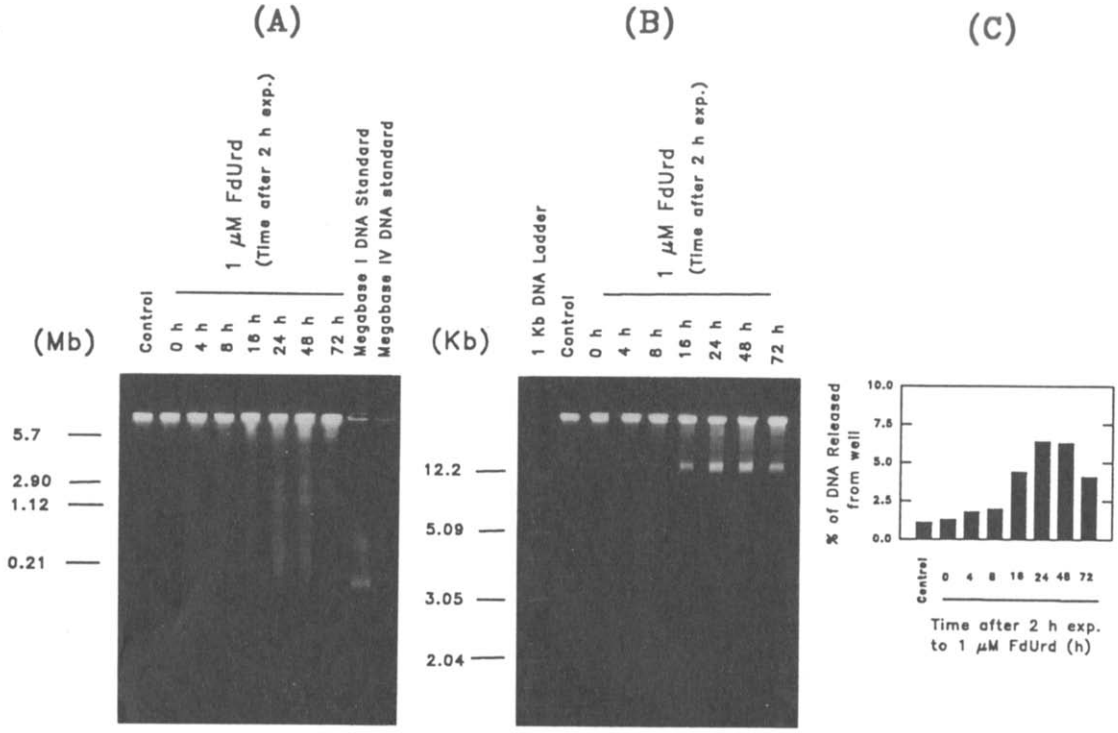

Fig. 3. Time-dependent induction of megabase DNA fragments induced by FdUrd in HCT-8 cells. Cells were exposed to 1 μM FdUrd for 2 hr, washed, and then allowed to grow in drug-free medium for the indicated times after treatment. Agarose plugs, containing DNA in drug-treated cells, were analyzed by PFGE (A) and CFGE (B), and the quantity of DNA released from the wells was determined (C), as described in Fig. 2.

[6RS]LV on the c-myc protein expression is shown in Fig. 6. In control HCT-8 cells, c-myc protein was detected at high levels. In contrast, lower levels of c-myc protein were observed in the drug-treated cells, demonstrating that the decline in c-myc protein expression was also concentration dependent, and [6RS]LV potentiated the suppression of c-myc protein expression.

The effect of FdUrd on c-myc protein expression was also time dependent (Fig. 7). A marked suppression of c-myc protein expression was observed at 16 hr post-drug treatment. The most pronounced decrease was seen after 24 hr following drug treatment and persisted up to 72 hr. These effects correspond in time and drug concentration to the observed effects of FdUrd on DNA fragmentation.

DISCUSSION

Although the precise mechanism(s) of FdUrd-induced cell death has not been delineated, DNA damage is considered an important determinant of FdUrd action [4, 6, 25–27]. The broad distribution of high molecular weight DNA fragments induced by FdUrd clustered at 0.1 to 5.7 Mb (Figs. 2 and 3). The formation of high molecular weight DNA fragments induced by FdUrd may be the result of inhibition of repair of spontaneously arising fragmentation of mature DNA and may also be due to the degradation of nascent DNA resulting from an imbalance in the deoxynucleotide triphosphate (dNTP) pools. Also, an elevated dUTP/dTTP ratio, resulting from inhibition of TS, could lead to misincorporation of dUTP into DNA and saturation of the uracil-*N*-glycosylase system causing strand breaks in nascent DNA. dNTP pool imbalance has been shown to act as a trigger for the activation or inhibition of oncogene expression [28]. More recently, it was proposed that fragmentation of nascent DNA and interference with DNA chain elongation could account for the observed effects with arabinosylcytosine [29, 30].

The observed DNA damage by FdUrd is unlikely to be related to its incorporation into cellular DNA, since similar levels of DNA damage and broad fragment size distribution were achieved with a new and specific TS antifolate inhibitor, ICI D1694, with no detectable incorporation into cellular DNA [8]. At equicytotoxic and pharmacologically achievable concentrations, both FdUrd and ICI D1694 produced similar TS inhibition (>95%) (data not shown). Moreover, 5-fluorouracil, which is known to incorporate into cellular RNA and DNA, but also results in temporal inhibition of TS, does not produce high levels of DNA damage [6].

The c-*myc* gene encodes a nuclear phosphoprotein of 439 amino acids [31, 32], and it is associated with control of cell proliferation and tumorigenesis

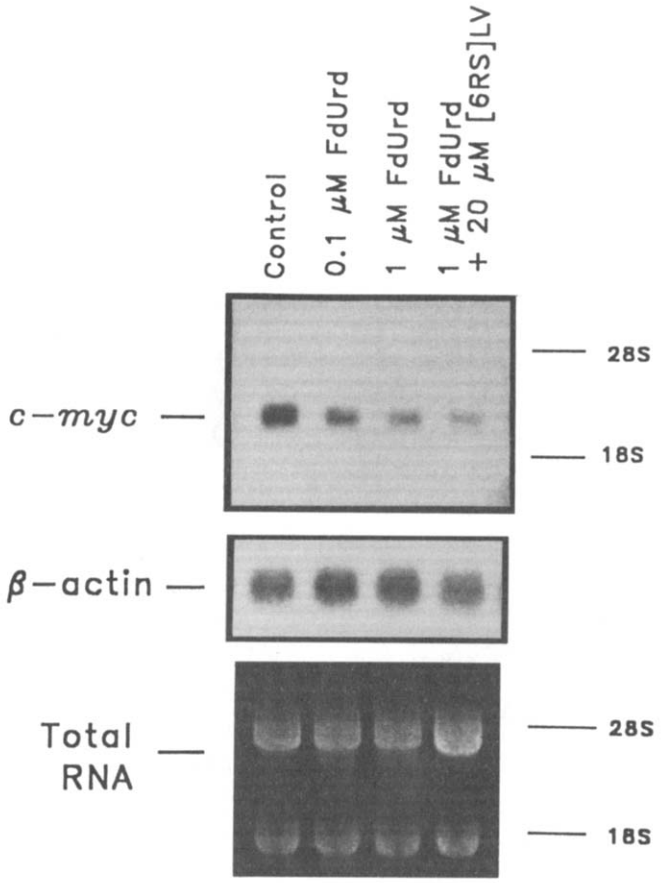

Fig. 4. Effects of FdUrd on c-*myc* oncogene expression. Cells were treated with 0.1 or 1 μM FdUrd with or without 20 μM [6RS]LV for 2 hr. At 24 hr following drug exposure, the cells were harvested. Total cellular RNA was isolated and analyzed by northern hybridization. Ten micrograms of RNA were loaded into each lane, and the hybridization conditions are described in the text. β-actin cDNA probe was hybridized to northern blots, and ethidium bromide staining of the gel was used to estimate the amount of RNA loaded in each lane.

[33, 34]. To examine whether c-*myc* expression is involved in the regulation of proliferation of HCT-8 cells exposed to FdUrd, we used northern and western blotting to assess c-*myc* RNA and protein expression and the expression of v-*myc* and H-*ras*. FdUrd treatment significantly suppressed the levels of c-*myc* transcript and protein, and [6RS]LV potentiated further the suppression of c-*myc* RNA and protein expression (Figs. 4 and 5), suggesting that the decrease in the c-*myc* expression by FdUrd treatment is related to the degree and duration of thymidylate synthesis inhibition. Furthermore, the decrease in steady-state c-*myc* RNA levels induced by FdUrd appears to be selective, since the agent had little effect on v-*myc* and H-*ras* transcription.

Although suppression of c-myc protein expression and DNA fragmentation induced by FdUrd preceded in time the observed cell growth inhibition, these results *per se* cannot prove a cause–effect relationship. Addition of dThyd up to 8 hr post-drug treatment, when no detectable DNA damage was observed, produced complete reversal of drug-induced effects. Addition of dThyd at the time that DNA fragmentation was observed failed to protect cells from any of the observed drug effects [6]. The results of [6RS]LV potentiation of FdUrd-induced DNA fragmentation and c-*myc* down-regulation, as well as the reversal of DNA damage by dThyd, strongly suggest an interrelationship between these factors and the observed growth inhibition.

The effect of FdUrd on c-*myc* expression could be due to differences in the half-life among different oncogenes. Thus, decreased synthesis coupled with a shorter half-life could result in the apparent decrease in the level of c-*myc* expression. This possibility cannot be ruled out on the basis of the results reported herein.

The observed suppression of c-*myc* expression caused by FdUrd is not accounted for by missense incorporation into DNA, since ICI D1694, an antifolate TS inhibitor, suppresses c-myc protein expression to a degree similar to the suppression by FdUrd (data not shown). Although DNA-damaging agents can produce suppression of c-*myc* gene

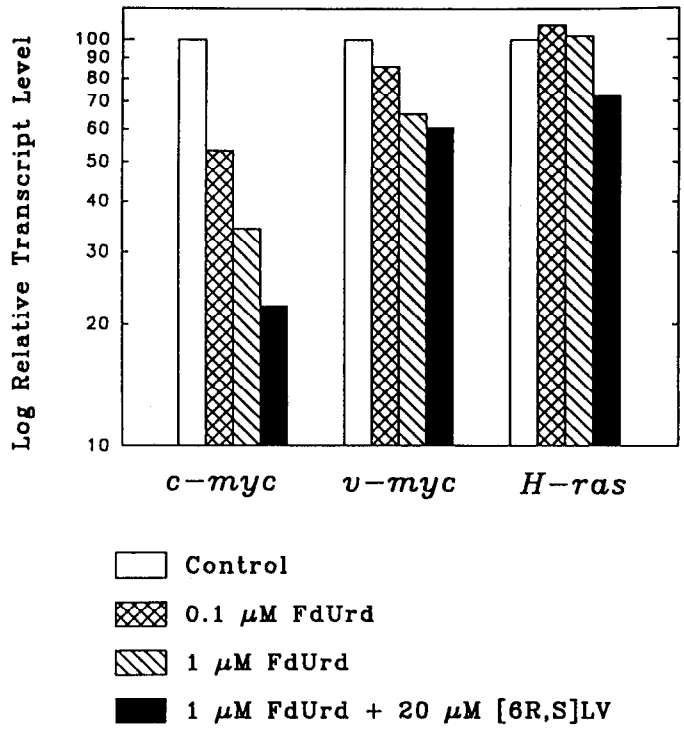

Fig. 5. Comparative quantitation of c-*myc*, v-*myc* and H-*ras* RNA levels. The relative levels of the c-*myc*, v-*myc* and H-*ras* hybrids were determined using densitometric scanning of a representative autoradiogram and expressed as the relative intensity (percentage of control).

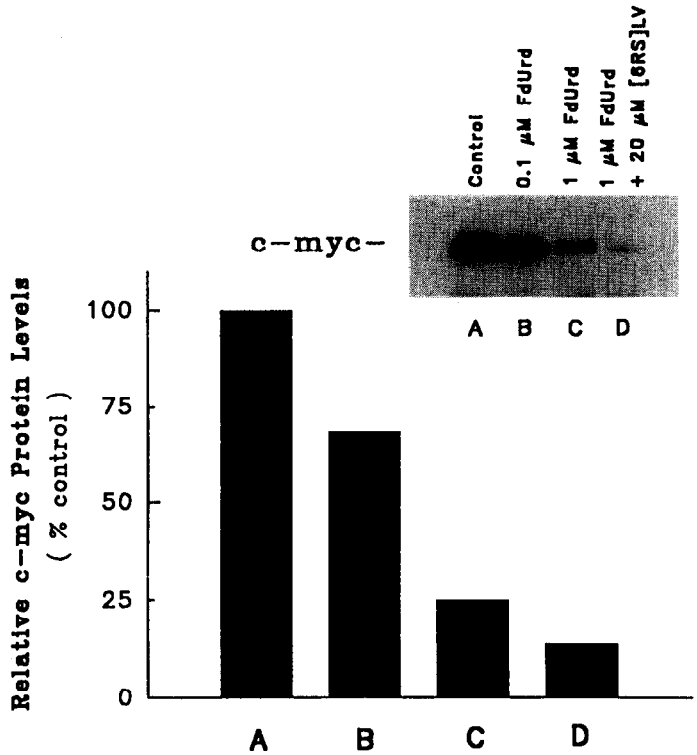

Fig. 6. c-myc Protein expression in HCT-8 cells exposed to various concentrations of FdUrd. The determination of c-myc protein expression was carried out in exponentially growing cells treated with 0.1 or 1 μM FdUrd alone or with 20 μM [6RS]LV. Western blotting was performed at 24 hr after drug treatment, as described in Materials and Methods.

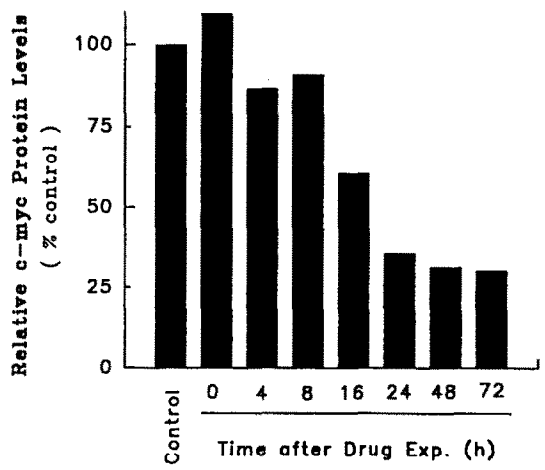

Fig. 7. Time course of FdUrd-induced suppression in c-myc protein expression in HCT-8 cells. The cells were treated with 1 μM FdUrd for 2 hr, washed twice and grown in drug-free medium for 0, 4, 8, 16, 24, 48 and 72 hr after drug exposure. Total cellular protein was extracted from each group, and 50 μg protein was used for western blot analysis.

expression, the cause of suppression of c-*myc* transcripts is yet unanswered. The alteration in c-*myc* mRNA expression may be due to the accumulation of DNA damage within the c-*myc* oncogene [15]. The suggestion is consistent with the observation in which c-*myc* expression is involved in secondary DNA fragmentation [35]. It is also possible that FdUrd incorporation into either small nuclear RNAs or pre-processed c-*myc* RNA can account for the decreased expression of c-*myc* transcripts or altered protein product. In this regard, positive results have been obtained using 5-fluorouracil [36–39].

A model proposed by Green *et al.** indicated that c-*myc* and p53 can promote both cell proliferation and cell death, through apoptosis. Preliminary results from our laboratory indicate that the up-regulation of p53 and the down-regulation of c-*myc* by FdUrd occur at the same time when maximum DNA damage occurs. It is possible, therefore, that a linkage between these factors exists for a direct or indirect drug effect. Furthermore, treatment with FdUrd may produce a depletion of certain growth factors that regulate the expression of c-*myc* and the activation of programmed cell death. These hypotheses need to be investigated to determine the potential therapeutic implications of these findings.

In brief, although the precise mechanisms of DNA fragmentation and c-*myc* down-regulation induced by FdUrd are not elucidated clearly, these effects precede the observed inhibition of cell growth. These effects were observed at pharmacologic and therapeutically achievable drug concentrations, and are consistent with the findings by Canman *et al.* [40], using the colon cancer cell line HT-29.

* Green DR, Bissonnette RP and Cotter TG, Apoptosis and cancer. *Principles and Practice of Oncology Updates* **8**: 1–14, 1994.

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