A Comparative Study of Some Properties of Chromatin from Normal Diploid and SV-40 Transformed Human Fibroblasts†

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ABSTRACT: The isolated chromatins of confluent monolayers of WI-38 human diploid fibroblasts and their SV-40 transformed counterparts 2RA cells have been compared. The chromatin of WI-38 cells can be distinguished on the basis of circular dichroism spectra from the chromatin of 2RA cells. In the 250–300-nm region, 2RA chromatin shows an increased positive ellipticity and a blue shift in respect to WI-38 chromatin. These differences in circular dichroism spectra between WI-38 and 2RA chromatins can be abolished by washing both chromatins with 0.25 M NaCl. Extraction with 0.25 M NaCl only removes a fraction of non-histone chromosomal proteins and no detectable histones. Histones and non-histone chromosomal proteins from both chromatins have been examined by gel electrophoresis. While no differences could be detected between histones from the two sources, non-histone chromosomal proteins from 2RA cells showed at least two bands that were absent from non-histone chromosomal proteins of WI-38. It is suggested that these differences may play an important role in determining the growth characteristics of WI-38 cells and their transformed counterparts, 2RA.

Chromatin isolated from interphase nuclei is a complex of DNA, histones and non-histone proteins, and some RNA. Following the pioneer work of Bonner and coworkers (Bonner *et al.*, 1968a), isolated chromatin is believed to retain most of the functions of chromatin in the intact cell. Recently a number of studies have compared the composition and some *in vitro* functional properties of chromatin isolated from normal tissues and from neoplasms originating from them (Grunicke *et al.*, 1970; Kostraba and Wang, 1971; Arnold *et al.*, 1973; Kadohama and Turkington, 1973). Particular attention has been focused on non-histone chromosomal proteins (Weisenthal and Ruddon, 1972; Yeoman *et al.*, 1973) which have recently been implicated as regulators of gene expression in general (Wang, 1968; Stellwagen and Cole, 1969a; MacGillivray, *et al.*, 1972; Stein *et al.*, 1974), and cell proliferation in particular (Baserga *et al.*, 1971; Baserga and Stein, 1971). However, in the studies mentioned above both the normal and the neoplastic tissues were a mixture of different types of cells which presumably contributed to a different extent to the structural and functional characteristics of chromatin and its chromosomal proteins. This applies also to a report by Wakabayashi and Hnilica (1973) who found immunological differences between the chromatin of normal rat liver and the chromatin of Novikoff hepatoma.

Previous studies from our laboratory have shown that anti-chromatin antibodies recognized differences between chromatins, and between non-histone chromosomal proteins (but not histones) of WI-38 human diploid fibroblasts and their SV-40 transformed counterparts, 2RA cells (Zardi *et al.*, 1973).

In this investigation we have extended our observations on WI-38 human diploid fibroblasts and their SV-40 transformed counterparts 2RA cells, and specifically we have compared some properties of the chromatins and chromosomal proteins isolated from these cells. Since the definition of chromatin varies from one laboratory to another, the term "chromatin" in this paper has an operational definition only and refers to the preparations obtained by the methods to be described. With this qualification, the results reported in this paper indicate that (1) chromatin from WI-38 cells can be distinguished from chromatin of 2RA cells, and (2) non-histone chromosomal proteins from WI-38 cells are different from non-histone chromosomal proteins of 2RA. Whether the alterations in chromatin structure occurring in 2RA cells are dependent on the changes occurring in non-histone chromosmal proteins, cannot yet be determined on the basis of our findings.

Materials and Methods

(*a*) *Cell Culture.* WI-38 human diploid fibroblasts were routinely purchased from Flow Laboratories (Rockville, Md.). The cells (between 24th and 28th passages) were plated either in 1-l. Blake bottles (surface of 210 cm^2) or in Falcon plastic T-flasks (surface of 75 cm^2), containing respectively 75 and 25 ml of Basal Medium Eagle as previously described (Rovera and Baserga, 1971). The experiments were performed 7 days later when the monolayers reached confluence.

SV40 transformed WI-38 (2RA cells) were a gift of Dr. Vincent J. Cristofalo, Wistar Institute, Philadelphia, Pa. The cells were grown in Falcon plastic T-flasks in Minimal Essential Medium (Microbiological Associates, Inc., Bethesda, Md.) plus 5% fetal calf serum, 2x BME vitamins and penicillin (200 units/ml), and streptomycin (94 μg/ml), supplemented with 10% CO_2 and 90% air at 37°. Under these culture conditions 2RA cells (which have gone beyond their 275th passage) form a confluent monolayer within 7 days after plating.

(*b*) *Harvest of Cells.* The monolayers were washed three times with Ca–Mg free Hank's Balanced Salt solution (Division of Becton, Dickinson and Company, Cockeysville, Md.), and scraped with a rubber policeman in the same solution. The cells were then harvested by centrifugation at 1500 rpm for 3 min in an International refrigerated centrifuge (PR6).

(*c*) *Cell Number and Other Analytical Procedures.* The number of cells in a confluent monolayer culture was deter-

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mined by counting the cells in a hemacytometer after trypsinization of the monolayers, as described under (d). The amount of DNA and proteins were determined respectively by the methods of Scott *et al.* (1956), and Lowry *et al.* (1951).

(*d*) *Chromosomes Preparation.* Preparation of metaphase chromosomes followed the method described by Moorhead *et al.* (1960) with slight modifications.

(*e*) *Isolation of Chromatin.* Clean nuclei were prepared by the method of Hymer and Kuff (1964). Chromatin was isolated from these Triton nuclei using the technique of Marushige and Bonner (1966), with slight modifications (Farber *et al.*, 1971). The cell pellets obtained after centrifugation were vortexed on Vortex-Genie (Scientific Industries Inc., Springfield, Mass.) with 8 volumes of 1% Triton X-100, 20 mM EDTA, 80 mM NaCl, pH 7.4. After vortexing, 3 volumes of 1 M sucrose in 20 mM EDTA and 80 mM NaCl (pH 7.4) were added and the nuclei were centrifuged. This step was repeated 3–4 times until clean nuclei were obtained without cytoplasmic contamination. The clean nuclei were washed twice with 5 ml of cold 0.15 M NaCl–0.01 M Tris (pH 8.3) and allowed to swell and lyse in 3 ml of cold, distilled water for 30 min. The nuclei were then gently homogenized with about 30 strokes of a Potter-Elvehjem homogenizer; the lysate was layered on to 1.7 M sucrose, stirred into the upper $\frac{2}{3}$ of the tube, and spun in an SW 39L Spinco rotor for 80 min at 37,000 rpm. The unsheared chromatin pellet was then resuspended in 0.01 M Tris buffer (pH 8.3).

(*f*) *Determination of Circular Dichroism.* (1) CHROMATIN. Chromatins isolated from confluent, quiescent cultures of WI-38 and 2RA cells were suspended in 0.01 M Tris (pH 8.3), dialyzed, and adjusted to about the same concentration (approximately 1 OD/ml at 260 nm). Circular dichroism (CD) was measured using a Jasco Model J-40 recording spectropolarimeter with CD only. The instrument was standardized as described by Simpson and Sober (1970). All experiments were carried out at 23° in nitrogen atmosphere in fused quartz 1-cm cells. The time constant was 4 sec, scanning speed below 1 cm/min, dynode voltage not over 400 V, and the precision in band wavelength was 0.3 nm. The maximum value of optical density ratio 400/258, a measure of light scattering, was 0.02. The mean ellipticity (θ) is expressed in deg cm^2 per dmol of nucleotide residue assuming the mean weight of a nucleotide as 330. Under our operating conditions, the overall error in repeated determinations of an identical sample was estimated to be 5% in the 250–300-nm region, and negligible at $\lambda < 250$ nm.

(2) PURE DNA. DNA from WI-38 and 2RA cells was isolated and purified according to the Marmur procedure (1961). The concentrations of DNA were adjusted to 1 OD/ml and circular dichroism spectra were taken under the same conditions described above for chromatin.

(3) CHROMATINS EXTRACTED BY 0.25 M NACL. After determination of circular dichroism the chromatins from WI-38 and 2RA cells were extracted with 0.25 M NaCl in 0.01 M Tris (pH 8.3) for 1 hr at 4°. Chromatins were then spun down at 37,000 rpm for 15 hr at 4° in a SW39L Spinco rotor. The salt-washed chromatin pellets were then resuspended in 0.01 M Tris buffer (pH 8.3) and circular dichroism spectra of salt-washed chromatins of WI-38 and 2RA were taken under the same conditions as described above.

(*g*) *Analysis of Supernatants from Salt-Washed Chromatins of WI-38 and 2RA Cells.* In one experiment WI-38 and 2RA cells were continuously labeled for 6 days with 1 μCi/ml of [^{3}H]leucine. Chromatins of WI-38 and 2RA cells were prepared and extracted with 0.25 M NaCl as described above. The supernatants of salt-washed chromatins were fractionated on a Bio-Rex 70 column (see below). In two other experiments WI-38 and 2RA cells were labeled (a) with 0.1 μCi/ml of [^{3}H]thymidine, or (b) with 0.1 μCi/ml of [^{3}H]uridine continuously for 6 days. In all cases the supernatants and the pellets of 0.25 M NaCl washed chromatins of WI-38 and 2RA cells were analyzed for acid-precipitable radioactivity by counting aliquots in a cocktail of Triton–toluene scintillation fluid (Patterson and Green, 1965), with a Packard TriCarb liquid scintillation spectrometer.

(*h*) *Dissociation of Chromatin.* The chromatin obtained after centrifugation through 1.7 M sucrose was resuspended by homogenization in 2.5 ml of 0.1 M sodium phosphate buffer (pH 7.0). For dissociation of proteins from DNA, chromatin suspensions were adjusted to 10 ml containing 6 M urea, 0.4 M guanidine-HCl, 0.1% β-mercaptoethanol, and 0.1 M sodium phosphate (pH 7.0), by addition of solid urea (Levy *et al.*, 1972). DNA was sedimented by centrifugation at 35,000 rpm at 4° for 40 hr, using a 50 Ti rotor in a Spinco Model L-65B ultracentrifuge. The supernatant contained the chromosomal proteins and trace amounts (1.2%) of DNA (Augenlicht and Baserga, 1973a). Approximately 75% of chromosomal proteins were dissociated from DNA under these conditions.

(*i*) *Separation of Histones from Nonhistone Chromosomal Proteins.* The chromosomal proteins were fractionated on a Bio-Rex 70 column (200–400 mesh, sodium form, 10.2 mequiv/g) equilibrated in 6 M urea, 0.4 M guanidine-HCl, and 0.1 M sodium phosphate (pH 7.0), as described by Levy *et al.* (1972).

(*j*) *Preparation of Histones from Chromatin by Extraction with H_2SO_4.* Histones were extracted from chromatin by the procedure described by Bonner *et al.*, (1968b).

(*k*) *Electrophoresis of Chromosomal Proteins.* Non-histone chromosomal proteins were analyzed on 10% acrylamide gels at pH 7.0 by the method of Weber and Osborn (1969). Non-histone chromosomal proteins obtained from the Bio-Rex 70 column were dialyzed for 5 hr at room temperature in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulfate and 0.1% β-mercaptoethanol. The protein solution was made 15% in sucrose and aliquots of 400 μl were electrophoresed in duplicate at 8 mA/gel at room temperature for 9 hr. Bromophenol Blue was used as the tracking dye. Both upper and lower chambers contained 0.1% sodium dodecyl sulfate and 0.1 M sodium phosphate at pH 7.0.

Histones were analyzed in urea–polyacrylamide gels in acidic conditions as described by Panyim and Chalkley (1969).

Staining of non-histone chromosomal proteins followed the method of Maizel (1971). The histone gels were instead stained for 1 hr with 1% Amido Black in 7% acetic acid (Shaw and Huang, 1970) and destained with acetic acid–methanol–water (1:5:10). The gels were scanned at 550 nm in a Gilford 2400 recording spectrophotometer with a linear transport device at a slit width of 0.35 mm.

(*l*) *Coelectrophoresis of Labeled Chromosomal Proteins.* For these experiments WI-38 cells were continuously labeled for 6 days with [^{3}H]leucine (1 μCi/ml), while 2RA cells were continuously labeled with [^{14}C]leucine (0.3 μCi/ml). The control groups were two sets of 2RA cells. One set of 2RA cells was continuously labeled for 6 days with [^{3}H]leucine (1 μCi/ml), and the other set with [^{14}C]leucine (0.3 μCi/ml), also for 6 days. The cells were harvested, pooled together, chromatin was prepared, and chromosomal proteins were fractionated as described above. Electrophoretic analysis of double-labeled nonhistone chromosomal proteins and histones followed the same procedures described above. In other experiments [^{3}H]tryptophan (1 μCi/ml) and [^{14}C]tryptophan (0.3 μCi/ml)

TABLE I: Saturation Density, DNA, and Protein Content of WI-38 and 2RA Cells.[a]

Cell Type	Cell No./cm^2	μg of DNA/cm^2	Protein/DNA in Chromatin
WI-38	55.4×10^3	0.4	2.24 ± 0.22
2RA	103.0×10^3	2.0	2.30 ± 0.25

[a] WI-38 and 2RA cells were grown in Falcon T-flask as described in Materials and Methods. When they reached saturation density, cells were trypsinized and counted in a hemacytometer. Chromatin was prepared as described in the text. Protein and DNA in whole cells were determined by the method of Lowry *et al.* (1951) and Scott *et al.* (1956), respectively.

were used to label both control and experimental cells. Since histones do not contain tryptophan (Stellwagen and Cole, 1969a), only non-histone chromosomal proteins were labeled in this case.

The gels, after electrophoresis, were immediately frozen in liquid nitrogen for a few seconds and sectioned into 2-mm slices with a manual slicing device. The slices were oxidized in an Intertechnique Oxymat, as described before (Augenlicht and Baserga, 1973a), and the radioactivity of ^{3}H and ^{14}C collected in separate scintillation vials was determined in a Packard liquid scintillation spectrometer.

Materials. Chemicals were purchased as follows: colemid (CIBA Pharmaceutical Company, Summit, N. J.); Triton X-100 (Sigma); sucrose (Schwarz/Mann, ultra pure); urea (Schwarz/Mann, ultra pure); guanidine-HCl (Schwarz/Mann, ultra pure); Bio-Rex 70 (Bio-Rad Laboratories, Richmond, Calif.); acrylamide (Eastman); bisacrylamide (Eastman); Coomassie Blue (Sigma); Amido Black (Sigma). Isotopes: [^{3}H]leucine (specific activity 30.8 Ci/mmol; [^{14}C]leucine (280 Ci/mol); [^{3}H]tryptophan (2.5 Ci/mmol); [^{14}C]tryptophan (53.5 Ci/mol); [^{14}C]adenosine triphosphate (49.3 Ci/mol); [^{3}H]uridine (28.3 Ci/mol); and [^{3}H]thymidine (6.7 Ci/mmol) were all purchased from New England Nuclear co. All other organic solvents were of reagent grade.

Results

Cell Number and Chromosome Number. Table I gives the saturation density of WI-38 and 2RA cells grown in Falcon T-flasks. For both cell types the saturation density was determined on the 7th day after plating and without change of medium. At confluence there are twice as many 2RA cells per cm^2 than WI-38. The amount of DNA per cm^2 is five times higher in 2RA than in WI-38 cells. Under these conditions, 7 days after plating, the turnover rate (new cells produced per 24 hr) is $^8/_{1000}$ and $^5/_{100}$ for WI-38 and 2RA, respectively.

The chromosome number in 2RA cells varied greatly. About 68% of metaphases had a chromosome number in the range of 61–80, and 19% had a chromosome number greater than 120. On the contrary, all metaphases of WI-38 cells had 46 chromosomes with a normal female karyotype (Weiss and Green, 1967).

Chromatin was isolated from either WI-38 or 2RA cells, as described in Materials and Methods. The absorption spectra were undistinguishable and the protein/DNA ratios were essentially the same (Table I). The circular dichroism spectra of the two chromatins are shown in Figure 1 (between 250 and 300 nm) and Figure 2 (between 200 and 250 nm). Chromatin

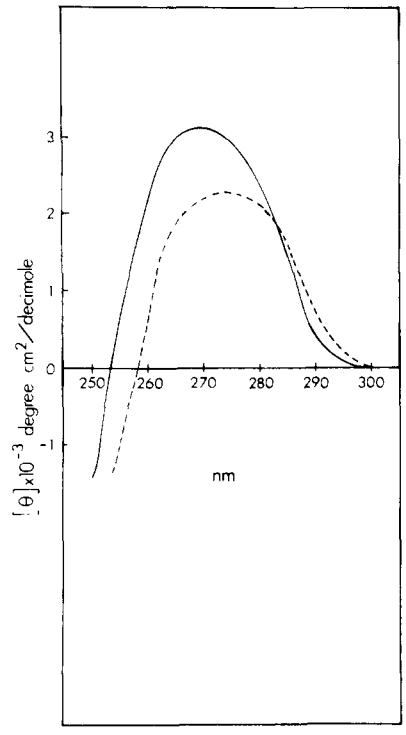

FIGURE 1: Circular dichroism spectra of chromatins from quiescent WI-38 and stationary 2RA cells, at wavelengths between 300 and 250 nm. CD spectra were measured at DNA residue concentrations of 48 μg/ml for WI-38 chromatin (- - - - - -) and 47 μg/ml for 2RA chromatin (—). [θ] was calculated as described in the text.

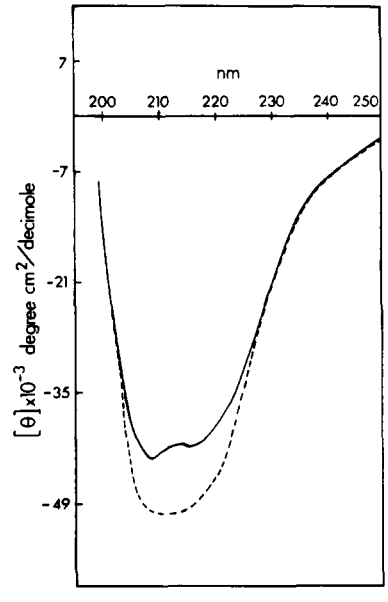

FIGURE 2: Circular dichroism spectra of chromatins from quiescent WI-38 and 2RA cells, at wavelengths between 250 and 200 nm. Same chromatins and same conditions as in Figure 1. (- - - - - -) WI-38 chromatin; (—) 2RA.

from WI-38 cells has a maximum positive ellipticity of +2300° cm^2/dmol, while negative ellipticity is centered around two bands with a maximum of 42,000° cm^2/dmol at 207 nm. 2RA chromatin shows an increased positive ellipticity (with a maximum of 3050° cm^2/dmol) and a blue shift (Figure 1). The curve in the $\lambda < 250$-nm region has a different shape and a maximum negative ellipticity of −50,700° cm^2/dmol.

This experiment has been repeated four times, and each time 2RA chromatin showed an increased positive ellipticity and a

TABLE II: Effect of Salt Extraction on RNA, DNA, and Protein Content of WI-38 and 2RA Chromatins.[a]

	[^{3}H]Uridine (cpm)		[^{3}H]Thymidine (cpm)		[^{3}H]Leucine (cpm)	
	S	P	S	P	S	P
WI-38	60 ± 34	6200 ± 340	2380 ± 320	240,820 ± 2020	10,200 ± 950	116,500 ± 1800
2RA	120 ± 30	70,080 ± 840	920 ± 45	256,760 ± 9200	9,554 ± 470	129,400 ± 2200

[a] S, supernatant; P, pellet. Cells were labeled and chromatin was prepared as described in Methods and Materials. The chromatins were extracted with 0.25 M NaCl, and the acid-precipitable radioactivity in the extracts and in the chromatin pellets was determined as described in the text. cpm refer to counts/minute in the extract and in the pellet from the same chromatin preparation.

blue shift in 250–300-nm region and a decreased negative ellipticity in the $\lambda < 250$-nm region. The absolute values varied ±10% around the mean values given in Figures 1 and 2, but the differences between the two chromatins persisted. Chromatin from quiescent 2RA cells showed a 32% (average) increase of positive ellipticity and a decreased negative ellipticity of 17% (average), compared to that of WI-38 chromatin.

Circular dichroism spectra of DNA from WI-38 and 2RA cells were identical (not shown). DNA from both sources has a positive symmetric CD band centered at 272 nm with a mean ellipticity $[\theta]_{272} = +7630°$ cm^2/dmol.

Salt Extraction of Chromatin. Previous studies have shown that the class of non-histone chromosomal proteins whose synthesis is increased when confluent monolayers of WI-38 fibroblasts are stimulated to proliferate bands in the low molecular weight region of polyacrylamide gels (Tsuboi and Baserga, 1972), and is extractable from chromatin by low concentrations of NaCl (Augenlicht and Baserga, 1973b). Concomitant with the extraction of this class of non-histone chromosomal proteins from stimulated WI-38 chromatin, there is a decrease in chromatin template activity to the level of chromatin from unstimulated control WI-38 (Augenlicht and Baserga, 1973b). We therefore attempted to determine whether extraction of chromatin from both sources with relatively low concentrations of NaCl could eliminate the differences in circular dichroism spectra of 2RA and WI-38 chromatins. Chromatins were isolated, washed with 0.25 M NaCl, and recentrifuged as described in Materials and Methods. The circular dichroism spectra of chromatins after extraction with 0.25 M NaCl are given in Figure 3 (between 250 and 300 nm) and Figure 4 (between 200 and 250 nm). In the 250–300-nm region, the differences are essentially abolished by extraction with 0.25 M NaCl, but some differences still persist in the $\lambda < 250$-nm region. Notice the dramatic decrease in negative ellipticity in Figure 4 (compared to Figure 2), although 0.25 M NaCl extracts only 8–12% of total chromosomal proteins (Augenlicht and Baserga, 1973b). In the 250–300-nm region, both chromatins show a decrease in positive ellipticity, the maxima being around +1900° cm^2/dmol.

Analysis of Salt Extract. In separate experiments WI-38 and 2RA cells were labeled with [^{3}H]thymidine, [^{3}H]uridine, and [^{3}H]leucine as described in Methods and Materials. The chromatin was prepared as usual and washed with 0.25 M NaCl. Table II shows that essentially no detectable [^{3}H]uridine or [^{3}H]thymidine radioactivity could be found in the 0.25 M NaCl extract. In contrast about 8.7 and 7% of acid-precipitable [^{3}H]leucine radioactivity were recovered in the supernatants of salt-washed chromatins of WI-38 and 2RA, respectively. The proteins recovered in the salt wash were analyzed on a Bio-Rex 70 column as described in Materials and Methods. In both cases the [^{3}H]leucine-labeled proteins eluted with the non-histone fractions. Figure 5 shows the only results obtained

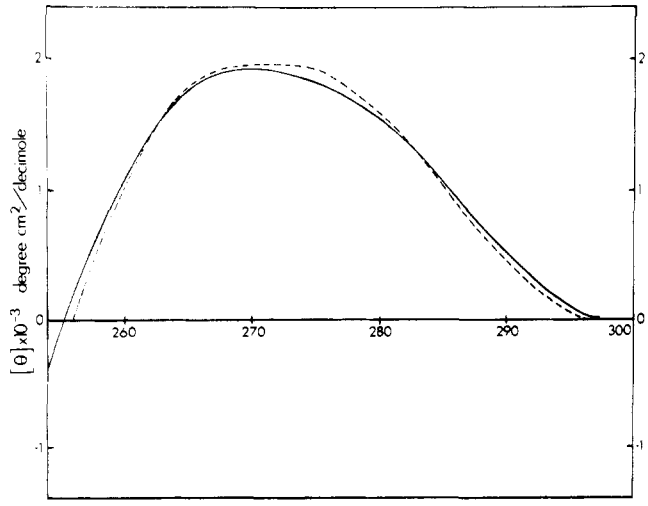

FIGURE 3: Circular dichroism spectra of chromatins washed with 0.25 M NaCl in 0.01 M Tris-HCl (pH 8.3) at wavelengths between 300 and 250 nm. The salt-washed chromatins were sedimented by centrifugation at 35,000 rpm for 15 hr in a SW39L Spinco rotor. The chromatin pellets were resuspended in 0.01 M Tris-HCl (pH 8.3) and CD spectra were taken under the same conditions as in Figure 1. (- - - - - -) WI-38 chromatin; (—) 2RA chromatin.

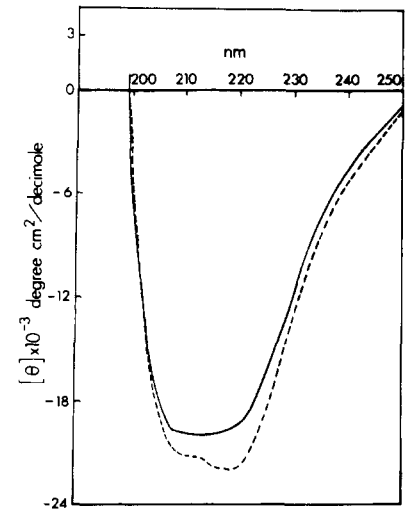

FIGURE 4: Circular dichroism spectra of WI-38 and 2RA chromatins washed with 0.25 M NaCl in 0.01 M Tris-HCl (pH 8.3) at wavelength between 250 and 200 nm. Same chromatins and same conditions as in Figure 3. (- - - - - -) WI-38 chromatin; (—) 2RA chromatin.

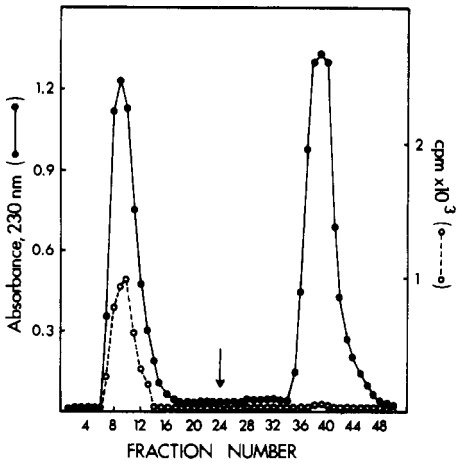

FIGURE 5: Bio-Rex 70 column chromatography of chromosomal proteins from 2RA cells. A 2 × 8 cm column of Bio-Rex 70 was loaded with the supernatant of a 0.25 M NaCl extract of chromatin from 2RA cells which were continuously labeled with [^{3}H]leucine for 6 days as detailed in the text. After washing with the equilibrating buffer which elutes the non-histone proteins, a step of 4 M guanidine hydrochloride was applied at the arrow. Histones elute in the second peak. An elution profile of dissociated total chromosomal proteins from 2RA chromatin serves as control to show the localization of non-histone and histone protein fractions. (●—●) absorbance at 230 nm; of dissociated total chromosomal proteins; (○ - - - - - ○) radioactivity of proteins extracted from chromatin by 0.25 M NaCl.

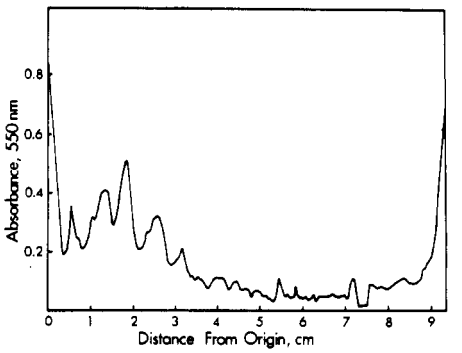

FIGURE 6: Absorbance profiles of standard non-histone chromosomal proteins from WI-38 cells after electrophoresis in sodium dodecyl sulfate polyacrylamide gels. Chromatin was isolated and non-histone chromosomal proteins were obtained from Bio-Rex 70 column chromatography as detailed in the test. Non-histone chromosomal proteins were analyzed on 10% sodium dodecyl sulfate polyacrylamide gels at pH 7.0 by the method of Weber and Osborn (1969). About 35–50 μg of proteins were applied to each gel and electrophoresis was performed in duplicate at 8 mA/gel at room temperature for 9 hr using Bromophenol Blue as tracking dye. After the electrophoretic run the gels were stained with 0.2% Coomassie Blue in 7% acetic acid at 25° overnight and destained with acetic acid–methanol–water (1:5:10) mixtures. The gels were scanned at 550 nm in a Gilford 2400 recording spectrophotometer.

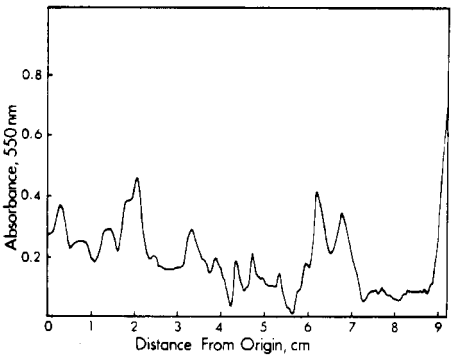

FIGURE 7: Absorbance profiles of stained non-histone chromosomal proteins of 2RA cells after electrophoresis in sodium dodecyl sulfate polyacrylamide gels. The method and the conditions for gel electrophoresis were exactly the same as in Figure 6.

with 2RA, but WI-38 gave essentially the same results (Baserga *et al.*, 1975).

Electrophoretic Profile of Histones. Histones were isolated from WI-38 and 2RA cells, as described in Materials and Methods. The gel electrophoretic profiles, as revealed by staining with Amido Black, were exactly similar for histones isolated from WI-38 and for histones isolated from 2RA cells (not shown). In order to determine whether there could be more subtle differences between histones from the two sources, WI-38 and 2RA cells were labeled respectively with [^{3}H]leucine and [^{14}C]leucine, as described in Materials and Methods. The cells were harvested and processed together. The radioactivity profiles of the gels and an analysis of the $^{14}C/^3H$ ratio revealed no detectable differences throughout the whole gels between histones from WI-38 cells, labeled with [^{3}H]leucine, and histones from 2RA cells, labeled with [^{14}C]leucine (not shown). These results confirm the findings of Zardi *et al.* (1973), that antibodies, anti-WI-38 chromatin could not recognize immunological differences between histones of WI-38 and 2RA cells.

Electrophoretic Profile of Non-Histone Chromosomal Proteins. Non-histone chromosomal proteins isolated from WI-38 and 2RA cells were resolved into many components by electrophoresis in the sodium dodecyl sulfate polyacrylamide gels. The stained gels revealed a highly heterogeneous banding pattern. Estimation of relative amounts of each individual band was made by scanning the stained gels at 550 nm. Figures 6 and 7 show the optical density profiles of stained gels of WI-38 and 2RA cells, respectively. The banding patterns of non-histone chromosomal proteins of WI-38 and 2RA cells are similar in regions of slow electrophortic mobility (high molecular weight). On the other hand, a distinguishing feature of two extra bands in the region of fast electrophoretic mobility (low molecular weight) is observed in non-histone chromosomal proteins isolated from 2RA cells. These two bands are either much smaller or undetectable in gels of non-histone chromosomal proteins from WI-38 cells.

Coelectrophoresis of Radioactively Labeled Non-Histone Chromosomal Proteins. The differences observed in the electrophoretic banding patterns of non-histone chromosomal proteins from WI-38 or 2RA could result from artifacts related to differences in solubility or to differential proteolytic activity during the isolation procedure. To answer this question we labeled WI-38 cells with [^{3}H]leucine and 2RA cells with [^{14}C]leucine, as detailed in Materials and Methods. When the cells formed confluent monolayers they were harvested and pooled together, and non-histone chromosomal proteins were isolated as described in Materials and Methods. As a control group we used two sets of cultures of 2RA cells, one labeled with [^{3}H]leucine and the other with [^{14}C]leucine. In the control gels in which non-histone chromosomal proteins from 2RA cells were labeled with either ^{3}H or ^{14}C, the ratio $^{14}C/^3H$ remained essentially the same, fluctuating throughout the whole gel around a ratio 0.4–0.5 (not shown). On the other hand, when non-histone chromosomal proteins of WI-38 and 2RA were coelectrophoresed together there was a marked difference in the $^{14}C/^3H$ ratio in the same region of the gel (Figure 8) in which differences were found in the stained gels. It seems therefore that these differences are not due to artifacts arising during the isolation procedure. A possibility that differences in these two regions may result from contamination of non-histones by histones in the process of fractionation on the Bio-Rex

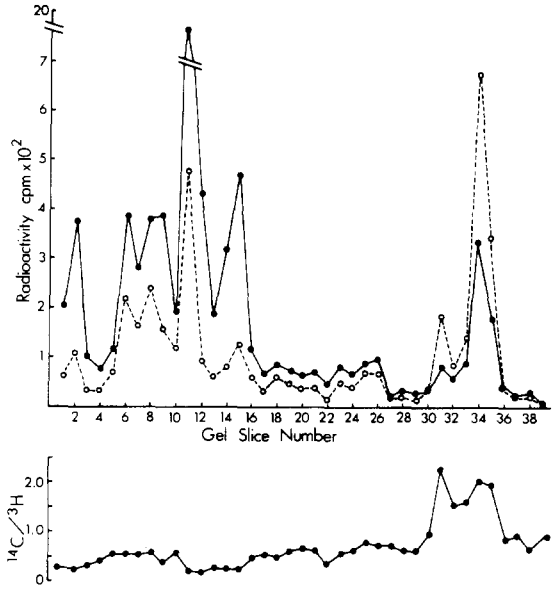

FIGURE 8: Electrophoretic radioactivity profiles of non-histone chromosomal proteins from WI-38 and 2RA cells. WI-38 cells were labeled with [^{3}H]leucine and 2RA cells with [^{14}C]leucine. The isolation procedure and analysis of non-histone chromosomal proteins are detailed in the text. (O - - - - - O) [^{14}C]leucine; (●—●) [^{3}H]leucine. The bottom of the figure gives the $^{14}C/^3H$ ratio.

70 column was considered. To eliminate this possibility, non-histone chromosomal proteins of WI-38 and 2RA cells were labeled respectively with [^{3}H]tryptophan and [^{14}C]tryptophan. The cells were pooled together after harvesting and the non-histone chromosomal proteins again isolated and electrophoresed as described above. Changes in the $^{14}C/^3H$ ratio in the region of low molecular weight proteins persisted even when non-histone chromosomal proteins were labeled with radioactive tryptophan (not shown).

Discussion

2RA cells are SV-40 transformed WI-38 fibroblasts that have different growth characteristics from the parent cell type. Our results show that the chromatin of stationary 2RA cells can be distinguished from the chromatin of quiescent WI-38 fibroblasts. Confluent monolayers of 2RA cells have a residual proliferative activity that is somewhat higher than in WI-38. However, the difference (5% *vs.* 1%) is too small to account for the differences in chromatin described in this paper.

The circular dichroism spectrum of 2RA chromatin is different from the circular dichroism spectrum of WI-38 chromatin. It is difficult to interpret these spectra, except to state that they indicate some differences in conformation between the two chromatins. Structural changes in chromatin that may be related to our present findings have been previously reported by Bolund *et al.* (1969), by Spelsberg *et al.* (1973) and by Baserga *et al.* (1975). Bolund *et al.* (1969) found a decreased thermal stability of chromatin in phytohemagglutinin-stimulated lymphocytes. Spelsberg *et al.* (1973) found an increased positive ellipticity in chromatin of estrogen stimulated chick oviduct that paralleled an increase in template activity. Finally, Baserga *et al.* (1975) described a marked increase in the positive ellipticity of chromatin from confluent monolayers of WI-38 cells 3 hr after stimulation by a nutritional change. In addition, Polacow and Simpson (1973), after fractionating rat liver chromatin into two fractions that differ in their transcriptional ability, showed that the more actively transcribing chromatin had an increased positive CD in comparison to the less actively transcribing fraction. These results seem to indicate that an increased positive ellipticity in the 250–300-nm region of circular dichroism spectra may indicate an increased transcriptional activity. In this respect it should be noted that progressive extraction of chromatin with increasing concentrations of salt, which causes an increase in the template activity of chromatin, also usually causes an increase in positive ellipticity toward that of protein-free DNA (Wagner and Spelsberg, 1971; Williams *et al.*, 1972; Simpson and Sober, 1970). It should also be noted that our θ values for chromatin are lower than most, but not all, values reported in the literature. The low values are probably due to the fact that we used unsheared chromatin, while most investigators shear chromatin before taking the CD spectra.

An attempt was made in our studies to investigate which chromatin components may be responsible for the conformational differences between the chromatins of 2RA and WI-38 cells. While we have not been able to show any detectable differences between histones of 2RA and histones of WI-38 cells, our findings show that non-histone chromosomal proteins of 2RA can be distinguished on the basis of gel electrophoresis from non-histone chromosomal proteins of WI-38. These findings confirm previous results in our laboratory showing that antibodies, anti-WI-38 chromatin could recognize and distinguish non-histone chromosomal proteins (but not histones) of WI-38 cells from those of 2RA cells, (Zardi *et al.*, 1973). In addition our findings show that when the two chromatins are washed with 0.25 M NaCl their circular dichroism spectra become undistinguishable, at least in the 250–300-nm region. A similar finding has been recently reported by Baserga *et al.* (1975) who showed that extraction with 0.25 M NaCl abolishes the differences in circular dichroism spectra between chromatin of quiescent WI-38 cells and chromatin of WI-38 cells stimulated to proliferate by a nutritional change. It has been reported in the literature that extraction of chromatin with such a low concentration of salt as 0.25 M NaCl does not remove histones (Johns, 1971; Simpson, 1973; Bolund and Johns, 1973). Our findings also indicate that extraction of 2RA chromatin with 0.25 M NaCl essentially removes only non-histone chromosomal proteins.

An increase in the synthesis of non-histone proteins and changes in their electrophoretic profiles have also been reported in several situations in which the growth rate of cells on their degree of differentiation is altered (Stellwagen and Cole, 1969b; Stein and Baserga, 1970; Smith *et al.*, 1970; Rovera and Baserga, 1971; Chung and Coffey, 1971; Levy *et al.*, 1973; Le Stourgeon *et al.*, 1973; Courtois *et al.*, 1974; Sanders, 1974). It would be tempting to speculate that non-histone chromosomal proteins may also be responsible for the difference in proliferating ability and other characteristics between WI-38 and 2RA cells (Cholon and Studzinski, 1974a,b). However, such conclusions on the basis of present findings would be premature, since we cannot rule out enzymatic modifications of nuclear proteins known to occur in cells stimulated to proliferate, such as phosphorylation (Kleinsmith *et al.*, 1966), acetylation (Pogo *et al.*, 1966), and methylation (Tidwell *et al.*, 1968).

The conclusions that can be drawn from our data are the following: (1) the chromatin of WI-38 cells can be distinguished on the basis of circular dichroism spectra from the chromatin of their SV-40 transformed counterparts, 2RA cells; (2) histones from WI-38 cells cannot be distinguished from histones of 2RA cells either on an immunological basis (Zardi *et al.*, 1973), or on the basis of gel electrophoretic profiles (present data); (3) non-histone chromosomal proteins from WI-38 cells

can be distinguished from non-histone chromosomal proteins of 2RA cells, both on the basis of immunogenicity (Zardi *et al.*, 1973), and on the basis of gel electrophoretic profiles (present data); (4) the difference in circular dichroism spectra between WI-38 and 2RA chromatins can be abolished by washing both chromatins with 0.25 M NaCl; (5) in the 0.25 M NaCl extract only non-histone chromosomal protens are detectable. The possibility that differences in the chromatin of 2RA and WI-38 cells may be due to modifications of histones and/or non-histone chromosomal proteins by acetylation, phosphorylation, methylation, or to other components of chromatin has not been ruled out by the present experiments. However, our data do lend support to the hypothesis that non-histone chromosomal proteins may be responsible for the differences in chromatin between WI-38 and 2RA cells.

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