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Induction of Stable Protein–Deoxyribonucleic Acid Adducts in Chinese Hamster Cell Chromatin by Ultraviolet Light†

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ABSTRACT: Ultraviolet (uv)-light-mediated formation of protein–DNA adducts in Chinese hamster cell chromatin was investigated in an attempt to compare chromatin alterations induced in vitro with those observed in vivo. Three independent methods of analysis indicated stable protein–DNA associations: (1) a membrane filter assay which retained DNA on the filter in the presence of high salt–detergent; (2) a Sepharose 4B column assay in which protein eluted coincident with DNA; and (3) a CsCl density gradient equilibrium assay which showed both protein and DNA banding at densities other than their respective native densities. Treatment of the irradiated chromatin with DNase provided further evidence that protein–DNA and not protein–protein adducts were being observed in the column assay. There is a fluence-dependent response of protein–DNA adduct formation when the chromatin is irradiated at low ionic strength and is linear for protein over the range studied. When the chromatin is exposed to differing conditions of pH, ionic strength, or divalent metal ion concentration, the quantity of adduct formed upon uv irradiation varies. Susceptibility to adduct formation can be partially explained in terms of the condensation state of the chromatin and other factors such as rearrangement, denaturation, and dissociation of the chromatin components. Besides providing information on the biological significance of these types of uv-induced lesions, this technique may be useful as a probe of chromatin structure.

A variety of photoproducts can be induced in cells upon exposure to ultraviolet (uv) light. Although it has been shown that pyrimidine dimers in DNA can lead to serious biological consequences, including cell death, the biological significances of other photo-induced products in the cell are relatively unknown. Previous studies with procaryotic or eucaryotic cells have demonstrated a decrease in extractability of DNA with increasing uv light fluences (Alexander and Moroson, 1962; Smith, 1962). This phenomenon was attributed to the formation of stable protein–DNA adducts. More recently, in an attempt to understand the photochemistry of these protein–DNA complexes, various workers have demonstrated the covalent linkage of amino acid molecules to the free bases of DNA, to synthetic polynucleotides, and to native DNA (Smith and Aplin, 1966; Smith and Meun, 1968; Smith, 1969, 1970; Varghese, 1973; Schott and Shetlar, 1974). Furthermore, it has been shown that a variety of protein molecules, including bovine serum albumin, DNA and RNA polymerases, aminoacyl tRNA synthetases, and ribosomal proteins, can be linked to nucleic acids via uv light (Smith, 1964; Markovitz, 1972; Schoemaker and Schimmel, 1974; Strniste and Smith, 1974; Gorelic, 1975). The biological consequences of uv-induced protein–DNA adducts are not well understood; yet, under certain experimental conditions, it has been reported that this type of lesion can be correlated with lethality in bacteria (Ashwood-Smith et al., 1965; Smith and O'Leary, 1967). Several other lines of evidence also indicate the possible involvement of proteins in the modification or inactivation of eucaryotic cells by uv light (Chu, 1965; Todd et al., 1968; Habazin and Han, 1970; Han et al., 1975).

The chromatin of eucaryotic cells is a heterogeneous complex of DNA with acidic (nonhistone) and basic (histone) nuclear proteins. Since it has been shown that uv-light irradiation of mammalian cells induces cross-links between protein and DNA (Alexander and Moroson, 1962; Habazin and Han, 1970; Han et al., 1975), this investigation was initiated to determine the possible role of chromosomal proteins in the cross-linking process. Using chromatin isolated from Chinese hamster cells and three independent methods of analysis, the results show a fluence-dependent relationship in the protein–DNA adducts formed. The degree of cross-linking is dependent upon the ionic environment of the

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chromatin when it is irradiated and may be correlated with the condensation state of the chromatin. Besides being a method of investigating the molecular sites of uv-light-induced protein–DNA complexes in chromatin, this technique may provide information concerning the morphology of chromatin.

Experimental Procedures

Tissue Culture. Chinese hamster cells (line CHO[1]) were grown in suspension at 37 °C in F-10 medium lacking calcium (Gibco) and supplemented with 15% heat-inactivated bovine cadet serum (Biocell Labs), 100 units/ml of penicillin G, and 100 μg/ml of streptomycin. To label chromatin DNA, cells were grown for three generations (16–18 h doubling time) to about 400 000 cells/ml in 1 l. of medium containing 100 μCi of [*methyl*-^{3}H]thymidine (13 Ci/mmol, ICN). To label chromatin protein, cells were grown in 1 l. of medium to about 200 000 cells/ml. The cells were harvested by centrifugation and resuspended in 500 ml of F-10 medium lacking lysine and containing dialyzed serum (dialysis was performed exhaustively against Earle's balanced salt solution). The cells were incubated an additional 8 h with 100 μCi of [^{14}C]lysine (279 mCi/mmol, New England Nuclear).

Chromatin Isolation and Analysis. All steps were carried out at 0–4 °C. Typically, 2–5 × 10^8 labeled cells were harvested by centrifugation at 300*g* for 10 min. The cellular pellet was washed with 40 ml of saline and centrifuged at 650*g* for 10 min three times. The procedure of Reeck et al. (1974) for isolation of HeLa nuclei was used for preparing CHO nuclei. Briefly, cells were suspended in 20 ml of 10 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 10 mM Tris[1] (pH 7.0) and disrupted with 12 strokes in a glass-to-glass homogenizer fitted with an "A" pestle, and the nuclei were harvested by centrifugation at 3500*g* for 20 min. This homogenization step was repeated. Purity of the nuclei was monitored by phase contrast microscopy. Chromatin was prepared from the purified nuclei by the decreasing ionic strength procedure of Shaw and Huang (1970). The swollen chromatin pellet was made to 0.1 mM Tris (pH 8.0) and stored on ice overnight. The chromatin was solubilized by shearing for 45 s in a Sorvall Omnimixer at the highest setting and was clarified by centrifugation at 10 000*g* for 35 min. The resulting supernatant was the final chromatin used in the experiments and was stored at 0 °C. Specific activities of the [^{3}H]TdR and [^{14}C]Lys chromatin[2] samples were 15 100 cpm/μg of DNA and 2700 cpm/μg of protein, respectively.

Total protein was determined by the method of Lowry et al. (1951) after first precipitating the chromatin with 11% trichloroacetic acid, washing the resultant pellet with ethanol and then ethanol–ether (3:1), and dissolving the pellet in 0.5 N NaOH (Paoletti and Huang, 1969). DNA was determined from the absorbance at 260 nm using the relationship of $A_{260nm}{}^{1\,cm}$ unit = 50 μg of DNA (Paoletti and Huang, 1969).

Irradiation with Ultraviolet Light. Irradiation with uv light was performed at room temperature using an unfiltered General Electric 30-W germicidal lamp (G30T8). The incident intensity was determined as described elsewhere (Barnhart and Cox, 1970). Samples of chromatin at a DNA concentration of 20–60 μg/ml (0.2–0.6 ml) were placed in 50-mm plastic Petri dish bottoms, covered with quartz windows, and positioned 17.5 cm under the light source. The corrected incident fluence at 254 nm was 6.1 J m^{-2} s^{-1}.

Filter Assay. [^{3}H]TdR chromatin samples (80 μl) either unirradiated or uv irradiated were mixed with 2 ml of either 1 mM Tris (pH 7.5), 1 mM EDTA, or 3 M NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, and 0.5% sodium lauroyl sarcosinate (SLS) and incubated at 37 °C for 20 min. The solutions were applied to individual presoaked Millipore filters (type HA) and washed under gentle suction (about 3 ml/min) with either 50 ml of 1 mM Tris (pH 7.5), 1 mM EDTA (low salt wash), or 3 M NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA (high salt wash). Filters exposed to the high salt wash were rinsed with 10 ml of the low salt mixture to remove residual salt from the filter. The filters were oven-dried (70 °C), placed in scintillation vials with 15 ml of Aquasol scintillation fluid (New England Nuclear), and counted in a Packard 2420 liquid scintillation system.

Column Assay. Samples of chromatin (controls and uv-irradiated) were adjusted to 2 M NaCl, 0.1% SLS, and incubated at 37 °C for 20 min before application to a 0.5 × 7.5 cm column of Sepharose 4B previously equilibrated with 2 M NaCl, 0.1% SLS, 1 mM Tris (pH 7.5). A sample not exceeding 0.1 ml was applied and eluted with the same buffer, and 0.1-ml fractions were collected directly into scintillation vials (to which was added 1 ml of water and 15 ml of Aquasol) and monitored for radioactivity. Columns were run at room temperature at a flow rate of 9 ml/h. The glass column had been pretreated with dichlorodimethylsilane, and concentrated solutions of bovine serum albumin and calf thymus histones were eluted through the resin prior to use in order to reduce adsorption of DNA and protein.

Cesium Chloride Density Gradient Equilibrium Assay. The methods of CsCl gradient analysis were similar to those previously described (Strniste and Smith, 1974); any modifications are described in the figure legends. Tubes were pretreated as previously described (Strniste and Smith, 1974) in order to reduce adsorption of the DNA and protein to the tube walls. Prior to fractionation, a water layer was carefully applied to the top of each gradient to ensure complete removal of materials situated at or near the top of the gradient. Fractions were assayed for radioactivity after adding 1 ml of water and 15 ml of Aquasol.

Results

Analysis of CHO Chromatin. The uv spectra of sheared, clarified chromatin (10 preparations) gave the following ratios: A_{260}/A_{320} = 14.4 ± 1.0 (standard error of the mean), A_{260}/A_{280} = 1.62 ± 0.01, A_{260}/A_{240} (max/min) = 1.38 ± 0.02, and A_{230}/A_{260} = 1.06 ± 0.02 (see Shaw and Huang, 1970). The chromatin in 0.25 mM EDTA, 0.1 mM Tris (pH 7.5) showed a broad melting profile typical of chromatin preparations, with a T_m of 77–78 °C and 30% hyperchromicity. The DNA prepared from chromatin showed a sharp melting profile, with a T_m of 44–45 °C and 36% hyperchromicity.

The protein/DNA mass ratio (seven preparations) was 2.17 ± 0.05. Urea gel analysis by the method of Panyim and Chalkley (1969) of the protein complement showed the five major histone bands and at least 15 distinct nonhistone

[1] Abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; SLS, sodium lauroyl sarcosinate; CHO, Chinese hamster cells.

[2] Chromatin preparations in which the DNA is labeled with [^{3}H]thymidine are referred to throughout the text as [^{3}H]TdR chromatin, whereas chromatin preparations in which the protein is labeled with [^{14}C]lysine are referred to as [^{14}C]Lys chromatin.

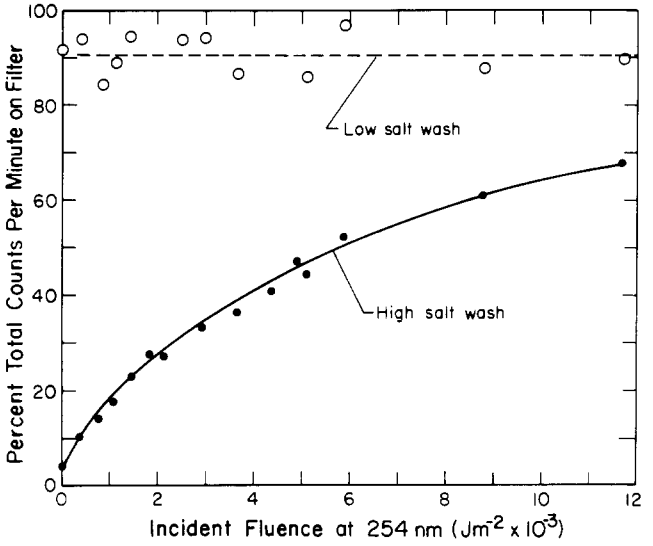

FIGURE 1: Formation of high salt-detergent-stable protein-DNA complexes in uv-light-irradiated CHO chromatin as detected by the membrane filter assay. [^{3}H]TdR chromatin at a DNA concentration of 21 μg/ml in 1 mM Tris (pH 7.5) was irradiated at 22 °C for a series of fluences (see Experimental Procedures for other details). The dashed line (--O--) represents stability of chromatin samples to the low salt washing procedure, whereas the solid line (—●—) represents formation of uv-light-induced, high-salt-stable protein-DNA complexes.

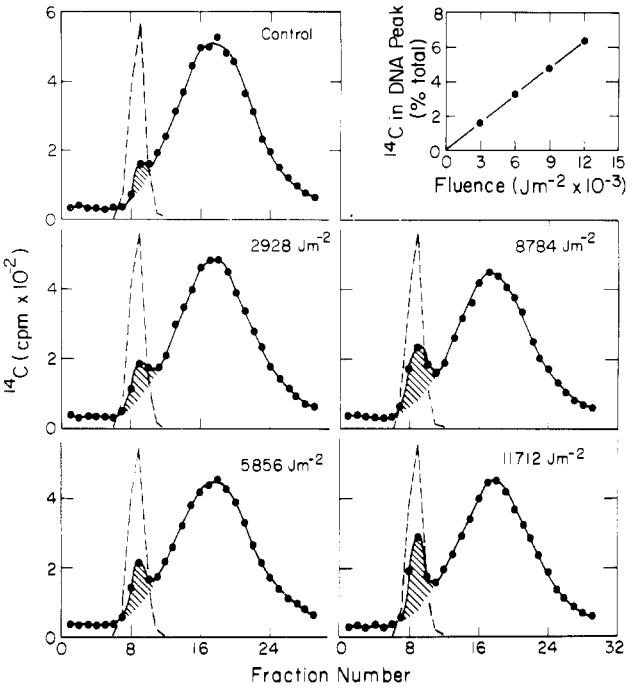

FIGURE 2: Column elution profiles of uv-light-irradiated [^{14}C]Lys chromatin. Samples of chromatin (200 μl) in 1 mM Tris (pH 7.5) at a DNA concentration of 60 μg/ml were uv irradiated at 22 °C. Aliquots containing 2-4 μg of DNA (4-8 μg of protein) were applied to a Sepharose 4B column, eluted, and assayed for radioactivity. Recoveries of input cpm were 75-85%. The solid line (—●—) reflects the ^{14}C cpm (protein profile), whereas the dashed line (---) reflects the elution profile of unirradiated DNA ([^{3}H]TdR chromatin) which was determined separately but in the same manner as for the [^{14}C]Lys chromatin. The percent of ^{14}C cpm associated with the DNA (hatched areas of profiles) is plotted in the summary as a function of uv light fluence after subtraction of the unirradiated control value.

bands. Fast-running bands characteristic of degradation products were absent. From densitometry, the histone/nonhistone mass ratio was estimated to be about two. Percentages of the individual histone bands could be determined more accurately and were found to be: H4, 18.7%; H2a + H2b, 48.8%; H3, 20.3%; and H1, 12.2% (total = 100%).[3]

Ultraviolet-Light-Induced Protein-DNA Adducts as a Function of Fluence. The usability and rapidity of a membrane filter assay for detection of protein-DNA complexes were first described by Jones and Berg (1966). The assay used in this report was similar to that outlined by Strniste and Smith (1974) (see also Experimental Procedures). DNA which is complexed with protein in chromatin is retained on Millipore filters (>90%) under conditions which minimize the dissociation of protein from DNA (i.e., low salt wash). However, prior exposure of the chromatin to high salt (3 M NaCl) and detergent (0.5% SLS) allows passage of the DNA through the filter (>95%). Addition of the detergent was found necessary to substantially reduce the retention of DNA to the filter. The reason(s) for this are not understood; however, it is possible that high salt exposure alone does not completely dissociate the chromatin, thus allowing entrapment of that DNA which is still partially associated with some protein. However, presence of the high salt and detergent did not reduce the capacity of the filter to bind protein. In experiments using [^{14}C]Lys chromatin, greater than 90% of the counts remained associated with the filter after high salt wash. Therefore, to analyze uv-light-induced protein-DNA adducts in chromatin, treatment of the irradiated chromatin with high salt and detergent was sufficient for dissociating DNA which is not linked to protein, thus allowing its passage through the filter, whereas DNA linked to protein does not dissociate and is thus trapped on the filter.

[3] Histone nomenclature used is that recommended by the Ciba Foundation Study Group.

Figure 1 shows the fluence-dependent formation of protein-DNA adducts in uv-light-irradiated chromatin as detected by the membrane filter assay. The data show a fluence-dependent nonlinear increase in amount of DNA trapped on the filter after the high salt wash. Chromatin stability in the low ionic strength irradiation buffer is evident from the low salt wash data. Neither the time necessary for irradiation nor the uv light itself interferes with retention of the chromatin DNA after low salt wash.

Molecular sieve chromatography is a useful tool for separating protein-DNA adducts from the bulk proteins. Sepharose 4B, with a fractionation range of 10^7 to 10^5 daltons, was considered optimum for our purposes. The dimensions of the column were sufficient to achieve good separation of DNA (exclusion volume $\geq 10^7$ daltons) from protein (salt elution volume $\leq 10^5$ daltons) while allowing for rapid analysis, each column run requiring about 20 min. For analysis, individual [^{14}C]Lys chromatin samples were irradiated for various incident fluences and run on the column as described in Experimental Procedures. The results of one of these experiments are shown in Figure 2. The unirradiated control invariably had some ^{14}C counts that remained with the DNA, even after high salt treatment, and corresponded to about 1-2% of the total protein. The most likely explanation for this is that the dissociation conditions employed were not sufficient to remove 100% of the chromatin proteins from the DNA. In contrast to the results of the filter assay with [^{3}H]TdR chromatin, the column assay with [^{14}C]Lys chromatin showed a linear increase in ^{14}C protein associated with DNA with increasing uv-light fluence over

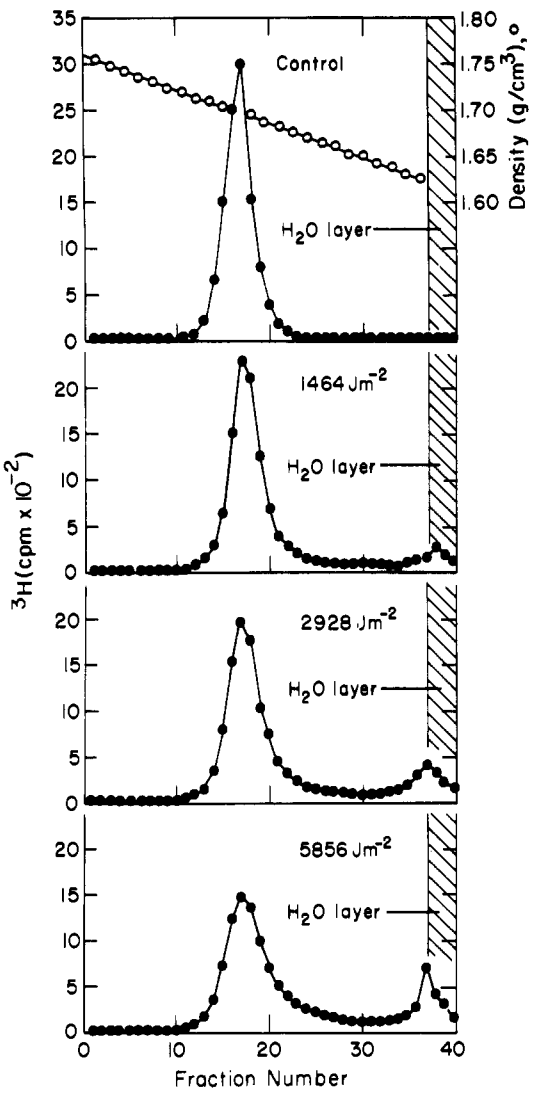

FIGURE 3: Cesium chloride density gradient equilibrium analysis of uv-irradiated [^{3}H]TdR chromatin. Samples of chromatin (21 μg of DNA/ml) in 1 mM Tris (pH 7.5) were irradiated with uv light for a series of fluences, adjusted to 3 M NaCl, 0.5% SLS, and incubated at 37 °C for 20 min. Aliquots of these samples (containing about 2.8 μg of DNA) were mixed with CsCl solutions to obtain a final average density of 1.70 g/cm^3. The mixtures were loaded into treated polypropylene tubes and centrifuged in a Beckman Type 50 Ti rotor at 34 000 rpm for 66 h at 20 °C. Recoveries of input cpm from the gradients, after adjusting for quenching effects due to the presence of CsCl, were 95–100%.

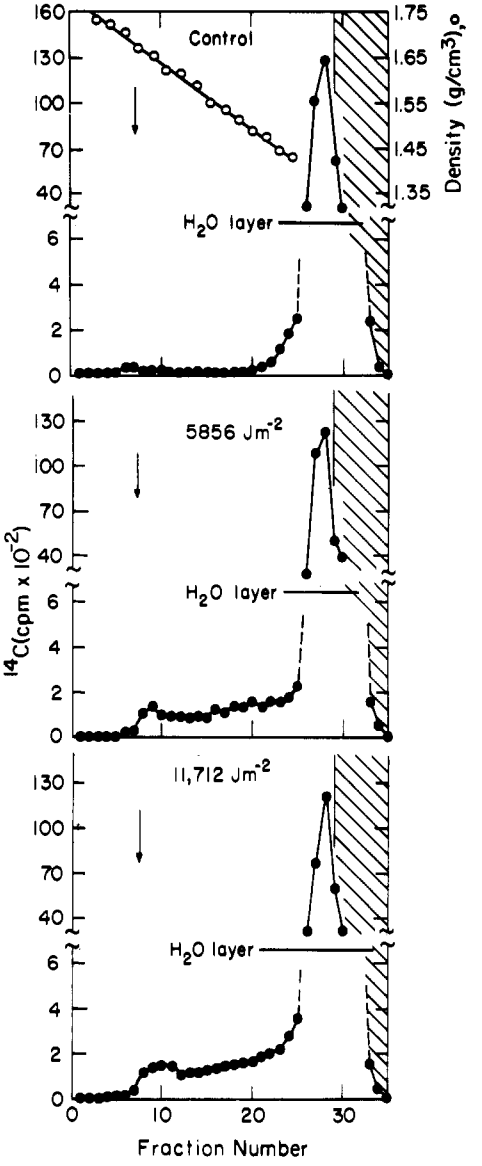

FIGURE 4: Cesium chloride density gradient equilibrium analysis of uv-irradiated [^{14}C]Lys chromatin. Samples of chromatin (60 μg of DNA/ml) in 1 mM Tris (pH 7.5) were uv irradiated for several fluences, and a 0.54-ml aliquot of the irradiated sample was mixed with 0.13 ml of 1 mM Tris (pH 7.5) and 0.005 ml of 30% SLS. A CsCl solution was added to this such that the final volume equaled 5 ml with an average density of 1.56 g/cm^3. Samples were loaded into treated polyallomer tubes and centrifuged at 44 000 rpm for 66 h at 20 °C using a Beckman SW 50.1 rotor. Recovery of input cpm from the gradients was greater than 90%. The arrows indicate the banding position of the peak fraction of unirradiated CHO DNA (using [^{3}H]TdR chromatin) present in each gradient.

the entire range studied. One percent of the total protein was linked to the DNA in chromatin per 1830 J m^{-2}.

Cesium chloride gradient analysis was employed not only for comparison to the results obtained from the filter and column assays but also because this method provides information on the density distribution of both protein and DNA in the uv-light-induced adducts. In Figure 3, the fluence-response with [^{3}H]TdR chromatin as determined by CsCl gradient analysis is shown. In this case, a "shallow" gradient ($\Delta\rho \simeq 150$ mg/gradient) using the fixed-angle 50 Ti rotor was used. From the profiles in Figure 3, it can be seen that there is a shift of some DNA from its native density to lighter densities. We calculate that the amount of material not banding at the native DNA density at each fluence is quantitatively similar to the results obtained by the filter assay (Figure 1).

Analysis of the distribution of uv-light-irradiated [^{14}C]Lys chromatin was performed on a "steep" gradient ($\Delta\rho \simeq 400$ mg/gradient) using the SW 50.1 swinging bucket rotor. The results in Figure 4 show that the irradiated samples have protein counts that appear near the native DNA equilibrium position and a distribution of counts in the middle of the gradient at densities between that of the DNA and protein, indicating heterogeneous protein-DNA complexes. As the fluence is increased, there is an addition of counts to all regions of the profile (excluding the bulk protein region). Summation of the counts in these regions gives comparable results to that obtained by the column technique. For example, the percentages of protein linked to DNA are: at 5856 J m^{-2}, 3.1% (CsCl gradient),

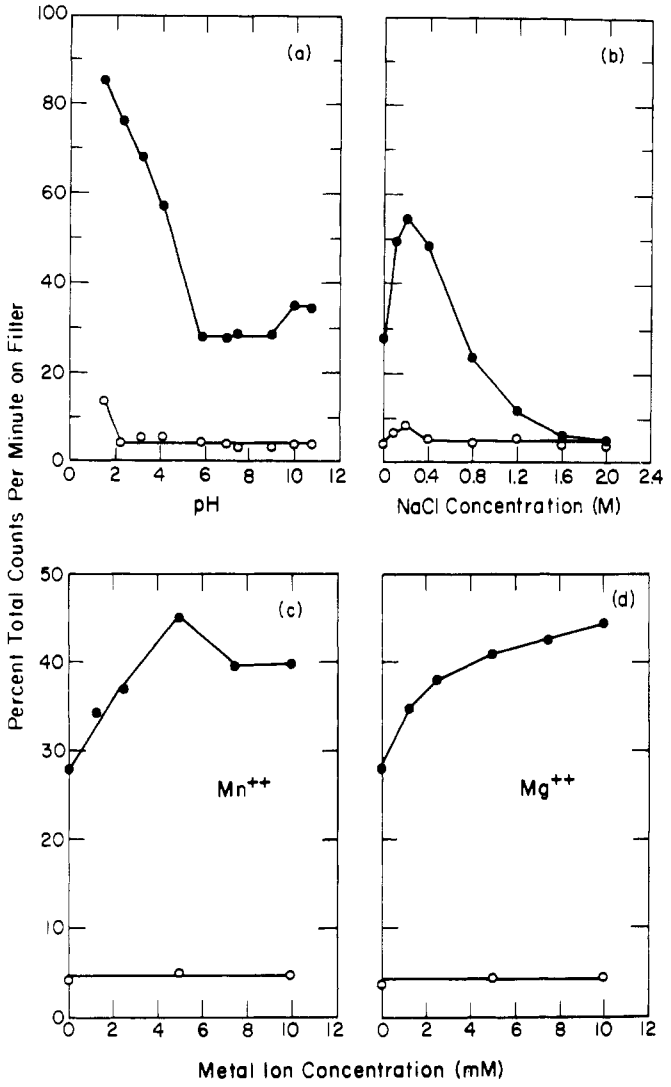

FIGURE 5: Formation of protein–DNA complexes in uv-irradiated [^{3}H]TdR chromatin as detected by the membrane filter assay as a function of (a) varying pH; (b) varying NaCl concentration; and (c and d) presence of $MnCl_2$ and $MgCl_2$, respectively, in the irradiation medium. The DNA concentration in all chromatin samples was 21 μg/ml. Samples were uv irradiated (2928 J m^{-2}) and assayed using the Millipore filter technique described in Experimental Procedures (high salt wash only). Ultraviolet-light-irradiated samples are noted by the closed circles (●), whereas unirradiated control samples are noted by the open circles (○).

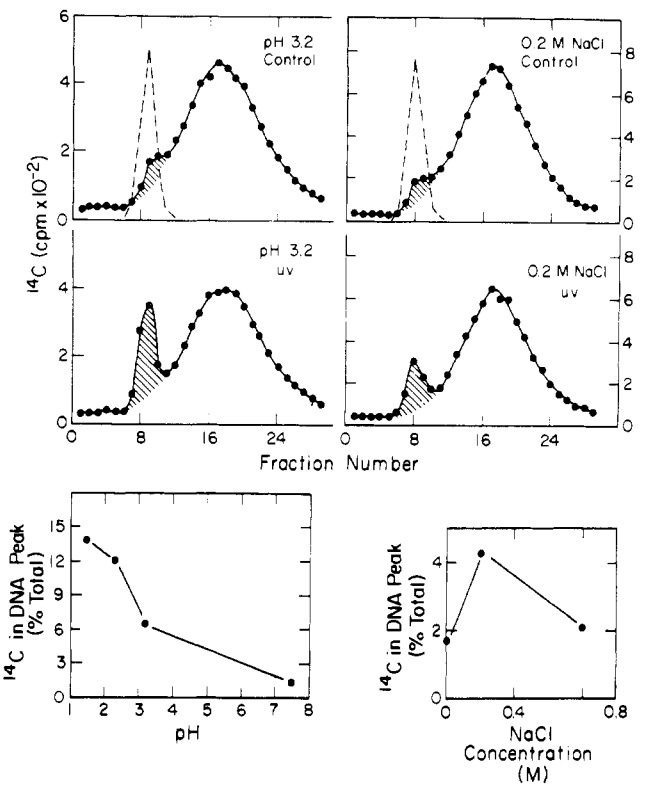

FIGURE 6: Column assay for uv-irradiated [^{14}C]Lys chromatin as a function of pH or ionic strength in the irradiation medium. The DNA concentration in all chromatin samples was 60 μg/ml. Samples were uv irradiated (2928 J m^{-2}) and analyzed by the column assay described in Figure 2 and Experimental Procedures. The solid line (—●—) reflects the ^{14}C cpm distribution (protein profile), whereas the dashed line (---) reflects the elution profile of DNA. The percent of ^{14}C counts associated with the DNA (hatched areas of the profile) is shown in the summary graphs. The appropriate control values have been subtracted for each point.

and 3.2% (column); at 11 712 J m^{-2}, 6.3% (CsCl gradient) and 6.4% (column).

Ultraviolet-Light-Induced Protein–DNA Adducts as a Function of pH. For studies of uv-light-induced protein–DNA cross-linking in chromatin as a function of pH, the irradiation medium was 1 mM Tris, and the pH was adjusted to the appropriate value with either HCl or NaOH. Samples were neutralized before subjecting them to the various assay procedures. The dramatic effect of pH on cross-linking using the filter assay (with [^{3}H]TdR chromatin) is shown in Figure 5a. There is little effect on adduct formation in the pH range 6.0–9.0 and only a slight increase above pH 9.0. As the pH is decreased from 5.0 to 1.5, there is a sharp and continual rise in the amount of DNA linked to protein, reaching a value three times that at neutral pH.

A similar set of experiments was performed using the column assay and [^{14}C]Lys chromatin. The results are presented in Figure 6. Only one profile is given (that for pH 3.2), but all data are summarized in Figure 6. The amount of protein linked to DNA increases tenfold as the pH is lowered from 7.5 to 1.5.

Cesium chloride density gradient equilibrium analysis of uv-light-irradiated [^{14}C]Lys chromatin at a few selected pH values confirmed this result (data not shown). Because of the closeness of the position of some of the linked material to the bulk protein peak, it was difficult to quantitate, but we estimate the amount of uv-light-induced protein linked to DNA at pH 3.2 or 2.3 to be 7.5 or 11.2%, respectively, of the total protein. This compares with 6.5 and 12.2% for those pH values, respectively, by the column technique. There are qualitative differences in the CsCl profiles at different pH values; for example, there is a decrease in material banding in the region of 1.70–1.60 g/cm^3 but a dramatic increase in the amount of material banding in the region 1.60–1.45 g/cm^3 as the pH of the medium is lowered.

Ultraviolet-Light-Induced Protein–DNA Adducts as a Function of Ionic Strength. For analysis of the effects of ionic strength, the chromatin samples were kept at 1 mM Tris (pH 7.5), adjusted to the appropriate NaCl concentration, and irradiated in the usual manner. The results with [^{3}H]TdR chromatin (filter assay) and with [^{14}C]Lys chromatin (column assay) are shown in Figures 5b and 6, respectively. It can be seen in Figure 5b that adduct formation increases as salt concentration is increased to about 0.2 M NaCl, peaking at a level twice that with no NaCl present. As the salt concentration is further increased, there

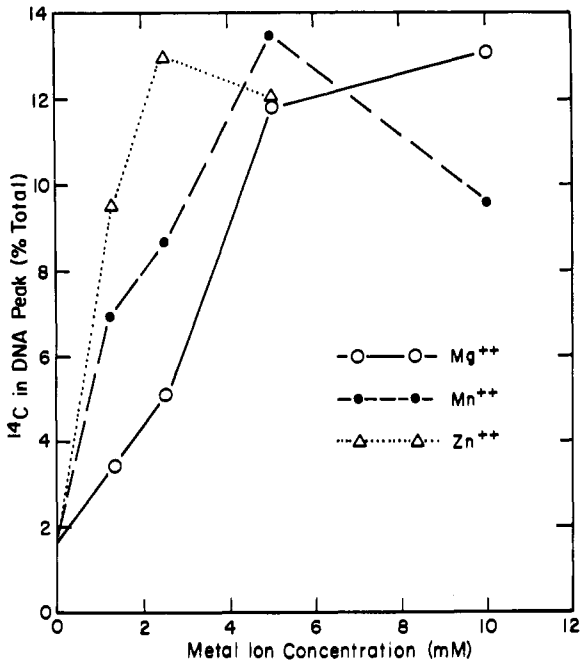

FIGURE 7: Summary of column assays for uv-irradiated [^{14}C]Lys chromatin as a function of the divalent metal ion concentration in the irradiation medium. Samples of [^{14}C]Lys chromatin in 1 mM Tris (pH 7.5) were adjusted to the desired divalent metal ion concentration and uv irradiated (2928 J m^{-2}) at a DNA concentration of 60 μg/ml. Column analysis was performed as described in Figure 2 and Experimental Procedures. The unirradiated control value has been subtracted from each point. The symbols used represent the various metal ions tested as shown in the figure.

is a continual decrease in linkage, reaching the unirradiated control values at 2 M NaCl. This result was confirmed by using the column assay ([^{14}C]Lys chromatin) at a few selected ionic strengths. A representative profile is shown in Figure 6, along with a summary of the data.

Ultraviolet-Light-Induced Protein-DNA Adducts as a Function of Divalent Metal Ion. For these studies, the chromatin in 1 mM Tris (pH 7.5) with either Mn^{2+}, Mg^{2+}, or Zn^{2+} included at the appropriate concentration was irradiated in the usual manner. Results with the filter assay ([^{3}H]TdR chromatin) for Mn^{2+} and Mg^{2+} are presented in Figures 5c and 5d, respectively. There is an enhancement of DNA linked to protein to a maximum level of not quite twofold as the metal ion concentration is increased. With Mn^{2+}, the maximum linkage is attained at a concentration of 5 mM, while with Mg^{2+}, it is reached at 10 mM (concentrations above 10 mM were not tested). Metal ion effects were also investigated by the column assay procedure ([^{14}C]Lys chromatin). The data for three metal ions are summarized in Figure 7. There is a dramatic increase in the percentage of protein linked to DNA when metal ions are present, reaching a maximum of about an eightfold enhancement for all three, but there are noticeable differences in the concentrations at which this level is reached, varying in the order $Zn^{2+} < Mn^{2+} < Mg^{2+}$.

DNase Treatment of the Irradiated Complexes. As additional proof that the observed phenomena represented a protein-DNA and not a protein-protein adduct, various uv-irradiated chromatin samples were subjected to DNase treatment. DNase I (bovine pancreas, Worthington DPFF) was treated with diisopropyl fluorophosphate according to Noltmann et al. (1961) to destroy any contaminating serine protease activity. The treated enzyme and the chromatin

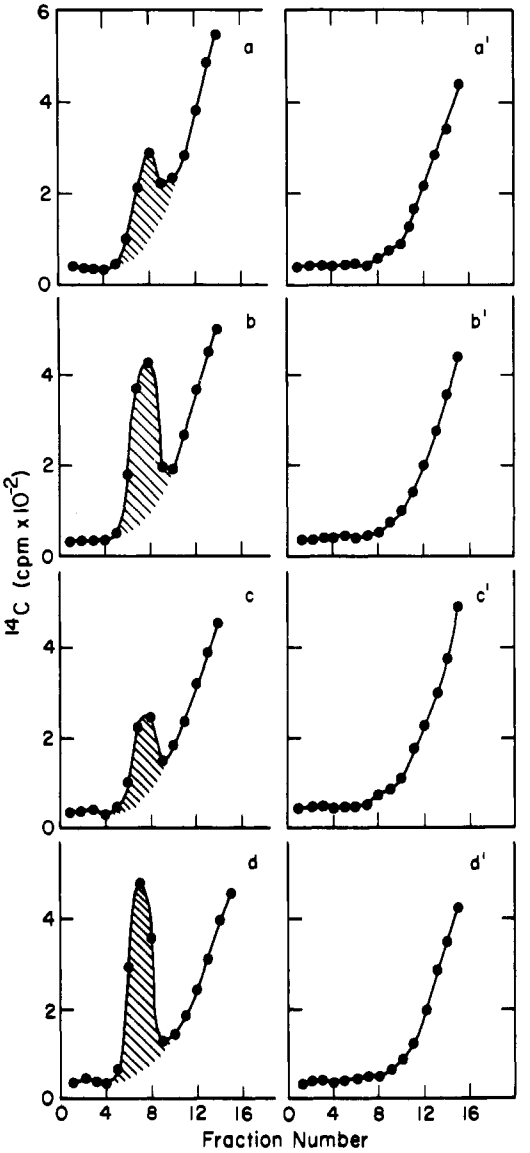

FIGURE 8: Column elution profiles of uv-irradiated [^{14}C]Lys chromatin with and without DNase I digestion. The various graphs represent (a) profile of [^{14}C]Lys chromatin exposed to 11 712 J m^{-2} in 1 mM Tris (pH 7.5); (a') a sample identical with (a) but treated with DNase I before analysis; (b) profile of [^{14}C]Lys chromatin exposed to 2928 J m^{-2} at pH 3.2; (b') a sample identical with (b) but treated with DNase I; (c) profile of [^{14}C]Lys chromatin exposed to 2928 J m^{-2} in 0.2 M NaCl, 1 mM Tris (pH 7.5); (c') a sample identical with (c) but treated with DNase I; (d) profile of [^{14}C]Lys chromatin exposed to 2928 J m^{-2} in 5 mM $MnCl_2$, 1 mM Tris (pH 7.5): and (d') a sample identical with (d) but treated with DNase I.

sample to be tested were adjusted to 2 mM $CaCl_2$, 10 mM $MgCl_2$, 50 mM Tris (pH 7.5). Enzyme was added to give 50 μg of DNase/μmol of DNA phosphate, and the mixture was incubated at 37 °C for 1 h before adjustment to 2 M NaCl, 0.1% SLS, and application to the column. It was determined that 60-min digestion was sufficient to remove all DNA from the exclusion volume of the column (fractions 6-10) and that there was no difference in the DNA elution profiles obtained with either unirradiated [^{3}H]TdR chromatin or chromatin exposed to 11 712 J m^{-2} in 1 mM Tris (pH 7.5). Figure 8 shows that DNase treatment removes all of the protein from the exclusion volume of the column, indicating that this protein material was associated with DNA and was not a result of protein-protein adducts or ag-

gregates. A similar treatment with RNase did not affect the protein elution profiles (data not shown).

Discussion

The evidence that the uv-light-induced phenomena we have observed are covalent protein–DNA adducts is as follows: (1) entrapment of DNA from irradiated chromatin samples on membrane filters and its high salt–detergent stability; (2) banding of both protein and DNA material at intermediate densities in CsCl equilibrium gradients; (3) elution of protein material coincident with DNA on the Sepharose 4B column and its stability to high salt and detergent treatment; and (4) DNase treatment of the irradiated complexes.

However, high fluences of uv light can denature DNA (Hagen and Keck, 1965), and it is known that certain histones associate very strongly to denatured DNA (Bartley and Chalkley, 1972). In a previous report concerning a uv fluence-dependent decrease in the deproteinization of deoxyribonucleoprotein, it was postulated that a majority of the adducts formed could be accounted for by a noncovalent interaction of protein to denatured DNA (Sklobovskaya and Ryabchenko, 1970). Using a variety of dissociation conditions, including that described by Sklobovskaya and Ryabchenko, we have observed that a combination of high salt (2 M or greater) and detergent results in the highest degree of dissociation of protein from DNA in irradiated chromatin (Strniste and Rall, unpublished data). Thus, we believe that, although the interaction of protein to denatured DNA in uv-irradiated chromatin could contribute to the quantity of adducts observed, only adducts maintained through covalent bonds could survive the dissociation conditions described in this paper. Although the specificity in the cross-linking of protein to DNA in uv-irradiated chromatin is not known, it may be similar to the covalent cross-links observed in previously described model systems (Smith and Aplin, 1966; Varghese, 1973; Schott and Shetlar, 1974).

The degree of cross-linking observed as a function of pH, ionic strength, and divalent metal ion possibly can be explained by the state of condensation of the chromatin in the particular environment. When chromatin is in a more condensed form, it is also more susceptible to protein–DNA adduct formation induced by uv light. Conversely, when the chromatin is in a more diffuse state, it is less susceptible to adduct formation. This seems reasonable merely upon consideration of proximity effects. The more compact the chromatin structure, the more intimate is the association (relationship) of the DNA with the chromatin proteins. It follows that, in this situation, the free radicals induced by uv light are more likely to effect covalent bond formation between the DNA and proteins.

This phenomenon is most clearly demonstrated in Figures 5b and 6, where the chromatin has been irradiated at various ionic strengths. As the salt concentration is raised from 0 to 0.2 M, the chromatin becomes more condensed, and this is reflected in the degree of cross-linking observed. The decrease in cross-linking beyond 0.2 M NaCl is a function of decondensation of the chromatin and/or selective removal of some chromatin proteins from the DNA. At 2 M NaCl, the irradiated chromatin value is equal to the unirradiated controls. At high ionic strength, greater than 90% of the proteins have been stripped from the DNA (Shaw and Huang, 1970), thus making them unavailable for cross-linking to the DNA. It is of interest to note that the data of Davies and Walker (1974) on the solubility of calf thymus chromatin in NaCl almost precisely reflect our data in Figure 5b (that is, it appears that the increase in adduct formation is directly related to the decrease in solubility of chromatin).

The marked increase in susceptibility of chromatin to cross-linking at acidic pH values can also be partly explained by an increase in condensation (decrease in solubility). The isoionic and isoelectric point of chromatin is near pH 5.0 (Davies and Walker, 1974), and chromatin is not very soluble below this point. Another possible explanation for increased adduct formation in acidic pH ranges is that there are many titratable groups on both the DNA and proteins below pH 5.0 (Walker, 1965) which may dramatically affect the interaction of protein and DNA in chromatin. Acid may render the DNA and protein more susceptible to uv-light-induced cross-links by masking or exposing charges (i.e., by denaturing) in such a manner that certain protein–DNA associations become more intimate and, therefore, more readily available to the adduct formation process.

While the state of condensation of the chromatin may be primarily responsible for the degree of cross-linking, another possible factor which may influence adduct formation is the rearrangement (migration) and exchange of proteins between DNA molecules. These phenomena are known to occur in chromatin in certain ionic environments (Ohlenbusch et al., 1967; Jensen and Chalkley, 1968; Clark and Felsenfeld, 1971; Ilyin et al., 1971; Van and Ansevin, 1973), some of which have been used in this study. It is likely that such rearrangements and exchanges affect the susceptibility of the protein and DNA to adduct formation, whether it be to enhance the process or to diminish it.

We have obtained some preliminary evidence which indicates differences in the degree to which histone and nonhistone chromosomal proteins are linked to DNA with uv light, suggesting that there is some specificity involved in the process (Strniste and Rall, unpublished observation). It is uncertain whether the nature or specificity of uv-light-induced linkages in vitro reflects the in vivo situation. Since the conditions of isolation and irradiation of the chromatin used in this study may not maintain the native in vivo structure of the chromatin [for example, Noll et al. (1975) report that mechanical shearing destroys much of the subunit structure of native chromatin], it will be interesting to compare in vitro results on the specificity of linkage with in vivo studies to see how chromatin structure is altered by isolation.

Since DNA in eucaryotic cells is associated with a variety of proteins, it seems likely that the uv-light-induced protein–DNA cross-links observed in irradiated cells consist, at least partially, of chromosomal protein–DNA complexes. It has been reported that there is about 1% of the DNA cross-linked to protein for every 10 J m^{-2} of uv light exposed to HeLa cells (Habazin and Han, 1970). From our filter assay data concerning the detection of protein–DNA adducts in uv-irradiated CHO chromatin, we estimate (from the initial slope of the curve in Figure 1) that there is about 0.1% of the chromatin DNA linked to protein per 10 J m^{-2}. This amount per 10 J m^{-2} could be artifactually low in light of the argument that retention of protein–DNA complexes to membrane filters may require a minimum number (greater than one) of linked protein molecules per DNA molecule (Braun and Merrick, 1975). Furthermore, when the chromatin was adjusted nearer to in vivo conditions by increasing the ionic strength or by adding divalent metal ions, the enhancement of cross-linking was such that the magnitude

in vitro approaches that value reported in the in vivo experiments of Habazin and Han (1970). However, a study comparing in vivo uv-light-induced protein–DNA adducts with in vitro uv-light-induced adducts is a necessity in order to confirm the biological significance of the data reported here.

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