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*REPRESSED AND ACTIVE CHROMATIN ISOLATED FROM INTERPHASE LYMPHOCYTES**

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These experiments involved the development of a procedure for isolating repressed and active chromatin from interphase calf thymus lymphocytes, and for studying the metabolism and morphology of such interphase chromosomal segments.

Up to 80 per cent of the deoxyribonucleic acid (DNA) of interphase thymus lymphocytes is inactive in messenger ribonucleic acid (RNA) synthesis,[1] and is apparently repressed,[2] correlating well with the differentiated state of these cells.[3] It has long been suggested that inactive chromosomal segments could be recognized cytologically during interphase and early prophase by their dense-staining characteristics, implying a condensed state (see ref. 4 for general review). Such condensed inactive chromosomal segments have been termed heterochromatin,[5–7] in contrast to segments in a more active, extended, diffusely staining state, termed euchromatin.

The heterochromatic sex-chromatin body of Barr[8] in female mammalian cells is composed of a segment of one X chromosome,[9–11] and the genes carried on this heterochromatic segment have been shown to be unexpressed in the phenotype of the cell.[12–14] The DNA of this heterochromatic X chromosome replicates later than that of other chromosomal segments,[15–17] and it has been shown that heterochromatic segments of interphase autosomes are much less active in RNA synthesis[6] and in DNA synthesis[18] than are euchromatic segments.

Much of the DNA of interphase thymus lymphocytes is visible cytologically as condensed, Feulgen-positive masses,[19, 20] resembling the heterochromatin of the inactive Barr sex-chromatin body. In this study it is shown that these condensed chromosomal segments can be isolated in mass from lymphocytes, that up to 80 per cent of the DNA of interphase lymphocytes is contained in these masses, and that the DNA, RNA, and protein metabolism of these condensed heterochromatin masses is decreased compared to that of the more-extended euchromatin fibrils.

Materials and Methods.—Previous isolations of chromatin: Chromatin represents interphase chromosomal segments and, as such, contains DNA, histones, nonhistone proteins, RNA, lipids, and perhaps polysaccharides.[21] The native association of such components is disrupted by marked departures of the isolation medium from isotonicity (salt concentration above 0.6 *M* or below 0.01 *M*) or from neutrality (above pH 8.0 or below pH 6.0),[22] imposing constraints upon the isolation procedure.[23] Previous methods of isolation of chromatin have utilized blending,[24, 25] grinding with sand[26] or with glass beads,[27] or ultrasonication[28, 29] of isolated nuclei that have been washed free of cytoplasm. In the method reported here, nuclear membranes, nuclear ribosomes, and soluble nuclear proteins were also removed before the isolation of chromatin fractions, and gelation was prevented throughout.

Isolation of interphase nuclei: A slight modification of the methods previously described[30] was used. Calf thymus lobes were brought to the laboratory in an ice-cold solution of 0.25 *M* sucrose within 1 hr of slaughtering. All subsequent operations other than incubation were performed at 2°C. The thymus of a single animal was selected, dissected free of adherent fat, connective tissue, and blood, and 100 gm of wet-weight tissue was finely minced. The mince was suspended in 1000 ml of 0.25 *M* sucrose–0.0033 *M* $CaCl_2$, and was homogenized at half speed in a Waring blendor 3 min. After filtering through cheesecloth, the homogenate was blended twice more for 3 min, with filtrations through flannelette, and was then centrifuged at 600 *g* for 7 min. The resultant nuclear pellet was washed once with 1000 ml of 0.25 *M* sucrose–0.0033 *M* $CaCl_2$, and was then suspended in 100 ml of an incubation medium of composition: 0.1875 *M* sucrose, 0.02 *M* glucose, 0.025 *M* Tris HCl, 0.0128 *M* NaCl, 0.0033 *M* $CaCl_2$, pH 7.1.

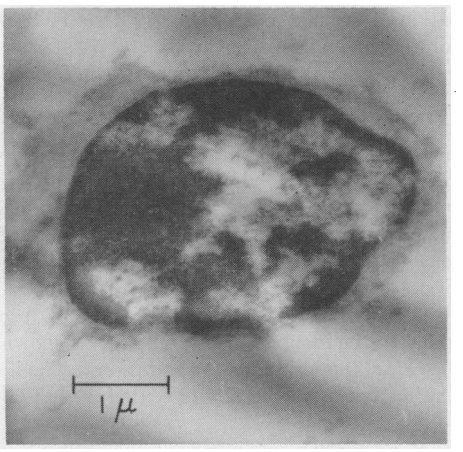
Incubation of nuclei: C^{14}-labeled precursors, such as thymidine-2-C^{14}, uridine-2-C^{14}, orotic acid-6-C^{14}, alanine-1-C^{14}, and lysine-1-C^{14} were added to the incubation suspensions, as shown in the legends to the tables, and were incubated at 37°C for 30 min in air with agitation. The incubation suspension was then added to 900 ml of cold incubation medium, and centrifuged at 600 *g* for 7 min.

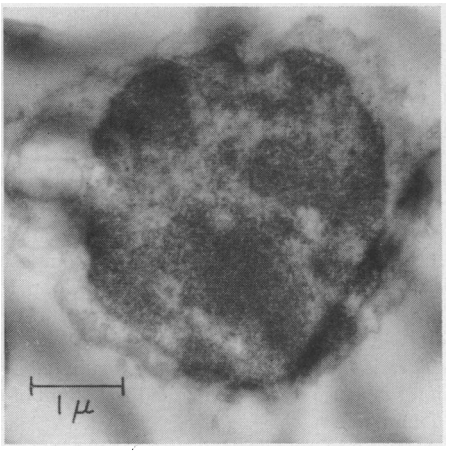
Isolation of subnuclear fractions: The supernatant incubation medium contained cytoplasmic fragments and nuclear membranes,[31] which were removed in this and a second washing in 1000 ml of incubation medium. The washed nuclear pellet was extracted three times with 1000-ml volumes of 0.01 *M* Tris HCl–0.0033 *M* $CaCl_2$, pH 7.1, removing nuclear ribosomes and neutral proteins.[31] The easily decantable slurry following the third Tris extraction was removed, and the residual extracted nuclei were resuspended in 1000 ml of cation-free 0.25 *M* sucrose, allowed to stand for 10 min to permit swelling of the nuclei, and were then sonicated for 5 sec at 20,000 cycles per second and 7 amp on a Branson S-75 sonifier. Such brief sonication is much less than the 7–20 min employed in previous isolations of chromatin.[28, 29] Previous studies of the effect of sonication on isolated DNA showed that 5 sec exposure had minimal effects on the physical[32] and biological[33] properties of protein-free DNA, and that the native association of histone with DNA, as in chromatin, further stabilizes DNA against physical change.[22]

The suspension was sonicated in aliquots of 60 ml at a time, and the resulting sonicate was centrifuged at 100 *g* for 5 min, with the supernate being filtered through four layers of flannelette to remove any aggregated material. This filtrate was centrifuged at 1000 *g* for 10 min, and the pellet collected as condensed (hetero-)chromatin masses. The supernate was then centrifuged at 3000 *g* for 30 min, and the pellet collected as the intermediate fraction. The supernate obtained was then centrifuged at 78,000 *g* for 60 min, and this pellet collected as extended (eu-)chromatin fibrils. The resultant clear supernate was then made 10 mM in calcium chloride and centrifuged at 1000 *g* for 10 min. This pellet was collected as particles in 10 mM calcium. The measurement of protein, DNA, RNA, and radioactivity was as previously described.[31]

Electron microscopy: Electron micrographs of the whole tissue mince before the addition of solutions, and of the various centrifuge pellets, were obtained after fixation of the tissues for 15 min in 1% OsO_4,[20] dehydration in alcohol, embedding in Epon,[34] sectioning at 800-A thickness on a Cambridge-Huxley microtome, and staining for 15 min in 1% uranyl acetate.[20] Such staining is needed to reveal fully the condensed (hetero-)chromatin masses as seen by phase contrast or ultraviolet microscopy.[35, 36] A Phillips model EM-100 A electron microscope was used, yielding microscopic magnifications to ×20,000. Subsequent magnifications were obtained photographically.

Results.—Electron micrographs of thymus tissue sampled before the addition of any preparative solutions (Figs. 1 and 2) reveal large masses of condensed (hetero-)chromatin arrayed peripherally toward the margins of the lymphocyte nucleus, with broad-based attachments to the nuclear membrane. These masses are seen at higher magnifications to be composed of a dense reticulum of 100-A fibrils, and are similar to those reported previously in thymus lymphocytes,[3, 19, 20] nucleated erythrocytes,[37] blood lymphocytes,[38] pancreas,[20, 39] liver,[40] von Kupfer cells and granulocytes,[41] lymphosarcoma cells,[36] Ehrlich ascites cells,[42] and as karyosomes[43] or chromocenters[5] in other well-differentiated cells. Fine strands of extended 50-A (eu-)chromatin fibrils are seen coursing between the

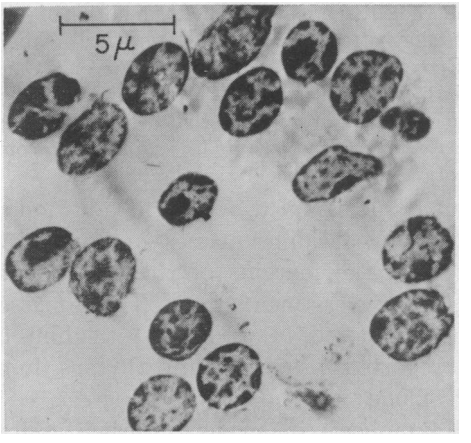


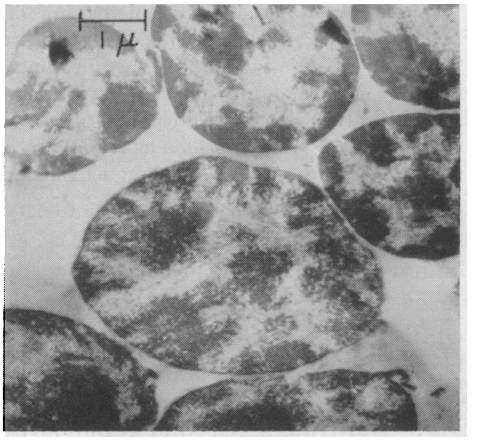


FIGS. 1 and 2.—Interphase calf thymus lymphocytes, before addition of preparative solutions. Masses of condensed (hetero-)chromatin contrast with fibrils of more extended (eu-)chromatin within the nucleus.

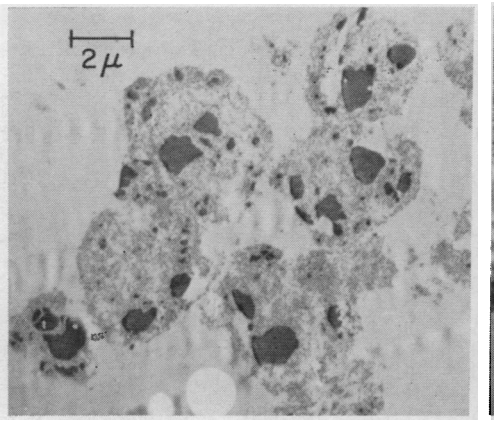
large condensed masses, and an occasional light-staining 0.5-μ nucleolus is seen, usually at the border of a condensed chromatin mass.[20]

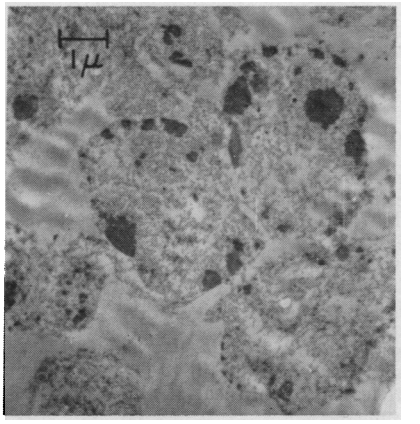
Electron micrographs of isolated nuclei following extraction of nuclear ribosomes and neutral proteins (Figs. 3 and 4) reveal these same features, but when the extracted nuclei are resuspended in cation-free isotonic sucrose (Figs. 5 and 6), the nuclei swell to twice their normal size, the extended chromatin fibrils are spread, and these are seen to course from the periphery of the condensed chromatin masses.[44, 45] Following brief sonication and isolation of the condensed (hetero-) chromatin masses (Figs. 7 and 8), their basic structure and dimensions remain unaltered, with some fragmented euchromatin fibrils still attached. Isolated euchromatin fibrils (Fig. 9) are seen to be free of condensed chromatin masses. The isolated particles in 10 mM calcium (Fig. 10) appear as aggregates of particles somewhat resembling those of isolated nucleoli[29] or their fragments. The intermediate



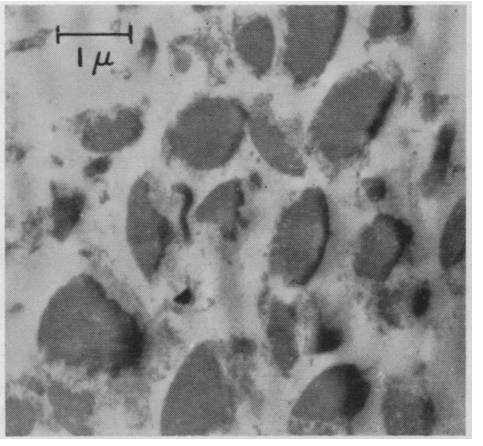


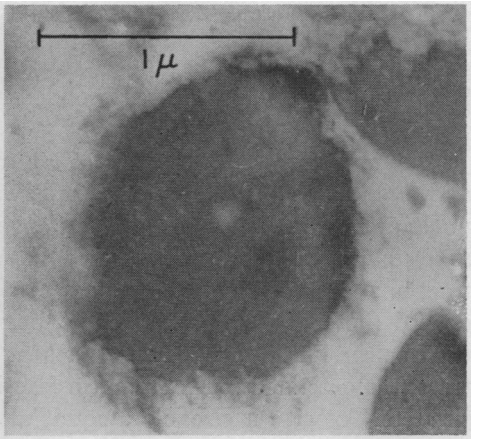
FIGS. 3 and 4.—Isolated lymphocyte nuclei after extraction of nuclear ribosomes. Masses of condensed (hetero-)chromatin, fibrils of more extended (eu-)chromatin, and an occasional light-staining nucleolus.





FIGS. 5 and 6.—Swollen nuclei in cation-free isotonic sucrose. Extended (eu-)chromatin fibrils are seen attached to the condensed (hetero-)chromatin masses, with fibril dispersion distally.





FIGS. 7 and 8.—Isolated condensed (hetero-)chromatin masses. A small number of euchromatic fragments remain attached to the masses.

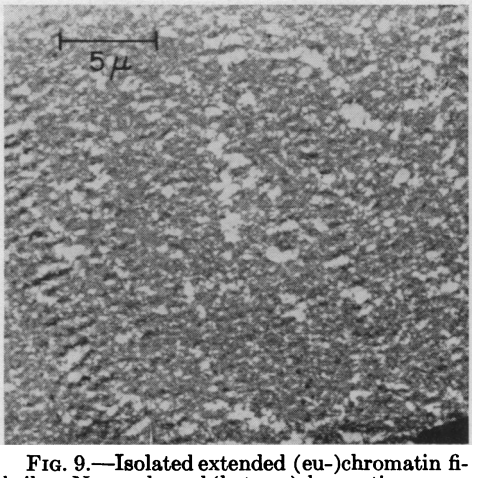


FIG. 9.—Isolated extended (eu-)chromatin fibrils. No condensed (hetero-)chromatin masses are found.

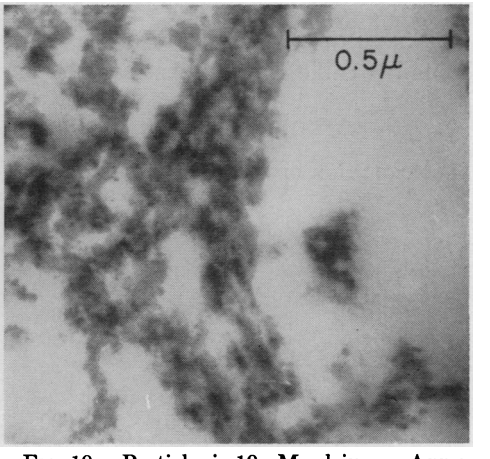


FIG. 10.—Particles in 10mM calcium. Aggregates of dense particles are evident, resembling those in isolated nucleoli.

TABLE 1

INCORPORATION OF OROTIC ACID-6-C^{14} INTO RNA OF SUBNUCLEAR FRACTIONS*

Fraction	Protein (mg)	DNA (mg)	RNA (mg)	Total counts (cts/min)	RNA, specific activity (cts/min/mg)	Composition DNA/RNA
Whole nucleus	1505.2	493.0	48.40	717,000	14,400	10.2
Cytoplasmic fragments and nuclear membranes	57.5	1.59	2.26	5,085	2,250	0.70
Nuclear ribosomes and pH 7.1-extracted proteins	253.6	14.37	24.73	38,200	1,543	0.58
Loose slurry after pH 7.1 Tris extraction	129.1	48.40	3.06	101,000	33,000	15.8
Condensed (hetero-) chromatin	481.5	259.26	7.14	71,855	10,600	36.3
Intermediate	136.8	47.39	3.10	63,230	20,400	15.3
Extended (eu-)chromatin	117.2	44.60	6.23	207,600	33,300	7.1
Particles in 10 mM calcium	65.9	8.95	1.75	106,000	60,600	5.1
Recovery	1237.6	424.44	48.27	592,970		
Per cent	82	86	97	83		

* Orotic acid-6-C^{14} (5.0 mg, 1.86 mC) added to suspension of isolated nuclei in 100 ml of incubation medium (see *Materials and Methods*).

fraction appears as a mixture of condensed (hetero-)chromatin masses and extended (eu-)chromatin fibrils.

The metabolism of DNA, RNA, and protein of the condensed chromatin masses and of the extended chromatin fibrils was studied by means of incubations of the nuclei with C^{14}-labeled precursors. Over 80 per cent of the protein, DNA, RNA, and counts incorporated into new RNA were recovered among the subnuclear fractions (Table 1). Up to 80 per cent of the nuclear DNA is found in the condensed (hetero-)chromatin fraction, but this fraction contains only 17 per cent of the nuclear RNA, and only 14 per cent of the newly synthesized RNA. By contrast, the extended (eu-)chromatin fraction contains the bulk of the newly synthesized RNA, with a sizeable amount also present within the particles in 10 mM calcium (Table 1). Little new RNA is found in the nuclear ribosomes, supporting previous evidence[1] that the chief RNA synthesis in isolated interphase lymphocyte nuclei is of the messenger type. Incubations incorporating thymidine-2-C^{14} into DNA and uridine-2-C^{14} into RNA (Table 2), or lysine-1-C^{14} and alanine-1-C^{14} into protein (Table 3), demonstrate that in each case, condensed (hetero-)chromatin

TABLE 2

INCORPORATION OF C^{14}-LABELED PRECURSORS INTO NUCLEIC ACIDS OF SUBNUCLEAR FRACTIONS

	DNA (mg)	Total counts* (cts/min)	DNA, specific activity (cts/min/mg)	RNA (mg)	Total counts† (cts/min)	RNA, specific activity (cts/min/mg)
Whole nucleus	344.0	228,200	664	40.30	350,000	8,680
Condensed (hetero-) chromatin	109.49	36,530	334	10.385	74,000	7,130
Intermediate	34.20	24,060	704	1.316	10,900	8,280
Extended (eu-)chromatin	35.85	43,950	1224	5.070	90,000	17,750
Particles in 10 mM calcium	5.55	6,020	1085	1.041	24,600	23,650

* Thymidine-2-C^{14} incorporated into DNA, after incubation of nuclei with 0.47 mg (50 μC) of isotope in 100 ml of incubation medium (see *Materials and Methods*).

† Uridine-2-C^{14} incorporated into RNA, after incubation of nuclei with 0.06 mg (7.5 μC) of isotope in 100 ml of incubation medium (see *Materials and Methods*).

TABLE 3

INCORPORATION OF C^{14}-LABELED AMINO ACIDS INTO PROTEINS OF SUBNUCLEAR FRACTIONS

	Protein (mg)	Total counts* (cts/min)	Protein, specific activity (cts/min/mg)	Protein (mg)	Total counts† (cts/min)	Protein, specific activity (cts/min/mg)
Whole nucleus	2118.0	107,200	51	1252.0	283,000	226
Condensed (hetero-)chromatin	500.0	3,670	7	357.2	11,950	34
Intermediate	102.2	4,100	40	97.4	11,950	123
Extended (eu-) chromatin	123.1	7,320	59	99.9	25,100	251
Particles in 10 mM calcium	22.3	1,641	74	22.4	6,744	301

* dl-Alanine-1-C^{14} incorporated into protein after incubation of nuclei with 3.72 mg (160 μC) of isotope in 100 ml of incubation medium (see *Materials and Methods*).
† dl-Lysine-1-C^{14} incorporated into protein after incubation of nuclei with 5.0 mg (95 μC) of isotope in 100 ml of incubation medium (see *Materials and Methods*).

metabolism is $^1/_3$ to $^1/_8$ as active as that of extended (eu-)chromatin. A similar correlation between condensation and inactivity, and between extension and activity has been demonstrated in polytene chromosomes[46] and in lampbrush chromosomes[47, 48] during interphase.

Discussion—The gene-inactivation hypothesis of cell differentiation postulates that as a cell differentiates, those genes not mediating the particular state of differentiation are inactivated by a repression mechanism.[1, 49–52] The present study confirms previous evidence[1, 2] that a large fraction of the DNA of interphase calf thymus lymphocytes is metabolically inactive, correlating well with the differentiated state of these cells.[3]

The isolation of repressed and active chromatin from interphase lymphocytes permits studies to elucidate the mechanism of repression in condensed (hetero)-chromatin. If this isolation procedure is gentle enough, the repressed and active chromatin fractions may demonstrate the native *in vivo* association of DNA, histones, nonhistone proteins, RNA, lipids, and other molecular species, permitting an assessment of their role in repression. The histone hypothesis of DNA inactivation is a plausible mechanism for the maintainence of chromatin repression.[1, 2, 25, 53] Current studies in progress[54] suggest that the active, extended (eu-)chromatin fibrils, as isolated by this procedure, have a deficiency of histone for their DNA content, as contrasted to the balance between histone and DNA in the inactive, condensed (hetero-)chromatin masses. A partial account of this study has been presented.[55]

Summary—Repressed and active chromatin can be isolated in mass from interphase calf thymus lymphocytes. Up to 80 per cent of interphase lymphocyte DNA is found in the repressed chromatin fraction. Repressed interphase DNA appears as condensed (hetero-)chromatin, while active interphase DNA appears as extended (eu-)chromatin fibrils. The extended (eu-)chromatin fraction contains the bulk of the rapidly labeled RNA of the nucleus.

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