Chromatin organization in the rat hypothalamus during early development

Stephen A. WHATLEY, Christine HALL and Louis LIM

Miriam Marks Department of Neurochemistry, Institute of Neurology, The National Hospital, Queen Square, London WC1N 3BG, U.K.

(Received 3 October 1980/Accepted 5 January 1981)

The organization of chromatin in neuronal and glial nuclei isolated from different brain regions of rats during development was studied by digestion of nuclei with micrococcal nuclease. A short chromatin repeat length (approx. 176 base-pairs compared with that of glial nuclei from foetal cerebral cortex (approx. 200 base-pairs) was present in hypothalamic neurons throughout the ages studied, which was similar to the repeat length of cortical neurons from 7- and 25-day-old animals (approx. 174 base-pairs). Whereas in cortical neurons the chromatin repeat length shortened from approx. 200 base-pairs in the foetus to approx. 174 base-pairs in the first postnatal week, the short chromatin repeat length of hypothalamic neurons was already present 2 days before birth, indicating that hypothalamic neurons differentiate earlier than cortical neurons during brain development.

The hypothalamus plays a unique role in the regulation of endocrinological activity and psychosexual behaviour, which is controlled by the concentration of circulating sex steroids. In addition, sex steroids determine the sexual differentiation of hypothalamic function, and therefore its subsequent hormonal responsiveness, during a critical period of neural differentiation (Gorski, 1971; Barraclough, 1973). Both the normal response of the hypothalamus and the sexual differentiation of this region are probably mediated by specific hormone receptors, which interact with the nucleus (McEwen *et al.*, 1974; White *et al.*, 1978, 1979). We have studied changes in nucleocytoplasmic relationships of the oestrogen receptor (White *et al.*, 1979) and of RNA (Hall & Lim, 1978) in the hypothalamus during development to clarify the involvement of the genome during sexual differentiation.

The organization of the structure of eukaryotic chromatin into regularly sized repeating units or nucleosomes is now well accepted (Hewish & Burgoyne, 1973; Felsenfeld, 1978). The length of DNA in the nucleosome varies between different organisms and tissues (Compton *et al.*, 1976; Morris, 1976; Noll, 1976), different cell types of the same tissue (Thomas & Thompson, 1978), or within the same cell type (Johnson *et al.*, 1976). This appears to be due to changes in the linker region of DNA between the basic 'core' particles (Kornberg, 1977; Felsenfeld, 1978). During development, the DNA repeat length of cortical neurons from rat and rabbit has been reported to shorten from approx. 200 base-pairs in the foetus to approx.

160 base-pairs in the first week of life (Ermini & Kuenzle, 1978; Brown, 1978). In comparison, the repeat length of DNA from adult cortical glial cells and cerebellar neurons is approx. 200 base-pairs (Thomas & Thompson, 1978). We were interested to discover whether short chromatin repeat length occurred in neurons of other regions of the forebrain. In this present study, we have investigated chromatin organization in the hypothalamus during development. This brain region is particularly interesting, since the nucleosomal linker region has been implicated in steroid-hormone-receptor-chromatin interaction in other hormone-responsive tissues (Massol *et al.*, 1978), and any shortening or lack of shortening of the linker regions could be a controlling factor in receptor-chromatin interaction.

Materials and methods

Materials

A.R.-grade chemicals were used in all cases. Micrococcal nuclease (EC 3.1.31.1) was purchased from Boehringer Corp. (London), Lewes, Sussex, U.K. Spermine and spermidine were purchased from Sigma (London) Chemical Co., Kingston upon Thames, Surrey. Endonuclease-*Hae* III restriction fragments of bacteriophage- ϕ X174 RF DNA were purchased from New England Biolabs (Beverly, MA, U.S.A.).

Animals

Female Wistar rats (Cobs strain) bred in our laboratory were used at 19 days gestational age

(2 days before birth), 7 days old and 25 days old. Foetal and 7-day-old rats were sexed by measurement of anal-genital distance. The animals were decapitated, their brains removed and dissected at 4°C to remove the cerebellum, cerebral cortex and hypothalamus, which was dissected as a block of tissue containing the hypothalamus, pre-optic area and amygdala.

Preparation of nuclei

Neuronal and non-astrocytic glial nuclei were prepared from the dissected tissue essentially as described by Thompson (1973), except that sucrose solutions contained 12 mM-MgCl₂ rather than 1 mM-MgCl₂. The resultant nuclear pellets were washed twice in 0.34 M-sucrose/buffer A (Hewish & Burgoyne, 1973) by centrifugation (5 min at 1000g and 4°C in a MSE Mistral centrifuge). Nuclei were counted and sized with a Coulter Counter (Coulter Electronics, Harpenden, Herts., U.K.) and the nuclear concentration was adjusted to 0.2×10^8 – 1×10^8 nuclei/ml in 0.34 M-sucrose/buffer A. The nuclear yields from the different brain regions as assayed by measurement of DNA (Burton, 1956) in the original homogenate compared with the recovery of the DNA in the final nuclear pellets (neuronal and glial) were $62 \pm 11\%$ ($n = 2$) for foetal animals and $48 \pm 16\%$ ($n = 5$) for post-natal animals. The yield did not differ significantly between different brain regions. The nuclei appeared intact on microscopic examination.

Nuclease digestion

Nuclear suspensions were made 1 mM with respect to CaCl₂, and the mixtures preincubated for 5 min at 0°C. Nuclei were digested with micrococcal nuclease (75 units/ 10^8 nuclei) for 400 or 650 s at 37°C (Ermini & Kuenzle, 1978). Digestion was terminated by the addition of 0.1 M-EDTA (sodium salt) to a final concentration of 10 mM and the samples were chilled on ice.

DNA extraction

DNA was analysed on horizontal 2.5%-polyacrylamide/0.5%-agarose slab gels in the buffer system of Loening (1967). After electrophoresis, the gels were stained with ethidium bromide (5 µg/ml) for 15 min, and DNA was observed under u.v. light and photographed through an orange filter (Kodak Wratten no. 22). The negatives were scanned at 560 nm with a Gilford (Oberlin, OH, U.S.A.) gel scanner for calibration of peak migration distances.

Results and discussion

Nuclei isolated from animals of various ages were isolated as described in the Materials and methods section. The nuclear fractionation procedure resulted

Fig. 1. Nuclear-size-distribution graphs from nuclei isolated from different brain regions

Neuronal (N) or glial (G) nuclei were isolated from (a) hypothalamus, (b) cerebral cortex and (c) cerebellum, and the size distributions determined as described in the Materials and methods section.

in two distinctly different size classes of nuclei for all brain regions, indicating an efficient separation of neuronal from glial nuclei (Fig. 1). Nuclei were digested with micrococcal nuclease and the resultant DNA fragments were analysed by electrophoresis, as described in the Materials and methods section. A comparison of the electrophoretic mobility of DNA fragments obtained from neuronal and glial nuclei of the hypothalamus, cerebral cortex and cerebellum is shown in Fig. 2. The bands from

Fig. 2. Photograph of a typical gel for determination of DNA repeat length

Neuronal (N) or glial (G) nuclei from the hypothalamus (H), cerebral cortex (C) and cerebellum (Cb) were digested and electrophoresed as described in the Materials and methods section. Abbreviations: *Hae*, restriction-nuclease digest of phage- ϕ X174-RF DNA; d, days.

Fig. 3. Typical size-calibration curve of DNA fragments run in polyacrylamide gels

Migration of the DNA fragments from endonuclease-digested ϕ X174 DNA was estimated by scanning the negatives of the gel photographs. Migration was measured as the distance between the origin and the peak maxima.

nuclei of cortical neurons 2 days before birth fell progressively backwards out of phase with those from cortical nuclei of later ages, and those from hypothalamic nuclei of different ages. This indicates a longer chromatin repeat length in foetal cortical neurons. Bands from cortical glia and cerebellar neurons at different ages were in phase with those of foetal cortical neurons, indicating a similar long repeat length.

Fig. 4. Graphs showing typical slopes for the determination of DNA repeat lengths in the nuclear populations studied

The mobilities of the nucleosome bands were estimated from photographs of the gels as the distance between the origin and the peak maxima on the scans of the negatives. The sizes of DNA fragments were estimated from calibration curves as shown in Fig. 3. The slope of the graph was derived by linear-regression analysis of the data, from which the DNA repeat length was determined. The upper abscissa scale DNA from cortical neurons of 7-day-old rats ($\blacktriangledown$) and foetuses 2 days before birth ($\triangle$), and the lower scale to DNA from hypothalamic neurons of 7-day-old rats ($\blacksquare$) and hypothalamic glia of 25-day-old rats ($\square$).

Photograph negatives were scanned and the sizes of the peak maxima determined after calibration with an endonuclease-*Hae* III-digested phage- ϕ X174-DNA marker (a typical calibration curve is shown in Fig. 3). The values for the DNA repeat lengths in the various nuclei were measured by taking the slope of the graph of band size against band number (Fig. 4), as described previously (Noll & Kornberg, 1977). Regression lines for the determination of DNA repeat lengths from the nuclei tested diverged from a common point of origin and were not simply displaced parallel to one another (Fig. 4). This indicates a true variation in repeat length rather than differences caused by variable exonucleolytic activity. The common size of the first fragment

Table 1. *DNA repeat lengths of nuclear preparations from neurons (N) and glia (G) of three different brain regions* Repeat lengths were determined by linear regression as described in the text and Fig. 4. Values shown are the means $\pm$ s.d. for three separate determinations of repeat length. Each regression analysis was performed on a minimum of four points. The r value did not fall below 1.00 for any analysis. For cortical neurons, (b) is significantly different from (a) ($P < 0.05$) and (b) and (c) are also significantly different from (a) ($P < 0.025$).

		Mean repeat length (base-pairs) measured by linear regression					
		Hypothalamus		Cortex		Cerebellum	
	Age (days)	N	G	N	G	N	G
(a)	-2	179 $\pm$ 10	—	195 $\pm$ 11	200 $\pm$ 4	—	—
(b)	7	174 $\pm$ 7	—	173 $\pm$ 7	212 $\pm$ 10	204 $\pm$ 9	205 $\pm$ 18
(c)	25	174 $\pm$ 9	214 $\pm$ 10	175 $\pm$ 11	207 $\pm$ 8	201 $\pm$ 5	207 $\pm$ 4

and the uniform migration of its leading edge in different samples (Fig. 2) also suggests a common-sized core particle between different samples. This is in agreement with previous conclusions (Ermini & Kuenzle, 1978; Brown, 1978). The determination of DNA repeat length was independent of nuclease-digestion time in the range 400–650 s.

The chromatin DNA repeat lengths for different classes of nuclei during development are shown in Table 1. During development, the chromatin repeat length of cortical neurons shortened from 195 $\pm$ 11 (2 days before birth) to 173 $\pm$ 7 base-pairs (7 days old). This latter repeat length was short compared with that of both cortical glia (approx. 206 base-pairs), cerebellar glia (approx. 206 base-pairs) and cerebellar neurons (approx. 203 base-pairs) during development. This short repeat length (Thomas & Thompson, 1978) and its appearance during development (Ermini & Kuenzle, 1978; Brown, 1978) are in agreement with previous reports. It is possible that the apparent decrease in chromatin repeat length displayed by cortical neurons during development may merely be due to the selection of different sub-populations of nuclei with different repeat lengths by the isolation procedure. This type of selection could cause an apparent change in the neuronal chromatin repeat length even if the repeat length of the neuronal sub-populations did not change during development. However, the high yield of nuclei by the procedure adopted (approx. 50%) makes the selection of sub-populations an unlikely explanation for the shift in the chromatin repeat length of cortical neurons. DNA from hypothalamic neurons 2 days before birth (179 $\pm$ 10 base-pairs), 7 days old (174 $\pm$ 7) and 25 days old (174 $\pm$ 9) also exhibited a short chromatin repeat length which was approximately the same as the repeat length of postnatal cortical neurons, and compares with a repeat length of 214 $\pm$ 10 base-pairs for hypothalamic glia (25 days old). However, in contrast with the cortex, hypothalamic neurons did not display a developmental shortening during the period studied, rather, shorten-

ing was completed by day 19 of gestation, a time at which regional differentiation of the hypothalamus has started (Hyyppä, 1969). This may indicate that the hypothalamic neurons mature earlier than cortical neurons during foetal development.

There does not appear to be a consistent correlation with change in nucleosome size. A short chromatin repeat length in cortical neurons has been ascribed to increased transcriptional activity relative to cortical glia (Thomas & Thompson, 1978) or linked to DNA replication (Ermini & Kuenzle, 1978). However, a correlation between transcriptional activity and a short repeat length is not universal (Compton *et al.*, 1976) and has been criticized for cortical neurons (Ermini & Kuenzle, 1978). In agreement with the suggestion that chromatin repeat length shortening is linked to DNA replication (Ermini & Kuenzle, 1978), hypothalamic neuroblast division is complete by day 19 of gestation (Ifft, 1972). However, a general correlation between DNA replication and chromatin repeat length does not seem to be valid for the brain, since in this present study actively dividing glia (cortex, 2 days before and 7 days after birth) did not display a short repeat length, nor was there a change in chromatin repeat length after neuronal division has ceased in the cerebellum.

The significance of chromatin shortening in the forebrain is therefore not yet clear, but in the hypothalamus it is not linked with the acquisition of hormone responsiveness. The shortening phenomenon appears rather to be linked with a general property of neuronal differentiation.

We thank the Medical Research Council, the Wellcome Trust and the Brain Research Trust for support. The assistance provided by Miss Diana Menzies and Mr. Mark Habershon is gratefully acknowledged.

References

- Barracough, C. A. (1973) *Handb. Physiol. Sect. 7* 2, 29–56

- Brown, I. R. (1978) *Biochem. Biophys. Res. Commun.* **84**, 285–292
- Burton, K. (1956) *Biochem. J.* **62**, 315–323
- Compton, J. L., Bellard, M. & Chambon, P. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 4382–4386
- Ermini, M. & Kuenzle, C. C. (1978) *FEBS Lett.* **90**, 167–171
- Felsenfeld, G. (1978) *Nature (London)* **271**, 115–121
- Gorski, R. A. (1971) in *Frontiers in Neuroendocrinology* (Martini, L. & Ganong, W. F., eds.), pp. 237–290, Oxford University Press, New York and London
- Hall, C. & Lim, L. (1978) *Biochem. J.* **176**, 511–521
- Hewish, O. R. & Burgoyne, L. A. (1973) *Biochem. Biophys. Res. Commun.* **52**, 504–510
- Hyypä, M. (1969) *Z. Anat. Entwicklungsgesch.* **129**, 41–52
- Ifft, J. D. (1972) *J. Comp. Neurol.* **144**, 193–204
- Johnson, E. M., Littau, V. C., Allfrey, V. G., Bradbury, E. M. & Matthews, H. R. (1976) *Nucleic Acids Res.* **3**, 3313–3329
- Kornberg, R. D. (1977) *Annu. Rev. Biochem.* **46**, 931–954
- Loening, U. E. (1967) *Biochem. J.* **102**, 251–257
- Massol, N., Lebeau, M., & Baulieu, E. (1978) *Nucleic Acids Res.* **5**, 723–738
- McEwen, B. S., Denef, C. J., Gerlach, J. L. & Plapinger, L. (1974) in *The Neurosciences, 3rd Study Program* (Schmitt, F. O. & Worden, F. G., eds.), pp. 599–620, MIT Press, Cambridge, MA
- Morris, N. R. (1976) *Cell* **8**, 357–364
- Noll, M. (1976) *Cell* **8**, 349–355
- Noll, M. & Kornberg, R. D. (1977) *J. Mol. Biol.* **109**, 393–404
- Thomas, J. O. & Thompson, R. J. (1978) *Cell* **10**, 633–640
- Thompson, R. J. (1973) *J. Neurochem.* **21**, 19–40
- White, J. O., Thrower, S. & Lim, L. (1978) *Biochem. J.* **172**, 37–47
- White, J. O., Hall, C. & Lim, L. (1979) *Biochem. J.* **184**, 465–468