Biochem. J. (1970) **120**, 435–437
Printed in Great Britain

Deoxyribonucleoproteins and the Tissue-Specific Restriction of the Deoxyribonucleic Acid in Chromatin

By THOMAS C. SPELSBERG* and LUBOMIR S. HNILICA
Department of Biochemistry, University of Texas M.D. Anderson Hospital and Tumor Institute, Houston, Tex. 77025, U.S.A.

(*Received 7 September* 1970)

Although histones appear necessary for the restriction of DNA (Bonner *et al.* 1968), their lack of tissue and species specificity point to the necessary presence of other factors in chromatin that are required for the highly organ-specific genetic restriction of the DNA. The present paper analyses the type of restriction of the DNA in native chromatin by analysing the type of restriction in hybrid chromatins composed of portions of the dissociated products of the chromatins from two different organs.

Techniques for the isolation of chromatin, the RNA synthesis *in vitro*, the isolation of RNA formed *in vitro* from chromatin template and the DNA–RNA hybridization have been reported elsewhere (Spelsberg & Hnilica, 1970*a*). Briefly, isolated rat (male Sprague–Dawley) liver and thymus nuclei (Blobel & Potter, 1966) were extracted three times with 80mM-NaCl–20mM-EDTA, pH 6.3, then once with 0.3M-NaCl and twice with 0.01×SSC† with a Teflon homogenizer. Each extraction was followed by centrifugation at $4000g_{av.}$ for 20min. The RNA synthesis *in vitro* was carried out for 4h. In each reaction 200μg of DNA or 600–1000μg of chromatin DNA was used as a template with 400–500 units of RNA polymerase from *Micrococcus luteus* (Nakamoto, Fox & Weiss, 1964). Each reaction mixture contained 400μmol of tris–HCl buffer, pH 8.0, 25μmol of $MgCl_2$, 10μmol of $MnCl_2$, 30μmol of 2-mercaptoethanol, 4μmol each of GTP, ATP and CTP, 2.35μmol of [^{3}H]UTP (50μCi/μmol; from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.), 10μmol of spermidine phosphate and 50μg of bentonite. The reactions were carried out in the presence of 0.1M-NaCl in a final volume of 5.0ml at room temperature. After incubation the reaction mixture was made 0.3M with respect to KCl, incubated for 20min and centrifuged at $2000g$ for 10min. This procedure releases 85–95% of the newly synthesized RNA with a little or no release of the endogenous chromatin RNA. The DNA–RNA hybridization method was essentially that of Gillespie & Spiegelman (1965). The annealing was carried out in 48% formamide–4×SSC, pH 7.0, at 37°C for 16h. Competition experiments involved incubation of the DNA on membrane filters with the competing RNA, followed by washing and ribonuclease treatment and finally incubation with the labelled RNA.

The hybrid chromatins were formed as follows. The chromatin was homogenized by hand in 2.0M-NaCl–50mM-sodium acetate, pH 6.0. The lower pH (6.0) was used in our experiments to prevent proteolysis and to more thoroughly extract all of the histones while leaving most of the non-histone proteins associated with the DNA (see Spelsberg & Hnilica, 1970*b*). The dissociated chromatin was centrifuged at $90000g_{av.}$ for 36h to sediment the DNA with associated non-histone proteins. The supernatants of histones along with RNA and about 5% of the non-histone proteins were carefully decanted from the pellets, precipitated by 70% $(NH_4)_2SO_4$ treatment, resuspended in 2.0M-NaCl–5.0M-urea–10mM-sodium acetate, pH 6.0, and used as solvents to resuspend the DNA–protein pellets of the other tissue. Reconstitution of the solubilized histone fraction of one organ to the DNA with acidic proteins of another organ was accomplished by gradual dialysis of this mixture from 2.0M-NaCl–5.0M-urea–50mM-sodium acetate, pH 6.0, to 0.4M-NaCl–5.0M-urea–50mM-sodium acetate, pH 6.0, over a 16h period. This was followed by a 6h dialysis against 50mM-sodium acetate buffer, pH 6.0, to remove urea. The chromatin in solution was sedimented by centrifugation at $30000g$ for 10min and subjected to the chromatin purification procedure in 80mM-NaCl–20mM-EDTA, pH 6.3. The native as well as reconstituted chromatin, resuspended in 0.01×SSC, was used as template for the RNA synthesis *in vitro*.

As reported by several authors, isolated chromatin exhibits organ-specific restriction of its DNA similar to that of native tissues (Huang & Bonner, 1965; Bonner *et al.* 1968; Paul & Gilmour, 1968;

* Present address: Division of Reproductive Biology, Department of Obstetrics and Gynecology, Vanderbilt University School of Medicine, Nashville, Tenn. 37203, U.S.A.

† Abbreviation: SSC, 0.15M-NaCl–15mM-sodium citrate, pH 7.0.

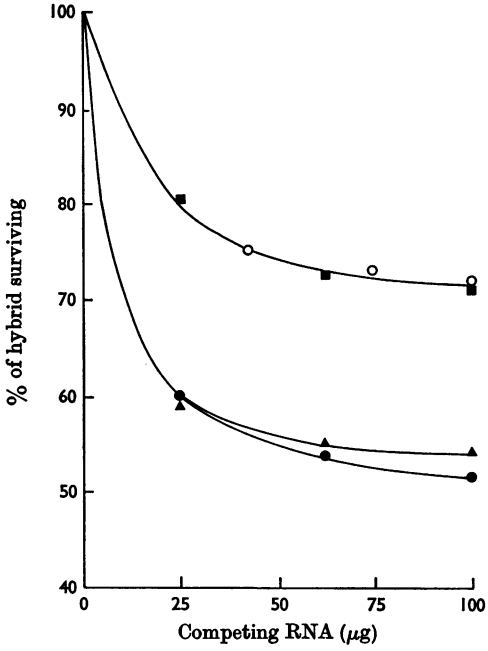

Fig. 1. Competitive hybridization of unlabelled RNA formed *in vitro* from the following templates: ●, native rat liver chromatin; ○, hybrid chromatin composed of histones from rat liver and DNA with associated non-histone proteins from rat thymus chromatin; ■, native rat thymus chromatin; ▲, hybrid chromatin composed of histones from rat thymus and the DNA with associated non-histone proteins from rat liver chromatin. The reconstitution was performed by dialysis at pH 6.0. The unlabelled RNA was made to compete against ^{3}H-labelled RNA formed *in vitro* from native rat liver chromatin template. The 100% hybridization represents 410 c.p.m. with 4 μg of DNA/filter.

Gilmour & Paul, 1969; Bekhor, Kung & Bonner, 1969; Smith, Church & McCarthy, 1969; Spelsberg & Hnilica, 1970*a*). Our results confirm this specificity of restriction. Competitive hybridization of RNA synthesized from reconstituted chromatin also demonstrated that the specificity of DNA restriction is retained in chromatin that has been reconstituted in the presence of 5.0 M-urea. Similar results were reported by Bekhor *et al.* (1969), Paul & Gilmour (1968) and Gilmour & Paul (1969). Our reconstituted chromatins contained quantitatively (histone/DNA ratio 1:1) and qualitatively (polyacrylamide-gel electrophoresis) all of the histones found in native chromatin. The non-histone protein content (non-histone protein/DNA ratio 0.8–1.0 in liver and 0.30–0.45 in thymus) in the reconstituted chromatin was found to be quantitatively similar to that in the native chromatin: the amount of non-histone protein in the hybrid chromatins was determined by the source of the DNA–non-histone protein used in the reconstitutions.

Unlabelled RNA, formed from the hybrid chromatin or native chromatin templates, was made to compete against labelled RNA formed from native rat liver chromatin template. In Fig. 1 the organ-specific restriction of DNA in the reconstituted hybrid chromatins appears to be determined by the DNA–non-histone proteins pelleted by centrifugation. Consequently the non-histone proteins or some other components associated with the DNA and not the histone-containing supernatants determined the pattern of restriction of the DNA in chromatin.

Experiments utilizing DNA–RNA hybridization in eucaryotes have certain limitations. If the RNA synthesized in nuclei of higher organisms were strictly heterogeneous, with no repetitious or closely similar nucleotide sequences, the large number of RNA species to be hybridized would make it impossible to obtain conclusive information in a reasonable length of time (Smith *et al.* 1969). Since a large portion of DNA in higher organisms was found to exist in a large number of either identical or highly similar copies (Britten & Kohne, 1968), these repetitive sequences allow a rather quick annealing of RNA to DNA. The redundant DNA sequences represent the major, if not total, fraction of the cell DNA involved in the hybrid formation in these experiments.

Previous evidence has indicated that either a special class of RNA (Huang & Bonner, 1965; Bonner *et al.* 1968; Bekhor *et al.* 1969) or the acidic proteins (Paul & Gilmour, 1968; Gilmour & Paul, 1969) of chromatin determine the pattern of restriction of the DNA. Our evidence here suggests that the total histone complement, along with some non-histone protein and RNA of chromatin extracted by 2.0 M-NaCl at pH 6.0, are not involved in the tissue-specific restriction of the DNA, but do participate in the general restriction of the DNA. The non-histone proteins and possibly RNA, remaining with the DNA after the salt extraction, specify this organ-specific restriction.

We thank Mrs Catherine Servance and Miss Susan Getz for their excellent technical assistance. The experimental work was supported by grants from the American Cancer Society (E-388), U.S. Public Health Service (CA-07746) and The Robert A. Welch Foundation (G-138).

Bekhor, I., Kung, G. M. & Bonner, J. (1969). *J. molec. Biol.* **39**, 351.

Blobel, G. & Potter, V. R. (1966). *Science, N.Y.*, **154**, 1662.

Bonner, J., Dahmus, M. E., Fambrough, D., Huang, R. C., Marushige, K. & Tuan, D. Y. H. (1968). *Science, N.Y.*, **159**, 47.

Britten, R. J. & Kohne, D. E. (1968). *Science, N.Y.*, **161**, 529.

Gillespie, D. & Spiegelman, S. (1965). *J. molec. Biol.* **12**, 827.
Gilmour, R. S. & Paul, J. (1969). *J. molec. Biol.* **40**, 137.
Huang, R. C. & Bonner, J. (1965). *Proc. natn. Acad. Sci. U.S.A.* **54**, 960.
Nakamoto, T., Fox, C. F. & Weiss, S. B. (1964). *J. biol. Chem.* **239**, 167.
Paul, J. & Gilmour, R. S. (1968). *J. molec. Biol.* **34**, 305.
Smith, K. D., Church, R. B. & McCarthy, B. J. (1969). *Biochemistry, Easton*, **8**, 4271.
Spelsberg, T. C. & Hnilica, L. S. (1970*a*). *Proc. 10th int. Cancer Cong., Houston* (in the Press).
Spelsberg, T. C. & Hnilica, L. S. (1970*b*). *Biochim. biophys. Acta* (in the Press).