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Sequence Complexity of Nuclear and Cytoplasmic Ribonucleic Acids from Clonal Neurotumor Cell Lines and Brain Sections of the Rat†

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ABSTRACT: The sequence complexity of both nuclear and cytoplasmic ribonucleic acids (RNAs) from rat brain is higher than the sequence complexity of these RNAs from other tissues. In addition, a higher percentage of the nuclear RNA sequences appear as cytoplasmic RNA in brain than in other organs [Chikaraishi, D. M., Deeb, S. S., & Sueoka, N. (1978) *Cell (Cambridge, Mass.) 13*, 111–121; Chikaraishi, D. M. (1979) *Biochemistry 18*, 3249–3256]. We would like to determine whether the high sequence complexity of brain RNAs is found in every cell of the brain or if this high complexity is due to the summation of lower, incompletely overlapping, complexities in the various cell types of the brain. We measured the sequence complexity of RNA fractions from both neurotumor cell lines and brain sections by saturation hybridization. Nuclear RNA from the central nervous system cell lines B103 and B27 hybridized to 14.5 and 13.5% of the unique-sequence DNA, respectively, while nuclear RNA from the peripheral neurotumor cell line RT4-D1 hybridized to 11.1% of the unique-sequence DNA. If asymmetric transcription is assumed, these hybridization values correspond to complexities of 29.0, 27.0, and 22.2% of the unique-sequence genome or 5.3×10^8, 4.9×10^8, and 4.0×10^8 nucleotides for the three cell lines. The sequences transcribed into nuclear RNA in these cell lines are included in the set of sequences transcribed in whole brain. Nuclear RNA from the five brain sections examined hybridized to from 14.7 to 16.3% of the unique-sequence DNA, within experimental error of the 15.6% found for whole brain. Total cytoplasmic RNA from B103 and B27 cell lines hybridized to 3.1 and 4.3% of the unique-sequence DNA, respectively, while released polysomal RNA hybridized to 3.2 and 3.9% for the two cell lines. These cytoplasmic complexities are equivalent in each case to ~25% of the sequences appearing in the nucleus of the cell line. In contrast, total cytoplasmic and released polysomal RNAs from whole brain were previously shown to represent 50–65% of the sequences appearing in the nucleus. Some brain sections show complexities for polyadenylated cytoplasmic RNA lower than the value for whole brain. In particular, this RNA fraction hybridized to 2.8% of the unique-sequence DNA for cerebellum as compared to 5.1% for whole brain. These data support the view that most of the sequences transcribed into nuclear RNA of the brain are found in every cell of the brain but that only one-fourth to one-third of these sequences are eventually translated in any given cell. Therefore, there must be a great deal of posttranscriptional selection in the brain.

The sequence complexity of RNA[1] from adult tissues of several rodents has been measured by saturation hybridization. In the rat (Shearer, 1977; Chikaraishi et al., 1978; Grouse et al., 1978; Kaplan et al., 1978; Tedeschi et al., 1978; Chikaraishi, 1979; Grady et al., 1979; Lee et al., 1979; Wilkes et al., 1979), mouse (Hahn & Laird, 1971; Brown & Church, 1972; Grouse et al., 1972; Bantle & Hahn, 1976; Grady et al., 1978; Kuroiwa & Natori, 1979; Van Ness et al., 1979), and rabbit (Brown & Church, 1972; Church & Schultz, 1974) the brain has consistently shown a higher complexity in both its nuclear and cytoplasmic RNAs compared to that found in other tissues. This relatively high sequence complexity for brain could either result from higher sequence complexity in every brain cell or from the summation of lower complexities with different transcripts in the various cell types of the brain.

Data from our laboratory have shown that, for the rat, brain nuclear RNA hybridizes to 15.5%, liver nuclear RNA hybridizes to 11%, and kidney, spleen, and thymus nuclear RNAs each hybridize to ~5% of the unique-sequence DNA. If asymmetric transcription is assumed, these nuclear RNAs represent 31, 22, and 10% of the unique-sequence genome, respectively. If the high complexity observed in the brain was due to a summation of different transcripts in each of many cell types, one might expect the average number of copies of a given sequence per cell to be lower for the brain than for other organs. This is not, however, observed. Brain, liver, and

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[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; hnRNA, heterogeneous nuclear RNA; rRNA, ribosomal RNA; mRNA, messenger RNA; HAP, hydroxylapatite; [^{125}I]DNA or unique-sequence [^{125}I]DNA, ^{125}I-iodinated unique-sequence DNA; PBS, phosphate-buffered saline; poly(A)+, polyadenylated; R_0t, RNA concentration (moles of nucleotide per liter) at t_0 × time (seconds); $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Cl_3CCOOH, trichloroacetic acid.

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kidney each have ~0.2 copies of most of their nuclear RNA sequences per cell (Chikaraishi et al., 1978).

In higher eukaryotes, cytoplasmic and polysomal RNA complexities are lower than the nuclear RNA complexity of a given tissue. In mouse and rat nuclear RNA has greater than or equal to 3 times the sequence complexity found in cytoplasmic RNA of most tissues (Birnie et al., 1974; Getz et al., 1975; Young et al., 1976; Kleiman et al., 1977; Chikaraishi et al., 1978; Grady et al., 1978; Chikaraishi, 1979). However, we have found that nuclear RNA from rat brain is only 1.5–2 times as complex as cytoplasmic RNA from rat brain (Chikaraishi, 1979). Similar observations have subsequently been made for mouse brain RNAs by Van Ness et al. (1979). The possibility that the high RNA complexity in brain is due to a composite of many cell types seems likely at the cytoplasmic level because of the increased ratio of cytoplasmic to nuclear RNA complexity in this organ.

To determine whether the high diversity in brain nuclear and cytoplasmic RNAs is due to each cell having the same high complexity or due to the summation of many cell types, each having lower complexities, we measured the sequence complexity of nuclear and cytoplasmic RNA from both clonal neurotumor cell lines and rat brain sections. By saturation hybridization to unique-sequence DNA, we find for all brain sections and two of the three neurotumor cell lines tested that the nuclear RNA complexity is close to that of the whole organ while the cytoplasmic RNA complexity is decreased.

Nuclear RNA from the central nervous system neurotumor cell lines B103 and B27 (Schubert et al., 1974) hybridized to 14.5 and 13.5% of the unique-sequence DNA, respectively, while nuclear RNA from the peripheral neurotumor RT4-D1 (Imada & Sueoka, 1978) hybridized to 11% of the unique-sequence DNA. Nuclear RNA from brain sections hybridized to from 14.5 to 16.5% of the unique-sequence DNA. All values are corrected for probe reactivity.

In a previous study, Kaplan et al. (1978) showed that regions of the rat brain (cerebral cortex, cerebellum, hypothalamus, and hippocampus) have poly(A)+ total RNA complexities similar to that of whole brain, as do three cultured neural cell lines, although their data show significantly greater differences between cell lines and brain than ours. Complexities similar to that of whole brain have also been observed for nuclear RNA from the cerebrum and cerebellum of the green monkey (Hahn, 1973).

The cytoplasmic RNA complexities of neurotumor cell lines reported here are significantly lower than that of the whole brain. Whereas brain cytoplasmic RNA hybridizes to 10% of the unique-sequence DNA and brain polysomal RNA 8% (Chikaraishi, 1979), total cytoplasmic and polysomal RNAs from B103 or B27 cell lines each hybridized to ~4% of the unique-sequence DNA. Certain brain sections, in particular the cerebellum, also have a decreased cytoplasmic RNA complexity compared to the total brain. These observations support the hypothesis that each cell of an organ transcribes quite similar sequences into nuclear RNA with much of the regulation of the eventual translation products occurring posttranscriptionally.

Experimental Procedures

Preparation of [^{125}I]DNA. Unique-sequence DNA was prepared, iodinated, and characterized as previously described (Chikaraishi et al., 1978; Chikaraishi, 1979). Briefly, the iodinated probe is ~475 bases in length. Greater than 90% of the probe can be driven into hybrid by sheared DNA with kinetics indicative of unique-sequence DNA. Less than 3% reassociates with repetitive sequence kinetics. After 3 months of storage at 4 °C, 10–15% of the ^{125}I radioactivity is no longer bound to acid-precipitable DNA. Therefore, we prepared a new probe every 2 months. After 125 h of hybridization at 68 °C, 8–10% of the ^{125}I radioactivity is released from acid-precipitable DNA. This radioactivity elutes from hydroxylapatite in the 0.03 M phosphate buffer fraction and is not included in our calculation of the percent hybridization.

Cell Culture. All cells were grown in Dulbecco's modified Eagle's medium (high glucose) supplemented with 50 units/mL each of penicillin and streptomycin. B103 and B27 cell lines (Schubert et al., 1974) were further supplemented with 12.5% horse serum and 2.5% fetal calf serum (FCS), RT4-D1 cells (Imada & Sueoka, 1978) were supplemented with 5% FCS, and HTC cells (Thompson et al., 1966) were supplemented with 10% calf serum. Cells were grown to confluency on 50- or 100-mm plastic cell culture dishes (Lux) at 36–37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was changed every 2–3 days. Cells were harvested by trypsinization, removed into a small volume of medium, and collected by centrifugation. They were then washed 1–2 times with phosphate-buffered saline (PBS; 2.68 mM KCl, 137 mM NaCl, 8.14 mM Na_2HPO_4, and 1.47 mM KH_2PO_4). Washed cells were immediately used for RNA extraction.

B103 Tumors. B103 cells from 1.5 confluent 50-mm plates were suspended in 0.2 mL of saline solution and injected subcutaneously in the shoulder of a 1–2-month-old rat. Rats were sacrificed in 2–3 weeks to recover ~1-cm round tumors. Fibrous tissue was removed and tumors were either quickly frozen in liquid nitrogen and stored at −70 °C, or, if cytoplasmic RNA was to be prepared, they were diced and used immediately. B27 cells were found to be nontumorigenic by using a variety of cell injection schemes with rats of several ages.

Brain Dissection. Rats were killed by cervical dislocation and the brains quickly removed and placed on a cold glass surface. The cerebellum was removed. The cerebral cortex was pulled back, excised laterally at the rhinal sulcus, and cut into thirds (anterior, parietal, and occipital). The region underlying the cortex, not including the cerebellum, midbrain, and stem, was then removed. All sections were kept in ice-cold PBS until the entire batch of 200 animals was dissected. Freezing tissues at this stage allows leakage of nuclear RNA into the cytoplasm and was therefore avoided.

Preparation of RNA. Nuclear and polyadenylated cytoplasmic RNAs were prepared from rat brain sections as previously described (Chikaraishi et al., 1978; Chikaraishi, 1979). For cell line or B103 tumor preparations, 4–7 g of tissue was homogenized (5 strokes) by using a Potter tissue grinder with a motor-driven pestle in either 0.14 M NaCl, 0.01 M Tris, pH 8.4, 1.5 mM $MgCl_2$ (Lindberg & Darnell, 1970), 0.1–0.2% Triton X-100, and 500 μg/mL spermidine or 0.2 M sucrose (Schwarz/Mann, RNase-free, pretreated with activated charcoal to remove impurities which absorb at 260 nm and diethyl pyrocarbonate to inactivate ribonucleases), 0.1 M NH_4Cl, 5 mM magnesium acetate, 20 mM Tris, pH 7.5, 1 mM dithiothreitol (Falvey & Staehlin, 1970), and 0.1–0.2% Triton X-100. The homogenate was spun at 13000*g* for 10 min. The upper two-thirds of the supernatant was used for either total cytoplasmic or released polysomal RNA preparation as previously described (Chikaraishi, 1979). Nuclei were completely resuspended in homogenization buffer, underlayed with 6 mL of homogenization buffer plus 0.6–1.0 M sucrose, and spun at 13000*g* for 10 min. The resulting pellet was resuspended in 0.14 M NaCl, 0.01 M Tris, pH 8.4, and

1.5 mM $MgCl_2$, and the nuclei were lysed with 1% sodium dodecyl sulfate ($NaDodSO_4$). Phenol–chloroform extractions were as previously described (Chikaraishi et al., 1978). The bulk of the DNA was removed from the ethanol precipitates by spooling on glass rods. Purified nucleic acid was then treated with 20 μg/mL column-purified DNase (see below) for 1 h in 1–2 mL of 10 mM Tris, pH 7.5, 10 mM $MgCl_2$, and 10 mM NaCl at 37 °C. Another aliquot of DNase was added and incubation continued an additional 1 h. The reaction was stopped by addition of 10 mM EDTA and 1% $NaDodSO_4$. Three milliliters of 0.1 M NaCl, 20 mM Tris, pH 7.5, 5 mM EDTA, and 0.1% $NaDodSO_4$ was added, and the RNA was extracted with phenol–chloroform–isoamyl alcohol (25:24:1) supplemented with 0.1% 8-hydroxyquinoline and saturated with 0.1 M Tris, pH 7.5, and then ethanol-precipitated. Intact RNA was separated from DNA oligonucleotides by passage over a 10-mL Sephadex G-100 column with a 0.5-mL Chelex 100 (Bio-Rad Laboratories) pad at the base, preequilibrated and extensively washed with 0.3 M NaCl and 0.01 M NaOAc, pH 6. The void peak was ethanol-precipitated, and the DNase treatment and Sephadex G-100 column separation were repeated. Two cycles of treatment were generally necessary and sufficient to completely remove DNA contamination from the RNA preparation. Purified RNA was stored as an ethanol precipitate.

DNase Purification. Worthington Biochemical Corp. "RNase-free" DNase 1 (code, DPFF), dissolved in 0.02 M NaOAc, pH 5.5, at 10 mg/mL, was applied to a 0.5-mL column of agarose–5′-[(*p*-aminophenyl)phosphoryl]uridine 2′(3′)-phosphate (Miles Yeda Lt.), preequilibrated with 0.02 M NaOAc, pH 5.5. Peak flow-through fractions were pooled and loaded on a second preequilibrated 0.5-mL column. (Maxwell et al., 1977.) The column-purified DNase was assayed for ribonuclease activity by testing the integrity of [^{32}P]rRNA on gels after incubation for 1 h at 37 °C with one aliquot of DNase. It was assayed for DNase activity as per the Worthington Catalog (April 22, 1978). This column purification of DNase was absolutely necessary because significant RNase contamination exists in these commercial preparations of DNase.

RNA–DNA Hybridizations. The hybridization and quantitation of hybrids by hydroxylapatite (HAP) chromatography were performed as previously described (Chikaraishi et al., 1978; Chikaraishi, 1979) except most reactions were performed in plastic microfuge tubes imbedded in sand and kept at 68 °C by a heat block. Under these conditions, collapse hybrids can be a problem after sampling due to the slow reequilibration to hybridization temperature. Therefore, samples were taken from the microfuge tubes which were immersed in a beaker of 68 °C water.

To ensure that we were assaying the formation of RNA–DNA rather than DNA–DNA hybrids, samples from later time points, taken into 1 mL of either 0.03 M phosphate buffer, pH 6.8 (1 M Na_2HPO_4 and 1 M NaH_2PO_4 combined to yield a 1 M stock solution, pH 6.8), or 0.05 M Tris, pH 7.5, were split, and half of the sample was treated overnight at 37 °C with 20 μg/mL RNase A (pretreated 10 min at 80 °C) (Galau et al., 1974), then made 0.03 M in phosphate buffer, pH 6.8, 0.2 M NaCl, and 0.06% $NaDodSO_4$, and chromatographed on HAP. A background reannealing of from 0.25 to 2.3% was observed in 5 of the 44 reactions reported in this paper. These background hybridizations have been subtracted from the data before plotting.

The S1 assay for brain section data was as previously described (Chikaraishi et al., 1978). The S1 assay for cell line hybridizations differed from the previously described procedure. The hybridization sample was taken into 100 μL of S1 buffer (0.3 M NaCl, 0.03 M NaOAc, pH 4.6, 1 mM $ZnCl_2$, and 18.5 μg/mL denatured calf thymus DNA). Samples (10 and 20 μL) were spotted onto GFA filters. Two microliters of S1 nuclease from *Aspergillus oryzae* (Miles Laboratories, 10^5 units/mL) was added to the balance of the sample and incubated 90 min (average) at 37 °C. A total of 50 μL of this digested sample was spotted onto a third filter. All filters were dried. The 20- and 50-μL samples were washed with 5% Cl_3CCOOH and used to determine the fraction of Cl_3CCOOH-precipitable counts rendered resistant to S1 nuclease during the hybridization. The 10- and 20-μL samples were used to determine the fraction of DNA which was Cl_3CCOOH precipitable. Background S1 resistance of 0.5–0.8% was routinely obtained for the unhybridized, single-stranded probe. For RNase controls the samples were taken into S1 buffer without NaCl. RNase A (10 μg/mL) was added along with the S1 nuclease before incubation at 37 °C. These reactions had a background S1 resistance of 1.5–2.5%. Background S1 resistance was subtracted from all data points.

Results

Hybridization of Cell Line Nuclear RNAs. B103 and B27 cell lines are derived from separate nitrosoethylurea-induced tumors of the central nervous system (Schubert et al., 1974) while RT4-D1 is derived from a nitrosoethylurea-induced peripheral neurotumor (Imada & Sueoka, 1978). B103 expresses neuronal properties while B27 and RT4-D1 exhibit glial properties. As shown in Figure 1 and Table I, B103, B27, and RT4-D1 nuclear RNAs hybridize to 14.5, 13.5, and 11.1% of the unique-sequence DNA, respectively, equivalent to 29.0, 27.0, and 22.2% of the complexity of unique-sequence DNA, assuming asymmetric transcription. These values are corrected for our probe reactivity which ranged from 90 to 97% (Galau et al., 1974). These hybridization values correspond to complexities of 5.3×10^8, 4.9×10^8, and 4.0×10^8 nucleotides for the three cell lines, equivalent to 120000, 110000, or 90000 distinct nuclear RNA sequences 4500 bases in length. The hybridization values for B103 and B27 are close to the 15.6% hybridization observed for total brain (Chikaraishi et al., 1978) while the hybridization of RT4-D1 nuclear RNA is significantly lower.

These levels of hybridization do not necessarily indicate that the same sequences are being expressed in both cell lines and brain. The degree of overlap between cell-line nuclear RNAs and brain nuclear RNA was determined by additivity experiments such as those of Figure 1C. In these experiments, cell-line nuclear RNA is hybridized to unique-sequence [^{125}I]DNA. At saturation, excess brain nuclear RNA was added to the same tube and the reaction continued to a new plateau. In some cases the reaction was split and brain nuclear RNA was added to half of the reaction, while a second aliquot of cell-line nuclear RNA was added to the other half of the reaction to confirm that the original plateau was a true saturation value.

Addition of brain nuclear RNA to a terminal RT4-D1 reaction resulted in a hybridization increase of 4.8% (Figure 1C), while a 1.5% increase was observed when brain nuclear RNA was added to a terminal B27 reaction and a 1% increase was observed when brain nuclear RNA was added to a terminal B103 reaction (data not shown). These experiments demonstrate that, within our level of detection, the sequences transcribed into nuclear RNA from cell lines are included in those found in whole brain nuclear RNA. The inclusion of neurotumor cell-line nuclear RNAs in nuclear RNA popula-

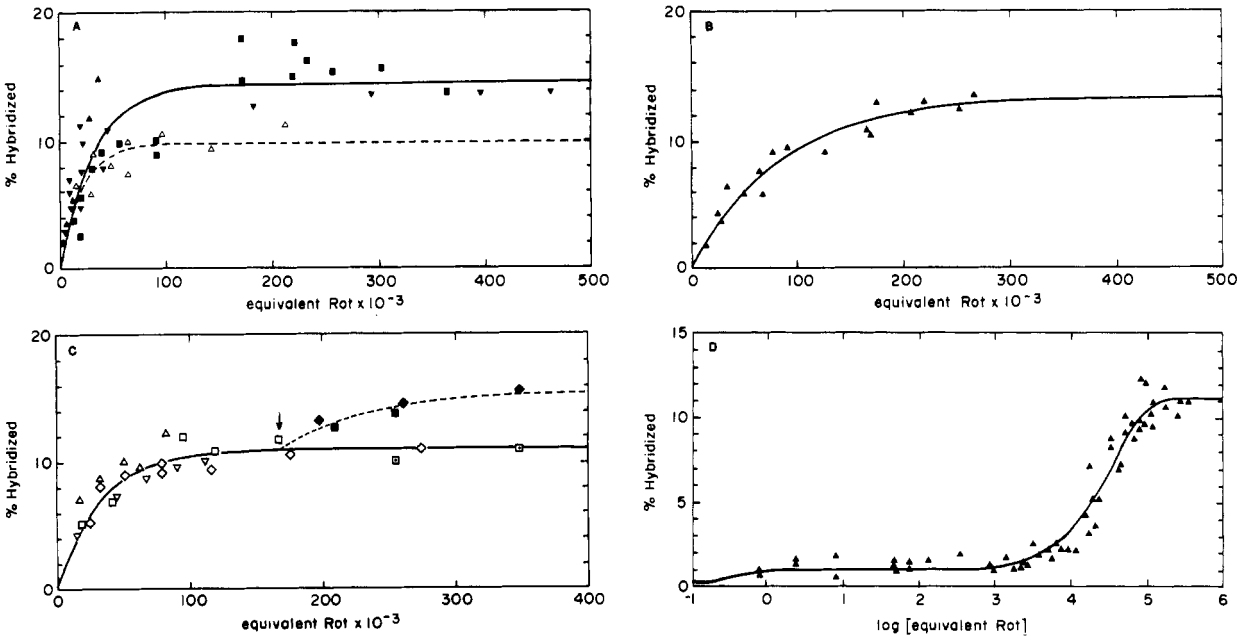

FIGURE 1: Hybridization of nuclear RNAs from cell lines. RNA was annealed to unique-sequence [^{125}I]DNA as described under Experimental Procedures. R_0t values are equivalent R_0t calculated by using the salt correction tables of Britten et al. (1974). We did not use the revised correction of Van Ness et al. (1979) to maintain consistency with previously reported data. Curves represent computer-derived best fits to the data by assuming first-order kinetics. Parameters are listed in Table I. (A) B103 nuclear RNA from three tumor (▲, ▼) and three cell-line (■) preparations was hybridized in eight separate reactions at concentrations of 2.0–47.1 mg/mL. Data points were analyzed by either S1 (▲) or HAP (▼, ■) as described under Experimental Procedures. Five DNA preparations were used. HTC nuclear RNA from two preparations was hybridized in two separate reactions at concentrations of 8.4 and 38.2 mg/mL (△). Data points were analyzed by HAP. Two DNA preparations were used. (B) B27 nuclear RNA from four preparations was hybridized in four reactions at concentrations of 8.1–22.6 mg/mL (▲). Data points were analyzed by HAP. Three DNA preparations were used. (C) RT4-D1 nuclear RNA was hybridized in four reactions at 7.6 (▽), 12.8 (□), 16.0 (△), and 16.6 (◇) mg/mL. At the R_0t indicated by the arrow, the 12.8 (reaction 1) and 16.6 mg/mL (reaction 2) reactions were split. Brain nuclear RNA was added to half of each reaction to a final concentration of 25 mg/mL for reaction 1 (■) and 30 mg/mL for reaction 2 (◆) with respect to brain nuclear RNA. RT4-D1 nuclear RNA was added to the other half of reaction 1 to a final concentration of 25 mg/mL with respect to new RT4-D1 nuclear RNA (⧈). Data points were analyzed by HAP. Three DNA preparations were used. (D) RT4-D1 nuclear RNA from four preparations was hybridized in eight separate reactions at concentrations of 0.78–16.6 mg/mL (▲). Data points were analyzed by HAP. Six DNA preparations were used. Note this is a log (R_0t) abscissa.

tion of the brain suggests that the high RNA complexities observed in these cell lines do not represent a nonspecific derepression of transcription in neoplastically transformed cells. This conclusion is further supported by the hybridization of nuclear RNA from HTC cells (Thompson et al., 1966), a liver cell line, to unique-sequence DNA (Figure 1A). This cell line has a nuclear RNA complexity close to that of normal liver [hybridizing to 9.9% of the unique-sequence DNA (Figure 1A) vs. 10.9% for liver (Chikaraishi et al., 1978)]. Furthermore, when HTC nuclear RNA is added to a terminal reaction of liver nuclear RNA with unique-sequence DNA, no increase in hybridization is observed (data not shown), indicating that, within detection, HTC nuclear RNA does not include sequences normally absent from liver nuclear RNA.

The curves shown in Figure 1 represent computer-derived best fits to the data by assuming pseudo-first-order kinetics. Our data for the nuclear RNAs are most consistent with a one-component curve. The hybridizations of B103, B27 RT4-D1, and HTC nuclear RNAs indicate average copy numbers of 0.17, 0.07, 0.15, and 0.26 copies/cell, respectively (Table I). These copy numbers are comparable to the average copy numbers of 0.20 and 0.27 copies/cell observed for brain and liver nuclear RNAs, respectively (Chikaraishi et al., 1978; recalculated in Table I). Average copy numbers cannot be rigorously compared due to the large variance in cytoplasmic RNA contamination of nuclear RNAs from different cell types and the possible change in reassociation kinetics at the high RNA concentrations used in these experiments (Van Ness et al., 1979).

To detect the possible existence of sequences of higher abundance, we assayed RT4-D1 nuclear RNA hybridizations at low R_0t values. Because of the low absolute levels of hybridization, these low R_0t samples were assayed in duplicate or triplicate. As shown in Figure 1D, this analysis shows that the RT4-D1 nuclear RNA is best fit by a two-component curve. The faster hybridizing component represents 8000 different RNA sequences. The kinetics for this component suggest an average copy number of 14 000 copies/cell. Although the data clearly indicate a faster hybridizing component, these kinetics cannot be accurate. With an RNA content of 10 pg/cell, the maximum average copy number for this class of sequences is 100 copies/cell.

Hybridization of Cytoplasmic RNAs from Cell Lines. Total cytoplasmic RNA, isolated from the postmitochondrial supernatant or polysomal RNA, isolated as puromycin-releasable polysomal RNA, was hybridized in RNA excess to unique-sequence [^{125}I]DNA. While whole brain cytoplasmic RNA hybridizes to 10% and whole brain polysomal RNA to 8% (Chikaraishi, 1979), these neural cell-line RNAs hybridized to a markedly lower degree. Total cytoplasmic RNAs from B103 and B27 cell lines hybridized to 3.1 and 4.3%, respectively, of the unique-sequence DNA while polysomal RNAs from B103 and B27 hybridized to 3.2 and 3.9% of the unique-sequence DNA (Figure 2 and Table II). In contrast, total cytoplasmic RNAs from liver and HTC cells hybridize to essentially the same degree. Total cytoplasmic RNA from liver hybridizes to 2.4% of the unique-sequence DNA (Chikaraishi, 1979) as compared to total cytoplasmic RNA from HTC cells which hybridized to 2.6% of the unique-sequence DNA. These levels of total cytoplasmic RNA hybridization correspond to complexities of 1.1×10^8, 1.6×10^8, and 9.5×10^7 nucleotides for B103, B27, and HTC cell lines, re-

Table I: Analysis of Nuclear RNA from Neurotumor and HTC Cell Lines

cell type	origin of cell	component	complexity: % hybridization ± SD[c]	complexity: nucleotides[d]	no. of different 4500-nucleotide sequences[e]	$R_0t_{1/2}$[f]	K_{obsd}[f]	K_{expd}[g]	fraction of RNA driving reaction (K_{obsd}/K_{expd})	av copy no./cell[h]
brain[a]		total	15.6 ± 0.5	5.7×10^8	1.3×10^5	2.1×10^4	3.3×10^{-5}	1.2×10^{-3}	0.027	0.20
B103	CNS[b]	total	14.5 ± 2.4	5.3×10^8	1.2×10^5	2.2×10^4	3.1×10^{-5}	1.3×10^{-3}	0.024	0.17
B27	CNS	total	13.5 ± 1.0	4.9×10^8	1.1×10^5	6.0×10^4	1.2×10^{-5}	1.4×10^{-3}	0.009	0.07
RT4-D1	PN[b]	total	11.1 ± 0.9	4.0×10^8	9.0×10^4					
		fast	1.0	3.6×10^7	8.1×10^3	2.7×10^{-1}	2.6	1.9×10^{-2}	136	1.38×10^4
		slow	10.1	3.7×10^8	8.2×10^4	2.6×10^4	2.7×10^{-5}	1.9×10^{-3}	0.015	0.15
liver[a]		total	10.9 ± 0.6	4.0×10^8	8.8×10^4	5.3×10^4	1.3×10^{-5}	1.7×10^{-3}	0.008	0.27
HTC	liver	total	9.9 ± 1.5	3.6×10^8	8.0×10^4	1.4×10^4	4.9×10^{-5}	1.9×10^{-3}	0.026	0.26

[a] Data from Chikaraishi et al. (1978). [b] CNS = central nervous system; PN = peripheral neurocell. [c] SD = root mean standard deviation calculated from least-squares analysis from a best-fit first-order equation. [d] Assumes that the rat genome is 2.8×10^9 nucleotides; 65% is unique sequence (McConaughy & McCarthy, 1970; Holmes & Bonner, 1974); assumes asymmetric transcription of DNA. [e] This assumes that the average hnRNA is 4500 bases long (Bantle & Hahn, 1976), which was measured as the number-average length of mouse brain poly(A)+ nuclear RNA and hence reflects the size of the abundant RNA molecules rather than the low abundance, complex class of RNAs. [f] $K_{obsd} = K_{observed}$ (liters per mole per second), calculated by least squares and best fit to a first-order equation from the data in Figure 1 by using a 9845B Hewlett-Packard desktop counter. $R_0t_{1/2} = \ln(2/K_{obsd})$. [g] $K_{expd} = K_{expected}$ (liters per mole per second), calculated by using a pure 2000-nucleotide sequence having a K_{expd} of 8.6×10^2 mol^{-1} s^{-1} L (Hastie & Bishop, 1976) as the standard and corrected by $f = 2.5$ for the rate reduction due to length disparity between tracer (475 nucleotides) and driver (3000 nucleotides) (Chamberlin et al., 1978). $K_{expd} = 2000(8.6 \times 10^2)/[2.5(\text{nucleotide complexity})]$. [h] Assumes that the mass of total RNA per cell is ~10 pg for cell lines, 7.6 pg for brain, and 25 pg for liver (Mandell et al., 1964; Winick & Nobel, 1965); 30% of the total RNA is isolated as nuclear RNA for tissues, and 20% is isolated as nuclear RNA for cell lines (see difference in procedures); the molecular weight of an RNA nucleotide is 330. Copy number per cell = [total amount RNA (g)/cell](fraction of RNA in nucleus)(fraction of RNA driving hybridization)/[[complexity of RNA (nucleotides)] × $330/(6.02 \times 10^{23})$].

Table II: Analysis of Total Cytoplasmic and Polysomal RNAs from Neurotumor and HTC Cell Lines

cell type	origin of cell	complexity: % hybridization ± SD[c]	complexity: nucleotides[d]	no. of different 1500-nucleotide sequences	$R_0t_{1/2}$[e]	K_{obsd}[e]	K_{expd}[f]	fraction of RNA driving reaction (K_{obsd}/K_{expd})	av copy no./cell[g]
			Total Cytoplasmic RNA						
brain[a]		10.0	3.6×10^8	2.4×10^5	6.3×10^4	1.1×10^{-5}	2.7×10^{-3}	0.0042	0.15
B103	CNS[b]	3.1 ± 0.6	1.1×10^8	7.5×10^4	7.6×10^3	9.1×10^{-5}	8.7×10^{-3}	0.0104	1.55
B27	CNS	4.3 ± 1.0	1.6×10^8	1.0×10^5	2.2×10^4	3.1×10^{-5}	6.0×10^{-3}	0.0052	0.53
liver[a]		2.4	8.7×10^7	5.8×10^4	2.3×10^4	3.0×10^{-5}	1.1×10^{-2}	0.0027	1.27
HTC	liver	2.6 ± 0.3	9.5×10^7	6.3×10^4	1.0×10^4	6.7×10^{-5}	1.0×10^{-2}	0.0066	1.14
			Polysomal RNA						
brain		8.0	2.9×10^8	1.9×10^5	4.6×10^4	1.5×10^{-5}	3.3×10^{-3}	0.0045	0.097
B103	CNS	3.2 ± 0.8	1.2×10^8	7.8×10^4	3.1×10^4	2.3×10^{-5}	8.0×10^{-3}	0.0028	0.19
B27	CNS	3.9 ± 0.7	1.4×10^8	9.5×10^4	2.3×10^4	3.0×10^{-5}	6.8×10^{-3}	0.0044	0.26

[a] Data from Chikaraishi (1979). [b-d] See corresponding footnotes in Table I. [e] Calculated from Figure 2 as described in Table I. [f] Same as Table I, footnote *g*, except driver = 1500 nucleotides and *f* therefore = 1.8. $K_{expd} = (2000)(8.6 \times 10^2)/[1.8(\text{nucleotide complexity})]$. [g] Assumes that the mass of total RNA per cell and the molecular weight of RNA nucleotide are as described in Table I, footnote *h*; 90% of total RNA is cytoplasmic RNA; 50% of cytoplasmic RNA is polysomal RNA. Copy number per cell = [total amount RNA (g)/cell](fraction of RNA in cytoplasm)(fraction of cytoplasmic RNA in this component)(fraction of RNA driving hybridization)/[[complexity of RNA (nucleotides)]330/(6.02×10^{23})].

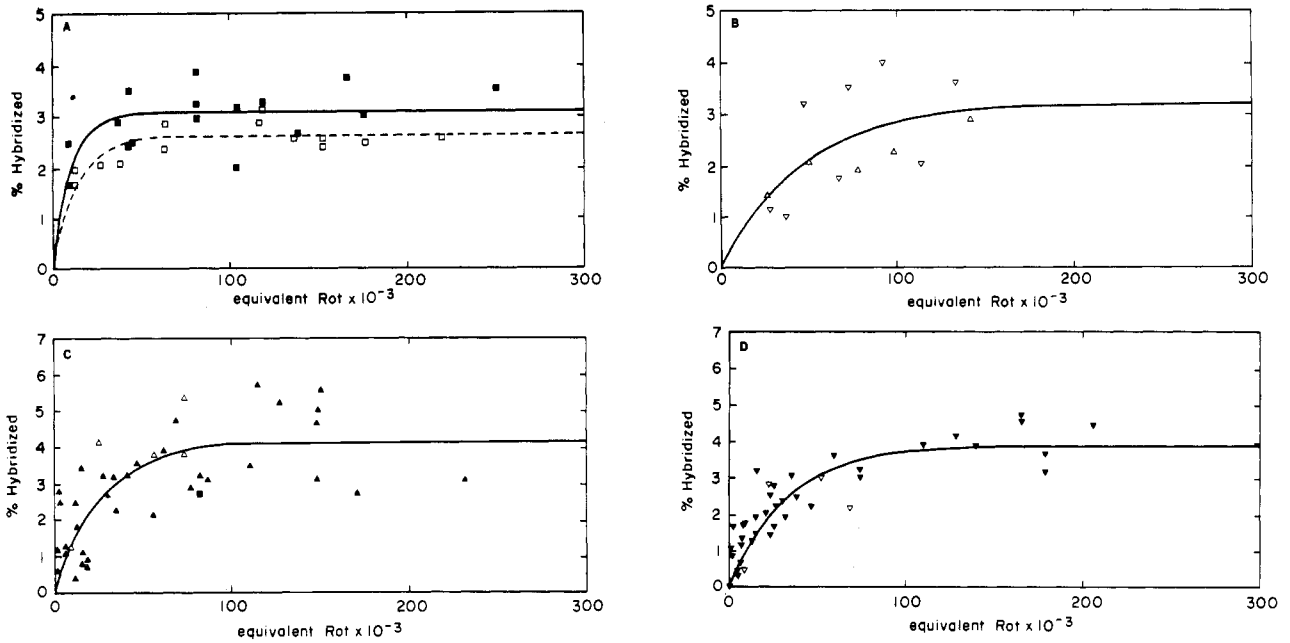

FIGURE 2: Hybridization of total cytoplasmic and polysomal RNAs from cell lines. RNA was annealed and data were analyzed as described in Figure 1. Parameters for curves are listed in Table II. (A) B103 total cytoplasmic RNA from one cell-line preparation was hybridized in three reactions at concentrations of 11.5, 21.4, and 22.1 mg/mL (■). HTC total cytoplasmic RNA from one preparation was hybridized in two reactions at concentrations of 17.0 and 23.9 mg/mL (□). Data points were analyzed by HAP. Two DNA probes were used in each case. (B) B103 polysomal RNA from one cell-line (△) and one tumor (▽) preparation was hybridized in three reactions at 5.9, 6.3, and 18.0 mg/mL. Data points were analyzed by HAP. Two DNA preparations were used. (C) B27 total cytoplasmic RNA from two preparations was hybridized in seven reactions at concentrations of 1.8–32.4 mg/mL. Data points were analyzed by S1 (△) or HAP (▲). Five DNA preparations were used. (D) B27 polysomal RNA from three preparations was hybridized in seven reactions at concentrations of 0.8–19.9 mg/mL. Data points were analyzed by S1 (▽) or HAP (▼). Four DNA preparations were used.

Table III: RNA Complexity of Rat Brain Sections

	nuclear RNA		poly(A)+ cytoplasmic RNA	
section	% hybridization[a]	complexity (nucleotides)[b]	% hybridization[c]	complexity (nucleotides)
anterior cerebral cortex	16.3	5.9×10^8	3.8	1.4×10^8
parietal cerebral cortex	16.2	5.9×10^8	3.6	1.3×10^8
occipital cerebral cortex	14.7	5.4×10^8	5.05	1.8×10^8
regions underlying cortex	15.6	5.7×10^8	5.05	1.8×10^8
cerebellum	15.1	5.5×10^8	2.8	1.0×10^8
whole brain	15.6	5.7×10^8	5.05	1.8×10^8

[a] Two preparations of each nuclear RNA were hybridized to a maximal R_0t of 120 000 for occipital cortex, cerebellum, and regions underlying the cortex and a maximal R_0t of 200 000 for all other sections. RNA/DNA ratios were 7000–14 000:1. Data points were analyzed by S1 analysis. The percent hybridization was calculated by a computer-derived best fit to the data assuming first-order kinetics. Values are corrected for a probe reactivity of 95%. [b] Complexity calculated as described in Table I. [c] Polyadenylated cytoplasmic RNA from each section was hybridized to a maximal R_0t of 6000 at RNA/DNA ratios of 500–750:1. These R_0t's are equivalent to an R_0t of 300 000 for total cytoplasmic RNA since only 2% of cytoplasmic RNA is polyadenylated. Data points were analyzed and the percent hybridization was calculated as described in footnote *a*.

spectively. This is enough information to encode 75 000, 100 000, and 63 000 different 1500-nucleotide messages.

The ratio of total cytoplasmic to nuclear RNA complexity is 0.21, 0.32, and 0.26 for B103, B27 and HTC cell lines, respectively. These values are similar to the values observed for organs such as kidney (0.34) and liver (0.23) which are composed of fewer cell types than brain. However, this ratio is 0.64 for the whole brain, supporting the idea that brain cytoplasmic RNA complexity represents the summed complexity of many different cell types, each utilizing as mRNA between one-fourth and one-third of the sequences present in the nucleus.

The average copy number of a cytoplasmic RNA sequence is ~0.15 copies/cell for whole brain compared to about 1.55 and 0.53 copies/cell for B103 and B27 neurotumor cell lines (Table II). This increase is consistent with the hypothesis that a given cytoplasmic RNA sequence is expressed in only a subset of the brain cells. Therefore, its average copy number in total brain is decreased due to dilution by cells that lack that given sequence. No significant difference is observed between the average copy number per cell for liver (1.27) and HTC cells (1.14).

Hybridization of RNAs Isolated from Brain Sections. Adult rat brains were dissected into five regions: the cerebellum, the anterior, parietal and occipital cerebral cortex, and the region underlying the cortex but not including the midbrain and stem. The nuclear RNA prepared from each region was hybridized in RNA excess to a trace amount of unique-sequence [^{125}I]DNA under conditions where no self-annealing of the [^{125}I]DNA probe occurred (Chikaraishi et al., 1978). Table III shows the results of these hybridizations and, for comparison, the hybridization of nuclear RNA from total brain. At saturation, each of the brain section nuclear RNAs anneals to approximately the same extent as total brain nuclear RNA. The saturation values of the brain sections range from 14.7 to 16.3% while that of whole brain is 15.6%. If asym-

metric transcription is assumed, these values represent a base sequence complexity of between 5.4×10^8 and 5.9×10^8 nucleotides for the brain sections as compared to $\sim 5.7 \times 10^8$ nucleotides for whole brain. Considering the fact that saturation values for various preparations of whole brain RNA can vary by as much as several percent (Chikaraishi et al., 1978), it is unlikely that the small differences seen among the various brain sections are significant. We therefore conclude that nuclear RNA from each section of the brain includes essentially all of those sequences found in nuclear RNA isolated from whole brain.

This is not, however, the case for poly(A)+ cytoplasmic RNA isolated from these brain sections. As shown in Table III, poly(A)+ cytoplasmic RNA isolated from whole brain, the occipital third of the cortex, and the region underlying the entire cortex saturated 5.1% of the unique-sequence [^{125}I]-DNA, while the anterior and parietal cortical regions had slightly lower hybridizations of 3.8 and 3.6%, respectively. The cerebellum had the lowest RNA diversity, hybridizing to only 2.8% of the unique-sequence DNA. This represents a range of complexities of between 1.0×10^8 and 1.8×10^8 nucleotides by assuming asymmetric transcription or between 67 000 and 120 000 different 1500-nucleotide sequences. This range of values indicates that the same poly(A)+ cytoplasmic RNA sequences are not expressed throughout the brain despite the consistency in the pattern of nuclear RNA representation. Therefore, these data support the hypothesis that the cytoplasmic RNA expression observed for whole brain represents a composite of different RNA sequence sets being expressed in the different cell types of the brain, with enough regional differences in cell types to cause these regional complexity differences.

Discussion

Nuclear RNA. We have measured the base sequence complexity of RNAs isolated from both neurotumor cell lines and brain sections by saturation hybridization to a high specific activity iodinated unique-sequence DNA probe. These studies have demonstrated that the majority of sequences transcribed into the nuclear RNA of whole brain are transcribed into the nuclear RNA both of clonal neurotumor cell lines and brain sections.

Whereas brain nuclear RNA hybridized to 15.6% of the unique-sequence genome (Chikaraishi et al., 1978), nuclear RNAs isolated from five brain sections hybridized to from 14.7 to 16.3% of the unique-sequence genome. Within the experimental error of this system, these values cannot be distinguished from the value obtained for brain. In the sheep, the nuclear RNA complexity of the same sections used in these studies has been analyzed (S. Deeb, personal communication). Sheep brain sections hybridize to between 18 and 19% of the unique-sequence DNA compared to 20% for whole sheep brain.

The cell lines used in these studies were isolated from nitrosoethylurea-induced tumors. B103 and B27 were isolated by Schubert et al. (1974) from separate tumors of the central nervous system. B103 exhibits properties characteristic of a differentiated neuronal cell such as an excitable membrane, α-neurotoxin binding (indicates acetylcholine receptors), inducible neuron morphology, neuronal-specific enzyme activities (i.e., glutamic acid decarboxylase), and low S100 protein (glial-specific) and high 14-3-2 protein (neuronal-specific) levels. B27, for the most part, exhibits properties typical of glial cells such as a lack of inducible neuron morphology, a nonexcitable membrane, and low, though detectable, 14-3-2 protein levels. However, B27 does not exhibit the increased S100 production found in some normal glial cells (Schubert et al., 1974).

The clonal cell line RT4-D1 was isolated as a glial subline of a peripheral nervous system tumor by Imada & Sueoka (1978). This tumor included pluripotent stem cell (RT4-AC) and stably differentiated neuronal (RT4-B and -E) and glial (RT4-D) cell types. These stably differentiated cell types also arise in culture from the stem cell. The clonal cell line used in this study was originally isolated as a clonal cell line of D-type cells from this tumor. RT4-D cell lines exhibit some properties found in glial and not in neuronal cells such as high S100 protein production (Imada & Sueoka, 1978) and the absence of voltage-dependent Na^+ influx (Tomozawa & Sueoka, 1978).

Nuclear RNA isolated from B103 and B27 cell lines is very close in nucleotide complexity to that of total brain, hybridizing to 14.5 and 13.5% of the unique-sequence DNA while nuclear RNA from RT4-D1 hybridized to 11.1% of the unique-sequence DNA. The central nervous system cell lines do not include from 1 to 1.5% of the unique-sequence DNA represented in brain while the peripheral neural cell line does not include 5% of the sequences found in brain. The cause of these differences has not been determined. They could be due to the neuronal vs. glial nature of the cell lines being used or due to the derivation from central vs. peripheral nervous systems.

The difference we see between nervous system cell lines and brain nuclear RNAs is similar to that seen by Kaplan et al. (1978) using poly(A)+ total RNA, although they consistently see a greater difference. This variance in results could be due to the use of nuclear vs. poly(A)+ total RNA. They find that the poly(A)+ total RNA from B104 neuroblastoma (Schubert et al., 1974) represents ~75%, C6 glimoa (deVillis & Brooker, 1973) represents ~80%, and primary glial cultures isolated from newborn rats represent ~61% of the sequences found in brain. In comparison, we found that B103 represents ~93%, B27 ~87%, and RT4-D1 ~71% of those sequences found in nuclear RNA from brain. On the average, neuronal cell lines represent 84% of the brain sequences and glial 75%, a difference of 9%, whose significance is debatable in view of the range of data values and variance in the derivation of cells being used.

In the case of the RT4-D1 cell line, we have clearly demonstrated a highly abundant component representing ~10% of the sequences expressed in this cell line. Abundant nuclear RNA sequences representing approximately the same fraction of the nuclear RNA complexity are observed in other systems, i.e., rat brain (Grouse et al., 1978), rat liver (Sippel et al., 1977; Reiners & Busch, 1980), AKR mouse embryo cells (Siegal et al., 1979), and mouse Friend cells (Getz et al., 1975; Mauron & Spohr, 1978). In many cases, abundant cytoplasmic RNAs are derived from abundant nuclear RNAs, though the relative nuclear abundance is generally lower than the cytoplasmic abundance (Sippel et al., 1977; Mauron & Spohr, 1978; Siegal et al., 1979; Reiners & Busch, 1980). This has been specifically shown for chick ovalbumin (Roop et al., 1978) and for heavy and light immunoglobulin chain RNAs (Schibler et al., 1978).

That the high nuclear RNA complexity of cell lines is not an artifact of transformation is supported by the observation that the nuclear RNA complexity of HTC cells is about the same as that of normal liver and the observation that the sequences appearing in the nuclear RNA in both neurotumor and HTC cell lines are included in the set of sequences normally appearing in their parental organs (Figure 1). Moyzis et al. (1980) have come to the same conclusions in a careful

study of the nuclear and poly(A)+ polysomal RNA complexities in normal and neoplastically transformed syrian hamster embryo cells, as have Supowit & Rosen (1980) in their analysis of poly(A)+ total RNA from normal and neoplastic mammary tissue. In contrast to these results, when the mouse cell lines AL/N and Balb 3T3 were compared to their virus-transformed derivatives, a 1.5-fold increase in total RNA complexity was observed (Grady & Campbell, 1973), and a 3-fold decrease in nuclear RNA complexity was observed in 12th-passage hepatoma cells compared to liver (Shearer, 1977).

Cytoplasmic and Polysomal RNAs. In contrast to the nuclear RNA, the complexity of cytoplasmic RNAs of either cell lines or some brain sections was significantly lower than the complexity of homologous RNA fractions isolated from the whole brain. Whereas brain poly(A)+ cytoplasmic RNA hybridizes to 5.1% of the unique-sequence DNA (Chikaraishi, 1979), poly(A)+ cytoplasmic RNA from brain sections hybridized to 2.8, 3.6, 3.8, 5.1, and 5.1% of the unique-sequence DNA for cerebellum, parietal cerebral cortex, anterior cerebral cortex, occipital cerebral cortex, and regions underlying the cortex, respectively. We feel that the decrease observed for cerebellum is particularly significant and may reflect the simple cellular organization and function of the cerebellum as compared to the cerebrum. That some of these sections have poly(A)+ cytoplasmic RNA complexities equal to that of whole brain may simply reflect the fact that within each of these brain sections there still is a vast diversity of cell types.

The difference between cell-line and whole brain cytoplasmic RNA complexities is much more pronounced. Whereas total cytoplasmic RNA from whole brain hybridizes to 10% of the unique-sequence DNA and polysomal RNA to 8% (Chikaraishi, 1979), B103 total cytoplasmic RNA hybridized to 3.1%, B103 polysomal to 3.2%, B27 total cytoplasmic to 4.3%, and B27 polysomal to 3.9% of the unique-sequence DNA. Although 64% of the nuclear RNA sequences appear in the cytoplasm of the brain, only ~26% appear in the cytoplasm of these neurotumor cell lines. The appearance of a similar percentage of the nuclear RNA in cytoplasmic RNA has been observed in kidney, liver (Chikaraishi et al., 1978; Chikaraishi, 1979), and HTC cells (Tables I and II).

We conclude that only one-third or less of the nuclear RNA sequences of a given cell appears in the cytoplasm of that cell and that the high cytoplasmic RNA complexity observed for rat brain is due to the summation of nonoverlapping sets of cytoplasmic RNA sequences originating from different cell types. Since the brain is undoubtedly the most complex of all organs in terms of its cellular organization and function, it is not surprising that various neural cell types might make different proteins to meet their particular needs. Since the nuclear RNA of clonal cell lines and different brain sections is so similar and overlapping, it appears that much of the regulation for cell-specific products may be posttranscriptional.

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Chemical Modifications of Atractyloside and Bongkrekic Acid Binding Sites of the Mitochondrial Adenine Nucleotide Carrier. Are There Distinct Binding Sites?†

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ABSTRACT: The nature of the binding sites for two specific inhibitory ligands of the ADP/ATP carrier in beef heart mitochondria has been investigated by means of chemical modifications with 2-hydroxy-5-nitrobenzyl bromide (HNB), a rather selective reagent for tryptophanyl residues, and 2,3-butanedione and phenylglyoxal, two reagents which modify arginyl residues. Atractyloside binding, but not bongkrekic acid binding, was rapidly inactivated by HNB. Atractyloside binding was also rapidly inactivated by butanedione and phenylglyoxal whereas bongkrekic acid binding was only slowly inactivated by these reagents. In all cases, inactivation decreased the number of high affinity binding sites without significant modification of the K_d value of the remaining sites; furthermore, specific protection of atractyloside or bongkrekic acid binding was afforded by preincubation with the homologous ligand. Inhibition of atractyloside binding by HNB was accompanied by the binding of HNB to the ADP/ATP carrier protein, as shown by examination of the spectral properties of the purified protein; protection against HNB inhibition by preincubation of mitochondria with atractyloside was correlated with a decrease in the amount of bound HNB. The sulfonium salt of HNB did not interfere with atractyloside binding. As HNB is a penetrable reagent in contrast to its sulfonium salt, the chemical reactivities of the two compounds being similar, it is inferred that the amino acid residues modified by HNB are located in the hydrophobic region of the ADP/ATP carrier molecule. Both atractyloside and bongkrekic acid bindings were inhibited by phenylglyoxal and butanedione, but the binding of atractyloside was inactivated at least 3 times faster than that of bongkrekic acid by these reagents. The reaction order with respect to phenylglyoxal concentration was 1 for inactivation of atractyloside binding and 2 for inactivation of the bongkrekic acid binding. Inactivation of atractyloside and bongkrekic acid binding by phenylglyoxal was studied as a function of the specific binding of [^{14}C]phenylglyoxal, sensitive to atractyloside and bongkrekic acid, respectively. Complete inactivation of atractyloside binding required the incorporation of 1 mol of [^{14}C]phenylglyoxal per mol of carrier dimer (M_r 60000), indicating a mechanism of half-site reactivity for the atractyloside site of the ADP/ATP carrier. Full inactivation of bongkrekic acid binding required at least twice as much phenylglyoxal. The data are compatible with the hypothesis that the ADP/ATP carrier protein is an asymmetric protein spanning the inner mitochondrial membrane, its asymmetry being reflected by distinct preexisting binding sites for atractyloside and bongkrekic acid.

The mitochondrial ADP/ATP carrier is specifically recognized and inhibited by two natural inhibitors, atractyloside and bongkrekic acid. These two inhibitors are characterized by a number of puzzling binding properties. They both bind specifically to the ADP/ATP carrier protein. They compete with each other for binding; yet, atractyloside is a nonpenetrant inhibitor which inhibits ADP/ATP transport when externally added to mitochondria whereas bongkrekic acid inhibits ADP/ATP transport only under conditions which favor its penetration into the matrix space of mitochondria (for review, cf. Vignais, 1976). In inside-out submitochondrial particles, the reverse situation holds (Lauquin et al., 1977), indicating that the binding asymmetry of the ADP/ATP carrier with respect to atractyloside and bongkrekic acid is not really due to the different permeability of the mitochondrial membrane to the two inhibitors but is inherent to the topography or the functioning of the carrier. Two hypotheses have been formulated to take into account the binding properties of atractyloside and bongkrekic acid. In the first case (Block et al., 1979), it is assumed that the carrier protein spans the membrane and possesses a binding site for atractyloside on the outer (cytosolic) face and another one for bongkrekic acid on the inner (matrix) face; the interaction between the two sites would then be indirect. In the second alternative (Klingenberg, 1976), it is supposed that atractyloside and

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