Influence of Chlorambucil, a Bifunctional Alkylating Agent, on DNA Replication and Histone Gene Expression in HeLa S₃ Cells

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ABSTRACT

We have examined the influence of chlorambucil, a bifunctional alkylating agent that inhibits cell proliferation, on DNA replication and histone gene expression in exponentially growing HeLa S₃ cells. During the period of treatment with chlorambucil (up to 3 days), neither transcription nor translation in general appeared to be affected, but the incorporation of [¹⁴C]thymidine into DNA was reduced to 15% of control values by the third day. The appearance of newly synthesized histones and non-histone proteins on chromatin was inhibited with a time course similar to that for inhibition of DNA synthesis. However, the representation of histone messenger RNA sequences in various cellular compartments did not appear to be affected by chlorambucil treatment, in contrast to the loss of histone messenger RNA sequences from polyribosomes following the more rapid inhibition of DNA and histone synthesis by 1-β-d-arabinofuranosylcytosine or hydroxyurea. The possibility is considered that chlorambucil interferes with histone gene expression at posttranscriptional or posttranslational levels. We also conclude that the inhibition of DNA synthesis by chlorambucil is most probably an indirect effect, a result of the inhibition of cells in the G₂ phase of the cell cycle.

INTRODUCTION

Bifunctional alkylating agents are used extensively in the chemotherapeutic treatment of neoplastic and autoimmune diseases. Once believed to exert their cytotoxicity primarily by preventing replication via cross-linking of the DNA helix (19), they have since been postulated instead to impair replication by inhibiting one of the cAMP² phosphodiesterases resulting in elevated intracellular levels of cAMP (48–50). It has been suggested that elevated levels of cAMP, which have been found in poorly replicating cells, affect replication in a manner not completely understood.

In either case, alkylation of intracellular molecules other than DNA does occur, resulting in impairment of key physiological processes and thus contributing to the cytotoxicity of alkylating agents. Alkylation affects respiration and glycolysis (reviewed in Ref. 55), and other metabolic processes such as transcription and translation can also be impaired. With regard to these parameters, considerable variation between different cell types is observed (11, 32, 54–56).

Transcription, if inhibited at all, is generally but not always impaired to a lesser extent than replication (56, 57). One might argue that the observed inhibition of transcription is caused by physical inactivation of the DNA template by cross-linking, but extensive cross-linking of DNA has not been found at physiologically significant concentrations of alkylating agent (reviewed in Ref. 56). It therefore is possible that it is not the alkylation of the nucleic acid fraction of chromatin that is responsible for inhibition of transcription but the nonnucleic acid components.

Chlorambucil, one of the alkylating agents, has been shown to interact preferentially with the protein fraction of chromatin (29). Chromosomal proteins are alkylated (29, 35) presumably via intra- and interpeptide linkages. Both the histone and the non-histone chromosomal proteins are alkylated. In addition, chromosomal proteins may be cross-linked to the DNA, as occurs during alkylation by nitrogen mustard (10). Not only are chromosomal proteins directly alkylated but their posttranslational modifications are impaired as well. For example, following treatment with alkylating agents, the phosphorylation of chromosomal proteins decreases (30). Alterations in posttranslational modifications of chromosomal proteins may have key functional significance, due to their involvement with transcriptional regulatory mechanisms (12, 14, 24, 33). Alkylation also has been reported to affect the synthesis of the chromosomal proteins. The synthesis of both the histone and non-histone chromosomal proteins is inhibited (27, 28, 58); the synthesis of histones, however, is more sensitive.

Because chromosomal proteins play an important role in cellular metabolism, further study of the influence of alkylating agents on their structural and functional properties is required. Chromosomal proteins contribute to the structural framework of chromatin via formation of nucleosomes (reviewed in Refs. 8 and 16), as well as to the higher-order arrangements of chromatin into chromosomes (1). In addition, chromosomal proteins appear to play a key role in gene regulation. The non-histone chromosomal proteins have been implicated in the transcriptional control of several gene sequences (2, 25, 38, 39, 41, 51–53). We have therefore examined the effect of the bifunctional alkylating agent, chlorambucil, on histone gene expression. Specifically, the representation of histone mRNA sequences in various intracellular compartments and the presence of newly synthesized histone proteins were studied in relation to DNA replication, since histone metabolism is greatly affected by the activity of the replication process. In addition, the expression of the histone genes is a cyclic phenomenon that occurs once each cell cycle (3, 4, 7, 9, 13, 26, 36–41, 43, 45), and might therefore be highly sensitive to inhibition by alkylation.

MATERIALS AND METHODS

Materials. Bovine serum albumin, S₁ nuclease, calf thymus histones, calf thymus DNA, Escherichia coli DNA, nucleoside phosphates.
triporphosphates, and *E. coli* tRNA were purchased from Sigma Chemical Co. (St. Louis, Mo.). NBT2 photographic emulsion was obtained from Eastman Kodak Co. (Rochester, N. Y.). [3H]Leucine (62 Ci/mmol), [3H]Juridine (27 Ci/mmol), [3H]thymidine (13 Ci/mmol), and Omnifluor were purchased from New England Nuclear (Boston, Mass.). [3H]Thymidine (57 Ci/mmol), [3H]dGTP (19 Ci/mmol), and [3H]dCTP (18 Ci/mmol), were obtained from Schwarz/Mann (Orangeburg, N. Y.). Reverse transcriptase was supplied by Dr. Joseph Beard (Life Sciences, Inc., St. Petersburg, Fla.). All other reagents, unless otherwise noted, were reagent grade.

**Cells.** HeLa S2 cells were grown at 37°C in suspension culture in Joklik-modified Eagle’s minimal medium supplemented with 7% (v/v) calf serum. When cells were grown in the presence of chlorambucil, the drug was added from a stock solution of 1 mg chlorambucil per ml in 95% ethanol:spinner salts (1:9). Chlorambucil was prepared immediately before addition to the cultures and was added daily. Cells grown in chlorambucil were not grown for more than 2 days without the addition of fresh medium or a complete change of medium. Cell densities were determined with a hemocytometer.

**DNA, RNA, and Protein Levels.** Unless otherwise stated, all steps were performed at 0-4°C. HeLa cells were washed twice with spinner salts, twice with 95% (v/v) ethanol, and once with 10% (w/v) trichloroacetic acid. RNA and DNA were solubilized as described by Schmidt and Thannhauser (34), and protein was solubilized in 0.1 N KOH. RNA was estimated by the orcinol reaction (23), with *E. coli* tRNA (50 µg/ml = 1 A260 unit) as the standard. The DNA was determined by the diphenylamine reaction (5), with calf thymus DNA (50 µg/ml = 1 A260 unit) as the standard, and protein was estimated by the method of Lowry et al. (21), with bovine serum albumin (E@280 = 6.67) as the standard.

**DNA, RNA, and Protein Synthesis.** In 5 ml of culture medium, 8 to 10 x 10⁸ HeLa cells were incubated for 30 min at 37°C with either 0.2 µCi [3H]thymidine, 5 µCi [3H]Juridine, or 4 µCi [3H]leucine per 5 ml. At the end of the incubation period, the cells were quickly centrifuged at 2000 x g for 5 min, washed twice with spinner salts, and washed once with 10% (w/v) trichloroacetic acid. The trichloroacetic acid precipitate was collected by centrifugation at 2000 x g for 5 min and dissolved in 500 µl of Protosol (New England Nuclear); 200 µl of the solubilized pellet were added to 3 ml of toluene:Omnifluor (1330:2500:168). Radioactivity was measured in a Beckman LS-230 scintillation counter.

**Autoradiography.** In 5 ml of culture medium, 8 to 10 x 10⁸ cells were incubated for 30 min at 37°C with 4 µCi of [3H]-thymidine. The cells were washed twice with spinner salts and suspended in a small volume of spinner salts:water (2:1). One drop of the cell suspension was placed on a clean slide, spread to a thin film, and allowed to dry. Autoradiography was performed as described by Kopriwa and Leblond (15), except that the NBT2 was diluted 1:2 with distilled water. Exposure was at room temperature for 3 days. The slides were developed for 2 min in D-19 photographic developer (Kodak) at room temperature, rinsed once with water, fixed in Kodak acid fixer for 5 min, and washed with running water for 10 min.

**Synthesis of Histones and Non-Histone Chromosomal Proteins.** In 10 ml of spinner salts:2% (v/v) fetal calf serum, 10⁸ cells were incubated with 50 µCi of [3H]leucine for 30 min at 37°C. Chromatin was isolated as described previously (41). Histones were extracted with 0.4 N H2SO4 and precipitated at -20°C by the addition of 3 volumes of ethanol. Precipitated histones were collected by centrifugation at 6000 x g for 30 min in a Beckman JA-20 rotor in a Beckman J-21 centrifuge, and dissolved in water. The amounts of histones and non-histone chromosomal proteins were estimated by the method of Lowry et al. (21), using calf thymus histones and bovine serum albumin, respectively, as standards. DNA levels were determined by the method of Burton (5) after DNA was extracted from the chromatim by heating at 90°C for 20 min in 5% trichloroacetic acid. Aliquots of the protein samples were added to 3 ml of Triton:toluene:Omnifluor (1330:2500:168) scintillation fluid, and radioactivity was estimated in a Beckman LS-230 liquid scintillation counter.

**Fractionation of Cells.** HeLa cells were washed twice in spinner salts and suspended in 1.5 mM MgCl2:10 mM NaCl:10 mM Tris-HCl (pH 7.0) for 20 min. The swollen cells were homogenized with a Dounce homogenizer (tight-fitting B pestle), and centrifuged at 10,000 x g for 15 min in a Beckman JA-20 rotor. The nuclei in the pellet material were further purified by 3 washes with 80 mM NaCl:20 mM EDTA:1% Triton (pH 7.2), and 2 washes with 150 mM NaCl:10 mM Tris (pH 7.2). The 10,000 x g supernatant was centrifuged at 100,000 x g for 90 min in a Beckman 60Ti rotor in a Beckman LS-65 centrifuge. The pelleted material constituted the polysomal fraction and the supernatant, the postpolyosomal fraction.

**Extraction of RNA.** RNA was extracted from the subcellular fractions with phenol and chloroform:isoamyl alcohol, as described previously (40).

**Isolation of Poly(A)⁺ Polysomal RNA.** Polysomal RNA was suspended in 10 mM Tris-HCl (pH 7.5):0.5 mM KCl, and applied to a 0.5- x 3-cm oligo(dG)·oligo(dC) cellulose (Collaborative Research) column previously washed with 1 volume of 0.1 N KOH, 2 volumes of water, and 20 volumes of 10 mM Tris-HCl (pH 7.5):0.5 mM KCl. RNA bound to the column was extensively washed with 10 mM Tris-HCl:0.5 mM KCl until adventitiously bound RNA ceased to be eluted. The poly(A)⁺ RNA was then eluted with 10 mM Tris-HCl (pH 7.5).

**Synthesis of cDNA.** Poly(A)⁺ RNA (5 µg) was incubated for 15 min at 46°C in 200 µl of 50 mM Tris-HCl (pH 8.3):20 mM 2-mercaptoethanol:9 mM MgCl2:30 mM NaCl:50 µM dCTP:50 µM dGTP, 50 µCi [3H]dGTP:200 µM dATP:200 µM dTTP, oligo(dG)·oligo(dC) (Pierce) (20 µg/ml), actinomycin D (20 µg/ml), and reverse transcriptase (220 units/ml). The reaction was stopped by the addition of 0.5% volume of 10% (w/v) sodium dodecyl sulfate, and 20 µg of *E. coli* DNA were then added. The cDNA was extracted twice with 1 volume of phenol and 1 volume of chloroform:isoamyl alcohol (24:1). NaOH was added to a final concentration of 0.33 N, and the sample was incubated at 37°C for 48 hr. The sample was neutralized with HCl and chromatographed on a Sephadex G-50 column (2 x 10 cm) with 1 ml 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.0). The void volume fractions containing the cDNA were pooled.

The size of poly(A)⁺ cDNA was estimated by sucrose gradient sedimentation (46). One volume of 1 N NaOH and 1 volume of 1 M NaCl were added to a small aliquot of cDNA, which was incubated at 37°C for 15 min and then centrifuged for 7 hr at 100,000 x g on a 5 to 20% sucrose gradient in 0.1 M...
The isolation of histone H4 mRNA (20), the addition of AMP residues to the 3' hydroxy terminus (47), and the synthesis of H4 histone cDNA has been described previously (6). Histone H4 cDNA was estimated to be 35 to 45% full-length copy, as determined by electrophoresis under denaturing conditions.

Hybridization of cDNA's with HeLa Cell RNA. Hybridization was carried out at 43° with the total polysomal poly(A) cDNA, and at 58° with the histone H4 cDNA in 15-μl capillary tubes containing 50% formamide:500 mM NaCl:1 mM EDTA:25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.0), variable amounts of RNA, and 150 pg of poly(A) cDNA or 17 pg of H4 cDNA. Samples were boiled for 5 min prior to hybridization. Hybrid formation was measured after digestion with single-stranded specific S1 nuclease (40) and precipitation with trichloroacetic acid.

RESULTS

Inhibition of Cell Growth. Initially, we examined the influence of chlorambucil on cell proliferation and on macromolecular biosynthesis in general in exponentially growing HeLa S3 cells. Treatment of exponentially growing HeLa S3 cells with chlorambucil at a concentration of 10 μg/ml resulted in inhibited population growth (Chart 1). During the initial 24 hr of growth in chlorambucil, the HeLa S3 cell density increased at a rate approximately 30% of that of cells not grown in chlorambucil. Thereafter, population growth ceased, although in some experiments growth continued for another day, but at an even slower rate (data not shown). In all experiments, the cell density underwent decline by the third day.

Concomitant with the cessation of population growth and subsequent decline, HeLa S3 cells underwent cytological changes which were apparent by phase contrast microscopy. By the third day, some cells still appeared normal in size and shape. A few of these cells apparently were capable of proliferating, as evidenced by the presence of bilobular structures associated with the terminal stages of cytokinesis. However, the majority of cells grown in chlorambucil appeared normal in shape, although they were larger than untreated cells. The diameter of these affected cells was approximately 2 to 3 times greater than normal. Visible in some of these enlarged cells was an extremely large nucleus. Regardless of size, all cells appeared to be viable, at least as judged by their ability to exclude trypan blue. Only the irregular, amorphous cellular remnants which were evident on the third day were stained by trypan blue. These trypan blue-staining remnants were at no time scored as cells when cell densities were determined. However, it should be emphasized that, despite these reservations, all data obtained from cells grown more than 48 hr in chlorambucil must be interpreted with extreme caution.

Synthesis and Accumulation of Macromolecules. The increase in cell volume of chlorambucil-treated cells can be attributed to cell growth without concomitant cytokinesis. The average amount of DNA per cell increased by 30% during the first 2 days of growth in chlorambucil (Chart 2). RNA and protein levels per cell increased to a slightly greater extent, RNA by 60% and protein by 55%. The apparent decrease in the levels of DNA, RNA, and protein by the third day may represent an overcorrection for material contributed by cellular debris. In correcting for cellular debris, we assumed that cell division in the treated cultures was negligible after the second day and that debris recovered with intact cells by low-speed centrifugation contained all of the macromolecules from any cells that lysed. Undoubtedly, some of the macromolecules did go into solution and were lost. This assumption, therefore, leads to an underestimation of the macromolecular content of cells treated for 3 days with chlorambucil. No corrections were needed for DNA, RNA, and protein content of cells during the first 2 days of treatment because of negligible cell lysis.

Treatment of HeLa cells with chlorambucil resulted in a progressive decrease in the incorporation of [3H]thymidine into DNA (Chart 3). By the third day of chlorambucil treatment, the rate of thymidine incorporation was 14% of that observed in untreated cells. This observed inhibition of [3H]thymidine incorporation was probably not due to the inability of the DNA precursor to enter the cell, since intracellular levels of acid-soluble [3H]thymidine were elevated approximately 2-fold during the first day of growth in chlorambucil and remained constant thereafter. However, the possibility must be considered
that the observed inhibition of \(^{14}\text{C}\)thymidine incorporation could be caused by dilution of the labeled precursor by elevated intracellular concentrations of unlabeled thymidine and that the rate of DNA synthesis may actually not be affected. However, the cell population does not increase after 24 hr of chlorambucil treatment (Chart 1), and the DNA level does not increase by more than 30% of that found in untreated, exponentially growing cells (Chart 2). It therefore appears that DNA synthesis in HeLa cells is impaired following chlorambucil treatment.

Perhaps the most straightforward interpretation of our results is that chlorambucil arrests HeLa cells in the G2 period of the cell cycle, and that the inhibition of DNA synthesis by the alkylating agent is an indirect effect. A G2 block is supported by the relatively slow and progressive time course of DNA synthesis inhibition and by the measured amounts of DNA per cell prior to and following chlorambucil treatment. The average DNA content of exponentially growing HeLa cells is 15 pg/cell and increases to 20 pg/cell after 2 days of chlorambucil treatment, a value twice that observed in G0, HeLa cells.

Although DNA synthesis is inhibited by chlorambucil, directly or indirectly, it is not inhibited with the same kinetics in all cells. The number of cells which were very active in DNA synthesis (>44 grains/cell), as determined by \(^{3}\text{H}\)thymidine labeling and autoradiography, decreased during growth in chlorambucil, but some DNA synthesis was still evident after 3 days of drug treatment (Chart 4). The DNA-synthetic capability, as measured by grain density in the autoradiographic film above the cells, was shifted to low to intermediate levels by the first day of growth in chlorambucil, and by the second day poorly replicating cells (5 to 19 grains/cell) began to accumulate. The decrease in the number of extremely poorly replicating cells (5 to 9 grains/cell) for the third day of alkylation was probably due to the narrow range of cells which were scored (the threshold was increased from 5 to 8 grains/cell for enlarged cells, which were in the majority). Our data are therefore consistent with a population of HeLa cells, heterogeneous with respect to sensitivity of DNA replication to chlorambucil. We cannot rule out the possibility that the very sensitive cells, which we refer to as poorly replicating, have in fact experienced a complete inhibition of DNA synthesis and that the activity inferred from the autoradiographs is due solely to an attempt of these cells to repair DNA damage induced by alkylation. In this case, chlorambucil would act much more rapidly than inferred from the data and could act on the entire cell population during one division cycle.

The increase during chlorambucil treatment in the amount of RNA per cell was apparently not due entirely to an increased rate of transcription. Transcription, as assayed by incorporation of \(^{3}\text{H}\)uridine into intact cells, increased by 17% during the first day of chlorambucil treatment, and remained at that elevated level for 2 days (Chart 3). While the increased \(^{3}\text{H}\)uridine incorporation can be construed to represent an increased rate of RNA synthesis, it may actually reflect an increased uptake of the labeled precursor. An increase in the amount of \(^{3}\text{H}\)uridine in the acid-soluble intracellular pool coincides with the observed increase in RNA synthesis (Chart 3). Thus, it appears that transcription in general may not, at least in a quantitative sense, be affected by chlorambucil. The possibility must therefore be considered that the increased RNA content per cell may reflect changes in stability of cellular RNA's or may simply be a reflection of an accumulation of cells in the G2 phase of the cell cycle.

Similar results were observed for protein synthesis in chlorambucil-treated cells. It appeared that incorporation of \(^{3}\text{H}\)leucine into intact cells increased by 100% during the first 3

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**Chart 3.** Synthesis of DNA, RNA, and protein in HeLa S3 cells grown in the presence or absence of chlorambucil. The incorporation of \(^{1}\text{C}\)thymidine (\(^{1}\text{C}\)Tdr), \(^{3}\text{H}\)uridine, and \(^{3}\text{H}\)leucine into their respective products was performed as described in "Materials and Methods." Points, averages of 5 to 6 individual experiments, each in duplicate, based on cpm/cell. Bars, 95% confidence limit of the mean. Top, incorporation into acid-insoluble material; bottom, incorporation into acid-soluble material. The incorporation into cells not grown in chlorambucil was set as 100%.

**Chart 4.** Autoradiographic analysis of control and chlorambucil-treated HeLa S3 cells following labeling with \(^{3}\text{H}\)thymidine. HeLa S3 cells, treated with chlorambucil for the indicated lengths of time, were briefly labeled with \(^{3}\text{H}\)thymidine and autoradiographed. The number of silver grains above labeled cells was determined by bright-field microscopy. For each sample, 150 labeled cells were examined.
days of chlorambucil treatment (Chart 3). However, a comparable increase in the amount of [3H]leucine in the acid-soluble intracellular pool was found during this period. Our results therefore suggest that protein synthesis in general is also quantitatively unaffected by chlorambucil treatment.

**Synthesis of Chromosomal Proteins.** Since the synthesis of histones is tightly coupled with DNA replication in many systems, we examined whether histone synthesis was affected by chlorambucil. Although the synthesis of most non-histone chromosomal proteins is not as tightly coupled to DNA replication (44), we have also examined their synthesis for comparison. In agreement with the data of Riches and Harrap (27, 28), the appearance of newly synthesized histones and non-histone chromosomal proteins on chromatin was depressed in chlorambucil-treated compared with control cells (Chart 5). Histone synthesis during the first 2 days was more sensitive to chlorambucil than was the synthesis of the non-histone chromosomal proteins. By the third day, however, both classes of chromosomal proteins were inhibited nearly equally, approximately 70% [this may be a conservative estimate, since the intracellular level of [3H]leucine increases following chlorambucil treatment (Chart 3)]. It should be noted that the time courses for chlorambucil-induced alterations in recruitment of chromosomal proteins and in DNA synthesis are approximately comparable. Thus, the coupling of DNA and histone synthesis found in HeLa cells does not appear to be altered by chlorambucil.

As shown in Chart 6, the amount of non-histone chromosomal protein associated with DNA (µg/µg DNA) increased substantially during chlorambucil treatment. In contrast, a limited increase in the accumulation of histones was observed, comparable to the reduced level of DNA synthesis.

**Metabolism of Histone H4 mRNA.** To further examine the influence of chlorambucil on histone gene expression, we have assessed the representation of histone mRNA sequences in various intracellular compartments in exponentially growing HeLa cells prior to and following chlorambucil treatment. RNA’s were isolated from the nucleus, polysomes, and postpolysomal cytoplasm of control and chlorambucil-treated cells, and hybridized in RNA excess to 3H-labeled H4 histone cDNA. As shown in Chart 7, the kinetics of hybridization of H4 histone cDNA with the 3 cellular RNA fractions was similar in chlorambucil-treated and control cells. This result suggests that there was neither an accumulation nor depletion during the course of drug treatment of H4 histone mRNA with respect to the non-H4 coding RNA. It therefore appears that neither the association of H4 histone mRNA with the polysomes nor the stability of H4 histone mRNA in the other cellular fractions was similar in chlorambucil-treated and control cells. This result suggests that there was neither an accumulation nor depletion during the course of drug treatment of H4 histone mRNA with respect to the non-H4 coding RNA. It therefore appears that neither the association of H4 histone mRNA with the polysomes nor the stability of H4 histone mRNA in the other cellular fractions was affected by chlorambucil during a period when significant effects on histone synthesis and DNA replication were observed. In contrast, when cells are treated with 1-β-D-arabinofuranosylcytosine or hydroxyurea, the rapid inhibition (within 30 min) of histone synthesis and DNA replication is accompanied by a loss of histone mRNA from the polyribosomes (42).

**Metabolism of Poly(A)+ mRNA.** Our inability to detect a
We have examined the influence of chlorambucil, a bifunctional alkylating agent, on DNA replication and histone gene expression in exponentially growing HeLa S3 cells. While the overall levels of RNA and protein synthesis did not appear to be altered by treatment with chlorambucil for periods of up to 3 days, there was an effect on DNA replication. DNA synthesis in HeLa cell cultures did not cease immediately upon addition of chlorambucil, but decreased progressively over a period of three days, while at the same time the average amount of DNA per cell increased to a G2 level (20 pg/cell). Our data are compatible with the hypothesis that chlorambucil does not directly inhibit DNA synthesis but that individual cells complete DNA synthesis in the presence of chlorambucil and are then arrested at some point in G2. Such an interpretation would be in agreement with the observations of Roberts (31). Interestingly, it has been reported that other alkylating agents also arrest cells in the G2 phase of the cell cycle (56).

Concomitant with the effect of chlorambucil on DNA synthesis was the apparent inhibition of the synthesis of the protein components of chromatin, as measured by the appearance of newly synthesized histone and non-histone protein on chromatin. It was expected that the synthesis of histones would be depressed, since in HeLa cells their synthesis is coupled with DNA replication. In these cells, it has been reported that histone mRNA’s are synthesized and translated only during the S phase of the cell cycle (3, 4, 7, 9, 13, 26, 36–41, 43, 45). As the cells reach the end of S phase, fewer histone mRNA’s are found on the polyribosomes (4), and during G2 there are few if any histone mRNA sequences in the nucleus or cytoplasm (4, 37). When DNA synthesis is inhibited by 1-β-D-arabinofuranosylcytosine or hydroxyurea, histone mRNA’s are lost from the polyribosomes, although it remains to be established whether, under these circumstances, histone mRNA’s are displaced from the polyribosomes or degraded in situ. Our data suggest that, although chlorambucil appears to block HeLa cells in the G2 phase of the cell cycle, the representation of H4 histone mRNA sequences in the nucleus and cytoplasm remains elevated, thus differing from the situation which normally occurs during the S-G2 transition and during inhibition of DNA synthesis by 1-β-D-arabinofuranosylcytosine or hydroxyurea. The same relative proportion of histone H4 mRNA to total polysomal RNA was found in control cultures and in those treated with chlorambucil for up to 48 hr. One interpretation of these results is that the chlorambucil-treated cell population is in G2 with respect to DNA content but that other biochemical events normally associated with G2 are not occurring. It is also reasonable to suspect that the mechanism by which chlorambucil brings about a perturbation in histone gene expression is not that which is operative following treatment with specific inhibitors of DNA synthesis. However, it is also possible that chlorambucil has some specific effect on transcription or stability of histone mRNA sequences.

It cannot be determined from the hybridization analysis whether or not the H4 mRNA’s on the polysomes in chlorambucil-treated cells are being actively translated. If the histone mRNA’s are inactivated in some way but remain on the polysomes, the inhibition of the appearance of newly synthesized histones on chromatin might be explained. It is doubtful that the histone mRNA’s are inactivated by cross-linking to the ribosomes, since translation in general is inhibited only minimally, if at all.

The possibility should be considered that the histone mRNA’s on the polyribosomes of chlorambucil-treated cells are still active but that the appearance of newly synthesized histones on chromatin is blocked. A decrease in the presence of newly synthesized histones associated with DNA in chlorambucil-treated cells may be due to the absence of newly synthesized examples of an effect of the chlorambucil-induced alteration in the representation of H4 histone mRNA sequences in the nucleus or cytoplasm of exponentially growing HeLa S3 cells was paralleled by the absence of an effect of the alkylating agent on poly(A)+ mRNA sequences (Chart 8). The representation of the poly(A)+ mRNA sequences in the nucleus and cytoplasm, as measured by RNA excess hybridization with homologous 3H-labeled poly(A)+ cDNA, was taken to reflect the total cellular mRNA population. The lack of a chlorambucil-induced effect on poly(A)+ mRNA sequences is consistent with essentially unaltered levels of incorporation of [3H]uridine into RNA’s in intact cells.

Taken together, these results suggest that chlorambucil does not quantitatively or qualitatively influence transcription in HeLa S3 cells.

**DISCUSSION**

We have examined the influence of chlorambucil, a bifunctional alkylating agent, on DNA replication and histone gene expression in exponentially growing HeLa S3 cells. While the overall levels of RNA and protein synthesis did not appear to be altered by treatment with chlorambucil for periods of up to 3 days, there was an effect on DNA replication. DNA synthesis in HeLa cell cultures did not cease immediately upon addition of chlorambucil, but decreased progressively over a period of...
DNA to serve as binding sites. The specific activity of the histone fraction relative to that of untreated cells would therefore decrease, since the histone:DNA ratio is the same in chromatin of treated and untreated cells. Alternatively, the increased amount of non-histone chromosomal proteins which binds to DNA during chlorambucil treatment (Chart 8; Ref. 28) may prevent binding of histones to chromatin, thus blocking an accumulation of histones beyond the level observed in non-drug-treated cells. Both of these models would predict an accumulation of histones in the nucleoplasm and/or cytoplasm during chlorambucil treatment of HeLa cells. We were unable to detect such a pool of histones by electrophoretic analysis of the nucleoplasmic and cytoplasmic proteins of chlorambucil-treated cells. Thus, whether or not histone mRNA sequences are translated remains an open-ended question. The possibility should also be considered that chlorambucil blocks posttranslational modifications of histones required for their interactions with the genome.

Our results suggest that transcription in general is not significantly altered, quantitatively or qualitatively, during the first 2 days of growth in the presence of chlorambucil. Data from pulse-labeling experiments with $[^3H]$uridine, which take the levels of acid-soluble radioactivity into account, suggest that chlorambucil does not modify the rate of RNA synthesis. Hybridization analysis of RNA's, using $[^3H]$-labeled poly(A)$^+$ cDNA as a probe, suggests that the representation of these RNA sequences is not changed in the nucleus or cytoplasm of chlorambucil-treated cells. The hybridization kinetics suggests that the relative amounts of the various RNA sequences are not affected by chlorambucil treatment, and the levels of hybridization suggest that all sequences represented in the DNA probe are found in both control and chlorambucil-treated cells.

It is reasonable to conclude from these results that genetic sequences transcribed prior to chlorambucil treatment are transcribed following treatment with the alkylating agent. However, we cannot eliminate the possibility that chlorambucil treatment renders sequences available for transcription which were not transcribed prior to drug treatment.

In conclusion, our results suggest that chlorambucil affects cell proliferation, DNA replication, and histone gene expression in HeLa $S_2$ cells. While the inhibition of DNA replication does not appear to be direct, the manner in which histone gene expression is altered by the alkylating agent remains to be resolved. Because of the involvement of histones with the structural and functional properties of the genome and the coupling of histone synthesis with DNA replication in many systems, understanding the mechanism by which chlorambucil modifies the expression of histone genes may be important. Additional information regarding the mechanisms of chlorambucil action at the cellular, biochemical, and molecular levels can be derived from further experiments using synchronized cell populations.

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