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Correspondence and requests for materials should be addressed to W.A. (e-mail: almers@mfz.mpmf-heidelberg.mpg.de).

Optical detection of a quantal presynaptic membrane turnover

Timothy A. Ryan*, Harald Reuter† & Stephen J Smith

Department of Molecular and Cellular Physiology, Stanford University Medical School, Stanford, California 94305, USA

† Department of Pharmacology, University of Bern, 3010 Bern, Switzerland

Exploration of the mechanisms and plasticity of synaptic transmission has been hindered by the lack of a method to measure single vesicle turnover directly in individual presynaptic boutons at isolated nerve terminals. Although postsynaptic electrical recordings have provided a wealth of invaluable basic information about quantal presynaptic processes¹, this approach has often proved difficult to apply at most central nervous system synapses^{2–6}. Here we describe the direct optical detection of single quantal events in individual presynaptic boutons of cultured hippocampal neurons. Using the fluorescent dye FM 1-43 as a tracer for presynaptic endocytosis^{7–10}, we have characterized

both evoked and spontaneous components of presynaptic function at the level of individual quanta. Our results are consistent with quantal interpretations of previous electrophysiological analyses^{1–6} and provide new information about the unitary membrane recycling event and its coupling to individual action potential stimuli, about spontaneous vesicle turnover at individual boutons, and about the numbers of vesicles recycling at individual boutons.

The strategy we used for optical detection of single presynaptic quanta builds upon earlier studies using the fluorescent dye FM 1-43 as a marker for synaptic vesicle turnover^{7–10}. So far there has been no direct evidence demonstrating that uptake and release of such a vesicle marker can occur in quantized units corresponding to single vesicles. Our approach has been to include refinements designed for the measurement of very weak presynaptic fluorescence signals. Single-molecule fluorescence detection^{11–14} has shown that detection of weak signals is limited primarily by the presence of background fluorescence in instruments and specimens. We used confocal laser scanning microscopy to minimize instrumental autofluorescence background, and sparse monolayer cell cultures and the briefest possible staining exposures to FM 1-43 to minimize specimen fluorescence backgrounds.

A scheme of our experimental protocol is shown at the top of Fig. 1. Figure 1a is a Nomarski image illustrating the site of a putative axo-dendritic synaptic junction (arrow). Figure 1b, c shows fluorescence images of the same field, acquired sequentially after FM 1-43 loading with a single action potential (AP) stimulus (image A in the schematic): both panels show a small area of bright fluorescence localized to the junction site—measurement reproducibility is excellent and is generally sustained for hours at unstimulated boutons. Figure 1d, e shows reduction in fluorescence produced by a long unloading train of APs (image B). The fluorescence signal ΔF is defined as the difference in bouton fluorescence measurements before and after unloading (that is, images A–B). This ΔF signal presumably represents the amount of dye taken up into a releasable vesicular pool during the specified load. A second series (Fig. 1f–h) with more stimulation during loading (20 AP) verifies the presence of a viable presynaptic bouton at the site of interest. The 20 AP load resulted in 16-fold greater uptake of releasable dye (Fig. 1i).

To test for the postulated quantization of FM 1-43 signals, we measured fluorescence values across a large number of minimally stimulated individual boutons. Figure 2a represents individual measurements from a representative experiment in which just 1 AP was stimulated: each vertical set of points represents three separate measurements from the same bouton (fluorescence after loading but before unloading, black circles; the residual non-releasable fluorescence measured after extensive unloading, white circles; and the intrinsic fluorescence of the synapse measured before loading (F_i), squares); solid, vertical lines represent ΔF , and dotted lines represent amplitudes of the residual fluorescence signal F_R . These data show that releasable fluorescence (ΔF) is ~ 10 times greater than F_i , but that F_R magnitude is in the same range as the minimal ΔF signals. Because F_R became visible after even the briefest dye exposures, regardless of whether any APs are fired, this weak, non-releasable fluorescence probably reflects a residual membrane staining with no special relationship to synaptic vesicle recycling. This interpretation of F_R is further supported by the lack of any significant bouton-by-bouton correlation between F_R and ΔF values (data not shown).

Figure 2b shows a histogram of the same ΔF values as in Fig. 2a. This histogram shows a distinct peak at a ΔF amplitude of 63 photoelectrons. There are also hints of secondary and tertiary peaks at double and triple that amplitude, but clearly the number of observations is inadequate for much certainty here. By pooling observations from several experiments, however, a multimodal response amplitude distribution becomes much more obvious. Figure 3a shows a frequency histogram of the different values of

* Present address: Department of Biochemistry, Cornell University Medical College, 1300 York Avenue, New York, New York 10021, USA.

ΔF measured from 409 individual boutons stimulated by 1, 2 or 3 APs. (The 2- and 3-AP stimulus runs were included to favour the possible occurrence of more multiple-quantum events.) The histogram shows a prominent peak centred at 60 photoelectrons, with higher-order peaks appearing precisely at integral multiples. This clearly multimodal response distribution is exactly as would be predicted from the hypothesis that releasable staining is taken up in quantal units corresponding to individual synaptic vesicles.

The solid line in Fig. 3a is a curve generated as the sum of four gaussian functions centred about exact integer multiples of the mean of the first peak (see Methods). The variance within individual peaks appears to be accounted for entirely by the expected photon shot-noise statistics associated with each measurement (see Methods). The peak spacing, F_Q , used to fit this histogram provides a factor that can now be used to calibrate any ΔF measurement in terms of an actual number of presynaptic quanta recycled ($N_Q = \Delta F/F_Q$). This calibration also indicates that the releasable fluorescence illustrated in Fig. 2b–e corresponds to a single quantum.

Our results may underestimate the numbers of quanta turned over by minimal AP stimuli. Dye exposures were limited here to ~ 7 s to minimize fluorescence backgrounds, whereas earlier work has shown that substantial AP-evoked endocytosis may trail stimulation with a half-time of as long as 20 s. Thus, this low-noise procedure may fail to label some number of late-endocytosing vesicles¹⁵.

It has long been known¹ that presynaptic quantal release can occur in a spontaneous as well as an evoked fashion. To characterize spontaneous vesicle turnover under our experimental conditions, we carried out experiments in which no APs were stimulated and in which calcium influx during any possible spontaneous APs (already minimized, if not eliminated by extracellular CNQX) were blocked by adding 100 μM extracellular Cd^{2+} . A dye exposure of 20 s gave the results shown in Fig. 3b, in which the frequency histogram of ΔF was measured at 212 different boutons from 5 different experiments. The distribution of ΔF values shows a prominent peak at 63 photoelectrons, and a second peak at twice that value. The positions of these peaks are similar to those obtained with AP stimulation (Fig. 3a). These data thus indicate that the endocytic uptake associated with spontaneous vesicular release and recycling is identical to that evoked by AP firing. These measurements also provide a means to assess frequencies of spontaneous release at individual boutons. Spontaneous recycling frequencies (f_s) were determined by measuring N_Q for a fixed dye exposure period, τ , from a population of identified presynaptic boutons. The frequency f_s , calculated for each bouton as N_Q/τ , varied considerably across boutons as well as different experiments. Average values of f_s ($\langle f_s \rangle$) determined from individual experiments spanned a tenfold range (0.01 s^{-1} – 0.1 s^{-1}).

This low frequency of spontaneous release indicates that spontaneous vesicle recycling must make only a very minor contribution

Figure 1 FM 1-43 visualization of vesicle turnover response to a single AP. Top, recycling vesicles were labelled by exposure to 15 μM extracellular FM 1-43 (Load) during AP stimulus. Thirty seconds of 20-Hz stimulation in a dye-free solution then released vesicular dye (Unload). **a**, A Nomarski image of hippocampal culture showing putative axo-dendritic synapse (arrow). Scale bar, 2 μm . **b**, Fluorescence of the same field following a single AP dye load; **c**, as **b**, but repeated 30 s later; **d**, **e**, as **b**, **c**, but after dye unloading; **f**, as **a**, at the beginning of a second loading/unloading sequence; **g**, as **b**, following a 20 AP load. The fluorescence signal is much brighter (although displayed here with lower-contrast gain) and additional fluorescent puncta are now apparent. **h**, As **g**, after unloading; **i**, comparison of ΔF (calibrated in photoelectrons) associated with 1 and 20 AP stimuli.

Figure 2 Fluorescence with a 1 AP loading stimulus, as measured from 67 individual boutons in a single experiment. **a**, Three measurements were made from each bouton: the intrinsic fluorescence before any dye exposure, F_i (filled squares); fluorescence after loading, F_L (filled circles); fluorescence after unloading, F_R (open circles). Length of solid vertical line corresponds to releasable fluorescence ΔF ; length of the dotted line to the dye-related residual fluorescence. **b**, A frequency histogram of the same ΔF data as in **a**.

to our measurement of evoked quantal activity. Figure 3c confirms this point: the number of quanta loaded at individual boutons by sequential stimulus episodes consisting of one and then five APs is in approximate proportion to the number of APs fired. If spontaneous quantal activity was important in dye uptake under these conditions, a disproportionately large signal would be expected from the single AP stimulus.

As a further test of the key expectation that F_Q should reflect the nature of the quantal event independently of its probability of occurrence, we reduced the probability of evoked release by using a low-calcium (0.5 mM) high-magnesium (3.5 mM) superfusate¹. Figure 3d shows that the resulting frequency histogram is still multimodal and that the quantal spacing F_Q is essentially unchanged from that observed at normal calcium/magnesium concentrations (2 mM each), even though the probability of release per AP was considerably reduced.

Estimates of the number of quanta composing the total recycling vesicle pool, N_{total} , were obtained by measuring the ratio $\Delta F_S/F_Q$ at individual boutons, where ΔF_S is the signal obtained for saturating load conditions (400 AP load). Figure 4a shows a representative frequency distribution of different N_{total} measured at 215 boutons

from three experiments and indicates that $\langle N_{\text{total}} \rangle = 127$. The fact that this number agrees closely with ultrastructural estimates of hippocampal vesicle pool sizes^{16,17} provides additional support for our conclusion that presynaptic ΔF quanta correspond to single presynaptic vesicles.

Measurements of quantal turnover per AP for brief-stimulus trains (7 AP), as well as N_{total} at the same synapses, were also made. For these experiments, longer dye exposures were used (Fig. 4) to minimize the possible underestimation of quantal turnover number, at the cost of modest but tolerable increases in background fluorescence noise. Data from such experiments indicate that the average fractional turnover of the entire pool per AP obtained in a single experiment was $0.47 \pm 0.03\%$ ($n = 54$), in close agreement with previous estimates obtained using macroscopic release assays of FM-1-43-labelled vesicles at hippocampal boutons¹⁸. A comparison of the quantal turnover to N_{total} at each synapse in a given experiment reveals a strong correlation between these two quantities (Fig. 4b). These results suggest that a single AP results in the uptake of between 0 and 3 quanta per bouton, with the average turnover across all competent boutons being 0.80 ± 0.08 quanta per AP. (Because of the possibility that turnover is still underestimated

Figure 3 Minimally stimulated fluorescence signal amplitudes. **a**, Histogram of ΔF values from 8 experiments (1, 2 or 3 AP load at 0.3 Hz). Solid line is a multiple gaussian with peaks at equal 60-photoelectron intervals and widths based on a photodetection noise model (see Methods). Dotted line is a single gaussian fitted to the background noise distribution (see Methods). **b**, Histogram of ΔF values from spontaneous vesicle turnover (212 boutons, 5 experiments). Solid-line fit from equation (1) (α_3, α_4 , both 0; $\mu = 63$ photoelectrons). **c**, Scaling of quantal turnover with AP number. Sequential measurements of ΔF from 1 AP and 5 AP loads of the same bouton were binned by nearest integral value of N_Q for 1 AP load ($n_1 = 44$, $n_2 = 28$, $n_3 = 7$, errors, s.e.m.). Dotted line has a slope of 5. **d**, Histogram for uptake evoked by 4, 5, 6 or 7 AP loads in low- Ca^{2+} , high- Mg^{2+} saline (119 synapses, 4 experiments). Solid line from equation (1), with $\mu = 58$ photoelectrons.

Figure 4 Estimates of total recycling pool size and fractional release per AP. **a**, Pool size distribution. The distribution of pool sizes (N_{total}) is shown in 3 experiments measuring each of 215 boutons. **b**, Quantal turnover/AP versus pool size. Three sequential measurements of ΔF were used to determine F_Q (1 AP), N_Q /AP (7 AP) and N_{total} (400 AP) in a single experiment. For the latter two experiments, dye exposures were extended to 15 and 60 s respectively. The scatter plot of N_Q /AP versus the total pool size N_{total} indicates a correlation between these two quantities (correlation coefficient, 0.68).

owing to the brevity of dye exposure relative to endocytosis delays, these number should be considered as lower limits.) If a strict, one-to-one coupling of unitary endocytic and exocytic events is assumed, our results indicate that a single AP may sometimes lead to multiple exocytic events at a single bouton^{19–21}. It is possible, however, that boutons with higher release rates actually comprise multiple active zones, and that evoked release per active zone might be limited to a maximum of one vesicle.

In summary, we have shown that dye uptake occurs in quantal steps during evoked and spontaneous presynaptic function, that such steps have the magnitude expected for uptake into a single synaptic vesicle, and that such endocytosis can proceed in minimum steps of one single quantum. Our findings strongly support endocytic vesicle recycling models of presynaptic function in central synapses. Until now, the idea that presynaptic endocytosis occurs in steps corresponding to single vesicles has been based on ultrastructural observations. The observed temporal proximity of single-quantum dye uptake events to single-AP stimuli also suggests that unitary exocytic and endocytic events are coupled closely in some way. Our optical method for presynaptic quantal analysis has several features that are likely to make it a very important complement to postsynaptic electrophysiological approaches: first, it is inherently insensitive to postsynaptic factors such as receptor saturation and modulation that can complicate interpretation of postsynaptic responses^{5,6,20,22}; second, it has an inherent ability to discriminate function at individual boutons, which is difficult with electrophysiological measurements^{19,23,24}; third, it offers high throughput because tens or hundreds of individual boutons can be analysed in parallel in a single imaging experiment; and finally, it is both convenient and amenable to long-term experimentation because no recording pipette needs to be introduced. We expect that these features will prove highly useful in the ongoing analysis of presynaptic function and plasticity.

Note added in proof: In a recent report²⁵ another example of multimodal FM1-43 distribution is indicated in the inset of Fig. 2. □

Methods

Most experimental details have been described¹⁸. Briefly, hippocampal CA1–CA3 regions were dissected from 4-day-old Sprague–Dawley rats, dissociated and plated onto Matrigel-coated coverslips and cultured for 3–5 weeks before use. Coverslips were mounted in a laminar-flow microscope chamber and continuously superfused with a saline solution consisting of 119 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 25 mM HEPES (buffered to pH 7.4), 30 mM glucose. Synaptic transmission was blocked by 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Research Biochemicals) 50 μM D,L-2-amino-5-phosphonvaleric acid (AP-5; Research Biochemicals) to prevent recurrent firing of unstimulated APs. Low-Ca²⁺ saline consisted of 0.5 mM CaCl₂ and 3.5 mM MgCl₂, but otherwise identical components. The dye-loading solution contained 15 μM FM 1-43 (Molecular Probes). For experiments to resolve individual quanta, dye exposures were kept brief (typically 7 s) to minimize background membrane staining. FM1-43 does not affect frequencies or amplitudes of spontaneous excitatory post-synaptic currents (EPSCs) measured in separate electrophysiological experiments (A. Bouron and H.R., unpublished results), indicating that the dye does not alter presynaptic function. APs were stimulated by passing 1-ms current pulses between electrodes placed at opposite ends of the perfusion chamber. In control experiments in which cells were stained with Fluo-3 AM, calcium imaging showed that neurons in our hippocampal cultures fired APs in response to 1-ms current pulses at a threshold field of ~4 V cm⁻¹ in both normal and low-Ca solutions. For all FM 1-43 experiments, field strengths of 10 V cm⁻¹ were used to ensure reliable action-potential firing. Simultaneous fluorescence and Nomarski imaging was performed with a modified Bio-Rand MRC 500 laser scanning unit coupled to a Zeiss IM-35 inverted microscope and a Nikon 40× 1.3 NA Fluor objective. Regions of culture coverslips were selected for imaging using a requirement that most boutons be in focus within a single image plane, as determined using both fluorescence and Nomarski images. If a few boutons lay outside a single focal plane, those boutons were eliminated from subsequent

analyses. For each image measurement, 2 frames were averaged using a dwell time of ~1.5 μs per pixel, and a sampling frequency of ~150 nm per pixel. The attenuated beam from an argon ion laser (200 microwatts at 488 nm) was used to slightly underfill the objective pupil. Fluorescence signals were corrected for dark current and calibrated in photoelectrons, using a factor obtained from fluctuation analysis of the photomultiplier output at different illumination intensities. Comparison of images like those in Fig. 1b, c with similarly acquired images of a subresolution fluorescent bead (50 nm diameter; data not shown) demonstrated that the apparent spatial extent of the synaptic fluorescence punctum is exactly as expected for a subresolution object such as a 50-nm vesicle, given the resolution limits of this confocal microscope.

Image analysis. Fluorescence measurements were made by averaging signals over a 4 × 4 pixel (0.36 μm²) region centred over bouton images. Boutons were selected for analysis of minimal-stimulus response based on their identification using long loading and unloading trains carried out at the end of each experiment. Boutons for which the fluorescence image shifted laterally by more than 4 pixels during the unloading (~10–20% of boutons in the field, probably resulting from motion of residual fluorescence) were also rejected. Boutons whose ΔF signal was within 1.5 standard deviations of the mean of the background noise distribution (≤26 photoelectrons) were considered to be below threshold and were eliminated, as were any boutons whose initial fluorescence values saturated the 8-bit digitization scale. The number of boutons scored as below-threshold decreased in proportion to the magnitude of the stimulus in a given experiment. Photodetection noise and the difference signal attributable to photobleaching were estimated from analysis of image-to-image variations across serially repeated images of minimally stained boutons. Noise data from 97 boutons obtained in this way were fitted with a single gaussian with s.d. = 14.5 photoelectrons. The photobleaching rate was ~5% per image. ΔF histograms were fitted with sums of gaussian curves with equally spaced peaks and widths set to approximate the variance expected from a poisson model of photodetection noise statistics as follows:

$$\sum_{i=1}^4 \frac{\alpha_i}{\sqrt{2\pi(i+2)\mu}} e^{-\frac{1}{2} \frac{(x-\mu)^2}{(i+2)\mu}} \quad (1)$$

where μ is the integral spacing of the means, α_i is the amplitude of each gaussian, and x is the position along the ΔF axis. The gaussian approximation of the underlying poisson process is valid as μ > 10. The standard deviation of each gaussian, i, was set to $\sqrt{(i+2)\mu}$, the expected dispersion arising from the two sequential measurements: image A consists of fluorescence from i stained vesicles, each contributing μ photoelectrons, plus a residual equal to ~μ photoelectrons; image B consists only of the residual of μ photoelectrons. As the histograms consist of a difference measurements of A–B, the total variance is the sum of variances from each measurement ((i × μ + μ) + μ). The unconstrained variables in this case are the individual amplitudes for each peak (α_i), and the centre of the first peak, μ. In contrast to classical quantal analysis¹, in which measurements on a given synapse are repeated serially, the peak amplitudes cannot be derived from poisson statistics as we are performing only a single measurement on each of an array of synapses of unknown statistical homogeneity.

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Correspondence and requests for materials should be addressed to T.A.R. at Cornell (e-mail: taryan@mail.med.cornell.edu).

Cloning and characterization of a mammalian proton-coupled metal-ion transporter

Hiromi Gunshin^{*}, Bryan Mackenzie^{*}, Urs V. Berger^{*}, Yoshimi Gunshin^{*}, Michael F. Romero[†], Walter F. Boron[‡], Stephan Nussberger^{*}, John L. Gollan[‡] & Matthias A. Hediger^{*§}

^{*} Renal Division, Department of Medicine, Brigham & Women's Hospital and Harvard Medical School, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115, USA

[†] Department of Cellular & Molecular Physiology, Yale University, New Haven, Connecticut 06510, USA

[‡] Gastroenterology Division, Department of Medicine, Brigham & Women's Hospital, and [§] Department of Biological Chemistry & Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, USA

Metal ions are essential cofactors for a wealth of biological processes, including oxidative phosphorylation, gene regulation and free-radical homeostasis. Failure to maintain appropriate levels of metal ions in humans is a feature of hereditary haemochromatosis¹, disorders of metal-ion deficiency, and certain neurodegenerative diseases². Despite their pivotal physiological roles, however, there is no molecular information on how metal ions are actively absorbed by mammalian cells. We have now identified a new metal-ion transporter in the rat, DCT1, which has an unusually broad substrate range that includes Fe²⁺, Zn²⁺, Mn²⁺, Co²⁺, Cd²⁺, Cu²⁺, Ni²⁺ and Pb²⁺. DCT1 mediates active transport that is proton-coupled and depends on the cell membrane potential. It is a 561-amino-acid protein with 12 putative membrane-spanning domains and is ubiquitously expressed, most notably in the proximal duodenum. DCT1 is upregulated by dietary iron deficiency, and may represent a key mediator of intestinal iron absorption. DCT1 is a member of the 'natural-resistance-associated macrophage protein' (Nramp) family^{3–5} and thus its properties provide insight into how these proteins confer resistance to pathogens.

In mammals, iron is thought to be transported into cells by transferrin-receptor-mediated endocytosis. However, since the available apo-transferrin in the intestinal lumen (that arising from biliary excretion) is insufficient to account fully for dietary iron

absorption⁶, it is likely that other non-receptor-mediated uptake systems exist in the intestine. Dietary iron comprises two forms, haem and non-haem iron. The bulk of intestinal non-haem iron is absorbed in the first portion of the duodenum, in which the acidic environment promotes solubilization of iron rendered in its 2+ reduced state by ferriredutase^{7,8} and ascorbate^{9,10}. How Fe²⁺ is subsequently absorbed however is poorly understood. The divalent cation transporters identified so far include the mono- or oligo-specific transporters derived from plants and yeast¹¹. In addition, mammalian divalent cation export systems have been described for zinc¹² and copper¹³, but a mammalian uptake system has not yet been identified.

We report the expression cloning, tissue distribution and initial characterization of a divalent-cation transporter, DCT1. By screening for iron uptake activity in oocytes, we isolated the DCT1 complementary DNA from a cDNA library prepared using duodenal messenger RNA from rats fed on a low-iron diet (Fe(-)D). *Xenopus* oocytes injected with Fe(-)D mRNA showed a 7-fold increase in ⁵⁵Fe uptake compared with water-injected (control) oocytes (Fig. 1). Following size-fractionation, maximal ⁵⁵Fe uptake activity was induced by a 4.0–4.5-kilobase (kb) mRNA fraction, from which we isolated a single cDNA (DCT1) which stimulated ⁵⁵Fe²⁺ uptake 200-fold (Fig. 1). The 4,409-base-pair (bp) DCT1 cDNA encodes a 561-amino-acid protein with 12 putative membrane-spanning domains (Fig. 2), predicted glycosylation sites in the fourth extracellular loop, and a consensus transport motif in the fourth intracellular loop^{3–5}. In bacteria, this motif is believed to participate in the interaction with ATP-coupling subunits; however its function is unknown and it is distinct from the nucleotide-binding fold of ABC transporters.

High-stringency northern-blot analysis of DCT1 transcripts (Fig. 3a) revealed prominent bands of 4.5 kb from proximal intestine, kidney, thymus and brain, and fainter bands in testis, liver, colon, heart, spleen, skeletal muscle, lung, bone marrow and stomach. By loading an equal amount of each mRNA, we found that DCT1 was expressed at a much higher level in proximal intestine than in

Figure 1 Uptake of 10 μ M ⁵⁵Fe in *Xenopus* oocytes injected with poly(A)⁺ RNA from normal or iron-deficient (Fe(-)D) rat duodenum or with RNA synthesized from DCT1 cDNA. Data are mean $\pm$ s.e.m. from 6–10 oocytes. Inset, relative ⁵⁵Fe uptake in oocytes injected with size-fractionated poly(A)⁺ Fe(-)D RNA. C, Control water-injected oocytes; 1, 2.0–3.0 kb poly(A)⁺ RNA; 2, 2.5–3.5 kb; 3, 3.3–4.0 kb; 4, 3.8–4.5 kb; 5, 4.4–5.7 kb; 6, 4.5–6.0 kb; 7, 4.8–6.4 kb; 8, 5.5–7.0 kb; and T, unfractionated poly(A)⁺ Fe(-)D RNA.