*RELATIONSHIP BETWEEN NUCLEAR GIANT-SIZE dRNA AND MICROSOMAL dRNA OF RAT BRAIN**

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Abstract.—(1) Among the DNA-like RNA (dRNA) species of rat brain, 35 per cent remain nuclear, whereas 65 per cent are found in the microsomes and may represent functional messenger RNA. It was estimated that 30,000–300,000 cistrons would thus code for messenger RNA in rat brain.

(2) No nuclear-specific dRNA with sedimentation characteristics similar to microsomal dRNA are detected.

(3) The nuclear large dRNA contain all the dRNA species of the brain.

(4) The presence of microsomal dRNA precursors and of nuclear restricted dRNA in large dRNA suggests the existence of a limited cleavage process and of a selection among the fragments for their transport from the nucleus to the cytoplasm. Such a selective mechanism could represent an important regulation step in the transfer of genetic information.

Introduction.—Rapidly labeled, large RNA with a base composition close to that of DNA was described in tissues with a low[1–4] or high mitotic index.[5–11] The large dRNA was found in the nucleus, whereas the cytoplasmic dRNA sedimented usually between 6 and 18*S*.[1, 3, 7–10, 12] However, large dRNA was also described in a microsomal fraction of rabbit brain.[13]

The nature and role of the large dRNA are unknown. In cells like erythrocytes,[1, 3, 4] synthesizing mainly hemoglobin, most of the messenger RNA should be found at 8–10*S*. The high amount of large dRNA found could not be explained by a requirement for the synthesis of high-molecular-weight protein. It was shown that the newly synthesized nuclear RNA had a very short half life compared to cytoplasmic RNA and that only a small fraction of the total RNA was found in the cytoplasm.[3, 4] Similar observations were made by Harris and Watts[14] for HeLa cells. Scherrer and co-workers[3] formulated several hypotheses to explain the observed facts. They postulated, for instance, that some large RNA could be nonfunctional, being representative of former stages in the evolution of the cell. An alternative hypothesis was that the hemoglobin messenger RNA could constitute the part of the larger molecule that is transferred to the cytoplasm. However, the experiments did not show a relation between the nuclear RNA species and those found with the polysomes.

Shearer and McCarthy,[15] working with L cells, demonstrated with hybridization techniques that 80 per cent of the dRNA species were never found in the cytoplasm. Competition experiments also ascertained the existence of RNA species restricted to the nucleus in normal or regenerating rat liver.[16] But neither the size nor the physiological significance of these nuclear-specific dRNA's were known. It would also be of interest to know whether the dRNA species

transferred to the cytoplasm are of the same size as their nuclear precursors or the product of partial degradation of a high-molecular-weight precursor.

Working with brain RNA's, we have tried to answer the following questions: (*a*) Is there any relationship between large dRNA and cytoplasmic messenger RNA and, if so, what is its nature? (*b*) Do nuclear-specific dRNA's occur in brain and, if they do, are they large or not?

As will be demonstrated, large dRNA's contain both nuclear-specific and cytoplasmic RNA species.

Materials and Methods.—1–2 mc of carrier-free $^{32}PO_4H_3$ (C.E.A., Saclay, France) neutralized and diluted in 0.14 *M* NaCl were injected intracisternally into rats. The animals were decapitated 15–17 hr later. RNA, either from lyophilized brain for total RNA or from a microsomal brain fraction for cytoplasmic RNA, was extracted by the sodium dodecyl sulfate (SDS) hot phenol method, deproteinized, and precipitated by ethanol as previously described.[17]

About 5 mg of total brain RNA was centrifuged (24,000 rpm, 14 hr, 2–3°) in a linear sucrose density gradient (4–20% sucrose on 10 mM Tris-HCl, pH 7.4, 0.1 *M* NaCl) in a SW 25.2 rotor of a Spinco centrifuge. 2-ml fractions were collected and aliquots were used for the determination of E_{260} mμ and of acid-insoluble radioactivity. Sedimentation coefficients were estimated according to Martin and Ames,[18] assuming that ribosomal RNA's sediment at 18 and 28*S*. The fractions corresponding to the following regions were pooled: region I, 50–32*S;* region II, 32–22*S;* region III, 22–6*S* (Fig. 1, *left*). The RNA's of each region were reprecipitated with ethanol and dissolved in 10 mM Tris-HCl, pH 7.4, 0.1 *M* NaCl, 5 mM $MgCl_2$. Contaminating DNA was eliminated by DNase (electrophoretically pure, Worthington) at 37° for 10 min at 2, 5, and 10 μg/ml, respec-

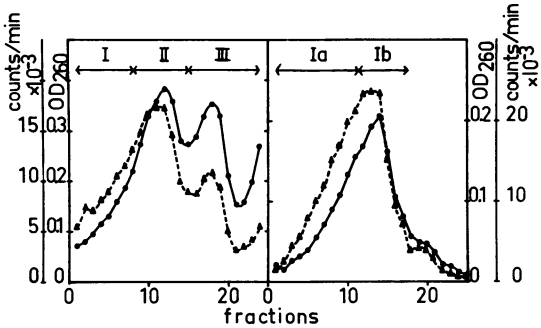

FIG. 1.—Sedimentation of total and giant-size RNA in sucrose density gradient. (*Left*) RNA was extracted from the brains of six rats by phenol at 60°, in the presence of 0.25% SDS. After deproteinization and precipitation with ethanol, the RNA solution was layered on a 4–20% sucrose linear gradient. Centrifugation was at 24,000 rpm (69,500 *g*) for 14 hr at 1–3° in a SW 25–2 swinging bucket rotor. 2-ml fractions were collected; E_{260} mμ (●—●) and acid-insoluble radioactivity (▲- - -▲) were determined on aliquots of each fraction. The 18*S* ribosomal RNA peak (fraction III) is contaminated with a little DNA that will be subsequently removed by DNase (see *Materials and Methods*). Fractions *I*, *II*, and *III* were pooled as indicated.

(*Right*) The RNA's of fraction *I* from three similar gradients were collected, pooled, and precipitated with ethanol. They were layered on a 4–20% sucrose. Centrifugation was at 19,500 rpm (38,600 *g*) for 14.5 hr at 1–3° in a SW 25–1 swinging bucket rotor. Fractions of 1 ml were collected; E_{260} mμ (●—●) and acid-insoluble radioactivity (▲- - -▲) were determined on aliquots. Fractions *Ia* and *Ib* were pooled as indicated.

tively, for regions I, II, and III. DNase was eliminated by treatment with phenol and a mixture of chloroform-isoamyl alcohol (4:1 v/v). After ethanolic precipitation, the RNA was redissolved in 10 mM Tris-HCl, pH 7.4, 0.1 *M* NaCl, and contaminating acid-soluble products, soluble RNA, and oligodeoxynucleotides resulting from DNase action were eliminated by passage through a column of Sephadex G 200[17] (Pharmacia, Uppsala, Sweden).

Preparations of microsomal RNA were also submitted to the Sephadex filtration. The necessity of this treatment of RNA before hybridization experiments has been demonstrated previously.[17]

The specific activity of the α-P of the free nucleotide pool was measured only on AMP, since 16 hr after the injection the isotopic equilibration of the nucleotide pool is reached in brain. The technique of extraction and chromatographic separation has already been described.[17]

Hybridizations were carried out according to Gillespie and Spiegelman.[19] The optimal incubation conditions for brain RNA were 67°, 16 hr in 4 SSC (0.6 *M* NaCl, 0.06 *M* sodium citrate).[17]

Results.—(a) *Preliminary remarks and experimental procedures:* We are considering here only RNA's with a base composition close to that of DNA; the messenger RNA's probably belong to this group. In previous experiments we had determined that total dRNA's were complementary to 1.2–1.4 per cent of the DNA.[14] Since the same experimental procedure has been used here, we shall recall briefly the main steps of this determination:

Radioactive RNA is required in order to detect RNA fixed on DNA after hybridization. If the specific activity of this RNA is known, the quantity of RNA complementary to DNA can be calculated. The specific activity of dRNA cannot usually be estimated by a direct method, since the preparations are contaminated with ribosomal RNA. An indirect method of calculation can be used. It is based on the following assumption: that the specific activity of the P of dRNA is identical to that of the α-P of the nucleoside triphosphates when isotopic equilibrium of the nucleotide pool is reached. The validity of this assumption for adult brain after intracisternal injection of labeled phosphates has been demonstrated.[17] The specific activity of the various fractions of dRNA studied in the present work has been calculated according to that of acid-soluble AMP. The labeling time was 15–16 hr. It can be assumed that all species of dRNA will be uniformly labeled, since they have been synthesized from a precursor pool isotopically equilibrated for several hours.

The radioactivity due to ribosomal RNA which is present in all the fractions must be subtracted from the total hybridized radioactivity before the quantity of hybridized dRNA can be estimated. The saturation of DNA by ribosomal RNA is reached at a relatively low RNA/DNA input. Under the conditions of our experiments, the hybridization level of ribosomal RNA reaches a plateau which corresponds to 0.15 per cent of the DNA. Once the specific activity of ribosomal RNA is known, the corresponding radioactivity fixed on DNA can be calculated. It amounts to less than 5 per cent of the total hybridized radioactivity in our experiments.

It must be emphasized here again that dRNA species whose production in the cell is low compared to that of the bulk dRNA species are probably not detected in our experiments. The most abundant species would indeed have saturated

the DNA before any significant involvement of the lesser species. The various fractions of the genome determined in the present work are therefore to be considered as minimal and relate to the relatively abundant dRNA species.

(b) *Saturation of DNA by microsomal RNA:* When microsomal RNA labeled with P^{32} for 15 hours is analyzed in sucrose density gradients, the profile of radioactivity is superimposable on that of the optical density. The radioactivity incorporated into dRNA is completely masked by the important labeling of rRNA. It can, however, be assumed that microsomal dRNA has the characteristics that were determined after shorter pulses[12] and that it sediments between 6 and 30*S* with a maximum at 10–15*S*.

Saturation curves were established with three different RNA preparations (Fig. 2). Saturation is reached when the RNA concentration in the medium is

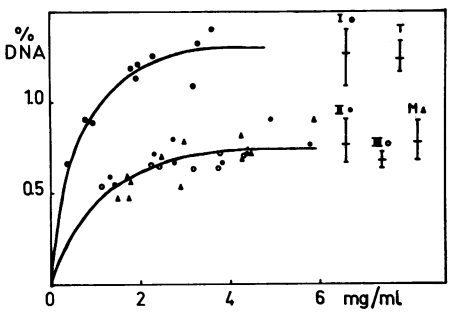

FIG. 2.—Saturation of DNA with RNA's of fractions, *I*, *II*, *III* and with microsomal RNA's. Hybridization was carried out under standard conditions (see *Materials and Methods*). 5–7 μg of DNA were hybridized with various concentrations of RNA of fractions *I* (◓), *II* (●), *III* (○), and microsomes (*M*▲).

According to the experiment, the hybridized radioactivity corresponded at saturation to 600–1500 cpm for RNA of fractions *I*, *II*, and *III* and 400–600 cpm for microsomal RNA. The quantity of RNA for 100 μg of DNA was calculated from these experimental data. The extreme and average values at saturation are represented. For comparison these same values are given for the totality of brain dRNA (*T*). Saturation was estimated to occur for RNA concentrations in the incubation medium of 2.0 mg/ml for fraction *I* and *T* and of 3.5 mg/ml for fractions *II*, *III*, and *M*.

about 3–4 mg/ml. This is about twice what was required for the saturation of DNA by total dRNA[17] and testifies to a low dRNA/rRNA ratio in the microsomes. The saturation plateau corresponds to 0.78 per cent of the DNA (extreme values 0.68–0.90%). For total dRNA, the saturation level is 1.24 per cent of the DNA; thus one can estimate that 0.46 per cent of the DNA codes for nuclear-specific RNA (nsRNA) and that about 65 per cent of the total dRNA species are found in the microsomes.

(c) *Saturation of DNA with dRNA fractions isolated according to their sedimentation coefficients:* The presence of RNA with a sedimentation coefficient higher than 28*S* is detected, even after a labeling time of 15 hours, by a slight displacement toward the bottom of the tube of the radioactivity profile in the 28*S* region. In the other parts of the gradients, the profiles of radioactivity and optical density are coincident, as was the case with microsomal RNA.

The possibility that the RNA's with a high sedimentation coefficient represent aggregates of smaller molecules rather than large RNA is suggested by experiments such as those of Hayes, Hayes, and Guérin;[20] Wagner, Katz, and Pen-

man;[21] Burdon;[22] or Bramwell and Harris.[23] In order to check this possibility, we have performed several experimental controls. RNA's were labeled for one hour because at this time there has been little incorporation of the radioactive precursor into ribosomal RNA. The results of these controls can be summarized as follows:

(*i*) The method of preparation of RNA does not affect the sedimentation profile of the labeled RNA. Our routine RNA preparation using hot phenol and SDS yields the same results as the alkaline pH method of Hadjivassiliou and Brawerman.[24]

(*ii*) A variation of the NaCl concentration (0–0.3 *M*) in the buffers used for sucrose gradient preparations does not affect the relative positions of rapidly sedimenting labeled RNA and 28*S* rRNA. At higher salt concentration, precipitation of rRNA occurs.

(*iii*) The divalent cations which could be present were complexed by 1.0 mM EDTA in the sucrose solutions. As above, this treatment does not affect the relative positions of the rapidly sedimenting RNA and the 28*S* rRNA.

(*iv*) The dissociation of possible hydrogen bonds by 4 *M* urea in the sucrose solutions does not result in an appreciable change in the sedimentation of the rapidly labeled RNA.

(*v*) 30–50*S* RNA's are not found in microsomal RNA which is extracted by the same method as total RNA's.

Although none of the arguments would be sufficient by themselves to ensure against the existence of aggregates, they strongly suggest that at least a part, if not the totality, of the rapidly sedimenting RNA's represent true large RNA's. Finally, a direct proof of the existence of such species has been provided by electron microscope examination.[4]

The RNA of the three zones defined under *Materials and Methods* and indicated by arrows in Figure 1 (*left*) were used to saturate DNA (Fig. 2). The saturation plateau is reached at RNA concentrations in the incubation medium of 2 mg/ml for fraction I RNA and of 3.5–4 mg/ml for fractions II and III RNA. The saturation level is 1.27 per cent of the DNA (from 1.09 to 1.40%) for fraction I, 0.77 per cent (from 0.66 to 0.91%) for fraction II, and 0.68 per cent (from 0.63 to 0.72%) for fraction III. Moreover, 1.15 per cent of the DNA was saturated in a reconstruction experiment where fractions I, II, and III were mixed in the incubation medium.

The saturation level of fraction I dRNA is similar to that of total dRNA. However, the RNA of fraction I could be contaminated with RNA of region II, a fact which would cast doubt on the validity of the saturation level determined for the large RNA. We have tried a further fractionation of these RNA's. Fractions I of three gradients were pooled, and the RNA was precipitated with ethanol and again subjected to sucrose density gradient centrifugation (Fig. 1, *right*). The RNA of region I*a* (40–55*S*) and I*b* (30–40*S*) saturated DNA when 1.21 and 0.87 per cent, respectively, of the DNA sites were occupied. The probability of a contamination of region I*a* RNA by 20–30*S* RNA's is, in these conditions, very low; and this result testifies that the high saturation plateau found for fraction I is not due to a contamination by RNA species sedimenting in region II.

The levels of saturation of DNA by fractions II and III dRNA and by microsomal dRNA are similar (0.77, 0.68, and 0.78%, respectively). Fraction III dRNA has the same sedimentation characteristics as the major part of microsomal dRNA and, being derived from total dRNA, certainly contains the cytoplasmic 6–22*S* dRNA. The fact that the average saturation level of microsomal dRNA is slightly higher than that of fraction III dRNA can be explained by the occurrence of 22–30*S* dRNA species in microsomes. When dRNA's of similar specific activities from fraction III and from microsomes were hybridized together, the hybridization level was the same as for microsomal dRNA alone; thus, the RNA species present were probably identical.

As for the RNA's of region II, they are most probably contaminated by the RNA of the two other fractions. The results obtained for this fraction will not be discussed further.

Discussion.—The existence of dRNA species sedimenting more rapidly than 28*S* ribosomal RNA has been first demonstrated in brain where the action of nucleolytic enzymes was avoided. Using the methods described, we find a relatively high degree of 30–50*S* labeled RNA.[2] Such rapidly sedimenting RNA was not found by us in a microsomal fraction,[12] although it was described in rabbit brain microsomes.[13] Since subcellular fractionation could not be performed on lyophilized brain, the absence of large RNA could be attributed to the action of degradative enzymes during the preparation of the microsomes. However, the presence of a ribonuclease inhibitor[25] in rat brain cytoplasm reduces this probability.

Several lines of evidence make us believe that the rapidly sedimenting RNA is large and does not represent a preparation artifact (see *Results*). Similar facts have been stressed for other animal tissues.[1, 3, 4, 8]

The saturation levels of DNA by large (fraction I) and total dRNA are, within experimental error, the same (1.24% of the DNA). This indicates that all dRNA species are present in rapidly sedimenting RNA. Microsomal dRNA is complementary to only 0.8 per cent of the DNA. It can be deduced that about 35 per cent of the dRNA species are nuclear-specific. We had formerly[17] estimated that 50,000–500,000 cistrons code for total dRNA in brain. Cytoplasmic messenger RNA would represent at most 33,000–330,000 cistrons. These relatively high figures suggest a redundancy of the cistrons similar to the known redundancy of ribosomal cistrons.[17]

Fraction III (6–22*S* RNA), which was prepared from total lyophilized brain, contains the microsomal RNA with the same sedimentation characteristics plus the low-sedimenting nuclear RNA. It saturates 0.7 per cent of the DNA as compared to 0.8 per cent for the total microsomal fraction, Reconstruction experiments demonstrated that most of the dRNA species were the same. The small and perhaps insignificant difference can be explained by the presence in microsomal RNA of species sedimenting between 20 and 30*S*. The data suggest furthermore that there is little, if any, nuclear-specific RNA in the 6–22*S* region.

The occurrence of high-molecular-weight precursors of microsomal dRNA and of nuclear-specific dRNA among the large RNA's has been demonstrated in our experiments. We do not know, however, if there are two different types of molecules with similar sedimentation characteristics or if a giant-size molecule

contains segments that would be transferred to the cytoplasm while others stay in the nucleus. The fate and role of nuclear-specific RNA is unknown. The absence of nuclear-specific species in the 6–22*S* region, together with the observation of Harris and Watts[14] and of Scherrer *et al.*,[3] suggests that these nuclear-specific RNA's are rapidly degraded in the nucleus. They could play a role in the regulation of gene transcription as suggested by Frenster.[26]

The relative amount of nuclear-specific RNA and cytoplasmic RNA species is probably variable in different cell types and dependent upon their physiological stage. Thus, in L cells, Shearer and McCarthy[15] found that only 20 per cent of the total dRNA were present in the cytoplasm. The authors suggest that in these cells with a high mitotic index, many dRNA species could be involved in mitosis and remain nuclear. Since mitoses do not occur at an appreciable rate in adult brain, another explanation must be sought to explain the occurrence of nuclear-specific RNA in nervous tissue. The existence of RNA species restricted to the nucleus was also demonstrated in liver.[16, 27] Some of these species disappeared in hepatomas.[27] Contradictory results were reported concerning the appearance of new nuclear RNA species during liver regeneration.[16, 27]

The relatively high percentage of dRNA species found in brain microsomes could be attributed to a contamination by nuclei or nuclear fragments. However, the mild tissue homogenization procedure (five strokes of the homogenizer pestle, driven by hand) that we have used makes such a contamination improbable. Moreover, large RNA found in purified brain nuclei (unpublished observations), in our hands, is absent from the microsomal fraction.

Our data lead us to assume the existence of two selection mechanisms in the nucleus: (*a*) Transformation of 32–50*S* into 6–22*S* RNA's requires a limited cleavage process; (*b*) transport toward the cytoplasm of specific RNA's requires a selection among the fragments and a protection of these selected fragments against the action of nucleases. These steps would contribute to a regulation mechanism of protein synthesis in animal cells at the level of the transport of RNA. The possibilities evoked by Scherrer and co-workers[3] and already noted in our introduction are compatible with our experimental data. Only the parts of the polycistronic precursors that are required for the immediate protein synthesis would be transported to the cytoplasm. The nuclear-specific RNA could represent (*a*) RNA of a former evolutive stage which is no longer necessary in adult tissue; (*b*) readily available messenger RNA coding for proteins specific to a function that the cell has to perform only erratically; or (*c*) polynucleotide sequences attaching messenger RNA cistrons to each other and released during the subsequent steps. The mechanism of cleavage of the giant-size RNA's and of selection of the fragments remains to be described.

Abbreviations: dRNA; DNA-like RNA; SDS: sodium dodecyl sulfate.

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