COMPARISON OF THE NUMBER OF RNA INITIATION SITES IN RAT BRAIN FRACTIONS ENRICHED IN NEURONAL OR GLIAL NUCLEI

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1. Introduction

Regulation of gene expression in eukaryotes appears to be exerted at least partially through selective transcription of specific DNA–chromatin sequences by endogenous RNA polymerases. As previously reported neurochemical studies revealed considerably higher RNA synthesis in mature neuronal- and astroglial- than in oligodendro- or microglial-enriched rat brain nuclear fractions [1–4]. More recent studies on the initiation of RNA synthesis support the view that chromatin proteins are involved in the selection of initiation sites for RNA polymerases [5,6]. Potentially the different rates of phosphorylation [7] and acetylation [8] of chromatin-bound neuronal and glial proteins as well as the differences in chromatin composition between both nuclear fractions [7,9] may be involved in the regulation of neuronal and glial transcription.

It is the main goal of this paper to present new insights in the mechanisms controlling the different degree of neuronal and glial chromatin-templated transcription. The experiments to be described deal with the quantitative determination of the number of initiation sites in neuronal- and glial-enriched rat brain nuclei using isolated rat brain RNA polymerase.

2. Methods

2.1. *Isolation of nuclei*

Rat brain nuclei were isolated from female albino Wistar rats weighing 160–180 g and purified by ultracentrifugation through 1.9 M sucrose with 1 mM $MgCl_2$ as described elsewhere [6]. Neuronal- (N) and glial- (G)enriched nuclear fractions were separated from isolated brain nuclei by discontinuous sucrose density gradient centrifugation [10]. The nuclear fractions obtained morphologically corresponded to those described previously [7,10] and characterized by [11,12]. The neuronal nuclei enriched fraction obtained as regards its composition is in good accordance with zone I and the glial nuclei enriched fraction with zone IV obtained by Austoker et al. [1] by means of zonal centrifugation. Since nuclei offer the advantage of chromatin in a minimally disturbed state [13] neuronal- and glial-enriched nuclei were used as templates for determination the number of RNA initiation sites with the aid of exogenous nuclear rat brain RNA polymerase.

2.2. *Preparation of rat brain nuclear RNA polymerase*

Preparation of nuclear brain RNA polymerase was exactly the same as described by Jacob et al. [14]. The enzyme preparation was standardized for its protein content and used as RNA polymerizing enzyme in assays in which the number of RNA initiation sites were quantitatively determined.

2.3. *Conditions for RNA synthesis*

The ionic composition of the assay mixtures was chosen to assure that RNA polymerase A and B would both be active during the incubation period and contained in a final volume of 50 µl the following components essentially as described by Reeder and Roeder [15]: 50 mM Na-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.9; 2 mM $MnCl_2$; 5 mM $Mg(OAc)_2$; 150 mM KCl; 2.5 mM dithiothreitol; 10%

glycerol; 1% bovine serum albumin; 1.0 mM each ATP, GTP, CTP, 0.4 mM [^{3}H]UTP (spec. act. 0.25 mCi/mmol) and 20 μl neuronal- or glial-enriched nuclei (3–5 μg DNA).

RNA polymerase B activity was determined by its sensitivity to low α-amanitin concentrations (2.5 μg/ml), the remaining α-amanitin-resistant activity was defined as RNA polymerase A activity. RNA polymerase C activity seems to be a minor component of the α-amanitin-resistant activity as revealed by addition of 150 μg α-amanitin/ml to the assay mix and was not measured separately. After equilibration the reaction was incubated at 25°C for various times. For kinetic studies 50 μl aliquots were pipetted onto Whatman 3 MM paper disks, precipitated in ice-cold 5% TCA with $Na_4P_2O_7$ and counted for radioactivity as described previously [16].

2.4. *Measurement of RNA initiation sites*

The number of initiation sites on neuronal and glial templates was titrated under conditions completely eliminating reinitiation using the technique described by Cedar and Felsenfeld [17] and Tsai et al. [5]. Either various concentrations of rat brain RNA polymerase preparation (0–10 μg protein) were incubated with fixed amounts of neuronal- and glial-enriched nuclei (10 μg DNA), or a fixed concentration of brain RNA polymerase preparation (5 μg protein) was incubated with varying amounts of neuronal- or glial-enriched nuclei (0–75 μg DNA) for 15 min in 50 μl assays containing: 50 mM Tris–HCl pH 7.9; 3 mM $MnCl_2$; 1.0 mM each ATP and GTP and 0.125 mM [^{3}H]UTP (spec. act. 0.8 mCi/mmol) to allow formation of stable initiation complexes [5]. Then RNA chain elongation was permitted by addition of 1.0 mM CTP and $MgCl_2$ and $(NH_4)_2SO_4$ in final concentrations of 5.0 respectively 400 mM. With increasing enzyme to template respectively template to enzyme ratios a saturation of high affinity RNA initiation sites is reached [18]. Under conditions preventing reinitiation and guaranteeing template saturation, the number of RNA chains initiated is equal to the number of RNA initiation sites on the template used and can be calculated from the saturation points of the titration curves (fig.2 A–C) [18]. The UMP incorporation into neuronal and glial nuclear RNA was determined by the paper disks method as described previously [16].

2.5. *Analytical procedures*

DNA was determined by the diphenylamine reaction [19] and protein by the method of Lowry et al. [20].

3. Results and discussion

Since incubations conducted at 25°C have proven to be more advantageous to that performed at 37°C [21], in vitro RNA synthesis in neuronal- and glial-enriched brain nuclei, previously isolated by the method described, was performed at 25°C. Conditions were chosen so that both RNA polymerase A and B activities were measured simultaneously in the same assay mix [15] and distinguished by their sensitivity toward α-amanitin (2.5 μg/ml). Figure 1 shows a typical time course of UMP incorporation into neuronal and glial nuclear RNA. In both nuclear brain fractions the UMP incorporation displays a biphasic time-dependent function with a rapid phase during the first 10 min followed by linear incorporation up to at least 90 min.

As verified by investigations using lysed nuclei

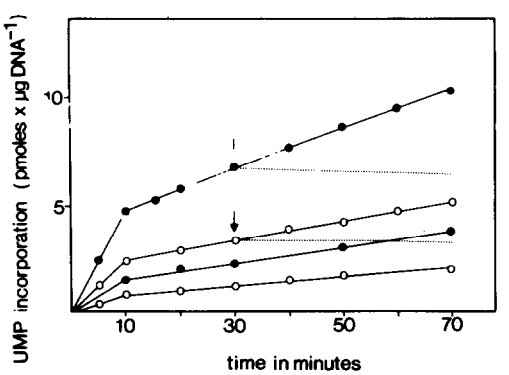

Fig.1. Kinetics of RNA synthesis of isolated neuronal- and glial-enriched rat brain nuclei. Incorporation was conducted at 25°C as described under 'Methods' with 5 μCi [^{3}H]UTP per assay of 50 μl. At 30 min after the initiation of neuronal and glial nuclear RNA synthesis actinomycin D (12.5 μg) and an excess of unlabelled UTP were added to the assay mixtures (arrows) and the stability of newly synthesized RNA was monitored for additional 40 min in both nuclear brain fractions (. . . . . .). Neuronal (●————●) and glial (○————○) nuclear RNA synthesis in the absence respectively neuronal (●- - - - - - - -●) and glial (○- - - - - - - -○) RNA synthesis in the presence of α-amanitin (2.5 μg/ml).

and increasing amounts of added nucleotide triphosphates (NTPs) the observed differences in UMP incorporation into neuronal and glial nuclear RNA are independent of endogenous nuclear NTP pool sizes and not due to different permeability of neuronal and glial nuclear membranes for added NTPs. Further differences in the stability of newly synthesized neuronal or glial nuclear RNA cannot account for the different transcriptional activities of both nuclear fractions as revealed by chasing experiments of [^{3}H] UMP-labelled neuronal and glial RNA in the presence of an excess of unlabelled UTP and actinomycin D (12.5 μg/assay) using the technique described previously [6] (see fig.1 arrows).

Since these apparently metabolic differences in neuronal and glial RNA synthesis may arise in part from differences in the number of initiation sites available for RNA polymerases, the total number of RNA initiation sites on neuronal and glial nuclear templates was quantitatively determined under conditions completely preventing RNA chain reinitiation. The number of high affinity initiation sites was measured by titrating either a fixed concentration of neuronal- or glial-enriched nuclei with increasing amounts of nuclear rat brain RNA polymerase, or by titration of a fixed concentration of RNA polymerase with varying quantities of neuronal- or glial-enriched nuclei. The number of both, high and low affinity initiation sites was examined by exposing a fixed concentration of neuronal or glial nuclei to a large excess of RNA polymerase [23]. Employing slight modifications of the technique developed by Cedar and Felsenfeld [17] and Tsai et al. [5] brain RNA polymerase and neuronal- and glial-enriched lysed nuclei were incubated under low ionic strength conditions only in the presence of three nucleotide triphosphates to allow formation of stable initiation complexes but to prevent excessive RNA chain elongation. After the initiation reaction is complete (15 min), nonspecific initiation and reinitiation were eliminated by addition of $(NH_4)_2SO_4$ up to a final concentration of 0.4 N [22]. Simultaneously the fourth nucleotide triphosphate is given to the assay mixture to permit RNA chain elongation of the RNA chains initiated immediately before for additional 15 min.

As can be seen from the RNA polymerase saturation curves (fig.2A) the final UMP incorporation obtained at the end of the elongation period increased

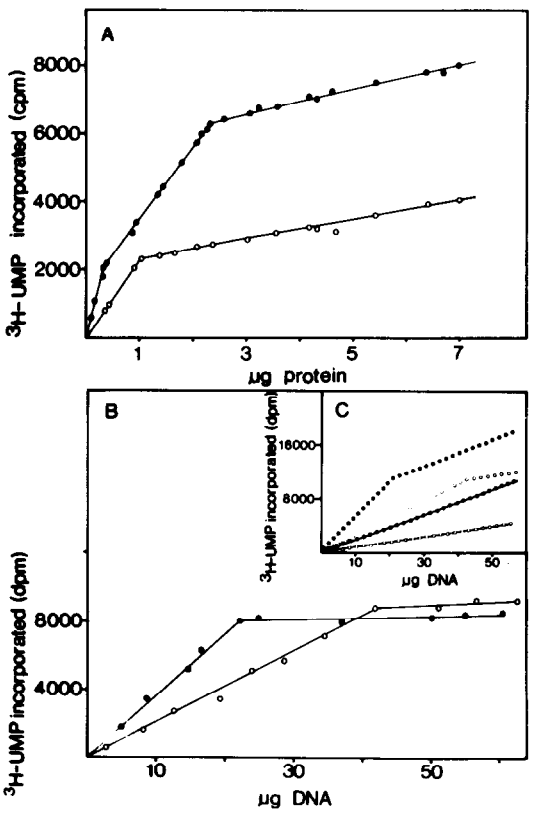

Fig.2 (A) RNA polymerase saturation curves on neuronal (●————●) and glial (○————○) nuclear templates. A fixed concentration of either neuronal- or glial-enriched nuclei (10 μg DNA) was incubated with increasing amounts of rat brain RNA polymerase (prepared according to Jacob et al. [14] and standardized by the protein content of the preparation). At the end of 15 min when the initiation reaction was complete RNA chain elongation was started by addtion of CTP, MgCl and $(NH_4)_2SO_4$ at final concentrations of 1.0 mM, 5 mM and 400 mM and the assay mixtures were further incubated at 25°C for 15 min. RNA synthesized was precipitated and counted as described under 'Methods'. (B) Net exogenous RNA polymerase activity of neuronal (●————●) and glial (○————○) enriched nuclei was obtained by subtracting endogenous RNA synthesis values from the corresponding values of endogenous plus exogenous RNA synthesis for neuronal- and glial-enriched nuclei. (C) Endogenous (●-●-●-●-●-●) and endogenous plus exogenous (● ● ● ● ● ●) RNA synthesis of neuronal enriched nuclei and endogenous (○-○-○-○-○-○) and endogenous plus exogenous (○ ○ ○ ○ ○ ○) RNA synthesis of glial enriched nuclei. Assays contained increasing amounts of lyzed neuronal- and glial-enriched rat brain nuclei either with or without a fixed concentration (5 μg protein) of rat brain nuclear RNA polymerase preparation. Incubation conditions were the same as described in fig.2A.

with the increasing amount of RNA polymerase added to a fixed concentration of neuronal or glial nuclei until the transition points are reached. These points of titration corresponded to the amount of RNA polymerase molecules required to saturate the available initiation sites on neuronal respectively glial templates.

In comparison to glial nuclei a greater quantity of RNA polymerase was needed to saturate the initiation sites present in neuronal nuclei. The amount of RNA polymerase needed to reach the titration point for neuronal nuclei was twice the amount required to saturate the glial initiation sites (fig.2A) indicating a greater number of high affinity RNA polymerase initiation sites on neuronal template. The validity of these results was confirmed by employing an alternative assay measuring the number of initiation sites by exposing fixed amounts of RNA polymerase to increasing amounts of neuronal- or glial-enriched nuclei (fig.2B–C).

In addition to the determination of the number of high affinity initiation sites, we quantified the total number of initiation sites including high and low affinity RNA initiation sites on neuronal and glial nuclear templates by adding a large excess of brain RNA polymerase to a fixed concentration of either neuronal- or glial-enriched nuclei. Under this condition the UMP incorporation into neuronal and glial nuclear RNA is a function solely of the available initiation sites present in neuronal and glial nuclei, independently of the enzyme concentration applied. The total number of initiation sites determined under enzyme excess conditions was found to be approximately 40% higher in both nuclear brain fractions as compared with values obtained by titration of fixed amounts of nuclei with increasing concentrations of RNA polymerase. Taken together, independently of the method applied a higher number of initiation sites available for RNA polymerase was determined on neuronal as compared to glial nuclear template.

In conclusion, the results outlined above clearly demonstrate a connection between the number of RNA initiation sites on the neuronal nuclear template and its transcriptional capacity both being higher than the corresponding values of the glial nuclear template.

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