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The Distribution of Active Genes (Globin) and Inactive Genes (Keratin) in Fractionated Chicken Erythroid Chromatin†

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ABSTRACT: Repeatable fractionation of sheared chromatin from purified populations of chicken erythroid cells has been achieved, based on the Bio-Gel procedure of Janowski et al. ((1972) *Karolinska Symp. 5,* 112). For reticulocytes, 3–5% of chromatin DNA is excluded from Bio-Gel A-50 m (peak I) and over 90% elutes in the included volume of the column (peak II). Peak I material has a higher protein/DNA ratio than peak II chromatin and the two fractions have melting profiles characteristic of "active" and "inactive" chromatin, respectively. In cells prelabeled with [^{3}H]uridine or [^{3}H]leucine there was very pronounced preferential association of radioactivity with the "active" peak I chromatin. The distribution of "active" (globin) and "inactive" (keratin) gene sequences in the DNA of fractions from peak I and peak II chromatin was determined with complementary DNA (cDNA) probes to chicken globin mRNA and chicken feather keratin mRNA. While slight enrichment for globin gene sequences was found in peak I (relative to DNA in these fractions), some 80% of the total globin hybrid formed was found in peak II fractions. Experiments with the keratin cDNA probe showed that these genes were equally distributed in both chromatin fractions rather than being confined to the "inactive" peak II material. The hybridization data in particular question the validity of claims for fractionation of chromatin into "active" and "inactive" material.

There is considerable biochemical and cytological evidence to support the general concept that in differentiated eukaryotic cells, a high percentage of the genome is transcriptionally inactive.

Rapid advances in the understanding of DNA sequence organization around specific eukaryotic genes can be expected with the advent of hybrid DNA technology and clonal selection of *Escherichia coli* containing the required hybrid DNA molecules (Wensink et al., 1974; Murray and Murray, 1974; Kedes et al., 1975). Nevertheless, a fundamental question in eukaryotes concerns the mechanism by which a small proportion of the genome, the euchromatin or "active" genes, is unravelled, so that transcription can occur. Although "active" chromatin is most easily seen cytologically (polytene puffs, lampbrush chromosomes, ribosomal DNA transcription), biochemical approaches aim at the isolation and identification of the molecules responsible for this activation process.

One approach involves reconstitution experiments with isolated chromatin components, and recent evidence suggests that nonhistone proteins are important in both cell-specific (Barrett et al., 1974) and cell-cycle-stage-specific (Stein et al., 1975) transcription.

Another approach is to attempt fractionation of chromatin in vitro, and to isolate and characterize the "active" component. Implicit in this approach is the assumption that the in vivo structure of the chromatin can be sufficiently preserved in vitro to permit fractionation. Specifically, it means that the more diffuse euchromatin segments should have different physicochemical properties from the more condensed heterochromatic material to allow separation by physical means.

The earliest attempts at chromatin fractionation employed a differential centrifugation procedure (Frenster et al., 1963; Frenster, 1965). Similar methods have been used by Chalkley and Jensen (1968), McCarthy et al., (1973), and Murphy et al. (1973).

Other approaches have been employed including DNase digestion followed by differential precipitation (Marushige and Bonner, 1971), thermal chromatography on hydroxylapatite (McConaughy and McCarthy, 1972), chromatography on ECTHAM-cellulose (Reeck et al., 1972; Howk et al., 1975), gel-exclusion chromatography (Janowski et al., 1972), density gradient centrifugation (Rickwood et al., 1974; Jackson and Chalkley, 1974), and thermal precipitation (Markov et al., 1975).

The results of Janowski et al. (1972) suggested, by a number of different criteria, that a successful fractionation of mouse liver chromatin into its transcriptionally active and inactive components had been achieved using a gel-exclusion chromatography technique. Passage of sheared chromatin through a Bio-Gel A-50m column resolved the chromatin into two components. The first component eluting at the exclusion volume of the gel had many of the properties expected for

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"active" chromatin while the other component appeared to represent "inactive" material.

Because the gel-exclusion procedure looked so promising, we have concentrated on this approach for the fractionation of avian erythroid chromatin. In particular we have attempted to make the procedure reproducible for a defined population of avian red cells (defined by buoyant density) and we have also employed highly specific complementary DNA (cDNA[1]) probes for genes which are being actively transcribed in red cells (globin cDNA) and those which are never active in red cells (chick feather keratin cDNA), as assays for "active" and "inactive" chromatin sequences.

Experimental Procedures

Cells. Avian reticulocytes obtained from the circulation and dividing erythroblasts from the bone marrow of highly anaemic hens were fractionated on isotonic bovine serum albumin gradients as previously described (Harlow and Wells, 1975). Labeling of RNA or protein in cells with [^{3}H]uridine (Schwarz/Mann, 49 Ci/mmol) or [^{3}H]leucine (Amersham/Searle, 18 Ci/mmol) was as described (Harlow and Wells, 1975).

Chromatin Preparation. Membrane-free nuclear bodies ("SNK" preparation of Harlow and Wells, 1975) were prepared from purified avian erythroid cells as described. These were suspended in 1 ml per original 1 ml of packed cells in 0.15 M KCl, 0.1 M $MgCl_2$, 1 mM 2-mercaptoethanol, 0.01 M potassium phosphate, pH 6.8 (the "high salt" buffer of Janowski et al., 1972), and homogenized (2 min at 48 000 rpm) in a cooled microchamber of the Sorvall omnimixer (Newton Conn.). The A_{260}^{1cm} of this suspension was measured (a portion was dissolved in 5 M urea–2 M NaCl) and adjusted to 25 units per ml with high salt buffer. The resulting suspension was sheared in an Aminco French Pressure Cell (American Instrument Co., Md.) at 4000–8000 psi (see Results and Discussion) and then centrifuged at 12 000*g* for 5 min to remove aggregated insoluble matter. This "pellet" represented about 30% of the DNA. No further material was precipitated if the original sheared suspension was centrifuged for up to 15 min at 12 000*g*; furthermore, the soluble sheared chromatin in the supernatant formed no additional precipitate if left on ice for 48 h.

Gel-Exclusion Chromatography. Experiments were carried out at 4 °C with Pharmacia K 16/70 columns. Bio-Gel A-50m (Bio-Rad Laboratories, Calif.) was mixed with degassed distilled water and the thin slurry allowed to pack from a reservoir. The columns were fitted with flow adaptors, inverted, and equilibrated with high salt buffer by pumping about 2 column volumes of the buffer through against gravity. Samples of sheared chromatin were loaded (20 or 40 A_{260}^{1cm} units per column) from below and the upward flow rate maintained at 9.5 ml/h. Usually four columns were run simultaneously and fractions of 1.8 ml collected. The 2–5% excluded "active" material is referred to as peak I and the major included "inactive" material as peak II chromatin. The amounts of DNA in peak I and peak II (relative to the A_{260}^{1cm} of each fraction) were estimated by the diphenylamine reaction (Burton, 1956). On this basis, the percentage of 260 nm absorbing material accounted for as DNA was 93% for peak I and 94% for peak II material.

Counting Procedures. In whole cell incorporation experiments, 0.2-ml portions were taken from incubations, washed onto Whatman GF/C discs with isotonic saline (2 × 10 ml), 5% trichloroacetic acid (2 × 10 ml), and ether (5 ml), dried, and counted by standard procedures. Radioactivity in chromatin fractions was determined by dispersing 0.2-ml portions in a mixture of toluene scintillant–Triton X-114 detergent (5:3, v/v), mixing thoroughly, and counting in a Packed Tri-Carb liquid scintillation spectrometer.

Melting Profiles of Chromatin. Dilute peak I material was concentrated 15-fold in a Sartorius membranfilter under vacuum (Sartorius membranfilter, SM 13200, Göttingen, Germany). This concentrated chromatin (0.5 ml) and the peak II material were equilibrated with 0.025 M phosphate (pH 6.8) by passage through a Sephadex G-25 column (1 × 18 cm) containing this buffer. Melting profiles of chromatin fractions were measured in 1-cm light-path quartz cuvettes in a Unicam SP 1800 spectrophotometer with associated heating block and XY recorder. The temperature was increased at a rate of 0.5 °C per min. The T_m and hyperchromicity of samples were determined from the trace and the melting profile was also replotted on normal probability paper (Knittel et al., 1968).

Protein/DNA Ratios. Protein was estimated using the micromethod of Schaffner and Weissmann (1973). DNA content was estimated using a value of $A_{260}^{1cm} = 22$ for a 1 mg/ml solution.

Preparation of Chromatin Fractions for Hybridization. To fractions from Bio-Gel profiles, sufficient calf thymus DNA (Sigma Chemical Co., Miss.) was added to bring the total to 200 μg of DNA. Sodium acetate, pH 4.0 (2.0 M), was added to a final concentration of 0.1 M followed by 2 volumes of ethanol and the mixture was allowed to stand for 14 h at −15 °C. The precipitated material was collected by centrifugation (10 000*g*, 10 min) and the pellet dried in vacuo. The dried pellet was suspended in 0.5 ml of Protease K digestion buffer (Gross-Bellard et al., 1973) containing 100 μg/ml of Protease K and the mixture incubated for 2 h at 37 °C. NaOH was added (final concentration, 0.3 M) and incubation continued for an additional hour to digest any RNA present; then samples were readjusted to pH 4.0 by the addition of 100 μl of 2.0 M sodium acetate, pH 4.0, and 160 μl of 1 N HCl. The volume was made to 2 ml with 0.1 M NaCl, 2 volumes of ethanol added, and material allowed to precipitate at −15 °C for 14 h. (All these manipulations were carried out within one tube for each sample to avoid losses on transfer.) The precipitate was collected after centrifugation (10 000*g*, 10 min) and dried in vacuo.

Hybridization of cDNA Probes to DNA. Two separate complementary ^{3}H-labeled probes were used. Chicken globin cDNA (specific activity 5.0 × 10^7 dpm/μg, 650 nucleotides long) was the gift of Mr. R. Crawford. Chicken feather keratin cDNA (Kemp, 1975) was the gift of Dr. D. Kemp.

The dry DNA pellets prepared from chromatin fractions were dissolved in 50 μl of hybridization buffer (0.18 M NaCl, 0.01 M Tris-HCl, pH 7.0, 0.001 M EDTA, pH 7.0, 0.5% sodium dodecyl sulfate). These were sonicated with cooling for 90 s (Daw "Soniprobe", 1130 A; setting 8; 3–4 mÅ) and then 2000 cpm of the appropriate cDNA probe was added to each fraction. The mix was taken up in 100-μl microcaps which were sealed, treated at 100 °C for 5 min, and then placed in a 60 °C water bath to allow reassociation to occur.

The reassociation reactions were taken to a C_0t of 3 × 10^3 relative to *chicken* DNA in the peak I fractions to ensure that the globin cDNA would reassociate to at least 80% even if there was no enrichment for globin genes in the peak I material (the

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; cDNA, complementary deoxyribonucleic acid; high salt buffer, 0.15 M KCl, 0.1 M $MgCl_2$, 1 mM 2-mercaptoethanol, 0.01 M potassium phosphate, pH 6.8; RNP, ribonucleoprotein.

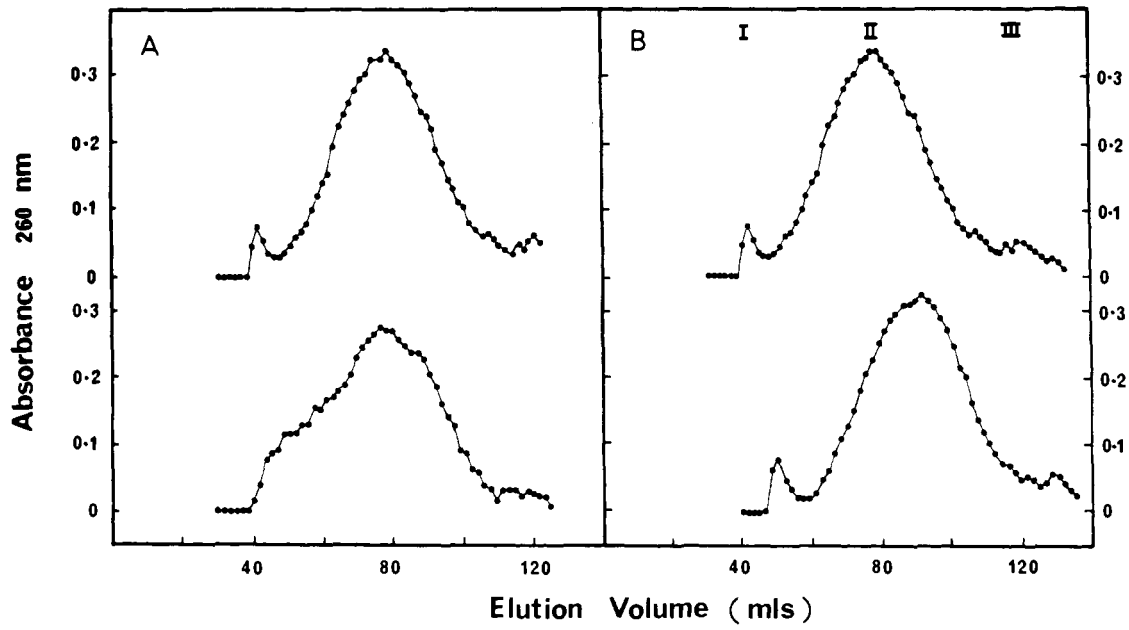

FIGURE 1: Reproducibility of chicken reticulocyte chromatin elution from Bio-Gel A-50m columns. Chicken reticulocyte chromatin sheared at 4000 psi was prepared for chromatography and 20 $A_{260}{}^{1cm}$ units per run chromatographed with upward flow-through Bio-Gel columns as described in Experimental Procedures. (A) The upper frame shows the standard elution profile obtainable with a "good" batch of Bio-Gel A-50m and the lower frame shows the profile obtained when a portion of the same chromatin is run immediately afterward on the same column. (B) The upper frame is a duplicate run of A, upper frame. After the run the column was repacked with the same Bio-Gel material and reequilibrated. The lower frame shows the elution profile of portion of the same preparation of chromatin chromatographed on the repacked column. Peak I is the excluded "active" chromatin and peak II the included "inactive" chromatin.

$C_0t_{1/2}$ value for globin cDNA to total chicken DNA is 1.2×10^3 in our hands). Doubling the time of reassociation (and thus the C_0t value) made no difference to the shape of the cross profile hybridization. The percentage of either globin or feather keratin cDNA hybridized in each fraction was determined by S_1 nuclease assays (Kemp, 1975). Control incubations of calf thymus DNA (200 μg) with globin or keratin cDNA were always performed. The maximum hybrid formed was 4%. The appropriate figure plus S_1 nuclease resistant cDNA radioactivity (3% of input) were subtracted.

Results and Discussion

In vivo, active chromatin segments are presumably interspersed with inactive portions, and it is therefore necessary to introduce breaks in the DNA in vitro (either by shear forces or limited DNase digestion) to allow segregation of these segments. In view of the lack of definitive data on the size of active fragments in vivo, we initially used a shearing force (4000 psi) to produce chromatin fragments containing double-stranded DNA of average molecular weight 1.4×10^6. This is unlikely to be much bigger than "gene size". (Some further consideration of the effect of shearing on chromatin fractionation is given below.)

It is relevant to consider the predicted result for avian erythroid chromatin fractionation into active and inactive portions. The hybridization results of McConaughy and McCarthy (1972) suggest that about 2% of this genome may be actively transcribed, and it was therefore anticipated that Bio-Gel fractionation should result in an excluded active peak representing about this percentage of the genome. The direct questions then were: (1) Could such fractionation be achieved? (2) Was it reproducible? (3) Were the two Bio-Gel fractions truly representative of active and inactive chromatin?

Reproducibility of Chromatin Fractionation on Bio-Gel A-50m. In attempts to repeat the results of Janowski et al. (1972) with chicken reticulocyte chromatin, the immediate problem encountered was lack of reproducibility. In particular the size of excluded active chromatin (peak I) relative to the inactive chromatin (peak II) was highly variable (from 5 to 25%). The results in Figure 1A show that even when portions of the same chromatin preparation were chromatographed simultaneously through several different (but similarly prepared) Bio-Gel columns there was variability in the elution profiles. The greatest source of variability appeared to be related to the number of times a particular column had been used. Since only about 75% of the applied material was recovered in the eluate from the column, it seemed possible that accumulated adsorbed material may have interfered with subsequent fractionation. The results in Figure 1B show that superimposable elution profiles could be obtained, if the agarose gel was removed from the column, washed and repoured, between experiments.

As shown in Figure 1B a typical fractionation profile consists of a small first peak, eluting at the exclusion volume of the gel and containing between 2 and 5% of the total material eluted from the column. Peak II contains by far the greatest proportion of the chromatin eluted while the small third peak, running at the total volume of the column, once again contains between 2 and 5% of the recovered material.

The kinds of profile shown in Figure 1B are extremely reproducible in the short term (see discussion below) provided that all preparative procedures are stringently controlled. In addition to the point above about repouring columns between each run, some other factors which are important for reproducibility of chromatin fractionation are the following.

1. Homogeneity of Cell Populations. Cells used in this study were relatively homogeneous since they were selected on the basis of buoyant density (Experimental Procedures). The nondividing cells also have the possible advantage of being naturally synchronized (in G_0 phase) since it is quite possible that unsynchronized dividing cell populations would give rise to more variable chromatin preparations (see also point 6).

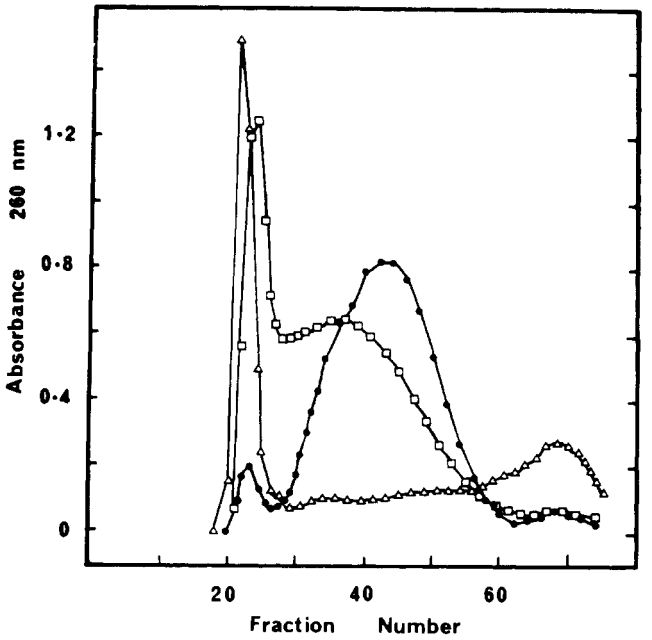

FIGURE 2: Elution profiles of chicken reticulocyte chromatin from different batches of Bio-Gel A-50m. Three identical columns were freshly packed with different batches of Bio-Gel A-50m and 20 $A_{260}{}^{1cm}$ units per column of chromatin chromatographed through them. The different symbols represent the elution profiles for each column. The "good" batch (filled circles) is the same as that used for profiles shown in Figure 1.

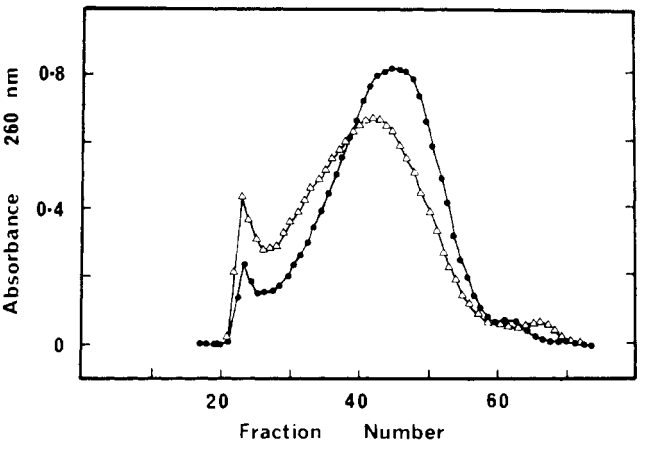

FIGURE 3: Shearing pressure and the elution profiles of chicken reticulocyte chromatin. Portions of a chromatin preparation were sheared at either 4000 psi or 6000 psi and chromatographed under standard conditions: (●—●) 6000 psi, sheared chromatin; (△—△) 4000 psi, sheared chromatin.

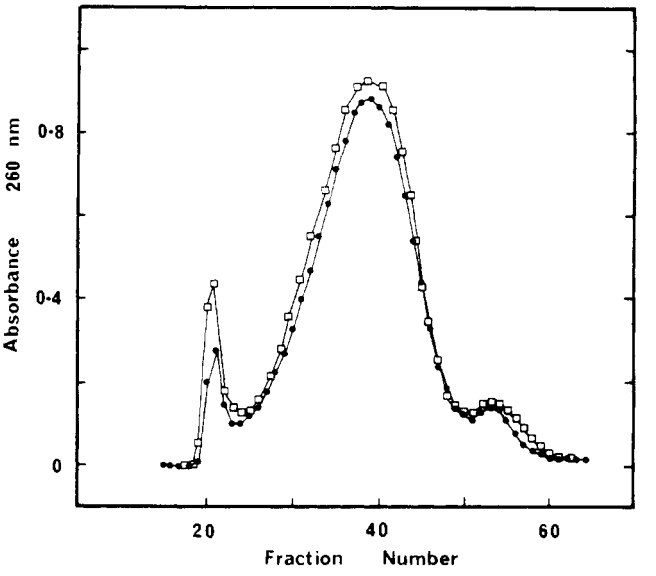

FIGURE 4: Elution profiles of chromatin prepared from chicken erythroblasts and reticulocytes. Chromatin from dividing erythroblasts and nondividing reticulocytes was chromatographed (each in duplicate) as described in Figure 1. (□—□) Erythroblast chromatin; (●—●) reticulocyte chromatin.

2. Clarification of Sheared Chromatin. (Removal of the Pellet, Experimental Procedures.) It was difficult to handle the chromatin without removal of the insoluble pellet (up to 30% of the DNA) prior to chromatography.

3. Quantity of Chromatin Applied to Column. For the 1 × 70 cm columns used, good resolution of chromatin was achieved for up to 40 $A_{260}{}^{1cm}$ units of sheared chromatin. With 50 units, clear distinction of peak I from peak II material was not obtained. The loading volume was usually 2 ml.

4. Batch of Bio-Gel Used. Within *one* batch of Bio-Gel A-50m, elution profiles for different preparations of avian reticulocyte chromatin were reproducible. As Figure 2 shows, between batches there was enormous variation. This profile change could be due to variation in the pore size between different batches of Bio-Gel, a smaller pore size excluding more of the sheared chromatin and so causing a larger proportion of the total material to elute at the exclusion volume of the column. Investigation of Sepharose 2B (Pharmacia, Uppsala, Sweden) suggested much less batch variation and equally good resolution of peak I and peak II chromatin.

5. Pressure Used to Shear Chromatin. Pronounced variation in the fractionation profile of reticulocyte chromatin may also be caused by alteration of the shearing pressure applied to chromatin. As Figure 3 shows, varying the shearing pressure from 4000 to 6000 psi resulted in a halving of the first peak size and a movement of the main peak material toward greater elution volumes. During the course of the experiments it was found necessary, due to the gradual deterioration of the Agarose gel from repeated washing and repouring, to increase the shearing pressure gradually from 4000 up to 800 psi (corresponding to double-stranded DNA molecular weights of about 1.4×10^6 and 0.9×10^6, respectively) in order to maintain a fractionation profile corresponding to that shown in Figure 1B. Of six batches tried, only two were originally "good" as judged by the success of obtaining profiles as in Figure 1B and these were used extensively. The deterioration of these batches was relatively slow and the increase in shearing pressure required to maintain profiles was applied gradually over a 6-month period.

6. Cell Type. The data in Figure 4 demonstrate that the type of cell from which the chromatin is obtained also affects the fractionation profile. Under identical conditions of preparation and chromatography, the chromatin originating from the dividing erythroblasts fractionates to yield a larger first peak (7.5%) than chromatin from reticulocytes (2–5%). This is not unexpected since the dividing erythroblast is more active transcriptionally than the nondividing reticulocyte (Williams, 1971; Harlow and Wells, 1975).

Physical Properties of Chicken Reticulocyte Chromatin Fractions. Some physical characteristics of isolated peak I and peak II material were investigated to determine whether the material corresponded in its properties to those expected for active and inactive chromatin.

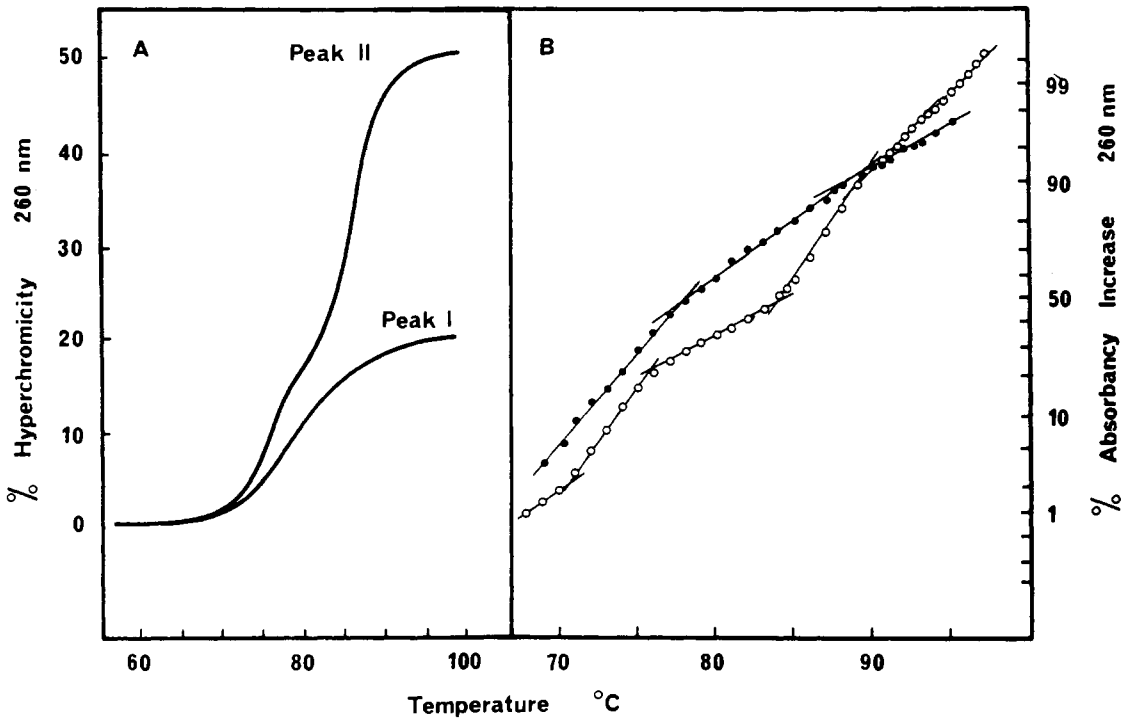

FIGURE 5: Thermal denaturation profiles of peak I and peak II reticulocyte chromatin. Material equivalent to peak I and peak II chromatin shown in Figure 1B was equilibrated with 0.025 M phosphate buffer (pH 6.8) as described in Experimental Procedures and hyperchromicity on thermal denaturation measured for each sample. (A) Hyperchromicity traces; (B) replot of data (Knittel et al., 1968). (●—●) Peak I chromatin; (○—○) peak II chromatin.

TABLE I: Protein/DNA Ratios of Peak I and Peak II Chromatin.[a]

Sample	DNA (μg/ml)	Protein (μg/ml)	Protein DNA
(A) Peak I	3.2	6.2	1.97
Peak II	17.3	24.8	1.44
(B) Peak I	4.1	8.5	2.08
Peak II	16.6	25.0	1.51
(C) Peak I	6.1	9.6	1.57
Peak II	35.9	50.0	1.39
Peak I mean			1.87 ± 0.16
Peak II mean			1.45 ± 0.03

[a] Chromatin from peak I or peak II regions (Figure 1B) was assayed for DNA and protein (see Experimental Procedures).

1. Protein/DNA Ratio. It has frequently been reported that template-active chromatin has a higher protein to DNA ratio than repressed chromatin (Frenster, 1965; Chalkley and Jensen, 1968; Marushige and Bonner, 1971). To determine whether such a difference in composition could be detected between the Agarose gel first and second peak chromatin fractions, the protein to DNA ratio of each of the components was examined. The results of three separate determinations are presented in Table I, and it is clear that peak I chromatin has a statistically greater protein to DNA ratio than peak II chromatin.

2. Thermal Denaturation. When a chromatin solution is heated, thermal denaturation occurs over a wider range of temperatures than for free DNA, and the mean melting temperature (T_m) of chromatin is generally several degrees higher than for DNA (Huang and Bonner, 1962). This presumably is the result of stabilization of the DNA helix by associated proteins, especially histones (Li and Bonner, 1971). Chromatin melting curves often exhibit the presence of several components, indicating different degrees of protein association at different regions of the DNA (Huang and Bonner, 1962). In fractionated chromatin, the highly condensed material displays a higher T_m than the extended material (Frenster, 1965; Duerksen and McCarthy, 1971). The thermal denaturation profiles obtained when peak I and peak II chromatin were melted, and the normal probability replots (Knittel et al., 1968) of these results are shown in Figure 5. Several differences between the melting profiles produced by the two fractions are evident. The peak I chromatin exhibited a hyperchromicity of 21%, while the corresponding value for the peak II material was 51%. T_m's for the first and second peak samples were 78 and 84 °C, respectively. Furthermore, it is clear from the normal probability replots of the two denaturation profiles shown in Figure 5B that major differences exist between the number and relative proportions of the melting components in the two fractions. These results are very similar to those of Frenster (1965) for calf thymus lymphocyte chromatin fractions obtained by differential centrifugation.

Distribution of Labeled RNA and Protein in Chromatin Fractions. The results of the previous section have shown that the peak I component of chromatin is different in physical properties from peak II chromatin and that it has some of the physical properties often associated with the active component of chromatin. The fractionation of radioactively labeled chromatin was one of the tests applied by Janowski et al. (1972) to determine whether the peak I material from Bio-Gel columns contained the transcriptionally active sequences. A similar approach has been applied here by labeling reticulocytes with [^{3}H]uridine or [^{3}H]leucine prior to chromatin preparation.

The elution profile and the position of ^{3}H label associated with the fractionated chromatin from cells prelabeled with [^{3}H]uridine are shown in Figure 6A. In agreement with the results of Janowski et al. (1972), it is quite clear that the only

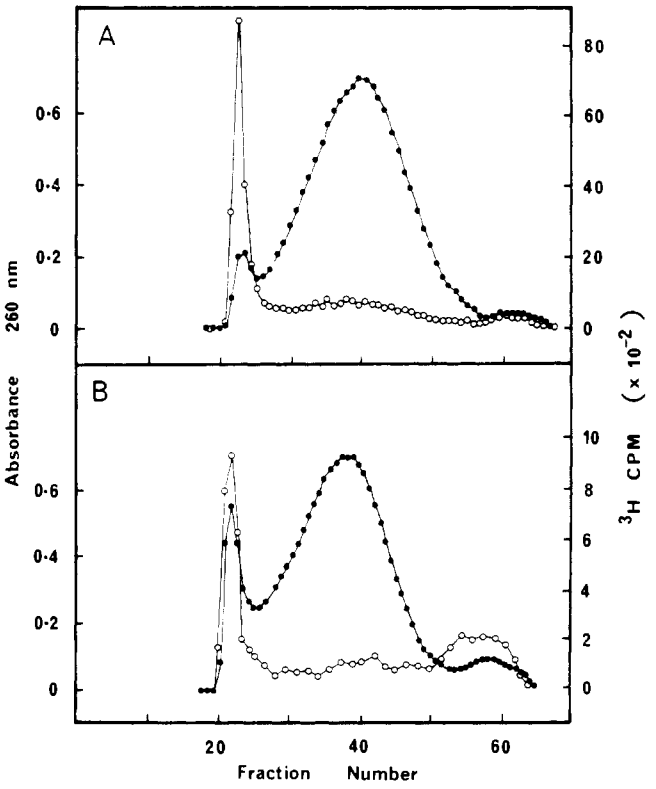

FIGURE 6: Association of labeled RNA and protein with reticulocyte chromatin. Chromatin prepared from reticulocytes prelabeled with [^{3}H]uridine or [^{3}H]leucine was fractionated on Bio-Gel columns. (●—●) A_{260} elution profile; (○—○) ^{3}H label. (A) From [^{3}H]uridine-labeled cells; (B) from [^{3}H]leucine-labeled cells.

major localization of labeled RNA on the profile coincides with peak I chromatin. In fact, 60% of the total radioactivity recovered was associated with the 4% of the chromatin that eluted as the first peak component. Relatively minor levels of label coincided with peak II and a small amount of radioactivity eluted at the total volume of the gel (presumably this represented material released from the chromatin).

Frenster et al. (1963) incubated thymocytes with ^{14}C-labeled amino acids prior to chromatin fractionation and showed that three to eight times more label was associated with euchromatin compared with heterochromatin. Since reticulocytes are active in the synthesis of chromosomal proteins, including histone f_2c (Appels and Wells, 1972), it might be predicted that newly synthesized chromosomal proteins would be associated with active, extended chromatin, rather than the inactive, highly condensed heterochromatin.

To test this prediction, reticulocytes were incubated with [^{3}H]leucine prior to chromatin fractionation. Figure 6B shows the radioactivity and elution profiles obtained from such a fractionation. Two peaks of radioactivity were observed, the prominent, sharp peak eluting with the peak I chromatin material while a second much broader peak of label appeared at the total volume of the column. Insignificant amounts of label were associated with the peak II chromatin fractions.

While the nature of the chromatin-associated proteins labeled during the [^{3}H]leucine incubation was not investigated, two classes of proteins would appear to be the most likely candidates: firstly, histone and nonhistone structural and regulatory proteins (perhaps those that are preferentially or uniquely associated with extended chromatin); and secondly ribonucleoprotein particle (RNP particle) proteins. Since RNP-particle proteins complex with newly synthesized RNA, they would be expected to elute at the same position as the RNA label. The broad peak of radioactivity that eluted at the total volume of the column probably represents chromatin-associated proteins released during the preparative procedures. (The high salt buffer containing 0.1 M $MgCl_2$ would assist dissociation.)

In summary, the preceding data show that peak I chromatin is different in physicochemical properties from the peak II material, and further that the peak I chromatin appears to be the metabolically active fraction. The preferential association of newly synthesized RNA with this peak I chromatin was pronounced.

Localization of Globin and Feather Keratin Gene Sequences in Chromatin Fractions. Globin messenger RNA is synthesized in the purified fraction of chicken reticulocytes used in this study (Harlow and Wells, 1975); therefore the globin genes of reticulocytes should be part of the active component of fractionated chromatin. A complementary DNA (cDNA) probe synthesized from purified avian globin mRNA can be used to detect globin genes in fractionated chromatin.

Reticulocyte chromatin from cells prelabeled with [^{3}H]-uridine was prepared and fractionated on Bio-Gel A-50m and the DNA from each of the fractions isolated and hybridized to globin cDNA (Experimental Procedures). As noted previously for other preparations, the newly labeled RNA is almost exclusively associated with the peak I material, but in contrast the results of the cross-profile hybridization, presented in Figure 7A, demonstrate clearly that the globin gene is not specifically localized to the peak I chromatin fractions. This result (repeated many times) casts some doubt on the previous assertion that the peak I chromatin is truly representative of active chromatin and, therefore, requires further comment. There are two difficulties in the interpretation of the cross-profile hybridization result with globin cDNA. The first concerns the possibility that the cDNA derived from adult globin mRNA templates hybridizes with fetal (and thus inactive) globin genes in the fractionated (adult) chromatin. The magnitude of this effect is impossible to determine since the precise relationships between chicken fetal and adult globin chains are not known (Brown and Ingram, 1974). However, the sheer magnitude of cDNA hybrid in the inactive peak II chromatin (about 80% of the total) makes it unlikely that the cross-profile hybridization shown in Figure 7A grossly distorts the distribution of active as opposed to inactive globin gene sequences on the basis of spurious hybridization to fetal genes.

The second difficulty in interpreting the cross-profile hybridization results with globin cDNA relates to the question of just what percentage of the cells used for chromatin preparation was actually involved in globin gene transcription. Avian reticulocytes were collected from a specific buoyant density fraction (Experimental Procedures) in order to avoid contamination by inactive cells, and while it is known that the cell population used synthesizes globin mRNA (Harlow and Wells, 1975) it would be very difficult to determine whether 100% of the cells do so. However, we do not believe that there is likely to be a large proportion of "inactive" cells in the reticulocyte population contributing to the large percentage of globin cDNA hybrid in chromatin peak II material, for as Figure 7B shows the fractionated chromatin from purified populations of dividing avian erythroblasts which are some four to five times more active in RNA synthesis than reticulocytes (Harlow and Wells, 1975) again showed a vast excess of globin cDNA hybridizing sequences in the peak II "inactive" fraction.

Because the change from the reticulocyte to the more active

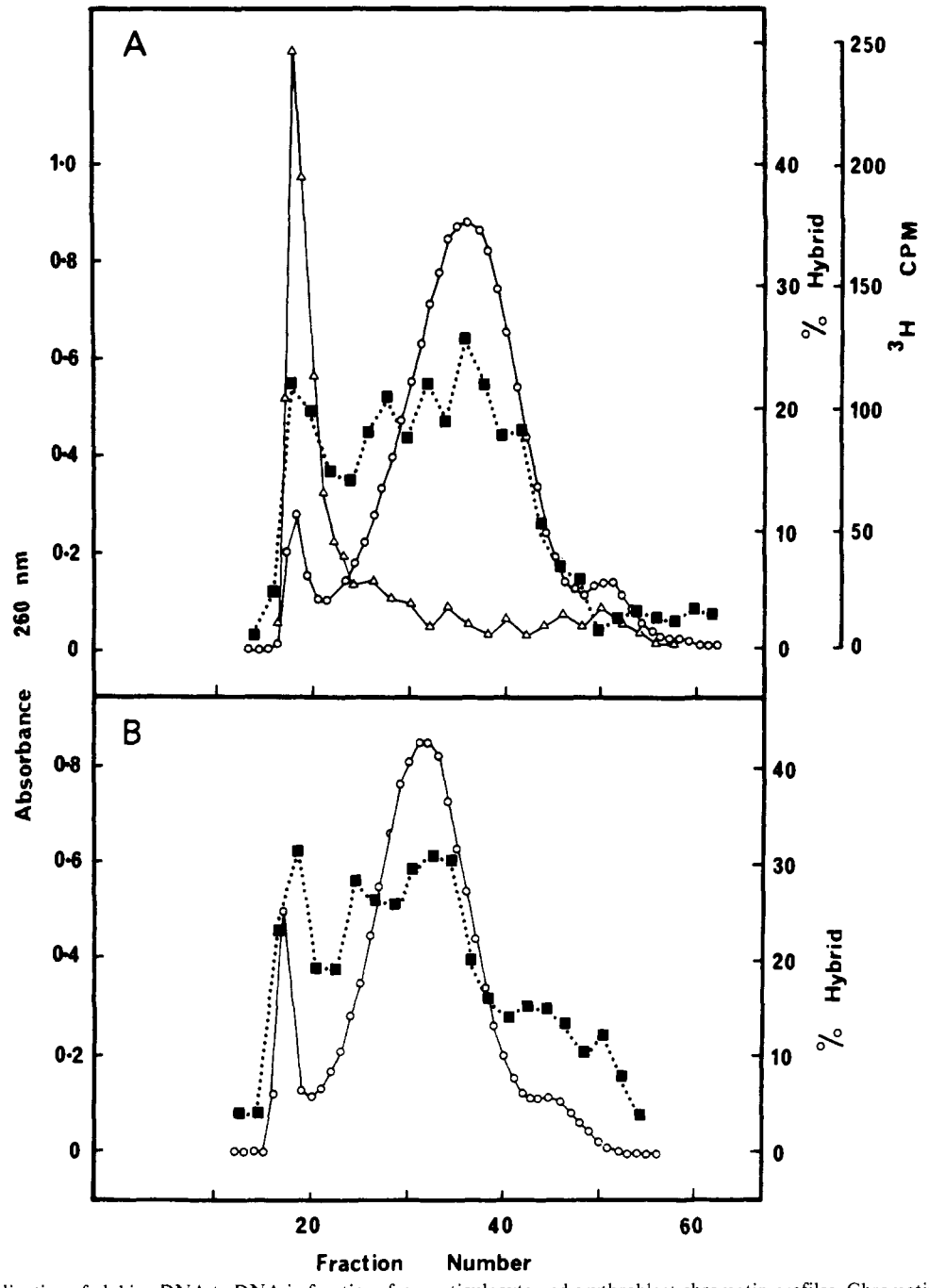

FIGURE 7: Hybridization of globin cDNA to DNA in fractions from reticulocyte and erythroblast chromatin profiles. Chromatin prepared from reticulocytes and erythroblasts was chromatographed on Bio-Gel columns and fractions prepared for hybridization as described in Experimental Procedures. Reticulocytes were prelabeled with [^{3}H]uridine. The percentage of chicken globin cDNA in hybrid form was assayed with S_1 nuclease. (O—O) A_{260} elution profile; (Δ—Δ) [^{3}H]uridine label; (■···■) percent globin cDNA hybrid. (A) Reticulocyte chromatin; (B) erythroblast chromatin.

erythroblast has produced virtually no change in the distribution of the globin genes across the fractionated chromatin profile, it is unlikely that the inactivity of the globin genes in reticulocyte chromatin is the major cause of the large amount of hybrid forming with the peak II fractions.

The use of a cDNA probe to chicken feather keratin is not subject to ambiguity. The keratin genes are not expressed in red cells and so the clear prediction, if Bio-Gel fractionation of avian erythroid chromatin really does give rise to active and inactive material, is that keratin cDNA hybridizing sequences should be absent or severely depleted in the peak I "euchromatin" and found almost exclusively in the peak II "heterochromatin". The results in Figure 8 show that no such segregation occurs and that the extent of hybrid formation appears to be directly proportional to the amount of DNA in the two fractions. This is an important negative result and supports the data with globin cDNA, suggesting that the chromatin fractionation is nowhere near as spectacular as might be believed from [^{3}H]uridine labeling data shown in Figures 6, 7, and 8.

Figure 9 expresses the cDNA hybridization data (derived from Figures 7 and 8) on the basis of percentage hybrid formed in the cross-profile hybridization per unit amount of DNA. For this purpose, the elution profile was arbitrarily divided into four sections A to D as shown in Figure 9A. Regions A and C are representative of peak I and peak II chromatin. This analysis

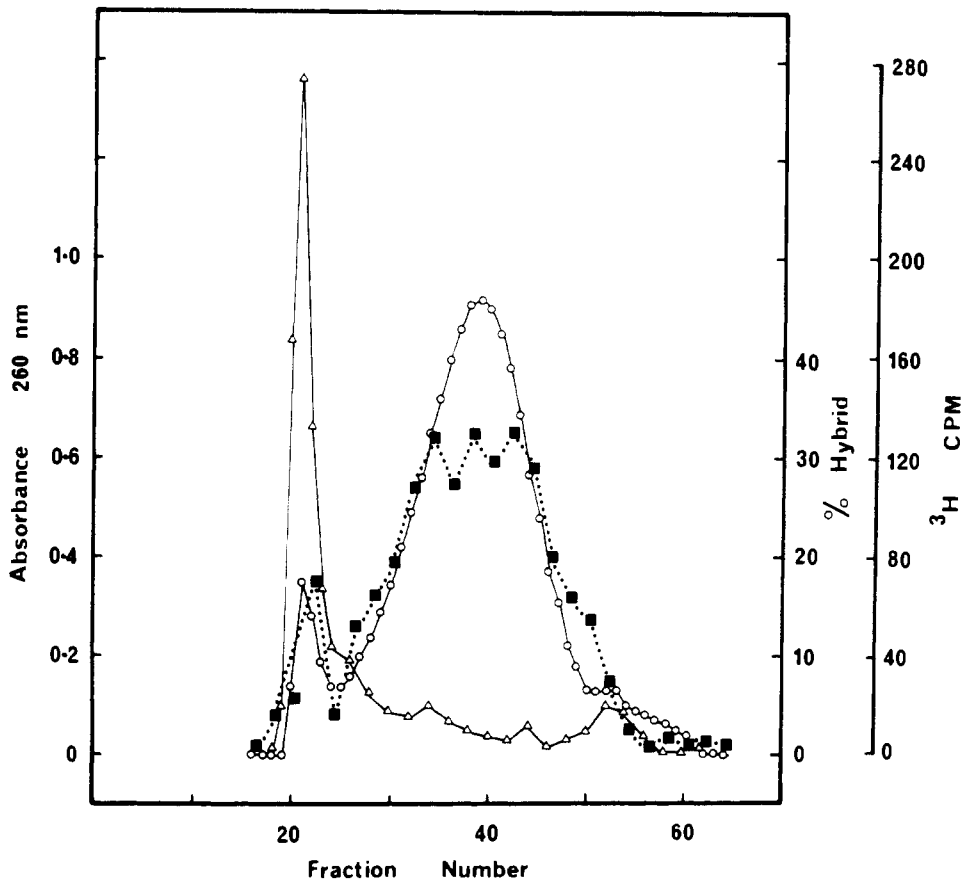

FIGURE 8: Hybridization of keratin cDNA to DNA in fractions from reticulocyte chromatin profile. The position of [^{3}H]uridine label and the extent of keratin cDNA hybridization to DNA from fractions of reticulocyte chromatin fractionated on Bio-Gel are as described for Figure 7. (O—O) A_{260} elution profile; (Δ—Δ) [^{3}H]uridine label; (■···■) percent keratin cDNA hybrid.

deemphasizes the total amount of hybrid in each region, but the data in Figure 9B suggest about a threefold enrichment of globin genes per unit of DNA in peak I (region A) compared with other regions of the profile. On the other hand, Figure 9C shows that there is virtually identical distribution of the keratin genes in all sections of the profile.

Superficially the data in Figure 9B suggest a useful enrichment for globin gene sequences (active chromatin). However, presentation of the data in this form can obscure the fact that the fractionation achieved is not useful in practice. That is, there is a threefold enrichment of globin genes in only about 4% of the DNA while the vast majority of these sequences are still present in the inactive material.

In every hybridization profile presented, the majority of the hybrid has always been associated with the main peak DNA. Although unlikely, it was possible that the cDNA probes cross-reacted with other sequences in the genome to such an extent that the main peak of hybrid formation was always found in those fractions containing the greatest amount of DNA. The data in Figure 10 show the results of an experiment in which globin cDNA probe was hybridized across the profile obtained when chicken DNA was fractionated on an actinomycin D–CsCl gradient. Clearly the main peak of hybrid does not coincide with the main peak of DNA. The globin genes are localized on the profile at a region of about 60% G + C content (unpublished data) compared with the main band position of 40% G + C. This indicates that the globin cDNA probe does give a true indication of the position of the globin genes and does not merely associate spuriously across a DNA profile.

The results of this section show that, in terms of separating active genes from inactive genes, the gel-chromatography fractionation procedure is not nearly as effective as many of the less direct tests would seem to suggest. The two independent tests employed using cDNA both show that the first peak is little different from the main peak in terms of its active gene content.

C_0t Analysis of DNA from Chromatin. Globin cDNA was hybridized to DNA derived from different fractions of chromatin to give a more precise quantitative estimate of the distribution of globin genes. In Figure 11 data are shown for hybridizations involving total chicken DNA, for DNA obtained from the discarded pellet prior to Bio-Gel chromatography of chromatin (Experimental Procedures) and for DNA isolated from the inactive peak II chromatin. Unfortunately, due to the very small porportion of the total chromatin eluting in the first peak, it proved impractical to prepare a C_0t curve using DNA from this material. (The technical problem of recovering very small amounts of DNA from a large volume of buffer containing phosphate and Mg^{2+} ions added to the impractibility).

Within the limits of experimental error the three curves are identical. The material discarded during the preparation procedure (pellet) is shown to have a globin gene content indistinguishable from that of whole DNA. Therefore the globin gene content of the column-fractionated chromatin has not been affected by the removal of this material. The peak II DNA is also indistinguishable from whole chicken DNA in terms of globin gene content. This reinforces the results obtained from the cross-profile hybridization experiments and indicates that specific elution of active globin gene sequences

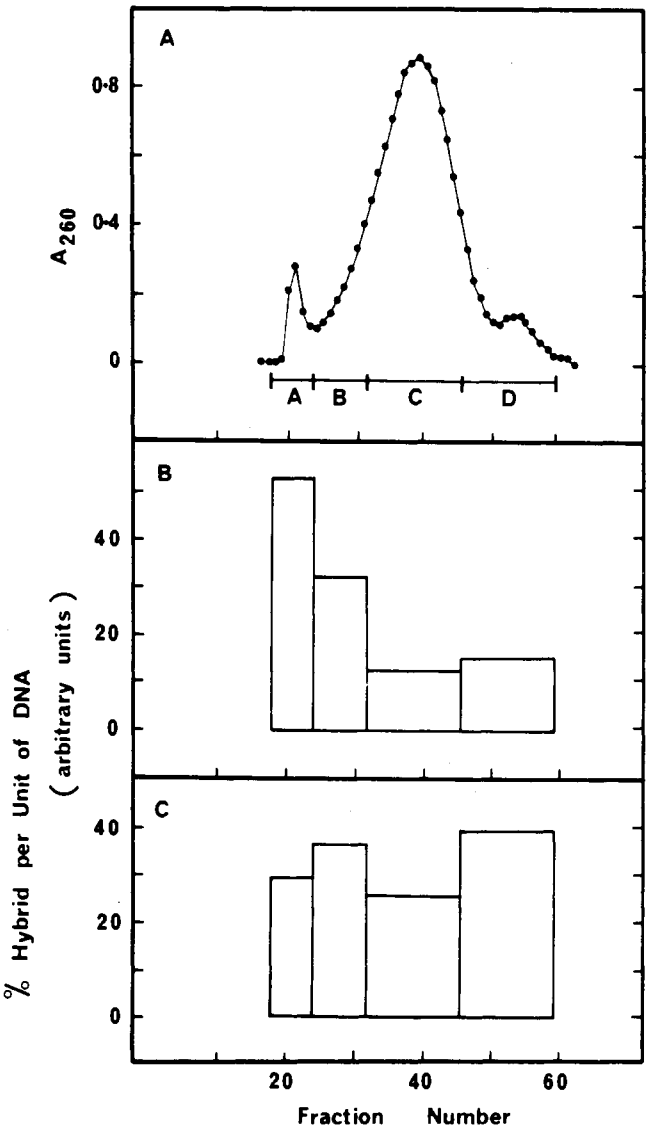

FIGURE 9: Distribution of globin and keratin gene sequences per unit of DNA in regions from reticulocyte chromatin profiles. The elution profile shown in A was divided into four regions A to D in which A represents peak I chromatin and C, peak II chromatin. The percent hybrid per unit of DNA was derived from data in Figures 7 and 8. (B) Globin gene sequence distribution; (C) keratin gene sequence distribution.

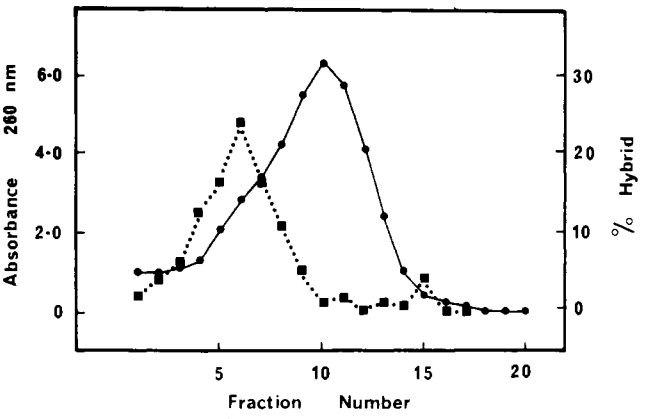

FIGURE 10: Position of globin gene sequences in an actinomycin D–CsCl gradient. Chicken DNA (1 mg; double-strand molecular weight ~5×10^6) was dispersed in a solution of final volume 12 ml, containing 0.01 M Tris-HCl (pH 8.4), 0.01 M NaCl, and 0.001 M EDTA, and 0.5 mg of actinomycin D was added. Solid CsCl was added and the final density of the solution was 1.600 at 20 °C. Centrifugation was at 32 000 rpm for 60 h at 20 °C in a type Ti-50 rotor. Fractions of 0.5 ml were collected, CsCl was removed after ethanol precipitation, and the DNA was hybridized with chicken globin cDNA. (●—●) A_{260} elution profile; (■···■) percent globin cDNA hybrid.

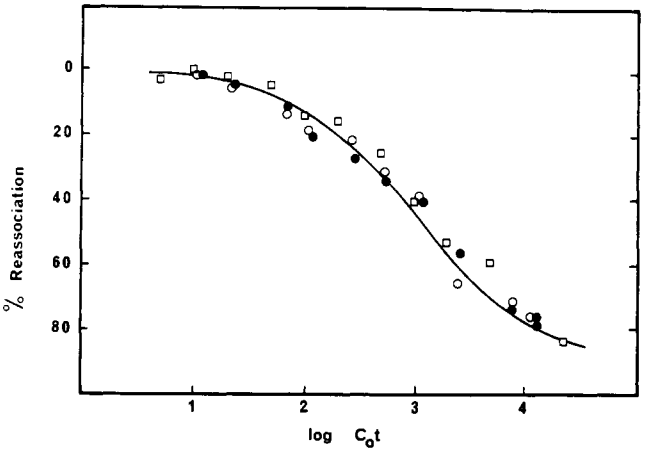

FIGURE 11: Reassociation kinetic analysis of globin cDNA with DNA from reticulocyte chromatin. Globin cDNA was mixed with sheared DNA and the percent hybrid determined at various times with S_1 nuclease. (○—○) Whole chicken DNA; (●—●) peak II chromatin DNA; (□—□) discarded "pellet" DNA.

in the peak I fractions has caused no observable reduction in the frequency of the globin gene in the bulk of the DNA.

General Discussion

It is possible to obtain repeatable fractionation of chicken reticulocyte chromatin on Bio-Gel A-50m columns in the high salt buffer described by Janowski et al. (1972), and the factors which are important for reproducibility have been discussed. There is no doubt that the active and inactive fractions thus obtained have different physicochemical properties and as such seem to correspond to euchromatin and heterochromatin derived from chromatin of different cell types by many different procedures. Nevertheless the central question is whether such fractions are truly representative of active and inactive chromatin. In this paper we have concentrated solely on results obtained using the high salt Bio-Gel fractionation procedure, but in fact we have investigated chicken erythroid chromatin fractionation in high and low salt buffers in sucrose gradients (Murphy et al., 1973) and by density gradient procedures (unpublished), and on the basis of cDNA hybridization results, we have not been able to obtain any appreciable enrichment for active sequences by any of these methods. This contrasts with the apparent spectacular enrichment for active chromatin if the nascent RNA-labeling results are used as a criterion.

Although the position of nascent RNA chains has been used extensively as a marker to indicate the position of active genes in fractionated chromatin (Janowski et al., 1972; Murphy et al., 1973), our results with both globin cDNA and keratin cDNA question this interpretation. In fact the absolute consistency with which we obtained nascent RNA label almost totally associated with peak I on Bio-Gel chromatography, no matter what the shape of the elution profile (data not shown), led us to wonder whether any other result was possible when chromatin was fractionated in this way.

In a recent article, Howk et al. (1975) described the fractionation of mouse fibroblast chromatin on ECTHAM-cellulose and, as shown previously by others for different chromatin preparations (Simpson and Reeck, 1973), found the

early eluting material to have a low template activity and the later active chromatin a high template activity for RNA polymerase in vitro. It is therefore significant that this criterion for active chromatin was not substantiated by hybridization studies with specific probes to active (Moloney leukemia type C) and inactive (mouse mammary tumor type B) viruses. This conclusion is entirely in agreement with our findings with respect to the localization of globin and feather keratin genes in chicken erythroid chromatin fractionated on Bio-Gel. It would be convenient simply to let the data for viral probes (Howk et al., 1975) reinforce our own conclusions. Unfortunately it is difficult to assess from the report of Howk et al. (1975) whether the type C virus sequence truly represented chromosomally located active genes and the type B virus sequence inactive genes in the fibroblast cells from which chromatin was prepared.

There is no doubt that two distinct fractions can be obtained from eukaryotic chromatin by a wide variety of techniques. It is much less certain that criteria for the active portion (protein/DNA ratios, melting profiles, template activity in vitro, and association of nascent RNA chains) necessarily describe the fraction of chromatin which is active in vivo. In principle the direct localization of specific genes in fractionated chromatin should be a better test for active and inactive chromatin.

The results obtained in this study show that chicken erythroid chromatin can be fractionated reproducibly on Bio-Gel A-50m. Despite the initial appealing results of labeled RNA being almost totally associated with 2–5% of the active chromatin DNA, hybridization data with globin cDNA strongly suggested that the gel exclusion technique did not give a substantial fractionation of chicken reticulocyte chromatin into its transcriptionally active and inactive components. The additional use of the feather keratin cDNA probe gave important negative results and supported this conclusion. Our more limited experience with other chromatin fractionation procedures (sucrose gradients and urografin gradients) also suggests that more stringent criteria should be applied to the designation of active chromatin fractions in other systems.

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