Gosule, L. C., & Schellman, J. A. (1976) *Nature (London) 259*, 333.
Grosjean, M. J., & Legrand, M. (1960) *C. R. Hebd. Seances Acad. Sci. 251*, 2150.
Holzwarth, G., & Holzwarth, N. A. (1973) *J. Opt. Soc. Am. 63*, 325.
Holzwarth, G., Gordon, D. G., McGinness, J. E., Dorman, B. P., & Maestre, M. F. (1974) *Biochemistry 13*, 126.
Jordan, C. F., Lerman, L. S., & Venable, J. H. (1972) *Nature (London), New Biol. 236*, 70–76.
Krauss, T. C. (1950) *Antennae*, McGraw-Hill, New York.
Maestre, M. F. (1970) *Mol. Biol. 52*, 543.
Maestre, M. F., Gray, D. M., & Cook, R. B. (1971) *Biopolymers 10*, 2537.
Nitayananda, R., Kini, U. D., Chandrasekhar, S., & Suresh, K. A. (1973) *Proceedings of the International Liquid Crystals Conference*, Bangalore, 325.
Reich, C., Maestre, M. F., Edmondson, S., & Gray, D. (1980) *Biochemistry* (preceding paper in this issue).
Schneider, A. S. (1973) *Methods Enzymol. 27D*, 270, 751.
Schneider, A. S., & Harmatz, D. (1976) *Biochemistry 15*, 4158.
Shapiro, J. T., Leng, M., & Felsenfeld, G. (1969) *Biochemistry 8*, 3219.
Shurcliff, W. A. (1962) *Polarized Light*, Harvard University Press, Cambridge, MA.
Suresh, K. A. (1976) *Mol. Cryst. Liq. Cryst. 21*, 315.
Tinoco, I., Jr., & Freeman, M. P. (1957) *J. Phys. Chem. 61*, 1196.
Tunis-Schneider, M. J., & Maestre, M. F. (1970) *J. Mol. Biol. 52*, 521.
Wiener, O. (1926) *Kolloidchem. Beih. 23*, 189.

Constrained Configuration of Double-Stranded Ribonucleic Acid in HeLa hnRNP and Its Relaxation by Ribonuclease D†

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ABSTRACT: Double-stranded RNA (dsRNA) sequences within hnRNP from HeLa cells have been probed by means of ethidium bromide binding as monitored by fluorescence enhancement. About 8% of total RNA present in these particles was able to intercalate ethidium bromide and therefore appeared to be double stranded. Isotherm binding plots were markedly biphasic, the first dye molecules being bound with an affinity constant ~100 times higher than that of authentic dsRNA. Evidence for anticooperative binding was obtained by comparing the data obtained with ethidium bromide and with another intercalating drug, 2,6-dimethylellipticinium acetate, whose unwinding angle is half that of ethidium. This anticooperative effect can be explained by the existence in native hnRNP of a constrained configuration of dsRNA sequences. This interpretation is supported by a very high polarization of ethidium bromide fluorescence. This highly rigid structure is maintained through interaction with hnRNP proteins as treatment with proteinase K completely abolishes the anticooperative effect. Furthermore, we have shown that a dsRNA-specific RNase (RNase D) purified from Krebs ascites cells [Rech, J., Cathala, G., & Jeanteur, Ph. (1976) *Nucleic Acids Res. 3*, 2055–2065] was able to relax the constraint without appreciably reducing the absolute amount of dsRNA sequences. Treatment of hnRNP by a combination of RNases A + T_1 resulted in the complete disappearance of ethidium binding, confirming our recent report on the presence in hnRNP of an RNase D in the masked state which becomes active only after removal of endogenous RNA [Rech, J., Brunel, C., & Jeanteur, Ph. (1979) *Biochim. Biophys. Res. Commun. 88*, 422–427].

Among the various modifications suffered by hnRNA[1] when being processed into mature mRNA, the most drastic one is certainly the removal of extensive sequences through a splicing mechanism (Breathnach et al., 1977; Tilghman et al., 1978a,b).

The enzymology of splicing involves to start with a cleaving step which must be exquisitely specific at least when it occurs within coding regions (Breathnach et al., 1977; Tilghman et al., 1978a). Among the variety of structural features in RNA which a specific nicking enzyme might possibly recognize, we have elected to focus our attention on dsRNA regions. The rationale underlying this approach is fourfold. First, a precedent exists in prokaryotic systems in which RNase III which exhibits in vitro specificity toward dsRNA (Robertson et al., 1968) has been unambiguously involved in the specific cleavage of primary transcripts of *Escherichia coli* rRNA (Dunn & Studier, 1973; Nikolaev et al., 1973) as well as of T7 mRNA (Dunn & Studier, 1973). Second, dsRNA regions which exist in hnRNA (Montagnier, 1968; Stern & Friedman, 1970; Harel & Montagnier, 1971; de Maeyer et al., 1971; Jelinek & Darnell, 1972; Kronenberg & Humphreys, 1972; Ryskov et al., 1972; Patnaik & Taylor, 1973; Jelinek et al., 1974; Monckton & Naora, 1974; Torelli et al., 1974; Robertson et al., 1977) are mostly absent from poly(A)-containing cytoplasmic mRNA (Jelinek & Darnell, 1972). Third, there is sequence complementarity between mRNA and hnRNA (Stampfer et al., 1972) whose double-stranded regions can form duplexes with mRNA (Naora & Whitelam, 1975; Ryskov et al., 1976a,b). This suggests that only one of the two strands of some double-stranded regions is lost during processing and therefore that important cleavage sites may

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[1] Abbreviations used: hnRNA, heterogeneous nuclear RNA; hnRNP, ribonucleoprotein particles containing hnRNA; mRNA, messenger RNA; dsRNA, double-stranded RNA; snRNA, small nuclear RNA; EB, ethidium bromide; DMEA, 2,6-dimethylellipticinium acetate.

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occur within them. Fourth, it has been reported that intercalating dyes specific for double-stranded structures seem to inhibit the cleavage of nuclear precursor RNAs (Snyder et al., 1971; Brinker et al., 1973; Yannarell et al., 1977; Chiu et al., 1980).

All hnRNAs being associated with proteins to form hnRNP during its nuclear residence, processing reactions must be approached at this level. As a prerequisite to the involvement of these duplex regions of hnRNA in some kind of processing signal, it had therefore to be established that they did also exist in hnRNP. Using *E. coli* RNase III as a reagent specific for dsRNA, it was shown recently that pulse-labeled RNA from hnRNP did contain such structures (Calvet & Pederson, 1977) essentially free of associated proteins (Calvet & Pederson, 1978). However, this kind of approach only takes into account the structure of pulse-labeled hnRNA and overlooks the presence in high abundance in hnRNP of snRNAs (Deimel et al., 1977; Northemann et al., 1977; Guimont-Ducamp et al., 1977) which have recently been shown to be hydrogen bonded to hnRNA either after deproteinization (Jelinek & Leinwand, 1978) or within hnRNP (Flytzanis et al., 1978). We have therefore chosen to address the more general problem of secondary structure of total RNA in hnRNP by using ethidium bromide (EB) fluorescence as a probe for double strandedness (Le Pecq & Paoletti, 1967). Using this approach, we have confirmed the presence in hnRNP of dsRNA regions and further demonstrated there a highly constrained configuration exhibiting a strongly anticooperative binding of EB. Complete relaxation of this constraint was obtained by treatment with exogenous RNase purified from Krebs ascites cells (Rech et al., 1976; J. Rech et al., unpublished experiments).

Experimental Procedures

Materials. RNase-free pancreatic DNase (code DPFF) and pancreatic RNase (code R) were from Worthington, T_1 RNase was from Sankyo, and proteinase K was from Merck. Ethidium bromide (EB) was obtained from Calbiochem and gave a unique fluorescent spot upon thin-layer chromatography (Le Pecq, 1972); 2,6-dimethylellipticinium acetate (DMEA) was synthesized and kindly provided by Dr. Dat Xuong. Concentrations of stock solutions of drugs were determined spectrophotometrically by using absorption coefficients of 5450 at 480 nm for EB (M_r = 427) and 27 280 at 315 nm for DMEA (M_r = 341).

RNase D from Krebs ascites cells was prepared by a modification (J. Rech et al., unpublished experiments) of a previously published procedure (Rech et al., 1976). Phage ϕ6, kindly provided by Dr. Van Etten, was grown on *Pseudomonas phase-olicola* as reported by Vidaver et al. (1973). RNA was prepared by phenol extraction in the presence of sodium dodecyl sulfate, followed by ethanol precipitation.

Preparation of hnRNP and Enzymatic Treatments. hnRNPs from HeLa cells were prepared as previously described (Blanchard et al., 1977) by using a slight modification of the sonication procedure described by Kish & Pederson (1975). hnRNPs were finally collected from a 45% (w/v) sucrose layer in buffer A (10 mM Tris-HCl, pH 7.4, at 25 °C, 0.13 M NaCl, 1.5 mM $MgCl_2$, and 6 mM β-mercaptoethanol) and stored frozen in −70 °C. All experiments were carried out in this buffer. Enzymatic treatments were as follows: (1) 10 μg/mL RNase-free pancreatic DNase for 30 min at 37 °C; (2) 50 μg/mL pancreatic RNase and 2 μg/mL RNase T_1 for 1 h at 37 °C; (3) 50 μg/mL proteinase K in the presence of 0.5% sodium dodecyl sulfate for 1 h at 37 °C. In this latter case, digested hnRNPs were phenol-extracted and RNA was recovered by ethanol precipitation, followed by redissolution in buffer A.

RNA determination was carried out by the orcinol method (Mejbaum, 1939) as modified for the presence of high amounts of sucrose (J. P. Zalta, personal communication).

Conditions for treatment with RNase D will be indicated in the legends to Figures 3 and 4.

Binding Studies. Fluorescence studies of dye binding were carried out by using a spectrofluorometer built in the laboratory and previously described (Paoletti, 1971). Excitation and emission wavelengths were respectively 520 and 600 nm for EB and 330 and 540 nm for DMEA.

We used two fluorescence cells, one with 3 mL of buffer A (blank cell) and the other with 3 mL of hnRNP solution in the same buffer (sample cell). A stock solution of dye (0.23 mM for EB and 0.18 mM for DMEA) was then added to each cell with a 5-μL micropipet. The final concentration of dye in the cuvettes was kept below 0.015 mM in order to avoid the inner filter effect. The light scattering due to hnRNP was determined in the solution without dye and subtracted from subsequent measurements. All experiments were carried out at 20 °C. For each concentration of dye, we obtain the fluorescence intensity of the blank cell (I_f) and of the sample cell (I_b). Prior to the experiment we determined the fluorescence intensity of a dye solution containing no hnRNP and the fluorescence intensity of the same concentration of dye bound to a large excess of hnRNP. The ratio of these two fluorescence intensities (V) was used to determine the concentations of bound and free dye at each point of the binding plot (Le Pecq & Paoletti, 1967).

If I_f is the fluorescence intensity of the free dye, VI_f is the fluorescence of the same concentration of dye completely bound to RNA. The fraction of dye bound to RNA under these experimental conditions is then

$$(I_f - I_l)/(V - 1)I_l$$

and the concentration of bound dye is equal to

$$C_b = C_t(I_f - I_l)/(V - 1)I_l$$

where C_t is the total concentration of dye in the medium. The concentration of free dye is then

$$C_f = C_t - C_b$$

From these data we can plot the Scatchard equation (Scatchard, 1949) $r/c = f(r)$ where $c = C_f$ and $r = C_b/[\text{DNA}]$.

If the sites for the drug are independent and of a single type, the plot representative of the Scatchard equation is a straight line whose equation is

$$r/c = K(n - r)$$

where K is the affinity constant for the binding and n is the number of dye molecules bound per nucleotide at saturation of dye. In the case of a biphasic representation of the equation $r/c = f(r)$, the plot was simulated by using two sets of independent parameters: K_1, n_1 and K_2, n_2 where K_1 and K_2 represent the affinity constants and n_1 and n_2 are the values of r at saturation of dye for the two types of sites. The determination of K_1, K_2, n_1, and n_2 has been previously described (Le Pecq & Festy, 1975). The calculations were performed by using a Hewlett-Packard 9810A calculator.

Polarization of Fluorescence. The polarization of fluorescence determinations was performed on the same apparatus as the one used for fluorescence determinations. Two polarizers (Polaroid Corp.) were mounted in the pathway of the light, one for the excitation and the other for emission.

The components of the polarized emitted light, I_{vv}, I_{vh}, and I_{hv}, were measured, and the coefficient of polarization was

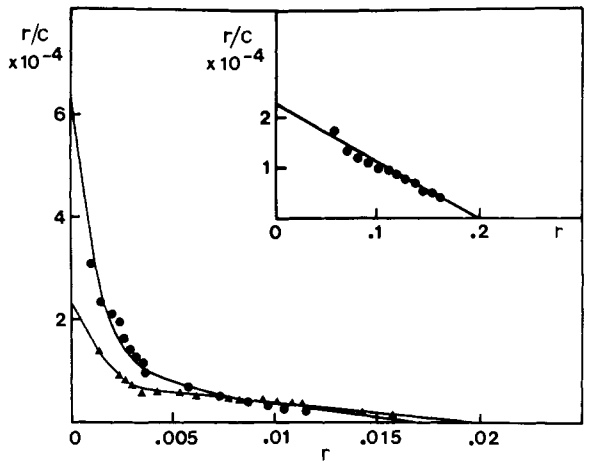

FIGURE 1: Scatchard plot of EB (●) and DMEA (▲) binding to native hnRNP. Experimental conditions have been described under Experimental Procedures. The hnRNP solution was 0.166 mM and 0.1 M in RNA respectively for the EB and DMEA experiments. (Insert): Scatchard plot of EB binding to phage $\phi 6$ RNA. Experimental conditions were as above except for the final concentration of $\phi 6$ RNA which was 3×10^{-6} M.

taken as equal to $p = (I_{vv} - I_{vh}t)/(I_{vv} + I_{vh}t)$ where t which is equal to I_{hv}/I_{hh} is a constant of the system under the conditions of the measurements. Under our conditions, t was found equal to 90.

As in the case of binding studies, the components of the fluorescence were measured for the free EB and for the dye bound to hnRNP. The concentration of RNA was equal to 1.66 mM, and the stock solution of EB had a concentration of 0.023 mM. Temperature was 20 °C. For each point, 5 μL of dye stock solution was added to 3 mL of buffer A or hnRNP solution in the same buffer, and the fluorescence intensities of the components of the emitted light were measured. At these concentrations on hnRNP, the correction due to free ethidium could be neglected (Paoletti & Le Pecq, 1969). A blank consisting of 3 mL of a solution of hnRNP at an RNA concentration equal to 1.66 mM was diluted with 5 μL of buffer at each measurement in order to correct for light scattering.

Results

Binding of EB and DMEA to hnRNP. hnRNPs prepared by sonication of isolated nuclei were purified through a triple sucrose cushion, and the heavier layer was selected so as to be enriched in particles containing least degraded hnRNA. The absence of contamination by ribosomes or preribosomes has been clearly ruled out by centrifugation in cesium chloride density gradients as detailed previously (Blanchard et al., 1977). When intercalative binding sites were titrated by EB fluorescence, the isotherm binding plot of Figure 1 was obtained. This biphasic curve can be fitted to a theoretical one computed on the basis of two independent sites characterized by the following parameters: $K_1 = 4 \times 10^7$ M^{-1}, $n_1 = 1.7 \times 10^{-3}$, $K_2 = 4 \times 10^5$ M^{-1}, and $n_2 = 1.44 \times 10^{-2}$, where K is the affinity constant and n is the number of dye molecules bound per nucleotide at saturation. These values were computed on the basis of the total RNA content of hnRNP, most of which is single stranded. By comparing the total number of sites ($n_1 + n_2 = 0.016$) to the n value of 0.2 measured for free double-stranded RNA from phage $\phi 6$ (insert to Figure 1), one can conclude that 8% of the RNA present in hnRNP is able to bind EB through intercalation and is therefore likely to be double-stranded RNA.

Considering this small value, it was mandatory to exclude a contamination by DNA, only a few percent of which could significantly contribute to the observed fluorescence. This possibility was ruled out by showing that the binding plot (not shown) of hnRNP treated with RNase-free pancreatic DNase was not significantly different from that of hnRNP incubated without enzyme.

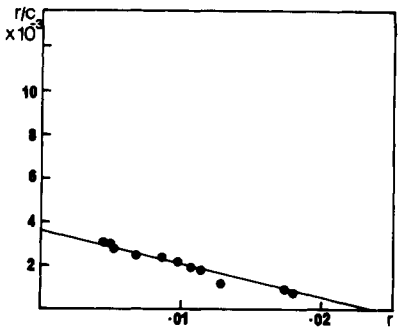

FIGURE 2: Scatchard plot of EB binding to RNA from deproteinized hnRNP. All experimental conditions were as in Figure 1.

When RNA from hnRNP was deproteinized by treatment with proteinase K, followed by ethanol precipitation, and isotherm binding plot (Figure 2) was obtained which no longer shows a biphasic appearance, reflecting the existence of a unique site with an affinity constant $K = 1.5 \times 10^5$ M^{-1}. Moreover, this value is quite close to that of site 2 in native hnRNP (4×10^5 M^{-1}) and also that exhibited by deproteinized authentic dsRNA from phage $\phi 6$ (3×10^{-1}) as can be derived from Figure 1. The total number of sites in deproteinized hnRNP has increased to $n = 2.5 \times 10^{-2}$. This could conceivably reflect masking of sites by proteins in native hnRNP, thereby casting a doubt on the validity of the 8% value for dsRNA in hnRNP. However, this possibility is not supported by the fact that micrococcal nuclease destroys all EB binding sites (not shown) as well as by previous reports by Calvet & Pederson (1977, 1978) indicating that double-stranded hnRNA in hnRNP is essentially free of proteins. On the other hand, we think that a more likely explanation would be the formation of intermolecular duplexes between hnRNA molecules upon incubation with proteinase K and ethanol precipitation. Such structures have actually been shown to occur with extraordinary ease even in a few minutes at room temperature in formamide and urea as used for electron microscopy (Fedoroff & Wellauer, 1977).

The biphasic nature of EB binding to native hnRNP (Figure 1) can be interpreted in terms of two models. The first one assumes the existence of two actually independent sites. Only elaborate explanations could, however, account for the fact that the site 1 has an affinity which exceeds that of naked dsRNA by ~2 orders of magnitude. Conversely, the second model postulates that all sites are equivalent but bind EB in a strongly anticooperative way. Such a situation would be highly reminiscent of that encountered with covalently closed circular DNA bearing negative superhelical turns (Vinograd et al., 1968). In this latter case, the first molecules of EB are bound with higher affinity than with linear DNA until all negative turns have been removed and then with a progressively lower affinity when positive turns are induced. This anticooperative effect results in a bimodal appearance of the isotherm binding plot. By analogy with the above situation, we suggest that dsRNA within hnRNP is under a topological constraint, resulting in a situation formally comparable to negative superhelical turns. In this latter case it was demonstrated (Davison, 1972) that the K_1/K_2 ratio for an intercalative ligand is an exponential function of the unwinding angle per bound dye molecule. According to this calculation, one should expect the K_1/K_2 ratio for a ligand with an un-

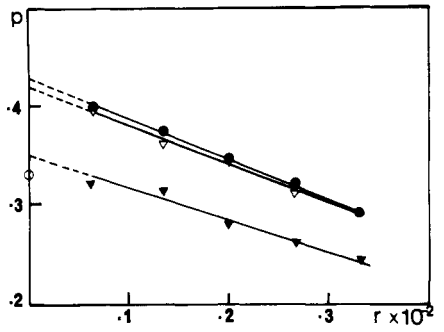

FIGURE 3: Variation of the coefficient of fluorescence polarization (p) of EB as a function of r for native hnRNP (●) and hnRNP incubated in the absence (▽) or in the presence (▼) of 10 μL of purified RNase D for 1 h at 37 °C. Experimental conditions were described under Experimental Procedures. (○) on the oridinate axis represents the extrapolated value of the fluorescence polarization for $r = 0$ obtained with either DNA or φ6 RNA.

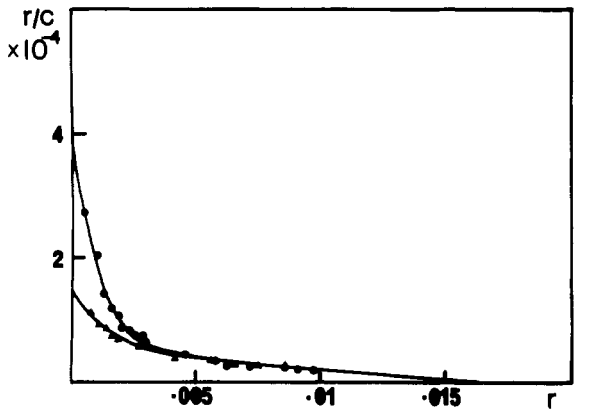

FIGURE 4: Scatchard plot of EB binding to hnRNP incubated in the absence (●) or presence (▲) of 10 μL of purified RNase D. The hnRNP solution was 0.166 mM in RNA. All experimental conditions have been described under Experimental Procedures.

winding angle half that of EB to be ~10 instead of 100 as observed for EB.

To this aim, we have therefore measured the binding parameters to native hnRNP of *N*-methylhydroxyellipticinium acetate for which an unwinding angle of 13° was determined (J. B. Le Pecq, unpublished results) by reference to the 26° value now accepted for EB (Wang, 1974). The Scatchard plot presented in Figure 1 still exhibits a biphasic, although less pronounced, appearance which can be fitted to a theoretical curve computed by using values of $K_1 = 4 \times 10^{-6}$ M^{-1} and $K_2 = 4 \times 10^5$ M^{-1}. As observed for EB, this latter value was similar to that determined with naked dsRNA (results not shown). This result exactly fulfills the above prediction which followed from the model of anticooperative binding resulting from a topological constraint of double-stranded RNA regions within hnRNP.

Fluorescence Polarization of EB Bound to hnRNP. The existence of such a topological constrained structure should be detectable by fluorescence polarization measurements of EB intercalated into the nucleic acid. The polarization coefficient of a fluorescent chromophore reflects the rotation undergone by this chromophore between excitation and emission. For EB it has been shown that the polarization coefficient (p) in rigid medium (infinite viscosity) is equal to 0.415 (Walh et al., 1970; Paoletti, 1971). If the molecule undergoes any rotational motion between excitation and emission, we will observe a depolarization of the emitted light, causing a decrease in the value of the polarization coefficient. When EB is intercalated into a double-stranded structure (DNA or RNA), it is subject to a rotation of the base pairs, and its polarization coefficient decreases from 0.415 to 0.320 [Wahl et al. (1970) and Figure 3]. Depolarization can be attributed to rotational Brownian motion as long as there is not interference by energy transfer between dye molecules. If such a transfer occurs, the depolarization will not reflect the motion of the dye but rather the rotation of the emitted light corresponding to the angle between two adjacent molecules of the dye. In order to eliminate this effect, we have to determine the polarization by extrapolating the results to no ethidium bound. When the dye is intercalated into a compact double-stranded nucleic acid, for example, into bacteriophage λ (Paoletti, 1971) or into chromatin DNA (Paoletti et al., 1977), the polarization coefficient is increased to a value of ~0.420.

We have measured the polarization coefficient p of EB bound to hnRNP at different concentrations of ethidium. The result is shown on Figure 3. At low values of the ratio of bound dye per nucleotide, the fluorescence polarization of the hnRNP-bound EB is higher than the one usually observed when bound to regular naked DNA or RNA. By extrapolating to r equals zero, we get a value of p equal to 0.415, identical with what is found for EB in a rigid medium. From this experiment we can conclude that the drug binds to regions of RNA in the particles, which are completely rigid and unable to undergo any Brownian motion along the axis perpendicular to the plane of bases. This is in good accordance with a constrained structure.

The regular decrease in fluorescence polarization with increasing r is most likely to correspond to an energy transfer between adjacent EB molecules. Indeed, although the r values used here are apparently low, they are actually quite high when compared to the actual number of sites. Even the first two points of this curve are already close to saturation of site 1 ($n_1 = 1.7 \times 10^{-3}$). Working at such low r values relative to this one as to prevent energy transfer was not practicable due to the too small fraction of dsRNA in hnRNP.

Binding of EB to RNase-Treated Particles. In order to confirm the double-stranded nature of the sites in hnRNP available for intercalative binding of EB, we have treated them with a combination of A + T_1 RNases, both specific for single-stranded RNA, and studied the binding of EB to these particles under the same conditions as in Figure 1. No binding of the dye to the particles, at least through intercalation, was observed, suggesting that the structures responsible for intercalation of the drug have been destroyed by RNases. This is surprising since double-stranded regions of hnRNA within hnRNP have been shown to be resistant to these RNases under strictly identical conditions (Calvet & Pederson, 1977). The explanation comes, however, from our recent finding (Rech et al., 1979) that hnRNPs do contain a dsRNA-specific RNase D active on poly(AU) which appears only after removal of endogenous RNA or after its digestion with RNases A + T_1. The absence of intercalation of EB in this experiment is therefore readily explained by the action of endogenous RNase D, subsequent to elimination of inhibitory single-stranded RNA by RNases A + T_1.

In order to check whether endogenous RNase D had any activity on dsRNA in native hnRNP, we have looked at the binding of the drug to particles incubated in the absence of any added RNases. The results are shown in Figure 4. As in the case of "native" hnRNP, the isotherm binding plot shows a strong anticooperative effect which can be simulated by using two sets of parameters: $K_1 = 2 \times 10^7$ M^{-1} and $K_2 = 3 \times 10^5$ M^{-1}; $n_1 = 1.5 \times 10^{-3}$ and $n_2 = 1.45 \times 10^{-2}$. The value of n at saturation of drug is equal to the one found for

"native" particles, i.e., $n = 1.6 \times 10^{-2}$.

It seems that autoincubation of the particles does not significantly alter their content in dsRNA but induces a slight decrease in the anticooperativity of binding. Such a change in anticooperativity suggests some relaxation of the constraints associated with dsRNA as confirmed by the slight but detectable decrease in fluorescence polarization observed in Figure 4. It therefore appears that endogenous RNase D in native hnRNP has some effect on the configuration of dsRNA within the particles. However, no significant degradative activity is observed, in keeping with the previously reported lack of degradation of exogenous poly(AU) to acid-soluble products (Rech et al., 1979). This observation prompted us to investigate the effect on supplementing native hnRNP with exogenous RNase D purified from Krebs ascites cells (Rech et al., 1976). A dramatic reduction of the anticooperative effect is observed in the isotherm binding plot which can be simulated with K_1 and K_2 values respectively equal to 7×10^6 and 3×10^5 M^{-1} (Figure 4). The value of n at saturation ($n = 1.56 \times 10^{-2}$) is not sufficiently different from either unincubated or autoincubated controls to allow further conclusions to be drawn.

The nearly complete suppression of anticooperativity strongly suggests that the constrained structure of dsRNA has been relaxed. Such a change in configuration should lead to a marked decrease in fluorescence polarization. This expectation is clearly fulfilled by the results of Figure 3 which shows that p has decreased to an extrapolated value for $r = 0$ of ~ 0.35. The further decline in p with increasing r due to energy transfer closely parallels that observed for either unincubated or autoincubated particles. Taken altogether, the above results point strongly to the existence of a constrained structure of dsRNA sequences which is maintained by proteins within hnRNP and which can be relaxed by a very limited cleavage by the dsRNA-specific RNase D.

Discussion

About 8% of total RNA in hnRNP is shown to bind EB by intercalation and therefore behaves as dsRNA. This value is somewhat higher than previously reported for hnRNA either naked (Jelinek & Darnell, 1972) or in hnRNP on the basis of resistance to single-strand-specific RNases and sensitivity to *E. coli* RNase III (Calvet & Pederson, 1977). It must be emphasized, however, that the present study is not restricted to pulse-labeled hnRNA but deals with the total RNA content of hnRNP including snRNAs (Deimel et al., 1977; Northemann et al., 1977; Guimont-Ducamp et al., 1977).

The most striking feature of the isotherm binding plot of EB to native is its marked biphasic appearance. This phenomenon can be interpreted in terms of two models.

The first model assumes the existence of two independent sites. The most plausible interpretation would then be as follows. The higher affinity site could be assigned to stretches of naked dsRNA freely accessible to EB. The lower affinity of site 2 could reflect the masking by proteins of otherwise genuine dsRNA. This model is, however, not tenable in view of the following considerations. On the first hand, dsRNA does not seem to be associated with proteins within hnRNP (Calvet & Pederson, 1977, 1978). On the other hand, the lower affinity site in the present case is the one whose affinity closely corresponds to that of naked RNA and masking by proteins cannot reasonably be expected to increase affinity for EB.

The second model postulates the existence of a unique site which binds EB anticooperatively in a way formally analogous to the situation found in covalently closed circular DNA (Vinograd et al., 1968). A strong support to this model was drawn from the observation that the K_1/K_2 ratio was dependent in an exponential way upon the unwinding angle of the intercalating ligand (Davidson, 1972) when comparing EB and DMEA. It must be emphasized that the strong anticooperative effect observed here reflects a superhelix density of dsRNA regions in hnRNP which is 5–6 times higher than that observed for covalently closed circular SV40 DNA (Bauer & Vinograd, 1970; Davison, 1972). dsRNA structures in hnRNP are supposed to be of the hairpin type (Jelinek & Darnell, 1972). Imposing topological constraints upon them implies that a structure exists which prevents its two ends from freely rotating one relative to the other. The complex protein moiety (Ducamp & Jeanteur, 1973; Brunel & Lelay, 1979) of hnRNP could easily play this role. Indeed, removal of proteins by treatment with proteinase K abolishes the anticooperativity and expectedly restores an affinity for EB which is comparable to that of either site 2 or $\phi 6$ RNA.

Should dsRNA be unable to freely rotate with reference to the bulky hnRNP structure, the fluorescence of EB should exhibit a high degree of polarization. This expectation is clearly fulfilled by the measurement of a polarization coefficient of 0.415 in native hnRNP equal to that previously observed in such constrained structures as DNA packed in bacteriophage λ heads (Paoletti, 1971) or in chromatin (Paoletti et al., 1977). We therefore conclude that dsRNA regions exist in hnRNP in a state of topological constraint most likely to be maintained by anchoring to particle proteins. Involvement of the latter in this role of topological clamp is not contradictory with the observation that dsRNA is not covered with proteins (Calvet & Pederson, 1977, 1978) as this model only requires that both ends of the dsRNA segment be bound to proteins.

Treatment of hnRNP with single-stranded specific RNases resulted in our hands in the complete elimination of EB binding sites, confirming the previous demonstration by Rech et al. (1979) of an RNase D in a mostly inactive form within hnRNP at least as far as the hydrolysis of an exogenous substrate was concerned. It should be pointed out that in our interpretation of the binding of EB to native hnRNP we assume that the drug binds in an intercalative manner to the double-stranded regions of hnRNA. However, it has been shown, using studies on tRNA crystals, that EB could bind nonintercalatively to tRNA (Liebman et al., 1977), and this type of binding could represent an alternative to the one we propose. Double-stranded regions of hnRNA are large enough to form tertiary structures able to bind the drug the same way as tRNA. In that case such tertiary structures, forming hydrophobic pockets and stabilized by proteins, could be responsible for the effects we describe. Whether the binding of the drug to these structures is intercalative or not will probably be answered once the tertiary structure of hnRNA is known in detail.

Especially interesting was the observation that treatment with purified RNase D from Krebs ascites cells (Rech et al., 1976) efficiently restores the free rotation of dsRNA in hnRNP as demonstrated by a drop in fluorescence polarization approaching that of free dsRNA. It should be emphasized that such an effect is obtained under conditions where little if any degradation of dsRNA occurs. Optimistically, such a nicking type of activity can be thought of as the cleavage step of a splicing reaction. Further work should now proceed with the examination of a defined population of hnRNP where cleavage reactions can be approached at the sequence level.

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References

Bauer, W., & Vinograd, J. (1970) *J. Mol. Biol. 54*, 281–298.
Blanchard, J. M., Brunel, C., & Jeanteur, Ph. (1977) *Eur. J. Biochem. 79*, 117–131.
Breathnach, R., Mandel, J. L., & Chambon, P. (1977) *Nature (London) 270*, 314–319.
Brinker, J. M., Madore, H. P., & Bello, L. J. (1973) *Biochem. Biophys. Res. Commun. 52*, 928–934.
Brunel, C., & Lelay, M. N. (1979) *Eur. J. Biochem. 99*, 273–283.
Calvet, J. P., & Pederson, T. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3705–3709.
Calvet, J. P., & Pederson, T. (1978) *J. Mol. Biol. 122*, 361–378.
Chiu, N. H., Bruszewski, W. B., & Salzman, N. (1980) *Nucleic Acids Res. 8*, 153–168.
Davidson, N. (1972) *J. Mol. Biol. 66*, 307–309.
Deimel, B., Louis, Ch., & Sekeris, C. (1977) *FEBS Lett. 73*, 80–84.
de Maeyer, E., De Maeyer-Guignard, J., & Montagnier, L. (1971) *Nature (London), New Biol. 229*, 109–110.
Ducamp, C., & Jeanteur, Ph. (1973) *Biochimie 55*, 1235–1242.
Dunn, J. J., & Studier, F. W. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3296–3300.
Fedoroff, N., & Wellauer, P. K. (1977) *Cell 10*, 597–610.
Flytzanis, C., Alonso, A., Louis, Ch., Krieg, L., & Sekeris, C. E. (1978) *FEBS Lett. 96*, 201–206.
Guimont-Ducamp, C., Sri-Widada, J., & Jeanteur, Ph. (1977) *Biochimie 59*, 755–758.
Harel, J., & Montagnier, L. (1971) *Nature (London), New Biol. 229*, 106–108.
Jelinek, W., & Darnell, J. E. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2537–2541.
Jelinek, W., & Leinwand, L. (1978) *Cell 15*, 205–214.
Jelinek, W., Molloy, G., Fernandez-Munoz, R., Salditt, M., & Darnell, J. E. (1974) *J. Mol. Biol. 82*, 361–370.
Kish, V. M., & Pederson, T. (1975) *J. Mol. Biol. 95*, 227–238.
Kronenberg, L. H., & Humphreys, T. (1972) *Biochemistry 11*, 2020–2026.
Le Pecq, J. B. (1972) *Methods Biochem. Anal. 20*, 41–86.
Le Pecq, J. B., & Paoletti, C. (1967) *J. Mol. Biol. 27*, 87–106.
Le Pecq, J. B, & Festy, B. (1975) *Biochim. Biophys. Acta 407*, 40–42.
Liebman, M., Rubin, J., & Sundaralingam, M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4821–4825.
Mejbaum, W. (1939) *Hoppe-Seyler's Z. Physiol. Chem. 258*, 117.
Monckton, P. B., & Naora, H. (1974) *Biochim. Biophys. Acta 335*, 139–154.
Montagnier, L. (1968) *C. R. Hebd. Seances Acad. Sci., Ser. D 267*, 1417–1420.
Naora, H., & Whitelan, J. M. (1975) *Nature (London) 256*, 756–759.
Nikolaev, N., Silengo, L., & Schlessinger, D. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3361–3365.
Northemann, W., Scheurlen, M., Gross, V., & Heinrich, P. C. (1977) *Biochem. Biophys. Res. Commun. 76*, 1130–1137.
Paoletti, J. (1971) Ph.D. Thesis, Paris University.
Paoletti, J., & Le Pecq, J. B. (1969) *Anal. Biochem. 31*, 33–41.
Paoletti, J., Magee, B. B., & Magee, P. T. (1977) *Biochemistry 16*, 351–357.
Patnaik, R., & Taylor, M. W. (1973) *Biochemistry 12*, 1990–1995.
Rech, J., Cathala, G., & Jeanteur, Ph. (1976) *Nucleic Acids Res. 3*, 2055–2065.
Rech, J., Brunel, D., & Jeanteur, Ph. (1979) *Biochem. Biophys. Res. Commun. 88*, 422–427.
Robertson, H. D., Webster, R. E., & Zinder, N. D. (1968) *J. Biol. Chem. 243*, 82–91.
Robertson, H. D., Dickson, E., & Jelinek, W. (1977) *J. Mol. Biol. 115*, 571–589.
Ryskov, A. P., Farashyan, V. R., & Georgiev, G. P. (1972) *Biochim. Biophys. Acta 262*, 568–572.
Ryskov, A. P., Kramerov, D. A., & Georgiev, G. P. (1976a) *Biochim. Biophys. Acta 447*, 214–229.
Ryskov, A. P., Tokarskaya, O. V., Georgiev, G. P., Coutelle, C., & Thiele, B. (1976b) *Nucleic Acids Res. 3*, 1487–1498.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–675.
Synder, A. L., Kann, H. E., & Kohn, K. W. (1971) *J. Mol. Biol. 58*, 555–565.
Stampfer, M., Rosbash, M., Huang, A. S., & Baltimore, D. (1972) *Biochem. Biophys. Res. Commun. 49*, 217–224.
Stern, R., & Friedman, R. M. (1970) *Nature (London) 226*, 612–616.
Tilghman, S. M., Tiemeir, D. C., Seidman, J. G., Peterlin, B. M., Sullivan, M., Maizel, J. V., & Leder, P. (1978a) *Proc. Natl. Acad. Sci. U.S.A. 75*, 725–729.
Tilghman, S. M., Curtis, P. J., Tiemeir, D. C., Leder, P., & Weissmann, C. (1978b) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1309–1313.
Torelli, U., Torelli, G., Cadossi, R., & Ferrari, S. (1974) *Haematologica 59*, 393–400.
Vidaver, A. K., Koski, R. K., & Van Etten, J. L. (1973) *J. Virol. 11*, 799–805.
Vinograd, J., Lebowitz, J., & Watson, R. (1968) *J. Mol. Biol. 33*, 173–197.
Wahl, Ph., Paoletti, J., & Le Pecq, J. B. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65*, 417–421.
Wang, J. C. (1974) *J. Mol. Biol. 89*, 783–801.
Yannarell, A., Niemann, M., Schumm, D. E., & Webb, T. E. (1977) *Nucleic Acids Res. 4*, 603–611.