H1\textsuperscript{0} RNA-binding Proteins Specifically Expressed in the Rat Brain\textsuperscript{*}

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During brain maturation, histone H1\textsuperscript{0} accumulates in both nerve and glial cells. The expression of this “linker” histone, the role of which still remains unclear, is a complex process, having both transcriptional and post-transcriptional regulatory components. In particular, the expression of H1\textsuperscript{0} in rat cortical neurons is regulated mainly at the post-transcriptional level, and unknown cellular proteins are likely to affect H1\textsuperscript{0} mRNA stability and/or translation. In looking for such factors, we tested the ability of rat brain extracts to protect H1\textsuperscript{0} RNA probe from degradation by T1 RNase. The results reported here demonstrate that rat brain contains at least one major (p40) and two minor (p110 and p70) binding factors, specific for H1\textsuperscript{0} RNA, all of which are much more or exclusively expressed in adult rat brain, when compared with other tissues. The binding of the factors is confined to a portion of the 3’-untranslated region (3’-UTR), which is highly conserved among murine and human H1\textsuperscript{0} mRNAs. These findings suggest that the proteins identified play a critical role in regulating the expression of H1\textsuperscript{0} histone in the brain of mammals.

During development and differentiation, cells undergo precise sequences of events that imply switching on or off different sets of genes, and even terminally differentiated cells respond to ever changing environmental conditions by modulating the transcriptional activity of many genes. As this often implies the need for remodeling chromatin (for review, see Ref. 1), it is not surprising that non-allelic isotypes of histones are synthesized in differentiated cells and enter chromatin, probably at topologically defined regions of the nucleus.

The H1 class of histones, also known as linker histones, are involved in organizing chromatin higher structures as well as in regulating specific gene expression (for discussion, see Ref. 2). In addition to a number of other pre-existing H1 subtypes, histone H1\textsuperscript{0} appears in cells, during terminal differentiation (3, 4) or after growth inhibition (5, 6). Although the role of this linker histone remains unclear, it has been proposed to localize specifically to the periphery of nucleus (7). Like other replacement histones, H1\textsuperscript{0} accumulates in postmitotic cells and is synthesized in the absence of DNA replication (8–11); however, transcription of the H1\textsuperscript{0} gene seems to be a replication-dependent event, at least in some cell types (12, 13). Regulation of H1\textsuperscript{0} expression is thus likely to be a complex process with both transcriptional and post-transcriptional components.

In maturing brain, H1\textsuperscript{0} accumulation was demonstrated in both neurons (11, 14–16) and glial cells (9). However, the role of H1\textsuperscript{0} in gene expression remains unclear.

We previously cloned two cDNAs encoding rat histones H1\textsuperscript{0} (17) and H3.3 (14), respectively. We used these cDNAs as probes to study the accumulation of the corresponding messages during rat brain development (14) and in cultured neurons (18). The effects of transcriptional inhibition by actinomycin D and the results of nuclear run-on experiments suggested that expression of both H1\textsuperscript{0} and H3.3 is regulated mainly at the post-transcriptional level (18). Post-transcriptional control processes often include regulation of mRNA localization, stability, and translation (18–31) and are mediated by several RNA-binding proteins (22, 26, 32–40). Therefore, it is likely that cellular factors, possibly expressed differentially in development, are involved also in histone mRNA binding and regulation in the brain.

The present study aimed, in particular, at the identification of proteins able to bind the mRNA encoding H1\textsuperscript{0} histone. We identified three brain-specific proteins that bind H1\textsuperscript{0} RNA probe with high specificity.

EXPERIMENTAL PROCEDURES

Preparation of Tissue Extracts—Fresh tissues from developing or adult rats were homogenized in nuclei buffer (NB: 0.32 M sucrose; 50 mM sodium phosphate buffer, pH 6.5; 50 mM KCl, 0.15 mM spermine; 0.15 mM spermidine; 2 mM EDTA and 0.15 mM EGTA), containing protease inhibitors (2 \mu g/mL aprotinin, 2 \mu g/mL antipain, 2 \mu g/mL leupeptin, 2 \mu g/mL pepstatin A, 1.0 mM benzamidine, and 1.0 mM phenylmethylsulfonyl fluoride, Sigma) and centrifuged at 1,000 \times g for 10 min at 4 °C. The supernatant was then used as such (post-nuclear extracts) or further centrifuged at 100,000 \times g for 60 min to separate the microsomal from the postmicrosomal (8–100) cell fraction. All the fractions, in NB, were split into aliquots and rapidly frozen in liquid nitrogen. Protein concentration was determined according to Lowry et al. (41).

Preparation of in Vitro Transcripts—The plasmids pMH1\textsuperscript{0} (17), pDH3 (obtained by ligation of the 5’ region of pDH 33–2 and the 3’ region of pDH 33–1 inserts, described in Ref. 14; it contains the entire sequence encoding the 1.2-kilobase H3.3 mRNA from rat) and pA1.3K (42) were linearized by restriction with BsmHI (pMH1\textsuperscript{0}) or HindIII (pDH3 and pA1.3K) and used as templates for in vitro transcription of both cold and \textsuperscript{32}P-labeled H1\textsuperscript{0}, H3.3, and c-erbA\textsuperscript{2} transcripts, respectively, from the T3 (pMH1\textsuperscript{0}) or the T7 (pDH3 and pA1.3K) RNA polymerase promoters (all the buffers and enzymes used for transcription were purchased from Promega). RNA was extracted once with phenol, twice with chloroform, and precipitated with ethanol and sodium acetate (0.3 M final concentration). The transcripts were collected by centrifugation at 10,000 \times g for 15 min, washed in 75% ethanol, and resuspended in distilled water. Small aliquots were used for counting and/or analysis on denaturing gels. On the basis of these analyses, we calculated the transcript concentration to be used in the next analyses.

T1 Nuclease Protection Assay—T1 nuclease protection assay was carried out, with modifications, according to the method described by Zaidi and Maltzer (43) and modified by Izquierdo and Cueva (44). Briefly, cell extracts (10–15 \mu g) were incubated for 10 min, at room temperature, with 0.5–1.0 \times 10^7 cpm (specific activity: 0.5–2.0 \times 10^7 cpm/pmol of RNA) of radiolabeled RNA, transcribed in vitro. Samples

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were then incubated for 30 min at 37 °C, with 100 units of T1 RNase (EC 3.1.27.3) to degrade the whole of the RNA except the portions protected by bound proteins; the extracts were finally exposed to a Spectrolite UV (254 nm) lamp (Aldrich Chemical Co., Inc.) for 15–20 min, in ice bath, to cross-link RNA to proteins. The covalent radioactive complexes were analyzed by denaturing electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide slab gels (PAGE), according to Laemmli (45), and the gels were directly exposed to x-ray film for autoradiography, with intensifying screens, for 12–18 h, at −70 °C. At the end of exposure, the gels were stained with Coomassie Brilliant Blue R-250 (Sigma) to confirm the loading of equal amounts of proteins per lane.

**Subcloning of Specific Regions of the H10 cDNA—**To localize, in the H10 RNA, the regions specifically involved in binding the factors identified, we subcloned different portions of the original pMH10 (EMBL accession number X70685; Ref. 17) into the Bluescript KS+ plasmid (Stratagene). In the first step, the insert of 1711 nucleotides was excised from the vector by EcoRI digestion and subcloned, by HindIII digestion, into one 1300-nt- and one 411-nt-long fragment (Fig. 5), both of which were subcloned into Bluescript. In the second step, we amplified 3 regions of the insert by polymerase chain reaction: 1) coding region (nt 1–496); 2) from nt 497 to nt 900 of the 3′-UTR; 3) from nt 1015 to nt 1230 of the 3′-UTR. To this aim we used the following primers: 1) 5′-AGCGAATTCATGATCGTGGCTGCCATCCAGGCA-3′; 2) 5′-AGCGAATTCCAAAGCTTGAGGGGAAAGGAAACGAACAACG-3′; 3) 5′-AGCGAATTCTCTCTTTGCTGGCTCTT-3′; 4) 5′-AGCGAATTCGCGTGGTGTTCGTCGGCTTGGGGACA-3′; 5) 5′-AGCGAATTCCTTGCTTGGGACA-3′; 6) 5′-CCCAGCTTGTAGATGGGG-3′; 7) 5′-AGCGAATTCTGCAGACGGTCGTAgg-3′; 8) 5′-AGCGAATTCCCTGGGAGGAAAGGAAACGAACAgg-T-3′; the 5′- and the 3′-primers used included EcoRI or HindIII site (underlined), respectively, to allow the oriented cloning of the amplified molecules. Subclones were sequenced, from both sides, by the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham).

**Affinity of Binding—**To quantify the affinity of the factors for RNA, identical amounts (15 μg) of post-nuclear lysates from brain at the 18th day of embryonal development (E18) were incubated with increasing amounts of radioactive 1300-nt-long transcript. Samples were then treated and analyzed as described above. After electrophoresis, the gel was exposed in the same cassette with a piece of nylon membrane on which known amounts of the same RNA, used for the binding reaction, had been spotted. After variable times of exposure, the film was scanned in a HP Scan jet 4CT and analyzed by the Sigmagel program, version 1.0 (Jandel Scientific). The planimetry of scans was finally used to calculate concentrations of RNA. To compare the intensities of bands corresponding to protein-RNA complexes with the known amounts of RNA, we substituted concentration of radioactive incorporated uridine (U) for RNA concentration (each molecule of the 1300-nt-long H10 RNA fragment contains 307 U residues) and assumed an arbitrary size of 40 nt (with an average of 10 U residues) for the fragment of RNA covalently bound to p40. Briefly, from the intensity of each band, it was calculated how many femtomoles of a 10 U-containing RNA fragment were bound; this number was assumed as the number of protein-RNA complexes per 15 μg of protein.

**RESULTS**

**Identification of H10 RNA-binding Factors—**Fig. 1 shows representative results of T1 RNase protection assays in which H10 and H3.3 in vitro transcripts were incubated with equal amounts (10 μg) of post-nuclear extracts from E18 rat brain. H10 RNA binds to one major (40 kDa: p40) and two minor (about 110 and 70 kDa, respectively: p110 and p70) proteins; these bands are clearly different, both for position and intensity, from those visible in the case of H3.3 RNA. Specificity of these bands is clearly different, both for position and intensity, from those visible in the case of H3.3 RNA. Specificity of these bands are confirmed uridine (U) for RNA concentration (each molecule of the 1300-nt-long H10 RNA, the regions specifically involved in binding the factors identified, we subcloned different portions of the original pMH10 (EMBL accession number X70685; Ref. 17) into the Bluescript KS+ plasmid (Stratagene).

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The presence of this protein might relate either to aging or to individual variation; however, we have not yet found any clear correlation between this protein and the physiological state of the animal.

Concerning localization of the factors identified, we found that the major band was enriched in the microsomal fraction, whereas the minor ones were more concentrated in the cytosolic S-100 fraction (Fig. 4).

**Identification of RNA Regions Involved in Binding to the Factors—**The first step toward the identification of the RNA region involved in binding was to test the binding capacity of two RNAs corresponding, respectively, to the first 1300 and to the last 411 nucleotides of the H10 RNA (Fig. 5). As shown in Fig. 6A, only the larger RNA (1–1300, in the figure) was able to bind to all the factors. This finding was also confirmed by experiments in which the binding of the whole H10 RNA was competed by a 10-fold excess of the longer RNA but not by the shorter one (1301–1711) (Fig. 6B).

Then we tested the binding capacity of three smaller RNAs corresponding to three different regions of the 1300-nt-long RNA: a) the coding region (nt 1–496); b) from nt 497 to nt 900

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1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; nt, nucleotide(s); UTR, untranslated region.
of the 3'-UTR, and c) from nt 1015 to nt 1230 of the 3'-UTR (see “Experimental Procedures” and Fig. 5). As shown in Fig. 7, the coding region (a) of H10 RNA did not bind to the factors at all (lane 2), while both RNAs (b and c) corresponding to portions of 3'-UTR (lanes 3 and 4) recognized the proteins. Scanning of the autoradiograms revealed that the intensity of the 40-kDa band bound to the entire 1300-nt-long RNA (lane 1) is equal to the sum of the intensities of the corresponding 40-kDa band detected with the two smaller RNAs (lanes 3 and 4). This finding suggests that p40 has binding sites for both regions of 3'-UTR of the H10 mRNA.

Binding Affinity—On the basis of the intensity of the spots reported in Fig. 8A (known amounts of the 1300-nt-long RNA; see “Experimental Procedures”) we calculated the amount of RNA bound to p40 (Fig. 8B). As the RNA concentration in the binding mixture was also known, we could calculate the apparent $K_D$ of the complexes by Scatchard analysis (Fig. 8C). $K_D$ values thus calculated, from different experiments, are in the range of 20–30 nM. As described under “Experimental Procedures,” we assumed an arbitrary length of 40 nt for the bound RNA. This size roughly represents an upper limit for the real, and still unknown, length of the sequence protected by p40: in the absence of cross-linking treatment, we did not notice, indeed, any radioactive band after denaturing electrophoresis on gels as concentrated as 15% polyacrylamide. On the contrary,

**Fig. 3.** Expression in the developing brain (A) and tissue specificity (B) of H10 RNA-binding factors. Post-nuclear extracts (10 μg) from different stages of development (A) or from different tissues of adult rat (B) were incubated with 1.0 × 10^6 cpm of radiolabeled RNA and subjected to T1 RNase protection assay. E, embryonal; P, postnatal day of development. M, muscle; H, heart; L, liver; S, spleen; K, kidney; B, brain.

**Fig. 4.** Intracellular distribution of H10 RNA-binding factors. Partially purified cell subfractions (10 μg each), from E18 fetal brain, were incubated with 0.5 × 10^6 cpm of radiolabeled H10 RNA. Pn, postnuclear; Mit, mitochondrial; Micr., microsomal; S100, postmicrosomal; −, without cell fraction.

**Fig. 5.** Schematic map of the rat H10 cDNA. Numbers indicate boundary nucleotides relevant to our analysis. $H$, recognition site for HindIII restriction enzyme; a) nt 1–496 coding region; b) 3'-UTR from nt 497 to nt 900; c) 3'-UTR from nt 1015 to nt 1230; 1300, entire 1300-nt-long RNA; 711, region from nt 397 to nt 496; 411, region from nt 901 to nt 1014; 1–496, nt 1 to nt 496 coding region; 900–1300, nt 901 to nt 1300.

**Fig. 6.** Identification, in the H10 transcript, of a 1300-nt-long fragment, responsible for the binding to factors. In A, results of T1 RNase protection assay performed with the full-length (1711 nt) transcript are compared with those obtained with two shorter RNAs, containing the first 1300 or the last 411 nt, respectively. In B, the protection assay with radiolabeled H10 RNA (1711 nt) was performed in the absence (first lane) or in the presence of 10:1 excess of one of the other of the shorter unlabeled RNAs.

**Fig. 7.** Identification, within the 1300-nt-long H10 RNA fragment, of shorter regions involved in binding to factors. Four different portions of the H10 RNA were tested by T1 RNase protection assay. The corresponding subclones, used as templates, were: 1) the insert containing the first 1300 nt of the pMH10; 2) the subclone corresponding to region a in Fig. 5; 3) the subclone corresponding to region b in Fig. 5; 4) the subclone corresponding to region c in Fig. 5.

**Fig. 8.** Calculation of the apparent $K_D$ for the binding of the 1300-nt-long H10 RNA to p40. E18 postnuclear extracts (15 μg) were incubated with increasing amounts (0.5, 1.0, 2.5, 25, and 50 nM, final concentration, respectively) of the 1300-nt-long RNA (B). The intensity of the signals obtained were compared with signals obtained from known amounts (0.5, 1.0, 2.0, and 5.0 fmol, respectively) of the same transcript, spotted directly on nylon (A). Results of calculations corresponding to samples 3–6 were used to draw the Scatchard plot reported in C. [PR], concentration of protein-RNA complex; [R], RNA concentration.
p40-bound RNA might be shorter than 40 nt and/or contain less than 10 U residues. Under these latter hypotheses, the number of protein-RNA complexes formed at a given RNA concentration could be underestimated, and the real $K_d$ values would be smaller than those reported here.

**DISCUSSION**

The generation of specific neuronal phenotypes depends on the synthesis and intracellular localization of specific regulatory as well as structural proteins. On the basis of several studies, it appears that the fine control of these aspects of protein metabolism depends largely on post-transcriptional regulation of the metabolism of the corresponding mRNAs (29, 46–53). Among the gene products whose concentration changes during neuronal cell maturation, differentiation-associated variants of both linker (such as H10) and core histones (such as H3.3) are of great interest, as their entering chromatin may further affect the transcriptional potential of the genome (54, 55).

In maturing brain, H10 accumulation seems to be regulated mainly at the post-transcriptional level (18). As increasing translational activation, and perhaps, enhanced degradation are (p70 and p110) factors that bind to H10 mRNA with high specificity, because the expression (and/or the binding ability) of these factors does not change significantly from E18 to adulthood, they are probably requested continuously both in differentiating and mature brain. In particular, one or more of these factors might be directly involved in enhancing H10 mRNA translation and degradation. This latter function would be consistent with the need of maintaining low concentrations of H11 mRNA, without inhibiting transcription of the H11 gene. Interestingly, the factors described here are predominantly or exclusively expressed in the brain. This suggests that the function of these proteins is linked to a pathway of H11 gene regulation specific to the brain. Furthermore this brain-specific function might have been conserved during evolution of mammals, as the factors identified recognize, in the 3′-UTR of H10 RNA, regions that contain blocks of sequences highly conserved among murine and human H11 genes (17).

It should be also noted that one (p40) of the H10 mRNA-binding factors is enriched in the microsomal subcellular fraction, while the other two proteins remain in the cytoplasm. Presumably, recruitment of H10 mRNA to ribosomes, its translational activation, and perhaps, enhanced degradation are functions managed by a complex formed by both soluble and anchored proteins. Anchoring the complex to membranes (possibly to the nuclear envelope) and/or to the cytoskeleton might have obvious advantages for localizing H10 mRNA to cytoplasmic regions from which newly synthesized H10 histone might easily reach specific chromatin domains.

Finally, it should be stressed that, to our knowledge, the factors identified in the present work represent the first example of brain-specific RNA-binding proteins that bind to a highly conserved region of one specific mRNA. The fact that this mRNA is the one encoding H10 protein adds interest to the finding, as further understanding of regulation of H10 synthesis might help to shed light on the role that this linker histone plays in chromatin remodeling in postmitotic cells.

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