Electron Spin Resonance for Detecting Polyadenylate Tracts in RNA's

Abstract. *Electron spin resonance is used to detect RNA's that contain polyadenylate tracts. The method depends on the ability of RNA's that contain polyadenylate sequences to associate with poly(2'-deoxy-2'-fluoro)uridylic acid, which has been spin-labeled with 4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidinooxyl. The formation of the hybridization product can be detected by monitoring the decrease in mobility of the spin probe.*

The presence of polyadenylate [poly(A)] tracts in eukaryotic messenger RNA's (mRNA) (*1*) and in 70*S* RNA of tumor viruses (*2*) is well established. The poly(A) tail seems to be located at the 3'-OH end of the messenger, and its purpose might be to confer some stability to mRNA during its intracellular transport and translation (*3, 4*).

We now report a novel assay for the detection and quantitation of poly(A) tracts in such RNA systems. It is based on monitoring the complex formation between spin (a stable organic free radical) labeled polyuridylate [poly(U)] or poly(2'-deoxy-2'-fluoro)uridylic acid [poly(dUfl)] and the poly(A) tail by electron spin resonance spectroscopy (ESR). The advantages of the spin-label assay for determining poly(A) tracts are rapidity and directness, since methods widely used so far for detecting hybrids of poly(A) and poly(U) usually involve their isolation by filtration onto membranes (*2*) or fiberglass (*5*) or by hydroxylapatite chromatography (*6*). Previous work has shown that ESR can detect the conformational changes of spin-labeled homopolyribonucleotides in aqueous solution (*7–9*). In Fig. 1 typical experimental and computer-simulated ESR spectra of spin-labeled poly(dUfl) and spin-labeled poly(dUfl) • poly(A) double strands are shown. The use of spin-labeled poly(U) instead of spin-labeled poly(dUfl) results in similar observations to those shown in Fig. 1. The spin-labeled poly(U) and poly(dUfl) had been chromatographed on Sephadex G-200, and the high-molecular-weight fractions with sedimentation values (s_{20}) of 6.9*s* and 12.3*s*, respectively, were used for our study. Poly(U) as well as poly(dUfl) were spin-labeled approximately to the same extent, the ratio of spin label to nucleotide being about 1 : 75. The ESR line shapes were analyzed according to the theory of Freed, Bruno, and Polnaszek (*10*), appropriately generalized to include completely asymmetric *g* and *A* tensors of the nitroxide radical (*11*). The simulated spectra are in good agreement with the experimental data (Fig. 1). For both single strands, isotropic Brownian rotational diffusion resulted in good simulations and correlation times (τ_R) of 3×10^{-10} and 3.5×10^{-10} second were determined for spin-labeled poly(U) and poly(dUfl), respectively.

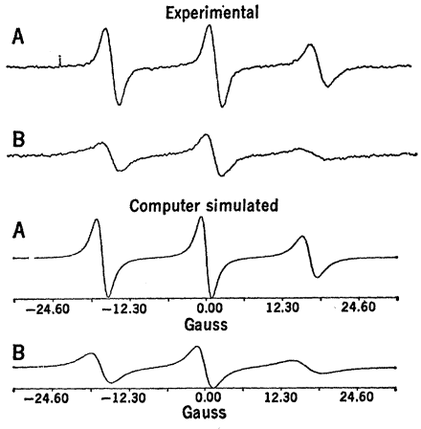

Fig. 1. Comparison of experimental and computer-simulated ESR spectra of spin-labeled poly(dUfl) (spectra A) and spin-labeled poly(dUfl) · poly(A) (spectra B). Poly(U) and poly(dUfl) were obtained from Miles Chemical Co., the latter having been synthesized according to (*13*). The spin label 4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidinooxyl was incorporated into poly(U) and poly(dUfl) according to (*7*). The ESR measurements were done in 0.01*M* tris · HCl, 0.01*M* NaCl, 0.001*M* EDTA, *p*H 7.5, with a Varian E-4 ESR spectrometer interfaced with a Fabri-Tek 1070 signal averaging computer. The spectra were usually time averaged 16 times. For the simulation of spectrum A an isotropic reorientation with $\tau_R = 3.5 \times 10^{-10}$ second was used, whereas spectrum B was simulated with axially symmetric reorientation and $\tau_{R_{||}} = 6.3 \times 10^{-10}$ and $\tau_{R_\perp} = 22.1 \times 10^{-10}$ second.

The effect of hybridization of spin-labeled poly(U) with poly(A) on the ESR line shape has been shown (*8*) to decrease the mobility of the spin label upon complex formation. Raising the temperature increased the mobility at the spin denaturation temperature T_m^{sp}, reflecting the temperature-dependent transition from double strands to single strands. The line shape of the spin-labeled complex has in the meantime also been simulated by application of Freed's theory (*11*), and it was noticed that the simulation of the spin-labeled duplex ESR spectra could be considerably improved by introducing axially symmetric Brownian rotational diffusion. Good simulations of the spin-labeled poly(U) • poly(A) were possible by choosing $\tau_{R_{||}} = 4.7 \times 10^{-10}$ and $\tau_{R_\perp} = 16.5 \times 10^{-10}$ second, taking as the symmetry axis of reorientation the molecular *y* axis of the nitroxide radical (not shown). Thus, the spin-labeled complex is not only characterized by a decrease in the mobility of the probe but in addition the probe undergoes an anisotropic motion, which is characteristic of the complex.

The above-discussed effect of a well-characterized motion of the nitroxide radical in a spin-labeled poly(U)• poly(A) complex applies also to a spin-labeled poly (dUfl) • poly(A) duplex. There too it was found that taking the *y* axis of the nitroxide radical as symmetry axis of reorientation would give good simulation data (Fig. 1). The

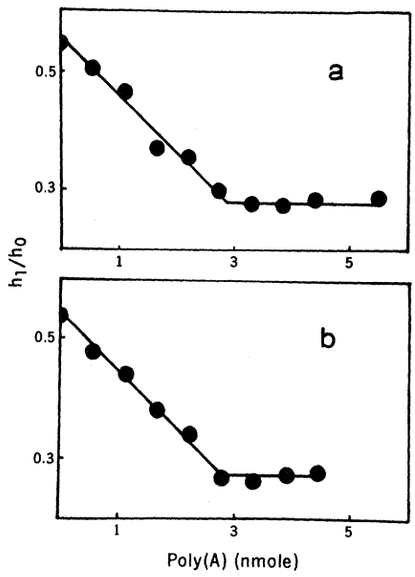

Fig 2. Electron spin resonance titration study of poly(A) with spin-labeled poly(dUfl) in 0.01*M* tris · HCl, 0.01*M* NaCl, 0.001*M* EDTA, *p*H 7.5, at 26°C. Poly(A) was obtained from Miles Chemical Co., and the spin-labeled poly(dUfl) was prepared as described in the legend to Fig. 1. The preparation of R17 RNA has been described (*12*). (a) Variation of the ratio of high field (h_{-1}) to center field (h_0) hyperfine components of 2.8 nmole of spin-labeled poly(dUfl) upon titration with a mixture consisting of R17 RNA and poly(A) at a molar ratio of 9 to 1, respectively. (b) Variation of the ratio of h_{-1} to h_0 of 2.8 nmole of spin-labeled poly(dUfl) upon titration with poly(A).

values for $\tau_{R_{||}}$ and $\tau_{R_{\perp}}$ were 6.3×10^{-10} and 22.1×10^{-10} second, respecpectively, indicating, as for the spin-labeled poly(U) • poly(A) complex, a three to four times faster rotational motion about the symmetry axis of reorientation. The molecular motion of the spin probes attached either to single or double strands has been reviewed (*12*).

The formation of double-stranded spin-labeled poly(U) • poly(A) or spin-labeled poly(dUfl) • poly(A) was also directly monitored by ESR spectroscopy by adding small portions of poly(A) of known concentration to a known amount of spin-labeled poly(U) or poly(dUfl). This is shown in Fig. 2, a and b, in the case of spin-labeled poly(dUfl). The data are plotted by taking the ratio of high field (h_{-1}) to center field (h_0) hyperfine components as a function of the number of nanomoles of poly(A) added. It was observed for spin-labeled poly(U) (not shown) as well as for poly(dUfl) that the ratio of the two hyperfine components remained constant as soon as both components of the hybridization product were present in equal amounts. The titration plot consists of two segments which form an intercept.

When R17 RNA, which is believed not to contain any long poly(A) tracts, was added to spin-labeled poly(dUfl), the ESR line shape remained unchanged. However, addition of a mixture of R17 RNA and poly(A) to a solution containing a known amount of spin-labeled poly(dUfl) caused characteristic ESR line shape changes. Figure 2a shows that the h_{-1}/h_0 ratio first decreases linearly and then remains constant. The two resulting segments form an intercept which corresponds to 2.8 nmole of poly(A), the amount of spin-labeled poly(dUfl) originally present in solution. Titration experiments in the presence of an excess of calf thymus DNA instead of R17 RNA gave the same plot (not shown). A titration with poly(A) alone is shown in Fig. 2b. The titration pattern as well as the position of the break point has not changed. In addition, the line shape of the ESR spectra after the break point is identical with the ESR spectrum of a spin-labeled poly(dUfl) • poly(A) complex obtained with mixing curves that were determined spectrophotometrically. As was previously observed in a slightly different buffer system (*13*), the mixing curves revealed two straight lines intersecting at 50 percent (on a molar basis) poly(dUfl).

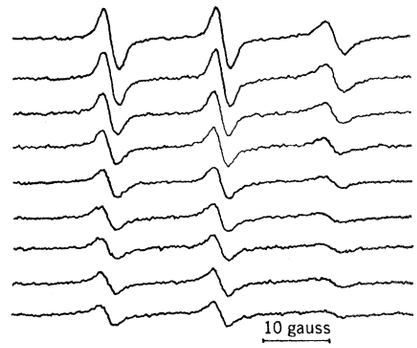

Fig. 3. Changes in ESR line shape of 4.2 nmole of spin-labeled poly(dUfl) upon addition of small amounts of rabbit globin mRNA in 0.01*M* tris · HCl, 0.01*M* NaCl, 0.001*M* EDTA, *p*H 7.5. From top to bottom the amount of mRNA added is: 0, 12.8, 25.6, 38.4, 51.2, 64, 76.8, 89.6, and 102.4 nmole. The rabbit globin mRNA had been prepared according to (*17*).

In order to trap a poly(A) tail in a viral or eukaryotic mRNA, one has to ascertain that the tail is freely accessible for hybridization with poly(U) or poly(dUfl). Recently we showed (*14*) that stacked adenylate segments which are not base-paired and which have an average length of 40 to 60 nucleotides were directly observable by circular dichroism spectroscopy in rabbit globin mRNA in the buffer used for this study. Subsequently it was shown by ESR spectroscopy that the presence of the poly(A) tail in this mRNA could be qualitatively determined with spin-labeled poly(U) as well as poly(dUfl) (*15*). Although the spin-labeled assay for detecting poly(A) tracts in RNA's gives satisfactory results with spin-labeled poly(U) and poly(dUfl), it was realized that for a quantitative determination of the poly(A) tracts it would be advantageous to use the polynucleotide analog. For the quantitation of poly(A) a titration is involved which increases the risk of nuclease contamination. It has been shown that poly(dUfl) is considerably more stable toward hydrolysis than is poly(U) (*13*), and in addition it seems to form only double strands with poly(A) (*13*). Thus, when poly(dUfl) is present in large excess at the beginning of the titration, the possibility of having triple strands formed is eliminated. The ESR line shape changes of 4.2 nmole of spin-labeled poly(dUfl) upon addition of small amounts of rabbit globin mRNA are shown in Fig. 3. After the addition of 64 nmole of mRNA, the overall line shape of the ESR signal does not change any further. The signal is characteristic of a spin-labeled poly(dUfl) • poly(A) complex. Plotting the data according to Fig. 2 reveals a break point which corresponds to an average poly(A) content of about 6.6 percent in rabbit globin mRNA, which can be readily hybridized with poly(dUfl).

Comparing this value with data obtained earlier by circular dichroism (CD) spectroscopy leads to an interesting observation. The CD work (*14*) revealed an average of about 8 percent of adenylic acid residues forming a homopolymer in mRNA prepared according to (*16*) where 20 percent or less of the mRNA did not bind to an oligodeoxythymidylate cellulose column. The mRNA used for the ESR study was made with a more recent procedure (*17*), and all the material now binds to the column. Although no systematic CD study was done on this material, preliminary CD data suggest that the average poly(A) content is certainly not lower than 8 percent in these new preparations and possibly higher. Therefore, slightly less of the poly(A) segment as apparently seen by CD spectroscopy is accessible to hybridization with spin-labeled poly(dUfl). It should be pointed out that a homogeneous size distribution of spin-labeled poly(U) or poly(dUfl) is used for the titration study. However, these chains are certainly substantially longer (molecular weight greater than 200,000) than the poly(A) segments in mRNA. Thus, one can expect besides hybridization unpaired poly(dUfl) as well as poly(A) segments due to interference from the segments other than poly(A) of the mRNA strands. This steric constraint effect could be made responsible for a systematic error in the determination of the average poly(A) content and might account for the small difference obtained between the current ESR studies and the earlier CD data (*14*). In case poly(dUfl) is not utilized efficiently, the percentage of average poly(A) content calculated would appear to be systematically too high, whereas the opposite is true if not all of the poly(A) segment is available for hybridization. Finally, it should be emphasized that this novel spin-label assay requires no more than 36 μg of poly(A)-containing RNA and has potential for direct quantitation of RNA tumor viruses.

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References and Notes

1. J. O. Bishop, M. Rosbash, D. Evans, *J. Mol. Biol.* **85**, 75 (1974); J. E. Darnell, W. R. Jelinek, G. R. Molloy, *Science* **181**, 1215 (1973).
2. D. Gillespie, S. Marshall, R. C. Gallo, *Nat. New Biol.* **236**, 227 (1972).
3. I. Slater, D. Gillespie, D. W. Slater, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 406 (1973).
4. F. Wilt, *ibid.*, p. 2345.
5. R. Sheldon, C. Jurale, J. Kates, *ibid.* **69**, 417 (1972).
6. N. G. Avadhani, M. Kuan, P. VanDer Lign, R. J. Rutman, *Biochem. Biophys. Res. Commun.* **51**, 1090 (1973).
7. A. M. Bobst, *Biopolymers* **11**, 1421 (1972).
8. Y.-C. E. Pan and A. M. Bobst, *ibid.* **12**, 367 (1973).
9. ——, *ibid.* **13**, 1079 (1974).
10. J. H. Freed, G. V. Bruno, C. F. Polnaszek, *J. Phys. Chem.* **75**, 3385 (1971).
11. S. A. Goldman, G. V. Bruno, C. F. Polnaszek, J. H. Freed, *ibid.* **56**, 716 (1972).
12. T. K. Sinha, Y.-C. E. Pan, A. M. Bobst, in preparation.
13. B. Janik, M. P. Kotick, T. H. Kreiser, L. F. Reverman, R. G. Sommer, D. P. Wilson, *Biochem. Biophys. Res. Commun.* **46**, 1153 (1972).
14. A. M. Bobst, Y.-C. E. Pan, D. J. Phillips, *Biochemistry* **13**, 2129 (1974).
15. A. B. Bobst, *Fed. Proc.* **33**, 1486 (1974).
16. J. B. Lingrel, R. E. Lockard, R. F. Jones, H. E. Burr, J. W. Holder, *Ser. Haematol.* **4**, 37 (1971).
17. J. Gorski, M. R. Morrison, C. G. Merkel, J. B. Lingrel, *J. Mol. Biol.* **86**, 363 (1974).
18. We thank J. B. Lingrel for the rabbit globin mRNA and also S. A. Goldman for supplying the computer program and helpful discussion.

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Light-Induced Changes in the Structure of Pigmented Granules in Aplysia Neurons

Abstract. *Pigmented granules in* Aplysia *neurons prepared in the dark contain material that appears to be composed of 50-angstrom globules and a precipitate, probably a calcium salt. On illumination the globules rearrange into paracrystalline arrays and membrane-like lamellae. The morphologic transformation may be related to calcium release from the granules, and the released calcium may mediate the light-evoked hyperpolarization described by others.*

The giant neurons of *Aplysia* hyperpolarize in response to light and have been studied as model photoreceptors (*1, 2*). Brown and Brown (*2*) have shown that the hyperpolarizing light response is due to an increase in the K conductance (G_K) of the membrane; they suggested that the action of light on G_K may be mediated by an increase in the cytoplasmic Ca concentration for two reasons: (i) the effect of light on G_K was mimicked by $CaCl_2$ injection (*3*), and (ii) pressure injection of the Ca chelator ethylene glycol-bis(aminoethylether)-*N,N'*-tetraacetic acid(EGTA) abolished the photoresponse. Since orange-yellow granules in the cytoplasm contain a pigment with an absorption maximum (*4*) at the same wavelength as the peak of the action spectrum of the light response, 490 nm (*1, 2*), it seems likely that the pigment mediating the light response is in the granules. I report here that the pigmented granules (*5–7*) are capable of accumulating or binding divalent cations, and that their contents undergo a light-induced change in substructure from globular particles about 50 Å in diameter to membrane-like lamellae. These morphological observations in combination with the physiological evidence suggest that release of Ca from the pigmented granules may mediate the hyperpolarizing light response and underlie the transformation from globule to lamella.

Dark-adapted neurons were prepared by dissecting the visceral or pleural ganglia from *Aplysia californica* under illumination by a photographic safe light with a dark red (Kodak 1A) filter; the light faced the ceiling about 2 m from the preparation. Whole ganglia were incubated for various lengths of time in artificial seawater (ASW) (*8*) in total darkness; they were then transferred under red light to fixative (*9*) for 1 hour before individual neurons were dissected out with usual illumination for the dissecting microscope. The isolated cells were fixed for an additional hour. Illuminated neurons were prepared by dissecting the ganglia from animals in room light. Ganglia were incubated in ASW in open beakers under a fluorescent desk lamp containing two Sylvania F15 T8-CW tubes positioned about 0.5 m from the ganglia. This supplied about 800 erg cm^{-2} sec^{-1} over the range of wavelengths from 435 to 570 nm. The temperatures of the solutions bathing ganglia in the light and in the dark were the same (about 22°C), and the illuminated solution did not become warmer with time. After incubation the illuminated whole ganglion was fixed for 1 hour, and the cell was then isolated and fixed for 1 hour longer.

Figure 1a shows a pigmented granule in a neuron prepared in the dark. It is bounded by a membrane, and its contents appear uniform in texture and in some areas seem to be made up of globules approximately 50 Å in diameter. A portion of the granule is more electron-opaque, and in many granules the darker area contains variable amounts of a scattered fine precipitate (*10*). The areas of different apparent densities and the precipitate are seen in unstained sections and are therefore not dependent on lead or uranyl stains. When unstained thin sections were floated on drops of 0.1*M* EGTA in tris(hydroxymethyl)aminomethane (tris) buffer, *p*H 8, the precipitate in the granules was removed, although floating sections on water or tris buffer alone did not remove the precipitate. This suggests that the precipitate contains Ca. In cells fixed in Ca-saturated phosphate buffer, a precipitate also was found just inside the limiting membrane of some granules (Fig. 1b). In cells that had been immersed for 1 hour in the dark in saline solution in which $SrCl_2$ had been substituted for $CaCl_2$, some of the pigmented granules contained a coarser precipitate often located just inside the limiting membrane. This apparent deposition of Sr in the granules further suggests that the granules are capable of binding or accumulating divalent ions (*11*). A high proportion of the pigmented granules of cells incubated in the light are converted to forms containing arrays of membrane-like lamellae (Fig. 1c). Images that can be interpreted as intermediate forms also occur (Fig. 1d), especially in cells illuminated for short times. These include paracrystalline arrays of globules (single arrow in Fig. 1d), small vesicular profiles embedded in areas of globules (double arrow in Fig. 1d), and small vesicles embedded in areas of globular units adjacent to membrane-like arrays in the same granule. In such composite forms there is often precipitate in the portion of the granule that retains the globular from, although precipitate is not visible among the membranous lamellae. The lamellae formed in pigment granules as a result of exposure to light have a typical double leaflet appearance, and pentalaminar regions of apparent membrane fusion also occur (inset, Fig. 1). The proportion of granules converted to membrane-like forms increases with the duration of illumination and decreases on return of cells from light to dark during incubation in ASW. It is not clear, however, whether the configuration of a single granule recovers from the effect of light or whether light-altered granules are replaced by newly formed granules in the dark.