# The Nature of Histone f1 Isolated from Polysomes†

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ABSTRACT: Histone f1 was extracted from the chromatin, nucleoplasm, and polysomes of cultured Chinese hamster cells and purified using preparative polyacrylamide gel electrophoresis. Pulse-labeling experiments demonstrated that f1 found in chromatin was the most newly synthesized, while f1 found in nucleoplasm was older and f1 found in polysomes was oldest. The f1 associated with polysomes had a much lower degree of phosphorylation than did chromatin f1. These differences indicated that polysomal f1 is a distinct metabolic fraction of f1 and may constitute a pool of non-chromatin-bound f1 suggested by previous experiments. Part of the f1 associated with the polysomes is extracted from the nucleus during cell fractionation and adsorbed to the polysomes. However, the reverse phenomenon also extensively occurs, resulting in a repartitioning of f1 during cell fractionation. Exhaustive extraction indicates that this pool of "old" f1 amounts to about 10% of total cell f1. Extraction of old f1 from the nucleus is dependent on a number of factors, including salt concentration of the buffer, sodium deoxycholate, and some unknown cytoplasmic factors.

In order to gain some insight into the specific biological function of the histone proteins, we have been studying the metabolic behavior of histone fractions in cultured Chinese hamster cells. These studies have indicated that histone fraction f1 is much more metabolically active than the other four histone fractions (f2b, f3, f2a1, and f2a2). Isotope dilution studies have shown that f1 turns over in the chromatin of exponentially growing cells, while the other histones do not (Gurley and Hardin, 1969, 1970). That is, f1 dissociates from the DNA and is replaced by newly synthesized f1. Inhibitor studies have shown that this f1 turnover continues even when mRNA synthesis is stopped with actinomycin D or when rRNA synthesis is stopped with the aminonucleoside of puromycin (Gurley and Hardin, 1970). Cell-cycle studies have also demonstrated that f1 turnover occurs in the $G_1$ phase when DNA synthesis is not occurring (Gurley *et al.*, 1972). However, f1 turnover was found to be stopped by X-irradiation (Gurley *et al.*, 1970a). It was demonstrated that 800 rads of X-irradiation inhibited both f1 turnover and f1 phosphorylation for the same period of time, suggesting that phosphorylation of f1 is functionally involved in its dissociation from DNA (Gurley and Walters, 1971).

There are several possible phenomena which could result in metabolic turnover of f1: (1) f1 may simply be degraded *in situ* by proteolytic enzymes and subsequently replaced; (2) f1 may exist in dynamic equilibrium between a soluble state and a DNA-bound state, in which case degradation and replacement of the soluble f1 would subsequently be observed as a reduction of f1 specific activity in isotope dilution studies using chromatin; and (3) f1 may be transported from the chromatin to some nonchromatin location within the cell and subsequently replaced on the chromatin by newly synthesized f1.

Cell-cycle studies on the metabolism of histones had suggested that a nonchromatin pool of histone f1 did exist (Gurley and Hardin, 1968, 1970). Other experiments had suggested that f1 turnover might be involved with stable RNA synthesis in some way (Gurley and Hardin, 1969). Those studies led us to search for histone f1 in the cytoplasm, which contains a large portion of stable RNA species. It was found that polysomes and ribosomes obtained from DNA-free cytoplasm did contain histone f1. This protein was isolated from purified cytoplasmic polysomes and positively identified as histone f1 by amino acid analysis and electrophoretic mobility (Gurley *et al.*, 1970b). Those experiments suggested that histone f1 might be involved in some form of nucleocytoplasmic transport phenomenon. As a result of those implications, we have further investigated the nature of histone f1 isolated from polysomes.

## 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.

To label histone f1, cells were cultured for two to three generations in growth medium containing 500 μCi of [$^3$H]lysine (2.9 Ci/mmol)/l. of culture. To pulse-label polysomal f1 with enough [$^{14}$C]lysine to be detectable in our small purified electrophoretic fractions, the exogenous lysine was removed from the medium so that it would not dilute the [$^{14}$C]lysine pulse. To do this, the $^3$H-labeled cells were resuspended in F-10 lacking lysine and allowed to grow exponentially for 10 hr to deplete the lysine contributed by the serum. [$^{14}$C]Lysine (310 Ci/mol) was then added for a 1-hr pulse (concentrations of [$^{14}$C]lysine are given for each experiment in the text). To measure the phosphorylation of histone f1, cells were labeled with $H_3{}^{32}PO_4$ (carrier free) using concentrations varying with the requirements of individual experiments.

*Preparation of Nuclei and Cytoplasm.* Cells were fractionated into cytoplasm and nuclei by a method employing the nonionic detergent Nonidet P-40 (Borun *et al.*, 1967a,b) and sodium deoxycholate. All operations were carried out at 1–3°. Cells ($3 \times 10^8$) were harvested by sedimentation and washed with 0.25 M sucrose. The cells were then suspended in 4 ml of either the hypotonic buffer, RSB [0.01 M NaCl–0.0015 M $MgCl_2$–0.01 M Tris, pH 7.4 (Penman, 1966)], or the isotonic buffer 0.13 M NaCl–RSB (0.13 M NaCl–0.0015 M $MgCl_2$–0.01

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M Tris, pH 7.4). This suspension is a critical part of the procedure and was performed by vortexing the cell-buffer mixture in a 12-ml conical centrifuge tube for 30 sec at full speed using an eccentric Vortex mixer (Scientific Products Co.). After standing 5 min, 0.5 ml of 10% (v/v) aqueous solution of Nonidet P-40 (Shell Chemical Co.) was added, and the suspension was immediately vortexed as above. After standing for 15 min in the presence of Nonidet, 0.5 ml of 5% (w/v) aqueous solution of sodium deoxycholate was added, and the suspension was immediately vortexed as above. After standing another 15 min in the presence of Nonidet and sodium deoxycholate, the suspension was vortexed once more. The nuclei were pelleted by centrifugation at 1000*g* for 10 min, and the supernatant cytoplasm was decanted.

*Microscopic Observation of Nuclei.* It has been demonstrated that light microscopy is inadequate to show that isolated cell nuclei are clean of cytoplasmic contaminants (Davison and Mercer, 1956). Use of the phase microscope was also found to be inadequate for judging the cleanliness of Chinese hamster nuclei. The cytoplasmic contaminants of these cells tend to remain uniformly encrusted around the nuclear membrane following nuclear isolation procedures, resulting in nuclei having good spherical morphology and no visible adherent material, as judged by phase-contrast microscopy, but having a high RNA-to-DNA ratio. Therefore, fluorescence microscopy using an Acridine Orange staining procedure was employed and found to be exceptionally vivid in demonstrating cytoplasmic contamination on isolated nuclei.[1] The method utilizes the fact that Acridine Orange qualitatively distinguishes between nuclear DNA, which fluoresces green, and cytoplasmic and nucleolar RNA, which fluoresce red (Bartalanffy, 1962). To monitor the degree of cytoplasmic contamination in our nuclear preparations, a drop of the nuclear suspension was mixed in 5 ml of 0.14 M NaCl plus 2 drops of an aqueous 0.5% Acridine Orange solution. After staining for 2 min, the nuclei were sedimented at low speed and resuspended in 0.5 ml of fresh 0.14 M NaCl. A drop of this nuclear suspension was observed in the fluorescence microscope equipped with an HBO-200 Osram lamp. Cytoplasmic contaminants were observed as red-fluorescing material attached to green-fluorescing nuclei.

*Fractionation of Nuclei and Cytoplasm.* To rupture the nuclei, the nuclear pellet was blended with 3 ml of 0.14 M NaCl for 15 sec at full speed (at 50,000 rpm) in an Omni-Mixer homogenizer equipped with a micro-homogenizer attachment (Ivan Sorvall, Inc.). The resulting homogenate was diluted to 10 ml with 0.14 M NaCl and centrifuged 30 min at 8000*g*. The supernatant nucleoplasm was decanted from the chromatin pellet. Histone f1 was prepared from both fractions as described below.

The cytoplasm was placed in a 30-ml centrifuge tube and underlaid with 1 ml of 1 M sucrose in either hypotonic or isotonic buffer. These samples were centrifuged 2.5 hr at 200,000*g* to pellet the polysomes, ribosomes, and ribosomal subunits. The supertanant cytoplasm was decanted from the polysomal pellet. Histone f1 was prepared from both fractions as described below.

*Preparation of Histone f1.* Histone f1 was prepared from all four fractions by extraction with 5% $HClO_4$, 0.74 N (Johns, 1964). The chromatin pellet was extracted 15 min, once with 1.6 ml of 5% $HClO_4$ and twice with 0.8 ml of 5% $HClO_4$. The extracts were recovered and pooled as previously described (Gurley and Hardin, 1968). The polysomal pellet was similarly extracted once with 0.8 ml of 5% $HClO_4$ and twice with 0.4 ml of 5% $HClO_4$, and the extracts were pooled. The nucleoplasm and cytoplasm solutions were made 5% in $HClO_4$ by adding concentrated $HClO_4$ (70%) and were then allowed to stand 2 hr. All four fractions were centrifuged 30 min at 8000*g*, and the clear supernatant extract was decanted. Trichloroacetic acid (100% w/v) was added to the extracts to bring them to 20% trichloroacetic acid. Histone f1 was precipitated overnight and recovered by centrifugation at 8000*g* for 15 min. The precipitate was washed once with 2.5 ml of acidified acetone (Johns, 1964) and once with 2.5 ml of acetone. The precipitate was then dissolved in 0.5 ml of water and lyophilized to dryness.

Preparative electrophoresis of these histone f1 preparations was performed using the method of Panyim and Chalkley (1969) adapted to a Canalco Pre-Disc apparatus designed for the continuous removal of protein from the bottom of the gel by a cross flow of buffer, as previously described by Gurley and Waters (1971). Effluent fractions from the electrophoresis column were counted in a Packard Tri-Carb spectrometer using Aquasol liquid scintillation fluid (New England Nuclear Corp.). Double-labeled samples, $^3H$ and $^{14}C$ or $^3H$ and $^{32}P$, were counted simultaneously in two channels by pulse height analysis.

## Results

*Histone f1 from Hypotonic Cytoplasm.* In order to examine the cytoplasm for evidence of nonchromatin histone f1, it was first necessary to isolate cytoplasm free from nuclear contamination. We chose the method of Borun *et al.* (1967a,b) for this purpose because this gentle procedure employs no homogenizer which might rupture the nuclei. Rather, the cytoplasm was dissolved away from the nuclei with the detergent Nonidet P-40, giving a cytoplasmic solution free from nuclear contamination (Borun *et al.*, 1967a,b). (In this report "hypotonic conditions" will refer to the buffer containing 0.01 M NaCl which was used to suspend cells for cell fractionation.)

When this method was applied to Chinese hamster cells, using a Nonidet concentration of 1% in hypotonic buffer, we found that a significant amount of red-fluorescing cytoplasm still coated the green-fluorescing nuclei when the preparation was treated with Acridine Orange. However, addition of deoxycholate at a final concentration of 0.5% completely removed this cytoplasm coat without rupturing the nuclei. A complete fractionation of Chinese hamster cells could then be obtained by centrifuging the detergent-treated cells and decanting the cytoplasm off the solid gelatinous nuclear pellet. The cytoplasm contained no nuclei or broken nuclear fragments and less than 0.3% of total cellular DNA, as measured by distribution of [$^3H$]thymidine in DNA isolated from the cell fractions as previously described (Gurley *et al.*, 1970b).

The nuclear pellet was further fractionated into chromatin and nucleoplasm and the cytoplasm into ribosome-free cytoplasm and a pellet containing all ribosome forms (called the polysome pellet). Histone f1 was prepared from each cell fraction, as described in the Materials and Methods section, and subjected to analytical polyacrylamide gel electrophoresis by the method of Panyim and Chalkley (1969). Histone f1 was detected in each fraction except the ribosome-free cytoplasm (Figure 1). It was also observed that extracts from the nucleoplasm, polysomes, and cytoplasm contained significant amounts of nonhistone proteins (Figure 1). Therefore, it was

[1] The authors are indebted to Mr. T. T. Trujillo for aid in applying this method to Chinese hamster cells.

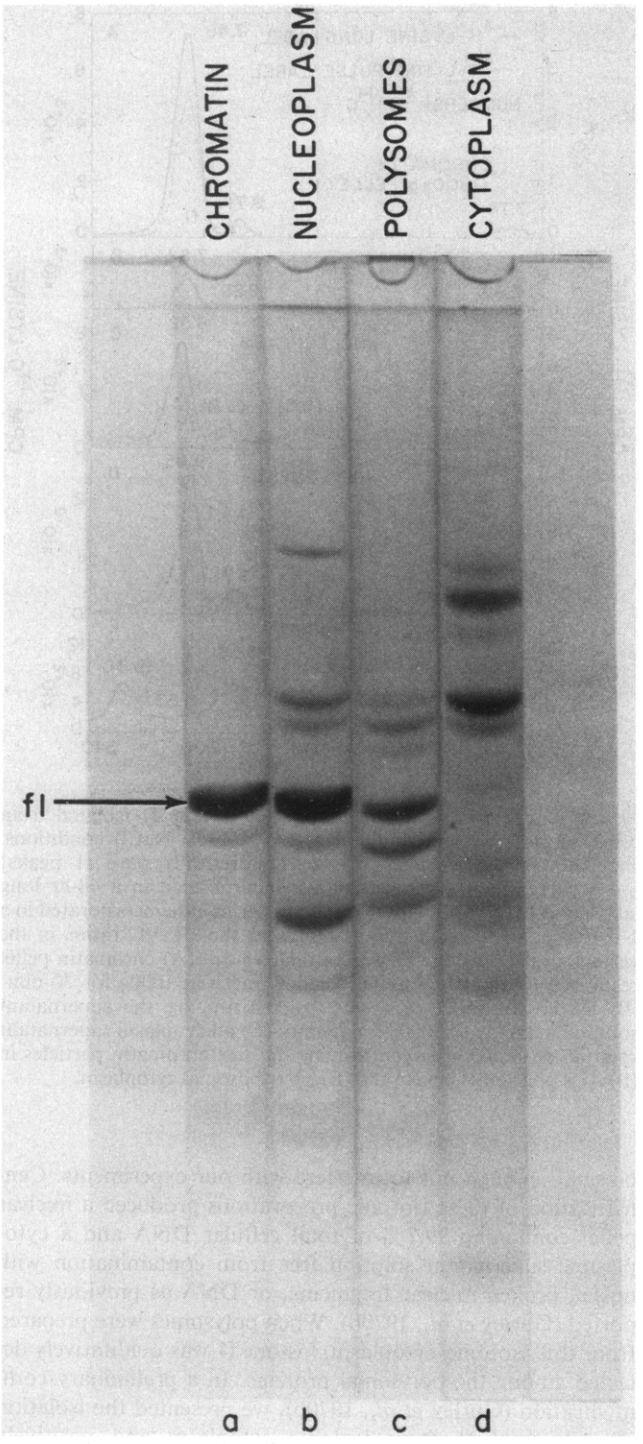


FIGURE 1: Electrophoregrams of histone f1 preparations from various cell fractions: (a) 25 $\mu$g of chromatin f1; (b) 25 $\mu$g of nucleoplasmic f1; (c) 25 $\mu$g of polysomal f1; and (d) 100 $\mu$g of ribosome-free cytoplasmic f1-like preparation. The electrophoresis method (Panyim and Chalkley, 1969) has been previously described (Gurley *et al.*, 1970b).

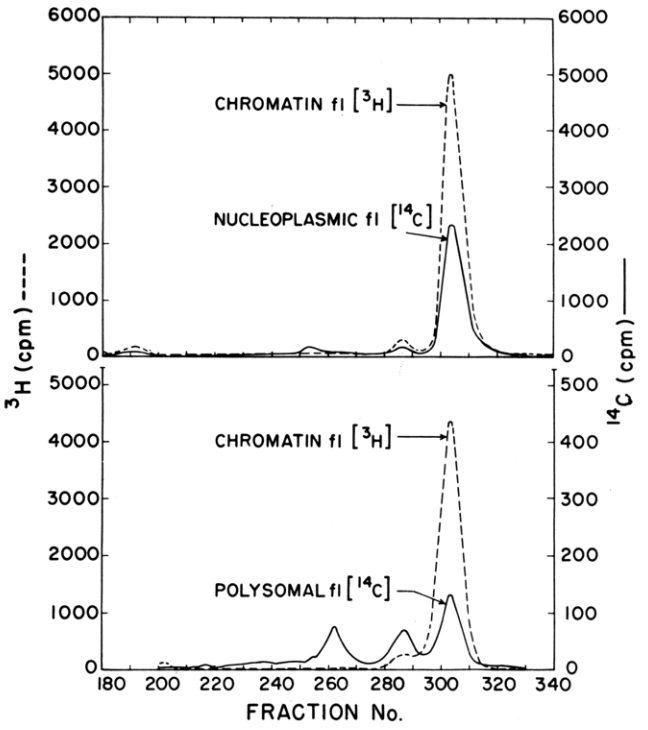


FIGURE 2: Preparative electrophoresis of histone f1 preparations from various cell fractions: (top) coelectrophoresis of [$^3$H]chromatin f1 (– – – –) in the same gel with [$^{14}$C]nucleoplasmic f1 (——); and (bottom) coelectrophoresis of [$^3$H]chromatin f1 (– – – –) in the same gel with [$^{14}$C]polysomal f1 (——).

necessary to remove these contaminants before making metabolic measurements on f1 from these cell fractions. This was accomplished by subjecting the extracted f1 preparations (seen in Figure 1) to preparative electrophoresis. The total radioactivity in only the f1 peak eluted from the bottom of the gel column was used for measuring f1 metabolism. Figure 2 presents an example of this method and demonstrates that nucleoplasmic and polysomal f1 have identical electrophoretic mobilities with chromatin f1 when subjected to electrophoresis in the same gel.

It has been shown that synthesis of histones occurs on small polysomes in the cytoplasm in HeLa cells (Robbins and Borun, 1967; Borun *et al.*, 1967a,b). To determine whether the histone f1 we isolated from polysomes was newly synthesized f1 not yet transported to the chromatin, we labeled cells with [$^3$H]lysine for two generations and then pulse labeled them with 250 $\mu$Ci of [$^{14}$C]lysine for 1 hr, as described in the Materials and Methods section. Histone f1 was extracted from the various cell fractions and subjected to preparative electrophoresis (Figure 3). The ratio of the long-label $^3$H counts per minute to the pulse-label $^{14}$C counts per minute was taken as a relative measure of the age of the proteins in the peaks. The chromatin contained an f1 with a $^3$H:$^{14}$C ratio of 1.68. The f1 recovered from the nucleoplasm had a higher ratio of 1.85, indicating that it was older than the chromatin f1. The f1 extracted from the polysomes had a ratio of 2.66, indicating that it was older than either the chromatin of nucleoplasmic f1. No f1 peak was observed in the ribosome-free cytoplasm. These measurements definitely indicated that polysomal f1 was not newly synthesized f1 but, rather, that the f1 isolated from polysomes was older than f1 found in the chromatin.

*Histone f1 from Isotonic Cytoplasm.* The use of hypotonic buffer during cell fractionation had an advantage over the use of isotonic buffer in that hypotonic buffer gave a complete fractionation into clean nuclei and nuclear-free cytoplasm, as previously mentioned. However, it was felt that the use of hypotonic buffer might possibly cause the isolation of histone f1 with polysomes for the following reasons: (1) some nucleoprotein might be solubilized in the nuclei during the low salt isolation procedures (Oth and Desreux, 1957) and subsequently f1 might be extracted into the cytoplasm; and (2)

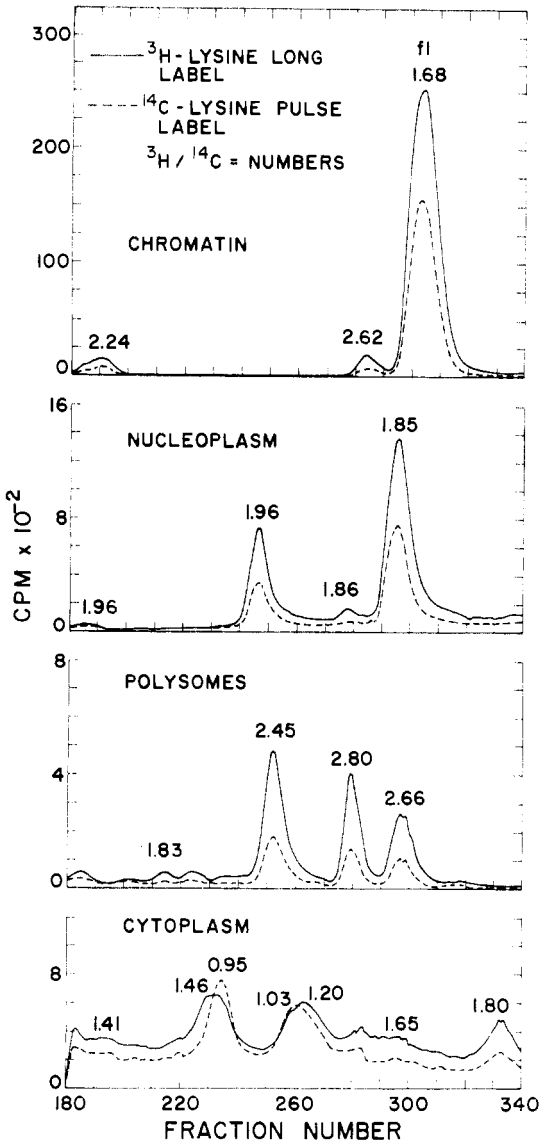


FIGURE 3: Preparative electrophoresis of histone f1 isolated from cell fractions prepared under hypotonic (0.01 M NaCl) conditions, showing the relative ages of electrophoretically pure f1 peaks: (——) [$^3$H]lysine counts per minute incorporated in a 32-hr long label; and (– – – –) [$^{14}$C]lysine counts per minute incorporated in a 1-hr pulse label. The numbers represent the $^3$H:$^{14}$C ratios in various peaks (the larger the ratio, the less newly synthesized f1 there was in the fraction).

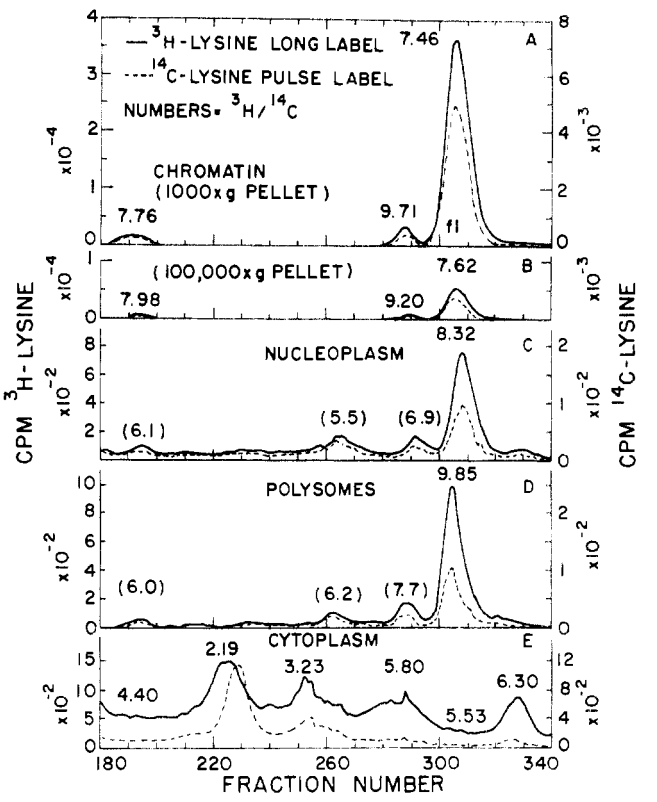


FIGURE 4: Preparative electrophoresis of histone f1 isolated from cell fractions prepared under isotonic (0.13 M NaCl) conditions, showing the relative ages of electrophoretically pure f1 peaks: (——) [$^3$H]lysine counts per minute incorporated in a 48-hr long label; and (– – – –) [$^{14}$C]lysine counts per minute incorporated in a 1-hr pulse label. The numbers represent the $^3$H:$^{14}$C ratios in the various peaks. Histone f1 was extracted from (A) chromatin pellet recovered by centrifuging the blended nuclei at 1000$g$ for 30 min; (B) chromatin pellet recovered by centrifuging the supernatant solution in (A) at 100,000$g$ for 15 min; (C) nucleoplasm supernatant solution decanted after centrifuging out fine chromatin particles in (B); (D) polysomal pellet; and (E) postribosomal cytoplasm.

polysomes, which are known to absorb extraneous proteins from the cytoplasm under low salt isolation conditions (Petermann and Pavlovec, 1961), might adsorb histone f1 extracted from the nucleus. The use of isotonic buffer in the cell fractionation procedure should eliminate these two possibilities, since nucleoprotein is essentially insoluble in 0.13 M NaCl (Oth and Desreux, 1957). Therefore, we decided to repeat the previous experiment using isotonic buffer in the cell fractionation procedure. (In this report "isotonic conditions" will refer to the buffer containing 0.13 M NaCl used to suspend cells for cell fractionation.)

Using Acridine Orange we found that, under isotonic conditions, the nuclei still contained a thin coat of red-fluorescing cytoplasm around the green-fluorescing nuclei, even after deoxycholate treatment. However, the degree of cytoplasmic contamination in the nuclear preparation was considered to be small enough not to interfere with our experiments. Centrifugation of these isotonic preparations produced a nuclear pellet containing 99.7% of total cellular DNA and a cytoplasmic supernatant solution free from contamination with nuclei, broken nuclear fragments, or DNA as previously reported (Gurley *et al.*, 1970b). When polysomes were prepared from this isotonic cytoplasm, histone f1 was qualitatively detected among the polysomal proteins. In a preliminary communication (Gurley *et al.*, 1970b), we presented the isolation of polysomal f1 from isotonic cytoplasm and positively identified this material as histone f1 based on solubility properties, electrophoretic mobility, and amino acid analysis. Histone f1 was isolated from large and small polysomes, from 80S ribosomes, and from 60S and 40S ribosomal subunits (Gurley *et al.*, 1970b).

Figure 4 presents results of an experiment similar to that in Figure 3 in which isotonic conditions were used for cell fractionation. In this experiment cells were labeled with [$^3$H]-lysine for three generations and pulse labeled with 250 $\mu$Ci of [$^{14}$C]lysine for 1 hr, as described in the Materials and Methods section. The large particulate chromatin (Figure 4a) removed from the blended nuclei by low-speed centrifugation contained an f1 with a $^3$H:$^{14}$C ratio of 7.46. The fine chromatin particles (Figure 4b) removed from the blend by high-speed centrifugation contained an f1 with a similar ratio of 7.62. The f1 recov-

TABLE I: Distribution of Histone f1 among Cellular Fractions.[a]

| Cell Fraction | Rel Distribution (%) Hypotonic Isolation | Isotonic Isolation |
|---|---|---|
| Chromatin f1 | 94.2 | 92.2 |
| Nucleoplasmic f1 | 5.0 | 2.4 |
| Polysomal f1 | 0.8 | 5.4 |
| Cytoplasmic f1 | — | — |

[a] Total radioactivity incorporated into the electrophoretic f1 peaks of each cell fraction was used to determine the relative distribution of histone f1.

TABLE II: Phosphorylation of Histone f1.[a]

| Cell Fraction | Long-Label Expt[b] $^3H:^{32}P$ | Pulse-Label Expt[c] $^3H:^{32}P$ |
|---|---|---|
| Chromatin f1 | 69.1 | 73.5 |
| Polysomal f1 | 131.0 | 96.0 |

[a] Relative amount of $^{32}PO_4$ incorporated into the electrophoretic histone f1 peaks of cell fractions prepared under isotonic (0.13 M NaCl) conditions. [b] Cells were grown exponentially for 40 hr in the presence of 500 $\mu$Ci of [$^3$H]lysine and 10 mCi of $H_3{}^{32}PO_4$ per l. of culture. [c] Cells were grown exponentially for 40 hr in the presence of 500 $\mu$Ci of [$^3$H]lysine/l. Then 20 mCi of $H_3{}^{32}PO_4$/l. was added for a 1-hr pulse.

ered from the nucleoplasm (Figure 4c) and polysomes (Figure 4d) had ratios of 8.32 and 9.85, respectively, indicating again that nucleoplasmic f1 was older than chromatin f1 and that polysomal f1 was older than all others. No peak was obtained in the f1 region in the ribosome-free cytoplasm (Figure 4e). These measurements further suggested that f1 might be migrating from the chromatin through the 100,000$g$ nucleoplasm to the polysomes *in vivo*. Such a migration could account for our previous measurements of histone f1 turnover in the chromatin (Gurley and Hardin, 1969, 1970; Gurley *et al.*, 1972).

A large quantitative difference was observed in the relative distribution of f1 among cellular fractions isolated under hypotonic conditions (Figure 3) and isotonic conditions (Figure 4). Contrary to expectations, there appeared to be a much greater recovery of f1 from polysomes isolated under isotonic (0.13 M NaCl) conditions (Figure 4d) than from polysomes isolated under hypotonic (0.01 M NaCl) conditions (Figure 3). Further investigations revealed that polysomal f1 recoveries amounting to 5–6% of total cellular f1 could be obtained under isotonic conditions (Table I). This is a sixfold increase in polysomal f1 using isotonic conditions over that obtained using hypotonic conditions.

*Polysomal f1 Phosphorylation.* Since phosphorylation appeared to play an integral role in f1 turnover (Gurley and Walters, 1971), we measured the degree of phosphorylation of chromatin and polysomal f1 by labeling cells with [$^3$H]lysine and $H_3{}^{32}PO_4$ for 40 hr. Following isotonic cell fractionation, the $^3H:{}^{32}P$ ratio in the electrophoretic f1 peaks was determined. The degree of polysomal f1 phosphorylation was found to be only 53% of chromatin f1 phosphorylation (Table II). When cells were labeled for 40 hr with [$^3$H]lysine and pulse labeled for 1 hr with $H_3{}^{32}PO_4$, the degree of polysomal f1 phosphorylation was 76% of chromatin f1 phosphorylation (Table II). From these two experiments, it appears that f1 associated with polysomes is dephosphorylated upon or subsequent to attaching to polysomes and that accumulation of phosphorylated f1 does not occur over a 40-hr period.

*Effect of Salt Concentration on Binding of f1 to Polysomes.* As a result of the large difference between amount of polysomal f1 isolated under hypotonic and isotonic conditions (Table I), the effect of salt concentration on f1 binding to polysomes was further investigated. Cytoplasm was first isolated from $^3$H-labeled cells using either hypotonic (0.01 M NaCl) or isotonic (0.13 M NaCl) buffer. The NaCl concentration of *isolated cytoplasm* was then adjusted to either 0.01, 0.13, or 0.5 M. Polysomes were prepared, and histone f1 was extracted and subjected to preparative electrophoresis (Table III). In the first experiment, it was found that, when hypotonic cytoplasm (first column) was raised to isotonic conditions (second column), the polysomes actually lost some of their f1 (third column) rather than obtaining the large increase in f1 usually seen under total isotonic conditions (standard). In the second experiment, when isotonic cytoplasm was lowered to hypotonic conditions, the large isotonic polysomal f1 recovery was unaffected rather than being greatly reduced. High salt concentrations dissociated f1 from polysomes regardless of the initial salt concentration. Therefore, the higher salt concentration of the isotonic cytoplasm did not bring about the large yield of polysomal f1 by increasing the binding of soluble cytoplasmic f1 to polysomes. Nor did the higher salt

TABLE III: Effect of NaCl Concentration on Isolation of Histone f1 from Polysomes.[a]

| Expt No. | Cytoplasm Isolation,[b] M NaCl | Polysome Isolation,[c] M NaCl | Polysomal f1 (cpm) |
|---|---|---|---|
| 1[d] | 0.01 | 0.01 | 6,411 |
| | 0.01 | 0.13 | 3,973 |
| | 0.01 | 0.50 | 0 |
| (standard) | 0.13 | 0.13 | 30,891 |
| 2[e] | 0.13 | 0.01 | 30,959 |
| | 0.13 | 0.50 | 0 |
| (standard) | 0.13 | 0.13 | 33,419 |

[a] Histone f1 isolated from the polysomes was subjected to preparative electrophoresis. The [$^3$H]lysine incorporated into the f1 peak was summed to measure the amount of f1. [b] Cytoplasm was first isolated using these conditions. [c] Isolated cytoplasm was then adjusted to these conditions, and polysomes were isolated. [d] One 4-l. culture was labeled two generations with [$^3$H]lysine and then divided into four 1-l. cultures, all equally labeled. Cytoplasm was then isolated from each culture as shown. [e] One 3-l. culture was labeled two generations with [$^3$H]lysine and then divided into three 1-l. cultures, all equally labeled. Cytoplasm was then isolated from each culture as shown.

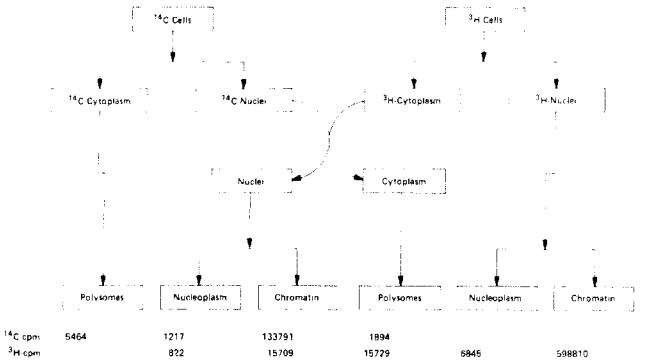


FIGURE 5: Exchange of histone f1 between nuclei and cytoplasm under isotonic (0.13 M NaCl) conditions at 2°. Nuclei labeled with [$^{14}$C]lysine were mixed with cytoplasm labeled with [$^{3}$H]lysine, as described in the text. The total radioactivity in the electrophoretic f1 peaks is shown below each cell fraction. No ribosome-free cytoplasmic f1 was detectable. In the cell fractions derived from the mixture, $^{14}$C counts per minute represents f1 derived from the nuclei, and $^{3}$H counts per minute represents f1 derived from the cytoplasm.

TABLE IV: Recovery of Polysomal Histone f1 from Cytoplasm at Each Step in the Isotonic Cell Fractionation Procedure.

| Culture No. | Cytoplasm Isolation | Polysomal f1,[a] $^{3}$H cpm | % of Control |
|---|---|---|---|
| 1 | Cells treated with Nonidet only | 1251 | 19.4 |
| 1 | Nonidet-treated nuclei further treated with Nonidet and sodium deoxycholate | 951 | 14.8 |
| 2 | Nonidet-treated nuclei further treated with Nonidet-cytoplasm, and sodium deoxycholate | 4884 | 75.8 |
| 3 | Cells treated with Nonidet and sodium deoxycholate (standard control) | 6445 | 100.0 |

[a] Polysomal histone f1 was subjected to preparative electrophoresis, and the $^{3}$H counts per minute in the electrophoretic f1 peak were summed.

concentration of isotonic cytoplasm facilitate the release of f1 from some inaccessible source within the hypotonic cytoplasm so that it could bind to polysomes. Thus, it appears that the effect of salt concentration upon amount of f1 recovered from polysomes may be a function of the presence or absence of nuclei.

*Nuclei–Cytoplasm Exchange of Histone f1.* To test the isotonic nuclei for extractable histone f1, the following experiment was performed as diagrammed in Figure 5. Two 1-l. cultures were labeled for 40 hr, one with 125 μCi of [$^{14}$C]-lysine and one with 500 μCi of [$^{3}$H]lysine. Nuclei and cytoplasm were isolated from each culture under isotonic conditions (0.13 M NaCl). The [$^{14}$C]nuclei were then mixed with the [$^{3}$H]cytoplasm and allowed to stand 15 min at 2°. The nuclei and cytoplhsm were then separated by centrifugation, and histone f1 was extracted from all four cell fractions and subjected to preparative electrophoresis. The total $^{14}$C and $^{3}$H in the f1 peaks is shown at the bottom of the diagram in Figure 5. It can be seen that [$^{3}$H]polysomes acquired only 1.4% of total $^{14}$C partitioning (136,902 cpm) with [$^{14}$C]nuclei. In contrast, [$^{14}$C]nuclei acquired 51.2% of total $^{3}$H partitioning (32,260 cpm) with [$^{3}$H]cytoplasm, with 95% of that acquired $^{3}$H being bound to the chromatin.

From an experiment of this design, one cannot determine the initial *in vivo* distribution of f1 between chromatin and polysomes because a redistribution of f1 between cellular compartments may have occurred immediately during the fractionation procedure. This experiment did demonstrate that only a very small amount of nuclear f1 extraction occurred *after* the initial fractionation had been made. In contrast to this, the experiment does indicate that, once the initial solubilization of cytoplasm has occurred, f1 associated with polysomes can be extensively dissociated and adsorbed onto chromatin of the suspended nuclei. Such redistribution of f1 among cellular compartments during isolation will complicate efforts to answer the question of *in vivo* location of histone f1. If histone f1 were indeed initially located on polysomes *in vivo*, its redistribution during isolation would extensively limit the amount of polysomal f1 isolatable.

*Effect of Detergents on Polysomal f1 Recovery.* Smart and Bonner (1971) have extensively investigated the extraction of histones from chromatin with deoxycholate. They reported that no histone I (f1) was removed from DNA at the concentration we used to isolate nuclei (0.012 M). To our knowledge, no one has investigated the extraction of histones with combined sodium deoxycholate and Nonidet. Therefore, the possibility that polysomal histone f1 might be extracted from the nucleus as a result of detergent solubilization was investigated next.

One 3-l. culture was labeled with 200 μCi of [$^{3}$H]lysine for 23 hr and then divided into three equally $^{3}$H-labeled 1-l. cultures. Each culture was then subjected to part of the isotonic cell fractionation procedure (Table IV). Culture 1 was processed through the Nonidet P-40 treatment. The cytoplasm was removed and polysomal f1 extracted. The nuclei were then resuspended in fresh isotonic buffer containing Nonidet, and deoxycholate was added. After 15 min, this buffer was removed and polysomal f1 extracted. Culture 2 was processed through the Nonidet treatment, and the cytoplasm was removed as in culture 1. However, the nuclei were then resuspended in unlabeled cytoplasm prepared from a comparable number of cells using only isotonic Nonidet. Deoxycholate was then added, and after 15 min this cytoplasm was removed and polysomal f1 was extracted. Culture 3 was treated according to the standard isotonic cell fractionation procedure described in the Materials and Methods section, including both Nonidet and DOC treatments. The cytoplasm was removed, and polysomal f1 was extracted as a standard control. Following preparative electrophoresis, recovery of f1 from each preparation was compared (Table IV).

Isotonic treatment of cells with *only* Nonidet solubilized approximately 90% of the cytoplasm, leaving a coat of cytoplasm around the nuclei as judged by Acridine Orange fluorescence. Sucrose gradient analysis (Enger and Tobey, 1972) of this Nonidet–cytoplasm indicated greater than 90% of the polysomes were recovered in this cytoplasmic preparation. However, the amount of histone f1 recovered from these polysomes (Table IV) was only 19.4% of the amount usually recovered when cells are treated with both Nonidet and deoxycholate (standard control). No significant amount of f1 was detected in the cytoplasm (similar to Figure 4e). When Nonidet-treated nuclei were further treated with isotonic

Nonidet and sodium deoxycholate to remove the remaining cytoplasm, only 14.8% of the standard amount of histone f1 was recoverable from the small polysomal pellet (Table IV). No [$^3$H]f1 was detectable in the polysome-free cytoplasm. Therefore, only 34.2% of the standard amount of f1 was recovered from polysomes when the preparation was performed stepwise. However, when the labeled Nonidet-treated nuclei were further treated with unlabeled isotonic Nonidet–cytoplasm containing polysomes and then sodium deoxycholate was added, 75.8% of the standard amount of [$^3$H]f1 was recovered from the polysomal pellet. No significant [$^3$H]f1 was detectable in the cytoplasm. Therefore, 95.2% of the standard amount of [$^3$H]f1 could be recovered when the stepwise preparation study was made with whole cytoplasm present during the sodium deoxycholate treatment.

From this study we must conclude that at least 75% of the polysomal f1 is derived from the nucleus as a result of treatment with isotonic (0.13 M NaCl) cytoplasm containing Nonidet and sodium deoxycholate. The curious thing about this is that the cytoplasm must be present. Since the isotonic Nonidet–sodium deoxycholate treatment alone (culture 1, Table IV) did not extract appreciable f1 into this buffer, the cytoplasm (culture 2, Table IV) must play an active role in extracting nuclear f1. Since neither the polysomes nor the cytoplasm in culture 2 (Table IV) contained appreciable f1, the role of the cytoplasm in extracting nuclear f1 must be more than just supplying polysomes to adsorb solvent-extracted f1.

Since the experiments in Figures 3 and 4 indicated that polysomal f1 had a different metabolic age from chromatin f1, we next performed similar experiments to see if age varied for the f1 extracted at different steps in the isolation procedure (Table V). A 1-l. culture was labeled with [$^3$H]lysine for 48 hr and then pulse labeled with 400 $\mu$Ci of [$^{14}$C]lysine, as described in the Materials and Methods section. These cells were first treated with isotonic Nonidet. After removing the cytoplasm, the nuclei were resuspended in unlabeled cytoplasm prepared using only isotonic Nonidet, and then sodium deoxycholate was added. After 15 min the cytoplasm was removed, and the nuclei were treated twice more by adding unlabeled cytoplasm (prepared with only isotonic Nonidet) and sodium deoxycholate. Finally, the nuclei were blended, and chromatin and nucleoplasm were obtained. Histone f1 was extracted from each fraction and subjected to preparative electrophoresis. The recovery and age of f1 from these fractions were compared (Table V). The polysomal f1 recovered with Nonidet alone was found to be older than chromatin f1 and had the same metabolic age as that obtained with the subsequent sodium deoxycholate treatment. From the [$^3$H]f1 recovered, it was observed that polysomal f1 was extracted from the nucleus in diminishing amounts with repeated treatments of fresh unlabeled isotonic cytoplasm containing Nonidet and sodium deoxycholate. After repeated treatments of the nuclei with the detergent cytoplasm, there was still a significant amount of nucleoplasmic f1 recovered that had a metabolic age older than chromatin f1 and only slightly younger than polysomal f1. The total polysomal f1 recovered amounted to 6.7% of chromatin f1, and the total f1 having a relatively old metabolic age compared to chromatin f1 (nucleoplasmic plus polysomal f1) amounted to 10.3% of chromatin f1.

*Affinity of Cell Fractions for Exogenous Histone f1.* As a result of the observation that polysomal f1 is extensively adsorbed by chromatin during preparation (Figure 5), we further studied the affinity of various cell fractions for histone f1. Cells (3 l.) were grown in the presence of 120 $\mu$Ci of [$^{14}$C]-lysine/l. for 42 hr. [$^{14}$C]Histone f1 was prepared by the method of Johns (Gurley and Hardin, 1968) and dissolved in 6 ml of isotonic buffer. Cells (5 l.) were also grown in 500 $\mu$Ci of [$^3$H]lysine/l. for 42 hr. This culture was then divided into five equally $^3$H-labeled 1-l. cultures. These cells were harvested by centrifugation, washed, and suspended in isotonic buffer containing various amounts of exogenous [$^{14}$C]histone f1 (Figure 6a). The cells were then fractionated using Nonidet P-40 and deoxycholate. Histone f1 was isolated from the four cell fractions and subjected to preparative electrophoresis. The recovery of endogenous [$^3$H]f1 and exogenous [$^{14}$C]f1 from the cell fractions is shown in Figure 6b–d.

Chromatin had the greatest capacity for exogenous [$^{14}$C]f1, adsorbing 43% of the total amount added at saturation (*i.e.*, when a 75% excess of f1 in the cell was added, see Figure 6b). This large increase in f1 on the chromatin did not result in a comparable displacement of endogenous [$^3$H]f1 (43%) from the chromatin, since no large loss of [$^3$H]f1 could be detected outside experimental error. Polysomes also showed a capacity for exogenous f1, adsorbing 4.6% of the total amount added when 75% of cell f1 was added (Figure 6c). Addition of exogenous f1 also resulted in a 100% increase in recovery of endogenous [$^3$H]f1 from polysomes. The recovery of exogenous [$^{14}$C]f1 from the cytoplasm was only 2% of the total amount added at saturation (Figure 6d). Since the cytoplasm is the fraction where one would expect to find the surplus unbound exogenous [$^{14}$C]f1, this recovery was very small, reflecting a 50% loss of total cpm of exogenous f1 added. Paik and Lee (1970) have reported finding a microsomal enzyme which very actively hydrolyzes histone f1. Part of this loss might result from such an enzyme; however, at least 30% of this loss is a reflection of nonhistone contaminants in such preparations of histone f1 extracted from chromatin (Gurley and Hardin, 1970; Gurley and Walters, 1971). A small amount of exogenous [$^{14}$C]f1 was also isolated from the nucleoplasm, but the amount was so highly variable that interpretation of the data was impossible.

The addition of exogenous [$^{14}$C]f1 resulted in a recovery of some endogenous [$^3$H]f1 in the cytoplasm (Figure 6d). As previously shown (Figures 3 and 4), an f1 peak could not be

TABLE V: Recovery and Metabolic Age of Histone f1 Isolated at Different Stages in the Isotonic Cell Fractionation Procedure.[a]

| Cell Fraction | Histone f1 Recovered: Long-Label $^3$H cpm | Histone f1 Recovered: Pulse-Label $^{14}$C cpm | Metabolic Age $^3$H:$^{14}$C Ratio |
|---|---|---|---|
| Chromatin | 206,127 | 101,511 | 2.03 |
| Nucleoplasm | 7,329 | 2,696 | 2.72 |
| Nonidet polysomes | 1,729 | 614 | 2.82 |
| First sodium deoxycholate polysomes | 6,960 | 2,442 | 2.85 |
| Second sodium deoxycholate polysomes | 3,543 | 1,041 | 3.40 |
| Third sodium deoxycholate polysomes | 1,672 | 588 | 2.84 |

[a] Total radioactivity incorporated into the electrophoretic f1 peaks of each cell fraction was used to determine the relative metabolic age (the larger the $^3$H:$^{14}$C ratio, the less newly synthesized f1 there was in the fraction).

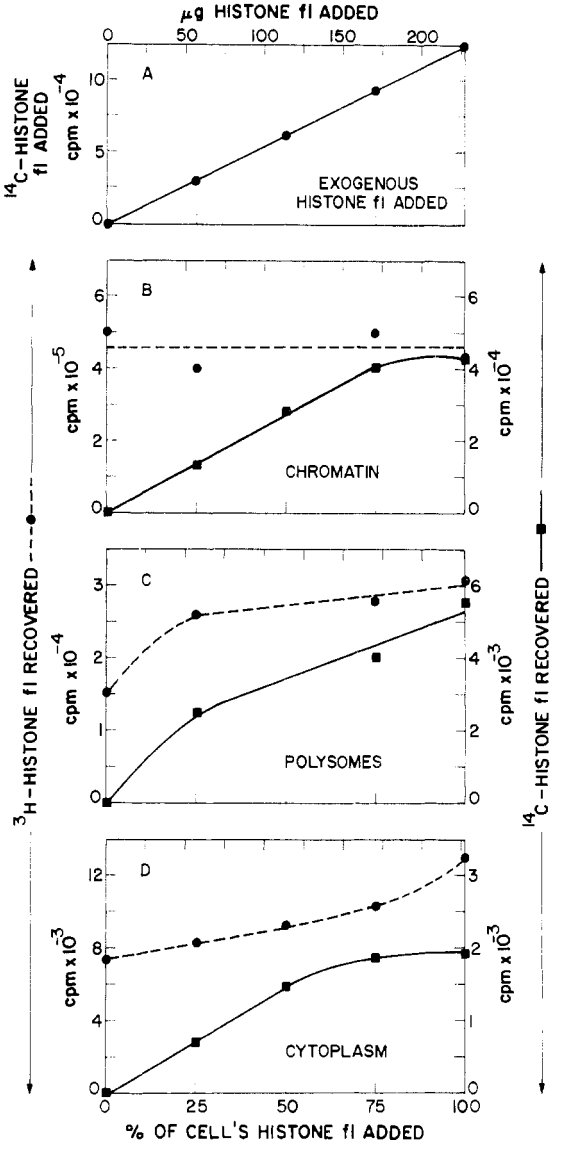


FIGURE 6: The effect of exogenous histone f1 on recovery of f1 from cell fractions. [$^{14}$C]Histone f1 was isolated and added to the cells suspended in isotonic buffer prior to addition of the detergents: (A) amount of exogenous [$^{14}$C]histone f1 added. The amount, in micrograms added to $3.3 \times 10^8$ cells, is shown on the top abscissa. The corresponding amount, in per cent of f1 recoverable from the cell, is shown on the bottom abscissa. (B–D) Recovery of endogenous [$^{3}$H]histone f1 (●) and exogenous [$^{14}$C]histone f1 (■) from chromatin, polysomes, and ribosome-free cytoplasm, respectively.

detected above background in the cytoplasm. However, when exogenous f1 was added in this experiment, a small amount of cytoplasmic endogenous [$^{3}$H]f1 could be recovered, amounting to 2% of total [$^{3}$H]f1. The increased recovery of endogenous cytoplasmic f1 and endogenous polysomal f1 might simply be the result of increasing yield by adding carrier f1. Another possibility is that the exogenous f1 may be sparing some of the f1 in the cytoplasm from destruction of the f1 hydrolyzing enzyme (Paik and Lee, 1970), thus making it possible for polysomes to adsorb more of it. Distinction between these and other possibilities will require further study.

## Discussion

The isolation from polysomes of histone f1 having a metabolic age and degree of phosphorylation different from that of f1 found in chromatin indicates that polysomal f1 is a distinct metabolic fraction of f1. Since the relative age of this polysomal f1 is older than that of chromatin f1, it is certain that this polysomal f1 is not newly synthesized histone.

We have made observations previously which suggested that histone f1 may exist in an extrachromatin pool not bound to DNA (Gurley and Hardin, 1968, 1970). It is possible that the "old" f1 isolated from polysomes in the above experiments constitutes that pool; however, it is impossible to determine from these experiments whether the *in vivo* location of such a pool is in the cytoplasm or in the nucleoplasm, or both. The uncertainty concerning this location arises from an apparent redistribution of f1 between the cellular compartments during cell fractionation. In experiments where $^{3}$H-labeled nuclei were treated with unlabeled cytoplasm containing polysomes, at least 75% of the polysomal f1 was extracted from the nuclei during cell fractionation. However, in experiments where $^{3}$H-labeled cytoplasm was mixed with $^{14}$C-labeled nuclei, it was demonstrated that at least 50% of cytoplasmic f1 could be transferred to the nuclear chromatin during cell fractionation. These two experiments suggest that there is a redistribution of f1 between nuclei and cytoplasm during cell fractionation so that it is impossible to determine the initial distribution of f1 by these techniques.

From experiments involving addition of exogenous f1 to cells prior to cell fractionation, it is clear that polysomes have a distinct affinity for histone f1 added *in vitro*. This affinity of polysomes for f1 probably explains why Dick (1968) observed that, when bean root tips were incubated with various histone fractions, the most lysine-rich fraction localized in the cytoplasm while the other histones localized in the nucleus. In the experiments reported here, part of the f1 associated with polysomes was extracted from the nucleus during cell fractionation and was adsorbed to the polysomes. It is not known whether the extracted f1 is nonspecifically adsorbed to polysomes or whether it is adsorbed because f1 is a natural component of polysomes which, therefore, have a specific adsorption site for f1. Neelin and Vidali (1968) could find no evidence of histones among the structural proteins of 80S ribosomes prepared from goose reticulocytes. Therefore, it is likely that histone f1 is not involved in the polysomes as a structural ribosomal protein. However, it is possible that f1 may have a more dynamic function which necessitates its presence on the whole polysomes of cells.

The repeated observation (Figures 3 and 4) that chromatin f1 is younger than nucleoplasmic f1 and that nucleoplasmic f1 is younger than polysomal f1 suggested that f1 may dissociate from the chromatin and enter the nucleoplasm and subsequently be transferred to the polysomes. Such a process would be consistent with our previous observations of chromatin f1 turnover (Gurley and Hardin, 1969, 1970). We have shown that X-irradiation will inhibit simultaneously both f1 phosphorylation and f1 turnover (Gurley and Walters, 1971). Those measurements suggested considering the hypothesis that histone f1 turnover occurs as a result of the DNA–f1 bond being weakened due to phosphorylation of the histone (Langan, 1971). Such an idea is compatible with the suggestion that *in vivo* histone displacement requires an active process (Evans *et al.*, 1970) possibly involving phosphorylation (Marushige *et al.*, 1969). It is also compatible with Sung and Dixon's (1970) suggestion that phosphorylation of histone I (f1) may be involved in DNA binding. The experiments in this report indicate that the "old" f1 found on the polysomes was phosphorylated much less than the chromatin-bound f1 and that phosphorylated f1 did not accumulate on polysomes over

a 40-hr period. If the polysomal f1 is that f1 lost from chromatin during f1 turnover, our phosphorylation experiments do not support the above hypothesis unless f1 is dephosphorylated before or upon attaching to the polysomes.

Another hypothesis to account for the observations in Figures 3 and 4 is that the turnover process of chromatin-bound f1 (Gurley and Hardin, 1969, 1970) involves specifically old f1 which forms an extractable pool of soluble f1 in the nucleoplasm. This pool may be specifically extracted from the nucleus and adsorbed to polysomes artifactually during cell fractionation. Repeated extractions of nuclei with cytoplasm (Table V) resulted in diminishing recoveries of old f1, suggesting that there was a selective extraction of one population of f1 molecules from the nucleus.

Finally, the isolation of old f1 from polysomes may result from the selective extraction of one member of a heterogeneous population of f1 directly from the chromatin. Ilyin *et al.* (1971) have observed that tRNA will specifically remove histone f1 from chromatin when the two are mixed *in vitro*. In the above experiments we found that some factor present in the cytoplasm was necessary to extract f1 from the nucleus and to transfer it to polysomes. Further experiments must be performed to see if that factor is tRNA. An involvement of tRNA in histone f1 turnover in chromatin *in vivo* would be compatible with certain observations we have previously made on f1 metabolism such as (1) the fact that naturally occurring f1 turnover stops when stable RNA synthesis stops in thymidine-blocked cells (Gurley and Hardin, 1969, 1970); and (2) the fact that f1 turnover continues when rRNA synthesis is specifically blocked by the amino nucleoside of puromycin (Gurley and Hardin, 1970).

Koslov and Georgiev (1970) have measured the rate and size of RNA synthesized on DNA with and without histone f1 present. They have found that f1 does not limit the *rate* of RNA synthesis but does limit considerably the *size* of RNA synthesized. These authors suggested that histone f1 is involved in stopping the movement of RNA polymerase along the DNA template. Such a control mechanism might also involve the removal of histone f1 and RNA from the chromatin, thus resulting in turnover of histone f1 which we have observed *in vivo* (Gurley and Hardin, 1969, 1970). We have shown that this f1 turnover occurs during the $G_1$ phase of the cell life cycle when no DNA synthesis was occurring (Gurley *et al.*, 1972).

Although it is very difficult at this time to interpret the above experiments in terms of biological function, the information obtained in these experiments should now make it possible to design rationally a method for the isolation of a metabolically unique fraction of histone f1 using polysomes as carriers. Such a method would facilitate the further investigation of (1) involvement of f1 turnover in RNA synthesis (Gurley and Hardin, 1969); (2) histone f1 nonchromatin pools (Gurley and Hardin, 1970); and (3) cessation of f1 turnover as a result of damage from X-irradiation (Gurley and Walters, 1971).

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