Response of Histone Turnover and Phosphorylation to X Irradiation*

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ABSTRACT: Previous studies have indicated that histone f1 turnover in cultured Chinese hamster cells could be stopped by X irradiation. Experiments were designed to determine whether there was any temporal correlation between this period of inhibited f1 turnover and the radiosensitive phosphorylation of f1. Significant rapid histone phosphorylation was detected only in fractions f1 and f2a2 after histones had been purified by either preparative polyacrylamide gel electrophoresis or IRC-50 chromatography. The phosphorylation of f2a2 was insensitive to X irradiation, while the phosphorylation of f1 was decreased by 50% 1 hr after irradiation with 800 rads.

The phosphorylation of f1 returned to 86% of control by 3-hr postirradiation. The normal turnover of f1 in chromatin was completely stopped for 3 hr after irradiation and then resumed at an accelerated rate. The concomitant reduction and recovery of f1 turnover and phosphorylation after irradiation suggest that these two biochemical properties of f1 are related to the same biological function of this histone. After irradiation, DNA replication continued at the same rate as in unirradiated cultures as measured by DNA mass increase. However, [^{3}H]thymidine incorporation into DNA was reduced by irradiation. Therefore, histone f1 phosphorylation does not appear to correlate with DNA replication *per se* but, rather, with some form of radiosensitive precursor incorporation into DNA.

A fundamental understanding of cellular radiosensitivity at the macromolecular level is necessary if a rational basis is to be established for the radiation treatment of malignant diseases. Experiments have recently been carried out in this Laboratory to determine how the metabolism of macromolecules in cultured Chinese hamster cells may be changed as a result of irradiation with relatively low doses of X radiation. These studies indicated that the turnover of histone f1, which occurs in the chromatin of exponentially growing cells (Gurley and Hardin, 1969, 1970), could be stopped for a short time by X irridiation (Gurley *et al.*, 1970a). It has also been reported (Ord and Stocken, 1968; Stevely and Stocken, 1968) that similar doses of radiation reduced the phosphorylation of histone f1. The following experiments were designed to determine whether there is any temporal correlation between the period of inhibited f1 turnover and reduction of f1 phosphorylation following irradiation. Such a correlation would suggest a possible functional relationship between these two biochemical processes.

Materials and Methods

Tissue Cultures. Chinese hamster cells (line CHO) were grown in suspension culture in F-10 medium lacking calcium (Ham, 1963), supplemented with 10% calf and 5% fetal calf sera and 100 units/ml of penicillin and 100 μg/ml of streptomycin (Tobey *et al.*, 1966). These cultures grew exponentially from 50,000 to 500,000 cells per ml with a generation time of 16–18 hr. The cell concentration was determined to a statistical precision of 1% or better using an electronic particle counter (Tobey *et al.*, 1967). To uniformly label their proteins, the cells were cultured for two generations in growth medium containing 50 μCi of reconstituted [^{14}C]protein hydrolysate[1]/l. of culture. To pulse label the cells with ^{32}P, 15 mCi of [^{32}P]H_3PO_4 [carrier-free (New England Nuclear)] was added to each liter of culture 1 hr prior to harvest.

Irradiation. Cultures (1 l.) were irradiated with 800 rads of X-rays as previously described (Walters and Petersen, 1968a). The response of this cell line to X irradiation has been studied in detail. Specific features of this response, including radiation-induced division delay, cell death, and macromolecular synthesis, have been reported earlier (Walters and Peterson, 1968a,b; Walters *et al.*, 1969, 1970; Walters and Tobey, 1970).

Preparation of Histones. Histone fractions f1, f2b, f3, f2a1, and f2a2 were obtained from the chromatin of Chinese hamster cells by the first method of Johns (1964) and Phillips and Johns (1965), as previously described for cultured cells by Gurley and Hardin (1968). Unfractionated histones were extracted from the chromatin with 0.4 N H_2SO_4 and recovered by precipitation with four volumes of ethanol.

Preparative Electrophoresis of Histones. The histone fractions from 2×10^8 to 5×10^8 cells were subjected to preparative disc electrophoresis by the method of Panyim and Chalkley (1969a) using a Canalco Prep-Disc apparatus (Canal Industrial Corp.) designed for the continuous removal of protein from the bottom of the gel by a cross-flow of buffer. A 4.5-cm high polyacrylamide gel (15% acrylamide–3 M urea–0.9 N acetic acid, pH 2.7) was cast in the annular space between the outside cooling jacket and center cooling post, giving a gel cylinder having an 0.8-cm i.d. and a 2.5-cm o.d. The gel was subjected to preelectrophoresis for 2 hr at 38 mA using 0.9 N acetic acid (pH 2.7) in all reservoirs and bottom cross-flow stream. The lyophilized histone was dissolved in 0.9 N acetic

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[1] Reconstituted [^{14}C]protein hydrolysate (Schwarz BioResearch, Inc.) is a solution containing the following U-^{14}C-labeled, purified L-amino acids: alanine, arginine, aspartic acid, glutamic acid, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, and valine.

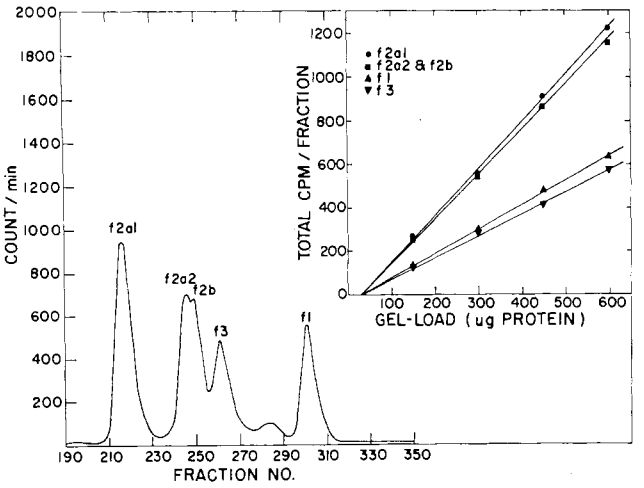

FIGURE 1: Preparative electrophoresis of ^{14}C-labeled Chinese hamster unfractionated histones on polyacrylamide gel. Insert shows that the total cpm in each histone fraction is proportional to its mass.

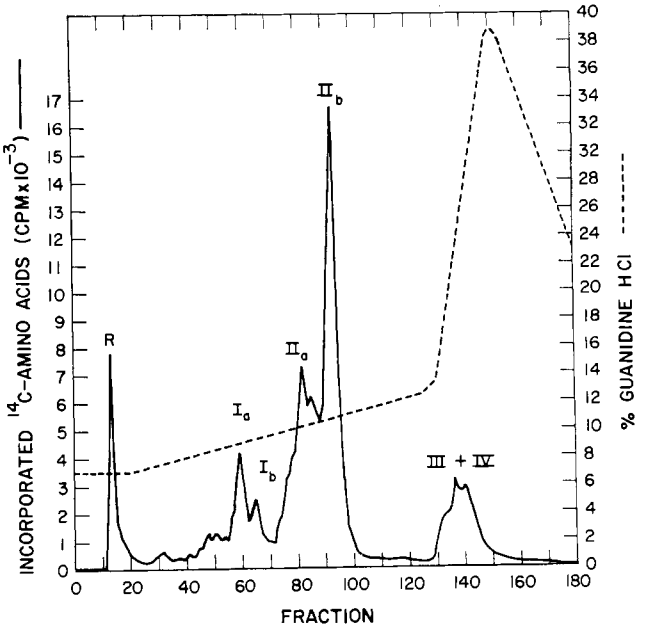

FIGURE 2: Chromatography of ^{14}C-labeled Chinese hamster unfractionated histones on Amberlite IRC-50 (Bio-Rex 70).

acid (pH 2.7) containing 50% (w/v) sucrose and applied to the top of the gel through a tube inserted through the anode buffer reservoir to the gel surface. Electrophoresis was performed for 11 hr at 12.5 mA while eluting protein from the bottom of the gel with a cross-flow of buffer at 1 ml/min. Fractions of the cross-flow buffer were collected every 2 min in liquid scintillation vials (Figure 1).

Chromatography of Histones. Histone fractions were further fractionated by column chromatography on Amberlite IRC-50 ion-exchange resin (Bio-Rex 70, Na^+ form, 200–400 mesh, Bio-Rad Laboratories) by the method of Rasmussen *et al.* (1962). Unfractionated histones were dissolved in 0.5 ml of 7% guanidinium chloride buffered with 0.1 M sodium phosphate (pH 6.8) for application to the column. However, the individual arginine-rich histone fractions were insoluble when added directly to buffered 7% guanidinium chloride. This problem was overcome by dissolving the amount of lyophilized histone fraction obtained from 2×10^8 to 5×10^8 cells in 0.25 ml of water. Then 0.25 ml of 14% (w/w) guanidinium chloride (Ultra Pure, Mann Research Laboratories) buffered with 0.2 M sodium phosphate (pH 6.5) was added to obtain a final 0.5-ml sample in 7% guanidinium chloride buffered with 0.1 M sodium phosphate (pH 6.8). The sample was applied to a 0.5×50 cm resin column which was equilibrated with 7% guanidinium chloride buffered with 0.1 M sodium phosphate (pH 6.8). The histone was eluted from the resin with a linear gradient of buffered 7–13% guanidinium chloride followed by buffered 40% guanidinium chloride at a flow rate of 2 ml/hr (Figure 2). Fractions of 0.5 ml were collected every 15 min in liquid scintillation vials.

Liquid Scintillation Counting. Aqueous histone solutions, effluent fractions from preparative electrophoresis, and effluent fractions from the IRC-50 column were counted in a Packard Tri-Carb spectrometer using Aquasol liquid scintillation fluid (New England Nuclear Corp.). ^{32}P and ^{14}C were counted simultaneously in two channels by pulse-height analysis.

DNA, RNA, and Protein Analysis. The total cell mass of DNA and RNA in 25-ml aliquots of culture was measured by a modified Schmidt–Thannhauser procedure (Enger *et al.*, 1968). The absorption of 260 mμ of the DNA and RNA hydrolysates was measured with a Beckman DU spectrophotometer. The protein in aqueous histone solutions was measured by the method of Lowry *et al.* (1951).

Results

When histones are extracted from chromatin, a large amount of phosphorus-containing nonhistone material is also obtained (for example, see Shepherd *et al.*, 1970). Therefore, before a study of the response of histone phosphorylation to irradiation could be made, it was necessary to obtain a method which would quickly remove these phosphorus-containing contaminants while providing a quantitative recovery of the histones. For this purpose, the electrophoretic method of Panyim and Chalkley (1969a,b), which separates all five major histone fractions on polyacrylamide gel, was adapted to use in a preparative electrophoresis apparatus (Figure 1). It was found that, when cells were grown in [^{14}C]amino acids for two generations and their histones then isolated and subjected to electrophoresis in this system, the recovery of ^{14}C in each histone peak was a quantitative measure of the protein subjected to electrophoresis above a lower limit of 30 μg of protein (see insert, Figure 1).

While this electrophoretic method provides complete separation of all five Chinese hamster histones on analytical gels (Gurley *et al.*, 1970b), band curvature was significant enough to make separation of f2a2 and f2b insufficient in the preparative system (Figure 1). To eliminate this problem, histones were first extracted from the chromatin by the method of Johns (1964) to produce histones f1 and f2b separated from the arginine-rich histones. Preparative electrophoresis of the arginine-rich histone complex provided adequate separation of its three histones f2a1, f2a2, and f3 in the absence of 2b (Figure 3). Therefore, cells prelabeled with [^{14}C]amino acids were pulse labeled for 1 hr with [^{32}P]PO_4 and their histones then isolated and subjected to electrophoresis as shown in Figure 3. It was found that histone f1 and some fraction in the arginine-rich complex were significantly phosphorylated in 1 hr while fractions f2a1, f3, and f2b were not. The phosphorylated fraction in the arginine-rich complex was found

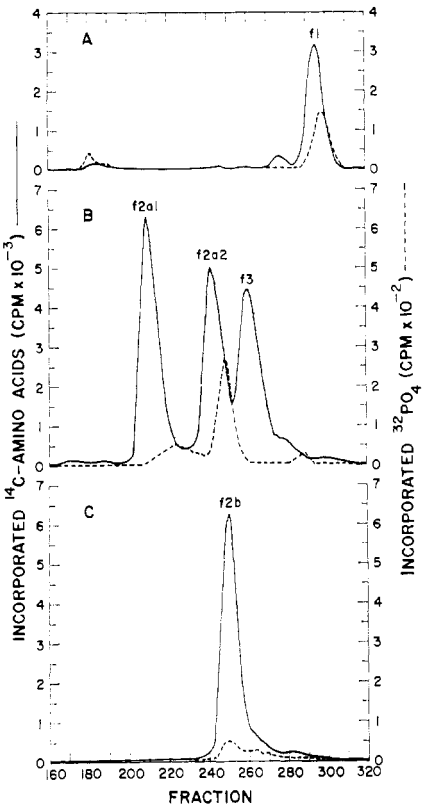

FIGURE 3: Preparative electrophoresis of histone fractions extracted from the chromatin of Chinese hamster cells labeled for two generations with [^{14}C]amino acids and pulse labeled 1 hr with [^{32}P]PO_4: (A) histone f1 extracted with $HClO_4$; (B) arginine-rich–histone complex extracted with ethanol; and (C) histone f2b extracted with HCl.

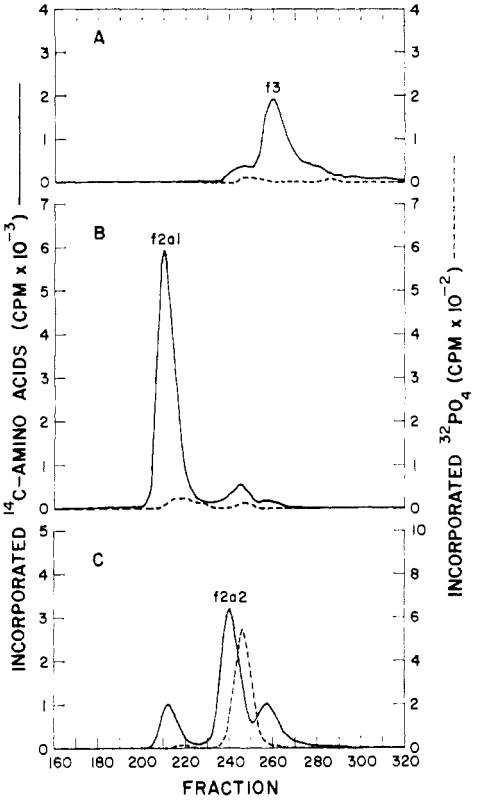

FIGURE 5: Preparative electrophoresis of ^{14}C-prelabeled, ^{32}P-pulse-labeled histone fractions subfractionated from arginine-rich–histone preparations similar to Figure 3b by the methods of Johns (1964) and Phillips and Johns (1965): (A) histone f3; (B) histone f2a1; and (C) histone f2a2.

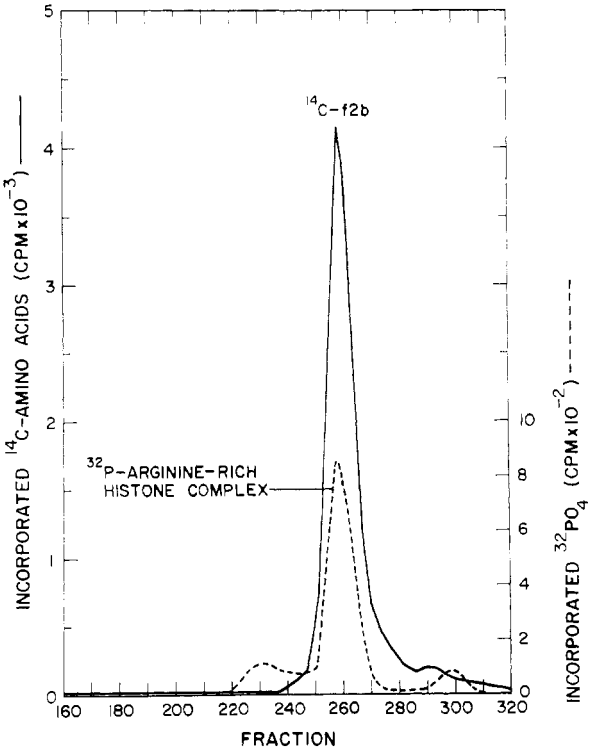

FIGURE 4: Preparative electrophoresis of a mixture of two histone preparations: (1) arginine-rich–histone complex similar to Figure 3b pulse labeled only with [^{32}P]PO_4, and (2) histone f2b similar to Figure 3c labeled only with [^{14}C]amino acids.

to have exactly the same mobility as f2b when a ^{32}P-labeled arginine-rich complex was mixed with ^{14}C-labeled f2b and both were subjected to electrophoresis in the same gel (Figure 4). It became necessary, therefore, to establish whether this phosphorylated fraction was an f2a2 histone migrating slower than the bulk of f2a2 because its charge was reduced by phosphorylation or whether it was an f2b histone which extracted with the arginine-rich complex for unknown reasons because it was phosphorylated.

When the arginine-rich complex was fractionated into its three components by the method of Johns (1964) and Phillips and Johns (1965) and each fraction was then subjected to electrophoresis, the phosphorylated fraction was found to stay associated with f2a2 (Figure 5). When all five histone fractions were subjected to chromatography on IRC-50, the phosphorylated arginine-rich histone was found to be associated with the major fraction of the f2a2 preparation (Figure 6). Furthermore, the phosphorylated fraction contained the same seven-peaked character as that of the bulk ^{14}C-labeled f2a2, although it chromatographed ahead of the ^{14}C-labeled f2a2 by several fractions. It appeared reasonable, therefore, that the phosphorylated histone in the arginine-rich complex was f2a2. Its faster elution rate on IRC-50 and its slower mobility on polyacrylamide gel compared to the ^{14}C-labeled f2a2 most likely reflect a decrease in positive charge due to its phosphorylation. The fact that the phosphorylated f2a2 trails the ^{14}C-labeled f2a2 (Figure 3B) by twice as many fractions as the phosphorylated f1 trails its ^{14}C-labeled f1 (Figure 3A) suggests that f2a2 contains more phosphorylated sites than f1.

To measure the effect of radiation on histone phosphoryla-

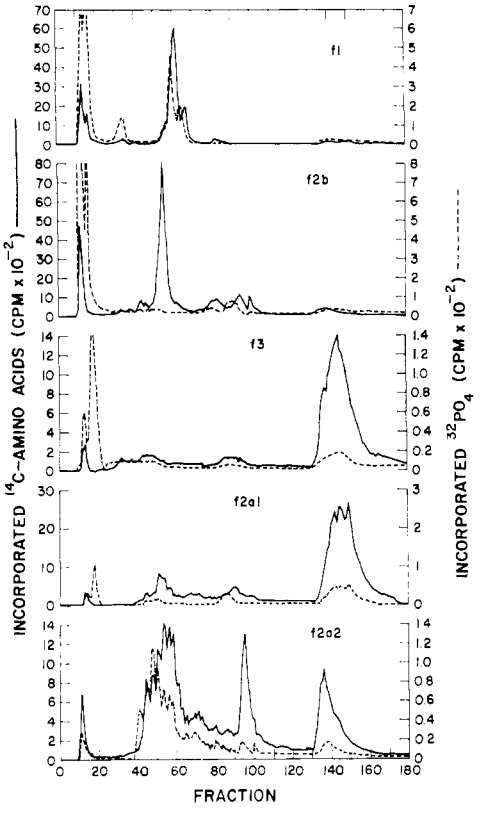

FIGURE 6: IRC-50 chromatography of histone fractions extracted from the chromatin of Chinese hamster cells labeled for two generations with [^{14}C]amino acids and pulse labeled 1 hr with [^{32}P]PO_4. The guanidinium chloride gradient was the same as in Figure 2: (A) histone f1; (B) histone f2b; (C) histone f3; (D) histone f2a1; and (E) histone f2a2.

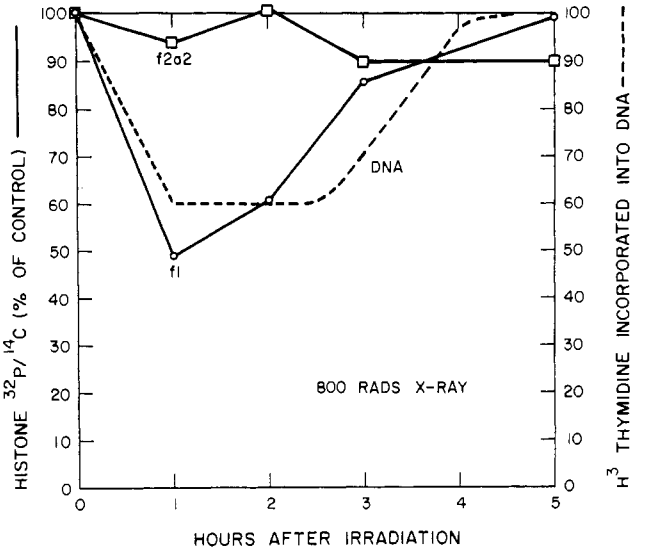

FIGURE 7: Response of histone phosphorylation in exponentially growing Chinese hamster cultures to 800 rads of X irradiation. Details of ^{14}C and ^{32}P labeling are described in the text.

tion, 1-l. cultures in exponential growth were labeled with [^{14}C]amino acids for two generations. The cultures were then irradiated with 800 rads of X-rays. At varying times after irradiation the cultures were pulse labeled with [^{32}P]PO_4 for 1 hr, the cells were harvested, and the histones were extracted and submitted to electrophoresis as in Figure 3. The total counts per minute of both ^{14}C and ^{32}P was determined for the f1 and f2a2 peaks. The degree of phosphorylation of each fraction was then expressed as the ratio of ^{32}P to ^{14}C (Figure 7). It was found that the phosphorylation of f1 was reduced to 50% of the nonirradiated control 1 hr after irradiation. By 3 hr after irradiation phosphorylation had returned to 86% of the control value. Histone f2a2 phosphorylation was not significantly affected by irradiation. In experiments to be published elsewhere (R. A. Walters, R. A. Tobey, and L. R. Gurley, 1970, unpublished data), we have shown that [^{3}H]-thymidine incorporation into DNA is affected by irradiation in the same manner as is f1 phosphorylation (Figure 7).

The effect of irradiation on cell division, RNA and DNA per cell, and histone mass and turnover has been described in a preliminary communication (Gurley *et al.*, 1970a). Those experiments suggested that histone f1 turnover was stopped for a short time by irradiation. Figure 8 illustrates more detailed measurements of this nature. Seven liters of cells was grown for two generations in the presence of [^{14}C]amino acids and then resuspended in medium containing no ^{14}C. The culture was divided into seven 1-l. cultures and irradiated with 800 rads (except the control). At time intervals after irradiation, samples were taken for histone fraction preparation and for determining the cell concentration and cellular DNA content. Irradiation with 800 rads delayed cell division 9 hr in cultures having a doubling time of 17.4 hr (Figure 8D). However, the mass of DNA in the irradiated cultures continued to increase at the doubling rate of the unirradiated culture (Figure 8C), indicating that irradiation did not inhibit the rate of DNA replication. This is in contrast to an expected decrease in DNA replication rate predicted from the effects of irradiation on [^{3}H]thymidine incorporation into DNA following irradiation (Figure 7).

The specific activities of histones decreased following irradiation as a result of dilution of the prelabeled histones with nonlabeled, newly synthesized histones (Gurley *et al.*, 1970a). The lines in Figure 8A and 8B represent the predicted decrease in specific activity if nonlabeled, newly synthesized histones were deposited in the chromatin at just the rate to maintain a constant histone to DNA ratio while DNA replication is occurring at the doubling rate of 17.2 hr and no turnover occurs. As previously observed (Gurley *et al.*, 1970a), the specific activities of f2a and f2b decreased at this predicted rate, indicating that this histones did not turn over following irradiation (Figure 8B). Histone f1, which is normally observed to turn over with a half-life of 74 hr in exponentially growing cultures (Gurley and Hardin, 1969), was found to exhibit no turnover for 3 hr immediately after irradiation (Figure 8B). It then resumed its turnover at a rate having a half-life of 31.1 hr. This rapid turnover rate following irradiation is the same as the turnover rate (31.2 hr) observed for histone f1 during thymidine inhibition of DNA synthesis (Gurley and Hardin, 1969, 1970). Thus, the turnover of f1 is stopped following irradiation for the same period of time that f1 phosphorylation is reduced by irradiation. Resumption of f1 turnover is coincident with resumption of f1 phosphorylation.

Discussion

Interest in the phosphorylation of histones has resulted from the possibility that this structural alteration might be involved with their biological function. The phosphorylation of histones has been shown to be energy dependent, occurring

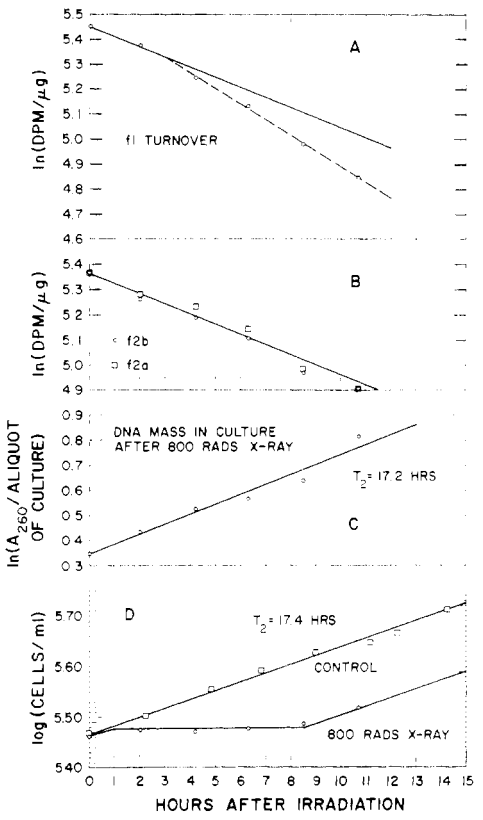

FIGURE 8: Response of histone f1 turnover in exponentially growing Chinese hamster cultures to 800 rads of X irradiation: (A) specific activity of prelabeled histone f1 following X irradiation, and (B) specific activity of histones f2a and f2b following X irradiation. Data points in parts A and B show actual rate of decrease in specific activity. The line is the theoretically predicted decrease in specific activity if histone synthesis continues uninhibited after irradiation with a doubling time of 17.2 hr and no turnover occurs. (C) Mass increase of DNA in the culture after irradiation; and (D) cell density in exponentially growing cultures and cultures exposed to 800 rads of X irradiation.

on the seryl residues after biosnythesis of the protein (Kleinsmith *et al.*, 1966; Ord and Stocken, 1966; Stevely and Stocken, 1966; Murray and Milstein, 1967; Langan and Smith, 1967; Langan, 1968). In the results reported above, significant rapid *in vivo* phosphorylation was detectable only in histones f1 and f2a2 after the histone fractions had been purified by either polyacrylamide gel electrophoresis or IRC-50 chromatography. In the case of f1, this came as no surprise, the active phosphorylation of f1 being in agreement with the reports of Langan (1968) and Ord and Stocken (1968). However, the rapid phosphorylation of f2a2 was different. Histone kinase had been shown to phosphorylate f1 and f2b to a much greater extent than the arginine-rich histones f2a and f3 *in vitro* (Langan, 1968). The f2b isolated from Chinese hamster cells, however, contained negligible ^{32}P in the purified f2b fraction. Ord and Stocken (1967) had found f3 to be phosphorylated but histone f2a to contain negligible phosphorus *in vivo* (Ord and Stocken, 1969). The f3 isolated from Chinese hamster cells, however, contained negligible ^{32}P in the purified f3 fraction.

The results in this report and the studies mentioned above have all reported two major phosphorylated histones *in vitro* and *in vivo:* f1 and some other fraction. Although the phosphorylation of any particular fraction may be tissue specific, as suggested by Gutierrez and Hnilica (1967), we feel that a major problem concerning which histone (other than f1) is phosphorylated may revolve about the ability to separate completely individual histone fractions from each other and from nonhistone phosphorus-containing material. The electrophoretic separation of histones f3 and f2b has been difficult to obtain in the past (for example, see Johns (1967)), but the method of Panyim and Chalkley (1969a) enables one to resolve f3 from other histone fractions. It is possible that the identical mobilities of f2b and phosphorylated f2a2 in polyacrylamide gels (Figure 4), along with the difficulties of separating f2b and f3, may have been misleading concerning which histone in the complex of intermediate electrophoretic mobility (f2a2, f2b, or f3) was phosphorylated. In addition, histones f1, f2b, and f3 contained significant quantities of contaminating phosphorylated nonhistone material in fraction R when chromatographed on IRC-50 (Figure 6), and the presence of such contaminants could obscure measurements on histone phosphorylation, as suggested by Shepherd *et al.* (1970). Of course, we cannot exclude the possibility that dephosphorylation of a specific histone fraction might occur during isolation by the action of a phosphatase like that reported by Meisler and Langan (1969), but in any event f2a2 rather than f2b and f3 was the only significantly measurable phosphorylated histone other than f1 in these Chinese hamster cells.

The cessation of f1 turnover in chromatin and the reduction in f1 phosphorylation immediately after irradiation, followed by the concomitant recovery of both turnover and phosphorylation 3 hr later, strongly suggest that both of these biochemical properties of f1 are related to the same biological function of this histone. The immediate response of these parameters to irradiation indicates that this function is close to the primary site of the radiation-induced lesion. The insensitivity of histone f2a2 phosphorylation acts as an internal control, indicating that the reduction of f1 phosphorylation is not due to some generalized radiation effect on phosphate uptake, pool size, or activation.

The time course of reduction and recovery of f1 phosphorylation after irradiation also closely resembled the time-course of reduction and recovery of [^{3}H]thymidine incorporation into DNA in Chinese hamster cells. Radiation-induced reduction of ^{32}P labeling of f1 has been reported in regenerating rat liver (Ord and Stocken, 1968; Stevely and Stocken, 1968), and it was suggested that f1 phosphorylation is required for DNA synthesis. However, in irradiated Chinese hamster cultures the rate of DNA replication, as measured by DNA mass increase, was not inhibited by 800 rads, nor was [^{32}P]P_i incorporation into DNA (R. A. Walters, R. A. Tobey, and L. R. Gurley, 1970, unpublished data) although [^{3}H]thymidine incorporation was. This same phenomenon has also been observed when synchronized Chinese hamster cells were irradiated in the S phase (R. A. Walters, R. A. Tobey, and L. R. Gurley, 1970, unpublished data). The results of this report, therefore, indicate that the relationship between the radiosensitive f1 phosphorylation and DNA synthesis is not as clear as it originally appeared. Histone f1 phosphorylation does not appear to correlate with DNA replication *per se* but, rather, with some form of radiosensitive precursor incorporation into DNA which does not reflect actual DNA replication.

The accelerated turnover rate of f1 (beginning 3 hr after irradiation) takes place while DNA replication is occurring at a normal rate. This same accelerated turnover rate of f1 has been observed in cultures treated with thymidine, with the amino nucleoside of puromycin, or with actinomycin D (Gurley and Hardin, 1969, 1970). Thus, rapid f1 turnover (half-life of 31–33 hr) can be measured in the presence or ab-

sence of DNA replication or when stable rRNA or unstable mRNA synthesis is inhibited. The function of this f1 turnover is still unknown. However, the association of f1 turnover with f1 phosphorylation suggests the possibility that it might be involved in some kind of nucleocytoplasmic transport. Dixon and Smith (1968) have shown that protamine is phosphorylated to facilitate its transport to DNA in developing sperm. Goldstein and Prescott (1968) have observed nuclear proteins migrating between interphase nuclei and cytoplasm. In this laboratory we have made observations which suggest that cells have the capacity to form a nonchromatin pool of histone f1 in the nucleus where it is available for exchange with the chromatin (Gurley and Hardin, 1970). We have also isolated and identified histone f1 in polysomes (Gurley *et al.*, 1970b), and Paik and Lee (1970) have isolated an enzyme from microsomes which specifically degrades f1. The temporal correlation between the radiosensitivity of f1 turnover and f1 phosphorylation suggests that such a nucleocytoplasmic transport function for f1 might be involved in radiation damage.

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