Association of Tissue-Specific Histones with Deoxyribonucleic Acid. Thermal Denaturation of Native, Partially Dehistonized, and Reconstituted Chromatins†

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ABSTRACT: First derivative thermal denaturation profiles were compared for chromatin samples prepared from chicken erythrocytes, chicken liver, and sea urchin (*Strongylocentrotus purpuratus*) sperm. Selective dissociation of various histone fractions, including tissue-specific F2c and γ histones, was manifested in characteristic changes of the thermal denaturation profiles. It was concluded that the binding of individual histone fractions to the DNA can be identified with particular temperatures of thermal denaturation. This observation was tested by denaturation experiments on hybrid chromatins. Chicken erythrocyte chromatin devoid of F2c histone and reconstituted with isolated liver F1 histone denatured like chicken liver chromatin. Conversely, chicken liver chromatin devoid of F1 histones and reconstituted with isolated F2c fraction exhibited a thermal denaturation profile characteristic of the chicken erythrocyte chromatin. In other words, the thermal denaturation profile of reconstituted chromatin was determined approximately by the sum of contributing histone fractions. The obvious distinctions recognized among the derivative thermal denaturation profiles of compositionally different nucleoproteins suggest that thermal denaturation sensitively detects variations in histone content and therefore is a valuable tool for the routine characterization of chromatin preparations.

The complexing of histones with DNA results in neutralization of a large portion of the negative charges on the phosphate groups of DNA and is accompanied by structural changes of the DNA. Recent studies indicate that the structure of chromatin is complex and concerns several levels of organization. The thermal denaturation profile of isolated chromatin was found to be either bimodal or multiphasic (Huang et al., 1964; Kurtz and Sinex, 1967; Ohlenbusch et al., 1967; Samis et al., 1968; Wilhelm and Champagne, 1969; Henson and Walker, 1970a,b; Shih and Bonner, 1970a,b; Spelsberg et al., 1971; Subirana, 1973; Bekhor, 1973), reflecting the melting of DNA regions stabilized to different extents by histones (Ohlenbusch et al., 1967; Henson and Walker, 1970a,b; Li and Bonner, 1971). Using methods for selective extraction of histones, Smart and Bonner (1971a) concluded that all classes of histones apparently contribute nearly equally to a wide range of physicochemical properties of chromatin, including its thermal denaturation. Their conclusions were further supported by the analysis of derivative thermal denaturation profiles of native and dehistonized chromatin preparations (Li and Bonner, 1971; Li, 1972; Li et al., 1973). The individual main regions of thermal stability were interpreted to reflect the denaturation of free DNA and DNA covered by nonhistone proteins or by less or more basic halves of histone molecules.

Shih and Bonner (1970a), Ansevin and Brown (1971), and Ansevin et al. (1971), however, reported that the individual histone fractions display a definite specificity in their contribution to the thermal denaturation profiles of reconstituted nucleohistones. In other words, each of the major histone fractions could be identified with a distinctive pattern of temperature, number, and magnitude of thermal transitions in the derivative thermal denaturation profile of simple nucleohistones. It is shown here that in various chromatin preparations histone fractions contribute characteristic patterns of thermal transition which are different in some cases from those observed for the simple nucleohistones, and that the distinctive thermal denaturation patterns of native chromatins are closely approximated by the sum of the patterns of contributing histone species.

Experimental Procedure

Erythrocytes and liver tissue were obtained from adult Leghorn chickens. Male *Strongylocentrotus purpuratus* sea urchins (Pacific Biomarine Supply Co., Venice, Calif.) were used to collect mature sperm.

Calf thymus Type I deoxyribonucleic acid, sodium salt, was bought from Sigma Chemical Co. (St. Louis, Mo., 63118). The ultrapure urea was purchased from Schwarz/Mann (Orangeburg, N.Y.). Acrylamide and bis(*N,N'*-methylenebisacrylamide) were the product of Bio-Rad Laboratories (Richmond, Calif.).

The isolation of chicken erythrocyte nuclei was described elsewhere (Tsai, 1973). Modified methods of Blobel and Potter (1966) and of Johnson and Hnilica (1970) were respectively employed for the isolation of chicken liver and sea urchin sperm nuclei. To prepare chromatin, isolated nuclei were homogenized gently by hand in 0.015 *M* NaCl–0.02 *M* EDTA–0.05 *M* $NaHSO_3$ (pH 6.3) (or 0.02 *M* NaCl–0.08 *M* EDTA (pH 6.3)) and centrifuged at 4000*g* for 10 min. The pellets were resuspended twice more in the same buffer and centrifuged. The resultant pellets were washed once in 0.3 *M* NaCl. The saline-washed pellets were washed two or three times with 0.01 × SSC, pH 7.0, by hand homogenization, suspended in 0.01 × SSC, pH 7.0 (1–1.5 mg of DNA/ml), and stored at −20° for further analysis.

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Selective Dissociation of Histones. (a) MAGNESIUM CHLORIDE. A series of magnesium chloride solutions (0.1, 0.2, 0.3, and 0.5 *M*) was used to dissociate histones. The dissociated histones were separated from the residual chromatins by centrifugation at 105,000*g* for 16 hr.

(b) SALTS IN UREA SOLUTION. Two sets of solution were used: (1) the solutions of 0.2 *M* NaCl in 4 *M* urea and 0.3 *M* NaCl in 8 *M* urea (Senshu, 1971a) and (2) the solutions of 0.05 *M* $MgCl_2$ in 4 *M* urea and 0.05 *M* $MgCl_2$ in 8 *M* urea. The isolated chromatin pellets were homogenized in these solutions (less than 0.3 mg of DNA/ml) and centrifuged at 105,000*g* for 40 hr. The upper four-fifths of all supernatants were carefully collected, dialyzed, and lyophilized. The lyophilized extracts were analyzed by polyacrylamide gel electrophoresis to determine which histone fractions were dissociated.

To study the thermal denaturation profiles of the histone-depleted chromatins, the pellets were carefully rinsed with ice-cold deionized water to prevent their contamination by dissociated histones, homogenized in 0.01 *M* EDTA (pH 7.0), and centrifuged at 30,000*g* for 45 min. This step was repeated once to assure complete removal of divalent cations which interfered with the thermal denaturation of chromatin. The sediments were then washed twice with 0.01 × SSC (pH 7.0), and centrifuged as above. Finally, the pellets were homogenized in small volumes of 0.01 × SSC, pH 7.0, to obtain a chromatin solution of about 1 mg of DNA/ml. Alternatively, the pellets of partially dehistonized chromatin were homogenized in 0.01 *M* EDTA (pH 7.0) and dialyzed against at least 20 vol of the same solution followed by four changes of 0.01 × SSC (pH 7.0) at 6-hr intervals.

Both dissociated and residual histones were analyzed by polyacrylamide gel electrophoresis with formic acid (Senshu, 1971b) or sodium dodecyl sulfate (Wilhelm et al., 1972) added to the electrophoretic media. Diphenylamine reaction was used to determine DNA (Burton, 1956) and the protein concentration was assayed by the Lowry et al. (1951) procedure. Calf thymus histone or bovine serum albumin was used as standard for acid-soluble or acid-insoluble protein analysis.

Dissociation and Reconstitution of Chromatin. Chicken liver and chicken erythrocyte chromatins were homogenized in 0.2 *M* $MgCl_2$ and the chromatin suspensions were centrifuged at 105,000*g* for 42 hr. The supernatants were collected and desalted rapidly in a Hollow Fiber beaker dialyzer (Dow Chemical Co.). The pellets were homogenized in 0.01 *M* EDTA (pH 7.0) and dialyzed against 20 vol of the same solution. This was followed by four changes of 0.01 × SSC (pH 7.0) at 6-hr intervals.

The reconstitutions were achieved by adding the 0.2 *M* $MgCl_2$ chromatin extracts (0.5 mg/ml of protein) to the partially dehistonized erythrocyte or liver chromatin (0.05 ml/ml of DNA). The amount of proteins added to every milligram of chromatin DNA was 0.3 or 0.5 mg. The speed of addition of histones was about 2 ml/hr with continuous stirring to prevent local accumulation of a high protein concentration and the precipitation of nucleoprotein (Ansevin and Brown, 1971). The reconstituted chromatins were precipitated in 0.1 *M* NaCl and washed several times with 0.01 × SSC, pH 7.0. The reconstitution of F2c nucleohistones was performed in the same way.

Thermal Denaturation of Chromatin. DNA, chromatin, and all other nucleoprotein preparations were denatured in an automated spectrophotometer in which the temperature of samples was increased at a rate of about 0.8°/min (Ansevin and Brown, 1971; Ansevin et al., 1971). Data on absorbance at 260 nm and cuvet temperature were analyzed by computer to obtain hyperchromicity at 260 nm, corrected for the thermal expansion of water, and the first derivative of hyperchromicity with respect to temperature. This first derivative of hyperchromicity was calculated at each point in succession from the equation of a quadratic or a cubic curve giving the best fit to 13 values centered at the given point. The denaturation solvent for all samples contained 5 m*M* cacodylate buffer (pH 7), 0.15 m*M* EDTA, and 3.6 *M* urea. The favorable resolving power of this medium for nucleoprotein complexes has been discussed (Ansevin and Brown, 1971; Ansevin et al., 1971, 1975). It should be noted that graphs of the first derivative of hyperchromicity vs. temperature (derivative denaturation profiles) contain all observed points but, for the sake of simplicity, only every fifth point has been emphasized by a symbol in the plots presented here.

Table I: Composition of Chicken Erythrocyte, Chicken Liver, and Sea Urchin Sperm Chromatins.

Chromatin	DNA	RNA	Acid-Soluble Proteins	Acid-Insoluble Proteins	Total Proteins
Chicken erythrocyte	1	0.01	0.98[a]	0.34	1.32[a]
			1.01[b]		1.35[b]
Chicken liver	1	0.01	0.93[a]	1.01	1.94[a]
			1.01[b]	1.12[b]	2.13[b]
Sea urchin sperm chromatin	1	0.001	0.93[b]	0.30[b]	1.23[b]

[a] Chromatin isolated with saline-EDTA (pH 6.3) solution. [b] Chromatin isolated with saline-EDTA (pH 6.3) solution containing $NaHSO_3$ to inhibit the possible proteolytic degradation of protein during the isolation of chromatin.

Results

Chemical Composition of Chromatins. The chemical compositions of chicken erythrocyte chromatin, chicken liver chromatin, and sea urchin sperm chromatin are summarized in Table I. They contain nearly equal amounts of acid-soluble proteins and DNA. The contents of acid-insoluble (nonhistone) proteins among these three chromatins varied from 0.3 to 1.1 (the ratio of protein to DNA). This variation was closely related to the in vivo biological activities of these tissues and supports the suggestion that more non-histone protein can be found associated with the biologically active chromatin than with less active chromatin (Marushige and Ozaki, 1967). The non-histone protein/DNA ratio was only 0.3–0.35 in the genetically repressed chicken erythrocyte and sea urchin sperm chromatin.

Thermal Stability of Isolated Chromatin. The plots of the first derivative of hyperchromicity against temperature provide a sensitive analysis of the thermal stability of chromatin; they are superior to simple plots of hyperchromicity against temperature both in the detail provided and the quantitation that may be achieved. Thermal denaturation profiles of chicken erythrocyte, chicken liver, and sea urchin

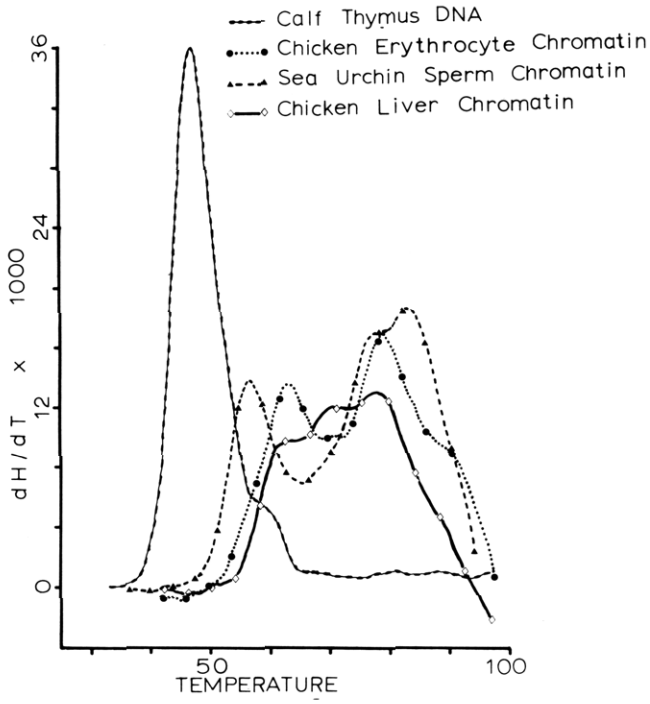

FIGURE 1: Thermal denaturation profiles of chicken erythrocyte, chicken liver, and sea urchin sperm chromatin.

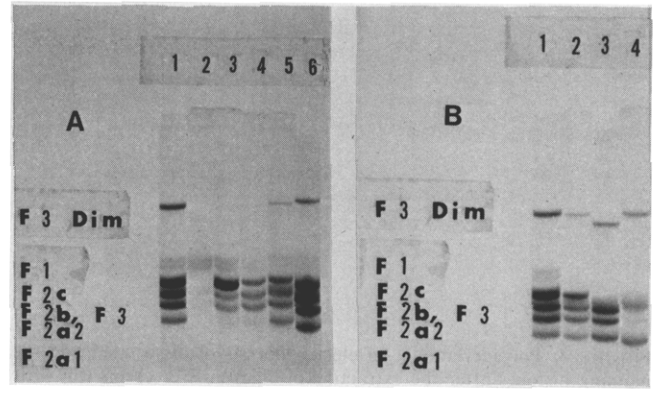

FIGURE 2: Polyacrylamide gel electrophoresis of dissociated and remaining histones after the extraction of chicken erythrocyte chromatin with $MgCl_2$ solutions. (a) 1, total histones; 2–6, histones dissociated with 0.1, 0.2, 0.3, 0.5, and 1.0 *M* MgCl, respectively. (b) 1, total histones; 2–4, histones remaining associated with DNA after the extraction with 0.1, 0.2, and 0.3 *M* $MgCl_2$, respectively. There were no histones left on the DNA after the extraction with 0.5 or 1.0 *M* $MgCl_2$.

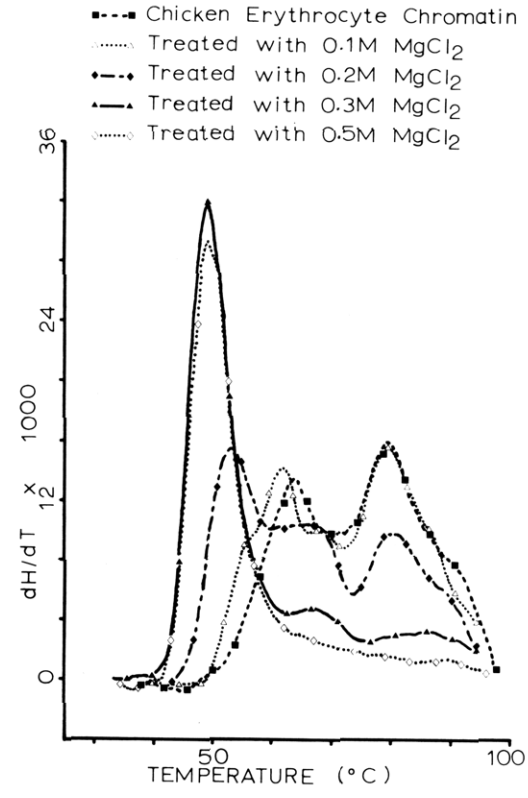

FIGURE 3: Thermal denaturation profiles of chicken erythrocyte chromatin after extraction with $MgCl_2$. Refer to Figure 2 for histones remaining in the residual chromatins.

sperm chromatins were compared to study the contribution of tissue-specific histones species to the thermal stability of DNA. The F2c fraction constitutes about 28–30% of all histones in chicken erythrocyte chromatin while F1 is present at a level of only 5–7%. However, about 20% of total histones in chicken liver chromatin are the F1 histones. The sea urchin sperm chromatin contains no F1 histone. A tissue-specific lysine- and arginine-rich histone fraction (γ histone) represents about 36% of the total histone content of the sperm chromatin. The derivative thermal denaturation profile of chicken liver chromatin is quite similar to that of rat liver chromatin (Ansevin et al., 1971) (Figure 1). The thermal denaturation profiles of chicken erythrocyte and sea urchin sperm chromatins are distinctly different, with two separate regions of thermal stability. It is likely that the area of the denaturation profile near 75° in the chicken liver chromatin reflects mainly the association of histone F1 with DNA. The low content of F1 histone in chicken erythrocyte chromatin is correlated with an absence of this middle peak in its thermal denaturation profile. Similarly, the absence of the very lysine-rich histones in sea urchin sperm chromatin is associated with a nearly complete separation of two major stages of thermal stability: an early peak representing weakly stabilized DNA and a larger, bimodal peak corresponding to a highly stabilized nucleoprotein.

Treatment of the chicken erythrocyte and chicken liver chromatins with 0.1 *M* $MgCl_2$ resulted in the selective dissociation of F1 histones. The F2c histone began to dissociate from chicken erythrocyte chromatin in 0.15 *M* $MgCl_2$. The 0.2 *M* $MgCl_2$ solution removed essentially all the F2c histone and also some of the F2b and F2a2 histones. The 0.3 *M* $MgCl_2$ solution dissociated all the F1 and F2c histones as well as most of the F2b and F2a2 histones; most of the F3 and F2a1 histones remained associated with the residual chromatin. In 0.5 *M* $MgCl_2$, all the histones were dissociated (Figure 2). Figure 3 shows the thermal denaturation patterns of magnesium chloride-treated chicken erythrocyte chromatin. The dissociation of F2c histone resulted in a substantial decrease in the more stabilized chromatin portion and a simultaneous increase in the portion of less stabilized chromatin. Further dissociation of the F2b and F2a2 histones resulted in the appearance of a free DNA peak. When the F1 histones were selectively removed from chicken liver chromatin using 0.1 *M* $MgCl_2$ (Figure 4), there was a prominent dip at the 75° region of the thermal denaturation pattern (Figure 5). After all the histones had been removed by 0.5 *M* $MgCl_2$, only the free DNA peak was observed by the thermal denaturation analysis, despite the association of residual amounts of non-histone proteins with the DNA in the remaining chromatin.

In the sea urchin sperm chromatin, 0.2 *M* $MgCl_2$ re-

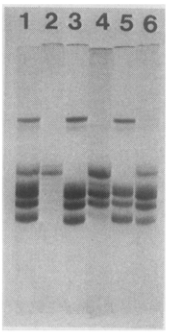

FIGURE 4: Polyacrylamide gel electrophoresis of dissociated and remaining histones after the extraction of chicken liver chromatin with $MgCl_2$ solutions. (1) Total histones, (2) histones extracted with 0.1 *M* $MgCl_2$; (3) histones remaining in the 0.1 *M* $MgCl_2$–residual chromatin; (4) histones dissociated by 0.2 *M* $MgCl_2$; (5) histones remaining in the 0.2 *M* $MgCl_2$–residual chromatin; (6) histones dissociated by 0.3 *M* $MgCl_2$. There were no histones left on the DNA after this extraction.

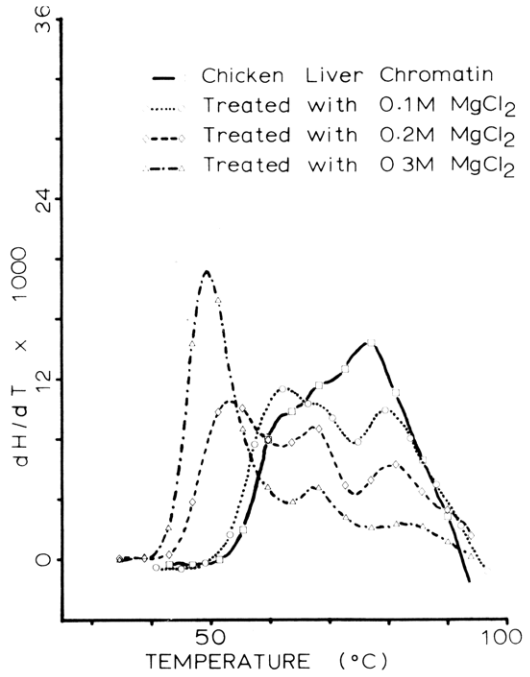

FIGURE 5: Thermal denaturation profiles of chicken liver chromatin after extraction with $MgCl_2$. Refer to Figure 3 for histones remaining associated in the residual chromatins.

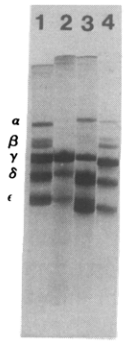

FIGURE 6: Polyacrylamide gel electrophoresis of dissociated and remaining histones after the extraction of sea urchin sperm chromatin with $MgCl_2$. (1) Total histones; (2) histones extracted with 0.2 *M* $MgCl_2$; (3) histones remaining in the 0.2 *M* $MgCl_2$–residual chromatin; (4) histones dissociated by 0.3 *M* $MgCl_2$. There were no histones left on the DNA after this extraction.

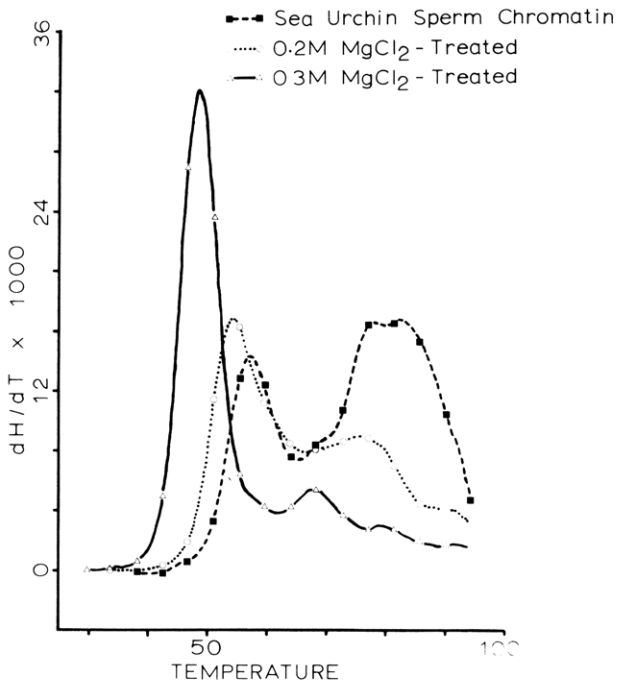

FIGURE 7: Thermal denaturation profiles of the $MgCl_2$-treated sea urchin sperm residual chromatins. Refer to Figure 6 for histones remaining associated in the residual chromatins.

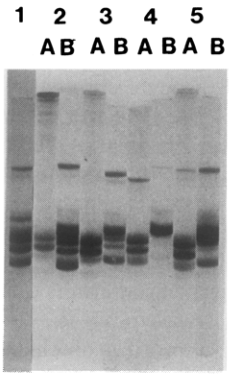

FIGURE 8: Polyacrylamide gel electrophoresis of dissociated (A) and remaining (B) histones after the extraction of chicken erythrocyte chromatin with NaCl–urea solutions. (1) Total histone; (2) A,B dissociation with 0–2 *M* NaCl–4 *M* urea; (3) A,B dissociation with 0.05 *M* $MgCl_2$–4 *M* urea; (4) A,B dissociation with 0.3 *M* NaCl–8 *M* urea; (5) A,B dissociation with 0.05 *M* $MgCl_2$–8 *M* urea.

moved mainly the tissue-specific histones (Figure 6). This treatment resulted in a great decrease in the more stable portion of chromatin, an increase in the less stable portion of chromatin, and an appearance of free DNA (Figure 7). However, the more stable portion of sea urchin sperm chromatin is partially exaggerated by an increase of turbidity at high temperature. After the treatment with 0.3 *M* $MgCl_2$, about 80% of the sea urchin sperm chromatin melted as pure DNA and a small peak around 68° was also observed in the residual chromatin. After treatment with 0.3 *M* $MgCl_2$, only traces of the histones remained in the residual chromatin.

The salt–urea solution dissociated F2b histone first, followed by F2a2, F2a1, F3, and finally by F1 histones together with F2c histone (Senshu, 1971a,b) (Figure 8). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed that more than 20 bands of non-histone proteins were dissociated from both chicken erythrocyte

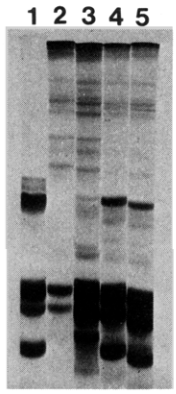

FIGURE 9: Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of chromosomal proteins after the extraction of chicken erythrocyte chromatin with NaCl–urea solutions. (1) Total histones (acid extract); (2–5) proteins dissociated in 0.2 *M* NaCl–4 *M* urea, 0.05 *M* $MgCl_2$–4 *M* urea, 0.3 *M* NaCl–8 *M* urea, and 0.05 *M* $MgCl_2$–8 *M* urea, respectively. Notice the large quantities of non-histone proteins extracted by these solvents.

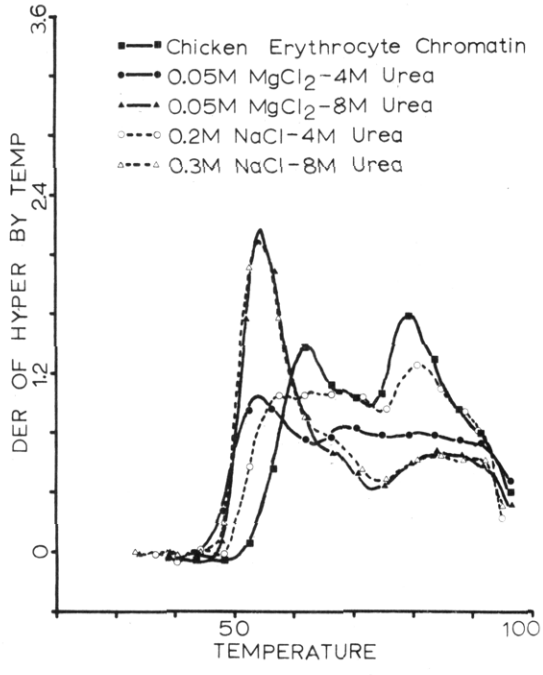

FIGURE 10: Thermal denaturation profiles of chicken erythrocyte residual chromatins after extraction with salt–urea solutions.

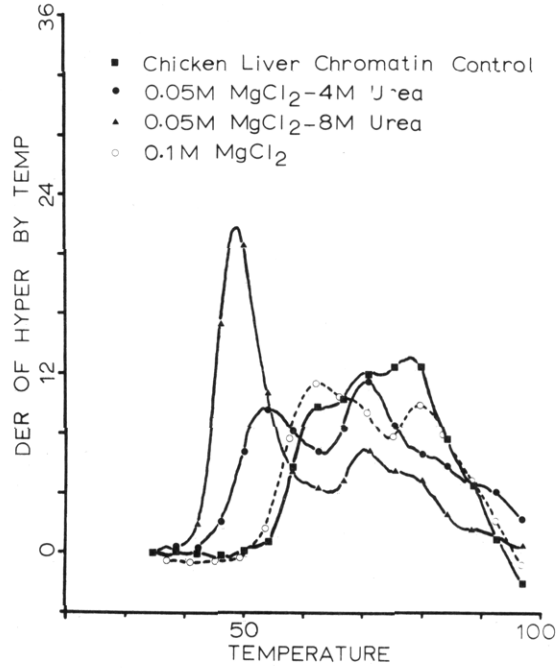

FIGURE 11: Thermal denaturation profiles of chicken liver residual chromatins after extraction with salt–urea solutions.

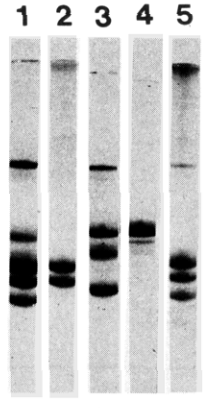

FIGURE 12: Polyacrylamide gel electrophoresis of dissociated and remaining histones of chicken liver chromatin after extraction with salt–urea solutions. (1) Total chicken liver histones; (2) histones extracted with 0.05 *M* $MgCl_2$–4 *M* urea; (3) histones remaining in chromatin after this extraction; (4) histones remaining in chromatin after extraction with 0.05 *M* $MgCl_2$–8 *M* urea; (5) extracted histones.

and chicken liver chromatin by these salt–urea solutions (Figure 9). The thermal denaturation profiles of the salt–urea extracted residual chromatins differed from those of the $MgCl_2$ treated residual chromatins. The solution of 0.2 *M* NaCl–4 *M* urea dissociated about 20–25% of the F2b and F2a2 histones from the chicken erythrocyte chromatin. This resulted in the lowering of both the less stable and the more stable peaks of the chromatin (Figure 10) and the appearance of a small peak of free DNA. When more of the F2b and F2a2 histones were dissociated with 0.05 *M* $MgCl_2$–4 *M* urea, a large portion of both the less stable and the more stable chromatin was shifted to a sharp peak of free DNA. After the treatment of chromatin with 0.3 *M* NaCl–8 *M* urea, or with 0.05 *M* $MgCl_2$–8 *M* urea, most of the histones were dissociated with the exception of the tissue-specific F2c fraction. The thermal denaturation pattern of the F2c histone-containing residual chromatin consisted of a broad, highly stabilized peak, a small, weakly stabilized peak, and a large peak of free DNA (Figure 10).

Dissociation of F2b and F2a2 histones from chicken liver chromatin with 0.05 *M* $MgCl_2$–4 *M* urea solution yielded a large peak of free DNA together with a prominent thermal transition centered at 70° (Figure 11). When F1 histone and a portion of the F3 histone remained in the residual chromatin after its treatment with 0.05 *M* $MgCl_2$–8 *M* urea (Figure 12), the thermal denaturation profile of the residual chromatin consisted of a large peak of free DNA, a small peak at 70–72°, and a small hump between 75 and 85°.

Reconstitution Experiments. Histones could be added slowly to diluted chromatin solution at low ionic strength to produce chromatin with more histones than the native chromatin (L. S. Hnilica and A. T. Ansevin, unpublished results). Similarly, histones could be added also to a partially dehistonized chromatin under the same conditions. In this study, both chicken liver and erythrocyte chromatins were

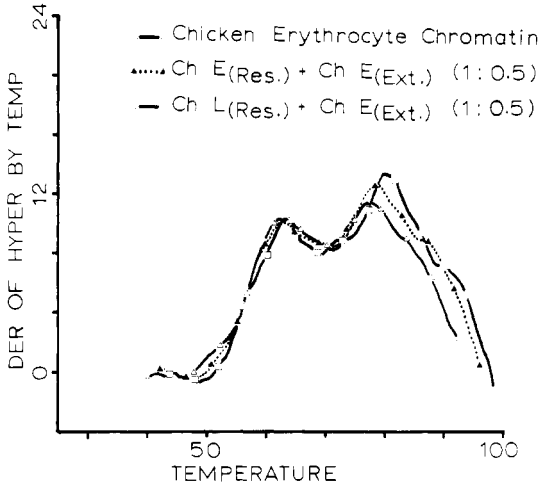

FIGURE 13: Thermal denaturation profiles of reconstituted chicken erythrocyte chromatins. The reconstitution was performed by adding 0.2 *M* $MgCl_2$-extract (containing all the F1 and F2c histones and some of the F2b and F2a2 histones) from chicken erythrocyte chromatin to the 0.2 *M* $MgCl_2$-extracted chicken erythrocyte or liver residual chromatin. The ratio of the added proteins to the DNA in the residual chromatin was 0.5:1.

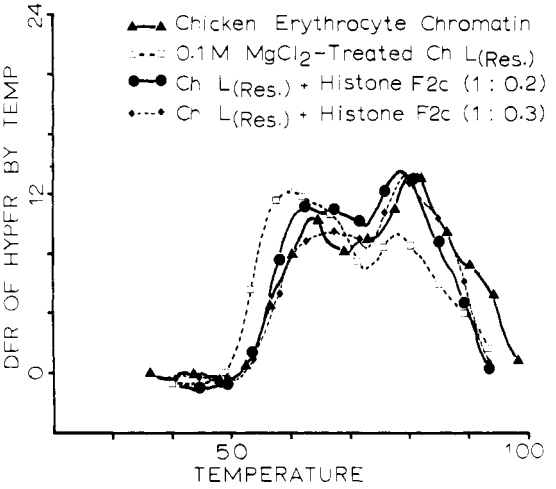

FIGURE 14: Thermal denaturation profiles of reconstituted chicken erythrocyte chromatins. The reconstitution was done by adding purified chicken erythrocyte F2c histone to the 0.1 *M* $MgCl_2$-extracted chicken liver residual chromatin (devoid of the F1 histones). The ratios of the protein added to the DNA in the residual chromatins are shown in the graph.

dissociated in 0.2 *M* $MgCl_2$, and then the dissociated histones were collected in the supernatant after ultracentrifugation. This procedure extracted all the F1 and F2c histones and some of the F2b and F2a2 histones from erythrocyte chromatin and all the F1 and some of the F2b and F2a2 histones from liver chromatin (Figures 2 and 4). After that, the pellets of residual chromatin from chicken erythrocytes or liver were reconstituted with their respective supernatants. The resulting reconstituted chromatins had thermal denaturation profiles similar to those of the native samples (Figures 13 and 15). In addition, hybrid chromatins were "reconstituted" from supernatant liver proteins and pelleted residual erythrocyte chromatin, and vice versa. In some cases, purified F2c histone was substituted for the mixed histone extract (Figure 14). As can be seen in Figures 13–15, the character of the thermal denaturation plots for the hybrid chromatins was determined principally by the chemical nature of the reconstituted histone fractions.

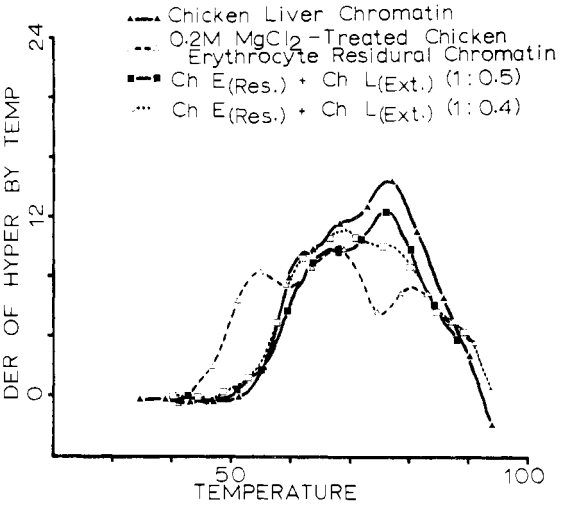

FIGURE 15: Thermal denaturation profiles of reconstituted chicken liver chromatins. The reconstitution was performed by adding 0.2 *M* $MgCl_2$-extract (containing all the F1 histones and some of the F2b and F2a2 histones) of chicken liver chromatin to the 0.2 *M* $MgCl_2$-treated chicken erythrocyte residual chromatin. The ratios of histones added to the DNA in the residual chromatin are shown in the graph.

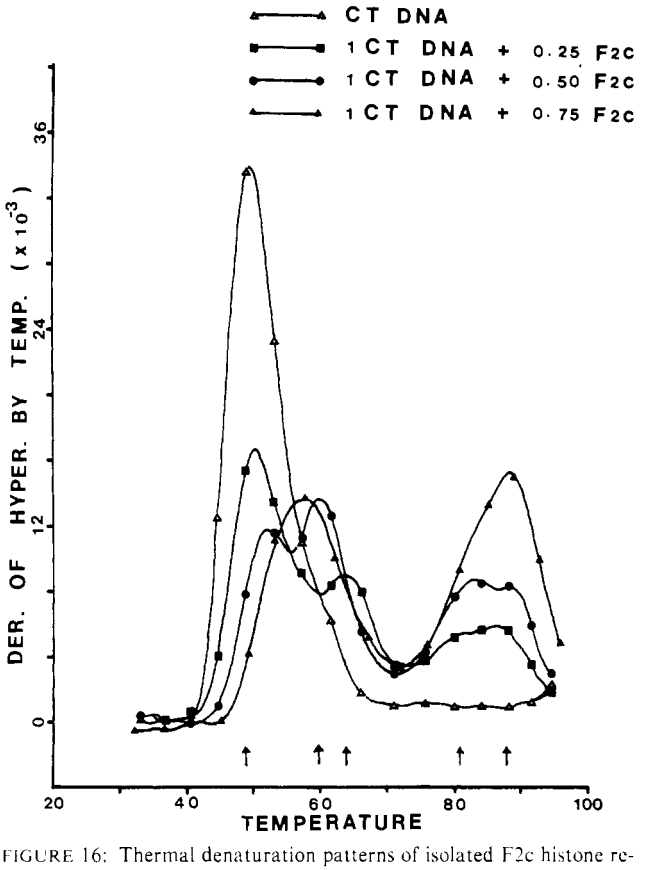

FIGURE 16: Thermal denaturation patterns of isolated F2c histone reconstituted to pure DNA. The protein/DNA ratios are shown in the graph.

The chicken erythrocyte residual chromatin assumed the character of chicken liver chromatin when its F2c histone was replaced with chicken liver F1 fraction. Conversely, the chicken liver residual chromatin gave a thermal denaturation profile like that of the erythrocyte chromatin if its F1 fraction was replaced by F2c histone.

Finally, the reconstitution of purified F2c histones to pure calf thymus DNA produced nucleohistones with distinctive thermal denaturation patterns, shown in Figure 16.

Table II: Apparent Thermal Transitions for Histones in Chromatin.

Histone Fraction	Approximate Transition Range at Half-max	Source of Nucleohistone	Obtained from
F1	71–79	CL	Figure 5 (1, 2)[i]
F1	71–79	CL	Figure 11 (1, 4)
F1	Broad[a]	CL	Figure 11 (3)
F2b	(59–64),[b] 71–85	CL	Figure 5 (2, 3)
F2a2	Similar to F2b	CL	Figure 11 (1, 2)
F2a1 + F3	63–72 80–92	CE	Figure 3 (4, 5)
F2c	71–85	CE	Figure 3 (2, 3)
F2c + others	(63–70), 77–95	CE	Figure 10 (4, 5)
F2c + others	(58–64), 73–84	CE	Figure 3 (2, 3)
γ	*d*, ≃77–89	SS	Figure 7 (1, 2)
γ	≃53–60, (77–84)	SS, SE	Figure 1 (3)[c]
F1	63–73 (77–83)[f]	Calf on rat	*e*
F2b	63–72	Calf on rat	*e*
F2a2	60–67, 75–83	Calf on rat	*e*
F2a1	(60–66), 78–88	Calf on rat	*e*
F3	(62–68), 73–83	Calf on rat	*e*
F2c	(56–68),[g] 77–92[h]	CE on calf	Figure 16

[a] Poorly resolved multiple components. [b] Numbers in parentheses in this column indicate minor stabilization area. CL = chicken liver, CE = chicken erythrocytes, SS = sea urchin sperm, SE = sea urchin embryos, calf on rat = calf thymus histones on rat thymus dehistonized chromatin. [c] Hnilica (1972), p 119. [d] Analysis of a possible low-temperature component of the profile could not be made in this case because of overlap with the free DNA melting region. [e] Ansevin and Brown (1971). [f] Pattern dependent on reconstitution conditions. [g] Variable within this range. [h] Highest concentration excluded. [i] Number in parentheses in last column refers to the curves examined within the given figure.

By curve analysis, at low histone to DNA ratio (0.25), three thermal transitions for nucleohistone could be observed (i.e., 64, 82, and 88°); 45% of the DNA was present in the free-melting form (48°). As more F2c was added, the highly stabilized areas merged to give a sharper peak with a maximum at 88°. At the same time, the more weakly stabilized transition became shifted to lower temperatures in an unexplained manner; this variability suggests that more than one factor may be involved in the weak stabilization detected within the temperature range 55–70°. In some ways, the variability is reminiscent of that found for the reconstitution of the very lysine rich fraction F1 (Ansevin et al., 1971; Van and Ansevin, 1973).

The contributions of the individual histones to the thermal denaturation profiles of chromatins analyzed in this study are summarized in Table II. In partial agreement with the observations of Li and Bonner (1971), we concluded that some, but not all, of the histones participate in the formation of both a moderately stabilized and a highly stabilized portion of chromatin in the present denaturation medium. To a crude approximation these peaks are found near 60 and 80°, respectively. It is possible that the melting region below 60°, found in sea urchin sperm, is caused by the presence of small gaps between adjacent histone-covered segments. The melting peak at 80° presumably originates from the most protected parts of the DNA molecules (Subirana, 1973; Li and Bonner, 1971). However, it must be emphasized that the less stabilized and more stabilized chromatin regions are not equivalent to the extended and the aggregated portions of chromatin. At the same time, our results indicate that F1 histone participates as a single peak in the derivative denaturation profile of complete chromatin preparations in urea–cacodylate denaturation medium. This statement probably applies also for the γ histone of sea urchin sperm and F2c histone of chicken erythrocytes. However, the existence of a single peak is less certain in these two cases because of ambiguity in the interpretation of the low-temperature region of sea urchin profiles and uncertainty about the quantitative contributions of F2b and F2a2 in curve 5 of Figure 10 or curve 3 of Figure 3. (More than F2c and F1 is extracted from erythrocyte chromatin by 0.2 *M* $MgCl_2$, as attested by gel patterns and by the similarity of curve 3 of Figure 3 with curve 3 of Figure 5.)

Discussion

The exact biological role of proteins in chromatin is not known. From the work of Skidmore et al. (1973), it appears that the arginine-rich histones are utilized in the maintenance of supercoiled DNA. The lysine-rich F1 and F2c histones may function in maintaining the higher organizational level of chromatin, perhaps condensing chromatin into a super-supercoiled structure (Bradbury et al., 1972). It has been suggested that the F1 histones are more exposed to the aqueous environment than are the other histone fractions (Smart and Bonner, 1971b). The fact that the F1 histones in chromatin are much more susceptible to proteolytic degradation (Panyim et al., 1968) than the other fractions supports this possibility. Also, the F1 histone fraction is the first to be dissociated from DNA by salts and acids in aqueous solution, even though this histone fraction has the largest number of basic amino acid residues available to interact with the phosphate groups of chromatin DNA.

The consideration of melting profiles of isolated chromatin might help to explain some aspects of chromatin structure. We have studied chicken liver chromatin (containing 20% F1 histones), chicken erythrocyte chromatin (containing only 5–7% F1 histones), sea urchin sperm chromatin (containing no F1 histones), F1 histone-depleted chicken liver chromatin, chicken liver chromatin after removal of essentially all its histones except for the F1 fraction, F2c depleted chicken erythrocyte chromatin, chicken erythrocyte chromatin after removal of most of the histones other than the F2c fraction, and reconstituted chicken liver, or erythrocyte chromatins. According to the evidence obtained from the thermal denaturation profiles of these preparations, the presence of F1 histones in native or extracted chromatin results in a thermal transition with an intermediate level of stability (a T_m peak around 75°). This is consistent with our observation in Figure 5 that a "dip" near 75° appeared

in the thermal denaturation profile of chromatin after it had been extracted with 0.1 *M* $MgCl_2$, and that a similar dip was found for chromatin kept at 4° for 6 days (Ansevin et al., 1971); it is known that the F1 histones are most susceptible to proteolytic degradation (Panyim et al., 1968). The prominent thermal transition of DNA–F1 histone complex seen here at about 75° is a feature distinguishing the F1 histones from all of the other histone fractions.

The F2c histone resembles F1 histones in its mode of dissociation. It is released at almost the same concentration of salts or acids as the F1 fraction. According to Bradbury et al. (1972), the removal of F2c histone does not unfold the supercoil structure of chromatin fibers. At low pH, histone F2c, in the absence of F1 histones, causes an abnormal aggregation of chromatin fibrils (Brasch et al., 1972). The F2c histone is not identical with F1 histone in its thermal stabilizing effect on chromatin. According to our studies (Figures 10 and 13–16), its association with DNA was expressed by the presence of a more highly stabilized thermal denaturation band centered near 80°. The higher stability of this band may be a consequence of the greater arginine content of the F2c fraction, since other arginine-rich histones (F2a1, F3, and the sea urchin sperm γ) also have components with a T_m of 80° or higher.

In this investigation, we have attempted to identify the approximate thermal regions for natural chromatin preparations to which different histones contribute and have extended the variety of histones considered to include two fractions apparently responsible for a virtually complete shutdown of the biochemical activity of nuclei, fraction F2c of erythrocytes (from chicken) and fraction γ of the sea urchin sperm.

Because of the limits imposed by an attempt to obtain information about a natural complex using a destructive analytical method, only empirical distinctions can be drawn from the data. Nonetheless, since these observations are reproducible, we believe that valuable deductions can be made. Results pertinent to the question of the contributions made by each histone fraction to the denaturation profile of chromatin are listed in Table II.

Similar to the suggestion of other investigators (Li and Bonner, 1971), we find that most histones contribute to the denaturation profile of nucleoproteins in more than one thermal region. However, the apparent contributions to natural and extracted chromatin preparations do not appear to be identical for all histones, even though a high and a low temperature region is found for several fractions. In some cases, the transitions deduced in extracted chromatin preparations were roughly similar to those observed with reconstituted nucleohistones; these include F2a2 and probably F2a1 and F3 histones. However, striking differences are found for F1, F2b, and probably F2c, between their contributions to chromatin profiles and those to reconstituted nucleohistone profiles. In chromatin that remained relatively intact, F1 was responsible for a single symmetrical component located at an intermediate temperature instead of the multiple-peaked profile observed for reconstituted F1 nucleohistone (Ansevin and Brown, 1971; Ansevin et al., 1971; Van and Ansevin, 1973) or when F1 remained on residual chromatin after the removal of other histones (curve 3, Figure 12). However, F2b histone appeared to make a bimodal contribution to the denaturation of chromatin observed here even though only a single peak was found for the simple nucleohistone reconstituted in earlier experiments (Ansevin and Brown, 1971). Considering these differences, we conclude that the thermal denaturation of simple nucleohistones containing lysine rich fractions of histones are not highly predictive of the contribution these individual histones make to whole chromatin.

Perhaps the most surprising feature of the chromatin denaturation profiles was the presence of poorly stabilized regions in chromatin preparations that are least active transcriptionally. In fact, Figure 1 shows that about 20% of the profile for sea urchin sperm denatures at lower temperatures than the most weakly stabilized regions of chicken liver chromatin, falling in a range that would be described as free or very weakly stabilized DNA (Ansevin et al., 1971, 1975). Chicken erythrocyte chromatin also has a surprising amount of poorly stabilized nucleohistone. This appeared to run counter to the general trend of results obtained by Getz et al. (1975), in which the exposure of weakly stabilized regions following the action of polyanions was correlated with the opening of the template for the synthesis of RNA. The identification of these thermally unstable regions in metabolically inactive chromatin, however, parallels that of Subirana (1973), who used a different thermal denaturation medium.

In line with chemical data, the observable difference between chicken liver and erythrocyte chromatins would seem to lie in the distinction between F2c and F1 histones; smaller quantitative differences in levels of F2b and F2a2, however, cannot be excluded on the basis of these particular experiments. We conclude that the shape of chromatin profiles is determined by the histone content of the chromatin (in the absence of significant quantities of polyanions) and suggest that the detection of different shapes for chromatin thermal denaturation profiles can be taken as evidence for qualitative or quantitative differences in their histone content. Because we have found that the analysis of derivative thermal denaturation profiles is a simple and relatively discriminating test of the composition of chromatin, we would like to reemphasize an earlier suggestion that such profiles be included as one of the standard means for characterizing chromatin preparations.

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Transcription of Bacteriophage T4 Genome *in Vitro*. Heterogeneity of RNA Polymerase in Crude Extracts of Normal and T4-Infected *Escherichia coli* B†

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ABSTRACT: In order to obtain RNA polymerase preparations carrying the necessary specificity determinants to transcribe the delayed-early genes of bacteriophage T4, crude extracts of uninfected and T4-infected *Escherichia coli* were fractionated in glycerol gradients of low ionic strength. In contrast to the reported sedimentation behavior of the purified enzyme, the RNA polymerase activity in crude extracts of normal and infected cells sedimented heterogeneously over a wide range of sedimentation coefficients. When the "heavy" (24–33 S) and "light" (14–20 S) regions of the gradient were precipitated with ammonium sulfate and recentrifuged, the former split into two subfractions, one again sedimenting heavy and the other sedimenting light. The latter did not split under the same conditions. The resulting subfractions from uninfected cell extracts had different thermal stabilities at 50° (half-lives ranging from 2–3 to 25 min) while those from T4-infected cell extracts were very thermolabile (half-life of 1–2 min). All the subfractions were more active on T4 DNA than on calf-thymus DNA. They also formed rifampicin-resistant, RNA chain initiation complexes with T4 DNA. Based on the kinetics of heat inactivation with T4 and calf thymus DNAs as templates and preferential transcription of T4 DNA, it is proposed that the T4-infected cell enzymes prepared as described here harbor heat-labile initiation factor(s). During infection the heavy sedimenting RNA polymerase activity disappears after 2.5 min at 37°. This appears to require phage-specific protein synthesis because (a) it does not happen in the presence of chloramphenicol and (b) it does not happen in T4 ghost-infected cells.

Four broad classes of RNA have been described in bacteriophage T4-infected cells of *Escherichia coli* called immediate-early, delayed-early, quasi-late, and late species of RNA depending upon the respective times of appearance and relative abundance at a given time in the infected cell. The immediate-early and delayed-early species could be distinguished by the fact that phage-specific protein synthesis is needed for the synthesis of delayed-early RNA *in vivo* (Grasso and Buchanan, 1969; Salser *et al.*, 1970) but not *in vitro*, using T4 DNA and purified *E. coli* RNA polymerase (Milanesi *et al.*, 1969). The apparent requirement for phage-specific protein synthesis to turn on delayed-early transcription *in vivo* has given rise to two models. The one proposed by Travers (1969, 1970) postulates specific initiation factor(s), analogous to the σ factor of *E. coli*. The other model postulates a putative anti-termination factor (Schmidt *et al.*, 1970; Brody *et al.*, 1970; Black and Gold,

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