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Characterization of cell cycle changes in polyamine-depleted HeLa cells

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Abstract
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DFMO reacts catalytically with and irreversibly inhibits the enzyme ornithine decarboxylase (ODC) thereby preventing the biosynthesis of the polyamines putrescine, spermidine, and spermine.

The depletion of polyamines in HeLa cells inhibits cellular processes such as DNA synthesis and proliferation. These processes could be reversed by the addition of spermidine 10-12 hours before S-phase DNA synthesis was initiated.

Nuclear reconstitution procedures were utilized to determine the location of the deficiency in polyamine-depleted cell nuclei. It was shown that the nuclear salt extract from polyamine-depleted cells was slightly deficient, whereas the salt-extracted nuclear template could not support DNA synthesis.

Nuclear salt extracts (NSE's) were assayed for DNA polymerase alpha activity. Results showed that polyamine-depleted cell nuclei contained only $\sim 50\%$ as much DNA polymerase alpha activity found in control cell nuclei. This deficiency could also be reversed by the addition of spermidine to polyamine-depleted HeLa cells 10-12 hours before S-phase DNA synthesis was initiated.

Since the activity in NSE's obtained from polyamine-depleted cell nuclei reacted similarly with various DNA templates, it appears that a quantitative rather than a qualitative deficiency exists between the DNA polymerase alpha of control and polyamine-depleted HeLa cell nuclei.

Salt-extracted whole cell lysates (LSE's) were assayed for total DNA polymerase alpha activity. Results indicate that LSE's from polyamine-depleted cells contained levels of DNA polymerase alpha activity comparable to control cell LSE's. These results indicated that a transport problem may exist which prevents DNA polymerase alpha from entering, or allows DNA polymerase alpha to exit freely from the nucleus in polyamine-depleted cells.

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Proliferation studies provided no evidence of the cells being synchronized via the DFMO block. This suggests that the polyamine deficiency does not block cells at a specific control site in the cell cycle. The cumulative evidence in this study indicates that polyamines are essential at several stages of cell cycle progression.

Keywords
Chemistry, Biochemistry

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Characterization of cell cycle changes in polyamine-depleted HeLa cells

Koza, Robert Anthony, Ph.D.

University of New Hampshire, 1989
CHARACTERIZATION OF CELL CYCLE CHANGES IN POLYAMINE-DEPLETED HELa CELLS

By

Robert A. Koza
B.A., North Adams State College, 1984
M.S., University of New Hampshire, 1986

DISSERTATION

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the Requirements for the Degree of

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in
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"...What a long strange trip it's been"
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Proliferation studies provided no evidence of the cells being synchronized via the DFMO block. This suggests that the polyamine deficiency does not block cells at a specific control site in the cell cycle. The cumulative evidence in this study indicates that polyamines are essential at several stages of cell cycle progression.
I. LITERATURE REVIEW

Introduction

The first documented evidence of the existence of polyamines came over 300 years ago when Antoni Van Leeuwenhoek described microscopic crystal structures in human semen. Over 200 years later, these structures were identified as phosphate salts of an organic base by P. Scheiner. Shortly thereafter, Ladenburg and Abel appropriately named this compound "spermidine" (1, 2, 3).

Scientific interest in polyamines was renewed in 1948 when Herbst and Snell showed that the diamine putrescine was required for the growth of Haemophilus parainfluenzae (4, 5). Since that time, much research has been undertaken to learn more about the essential physiological roles of polyamines in cellular processes.

The most commonly found polyamines in eukaryotic cells are the diamine putrescine \([\text{NH}_2(\text{CH}_2)_4\text{NH}_2]\), the triamine spermidine \([\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}_2]\), and the tetraamine spermine \([\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}_2]\). Another common polyamine, the diamine cadaverine \([\text{NH}_2(\text{CH}_2)_5\text{NH}_2]\), seems to be biologically insignificant as compared with the other polyamines found in eukaryotic cells.

In this literature review, I will briefly summarize the
biosynthesis and regulation of polyamine concentrations in mammalian systems. I will also review research which has been done to establish the biological significance of polyamines in living organisms.

**Polyamine Biosynthesis and Regulation**

Polyamine biosynthesis and the regulation of polyamine biosynthesis is described in several excellent review articles (6-9). Since animals, plants, and bacteria produce polyamines by slightly modified biosynthetic pathways, I will discuss those which are pertinent to the work I have performed on mammalian cell systems.

The only polyamine biosynthetic pathway found in mammalian cells is that from ornithine to putrescine via the rate limiting enzyme ornithine decarboxylase (ODC). Ornithine, a byproduct of the urea cycle, is produced from the conversion of arginine to ornithine by the enzyme arginase. Unlike most urea cycle enzymes, arginase is found in many extrahepatic tissues, thus insuring the availability of ornithine for polyamine biosynthesis (6). It is also interesting to note that arginase activity has been found to correlate closely with spermidine and spermine concentrations (10).

ODC is a pyridoxal phosphate-dependent enzyme, has an extremely short half-life (10-30 min.), and is highly acidic. Interestingly, there is some evidence which
indicates that a correlation may exist between protein acidity and rate of degradation. Although ODC activity is very low in non-growing cells, it can be stimulated rapidly and many-fold by hormones, growth factors, and other agents that stimulate cell growth and proliferation. Because of the need for a cellular system to rapidly induce or inhibit the biosynthesis of polyamines, many mechanisms have been suggested for controlling ODC activity. Some of these include post-translational modifications such as phosphorylation (12, 13), polyamine-induced production of antizymes (14, 15), polyamine-dependent control of transcription and/or stability of ODC mRNA (16, 17), and polyamine-mediated translational control of ODC mRNA (18-21).

Putrescine can be converted to spermidine and finally to spermine by subsequent additions of aminopropyl groups [-\((\text{CH}_2)_3\text{NH}_2]\) from decarboxylated S-adenosylmethionine. These transfer reactions are catalyzed by spermidine synthase and spermine synthase respectively. Decarboxylated S-adenosylmethionine is synthesized from S-adenosylmethionine via the enzyme S-adenosylmethionine decarboxylase.

Spermine and spermidine can be interconverted to spermidine and putrescine by the enzymes spermidine-N\(^1\)-acetyltransferase and polyamine oxidase (8). Spermidine-N\(^1\)-acetyltransferase uses acetyl coenzyme A to acetylate both spermine and spermidine to produce the products N\(^1\)-acetyl spermine and N\(^1\)-acetyl spermidine respectively. Polyamine oxidase then cleaves off an N-acetylpropionaldehyde group to
produce spermidine and putrescine. Figure 1 shows a summary of the biosynthesis and interconversion of polyamines in mammalian cells.

The degradation of putrescine can occur by two pathways. It can be oxidized via diamine oxidase to produce γ-amino-butyraldehyde, or it can be acetylated by a microsomal enzyme and then oxidized by the enzyme monoamine oxidase. Although both of these pathways can eventually lead to the synthesis of the neurotransmitter γ-aminobutyrate (GABA), the latter pathway is especially important in the brain where very small amounts of diamine oxidase are present (22).

**Interactions of Polyamines with Macromolecules**

Since the polyamines spermine, spermidine, and putrescine are polycationic in nature, it has been suggested that they may associate with polyanionic molecules such as nucleic acids.

Mandel (23) showed that the denaturation temperature (Tm) of DNA increased in the presence of spermine. This suggested that spermine may play a role in stabilizing the DNA structure by binding to its negatively charged phosphodiester backbone. Liquori et al. (24) proposed that polyamines formed a complex with DNA based on results of X-ray analysis of crystallized spermine hydrochloride. It was postulated that this binding occurred in the narrow or minor
FIGURE 1

SUMMARY OF POLYAMINE BIOSYNTHESIS AND INTERCONVERSION IN MAMMALIAN CELLS

Arginine
1 → Urea
Ornithine
2 → CO₂
Putrescine

Decarboxylated S-adenosylmethionine
3 → CO₂
5'-Methylthioadenosine

S-adenosylmethionine
3 → CO₂

Decarboxylated S-adenosylmethionine
5 → 5'-Methylthioadenosine

Arginase
2. Ornithine decarboxylase
3. S-adenosylmethionine decarboxylase
4. Spermidine synthase
5. Spermine synthase
6. Spermidine-N1-acetyltransferase
7. Polyamine oxidase

Spermidine

N₁-acetylspermidine
6

Spermine

N₁-acetylspermine
6

5
groove of DNA because of the various electrostatic and hydrophobic forces involved.

After studying X-ray analysis of DNA-polyamine complexes, Suwalsky et al. (25) suggested that a combination of several models existed for the binding of polyamines to DNA. It was proposed that polyamines bind to both the narrow and the major grooves of DNA. Further support for this was provided by Drew et al. (26), who used electron density mapping techniques to show that the major groove of the dodecamer d(CpGpCpGpApApTpTpCpGpCpGp) binds a single spermine molecule. It was proposed that the two [-NH$_3^+$] groups of spermine formed salt links with phosphate oxygen atoms while one of the two interior [-NH$_2^+$] groups forms a hydrogen bond with the O-6 of a guanine residue. Wemmer et al. (27) also studied the binding of spermine to d(CpGpCpGpApApTpTpCpGpCpGp) and suggested that this polyamine-DNA interaction is extremely short-lived under physiological conditions. It was suggested however, that a complex containing DNA, polyamine, and an associated protein may be relatively stable.

Baeza et al. (28) used electron microscopic techniques to demonstrate that DNA can be condensed or compacted in the presence of spermidine. This compacted DNA was found to be resistant to DNase I, and exhibited higher transcriptional activity than naked DNA when using E. coli DNA-dependent RNA polymerase. In view of these findings, Srivenugopal et al. (29) used spermidine analogs with the structure
\[
\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_x\text{NH}_2, \text{ where } X=5-8 (X=4 \text{ for spermidine}), \text{ to aggregate negatively supercoiled Col E1 DNA. } ^1\text{H} \text{ and } ^{31}\text{P-NMR spectroscopic studies indicated that the spermidine analogs formed much less rigid DNA aggregates than those formed by spermidine.}
\]

A study by Behe et al. (30) showed that only micromolar concentrations of spermine and spermidine were needed to change the oligonucleotide (dm\(^5\)C-G) from a right-handed B-configuration to a left-handed Z-configuration. Thomas and Kiang (31) found that the presence of 150 \text{uM spermidine increased the binding of estrogen receptor to the oligonucleotide sequences poly(dA-dC).poly(dG-dT), poly(dA-dT).poly(dA-dT), and to calf thymus DNA 22-fold, 3-fold, and 6-fold respectively. It was proposed that this increase in binding affinity resulted because spermidine facilitated the conversion of certain polynucleotide sequences to the Z-DNA form.}

Several investigators have shown that polyamines may act to stabilize RNA as well as DNA. Early studies by Colburn et al. (32) showed that spermine and Mg\(^{++}\) were both necessary to stabilize functional 70S ribosomes of \textit{E. coli}. Further evidence by Raina and Teleranta (33) showed that the concentration of cations in the medium was very important in allowing rat liver microsomal particles to take up and release polyamines. It was also shown that the distribution of polyamines in the cell closely correlated with RNA distribution. Therefore, it was suggested that RNA-
containing structures may be stabilized by polyamines. Stevens and McCann (34) provided further evidence of polyamine-RNA interactions by showing that almost 98% of the intracellular spermine of *Bacillus stearothermophilus* was bound to ribosomes and mRNA.

Balton and Kearns (35) used NMR techniques to show that yeast tRNA\(^{Phe}\) became much more stable when in the presence of spermine or spermidine. They also showed that approximately 3 molecules of spermine or spermidine were needed to stabilize each tRNA molecule. However, the diamines putrescine and cadaverine were found to be ineffective in stabilizing the tRNA\(^{Phe}\) structure. Another study by Nothig-Laslo et al. (36) involving yeast tRNA\(^{TYr}\), suggested that spermine and spermidine exhibited their stabilizing effects by binding to the anti-codon loop of the tRNA molecule.

Glaser and Gabbay (37) used the synthetic double-stranded RNA polymers rA-rU and rI-rC to show stabilizing effects by spermine and spermidine. However, when polyamine derivatives containing hydrophobic substituents were used, the stabilizing effects were greatly diminished.

Polyamines have been implicated by many investigators to be essential for normal DNA synthesis and replication. Much of this research has been directed at either the stimulation of DNA polymerase activity by polyamines and/or the polyamine activation of the DNA template to allow DNA synthesis to occur.

An early study by Brewer and Rusch (38) showed that the
Addition of $5.7 \times 10^{-4}$ M spermine to a preparation of nuclei isolated from *Physarum polycephalum* stimulated DNA synthesis many-fold. It was proposed that this stimulation occurred because spermine activated the DNA polymerase present in the nuclei. Wichner et al. (39) demonstrated that the addition of spermidine to a complex containing *E. coli* DNA polymerase III, a copolymerase, and a primer fragment caused a 10-fold increase in DNA replication.

Another study by Schekman et al. (40) showed that both spermidine and DNA unwinding protein were necessary for the DNA polymerase III holoenzyme to replicate φX174 single-stranded circular DNA. However, either the DNA unwinding protein or spermidine was required to replicate DNA from the bacteriophages M13 and G4. These results suggested that the DNA unwinding protein and spermidine acted on the DNA at different stages of DNA synthesis initiation and/or elongation.

Yoshida et al. (41) isolated DNA polymerase alpha and beta from calf-thymus DNA and investigated the effect of polyamines on polymerase activity. In DNA replication systems containing either natural or synthetic single-stranded DNA, it was shown that DNA polymerase alpha and beta could be stimulated to the same extent by the addition of 0.5-1.0 mM spermidine or 2-10 mM spermine. However, polyamine derivatives with altered terminal amino groups had no stimulatory effects on these polymerases. This suggested that stimulatory effects by polyamines on DNA polymerases
may be due to specific interactions between the polyamine terminal amino groups and the DNA template.

By studying the kinetics involved in the stimulation of KB cell DNA polymerase alpha by polyamines, Fischer and Korn (42) suggested that polyamines may destabilize the polymerase-nucleic acid interaction at the polymerase-primer terminus. It was shown that when DNA concentrations were low, spermidine had both stimulatory and inhibitory effects. This could be due either to a decrease in non-specific binding of the polymerase on areas of the DNA distant from the 3'-OH terminus, or by causing a general destabilization of the polymerase-DNA complex thus allowing both the forward and reverse reactions to occur more readily. The second possibility was favored because it had been shown that polyamines did not affect the binding of DNA polymerase alpha to a single strand of DNA, but did affect the interaction between DNA polymerase alpha with competing DNA molecules.

Finally, Geiger and Morris (43) studied the rate of DNA replication fork movement in polyamine-deficient E. coli cells by using DNA autoradiographic techniques. It was shown that polyamine-deficient cells exhibited only one-half of the rate of replication as compared with polyamine-supplemented cells. Also, if spermidine was added to the polyamine-deficient cells, the rate of DNA synthesis was increased. When homologs of spermidine ([NH₂(CH₂)₃NH(CH₂)ₓNH₂] where x=5-8) were added to the
polyamine-deficient cells, some stimulation of DNA replication was seen when $X=5$, but not when $X=6-8$. This suggests that the triamine spermidine was specifically required for normal replication fork movement.

Polyamines have been shown to play a significant role in DNA transcription reactions. Both in vitro and in vivo studies have shown that polyamines may affect RNA synthesis either by stabilizing the DNA-DNA-dependent RNA polymerase-RNA complex, or by regulating the activity of DNA-dependent RNA polymerases.

Janne et al. (44), after isolating DNA-dependent RNA polymerases I and II from pig nuclei, performed in vitro assays using double-stranded DNA to determine whether polyamines could modulate RNA polymerase activity. RNA polymerases I and II were found to be stimulated with 2.5 mM and 5-10 mM spermidine, or 0.5-2.0 mM and 1-5 mM spermine respectively. Since it was shown by sucrose density gradients that the polyamine-supplemented reactions produced a greater amount of long RNA transcripts, it was proposed that polyamines stimulated the elongation of RNA by stabilizing the transcription complex of DNA, DNA-dependent RNA polymerase, and RNA rather than by increasing the rate of initiation for transcription.

Another in vitro experiment by Nuss and Herbst (45) showed that spermidine stimulated the dissociation of RNA polymerase from the DNA template and initiated new RNA transcription in an assay containing E. coli DNA-dependent
RNA polymerase and T4 DNA. Sedimentation analysis results suggested that spermidine had no affect on the size of the RNA transcripts. Since these investigators had also shown that spermidine had no affect on the release of RNA products from the transcription complex (DNA, RNA polymerase, and RNA), it was proposed that polyamines may play a role in reversing the inhibition of RNA synthesis by RNA products.

Rose and Jacob (46) studied the effects of varying amounts of polyamines in an in vitro assay containing purified polyadenylation polymerase, rat liver polyadenylic acid (poly A), nuclear RNA, E. coli tRNA, and other factors. It was shown that 0.4-1.2 mM spermine, 7.5 mM spermidine, or 15 mM putrescine inhibited the polyadenylation reaction. Since these same polyamine concentrations were shown to enhance RNA synthesis by DNA-dependent RNA polymerase II, these authors proposed that polyamines may be essential in the regulation of mRNA synthesis.

Igarashi et al. (47) showed that 5 mM spermine, spermidine, or putrescine stimulated the synthesis of tRNA^{Ile}, but did not affect and sometimes even inhibited the synthesis of glutamyl, leucyl, lysyl, and methionyl tRNA's in rat liver. The mechanism involved in the polyamine stimulation of tRNA^{Ile} was studied further by Igarashi et al. (48), and it was suggested that polyamines prevented the complex containing tRNA^{Ile} synthetase, isoleucine, and AMP from being inhibited by tRNA.

Many investigators have also shown that polyamines can
affect *in vivo* RNA synthesis. Much of this research has been performed on regenerating tissues or rapidly growing and developing organisms such as the larvae of *Drosophila melanogaster*. In an early study by Dykstra and Herbst (49), partial hepatectomies were performed on rats and the rats were injected with $^3$H-putrescine and $^3$H-spermidine to observe the rate of polyamine uptake in the regenerating liver tissue. In 48-72 hours following the partial hepatectomies, it was observed that putrescine and spermidine uptake was increased 3 to 5-fold and 2-fold respectively as compared to normal rat livers. Since it was also shown that increased RNA synthesis occurred concurrently with increased polyamine uptake, it was suggested that polyamines may play a role in the stimulation of RNA synthesis in regenerating rat liver tissue.

Herbst et al. (50) used *Drosophila* larvae grown in medium supplemented with 1-10 mM spermidine or putrescine to determine whether the rate of RNA synthesis could be affected. After 72 hours, it was shown that the larvae grown in spermidine-supplemented medium incorporated 3 times more $^3$H-uridine into RNA than the control larvae, however; larvae grown in putrescine-supplemented medium exhibited an inhibition of RNA synthesis. In another approach by the same authors, spermine or spermidine was added to an *in vitro* assay containing an *E. coli* DNA-dependent RNA polymerase and a variety of double-stranded DNA templates. If this assay was performed under low ionic strength
conditions, it was shown that RNA synthesis could be stimulated 3 to 4-fold with 0.25 mM spermine or 2.5 mM spermidine. If optimum ionic concentrations were used, 2-6 mM spermine or spermidine was found to stimulate RNA synthesis 2-fold. In view of these data, it was proposed that spermidine may stimulate RNA synthesis by stabilizing the active transcription complex.

Byus and Herbst (51) grew Drosophila larvae in polyamine-supplemented medium to determine the effects of polyamines on the synthesis of 4S, 18S, and 28S RNA. It was shown that $^3$H-uridine incorporation was increased 30-150% when larvae were grown in medium supplemented with 1-5 mM spermidine. However, if the larvae were grown in medium supplemented with 1-100 mM putrescine or 120 mM spermidine, the incorporation of $^3$H-uridine into these RNA's was inhibited. It was also shown by these investigators that the addition of polyamine did not modify the uptake of $^3$H-uridine or its conversion to $^3$H-UTP, nor did it affect the turnover rate of the RNA.

Many investigators have studied the in vitro and in vivo effects of polyamines on protein synthesis. Some of these effects involve polyamine-interactions with ribosomes and various initiation complex subunits, and increased accuracy of tRNA codon-anticodon base pairings.

It was shown by Igarashi et al. (52) in an in vitro experiment that spermidine was able to stimulate the incorporation of phenylalanine into E. coli protein.
Further studies showed that the added polyamine seemed to stimulate poly (U) binding to ribosomes to a greater extent than tRNA^Phe binding. Since other evidence indicated that polyamines interact with ribosomal subunits, it was proposed that polyamines affect the initiation complex.

Hunter et al. (53) used a cell-free wheat germ system and exogenous tobacco mosaic virus (TMV) mRNA to demonstrate that both spermidine and spermine stimulated protein synthesis. It was shown that the addition of polyamines increased polypeptide chain elongation 2-fold, and increased the amount of complete translation products. However, contrary to reports by Igarashi et al. (52), there seemed to be no evidence suggesting that polyamines stimulated the initiation rate of translation. It was therefore proposed by these authors that polyamines did not affect the rate of initiation, but rather increased production of complete mRNA transcripts before degradation occurred.

Igarashi et al. (54) isolated 50S ribosomal subunits from E. coli and reconstituted 70S ribosomes by adding 30S ribosomes containing different combinations of 16S RNA core particles and proteins derived from the 30S subunits isolated from a polyamine-deficient E. coli mutant grown in the presence or absence of polyamines. These reconstitution experiments seemed to suggest that decreased protein synthesis in polyamine-deficient E. coli mutants grown in the absence of polyamines was due to both a deficiency in the 30S subunit proteins and decreased methylation of the
16S RNA core particles. It was proposed that polyamines were necessary in stabilizing 16S RNA core particles for methylation to occur. After methylation of the 16S RNA core particles, 30S subunits could be assembled by the addition of the 30S-derived proteins. Igarashi et al. (55) later showed that the protein fraction of the 30S ribosomal subunits isolated from polyamine-deficient E. coli mutants grown in the absence of polyamines were deficient in S1 proteins and a 55 Kdal protein. It was also shown that if the 16S RNA core particles of the 30S subunits were methylated, the amounts of these proteins were increased. These data indicated that a correlation exists between the amount of methylation of the 16S RNA core particle and the amounts of S1 and 55 Kdal proteins in the 30S ribosomal subunits.

Since it had been shown that spermidine enhanced the amount of complete proteins which are produced, Abraham et al. (56) used a cell-free wheat germ system with poly (U) or TMV mRNA to determine whether spermidine played a role in translational fidelity. Although poly (U) should only produce polyphenyalanine chains (UUU or UUC), the fidelity of translation can be determined by the amount of accidental leucine (UUG or UUA) incorporated into the polypeptide in the presence or absence of spermidine. Results showed that the presence of spermidine caused a 10-fold decrease in the incorporation of leucine. Since the erroneous reading of some codons can actually terminate the synthesis of a protein, another experimental approach by these researchers
was to use TMV mRNA and $^{35}$S-methionine to determine whether polyamines and/or Mg$^{++}$ can affect the amount of complete proteins produced. Evidence suggested that the addition of polyamines increased the production of complete proteins and decreased the production of incomplete proteins. It was proposed that spermidine enhanced the fidelity of translation by stabilizing the tRNA anticodon, therefore increasing correct codon-anticodon associations.

By using a cell-free wheat germ system with natural protamine mRNA isolated from the testis of rainbow trout, Ito and Igarashi (57) showed that spermidine increased the fidelity of translation by decreasing the misreading of tRNA codons. Misreading seemed to occur more readily with the 5'-base of the codon (high-frequency misreading) than with the middle base of the codon (low-frequency misreading). Although spermidine seemed to prevent both types of misreading from occurring, it seemed to have a much greater effect in preventing low-frequency misreadings. These authors proposed that polyamines may be significant for insuring that correct aminoacyl-tRNA binding to ribosomes takes place.

Several investigators have obtained evidence that polyamines may affect DNA structure and stability by interacting with the histones and nucleosome core particles associated with the DNA. Many of these interactions seem to involve post-translational modifications such as ribosylation, phosphorylation, and acetylation.
In vitro studies by Byrne et al. (58), have shown that ADP-ribosylation of H1 histones in HeLa cell nuclei can be stimulated 1-3 fold with 1-2 mM spermine, spermidine, or putrescine. These authors proposed that H1 histones from neighboring chromatin fibers may be linked to one another via poly-ADP ribose bridges causing condensation of the chromatin. Since an enzyme exists which is capable of hydrolyzing the poly-ADP ribose chains, and no increase of poly-ADP ribose was found to occur, these authors suggested that intermittent condensation and relaxation of specific areas of DNA may regulate DNA synthesis and replication.

Perrela and Lea (59,60), in support of Byrne et al. (58), also showed that 1 mM spermine increased ADP-ribosylation of H1 histones 2-fold. However, it was also shown that the addition of spermine caused a decrease in the ADP-ribosylation of the other histones. It was therefore proposed that ADP-ribose is redistributed among the histones. It was speculated that this may cause certain histone-DNA interactions to weaken because of the interactions of negatively-charged poly-ADP ribose with the highly basic areas of the H1 histones. The weakened interaction between H1 histones and DNA may cause a relaxation of the chromatin, which may in turn allow DNA replication to occur.

Lough (61) studied the effects of spermine on the phosphorylation of basic nuclear proteins extracted from nuclei isolated from both proliferation stage and myotube (post-mitotic) stage chicken myoblast cells. In both cell types,
it was shown that 2 mM spermine increased phosphorylation of most phosphoproteins 1.5-2.0-fold. However, histones H1a and H3 from myotube cell nuclei exhibited 3.5 and 9-fold increases in phosphorylation respectively in the presence of spermine. Although the mechanism of this specific increase of phosphorylation is unknown, it was stated by the author that this may be the first demonstration that polyamines may be able to modulate histone function by promoting their phosphorylation at various times of cell growth.

The interactions of polyamines and acetylpolyamine derivatives with nucleosome core particles was studied by Morgan et al. (62). Thermal denaturation studies showed that tetra- and triamines were 2-3 times more effective than diamines, and acetylpolyamines were less effective than unmodified polyamines with equivalent charge, in stabilizing nucleosome core particles. Results of circular dichroism studies suggested that these polycations are capable of changing the DNA twist and/or folding. These authors suggested that in vivo DNA replication and transcription events may be facilitated by the acetylation of polyamines and histones which subsequently lowers the stability, and changes the conformation of the nucleosome core.

Along with evidence suggesting that polyamines interact with chromatin-associated histones or nucleosomes, many investigators have shown that several non-histone proteins found in the nucleus or nucleolus may be affected by these polycations.
Atmar, Daniels, and Kuehn (63) incubated nuclei and nucleoli isolated from *Physarum polycephalum* with $[^{32}P]$ phosphate in the presence or absence of 1 mM total polyamines to determine the effects of polyamines on protein phosphorylation. It was shown that phosphorylation was enhanced 6-30 fold depending on the combination of polyamines used. The separation of the proteins using gel electrophoresis indicated that polyamines specifically stimulated the phosphorylation of proteins with molecular weights of 14, 27, 52, and 70 Kdal to a greater extent than other proteins. It was suggested by these authors that the polyamine-stimulated phosphorylation of these proteins seems to be very specific. It was also noted that the two most highly phosphorylated proteins, the 52 and 70 Kdal proteins, were found primarily in the nucleolus where ribosomal RNA synthesis occurs.

Kuehn et al. (64) further characterized this acidic 70 Kdal nucleolar phosphoprotein and found that it stimulated ribosomal RNA synthesis by RNA polymerase I 5-fold. It was suggested that this protein, whose phosphorylation is stimulated by polyamines, may be able to regulate transcription and ribosomal RNA synthesis. In a later study, Daniels, Atmar, and Kuehn (65) isolated and purified a 26 Kdal protein kinase which catalyzed the transfer of a phosphate from $[^{32}P]$ to the previously studied 70 Kdal protein (63,64). The activity of this protein kinase was stimulated as much as 60-fold by spermine and spermidine.
It was proposed that polyamines may regulate rRNA gene transcription by modulating the activity of a highly specific protein kinase.

An interesting report by Atmar and Kuehn (12) gives evidence which suggests that the acidic 70 Kdal nucleolar phosphoprotein and its specific 26 Kdal polyamine-stimulated protein kinase are in fact ornithine decarboxylase and its associated antizyme respectively.

Chen and Verma (66) studied the effect of polyamines on endogenous protein phosphorylation in nuclear fractions of mouse neuroblastoma cells. It was shown that 1 mM spermine inhibited the phosphorylation of both a 120 Kdal and a 11 Kdal protein. Further studies using C6 glioma cells, L5 myoblasts, S91 melanoma cells, PC12 cells, and IMR-90 human fibroblasts also indicated that the phosphorylation of a 11 Kdal protein could be inhibited with 1 mM spermine. However, only the mouse neuroblastoma cells exhibited inhibited phosphorylation of the 120 Kdal protein suggesting that this phosphorylation is a neuroblastoma cell-specific event. Since the phosphorylation of the 11 Kdal protein can be inhibited by spermine in several different cell lines, these authors suggested that this protein may play a significant role in spermine-regulated cellular processes.

Further research by Verma and Chen (67) indicated that spermine affected the phosphorylation of mouse neuroblastoma 11 and 10 Kdal nuclear proteins by inhibiting a specific 37 Kdal nuclear protein kinase (NI kinase). It was shown that
the activity of this kinase on the 11 and 10 Kdal proteins was completely inhibited by 1 mM spermine; however, 10 mM putrescine, 2 mM spermidine, 5 mM arginine, 10 mM NH₄Cl, 1 mM N¹-acetylspermine, and 1 mM Co(NH₃)₆³⁺ did not affect this reaction. This suggests that the inhibition by spermine is not due to charge or counter-ion effects. The authors proposed that the 11 and 10 Kdal proteins, along with polyamines as regulatory factors, may be important in gene regulation.

Friedman (68) published evidence that polyamines may also stimulate nuclear protein phosphatase activity. It was shown that spermine was able to activate protein phosphatase activity 2-4 fold on sonicated HeLa cell nuclei. Further studies using phosphatase inhibitors characterized this nuclear protein phosphatase as protein phosphatase I. It was suggested that because protein phosphatase I activity can be activated by physiological concentrations of spermine, polyamines may serve a purpose in the regulation of nuclear protein activity by dephosphorylation as well as phosphorylation events.

The use of eukaryotic cell cultures instead of animal tissue systems gave researchers another means to study the specific effect(s) of polyamines on cellular processes. The advantages of using eukaryotic cell cultures are that they can be easily manipulated, their environments can be carefully controlled, and they can easily be synchronized using procedures described by Mueller and Kajiwara (69), and Goyns
The inhibition of the polyamine biosynthetic enzymes S-
adenosylmethionine (SAM) decarboxylase, ornithine decarbo-
oxylase (ODC), and spermidine and spermine synthase can be
achieved by the additions of methylglyoxal bis(guanyl-
hydrazone) (MGBG), α-difluoromethylornithine (DFMO) and α-
methylornithine (α-MO), and 5'-substituted adenosines
respectively to cell cultures (71).

Fillingame and Morris (72) studied the effect of the
polyamine biosynthesis inhibitor MGBG on concanavalin A (Con
A)-stimulated lymphocytes. It was shown that Con A-stimu-
lated lymphocytes exhibited increased DNA synthesis and
increased cellular contents of RNA, protein, and polyamines.
Since the increased contents of RNA and polyamines occurred
6, and 8-10 hours respectively following Con A stimulation,
it was suggested that polyamines may play a role in RNA
stabilization. However, the addition of MGBG to the lympho-
cyte cultures was shown to deplete the spermidine and
spermine contents of the cells, but did not affect RNA
synthesis or processing. This led to the suggestion that no
correlation existed between increased polyamine content and
increased RNA biosynthesis in Con A-stimulated lymphocytes.

Using the same lymphocyte system, Fillingame et al. (73)
showed that added MGBG decreased both DNA synthesis and the
concentrations of spermidine and spermine. Since the
addition of exogenous spermine and spermidine to the cell
system reversed this inhibition, it was suggested that DNA
synthesis in lymphocytes was dependent upon spermidine and spermine.

Knutson et al. (74), studying nuclei isolated from Con A-activated lymphocytes, showed that nuclei isolated from cells treated with MGBG exhibited a 50% decrease in DNA synthesis. These investigators also proposed that decreased DNA synthesis may be directly related to decreased cellular spermine and spermidine levels. The addition of either spermidine or sonicated control cell nuclei to the MGBG-treated cell nuclei did not reverse this inhibition.

Krokan and Eriksen (75) studied the effects of polyamine-depletion in HeLa cells treated with MGBG. HeLa cells which were treated with MGBG for 20 hours exhibited a 50% decrease in spermine and spermidine levels, however; the levels of putrescine increased 200%. The MGBG-treated HeLa cells also showed a reduction in \(^3\)H-thymidine, \(^3\)H-uridine, and \(^{14}\)C-leucine incorporation into DNA, RNA, and protein respectively. However, if exogenous polyamines were also added to the cell culture during the 20-hour MGBG-treatment, no reduction of DNA, RNA, or protein synthesis occurred. The same authors showed that nuclei isolated from MGBG-treated HeLa cells also exhibited a decrease of DNA synthesis similar to that seen in whole cells. This inhibition of DNA synthesis could not be reversed by the addition of spermine or spermidine to the in vitro nuclear system.

In order to study the effects of MGBG-treatment on DNA synthesis, these authors used bromodeoxyuridine labeling
techniques to determine DNA chain elongation rates. Since these studies indicated that the effects of MGBG inhibition were small, it was suggested that spermidine and spermine reduction caused a decrease of active DNA replication units and DNA initiation sites. In another study, these investigators used autoradiography techniques to determine whether the decrease of DNA synthesis is caused by a decrease of the number of cells in S-phase (DNA synthesis) of the cell cycle. It was shown that there was no decrease in the number of cells in S-phase; however, the polyamine-deficient cells proceeded through the cell cycle at a much slower rate.

Since it has been shown that MGBG may be "toxic" to many cells by directly inhibiting DNA synthesis, RNA synthesis, and cellular respiration, the results of experiments using this polyamine biosynthesis inhibitor must be interpreted with caution (76).

Mamont et al. (77) showed that rat hepatoma tissue culture (HTC) treated with the ornithine decarboxylase inhibitor α-methylornithine (α-MO) prevented putrescine synthesis, lowered the endogenous concentrations of putrescine and spermidine, and decreased rates of DNA synthesis and proliferation. However, these effects of α-MO could also be reversed by the addition of either spermidine, spermine, or putrescine to the cell culture.

Another study by Mamont et al. (78) showed that the ornithine decarboxylase inhibitors α-MO and DFMO were found
to lower putrescine and spermidine concentrations, and reduce the proliferation rates of both rat HTC cells and mouse leukemia cells. HTC cells treated with DFMO were shown not to exhibit the normal increase of ornithine decarboxylase that is usually found after proliferation. The inhibitory effects of DFMO were found to be reversed by the addition of either 10 uM putrescine or spermidine, or 1 uM spermine to the cell cultures.

Seyfried and Morris (79) found that 1 mM DFMO added to a cell culture of Con A-activated bovine lymphocytes inhibited $^3$H-thymidine incorporation into DNA. It was shown that polyamine biosynthesis was inhibited 90% and both putrescine and spermidine levels were significantly lowered. However, 1 mM DFMO did not affect in vivo protein or RNA synthesis. These results indicate that DFMO, as does MGBG, decreased polyamine levels, and inhibited DNA synthesis and proliferation.

Sunkara et al. (80) treated the two normal cell lines PA2 (human fibroblasts), mouse 3T3 cells, and the 3 transformed cell lines CHO, HeLa, and SV3T3 with 8 uM MGBG to determine at which stage(s) of the cell cycle these cells accumulate. Although spermine and spermidine concentrations were found to be significantly reduced in all of the cell lines, examination of the prematurely condensed chromosomes of the polyamine-depleted cells indicated that normal cells accumulated mainly in early G1-phase while transformed cells accumulated in S-phase. These data suggest that polyamines
are essential for normal cells to progress through the cell cycle while transformed cells will continue to progress through the cell cycle but will not complete DNA synthesis. Upon further examination of the condensed chromosomes of the transformed cell lines, these authors also suggested that polyamines may affect DNA chain initiation.

Seidenfeld et al. (81) also studied the effects of polyamine depletion by DFMO on the cell cycle phase distributions of five human carcinoma cell lines. The addition of 1-5 mM DFMO to the HuTu-80 (stomach), HT-29 (colon), MCF-7 (breast), A-427 (lung), and ME-180 (cervix) cell lines caused significantly reduced polyamine concentrations and inhibited proliferation rates. DNA histograms showed that all of the cell lines except ME-180 had altered cell cycle phase distributions where the cells seemed to accumulate in G1 phase. The addition of 5-50 uM putrescine had no effect on the ME-180 cell line, but reversed the altered phase distributions of the other cell lines. These observations suggested that some human carcinoma cell lines are more dependent than others on the amount of polyamines needed to progress normally through the cell cycle. These authors also suggested that human carcinoma cell lines may react differently to the DFMO-induced inhibition of polyamine biosynthesis.

In our laboratory, Herbst and Branca (82) showed that both DNA synthesis and proliferation could be inhibited in HeLa cells grown in the presence of the ODC inhibitor DFMO.
while simultaneously inducing the production of ODC antizyme with 1,3-diamino-2-propanol. In vitro DNA synthesis assays of nuclei isolated from these polyamine-deficient cells also showed inhibited DNA synthesis. The addition of 40 μM spermidine to a polyamine-depleted HeLa cell culture before the initiation of S-phase in synchronized cells reversed this inhibition. However, as seen by previous investigators (73,74), this inhibition could not be reversed by the addition of polyamines to the in vitro DNA synthesis assay of nuclei isolated from polyamine-deficient cells.

In another study, Herbst and Elliott (83) added polyamines or polyamine analogs to HeLa cell cultures depleted of polyamines by DFMO to determine whether inhibited DNA synthesis in nuclei isolated from these cells could be reversed. The nuclei isolated from cell cultures supplemented with 10 μM spermine, spermidine, or putrescine 24 hours before S-phase was initiated exhibited complete reversal of the inhibition of DNA synthesis. When spermidine analogs were used (NH₂(CH₂)₃NH(CH₂)ₓNH₂), slight stimulatory effects were seen when X=5, but not when X=3, or 6-8. These authors also tried to stimulate DNA synthesis in nuclei isolated from polyamine-depleted HeLa cells by adding various concentrations and combinations of polyamines to the in vitro DNA synthesis assay. However, as seen previously (73,74,81), the addition of polyamines to the in vitro DNA synthesis assay of polyamine-depleted nuclei had no stimulatory effect on DNA synthesis.
More recently, Gallo, Koza, and Herbst (84) compared the levels of polyamines and DNA synthesis in control cells and cells depleted of polyamines by DFMO. It was shown that control cells had a synchronous peak of DNA synthesis at 3 hours, and the highest concentrations of polyamines at 6-9 hours after the initiation of S-phase, whereas the polyamine-depleted cells exhibited inhibited DNA synthesis, no detectable putrescine, and an 80% decrease in spermine and spermidine. Although intracellular polyamine pools were replenished when polyamines were added to polyamine-depleted cells at the time S-phase was initiated, DNA synthesis was only partially restored with a 3-6 hour delay in reaching the maximum rate. If 20 μM spermidine was added to polyamine-depleted cell cultures at least 10 hours before S-phase was initiated, DNA synthesis was almost totally restored with a normal peak of DNA synthesis 3 hours after the initiation of S-phase. It was suggested that this reversal of inhibited DNA synthesis by the addition of exogenous polyamines may be a time-dependent process requiring at least 10 hours. It was proposed that this 10 hour period may be essential for the cells to synthesize and replenish replication factors, and/or to organize replication factors into active replication complexes.

In my M.S. degree thesis research entitled "The Role of Polyamines in HeLa Cell DNA Synthesis" (85), I further examined the time-period needed to reverse the inhibition of DNA synthesis and proliferation in polyamine-depleted HeLa
cells. It was determined that 20 uM spermidine must be supplemented to a cell culture being depleted of polyamines by DFMO at least 10 hours before S-phase is initiated for normal DNA synthesis and cell proliferation to occur. Nuclear fractionation and reconstitution studies such as those described by Brun and Weissbach (86), and Enomoto et al. (87) were also utilized in the hopes of characterizing the site of deficiency in DNA synthesis in polyamine-depleted HeLa cell nuclei. Isolated control and polyamine-depleted HeLa cell nuclei were fractionated by salt extraction techniques and then reconstituted in various combinations to determine whether the salt-extracted soluble enzymes or the salt-extracted nuclei (SEN) remaining were deficient in supporting DNA synthesis. If the salt extracts of polyamine-depleted cell nuclei were reconstituted with control cell SEN, some stimulation of DNA synthesis was seen; however, salt extracts of control cell nuclei were unable to stimulate DNA synthesis when reconstituted with polyamine-depleted HeLa cell SEN. This suggested that the chromatin of polyamine-depleted cells cannot serve as a template for DNA synthesis. Since salt extracts obtained from polyamine-depleted cells only partially stimulate DNA synthesis as compared with control cell nuclear salt extracts when reconstituted with control SEN, it was suggested that a deficiency also exists in the soluble enzyme fraction of polyamine-depleted cell nuclei. Further studies indicated that the activity of DNA polymerase alpha
in polyamine-depleted cell nuclear salt extracts was only about 50% of the activity in control cell nuclear salt extracts. From these data, it was proposed that the recovery of HeLa cells from the effects of polyamine-depletion requires reversal of the deficiencies in both the soluble enzyme activity, i.e., nuclear DNA polymerase alpha, and in the chromatin template.

My dissertation research, "Characterization of Cell Cycle Changes in Polyamine-depleted HeLa Cells", is an extension of studies completed for my M.S. degree thesis (85). Since it had been shown that both the nuclear salt extracted enzyme(s) and the chromatin template remaining were defective in polyamine-depleted HeLa cells, experiments were performed to characterize these deficiencies. Also, the time period required to reverse the deficiencies in polyamine-depleted HeLa cell nuclei was examined further.

The activity of DNA polymerase alpha on "activated" vs heat-denatured DNA templates and immunoprecipitation studies were utilized to determine whether the deficiency of DNA polymerase alpha in polyamine-depleted HeLa cell nuclei was caused by an incomplete holoenzyme complex. Although immunoprecipitation results were inconclusive, the use of the two different DNA templates suggested that a quantitative rather than a qualitative deficiency exists in DNA polymerase alpha extracted from polyamine-depleted HeLa cell nuclei.

A comparison was also made between DNA polymerase alpha
activity extracted from whole HeLa cells and isolated HeLa cell nuclei. These experiments seemed to indicate that either a "transport" problem may exist in polyamine-depleted cells preventing DNA polymerase alpha from entering the nucleus from the cytosol, or the DNA polymerase alpha is able to exit freely from the nucleus in polyamine-depleted cells.

In an attempt to determine the deficiency in the chromatin template, a comparison was made between histones extracted and partially purified from both control and polyamine-depleted cell nuclei. Upon analyzing the partially purified histone preparations utilizing polyacrylamide gel electrophoresis, it was determined that the defect of chromatin in polyamine-depleted cell nuclei was not due to a lack of histones.

The in vitro phosphorylation of nuclear proteins was studied at various time periods before the initiation of S-phase to determine whether a difference exists between nuclei isolated from control or polyamine-depleted HeLa cells. Results indicated that control cell nuclei exhibited a substantial decrease in the phosphorylation of an 31 kdal nuclear protein as cells approached S-phase. However, nuclei isolated from cells undergoing polyamine-depletion by DFMO continually phosphorylated this same protein. It was also shown that if the polyamine-depleted cell culture was supplemented with 20 uM spermidine 12 hours before S-phase, the nuclear protein phosphorylation pattern obtained was
similar to that obtained from control cell nuclei.

Several other experiments were performed to further characterize the time period required to reverse the effects of polyamine-depletion. The results of these experiments suggested that 20 μM spermidine must be supplemented into a polyamine-depleted cell culture at least 10-12 hours before S-phase to fully reverse these deficiencies. It is apparent that cells inhibited by polyamine-depletion are not arrested at only a single control site in the cell cycle, but rather are arrested throughout a broad area of at least 10-12 hours which also encompasses late G1-phase.
II. MATERIALS AND METHODS

Maintainence of Stock and Experimental HeLa Cell Cultures

Stock HeLa S₃ cell cultures were obtained from Dr. Sheldon Penman (Dept. of Biology, MIT, Cambridge, MA), and maintained as suspension cultures in spinner flasks incubated at 37°C and 140 rpm in a rotary shaker-incubator (Psychrotherm; New Brunswick Scientific Co. New Brunswick, NJ).

The stock and experimental suspension cultures were grown in Ca²⁺- and Mg²⁺-free modified Basal Minimum Eagles (BME) medium which was supplemented with 10% v/v horse serum, 0.1 mM glycine, 0.1 mM serine, 0.01 mM myoinositol, 2.0 mM glutamine and 0.1% w/v Pluronic F-68. The addition of the detergent Pluronic F-68, and restricting the levels of Ca²⁺ and Mg²⁺ in the medium, prevented the cells from clumping and adhering to the culture flasks.

The water used for the preparation of the modified BME medium was deionized using a Milli-Q purification system (Millipore Corp., Bedford, MA), glass distilled, and sterilized by autoclaving. Aliquots of the following concentrated stock solutions were added to the sterile water: Earle's Balanced Salt Solution (EBSS), vitamins, amino acids, sodium bicarbonate, Pluronic F-68, glycine-serine-myoinositol, glutamine, and the antibiotics Penicillin G and
Streptomycin.

Irradiated horse serum, which contains essential growth factors, was added to the medium used for maintaining cell growth, but was replaced with an equal volume of sterile water in medium used for washing cell pellets. Table 1 shows the composition of the modified BME medium.

All of the culture medium components were obtained from the Grand Island Biological Corp. (GIBCO; Grand Island, NY) except for Pluronic F-68 (Wyandotte Chemical Corp., Parsippany, NJ), glycine and myoinositol (Calbiochem-Behring Corp., La Jolla, CA), glutamine and serine (Sigma Chemical Co., St. Louis, MO).

Aseptic manipulations involving cell cultures, preparation of the modified BME medium, etc., were performed in a laminar flow hood (The Baker Co. Inc., Sanford, ME). Stock suspension cultures were maintained daily by removing 50% of the high density cell suspension (5-6 X 10^5 cells/ml) and adding an equal volume of fresh modified BME medium. Cell cultures were scaled up for experiments by transferring aliquots removed from the stock cultures to a flat-bottomed boiling flask and an equal volume of BME medium was added daily until a desired volume of culture was obtained.

A physiological pH of approximately 7.4 was maintained in both the stock and experimental cultures by using a bicarbonate (HCO_3^-)-CO_2 buffering system which maintains an equilibrium between HCO_3^-1, H_2CO_3 (carbonic acid), CO_2 (aq), and H_2O. The bicarbonate was supplied by the addition of
<table>
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<sup>a</sup>Amount in 1 liter of prepared medium

<sup>b</sup>U= units
sodium bicarbonate (NaHCO₃) to the modified BME medium, while the CO₂ was provided by HeLa cell respiration and by subjecting the closed cell culture systems to a stream of gas containing 95% air and 5% CO₂ before incubation. The CO₂ atmosphere allows an equilibrium to occur between CO₂ (g) and CO₂ (aq), therefore maintaining a pH near 7.4.

Cell culture flasks (ie. spinner flasks, flat-bottomed flasks) were siliconized with a 1% solution of dichlorodimethylsilane (Aldrich Chemicals, Milwaukee, WI) in benzene to prevent cells from sticking to the glass surfaces. Culture flasks were desiliconized by rinsing with a 10:1 solution of 95% ethanol v/v:50% KOH w/v and, after allowing to air dry for 30 minutes, and rinsing with tap water. The desiliconized glassware and other glassware (ie. pipettes, medium and solution bottles) were cleaned by soaking overnight in an acid bath containing concentrated sulfuric acid and the oxidizing agent NoChromix (Godax Laboratories, New York, NY) before being thoroughly rinsed with tap water and finally with distilled water.

As a means of preventing cell culture contamination, two stock spinner cultures were maintained simultaneously and their cell populations were monitored continuously. Cell populations were monitored by diluting aliquots of the cell culture with saline and counting the cells using a Royco automatic cell counter (Royco Instruments, Menlo Park, CA). If there was any evidence of cell culture contamination (i.e., unusually low cell population) the cell cultures were
examined microscopically to determine if contamination was present.

Cell viability was determined microscopically by examining an aliquot of cell suspension which had been stained with erythrosin B (0.08% erythrosin B in 0.4% NaCl). Non-viable cells stain red whereas viable cells remain unstained.

The stock suspension cultures were trypsinized approximately every two weeks to remove dead cells and cellular debris. This was accomplished by centrifuging (800 rpm/10 min./25°C) 100 ml of high density cell suspension (5-6 X 10^6 cells/ml), and resuspending the cell pellet in 20 ml of 0.05% trypsin (3600 NF units/mg; Calbiochem, La Jolla, CA) in saline A (20 mg/ml phenol red, 0.4 mg/ml KCl, 8.0 mg/ml NaCl, 1.0 mg/ml glucose, 0.35 mg/ml Na₂CO₃). The suspension was incubated in a 37°C water bath for 7 minutes after which an equal volume of fresh modified BME medium was added to stop proteolysis. The incubated suspension was centrifuged (800 rpm/10 min./25°C) and the pellet was resuspended in fresh modified BME medium at a cell density of approximately 3.5 X 10^5 cells/ml. The cell culture was transferred to a spinner flask, gassed with 5% CO₂:95% air for 1 minute and returned to the Thelco convection incubator. When normal logarithmic cell growth had resumed (approximately 2-3 days), the newly trypsinized cell suspension was divided into two aliquots and maintained as duplicate spinner culture stocks.
Cell cultures used for experimental purposes were first trypsinized and then resuspended at a density of 120,000 cells/ml for control cultures and 150,000 cells/ml for cultures to which 1 mM DFMO was added to inhibit polyamine biosynthesis. The experimental cell cultures were synchronized using the thymidine-block procedure developed by Mueller and Kajiwara (69). Thymidine (1 mM) (Calbiochem Corp. La Jolla, CA) was added to the cell cultures after they had resumed log phase growth (32 hours) and the cells were allowed to grow for 16 hours in the presence of thymidine. At this time (48 hours), the thymidine-supplemented modified BME medium was removed by centrifugation (800 rpm/10 min./25°C) and the cells were washed 2X with 1/2 culture volumes of serum-free modified BME medium. The cell pellets were resuspended in 1 culture volume of fresh modified BME medium and the cells were allowed to grow for 8 hours before another addition of 1 mM thymidine (56 hours). The cells were again grown for 16 hours in the presence of thymidine before being washed as described above. The cell pellets were resuspended at a desired concentration to initiate S-phase of the cell cycle (72 hours).

**Determination of Cell Proliferation**

HeLa cell culture densities were measured at desired times following the release of the second thymidine block.
and the initiation of S-phase DNA synthesis to determine cell proliferation. Aliquots of the cell cultures were diluted with saline and the cells were counted using a Royco automatic cell counter. Cell cultures specifically set up for proliferation studies were adjusted to a cell density of 300,000 cells/ml at the initiation of S-phase. Cell proliferation, as indicated by a doubling of the cell population, was monitored in the synchronized cell culture experiments of this study.

Assay of DNA Synthesis in HeLa Cell Lysates

HeLa cell lysates were utilized in some experiments to assay DNA synthesis at various time intervals following the initiation of S-phase. A desired volume of cell culture (@ 500,000 cells/ml) was centrifuged (800 rpm/10 min./25°C) to remove the modified BME medium. The cell pellet was resuspended in 1 ml buffer A (2 mM EGTA, 10 mM HEPES, 0.1 mM PMSF, 3 mM MgCl₂, 2 mM DTT) per 5 x 10⁶ cells and transferred to an appropriately-sized Corex tube. The suspension was centrifuged (2000 rpm/5 min./4°C) and the supernatant was removed. The cell pellet was resuspended in 1 ml buffer A per 25 x 10⁶ cells and transferred to an appropriately-sized Dounce homogenizer. The Dounce homogenizer containing the cell suspension was maintained in an ice bath for 7 minutes before 30 pestle strokes were applied to lyse the cells. The lysate was examined microscopically to determine
whether lysis was complete.

100 ul aliquots of lysate (8 2.5 X 10^6 cell equivalents) were transferred to four replicate 13 ml Corex tubes to which were added the following assay ingredients: 100 ul Weissbach assay mix (125 mM Tris.HCl (pH 8.1), 20 mM MgCl_2, 16.25 mM ATP, 125 mM NaCl, 5 mM DTT, and 0.25 mM each of dATP, dCTP, dGTP, dTTP), 25 ul buffer A, and 25 ul (2 uCi) [^3H]-dTTP (10-20 Ci/mmol).

Triplicate assay tubes were incubated in a 37°C water bath for 30 minutes. One of the four replicate assay tubes was kept on ice and utilized as an unincubated blank. Following the incubation period, the assay tubes were chilled on ice, and the contents of each tube were transferred to 24 mm GFC glass-microfiber filters (Whatman Ltd. Maidstone, England) on a filtering manifold (Millipore Corp. Bedford, MA) using two 3 ml aliquots of buffer A. This was filtered with a low vacuum, following which, 15-20 ml of cold 4% w/v HClO_4 was added to each filter well and allowed to pass through the filters without applying a vacuum. The filters were removed from the filtering manifold, placed in liquid scintillation vials, and dried under a heat lamp. 10 ml of liquid scintillation fluid (0.392% w/v PPO and 0.008% w/v bis-MSG in toluene) were added to each vial and the [^3H]-dTMP incorporated into the precipitated DNA was measured using a Beckman Model LS-7000 liquid scintillation spectrometer. The data, recorded as the mean of three replicate assay tubes, did not exceed a variability of ±10%
of the individual assay tubes.

**Assay of DNA Synthesis in HeLa Cell Nuclei**

Nuclei, isolated from HeLa cells, were assayed at various time periods following the initiation of S-phase DNA synthesis. The same procedures were used as in the Materials and Methods (Assay of DNA Synthesis in HeLa Cell Lysates) except that after the initial buffer A wash, the cell pellets were resuspended in 1 ml buffer A containing 0.025% Triton X-100 per 50 X 10⁶ cells and transferred to appropriately-sized pre-chilled Dounce homogenizers. The Dounce homogenizers containing the cell suspensions were placed in an ice bath for 7 minutes before 20 pestle strokes were applied to lyse the cells. An equal volume of buffer A containing 0.075% Triton X-100 was added to the homogenates to provide a final volume of 1 ml buffer A containing 0.05% Triton X-100 per 25 X 10⁶ cell equivalents. The lysate was transferred to an appropriately-sized Corex tube, centrifuged (2000 rpm/5 min./4°C), and the supernatant was removed. The pellet of nuclei was resuspended in 1 ml buffer A per approximately 10 X 10⁶ nuclei and the nuclei were counted using a hemacytometer. Aliquots of the nuclear suspension containing 2.5-3.0 X 10⁶ nuclei were pipetted into four replicate 13 ml Corex assay tubes. The tubes were centrifuged (2000 rpm/5 min./4°C) and the supernatant was removed.
The nuclear pellets were resuspended and the following additions were made to the assay tubes: 100 ul "modified" Weissbach assay mix (-NaCl), 75 ul buffer A containing 0.3 M KCl, 50 ul buffer A, and 25 ul \[^{3}H\]-dTTP (2 uCi/assay tube). The same assay procedures utilized in the Materials and Methods (Assay of DNA Synthesis in HeLa Cell Lysates) were used to determine the quantity of \[^{3}H\]-dTMP incorporated into the DNA. The data, recorded as the mean of three replicate assay tubes, did not exceed a variability of ±10% of the assay data in individual tubes.

The buffer A components EGTA, PMSF, and HEPES were obtained from the Sigma Chemical Co. (St. Louis, MO). DTT and MgCl\(_2\) were obtained from Calbiochem-Behring Corp. (La Jolla, CA) and the J. T. Baker Chemical Co. (Phillipsburg, NJ) respectively.

Triton X-100 and [methyl-\(^{3}H\)]-deoxythymidine-5'-triphosphate: tetra-sodium salt (\[^{3}H\]-dTTP) were obtained from New England Nuclear Corp. (Boston, MA). The ATP and dNTP's used in the in vitro assay for DNA synthesis were obtained from the Calbiochem-Behring Corp. (La Jolla, CA) and Pharmacia P-L Biochemicals Inc. (Piscataway, NJ) respectively.

Assay of DNA Polymerase Alpha in HeLa Cell
Nuclear Salt Extracts

Nuclei were isolated, resuspended in buffer A, and
counted according to Materials and Methods (Assay of DNA Synthesis in HeLa Cell Nuclei). Following this, the Corex tube containing the nuclear suspension was centrifuged (2000 rpm/5 min./4°C) and the buffer A supernatant was discarded. The nuclear pellet was then resuspended in 1 ml buffer A containing 0.3 M KCl per 100 X 10^6 nuclei and extracted on ice for 30 minutes with occasional gentle agitation. The Corex tube containing the nuclear suspension was centrifuged (2000 rpm/5 min./4°C) and the supernatant obtained was saved as the nuclear salt extract.

The nuclear salt extract was assayed for DNA polymerase alpha activity by pipetting 10 ul aliquots (approximately 1 X 10^6 nuclear equivalents) of nuclear salt extract into four replicate small assay tubes to which was added the following: 20 ul Enomoto assay mix (5 mM DTT, 6.24 mM MgCl_2, 0.5 mg/ml bovine serum albumin (BSA), 125 mM Trizma-base; pH 8.1), 10 ul radioactive nucleotide mix (500 uM each of dATP, dCTP, and dGTP, 100 uM dTTP, 0.5 uCi [^3]H]-dTTP, and 10 ul (1 mg/ml) "activated" or heat-denatured calf thymus DNA. If less than 10 ul of nuclear salt extract were initially pipetted into the small assay tubes, the balance was made up with buffer A containing 0.3 M KCl.

Three of the replicate assay tubes were incubated in a 37°C water bath for 30 minutes. An unincubated blank assay tube was held in an ice bath during this period.

Following the incubation period, the contents of each assay tube were transferred onto separate 3/4 inch square
Whatman #1 filter papers. The filter papers were placed into 1 volume (10 ml/filter paper) 5% cold trichloroacetic acid (TCA) in an ice bath and allowed to remain in this solution for 10 minutes with occasional swirling. The TCA was removed by aspiration and the filter papers were washed 2X with additional 1/2 volumes of cold 5% TCA. The filter papers were then washed with 1 volume of 95% ethanol, placed into liquid scintillation vials, and dried under a heat lamp. 10 ml of liquid scintillation fluid were placed into each vial. The $^3$H-dTMP incorporated into the "activated" or heat-denatured calf thymus DNA by DNA polymerase alpha was measured using a Beckman LS-7000 Liquid Scintillation Spectrometer. The data, recorded as the mean of three replicate assay tubes, did not exceed a variability of ±10% of the assay data in individual tubes.

The "activated" calf thymus DNA and bovine serum albumin (BSA) were obtained from the Sigma Chemical Co. (St. Louis, MO). The calf thymus DNA used in the preparation of heat-denatured DNA was obtained from Calbiochem Corp. (La Jolla, CA).

Heat-denatured calf thymus DNA was prepared by a method described by Baril et al. (88). A solution of calf thymus DNA (1 mg/ml) in 10 mM Tris-HCl (pH 7.5) was prepared. The DNA was denatured by heating for 10 min. in a boiling water bath followed by rapid cooling in an ice bath. Small aliquots of this solution were made and stored frozen at -20°C until used.
Assay of DNA Polymerase Alpha in HeLa Cell Lysate Salt Extracts

DNA polymerase alpha activity was also assayed in HeLa cell lysate salt extracts. This was done using the procedures described in Materials and Methods (Assay of DNA Synthesis in HeLa Cell Lysates) except that following the initial buffer A wash, the cell pellet was resuspended in 0.8 ml buffer A per 100 X 10^6 cells before being transferred to a pre-chilled Dounce homogenizer.

The Dounce homogenizer containing the cell suspension was maintained in an ice bath for 7 min. before 30 pestle strokes were applied to lyse the cells. Following this, 0.2 ml of buffer A containing 1.5 M KCl was added to the lysate to give a final volume of 1 ml buffer A containing 0.3 M KCl per 100 X 10^6 cell equivalents. The lysate was transferred to an appropriately sized Corex tube and extracted in an ice bath for 30 min. before being centrifuged (2000 rpm/5 min./4°C). The supernatant was saved as the lysate salt extract.

5-10 ul aliquots of lysate salt extract were pipetted into four replicate assay tubes and assayed for DNA polymerase alpha activity as described in Materials and Methods (Assay of DNA Polymerase Alpha activity in HeLa Cell Nuclear Salt Extracts).

The amount of [^3H]-dTMP incorporated into acid precipi-
tated DNA was determined with a Beckman LS-7000 liquid scintillation spectrometer. The data, recorded as the mean of three replicate assay tubes, did not exceed a variability of ±10% of the assay data in individual tubes.

Assay of DNA Polymerase Alpha in HeLa Cell Cytosolic Fractions

HeLa cell cytosolic fractions were obtained by utilizing similar procedures described in Materials and Methods (Assay of DNA Polymerase Alpha Activity in HeLa Cell Lysate Salt Extracts) except that the cell pellet was resuspended in 1 ml buffer A per 100 X 10^6 cells after the initial buffer A wash. This suspension was transferred to an appropriately sized pre-chilled Dounce homogenizer and maintained in an ice bath for 7 min. before 30 pestle strokes were applied to lyse the cells. The suspension was transferred to an appropriately sized Corex tube and centrifuged (2000 rpm/5 min./4°C). The supernatant was saved as a crude cytosolic fraction.

5 ul aliquots of the crude cytosolic fraction were pipetted into four replicate small assay tubes along with 5 ul buffer A containing 0.6 M KCl (ie. total volume of 10 ul containing 0.3 M KCl, therefore maintaining 60 mM KCl in the assay). DNA polymerase alpha activity was assayed according to Materials and Methods (Assay of DNA Polymerase Alpha Activity in HeLa Cell Nuclear Salt Extracts).
The amount of $[^{3}H]\text{-dTMP}$ incorporated into acid precipitated DNA was determined with a Beckman LS-7000 liquid scintillation spectrometer. The data, recorded as the mean of three replicate assay tubes, did not exceed a variability of $\pm 10\%$ of the assay data in individual tubes.

**Extraction and Partial Purification of Histones from HeLa Cell Nuclei**

Histones were extracted from HeLa cell nuclei and partially purified according to procedures developed by Lough (61). Nuclei were isolated and counted using procedures described in Materials and Methods (Assay of DNA Synthesis in HeLa Cell Nuclei). The nuclear suspension was centrifuged (2000 rpm/5 min./$4^\circ\text{C}$) and the buffer A supernatant was removed. The nuclear pellet was resuspended in 1 ml of histone extraction buffer (HEB) ($160 \text{ mM NaCl, 1 mM PMSF, 5 mM NaHSO}_3$) per $100 \times 10^6$ nuclei and an aliquot of concentrated $\text{HCl}$ was added to bring the final $\text{HCl}$ concentration to $0.5 \text{ N}$. This suspension was transferred to an appropriately sized pre-chilled homogenizer and 10 pestle strokes were applied to lyse the nuclei. The nuclear lysate was held on ice for 30 minutes before being transferred to a small Corex tube and centrifuged (2000 rpm/5 min./$4^\circ\text{C}$). The supernatant containing acid soluble proteins was saved and the remaining pellet was extracted again on ice with 1 volume of $0.25 \text{ N HCl}$ (1 ml per $100 \times 10^6$ nuclear equiva-
lents) for 15 minutes. This suspension was centrifuged (2000 rpm/5 min./4°C) and the supernatant was pooled with the extract obtained in the previous step. The combined histone extracts were placed into a 3500 MW cutoff dialysis tube and dialyzed overnight in deionized glass distilled water at 4°C.

The dialyzed extract was transferred to a small Corex tube, shell frozen using liquid nitrogen, and lyophilized to dryness. Following this, the dried protein fraction remaining was solubilized in 10 ul of sample buffer (62.5 mM Tris-HCl (pH 6.8), 2.3 % w/v sodium dodecyl sulfate (SDS), 10% v/v glycerol, 5% v/v B-mercaptoethanol, 0.04 mg/ml bromophenol blue) per 3 X 10^6 nuclear equivalents. Small aliquots were removed and stored frozen at -20°C until used.

The partially purified histone preparation was analyzed using discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (89). Aliquots containing 1.5-6 X 10^6 nuclear equivalents of histone were placed on a 1 mm cross sectional 18% acrylamide SDS-slab gel along with high and low molecular weight protein standards and electrophoresed at 15 mA until the tracking dye (bromophenol blue) reached the bottom of the gel. The gel was stained with Coomasie brilliant blue (0.25% in 10% v/v glacial acetic acid and 50% v/v methanol in water) to resolve the protein bands. Photographs of the gel were taken utilizing Polaroid 55 Professional Land Film.

The 3500 MW cutoff dialysis tubing was obtained from
Spectrum Medical Industries Inc. (Los Angeles, CA). The reagents used for SDS-PAGE including ammonium persulfate, acrylamide, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), glycine, bromophenol blue, and B-mercaptoethanol were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulfate and the molecular weight protein standards were obtained from Biorad Co. (Richmond, CA). Coomasie brilliant blue G-250 was obtained from Eastman-Kodak Co. (Rochester, NY).

In Vitro Phosphorylation Assay of HeLa Cell Nuclear Proteins

The in vitro phosphorylation of HeLa cell nuclear proteins was studied at various time periods of the cell cycle. This was done by procedures developed by Verma and Chen (67). Nuclei were isolated and counted at a desired time period by procedures described in Materials and Methods (Assay of DNA Synthesis in HeLa Cell Nuclei). After counting the nuclei, the nuclear suspension was centrifuged (2000 rpm/5 min./4°C) and the supernatant was removed. The nuclear pellet was resuspended in 1 ml buffer A per 100 X 10^6 nuclei and 100 ul aliquots (approximately 10 X 10^6 nuclei) were pipetted into pre-chilled Eppendorf tubes along with 50 ul of phosphorylation assay mixture (3 mM EGTA, 150 mM KF, 30 mM magnesium acetate, 3 mM sodium molybdate, 1 mM PMSF, and 15 uM (3.1 X 10^7 cpm/nmol) [*-32P]-ATP). The Eppendorf assay tubes containing 150 ul of assay components
were incubated for 30 minutes at room temperature. After incubation, 30 ul of stop solution (12% w/v SDS, 0.5 M Tris-HCl, pH 9.0, 5 mM EDTA, 25% w/v sucrose, 10% v/v B-mercaptoethanol, and 0.1% w/v bromophenol blue) was added to each assay tube. The assay tubes were placed into a boiling water bath for 3 minutes, quick-frozen with liquid nitrogen, and stored at -70°C until used.

The phosphorylation assay mixture was analyzed using discontinuous SDS-PAGE as described by Laemmli (90). Aliquots of assay samples were applied to a 1 mm cross sectional 15% acrylamide SDS-slab gel along with molecular weight standards and partially purified histone preparation (see Materials and Methods: Extraction and Partial Purification of Histones from HeLa Cell Nuclei) and electrophoresed at approximately 20-25 mA until the tracking dye reached the bottom of the gel. The gel was stained and destained as described in Materials and Methods (Extraction and Partial Purification of Histones from HeLa Cell Nuclei) and vacuum dried onto Whatman filter paper using a Hoefer Scientific Instruments (San Francisco, CA) Model SE1140 slab gel dryer. An autoradiograph was made by exposing the dried gel on Kodak X-ray film (Eastman-Kodak Co., Rochester, NY) for an appropriate period of time.

[γ-32P]-Adenosine 5'-triphosphate tetra(triethylammonium) salt ([γ-32P]-ATP; 3000 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, MA). The chemicals used in the phosphorylation assay and stop solution such as EDTA,
KF, magnesium acetate, and sodium molybdate were obtained from Fisher Scientific Co. (Fair Lawn, NJ). Sucrose was obtained from Schwarz/Mann (Orangeburg, NY).

**Immunoprecipitation of DNA Polymerase Alpha from HeLa Cell Lysate Salt Extracts**

Procedures used for the immunoprecipitation of DNA polymerase alpha from lysate salt extracts obtained from $[^{35}\text{S}]$-methionine labeled HeLa cells were developed in collaboration with Dr. Andrew P. Laudano, Dept. of Biochemistry, University of New Hampshire (Durham, NH). When DNA synthesis was initiated, 5 X $10^6$ HeLa cells were resuspended in 10 ml of methionine-free modified BME medium supplemented with 0.5 mCi of $[^{35}\text{S}]$-methionine and grown in a rotary shaker-incubator for 3-4 hours at 37°C. Following this, lysate salt extracts were prepared using procedures described in Materials and Methods (Assay of DNA Polymerase Alpha in HeLa Cell Lysate Salt Extracts) except that the cell pellet was resuspended in 0.4 ml of buffer A per 5 X $10^6$ cells before being homogenized. After the cells were Dounce homogenized, 0.1 ml of buffer A containing 1.5 M KCl was added to the lysate and this was maintained on ice for 30 minutes with occasional swirling. The lysate was then transferred to a small Corex tube and centrifuged (2000 rpm/5 min./4°C) and the supernatant was saved as the lysate salt extract. The lysate salt extract was transferred into
Eppendorf tubes, quickly frozen with liquid nitrogen, and stored at -70°C until used.

The lysate salt extracts were pre-cleared with protein A-agarose-nonimmune IgG to remove non-specific binding proteins. The protein A-agarose-nonimmune IgG complex was made by adding 0.5 ml of nonimmune rabbit serum to 1.0 ml of a 50% v/v suspension of protein A-agarose in immunoprecipitation wash buffer (buffer A containing 0.15 M KCl and 0.05% v/v Triton X-100) in a conical centrifuge tube. This suspension was mixed by occasional and gentle agitation for 30 minutes at 4°C. The suspension was then washed with 10 ml of immunoprecipitation wash buffer and briefly centrifuged. The supernatant was removed by aspiration and the agarose pellet was washed 2 more times as described above. Following the final wash, the protein A-agarose-nonimmune IgG pellet was resuspended in 0.5 ml of immunoprecipitation wash buffer to give a 50% v/v suspension. An aliquot of 0.4 ml of the 50% v/v suspension of protein A-agarose-nonimmune IgG was placed into an Eppendorf tube and briefly centrifuged. The supernatant was removed by aspiration and the agarose pellet was resuspended in 0.2 ml of thawed lysate salt extract. This suspension was maintained at 4°C with occasional and gentle agitation. Following this, the suspension was briefly centrifuged and the supernatant was removed and saved as the pre-cleared lysate salt extract.

The immunoprecipitation assay was performed by adding the following into each Eppendorf assay tube: 50 ul pre-cleared
lysate salt extract, 50 ul immunoprecipitation wash buffer or radioimmunoprecipitation assay buffer (RIPA) (0.01 M potassium phosphate buffer (pH 7.4), 0.15 M NaCl, 0.1% w/v SDS, 1% v/v Nonidet-P40, and 1% w/v sodium deoxycholate), 20 ul (3 mg/ml) ovalbumin, and 10 ul nonimmune serum or anti-DNA polymerase alpha IgG preparation. In order to show specific IgG interactions with DNA polymerase alpha, blocking assays were occasionally performed by adding an aliquot (30 ug) of purified DNA polymerase alpha core enzyme to assay tubes containing the anti-DNA polymerase alpha IgG. The Eppendorf assay tubes were maintained at 4°C for 1-2 hours with occasional and gentle agitation. Following this, 50 ul of a 50% suspension of protein A-agarose in immunoprecipitation wash buffer was added to each Eppendorf assay tube. This was maintained at 4°C for another 30 minutes with occasional and gentle agitation. The suspension was briefly centrifuged and the supernatant was removed by aspiration. The protein A-agarose complex was washed 4 times by adding 1 ml of RIPA buffer to each Eppendorf assay tube, briefly vortexed and centrifuged, and the supernatant was removed by aspiration. The protein A-agarose complex was resuspended in 25 ul sample buffer (62.5 mM Tris-HCl (pH 6.8), 2.3% w/v SDS, 10% v/v glycerol, 5% v/v B-mercaptoethanol, and 0.04 mg/ml bromophenol blue), boiled for 3 minutes, centrifuged briefly, and stored at -70°C until used.

Samples of the thawed [35S]-methionine labeled immuno-
precipitates were analyzed by using discontinuous SDS-PAGE as described by Laemmli (89). Aliquots of the samples were placed on a 7.5-8% acrylamide SDS-slab gel and electrophoresed at 20-25 mA until the tracking dye reached the bottom of the gel. The gel was stained and destained according to procedures described in Materials and Methods (Extraction and Partial Purification of Histones from HeLa Cell Nuclei). The gel was then washed for 30 min. with distilled water and allowed to soak for 30 min. in a 1 M solution of sodium salicylate. The gel was vacuum dried and exposed on X-ray film as described in Materials and Methods (In Vitro Phosphorylation Assay of HeLa Cell Nuclear Proteins).

\[^{35}S\]-methionine (>800 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, MA) or ICN Radiochemicals (Irvine, CA). Protein A-agarose was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). The components used in the preparation of RIPA buffer were obtained as follows: Nonidet P-40, Bethesda Research Laboratories (Gaithersburg, MD), potassium phosphate, J. T. Baker Co. (Philipsburg, PA), and sodium deoxycholate, Sigma Chemical Co. (St. Louis, MD).

The purified DNA polymerase alpha core enzyme and rabbit anti-DNA polymerase alpha polyclonal IgG preparations were obtained from Dr. Earl F. Baril, The Worcester Foundation for Experimental Biology (Shrewsbury, MA). The mouse anti-DNA polymerase alpha monoclonal IgG preparations were
obtained from Dr. Robert Hickey, The Worcester Foundation for Experimental Biology (Shrewsbury, MA). The rabbit non-immune serum was obtained from Dr. Andrew P. Laudano, The University of New Hampshire (Durham, NH).
III RESULTS AND DISCUSSION

S-phase DNA Synthesis in Synchronized Control and DFMO-Inhibited HeLa Cells

HeLa cells were synchronized for S-phase DNA synthesis using the double thymidine (Tdr) block procedure (see MATERIALS AND METHODS). TABLE 2 shows a summary of the synchronization procedure and procedures developed to deplete cells of polyamines by the addition of 1 mM ad-difluoromethylornithine (DFMO).

Cells were assayed for DNA synthesis by measuring \[^{3}\text{H}]\text{-Tdr incorporation at 3 hour intervals following the initiation of S-phase by using procedures described in my M.S. Thesis entitled: The Role of Polyamines in HeLa Cell DNA Synthesis (1986), (see MATERIALS AND METHODS; Analysis of DNA Synthesis in Synchronized HeLa Cells).

As shown in FIGURE 2, synchronized HeLa cells had a peak of DNA synthesis approximately 3 hours following the initiation of S-phase DNA synthesis at 0h (i.e. the removal of the 2nd Tdr block at 72 hours is equal to 0h).

The cells grown in the presence of the polyamine biosynthesis inhibitor DFMO exhibited both severely inhibited and asynchronous DNA synthesis.
### TABLE 2

**SYNCHRONIZATION AND POLYAMINE-DEPLETION OF HELA CELL CULTURES**

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>0h</th>
<th>32h</th>
<th>48h</th>
<th>56h</th>
<th>72h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>BME(^a)</td>
<td>Tdr(^b)</td>
<td>BME(^c)</td>
<td>Tdr</td>
<td>BME</td>
</tr>
<tr>
<td>DFMO(^d)</td>
<td>BME+ DFMO</td>
<td>Tdr DFMO</td>
<td>BME+ DFMO</td>
<td>Tdr DFMO</td>
<td>BME+ DFMO</td>
</tr>
</tbody>
</table>

\(^a\) Basal Modified Eagle's Medium  
\(^b\) Thymidine  
\(^c\) At 48 and 72 hours, the cells are washed 2X with serum-free BME before being resuspended in fresh BME  
\(^d\) DFMO = α-difluoromethylornithine
FIGURE 2

S-PHASE DNA SYNTHESIS IN SYNCHRONIZED CONTROL AND DFMO-INHIBITED HELA CELLS

Control (○) and DFMO-treated (△) HeLa Cells were synchronized by the double thymidine block procedure. DFMO cells were grown in medium containing 1 mM DFMO throughout the synchronization procedure. Cells were isolated at 3-hour intervals following the initiation of S-phase DNA synthesis at 0 hours and assayed for DNA synthesis by [^3H]-thymidine (Tdr) incorporation into DNA. See M.S Thesis entitled: The Role of Polyamines in HeLa Cell DNA Synthesis, (1986). (MATERIALS AND METHODS; Analysis of DNA Synthesis in Synchronized HeLa Cells)
Spermidine (20 uM) was supplemented to DFMO-inhibited HeLa cells -10h, -8h, and -6h (i.e. 62h, 64h, and 66h into the synchronization procedure) prior to S-phase DNA synthesis to determine the period of time needed to reverse DFMO-inhibited HeLa cell DNA synthesis. The designations DFMO/SD62, SD64, and SD66 were used to identify these cultures. Following the initiation of S-phase DNA synthesis at 0h (72h), the cell cultures were again supplemented with spermidine and assayed for DNA synthesis by measuring [3H]-Tdr incorporation into DNA at 3 hour intervals as described in my M.S. Thesis entitled: The Role of Polyamines in HeLa Cell DNA Synthesis (1986), (see MATERIALS AND METHODS; Analysis of DNA Synthesis in Synchronized HeLa Cells). The Results shown in FIGURE 3 indicate that the addition of spermidine (20 uM) to DFMO-inhibited HeLa cells 10 hours prior to S-phase DNA synthesis (i.e. DFMO/SD62) fully reversed this inhibition. Both DNA synthesis and the synchronization pattern were comparable to control HeLa cells. However, when spermidine was added to DFMO-inhibited HeLa cells at later times of the synchronization protocol (i.e. DFMO/SD64 and DFMO/SD66), DNA synthesis remained inhibited and asynchronous to various degrees.

These results indicated that the reversal of inhibited DNA synthesis in polyamine-depleted HeLa cells is a time-dependent process requiring at least 10 hours. It is
FIGURE 3

REVERSAL OF THE DFMO-INDUCED INHIBITION OF HELA CELL DNA SYNTHESIS BY SPERMIDINE

Control (○), DFMO (△), DFMO/SD_{62} (■), DFMO/SD_{64} (●), and DFMO/SD_{66} (▲) treated HeLa cells were synchronized using the double thymidine block procedure. 20 μM spermidine was added to and maintained in the respective cultures at the time of the synchronization procedure indicated. Cells were isolated at 3-hour intervals following the initiation of S-phase DNA synthesis and assayed for DNA synthesis by [^{3}H]-Tdr incorporation into DNA. (See MATERIALS AND METHODS: Analysis of DNA Synthesis in Synchronized HeLa Cells, [85])
suggested that polyamines may be necessary for the synthesis or organization of DNA replication factors into active replication complexes required for normal S-phase DNA synthesis. Since the addition of spermidine later in the synchronization procedure failed to restore a synchronized peak of DNA synthesis 3 hours after initiating S-phase DNA synthesis, it also may also be suggested that polyamines are essential for the normal traverse of cells through G1-phase and into S-phase of the cell cycle.

Determination of the Critical Time Period of the HeLa Cell Cycle for Polyamine Enrichment

Spermidine (20 uM) was supplemented to HeLa cell cultures at various time periods and lengths of time prior to the initiation of S-phase DNA synthesis in an attempt to determine the critical time period in which spermidine must be present to reverse DFMO-inhibited HeLa cell nuclear DNA synthesis. Spermidine was maintained in the cell cultures for lengths of time ranging from 16 hours (i.e. DFMO/SD_{56-72}), 4 hours (i.e. DFMO/SD_{56-60}), and 1 hour (i.e. DFMO/SD_{56-57}, SD_{58-59}, SD_{60-61}, and SD_{62-63}) to determine whether 1-hour exposures of DFMO-inhibited HeLa cells to spermidine can be effective in reversing the DFMO-inhibited DNA synthesis.

Nuclei were isolated from the various cell cultures 3 hours after the initiation of S-phase DNA synthesis. DNA synthesis was assayed by measuring \(^{3}H\)-dTMP incorporation
into DNA. (see MATERIALS AND METHODS)

The results in TABLE 3 show that nuclei isolated from DFMO-inhibited HeLa cells exhibited only approximately 25% of the amount of DNA synthesis as seen in control nuclei. The addition of spermidine at different time periods and lengths of time reversed the inhibition of nuclear DNA synthesis from 44% to >100% as compared to control cell nuclei.

Although these results are inconclusive as to the critical time period of the cell cycle in which polyamine must be added to reverse the DFMO-inhibition of HeLa cell nuclear DNA synthesis, they do suggest that polyamines must be added to the cell culture at least 10 hours before S-phase to fully reverse the DFMO-inhibited DNA synthesis.

The results in TABLE 3 also show that the addition of spermidine for only 1 hour time-periods seemed to be as effective in reversing DFMO-inhibited DNA synthesis as extended time periods in the presence of spermidine. This seems to indicate that cellular pools of polyamines can be rapidly replenished by polyamine-depleted HeLa cells resulting in normal S-phase DNA synthesis.
TABLE 3

DETERMINATION OF THE CRITICAL TIME PERIOD OF THE HE LA CELL CYCLE FOR SPERMIDINE ENRICHMENT TO REVERSE THE DFMO-INDUCED INHIBITION OF HE LA CELL NUCLEAR DNA SYNTHESIS

Control and DFMO HeLa cells were synchronized using the double thymidine block procedure. Spermidine (20 μM) was supplemented to the cell cultures at the time of the synchronization period indicated. Nuclei were isolated 3 hours into S-phase and assayed for [³H]-dTMP incorporation into DNA. (see MATERIALS AND METHODS)

<table>
<thead>
<tr>
<th>Source of Nuclei</th>
<th>pMol [³H]-dTMP incorp./10⁶ nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>exp.1</td>
</tr>
<tr>
<td>Control</td>
<td>6.2</td>
</tr>
<tr>
<td>DFMO</td>
<td>1.5</td>
</tr>
<tr>
<td>DFMO/SD6-72,72</td>
<td>4.3</td>
</tr>
<tr>
<td>DFMO/SD56-60</td>
<td>---</td>
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<tr>
<td>DFMO/SD56-57</td>
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<tr>
<td>DFMO/SD58-59</td>
<td>---</td>
</tr>
<tr>
<td>DFMO/SD60-61</td>
<td>---</td>
</tr>
<tr>
<td>DFMO/SD62-63</td>
<td>---</td>
</tr>
</tbody>
</table>

SD = spermidine
Reversal of the DFMO-induced Inhibition of HeLa Cell Proliferation by Spermidine

Spermidine (20 uM) was supplemented to DFMO-inhibited HeLa cell cultures at various times prior to the initiation of S-phase DNA synthesis (72h) to determine the time period needed to reverse DFMO-inhibited HeLa cell proliferation.

The results in FIGURE 4 indicate that spermidine must be supplemented to a DFMO-inhibited HeLa cell culture at least 10 hours before S-phase is initiated to reverse the DFMO-inhibition of proliferation. These results coincide well with the results obtained for the reversal of HeLa cell DNA synthesis by spermidine supplementation.

These results suggest that the reversal of the inhibition of proliferation of DFMO-inhibited HeLa cells by spermidine is also a time-dependent process which requires at least 10 hours.

Reversal of the DFMO-induced Inhibition of DNA Polymerase Alpha Activity by Spermidine

Spermidine (20 uM) was supplemented to a polyamine-depleted HeLa cell culture 10 hours before the initiation of S-phase DNA synthesis (i.e., 62 hours into the synchronization procedure) to determine whether DFMO-inhibition of DNA polymerase alpha activity could be reversed. HeLa cell nuclear salt extracts (NSE) were assayed for DNA polymerase alpha activity at 3-hour
FIGURE 4

REVERSAL OF THE DFMO-INDUCED INHIBITION OF HEla CELL PROLIFERATION BY SPERMIDINE

Control (○), DFMO (△), DFMO/SD62 (□), DFMO/SD64 (●), DFMO/SD66 (▲), and DFMO/SD72 (□) treated HeLa cells were synchronized using the double thymidine block procedure. 20 uM spermidine was added to and maintained in the respective cultures at the time of the synchronization procedure indicated. Cells were counted at 3-hour intervals beginning 9 hours after S-phase DNA synthesis was initiated by the use of a Royco automatic cell counter. (see MATERIALS AND METHODS)
% PROLIFERATION

TIME (h)
intervals following the initiation of S-phase DNA synthesis by measuring the incorporation of $[^3H]$-dTMP into "activated" calf thymus DNA templates.

The results in FIGURE 5 show that a peak of DNA polymerase alpha activity of about the same magnitude occurs in both control and DFMO/SD$_{62}$ NSE's at approximately 3-6 hours following the initiation of S-phase DNA synthesis. The NSE obtained from polyamine-depleted HeLa cells exhibited only 50% of the activity of the control HeLa cell NSE.

These results correlate well with results obtained for the reversal of DFMO-inhibited HeLa cell DNA synthesis and proliferation. It seems that at least 10 hours is also needed for polyamine-depleted HeLa cells to reverse DFMO-inhibited DNA polymerase alpha activity.

**Viability of HeLa Cells in a Thymidine-Synchronized Cell Culture**

Studies were performed on control, DFMO, and DFMO/SD$_{60}$ cell cultures at various times following the initiation of S-phase DNA synthesis to determine if inhibited cellular processes in polyamine-depleted cells actually occur because of a decrease in the number of viable cells present.

The results in TABLE 4 show that the viability rates of synchronized control and polyamine-depleted cells were 89% and 85% respectively 24 hours after S-phase. These results suggest that loss of viability in polyamine-depleted cells
FIGURE 5

REVERSAL OF THE DFMO-INDUCED INHIBITION OF DNA POLYMERASE ALPHA ACTIVITY BY THE ADDITION OF SPERMIDINE

Control (○), DFMO (△), and DFMO/SD₆₂ (■) treated HeLa cells were synchronized using the double thymidine block procedure. Spermidine (20 μM) was added to and maintained in the DFMO/SD₆₂ cell culture at the time of the synchronization procedure indicated. Nuclei were isolated at S-phase and at 3-hour intervals following the initiation of S-phase DNA synthesis. Nuclei were salt-extracted for 30 min. with buffer A containing 0.3 M KCl and the nuclear salt extracts were assayed for DNA polymerase alpha activity. (see MATERIALS AND METHODS).
Control, DFMO, and DFMO/SD<sub>60</sub> HeLa cells were synchronized using the double thymidine block procedure. Spermidine (20 μM) was added to and maintained in the DFMO/SD<sub>60</sub> cell culture 60 hours into the synchronization procedure (i.e. 12 hours before S-phase DNA synthesis is initiated). Cells were monitored for viability at the times indicated following the initiation of S-phase DNA synthesis (0h). (see MATERIALS AND METHODS)

<table>
<thead>
<tr>
<th>Source of Cells</th>
<th>% Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0h</td>
</tr>
<tr>
<td>Control</td>
<td>96</td>
</tr>
<tr>
<td>DFMO</td>
<td>92</td>
</tr>
<tr>
<td>DFMO/SD&lt;sub&gt;60&lt;/sub&gt;</td>
<td>95</td>
</tr>
</tbody>
</table>

SD= spermidine
is not a major factor in the inhibition of DNA synthesis, proliferation, and DNA polymerase alpha activity.

Summary of Reconstitution of HeLa Cell Subnuclei

One of the aims of my M.S. Thesis entitled: The Role of Polyamines in HeLa Cell DNA Synthesis, (1986), was to try to establish the location of the deficiency of DNA synthesis caused by polyamine-depletion via DFMO. This was done by performing nuclear reconstitution experiments such as those described by Brun and Weissbach (86), Enomoto et al. (87), and in my M.S. Thesis (85) (see MATERIALS AND METHODS; Fractionation and Reconstitution of Isolated HeLa Cell Nuclei), where HeLa cell nuclei are fractionated into 2 "inactive" components by salt-extraction and then reconstituted to regain an "active" DNA synthesizing unit. The 2 components, nuclear salt extract (NSE) and salt-extracted nuclei (SEN) of both control and DFMO-inhibited HeLa cells can be reconstituted in various combinations to determine which fraction of the DFMO-inhibited cell was most deficient.

A summary of several experiments are shown in FIGURE 6. When the NSE obtained from DFMO-inhibited HeLa cells is reconstituted with control HeLa cell SEN, a slight increase in DNA synthesis is observed. However, NSE obtained from control HeLa cells could not stimulate DNA synthesis in DFMO-inhibited HeLa cell SEN. This suggests that a partial
Control (C) and DFMO-treated (D) HeLa cells were synchronized using the double thymidine block procedure. Nuclei were isolated and fractionated by salt extraction 3 hours into S-phase. Whole nuclei, salt-extracted nuclei (SEN), and SEN reconstituted with 3X nuclear salt extract (SE) equivalents were assayed for $[^3\text{H}]-\text{dTMP}$ incorporation into DNA. (see MATERIALS AND METHODS: Fractionation and Reconstitution of Isolated HeLa Cell Nuclei, [85])
deficiency exists in the soluble enzyme fraction of DFMO-
inhibited cell nuclei; however, a major deficiency seems to
exist in the DNA template of DFMO-inhibited HeLa cell SEN.
Since it also has been shown (85) that the DNA polymerase
alpha activity in DFMO-inhibited HeLa cell nuclei is only 
50% as compared to control cell nuclei, a major thrust of
my dissertation research was to examine the deficiency of
DNA polymerase alpha in polyamine-depleted HeLa cells.
Also, several experiments were performed to try to
characterize the deficiency which is found in the polyamine-
depleted HeLa cell nuclear template.

DNA Polymerase Alpha Activity of Control and DFMO Nuclear
Salt Extracts on "Activated" and Heat-denatured Calf Thymus
DNA

Several investigators have used various DNA template
preparations to study the DNA polymerase alpha holoenzyme or
subunit fractions (90-93). It has been shown that DNA
polymerase alpha holoenzyme preparations have the ability to
synthesize DNA on templates containing a high base/primer
ratio (i.e., heat-denatured DNA), whereas an incomplete
enzyme lacking subunits such as RNA primase or C1C2 primer
recognition proteins would be inhibited. Templates
containing a low base/primer ratio (i.e., DNase I "activated" DNA) support similar rates of DNA synthesis with
either the DNA polymerase alpha holoenzyme or the DNA
polymerase alpha core enzyme.
Since it has been shown that there is a deficiency of DNA polymerase alpha in DFMO-inhibited HeLa cells (85), several studies were performed using both "activated" and heat-denatured DNA templates in an attempt to determine whether a subunit (i.e., DNA primase or C1C2 primer recognition protein) deficiency exists in NSE's obtained from these cells.

The results in TABLE 5 indicate that the NSE's obtained from DFMO-inhibited HeLa cells 3 hours after S-phase initiation show no significant decrease in the utilization of heat-denatured DNA as a template. This suggests that the deficiency of DNA polymerase alpha in DFMO-inhibited cells is not due to a lack of subunit integrity, but rather to a decreased level of enzyme, or enzyme activity due to post-translational modifications such as described by Donaldson and Gerner (94).

DNA Polymerase Alpha Activity of NSE's and LSE's of Control and DFMO HeLa Cells on "Activated" Calf Thymus DNA Templates

DNA polymerase alpha assays were also performed on salt-extracted whole cell lysates (LSE) from control and DFMO-inhibited HeLa cells. The LSE's obtained contain DNA polymerase alpha from both the cytosol fraction and nucleus of the cells.

TABLE 6 shows results of 4 experiments in which both NSE's and LSE's obtained from control and DFMO-inhibited HeLa cells were assayed for DNA polymerase alpha activity on
TABLE 5

DNA POLYMERASE ALPHA ACTIVITIES OF CONTROL AND DFMO-INHIBITED HEla CELL NUCLEAR SALT EXTRACTS ON "ACTIVATED" AND HEAT-DENATURED CALF THYMUS DNA

Control (C) and DFMO-treated (D) HeLa cells were synchronized using the double thymidine block procedure. Nuclei were isolated 3 hours after the initiation of S-phase DNA synthesis and saltexttracted for 30 min. using buffer A containing 0.3 M KCl. Nuclear salt extracts (NSE) were assayed for DNA polymerase alpha activity using either "activated" (Act.) or heat-denatured (H.D.) calf thymus DNA templates. (see MATERIALS AND METHODS)

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>exp.1</td>
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<tr>
<td>Control (Act.)</td>
<td>23 (100)</td>
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<td>DFMO (Act.)</td>
<td>9.2 (40)</td>
</tr>
<tr>
<td>Control (H.D.)</td>
<td>23 (100)</td>
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<tr>
<td>DFMO (H.D.)</td>
<td>7.3 (32)</td>
</tr>
</tbody>
</table>

NE= nuclear equivalents
(numbers in parentheses are the normalized results with activity of control NSE= 100)
TABLE 6

DNA POLYMERASE ALPHA ACTIVITIES OF CONTROL AND DFMO-INHIBITED HELA CELL NUCLEAR AND LYSATE SALT EXTRACTS ON "ACTIVATED" CALF THYMUS DNA

Control (C) and DFMO-treated (D) HeLa cells were synchronized using the double thymidine block procedure. Nuclei were isolated and lysates were made 3 hours after the initiation of S-phase DNA synthesis. Both the nuclei and the lysates were salt-extracted for 30 min. with buffer A containing 0.3 M KCl. Nuclear salt extracts (NSE's) and lysate salt extracts (LSE's) were assayed for DNA polymerase alpha activity using a "activated" (Act.) calf thymus DNA template. (see MATERIALS AND METHODS)

<table>
<thead>
<tr>
<th>Source of NSE</th>
<th>pMol $[^{3}H]-$dTMP incorp./$10^6$ NE/CE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>exp.1</td>
</tr>
<tr>
<td>Control (Act.)</td>
<td>14 (100)</td>
</tr>
<tr>
<td>DFMO (Act.)</td>
<td>9.7 (69)</td>
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</table>

<table>
<thead>
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<th>Source of LSE</th>
<th>pMol $[^{3}H]-$dTMP incorp./$10^6$ NE/CE</th>
</tr>
</thead>
<tbody>
<tr>
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<td>19 (100)</td>
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<tr>
<td>DFMO (Act.)</td>
<td>17 (90)</td>
</tr>
</tbody>
</table>

NE/CE= nuclear equivalents/cell equivalents
(numbers in parentheses are the normalized results with activity of control NSE's and LSE's= 100)

80
"activated" calf thymus DNA 3 hours after S-phase DNA synthesis was initiated.

As seen by these results, NSE's obtained from DFMO-inhibited HeLa cells had only ~50% of the DNA polymerase alpha activity as compared to control NSE's. However, LSE's from both control and DFMO-inhibited HeLa cells contained similar DNA polymerase activity levels. These results indicate that although NSE's obtained from DFMO-inhibited HeLa cells have inhibited DNA polymerase alpha activity, whole cell LSE's obtained from the same cells contain as much DNA polymerase alpha as control whole cell LSE's. This suggests that polyamine-depletion by DFMO may prevent DNA polymerase alpha from being transported into the nucleus from the cytosol, or allow the DNA polymerase alpha to exit freely from the nucleus.

DNA Polymerase Alpha Activity of NSE's and LSE's of Control and DFMO HeLa Cells on Heat-denatured Calf Thymus DNA

NSE's and LSE's obtained from both control and DFMO-inhibited HeLa cells were assayed for DNA polymerase alpha activity on heat-denatured calf thymus DNA templates 3 hours after S-phase was initiated. This was performed to determine whether the DNA polymerase alpha obtained from control and polyamine-depleted HeLa cell LSE's is a complete enzyme (holoenzyme), or lacked specific subunits such as DNA primase or C1C2 primer recognition proteins.

The results in TABLE 7 show that NSE's obtained from
DFMO-inhibited cells contain less than 50% of the DNA polymerase alpha activity as control NSE's. However, LSE's from DFMO-inhibited cells contained as much DNA polymerase alpha activity as LSE's from control cells. These results, along with the results previously discussed in TABLE 6, suggest that the DNA polymerase alpha enzyme is complete in both control and polyamine-depleted HeLa cells; however, a transport problem may exist in polyamine-depleted HeLa cells which may prevent DNA polymerase alpha from entering the nucleus, or allowing it to exit freely from the nucleus.

**Time Course Experiment Examining DNA Polymerase Alpha Activities of Control and DFMO-inhibited HeLa Cell Nuclear and Lysate Salt Extracts on "Activated" Calf Thymus DNA**

A time course experiment was performed in which NSE's and LSE's from both control and DFMO-inhibited HeLa cells were assayed for DNA polymerase alpha on "activated" calf thymus DNA templates 12h and 6h before S-phase DNA synthesis was initiated (i.e. -12h and -6h), at the time S-phase DNA synthesis is initiated (i.e. 0h), and at 3 hour intervals following S-phase DNA synthesis initiation (i.e. 3h, 6h, and 9h). (TABLE 8)

This experiment was performed in an attempt to determine whether a pattern exists between LSE and NSE DNA polymerase alpha which may indicate a "transport" problem between the cytosol and nucleus of polyamine-depleted HeLa cells.
Control (C) and DFMO-treated (D) HeLa cells were synchronized using the double thymidine block procedure. Nuclei were isolated and lysates were made 3 hours after the initiation of S-phase DNA synthesis. Both the nuclei and lysates were salt-extracted for 30 min. with buffer A containing 0.3 M KCl. Nuclear salt extracts (NSE's) and lysate salt extracts (LSE's) were assayed for DNA polymerase alpha activity using a heat-denatured (H.D.) calf thymus DNA template. (see MATERIALS AND METHODS)

<table>
<thead>
<tr>
<th>Source of NSE</th>
<th>pMol $[^{3}H]$-dTMP incorp./$10^6$ NE/CE</th>
<th>exp.1</th>
<th>exp.2</th>
<th>exp.3</th>
<th>exp.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (H.D.)</td>
<td>20 (100)</td>
<td>16 (100)</td>
<td>22 (100)</td>
<td>13 (100)</td>
<td></td>
</tr>
<tr>
<td>DFMO (H.D.)</td>
<td>7.6 (38)</td>
<td>6.7 (42)</td>
<td>9.0 (41)</td>
<td>6.0 (46)</td>
<td></td>
</tr>
<tr>
<td>Source of LSE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (H.D.)</td>
<td>33 (100)</td>
<td>19 (100)</td>
<td>21 (100)</td>
<td>28 (100)</td>
<td></td>
</tr>
<tr>
<td>DFMO (H.D.)</td>
<td>26 (79)</td>
<td>18 (95)</td>
<td>22 (105)</td>
<td>23 (82)</td>
<td></td>
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</table>

NE/CE= nuclear equivalents/cell equivalents

(numbers in parentheses are the normalized results with activity of control NSE's and LSE's= 100)
Control and DFMO-treated HeLa cells were synchronized using the double thymidine block procedure. Nuclei were isolated and lysates were prepared at the times of the cell cycle indicated (i.e. 0h= initiation of S-phase DNA synthesis). Both nuclei and lysates were salt-extracted for 30 min. with buffer A containing 0.3 M KCl. Nuclear salt extracts (NSE's) and lysate salt extracts (LSE's) were assayed for DNA polymerase alpha activity using an "activated" calf thymus DNA template. (see MATERIALS AND METHODS)

<table>
<thead>
<tr>
<th>Source of NSE</th>
<th>pMol [3H]-dTMP incorp./10⁶ NE/CE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-12h</td>
</tr>
<tr>
<td>Control</td>
<td>9.2</td>
</tr>
<tr>
<td>DFMO</td>
<td>8.5</td>
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<table>
<thead>
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<th>Source of LSE</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.6</td>
</tr>
<tr>
<td>Control</td>
<td>7.6</td>
</tr>
</tbody>
</table>

NE/CE= nuclear equivalents/cell equivalents

NOTE: 0h= initiation of S-phase DNA synthesis
As seen previously (TABLES 6 and 7), the LSE's obtained from both control and DFMO-inhibited HeLa cells exhibited comparable DNA polymerase alpha activity at all times of the cell cycle. The activity of DNA polymerase alpha in the NSE obtained from DFMO-inhibited HeLa cells 12 hours before S-phase DNA synthesis was initiated was similar to that of control HeLa cell NSE, however, as the cells approached and proceeded through S-phase, DFMO-inhibited HeLa cell NSE's exhibited a decrease of DNA polymerase alpha activity when compared to control cell NSE's.

Also, both NSE's and LSE's from both cell types exhibited a concommitant increase of DNA polymerase alpha activity at approximately 3-6 hours after intitiation of S-phase.

**Attempted Immunoprecipitation of the DNA Polymerase Alpha Holoenzyme**

In another attempt to study whether a deficiency exists in the DNA polymerase alpha holoenzyme complex (640 Kdal) in the NSE or LSE of DFMO-inhibited HeLa cells, immunoprecipitation studies were performed employing polyclonal and monoclonal antibodies directed against the DNA polymerase alpha core enzyme (180 Kdal). (see MATERIALS AND METHODS) Although some evidence (data not shown) was obtained which indicated that the 180 Kdal core protein could be immunoprecipitated by using a rabbit polyclonal antibody obtained from Dr. Earl F. Baril (The Worcester Foundation for Experimental Biology, Shrewsbury, MA), all
attempts at immunoprecipitating the holoenzyme along with its subunits (i.e. DNA primase, C1C2 primer recognition proteins, etc.) have failed.

Two possible explanations for this failure could be that the multiunit enzyme complex is very unstable when utilizing these procedures, or that the antibody interactions with the core enzyme may cause the subunits to dissociate from the enzyme complex.

Cell Cycle Dependent Phosphorylation of a Specific 31 Kdal Nuclear Protein

Several investigators have shown that polyamines may regulate phosphorylation events, which may in turn affect cellular processes such as gene regulation and the normal progression of cells through the cell cycle (66, 67).

An *in vitro* nuclear protein phosphorylation experiment was performed on control, DFMO-inhibited, and DFMO-inhibited HeLa cells supplemented with spermidine 12 hours before S-phase DNA synthesis (DFMO/SD60) to determine whether a difference exists in the nuclear protein phosphorylation patterns.

The results in FIGURE 7 show a 31 Kdal nuclear protein which became less phosphorylated as both control and DFMO/SD60 cells approached S-phase. However, DFMO cells phosphorylated this 31 Kdal protein throughout this entire time period. These results suggest that this cell cycle
FIGURE 7

CELL CYCLE DEPENDENT PHOSPHORYLATION OF A SPECIFIC 31 KDAL NUCLEAR PROTEIN

Control (C), DFMO (D), and DFMO/SD₆₀ (Dₛ₆₀) treated HeLa cells were synchronized using the double thymidine block procedure. Spermidine (20 uM) was added to and maintained in the DFMO/SD₆₀ cell culture at the time of the synchronization procedure indicated. Nuclei were isolated and assayed for in vitro nuclear protein phosphorylation 12h (lanes 1-3), 6h (lanes 5-7), and 0h (lanes 9-11) before S-phase was initiated. (see MATERIALS AND METHODS). The arrow indicates the site of the 31 Kdal nuclear protein.
event may be essential for cells to progress normally into S-phase DNA synthesis. Polyamine-depleted HeLa cells supplemented with spermidine 12 hours prior to the initiation of S-phase DNA synthesis were able to reverse this phosphorylation event before S-phase DNA synthesis is initiated.

**Isolation and Partial Purification of Histones from Control and Polyamine-depleted HeLa Cell Nuclei**

In an attempt to study the deficiency in the DNA template which prevents DFMO-inhibited HeLa cell nuclear DNA synthesis, procedures were utilized to extract and partially purify histones from both control and DFMO-inhibited HeLa cells 3 hours after S-phase was initiated. (see MATERIALS AND METHODS)

Histone preparations were electrophoresed on 18% SDS-PAGE, stained, destained, and the gel was photographed.

FIGURE 8 shows histone patterns prepared from both control (lanes 1-3) and DFMO-inhibited (lanes 7-9) HeLa cells. These results suggest that no major deficiency exists in the nuclear histone levels of polyamine-depleted HeLa cells. From the molecular weight standards, histones H1a, H1b, H3, H2b, H2a, and H4 could be resolved with molecular weights of approximately 34.5, 33.0, 18.7, 17.3, 16.5, and 14.0 Kdal respectively.
Control and DFMO-treated HeLa cells were synchronized using the double thymidine block procedure. Three hours after the initiation of S-phase DNA synthesis, nuclei were isolated from these cells and histones were extracted and partially purified. The histone preparation was solubilized in sample buffer and electrophoresed on 18% SDS-PAGE. Control lanes 1, 2, and 3 contain 1x, 2x, and 4x equivalents of sample buffer containing histones. DFMO lanes 7, 8, and 9 contain 1x, 2x, and 4x equivalents of sample buffer containing histones extracted from these cells. (see MATERIALS AND METHODS)
Attempt to Determine whether DFMO-inhibited Non-Thymidine Synchronized HeLa Cells are Blocked at a Specific Stage of the Cell Cycle

To determine whether DFMO blocks HeLa cells at a specific stage of the cell cycle, cells were grown in the presence or absence of 1 mM DFMO until the DFMO-inhibited cell culture stops proliferating (96h). At this time, the DFMO is removed from the inhibited cell culture and 20 uM spermidine was added to initiate growth of the cells (DFMO/SD0/-DFMO0). Cells were then counted at various time periods to determine the rate of proliferation.

FIGURE 9 shows the proliferation rates of control cells and DFMO/SD0/-DFMO0 cells after removal of DFMO and addition of spermidine.

Since DFMO/SD0/-DFMO0 cells show no synchronous proliferation after almost 40 hours, it seems highly unlikely that DFMO blocks these cells at a single site in cell cycle progression. However, it is very likely that DFMO blocks the cells at several different sites of cell cycle progression through late G1-phase to the beginning of S-phase.

Viability of Control, DFMO, and DFMO/SD60 HeLa Cells in a Non-synchronized Cell Culture

In order to discount the possibility that a DFMO-induced synchronization of HeLa cells cannot be achieved because of loss of viability in DFMO-inhibited HeLa cells, experiments
HeLa cells were grown for 96 hours in the presence and absence of 1 mM DFMO. Every 24 hours the cells were set at a density of 300,000 cells/ml. After 96 hours, the cells grown in the presence of DFMO were washed and supplemented with spermidine (20 μM). These cells were labeled DFMO/SD₀/-DFMO. After washing and supplementing the cells with spermidine (Oh), the cells were counted with the use of a Royco automatic cell counter at the times indicated to determine their proliferation rates. (see MATERIALS AND METHODS)
were performed in which cell viability was monitored extensively. The results in TABLE 9 show that in a non-thymidine synchronized cell system, cells grown in the presence of 1 mM DFMO for 96 hours maintained almost 90% of their viability. Control and DFMO-inhibited HeLa cell cultures supplemented with spermidine after being in the presence of DFMO for 60 hours were 98% and 96% viable respectfully. These results suggest that loss of viability in DFMO-inhibited HeLa cells is not a major factor in the inhibition of proliferation.
TABLE 9

VIABILITY OF HELA CELLS IN A NON-SYNCHRONIZED CELL CULTURE

Control, DFMO-treated, and DFMO-treated cell cultures supplemented with spermidine (20 uM) at 60h (DFMO/SD60) were grown for 96 hours in the indicated medium while maintaining a cell density of approximately 300,000 cells/ml. During this period, aliquots of cells were removed at the times indicated and monitored for cell viability. (see MATERIALS AND METHODS)

<table>
<thead>
<tr>
<th>Source of Cells</th>
<th>% Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24h</td>
</tr>
<tr>
<td>Control</td>
<td>97</td>
</tr>
<tr>
<td>DFMO</td>
<td>97</td>
</tr>
<tr>
<td>DFMO/SD60</td>
<td>---</td>
</tr>
</tbody>
</table>

SD= spermidine
IV SUMMARY

HeLa cells depleted of polyamines by DFMO exhibit inhibited and asynchronous DNA synthesis, blocked cell proliferation, and reduced nuclear DNA polymerase alpha activity. The addition of 20 μM spermidine to the culture medium of the polyamine-depleted HeLa cells at least 10-12 hours prior to the initiation of S-phase (60-62 hours of the synchronization protocol) was found to fully reverse these inhibited processes. However, these processes could not be fully restored by the addition of spermidine to polyamine-depleted cells at time periods closer to the initiation of S-phase (i.e. 8 or 6 hours). These results suggest that the reversal of these inhibited processes by the addition of spermidine is a time-dependent process requiring at least 10-12 hours.

The critical time period of polyamine-enrichment to reverse inhibited DNA synthesis in nuclei isolated from polyamine-depleted HeLa cells was also studied by adding spermidine to culture medium of polyamine-depleted HeLa cells for time-periods ranging from 16 hours to 1 hour. Although results were inconclusive as to the critical time period of the cell cycle in which polyamine must be added to reverse this inhibition, the data suggest that polyamines must be added to the cell culture at least 10 hours prior to...
S-phase to fully restore DFMO-inhibited nuclear DNA synthesis. Furthermore, "pulse" additions of spermidine for time-periods as short as 1 hour were as effective in reversing DFMO-inhibited DNA synthesis as extended time-periods in the presence of spermidine suggesting that cellular pools of polyamine are rapidly replenished.

A summary of nuclear reconstitution data previously published (85) and confirmed in this study shows that the nuclear salt extract (NSE) obtained from polyamine-depleted HeLa cell nuclei is deficient in reconstituting DNA replication in salt-extracted nuclei (SEN) obtained from control cells. However, NSE from control cell nuclei had no effect in reconstituting DNA replication in SEN obtained from polyamine-depleted cells. This suggests that the soluble proteins required for DNA synthesis in polyamine-depleted HeLa cell nuclei have somewhat reduced activity while the DNA template of these nuclei cannot be utilized for DNA synthesis.

Since the activity of DNA polymerase alpha in NSE's obtained from polyamine-depleted HeLa cell nuclei was reduced ~50% as compared with control NSE's, a large part of my dissertation research was directed towards the characterization of this apparent deficiency in DNA polymerase alpha of polyamine-depleted HeLa cells.

Two experimental systems were utilized to determine whether a deficiency existed in the DNA polymerase alpha holoenzyme complex of polyamine-depleted cells. One
approach was to determine the activity of DNA polymerase alpha in both control and polyamine-depleted HeLa cells on DNA templates containing high base/prime and low base/prime ratios (i.e. heat-denatured and DNAse I "activated" calf thymus DNA respectively). If the DNA polymerase alpha of the polyamine-depleted HeLa cells was deficient in subunits such as DNA primase or C1C2 primer recognition proteins, decreased activity on heat-denatured DNA as a template for DNA synthesis would be observed.

Since NSE's obtained from polyamine-depleted HeLa cells did not exhibit a significant decrease in the utilization of heat-denatured DNA as a template, it was concluded that the deficiency of DNA polymerase alpha in the nuclei is not due to an incomplete DNA polymerase alpha holoenzyme, but rather to decreased enzyme protein synthesis. Another possible interpretation is that the DNA polymerase alpha activity is decreased because the polyamine-depleted cells are unable to regulate the enzyme activity by post-translational modifications.

The second approach involved immunoprecipitation procedures. Polyclonal and monoclonal antibodies specific for the 180 Kdal DNA polymerase alpha core enzyme were used in an attempt to immunoprecipitate the holoenzyme complex. However, the antibodies did not immunoprecipitate the DNA polymerase alpha core enzyme along with associated subunits. Possible explanations for this failure were that the DNA polymerase alpha holoenzyme complex was very unstable and
easily disrupted, or that the antibody interactions with the DNA polymerase alpha core enzyme caused the other subunits to dissociate from the enzyme complex.

Total cellular (cytosolic and nuclear) DNA polymerase alpha activity in control and polyamine-depleted HeLa cells was studied by extracting crude lysates of cells with 0.3 M KCl. The lysate salt extracts (LSE's) obtained were assayed for DNA polymerase alpha activity on both "activated" and heat-denatured calf thymus DNA templates. LSE's of control and polyamine-depleted HeLa cells exhibited similar DNA polymerase alpha activity on both DNA templates. From these results it appears that an abnormality exists in polyamine-depleted HeLa cells which may affect the entering of release of DNA polymerase alpha from the nucleus. Since control and polyamine-depleted cell LSE's utilized heat-denatured DNA templates for DNA synthesis, it also is apparent that specific subunits such as DNA primase or $C_1C_2$ primer recognition proteins were present in the DNA polymerase alpha complexes of both cell lysates.

LSE's and NSE's from both control and polyamine-depleted cells were assayed for DNA polymerase alpha activity on "activated" DNA templates at various times of the cell cycle in an attempt to determine if the entry or release of DNA polymerase alpha from the nucleus in polyamine-depleted cells was poorly regulated. Although results were inconclusive, the activity of DNA polymerase alpha in LSE's of both control and polyamine-depleted cells remained
comparable throughout the cell cycle. Furthermore, both the NSE's and LSE's from control and polyamine-depleted cells exhibited a peak of DNA polymerase alpha activity approximately 3-6 hours following the initiation of S-phase.

Studies were performed with nuclei prepared at various time-periods prior to the initiation of S-phase to determine whether a difference exists between polyamine-depleted cell nuclei and control cell nuclei in nuclear protein phosphorylation. It was shown that a protein with a MW of approximately 31 Kdal was phosphorylated extensively in control nuclei 12 hours before S-phase. The same protein was phosphorylated approximately half as much in polyamine-depleted cell nuclei as compared to control cell nuclei. This protein phosphorylated to a reduced extent as control nuclei approached S-phase, but was continually phosphorylated in the DFMO nuclei. The addition of spermidine to cell cultures containing DFMO 12 hours before S-phase prevented the phosphorylation of this protein comparably to the control nuclei. These results indicate that polyamines are essential for the regulation of the phosphorylation of a specific protein during the cell cycle. This could be due either to a lack of the protein or protein kinase, or an inhibition of specific protein kinase activity by polyamines.

To further study the deficiency in the nuclear template of polyamine-depleted HeLa cells, histones were extracted from control and polyamine-depleted HeLa cell nuclei,
partially purified, and analyzed by SDS-PAGE. Results indicated that no difference existed in the type or quantity of histones extracted from polyamine-depleted versus control cell nuclei.

Experiments were performed to determine whether polyamine-depleted HeLa cells are arrested at a specific time of the cell cycle. Cells were grown in the presence of DFMO for 96 hours until proliferation ceased. After DFMO was removed and spermidine was added to the cell culture, proliferation studies were performed to determine whether the cells would proliferate synchronously. The results revealed that synchronous proliferation did not occur suggesting that polyamine-depleted HeLa cells are arrested throughout a broad region of the cell cycle encompassing G1 phase and early S-phase. Another explanation for these results was that the polyamine-depleted cells were non-viable due to the extended time period in the presence of DFMO. To study this question, cells were examined for viability during growth in the presence of DFMO for 96 hours. It was shown that ~90% of the polyamine-depleted HeLa cells were viable after 96 hours in the presence of 1 mM DFMO suggesting that the lack of synchronous proliferation is not due to decreased viability of polyamine-depleted cells.

Cell viability studies were also performed following the initiation of S-phase DNA synthesis in thymidine synchronized cells. It was found that cells maintained in
the presence of DFMO 24 hours after S-phase was initiated remained 85% viable as compared to 89% viability for control cells. These results suggest that the inhibition of DNA replication in polyamine-depleted HeLa cells was not due to decreased cell viability.
LIST OF REFERENCES


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