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Binding of an RNA Trafficking Response Element to Heterogeneous Nuclear Ribonucleoproteins A1 and A2*

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Jianguo Shan, Kim Moran-Jones, Trent P. Munro, Grahame J. Kidd, Donald J. Winzor, Keith S. Hoek, and Ross Smith‡

From the Biochemistry Department, The University of Queensland, Queensland 4072, Australia

Heterogeneous nuclear ribonucleoprotein (hnRNP) A2 binds a 21-nucleotide myelin basic protein mRNA response element, the A2RE, and A2RE-like sequences in other localized mRNAs, and is a *trans*-acting factor in oligodendrocyte cytoplasmic RNA trafficking. Recombinant human hnRNPs A1 and A2 were used in a biosensor to explore interactions with A2RE and the cognate oligodeoxyribonucleotide. Both proteins have a single site that bound oligonucleotides with markedly different sequences but did not bind in the presence of heparin. Both also possess a second, specific site that bound only A2RE and was unaffected by heparin. hnRNP A2 bound A2RE in the latter site with a K_d near 50 nM, whereas the K_d for hnRNP A1 was above 10 μM. UV cross-linking assays led to a similar conclusion. Mutant A2RE sequences, that in earlier qualitative studies appeared not to bind hnRNP A2 or support RNA trafficking in oligodendrocytes, had dissociation constants above 5 μM for this protein. The two concatenated RNA recognition motifs (RRMs), but not the individual RRMs, mimicked the binding behavior of hnRNP A2. These data highlight the specificity of the interaction of A2RE with these hnRNPs and suggest that the sequence-specific A2RE-binding site on hnRNP A2 is formed by both RRMs acting *in cis*.

The family of more than 20 heterogeneous nuclear ribonucleoproteins (hnRNPs)[1] appears to play diverse roles in the post-transcriptional processing of hnRNA and subsequent packaging, transport, and translation of mRNA (1–4). hnRNPs A1, A2, B1, B2, C1, and C2 are the major components of 40S "core particles," studied primarily in HeLa cells, which are thought to package nascent ssRNA in the nucleus in a histone-like fashion (5–10). The hnRNP A/B proteins have a modular structure with two N-terminal RNA recognition motifs (RRMs) followed by a glycine-rich region (Fig. 1) (11). The three-dimensional structures of the tandem RRMs of hnRNP A1, which are assumed to be the principal mediators of the RNA-protein interactions, have been determined (12, 13). Although the three-dimensional structures of other hnRNPs of the A/B family have not been determined, their RRMs share sufficient sequence identity with those of hnRNP A1 to suggest that they share the same tertiary fold: a four-stranded antiparallel β-sheet flanked by two helices. This module adopts a comparable fold in the RRMs of more distantly related proteins, such as U1A (14–17).

Although a number of the hnRNPs have a general role in intranuclear packaging of RNA, some also manifest sequence-specific binding (1, 4, 18–22). In addition to being a major component of core particles (23), hnRNP A2 binds a telomeric sequence (24, 25, 43) and a small RNA segment known to be necessary and sufficient for transport of the message encoding myelin basic protein in the cytoplasm of oligodendrocytes, the A2RE (26–28). The binding of hnRNP A2 to A2RE has been shown, by mutational and antisense approaches, to be essential for cytoplasmic RNA trafficking in oligodendrocytes (29).

Although hnRNP A2 has been shown to bind A2RE (29–31), the number of binding sites, their affinities, and location are unknown. We have used a resonant mirror biosensor and UV cross-linking gel mobility shift assays to investigate the binding of the A2RE and other oligoribonucleotides to recombinant human hnRNPs A1 and A2 and rat hnRNP A2. These proteins are shown to possess a single site that can associate with oligonucleotides with widely different base sequences. Both also possess a second site that is specific for A2RE, with considerably stronger binding to hnRNP A2 than hnRNP A1. Heparin inhibits the nonspecific interaction but not the binding of the A2RE to its specific site. We also show that an oligonucleotide consisting of the 5′ 11 nucleotides of A2RE binds as strongly as the 21-mer, thereby defining more precisely the dimensions of the segment of the A2RE that is recognized by hnRNP A2 and identifying one of the smallest single-element RNA-localization motifs. Finally, experiments with expressed and purified modules of hnRNP A2 indicate that the specific A2RE-binding site is formed by the two RRMs acting in concert, whereas single RRMs are capable of interacting only weakly or nonspecifically with RNA.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—hnRNP A1 was expressed in *Escherichia coli* and purified from the centrifuged cell lysate by passage through columns of DEAE-cellulose (Whatman Bioscience, Cambridge, United Kingdom), AGPoly(U) (Amersham Pharmacia Biotech), and phenyl-Superose (Amersham Pharmacia Biotech) columns (32). Human hnRNP A2 was expressed in *E. coli* and purified as described previously (29). Rat hnRNP A2 was isolated from brain extracts by using superparamagnetic particles bearing A2RE (30). Briefly, brains were removed from 21-day-old Wistar rats and homogenized. The homogenate was centrifuged at 13,000 × *g* for 25 min at 4 °C, and the supernatant was collected. 0.5 mg of streptavidin-coated magnetic particles (Roche Molecular Biochemicals) was incubated on ice for 10 min with 2 μg of biotinylated A2RE or dA2RE. Unbound RNA was washed off, and about 5 mg of brain protein was added to the magnetic particles and incubated for 30 min on ice. Heparin was used to reduce nonspecific binding. Then

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‡ To whom correspondence should be addressed: Biochemistry Dept., University of Queensland, Qld 4072, Australia. Tel.: 61-7-3365-4627; Fax: 61-7-3365-4699; E-mail ross@biosci.uq.edu.au.

[1] The abbreviations used are: hnRNP, heterogeneous nuclear ribonucleoprotein; A2RE, 21-ribonucleotide hnRNP A2 response element; A2RE11, 5′ 11-ribonucleotide segment of A2RE; HPLC, high pressure liquid chromatography; RRM, RNA recognition motif.

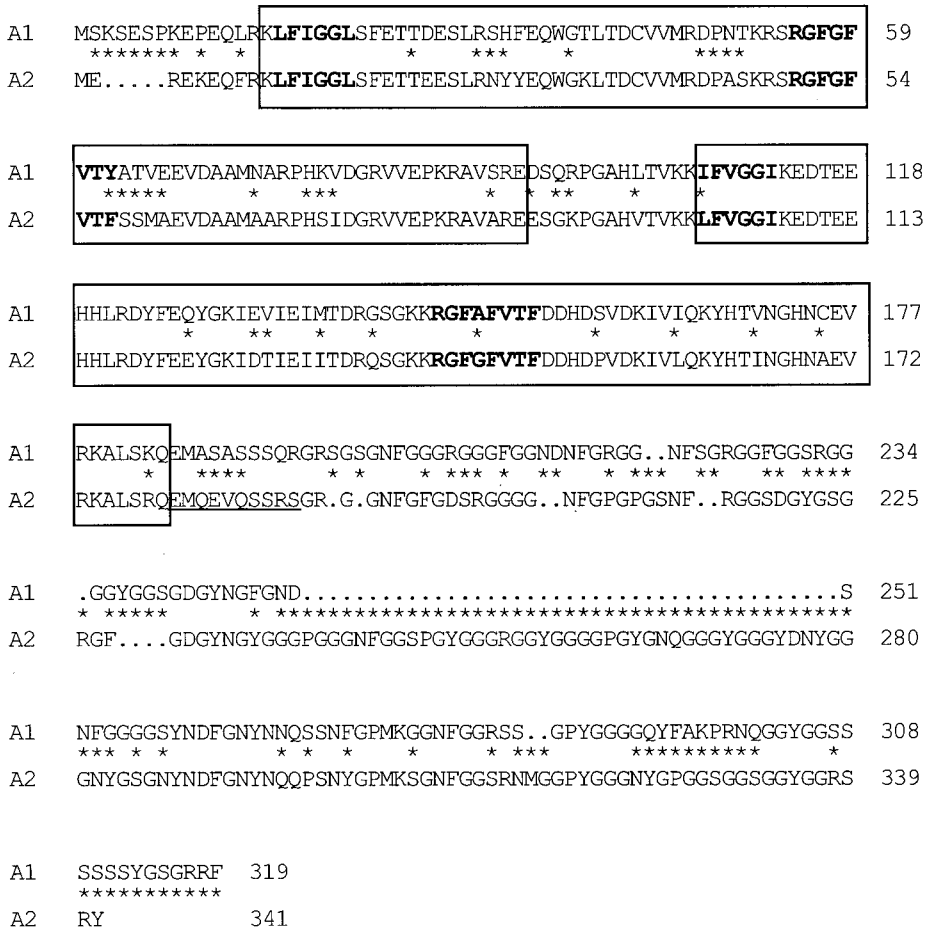

FIG. 1. **Comparison of the primary structures of hnRNPs A1 and A2.** These proteins have two RRM domains, in *boxes*, followed by a glycine-rich domain. The two highly conserved sequences within the RRMs, the hexameric RNP2 and the octameric RNP1, are in *bold letters*. *Asterisks* mark non-identical residues, and the residues 180–189 of hnRNP A2 are *underlined*. The RRM domain limits are those of Kozu *et al*. (50).

the particles were washed to remove unbound proteins, and the bound proteins were released by incubation in 100 μl of 30% acetonitrile, 0.1% trifluoroacetic acid and purified by reverse-phase HPLC. Segments of hnRNP A2 were expressed, also in *E. coli* BL21(DE3), with cleavable hexahistidine and S·Tag™ using the vector pET30b+ (Novagen, Madison, WI). The human hnRNP A2 fragments generated were residues 1–89, 101–179, 1–179, and 1–189, each with the N-terminal extension Ala-Met-Ala-Ile-Ser from the expression vector. Chromatography on a metal affinity resin (Talon, CLONTECH, Palo Alto, CA) was used to isolate the fusion proteins from cell lysates, and they were then cleaved with 1 unit of recombinant enterokinase (Novagen)/50 μg protein for 16 h at 25 °C. The resultant proteins were purified by reverse-phase HPLC on a C18 column (Vydac, Hesperia, CA) using gradients of acetonitrile in 0.1% trifluoroacetic acid. Fractions containing protein were lyophilized and finally dissolved in water. The identity and purity of all proteins was established by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and by electrospray mass spectrometry on a PerkinElmer Sciex 165 spectrometer: all were judged to be better than 90% pure. Rat hnRNP A2 had a mass of 36,086 ± 10: as the calculated average mass for rat hnRNP A2 is 36,040 (including assumed N-terminal acetylation as the protein is known to be N-terminally blocked), it appears that this protein has additional post-translational modification, the nature of which cannot be deduced from the mass values alone. In parallel experiments in which rat brain hnRNP A2 was substituted for the recombinant human protein, the biosensor responses were quantitatively similar, suggesting that the post-translational modification of the rat protein does not strongly influence oligonucleotide binding.

Oligonucleotides—A2RE (GCC AAG GAG CCA GAG AGC AUG), A2RE11 (GCC AAG GAG CC), NS1 (CAA GCA CCG AAC CCG CAA CUG), NS2 (AGA AAG AGA CCC UCA CGA CUG), corresponding oligodeoxyribonucleotides (dA2RE and dNS1), and A2RE11 oligoribonucleotides mutated in single positions (C3G, G7A, A8G, G9A (29)) were synthesized and purified by Oligos Etc. (Wilsonville, OR) with or without 3′ biotinylation. The concentrations of oligonucleotide solutions were calculated from the measured absorbance at 260 nm (33) with allowance for the presence of biotin where appropriate.

Biosensor Preparation—An IAsys resonant mirror biosensor (Affinity Sensors, Cambridge, UK) was used to determine the equilibrium affinities for the interactions of hnRNPs with A2RE and other related oligonucleotides, NS1 and NS2 being chosen as controls to distinguish specific from nonspecific binding. Although it is customary to maximize the response by attaching the smaller of the two interacting molecules to the biosensor surface, in some experiments we chose to immobilize the hnRNP A2 (M_r 36,000) rather than the RNA (M_r 7200 maximum). Immobilization of the protein not only avoided the possibility of degradation of the potentially labile RNA on the sensor surface but also allowed investigation of the binding of different RNAs on a single cuvette. Purified hnRNP A2 was covalently attached to the carboxymethyldextran-coated sensing surface of the biosensor cuvette using the standard 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide/*N*-hydroxysuccinimide (Advanced Laboratory Solutions, Melbourne, Australia) procedure for random coupling via amino groups of the protein (34). The protein was prepared as 0.1 g/liter solutions at pH 5.0 in 10 mM acetate buffer for attachment to the cuvette; sedimentation equilibrium experiments yielded a linear ln c *versus* r^2 plot with a slope that indicated that hnRNP A2 has a molecular weight of 36,000 ± 4000 under these conditions. It may therefore be assumed that the oligonucleotides bind to monomeric protein. After removal of the uncoupled hnRNP A2 by washes with phosphate-buffered saline (10 mM sodium phosphate, 138 mM NaCl, 2.7 mM KCl), pH 7.4, containing 0.05% v/v Tween 20 (Sigma) (PBS/T), the residual activated ester groups were inactivated by exposure to ethanolamine. The cuvette was then washed with PBS/T buffer containing 2 M NaCl to remove any noncovalently bound protein, a step that had no effect on the ability of the immobilized hnRNP to interact with the A2RE. The typical response of 1200–2600 arc s for the immobilized human hnRNP A2 on different cuvettes corresponds to the coupling of 8–17 ng/mm^2 of sensor surface. The corresponding values for the rat protein were 3000 arc s and 20 ng/mm^2.

In other experiments biotinylated oligodeoxyribonucleotides were attached to cuvettes bearing avidin (Sigma) prepared as discussed below. The hnRNPs appear to have equal affinity for equivalent oligoribonucleotides and oligodeoxyribonucleotides, and the use of 2′-deoxynucleotides therefore yields more stable biosensors without affecting

the binding experiments. After washing the biosensor cuvette with PBS/T, equivalent amounts of biotinylated dA2RE or dNS1 were added to cuvettes and left until the responses reached equilibrium (~20 min). 5 mM NaOH was used to remove unbound oligonucleotide. The cuvettes were then washed extensively with PBS/T to establish a base line before hnRNP solutions of different concentrations in PBS/T were added.

Binding of hnRNP to Immobilized Oligonucleotide—Because of the limited solubility of the hnRNPs and the fragments of hnRNP A2, the protein solutions were prepared by 50–100-fold dilution of concentrated stock solutions into PBS/T shortly before use in the biosensor. Freshly diluted solutions were used for each measurement. The concentrations of the proteins in these solutions were determined by amino acid analysis of 24-h acid hydrolsates with norleucine as the internal standard; the amounts of several of the stable amino acids were used with the known amino acid sequences to calculate the molar concentrations. Binding of protein was monitored at 0.4-s intervals at 25 °C in PBS/T until equilibrium had been attained (~15 min). After binding measurements, removal of the cuvette contents was followed by a rinse with PBS/T. The cuvettes were regenerated by removal of bound protein with 5 mM NaOH before washes with PBS/T to re-establish the base line for the next experiment.

Binding of RNA to Immobilized hnRNP A2—PBS/T (100 μl) was first placed in the hnRNP A2-modified cuvette and the response monitored for 5 min to establish a base line, after which aliquots of a stock solution of RNA in PBS/T were added to achieve a range of concentrations in the liquid phase. The binding of RNA was monitored routinely for 10–15 min, by which time equilibrium had been attained. Removal of the cuvette contents was followed by rinsing with PBS/T. The cuvettes were regenerated by removal of bound RNA with PBS/T containing 2 M NaCl before washes with PBS/T to re-establish the base line for the next experiment. Competition experiments followed the same general protocol except that mixtures containing a fixed concentration of A2RE (1 μM) and a range of soluble hnRNP A2 concentrations were placed in the cuvette for monitoring of the association phase until attainment of equilibrium. Subsequently, the cuvette was washed with PBS/T-2 M NaCl to remove the RNA and protein, and the base line re-established after washing the cuvette with PBS/T. In a third series of experiments designed to determine the effect of heparin on interaction between oligoribonucleotides and immobilized hnRNP A2, the glycosaminoglycan (from porcine intestinal mucosa, sodium salt, M_r 500 000; Sigma) was included in the buffer at a concentration of either 1 or 10 g/liter.

Analysis of Data—In keeping with the procedure adopted in most biosensor studies with the IAsys instrument (35), the approximation has been made that the extent of complex formation between added oligoribonucleotide (O) and the immobilized hnRNP A2 is sufficiently small to allow substitution of the total RNA concentration $(C_O)_{tot}$ for its free counterpart, C_O. The reliability of this approximation was checked by means of the following expression (Equation 1),

$$C_O = (C_O)_{tot} - (100V)[R_{eq}/(M_O\ 0.8 \times 815{,}000)] \quad \text{(Eq. 1)}$$

where V is the volume of liquid in the cuvette (μl), M_O is the RNA molecular weight (7200 for A2RE), and the calibration constant is taken as 80% of that for proteins (36) to accommodate the difference between the specific refractive increments of protein and nucleic acids.

Estimates of thermodynamic dissociation constant, K_d, for the interaction of oligoribonucleotides with immobilized hnRNP A2 were obtained by nonlinear regression analysis of the concentration dependence of the equilibrium response, R_{eq}, in terms of the rectangular hyperbolic relationship (see Equation 2),

$$R_{eq} = R_{max}C_O/(K_d + C_O) \quad \text{(Eq. 2)}$$

to obtain as two curve-fitting parameters the magnitudes of K_d and R_{max}, the maximal response associated with saturation of all immobilized hnRNP A2 sites (37). An equivalent analysis was performed for experiments in which oligodeoxyribonucleotide was immobilized on the biosensor cuvette.

Because the equilibrium constant in Equation 2 refers to the interaction of oligoribonucleotide with a chemically modified (immobilized) form of hnRNP A2, competition experiments were performed to obtain a quantitative characterization that refers unequivocally to the interaction between A2RE and hnRNP A2 in solution. Competitive binding assays involving the measurement of binding responses for mixtures of oligoribonucleotide and hnRNP A2 were analyzed by determining the free oligoribonucleotide concentration, C_O, on the basis of the measured equilibrium response and a calibration plot, R_{eq} *versus* C_O, constructed from the data for oligoribonucleotide alone. Knowledge of C_O for a mixture with total oligoribonucleotide and hnRNP A2 concentrations,

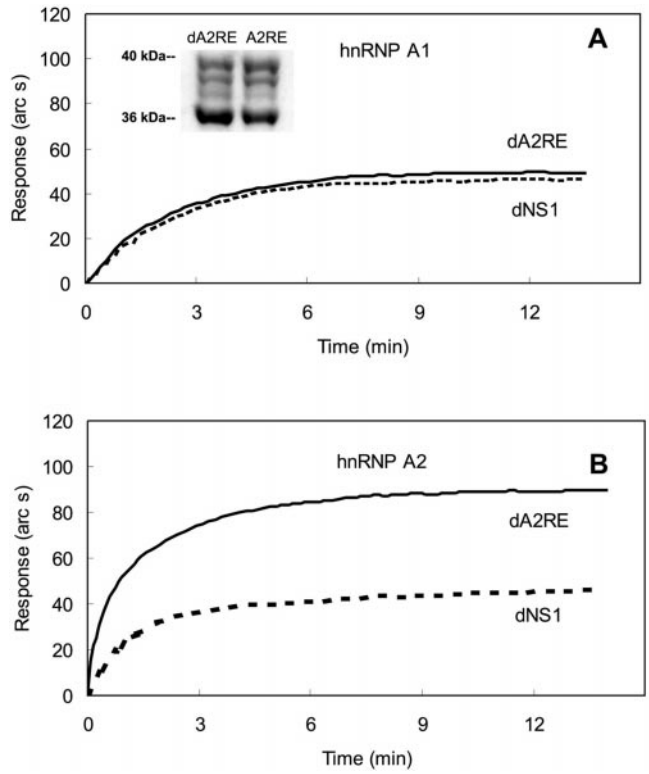

FIG. 2. **hnRNP A2 shows sequence-specific oligonucleotide binding in biosensor.** dA2RE and dNS1 were immobilized on the two compartments of a single biosensor cuvette; for both the biosensor response on addition of the oligonucleotide was 260 arc s, indicating that equal amounts were immobilized. Solutions of hnRNPs A1 and A2 in PBS/T were added, in separate experiments, to yield a final concentration of 4 μM. The data reveal a 2-fold greater binding of hnRNP A2 (*B*) compared with hnRNP A1 (*A*) to dA2RE. hnRNP A1 binds the nonspecific oligonucleotide dNS1 and dA2RE equally. In this experiment the immobilized oligonucleotides were DNA rather than RNA, to lessen the chances of degradation. *Inset* in *A*, Coomassie Blue-stained SDS-polyacrylamide gel showing equivalent binding of rat brain hnRNP A2 (36 kDa) to A2RE and dA2RE. The positions of standard proteins are shown at *left*. Biosensor experiments (Figs. 5 and 6) have also shown that the binding to the hnRNPs is not affected by the presence or absence of the 2′-OH group.

$(C_O)_{tot}$ and $(C_H)_{tot'}$ respectively, allowed evaluation of the binding function, r (see Equation 3),

$$r = [(C_O)_{tot} - C_O]/(C_H)_{tot} \quad \text{(Eq. 3)}$$

Analysis of the resulting [r, C_O] data in terms of the rectangular hyperbolic relationship (see Equation 4)

$$r = n(C_O)/(K_d + C_O) \quad \text{(Eq. 4)}$$

was then used to determine not only the dissociation constant for the interaction between oligoribonucleotide and hnRNP A2 in solution but also n, the number of binding sites for oligoribonucleotide on hnRNP A2.

Electrophoretic Mobility Shift Assays—Recombinant and rat proteins were obtained as described above. Rat brain proteins to be used in UV cross-linking experiments were removed from A2RE-linked magnetic particles by incubation in 300 mM $MgCl_2$ for 1 h at 4 °C. Eluted proteins were dialyzed against 10 mM HEPES, pH 7.5, containing 40 mM NaCl and 5% glycerol and used directly or subjected to reverse-phase HPLC to isolate the hnRNP A2.

Equimolar amounts of oligoribonucleotide, ^{32}P-labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, MA), and equimolar amounts of each protein were mixed in binding buffer (10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 4% w/v gylcerol, 0.1% w/v Triton X-100, and 1 mM dithiothreitol) and incubated for 30 min on ice. The reaction mixtures were then irradiated with 250 mJ of 254 nm light in a Bio-Rad GS Genelinker UV chamber. These samples were run on 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels that were exposed to autoradiography film.

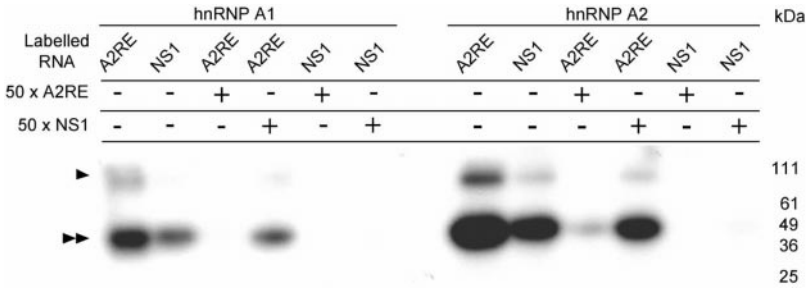

FIG. 3. **UV cross-linking experiments show sequence-specific A2RE binding.** Electrophoretic mobility-shift assays show binding of A2RE and NS1 to hnRNPs (band marked by *double arrowhead*). 1.4 nmol of ^{32}P-labeled A2RE or NS1 oligoribonucleotides were added separately to purified 1 pmol of recombinant hnRNPs A1 or A2 and UV cross-linked, in the absence of heparin, prior to analysis by SDS-polyacrylamide gel electrophoresis and autoradiography. Competition experiments show that, for both proteins, a 50-fold excess of unlabeled A2RE competes with both labeled A2RE and NS1, whereas a 50-fold excess of unlabeled NS1 competes only with labeled NS1. The bands of unbound oligonucleotide, which ran with the marker dye, are not shown. The upper bands visible in most lanes (*single arrowhead*) were not obtained consistently and may arise from dimer formation The positions of marker proteins, with molecular masses in kilodaltons, are shown at *right*.

RESULTS

hnRNP A2 Possesses Sequence-specific and Nonspecific RNA-binding Sites—Biosensor data (Fig. 2) were recorded for non-saturating concentrations of hnRNPs A1 and A2 (purity shown in Fig. 10) with A2RE and an equivalent oligonucleotide in which the base sequence had been randomly modified (NS1) (30), immobilized in the two compartments of a biosensor cuvette. The biosensor responses showed that these hnRNPs bound both oligonucleotides; however, there were marked differences between them. Both bound NS1 to a similar extent and hnRNP A1 also produced an almost equal response with A2RE, but the response with A2RE and hnRNP A2 was 2-fold higher, suggesting that it possesses site(s) that bind NS1 and others that bind A2RE but not NS1. These experiments were performed with oligodeoxyribonucleotides rather than oligoribonucleotides immobilized on the biosensor to enhance the biochemical stability of the biosensor cuvette during repeated usage. We have evidence from a number of approaches that the presence or absence of the 2′-hydroxyl group does not influence the hnRNP-oligonucleotide interactions; for example, the *inset* in Fig. 2*A* shows equivalent binding of rat brain proteins (predominantly hnRNP A2) to magnetic particles bearing A2RE and dA2RE.

The conclusion drawn above was explored further by using UV cross-linking electrophoretic mobility shift assays (Fig. 3). ^{32}P-Labeled oligoribonucleotides were cross-linked to bound proteins by exposure to UV light and then subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. In accord with the above biosensor results, hnRNP A2 was found to bind both A2RE and NS1, with a clear preference for the former. This binding to A2RE was largely eliminated by addition of a 50-fold excess of unlabeled A2RE but not by an equivalent amount of unlabeled NS1. Labeled NS1 did not bind in the presence of a 50-fold excess of unlabeled NS1 or A2RE. Purified rat brain hnRNP A2 and the mixture of rat brain A2RE-binding proteins eluted from the magnetic particles manifested similar behavior (data not shown), except that the latter did not show any binding to NS1; this is in contrast to the recombinant proteins that bind NS1, albeit less strongly than they bind A2RE. hnRNP A1 bound both A2RE and NS1, though more weakly to both than hnRNP A2. The binding of hnRNP A1 to A2RE was stronger than to NS1, suggesting sequence-specific binding that is weaker than that of hnRNP A2. As for hnRNP A2, the association of hnRNP A1 with both A2RE and NS1 was eliminated on addition of a 50-fold excess of A2RE, but only NS1 binding was eliminated by a 50-fold excess of NS1.

The inference that hnRNP A2 possesses a strong binding site for A2RE that does not bind other oligoribonucleotides of the same length and base composition was confirmed by modifying the experiment depicted in Fig. 2. hnRNP A2 was immobilized on a cuvette and a saturating concentration of one of two nonspecific oligoribonucleotides (10 μM NS1 or NS2) or A2RE (500 nM) added. As anticipated from the data in Fig. 2, the

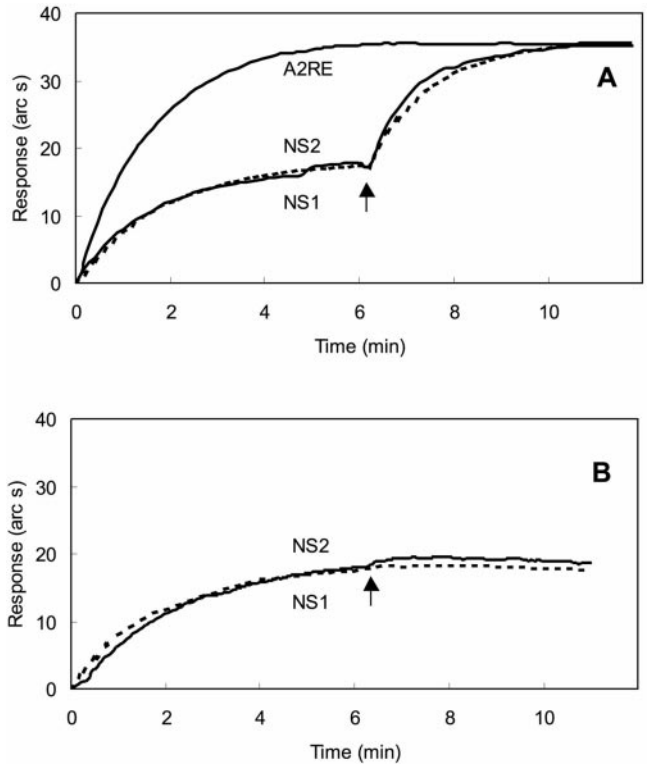

FIG. 4. **hnRNP A2 possesses sequence-specific and nonspecific RNA binding sites.** The data in Fig. 2 show that hnRNP A2 binds more A2RE than hnRNP A1. Here we show that hnRNP A2 possesses sites that are sequence-specific and nonspecific. Comparison of the IAsys biosensor time course for the binding of A2RE (to 500 nM) to immobilized hnRNP A2 with that for oligoribonucleotides with the same composition but scrambled sequence (NS1 and NS2; to 10 μM). *A*, A2RE, or NS1 or NS2, was added to the cuvette at time 0. After attainment of binding equilibrium with the immobilized hnRNP A2, A2RE sufficient to increase its concentration by 500 nM was added to each cuvette at the time indicated by the *arrow*. Only the hnRNP A2 previously equilibrated with NS1 or NS2 showed increased binding on addition of the second aliquot of oligonucleotide, suggesting that the protein possesses sites that bind A2RE but not equivalent oligonucleotides with scrambled sequences. *B*, in a parallel experiment, 10 μM NS1 or NS2, rather than A2RE, was added at the time indicated by the *arrow* after the attainment of equilibrium. Addition of further NS1 or NS2, in contrast to A2RE, results in only a minor increase in binding at the subsaturating concentrations used.

response with A2RE was double that with NS1 or NS2. After attainment of equilibrium (*arrow*, Fig. 4*A*), sufficient A2RE was added to each biosensor to raise its concentration by 500 nM. This addition led to little change in the response on addition to A2RE but to a doubling of the biosensor response on addition of A2RE to the cuvette previously containing only NS1 or NS2. By contrast, addition of a further aliquot of NS1 or NS2 to the cuvettes previously equilibrated with these oligoribonucleotides did not further increase the biosensor response (Fig. 4*B*).

Heparin Eliminates Binding at the Nonspecific Site—Additional evidence for the description of the two classes of binding sites as nonspecific and specific was obtained by examining the effects of addition of the polyanionic glycosaminoglycan, heparin, to the solution phase. Heparin (1 g/liter), which is commonly used to counter nonspecific protein-RNA interactions, was added to solutions of the hnRNPs in biosensor cuvettes bearing immobilized dA2RE or dNS1. Heparin diminished the binding to both DNAs, halving the biosensor response from the dA2RE with hnRNP A2 (Fig. 5*C*) and almost eliminating the binding of dA2RE to hnRNP A1 (Fig. 5*A*) and of NS1 to both proteins (Fig. 5, *B* and *D*). A 10-fold increase in the heparin concentration, to 10 g/liter, did not further diminish the response to the A2RE (data not shown). In view of these results and those described in the previous section, it is unlikely that A2RE binds only to sequence-specific sites distinct from the NS-binding sites. Thus, the site occupied by NS1 or NS2 may also accommodate A2RE, and it can therefore be concluded that in addition to at least one nonspecific site, there is an equal number of sites on hnRNP A2 that bind the A2RE selectively.

Dissociation Constants for the RNA Interactions—The binding curves in Fig. 5 were used to calculate dissociation constants by nonlinear regression analysis using Equation 2. Results obtained in the absence of heparin were used to calculate K_d for hnRNP A1 with A2RE and NS1, and for hnRNP A2 with NS1, values that reflect binding to the nonspecific sites (Table I). The data for A2RE binding to hnRNP A2 were fitted using a two-site model, yielding K_d values of 59 and 220 nM for the specific and nonspecific sites, respectively. The value of K_d, 87 nM, obtained for hnRNP A2 in the presence of heparin (Fig. 5*C*) reflects binding to the specific site only. It is a little higher than the values obtained in the absence of heparin, perhaps as a result of the large increase in ionic strength that results from addition of this polyanion.

The above dissociation constants, derived from experiments in which the oligodeoxyribonucleotides were immobilized, are in agreement with the values calculated from experiments in which hnRNP A2 was immobilized and A2RE added in the absence of heparin (Fig. 6*A* and Table I). Thus, the binding constants appear to be uninfluenced by the tethering of the molecules to the biosensor, a conclusion reinforced by competition experiments outlined in the next section. Experiments with rat brain hnRNP A2 paralleled those with the recombinant human protein, with nonspecific and specific binding again evident and a K_d for the latter of ~15 nM, about 3-fold lower than for the human protein (Fig. 6*B*).

Interaction between hnRNP A2 and A2RE in Solution—To examine the interaction between hnRNP A2 and A2RE in solution, experiments were performed in which solutions containing various concentrations of human hnRNP A2 and a fixed concentration of A2RE were added to a cuvette with human hnRNP A2 immobilized on the sensor surface. Addition of soluble hnRNP A2 to this cuvette, in the absence of A2RE, caused little change in the biosensor response. The decrease in equilibrium response with increasing concentrations of hnRNP A2 in the A2RE-containing mixture (Fig. 7*A*) thus reflects competition between the soluble and immobilized forms of the protein for A2RE and hence establishes specificity of oligoribonucleotide binding to the cuvette sensor surface. Furthermore, the use of Equation 3 for evaluation of the binding function for each reaction mixture allows conclusions to be drawn about the strength and stoichiometry of the interaction between oligoribonucleotide and hnRNP A2. Nonlinear regression analysis of the dependence of binding function upon A2RE concentration in terms of the hyperbolic expression in Equation 4 is consistent with the existence of two sites with an intrinsic dissociation constant of 79 (±3) nM. However, from the Scatchard plot of the

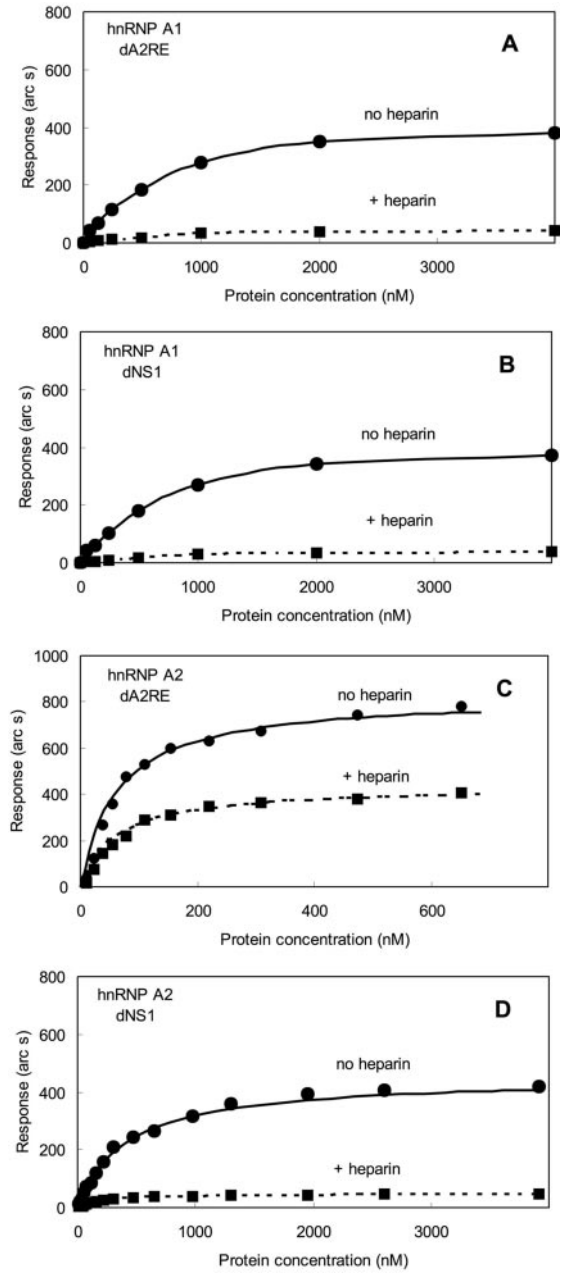

FIG. 5. **Effect of heparin on binding of oligonucleotides to hnRNPs A1 and A2.** Equilibrium biosensor responses, with dA2RE (*A*, *C*) or dNS1 (*B*, *D*) immobilized on the surface of an IAsys cuvette, as a function of solution protein concentration. Data were recorded for hnRNPs A1 (*A*, *B*) and A2 (*C*, *D*) in the absence (*solid lines*) and presence (*dashed lines*) of 1.2 g/liter heparin. Heparin eliminates nonspecific binding (*A–C*) but does not interfere with A2RE binding to the specific site (*C*). The data were fitted to Equation 2 assuming a single binding site (*A*, *B*, *D*, and the *lower curve* of *C*) or a modified form of this equation for two sites (*upper curve* of *C*). The K_d values derived from these curves are presented in Table I.

TABLE I
Binding of hnRNPs A1 and A2 to oligonucleotides

Dissociation constants (K_d in nM) were calculated from resonant mirror biosensor measurements using a single-site binding equation for NS1 and two binding sites for A2RE. Experiments were performed with either oligonucleotide or hnRNP immobilized on the sensor cuvette.

	Immobilized oligonucleotide			Immobilized protein	
	dNS1	dA2RE		A2RE	
		Specific	Nonspecific	Specific	Nonspecific
Human hnRNP A1	438 ± 69	>10,000	431 ± 67	ND[a]	ND
Human hnRNP A2	246 ± 29	59 ± 19	220 ± 44	42 ± 4, 45 ± 5[b]	250 ± 43, 285 ± 41[b]
Rat hnRNP A2	ND	ND	ND	15 ± 2	225 ± 36

[a] ND = not determined.
[b] Averaged from competition experiment data presented in Fig. 7*B*.

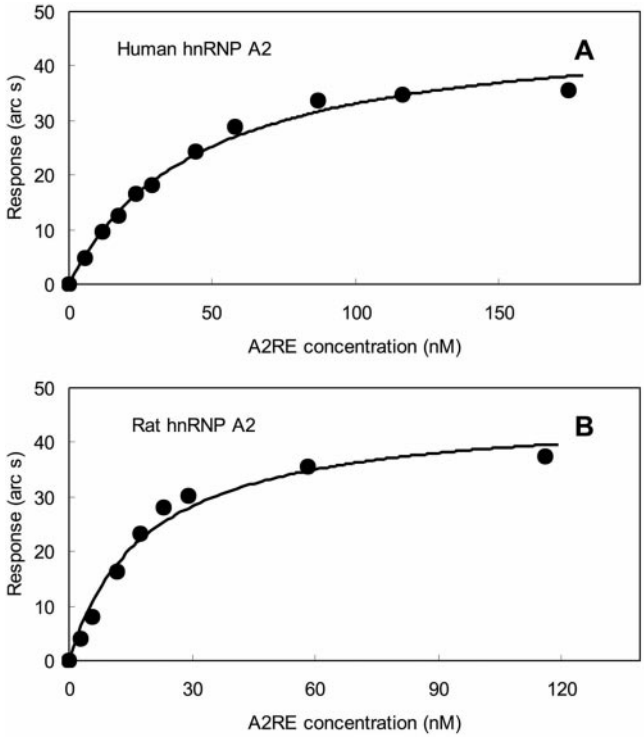

FIG. 6. **A2RE binding to immobilized hnRNP A2.** Solutions of A2RE were added to human recombinant (*A*) and rat brain (*B*) hnRNP A2 and the equilibrium biosensor responses recorded. The data were fitted to a two site model, yielding the K_d values presented in Table I. Because the ligand in solution in these experiments is A2RE, which has a mass 6-fold lower than hnRNP A2, the responses are smaller than those in Fig. 5.

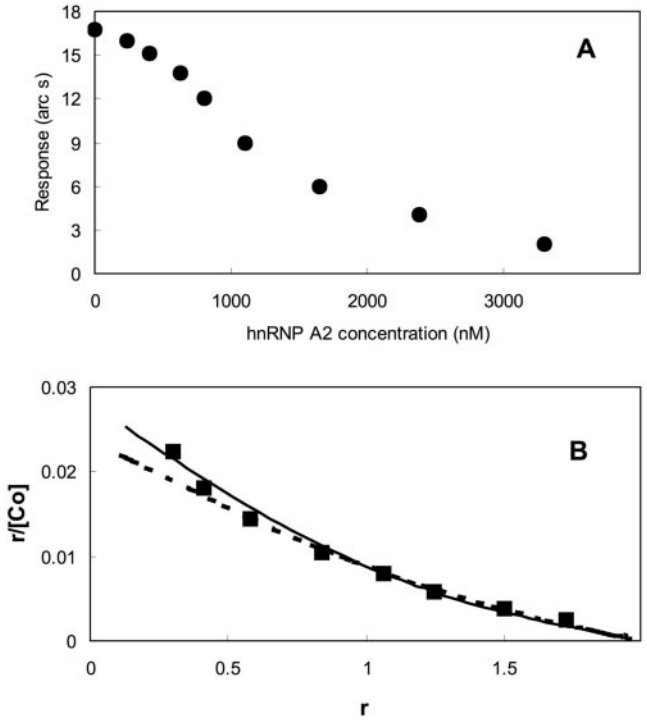

FIG. 7. **Competitive binding data for the interaction of A2RE with human hnRNP A2 in solution.** Solutions containing A2RE (1 μM) and hnRNP A2 (233–3300 nM) were placed in an IAsys cuvette with hnRNP A2 attached to the sensor surface. *A*, dependence of equilibrium binding response upon the concentration of hnRNP A2 included in the solution. *B*, Scatchard linear transform of the competitive binding data. Lines are simulated plots for two-site systems with different affinities ascribed to the two sites: K_1 = 40 nM, K_2 = 320 nM (*solid line*); K_1 = 50 nM, K_2 = 250 nM (*dotted line*). The units of r/C_O are nM^{-1}.

same data (Fig. 7*B*) it is apparent that the binding is poorly described by this single rectangular hyperbolic relationship. Better descriptions are obtained by invoking different dissociation constants for the two sites, as is evident from the simulated curves for the situation where K_1 = 40 nM and K_2 = 320 nM (*solid line*), or where K_1 = 50 nM and K_2 = 250 nM (*dotted line*). The most important point to emerge from Fig. 7*B* is the overall stoichiometry of the interaction (n = 2). Taken in conjunction with the results presented in Figs. 5 and 6, this finding establishes that there is a single site on hnRNP A2 that binds oligoribonucleotides irrespective of their sequence and one additional site that binds A2RE, but not other oligoribonucleotides, with the same composition but scrambled nucleotide sequences.

Interaction of hnRNP A2 with a Shorter Segment of A2RE—The 21-nucleotide A2RE segment has been shown, by deletion analysis, to be necessary and sufficient for myelin basic protein mRNA transport in cultured oligodendrocytes (26). To define more closely the protein-binding segment of the A2RE, we earlier conducted affinity partition experiments with three overlapping biotinylated 11-nucleotide fragments of the A2RE attached to streptavidin-labeled magnetic beads (29). Those qualitative experiments indicated that the binding of the segment comprising the first 11 nucleotides from the 5′ end (A2RE11) to hnRNP A2 is similar to that for the intact 21-nucleotide A2RE. That observation has now been quantified by means of the biosensor.

At saturating concentrations of A2RE11 the response was similar to that obtained with the A2RE and again was halved in 1 g/liter heparin (Fig. 8*A*). In addition, the dissociation constant derived from experiments with a range of AR2E11 concentrations was 44 nM (Fig. 8*B*), which effectively duplicates the K_d of 42–45 nM obtained for A2RE binding to immobilized hnRNP A2. Inasmuch as these results exhibit a quantitative parallel with those for the intact A2RE, it is concluded that the 11-base oligoribonucleotide suffices to occupy either the nonspecific or the specific site on hnRNP A2.

Binding of Point Mutants of A2RE11—Earlier experiments in which rat brain protein binding to RNA immobilized on

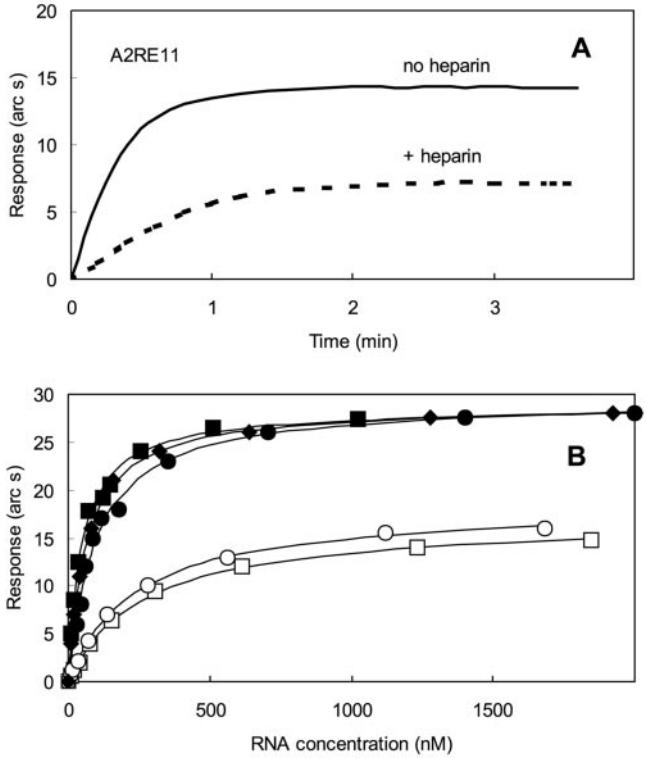

FIG. 8. **Mutations in the A2RE11 sequence that affect RNA transport eliminate binding to hnRNP A2.** *A*, biosensor response on addition of a saturating concentration of A2RE11 to immobilized hnRNPA2. In the absense of heparin the response mirrors that with A2RE. In the presence of 1 g/liter of heparin, the response is halved. *B*, responses from a biosensor bearing immobilized hnRNP A2 as a function of the concentration of added oligoribonucleotide. These data reinforce the earlier conclusion from qualitative magnetic particle affinity assays and RNA transport experiments (29) that mutations that interfere with RNA transport also reduce binding to hnRNP A2. The A2RE mutations C3G (♦) and G7A (●) reduce binding slightly compared with A2RE11 (■), but the two mutations A8G (○) and G9A (□), which markedly affect RNA transport, increase the K_d for hnRNP A2 binding by at least 50-fold. The lines are the best fits to the data assuming one (A8G and G9A) or two (A2RE11, C3G and G7A) sites, which yielded the dissociation constants in Table II.

magnetic particles was measured semiquantitatively had shown that single-base mutations of the A2RE11 sequence had markedly varying effects, some not affecting binding to hnRNP A2 and others eliminating the association. These results were confirmed in biosensor experiments with four point-mutated A2RE11 sequences: C3G, G7A, A8G and G9A (29). The binding of C3G and G7A was quantitatively similar to that A2RE11, whereas the binding of the other two mutants was indistinguishable from that of NS1 (Fig. 8*B*). From simulation of these binding curves it was deduced that the A8G and G9A mutations increased K_d by more than 50-fold for the specific site (Table II).

A2RE-binding Site Location—Many hnRNPs possess multiple RRMs. HnRNP A/B subfamily members contain two N-terminal RRMs, both of which bind RNA. The C-terminal glycine-rich tail has also been implicated in RNA binding. To locate the A2RE binding site on hnRNP A2, we expressed and purified several segments (Fig. 10*A*) and used them in biosensor cuvettes bearing dA2RE and dNS1 (Fig. 9), and in UV cross-linking experiments (Fig. 10).

In biosensor experiments the N-terminal RRM (residues 1–89) showed equivalent binding to both oligonucleotides (Fig. 9*A*), and A2RE binding to the second RRM (residues 101–179) was only slightly above that of NS1 (Fig. 9*B*), compared with hnRNP A2 used in the same cuvette (Fig. 9*C*). Thus, they bind RNA nonspecifically and show little or no evidence of specific A2RE binding. In contrast, the concatemer of the two RRMs showed substantially higher binding to A2RE than to NS1 (Fig. 9*D*) and thus has a specific A2RE-binding site in addition to its capacity for nonspecific binding. However, even this concatemer did not quantitatively mimic the binding properties of hnRNP A2, but the addition of a further 10 residues (180–189; EMQEVQSSRS) increased the binding (Fig. 9*E*) to a level comparable with that of the intact protein (Fig. 9*F*; Table III); the equivalent segment of hnRNP A1 causes a 100-fold increase in the binding of poly[r(eA)] (38). The effects of heparin on the hnRNP A2 fragments were not as readily interpreted as for the whole protein. In contrast to its effects on hnRNP A2, addition of heparin to RRM1 or RRM2 lowered, but did not eliminate, binding.

UV cross-linking experiments were performed with equimolar amounts of hnRNPs A1 and A2 and the A2 fragments. In contrast to the biosensor experiments, both proteins manifested specific and nonspecific binding, with weaker binding to hnRNP A1 than to hnRNP A2 (Fig. 10*B*). Weak binding of A2RE and NS1 to residues 101–179 (Fig. 10*C*) but not to residues 1–89 (data not shown) was detected only after extended exposure of the autoradiographs. The segments 1–179 and 1–189 bound both A2RE and (more weakly) NS1. At equivalent molar concentrations A2RE binding to the intact protein still exceeded that to residues 1–189. Two differences between the biosensor and UV cross-linking results are the comparable binding of hnRNPs A1 and A2 to A2RE and the marked difference in binding to the polypeptide comprising residues 1–189 and the intact proteins in the latter experiments; these results contrast with the weak specific binding of hnRNP A1 to A2RE, and the comparable binding of residues 1–189 and hnRNP A2, in the biosensor. Although no definitive explanation can at present be given for these differences, we note that the biosensor data reflect the extant equilibria, whereas the cross-linking data come from nonequilibrium measurements that may be influenced by more efficient cross-linking, perhaps to the C-terminal glycine-rich domain.

DISCUSSION

We have established that expressed human hnRNP A2 possesses at least two sites for binding oligonucleotides. One recognizes RNA molecules with widely differing sequences, but with similar dissociation constants, the second is specific for A2RE. The dissociation constants for both sites are typical of RRM-containing proteins (20, 39, 40) and are within an order of magnitude, but the discrimination between A2RE and other sequences for the specific binding site on hnRNP A2 is effectively absolute (Fig. 5). No binding of NS1 or NS2 to the specific site was detected even at the highest oligonucleotide concentrations used in the absence of heparin. On the other hand, the A2RE retained its affinity for the specific site in the presence of heparin concentrations (10 g/liter), which eliminated binding at the nonspecific site. The ability of heparin to block just one site, and the clear discrimination between oligonucleotides possessing and lacking an A2RE-like sequence observed for the other site, signify a fundamental difference in the nature of the two binding regions on hnRNP A2.

Most RRMs have been assumed to have a basal level of nonspecific affinity for RNA, although only in a few instances has this been proven (41). As constituents of core particles, hnRNP A1 and hnRNP A2, in particular, have been proposed to have roles in intramolecular RNA packaging, which would presumably be independent of RNA sequence. This hypothesis is partly supported by our data. Both isolated RRMs of hnRNP A2 manifest nonspecific binding of comparable strength, although the intact protein appears to have a single site for

TABLE II
Effect of A2RE point mutations on hnRNP A2 binding

Oligoribonucleotides having point mutations were added to a biosensor cuvette bearing immobilized hnRNP A2. The biosensor responses (Fig. 8*B*) were fitted to a two-site model, yielding dissociation constants (nM) for specific and nonspecific sites.

	C3G	G7A	A8G	G9A	A2RE11
Specific	62 ± 10	92 ± 18	>5000	>5000	44 ± 7
Nonspecific	248 ± 44	262 ± 41	233 ± 44	250 ± 42	244 ± 31

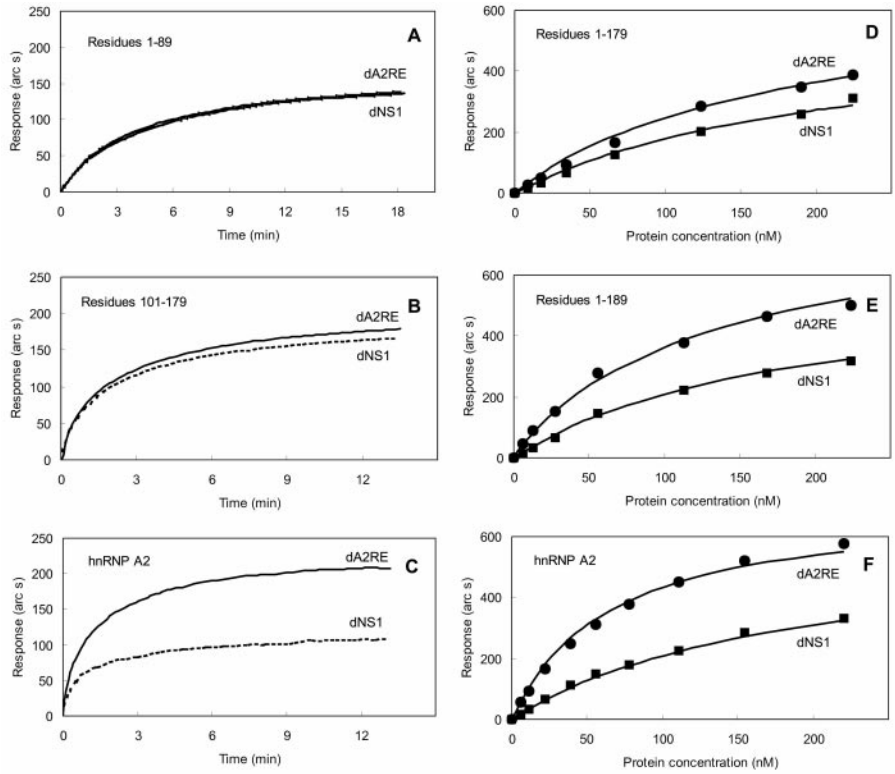

FIG. 9. **Specific binding site of hnRNP A2 requires both RRMs.** Measurements of the binding of hnRNP A2 and fragments to biosensor surfaces bearing dA2RE or dNS1. Both RRM1 (326 nM) and RRM2 (293 nM) manifest equivalent binding to A2RE and NS1 (*A* and *B*), indicating that they bind A2RE very weakly, if at all, compared with the intact protein (30 nM in *C*). The data for the individual RRMs were recorded at a single concentration corresponding to the maximum used with intact hnRNP A2 in earlier experiments. Because no specific binding was evident, experiments were not performed at other protein concentrations. The concatenated repeats (RRM1 + RRM2) showed some specific binding (*D*), but the RRMs with the 10 additional flanking residues (*i.e.* 1–189; *E*) approached the binding of intact hnRNP A2 (F). The curves in *D*--*F* represent the best fit to the data assuming one (NS1) or two (A2RE) sites (see Table III).

nonspecific interaction with even the relatively short A2RE. However, in contrast to the recombinant proteins, hnRNP A2 isolated from rat brain protein extracts shows little nonspecific binding in electrophoretic mobility shift assays performed in the absence of heparin (29). This difference between the human and rat proteins does not necessarily exclude the existence of nonspecific binding sites on the rat hnRNPs; the nonspecific RNA-binding sites on these proteins may, for example, already be occupied in the tissue extracts, thus precluding the binding of added radiolabeled, nonspecific oligonucleotide. Alternatively, there may be conformational differences between the rat brain protein isolated using only the magnetic particles and the proteins isolated by reverse-phase HPLC that generate the nonspecific site. But the physiological significance and detailed analysis of this nonspecific binding is beyond the scope of this communication.

Although some hnRNPs have high affinity for oligonucleotides regardless of sequence, they may also have additional, more specific functions (23, 42). hnRNP A2 binds the telomeric sequence TTAGGG (24, 25, 43), which bears no relationship to the A2RE sequence and is therefore unlikely to mimic the A2RE binding detected in this study. Because heparin was not used in the studies with telomeric DNA, it is possible that the TTAGGG sequence binds with an atypically high affinity to the nonspecific site; alternatively, it may interact with a specific site on hnRNP A2 other than the one described here.

The size of the A2RE-binding site on hnRNP A2 was earlier suggested to be around 11 nucleotides, based on an analysis of the effects of mutations within the A2RE11 (29). This conclusion is reinforced by the data presented here: several single base mutations within A2RE11 increased the dissociation constant by more than 50-fold. Examination of the A2RE sequence reveals an interesting near-repetition of the A2RE11 sequence that results in significant binding of the 3′ 11 nucleotides and the middle 11 nucleotides of A2RE to hnRNP A2 (29). A loose analogy may be drawn with the parallel and antiparallel tandem repeats found in the DNA segments recognized by transcription factors, but the repeats in A2RE are single-stranded, imperfect, and overlapped. Furthermore, although the A2RE has, as a result of this repetition, potentially more than one binding site, only one molecule of hnRNP A2 is bound.

Unless the interaction with hnRNP A2 has a pronounced effect on its secondary structure, the protein-bound A2RE is likely to be single-stranded. Calculations using the program mfold (44) indicate that neither A2RE nor A2RE11 and its mutants forms stable secondary structure, with the exception of A8G, which can form 3 base pairs. Therefore, although these calculations are more reliable for larger oligonucleotides, perhaps the closest parallels can be drawn between A2RE-hnRNP A2 and the RNA complexes formed by Sex-lethal protein and the N-terminal tandem RRMs of poly(A) binding (PABP) protein. Nine nucleotides of a 12-nucleotide segment of single-stranded *transformer* mRNA bind in a V-shaped cleft formed by the tandem RRMs of Sex-lethal protein (45, 46). Seven consecutive nucleotides of poly(A) are bound in a narrow trough formed by the β-strands of the RRMs of PABP (46, 47). The stretches of RNA bound to these proteins, 7–9 nucleotides, are comparable in length with A2RE11. In both of these complexes,

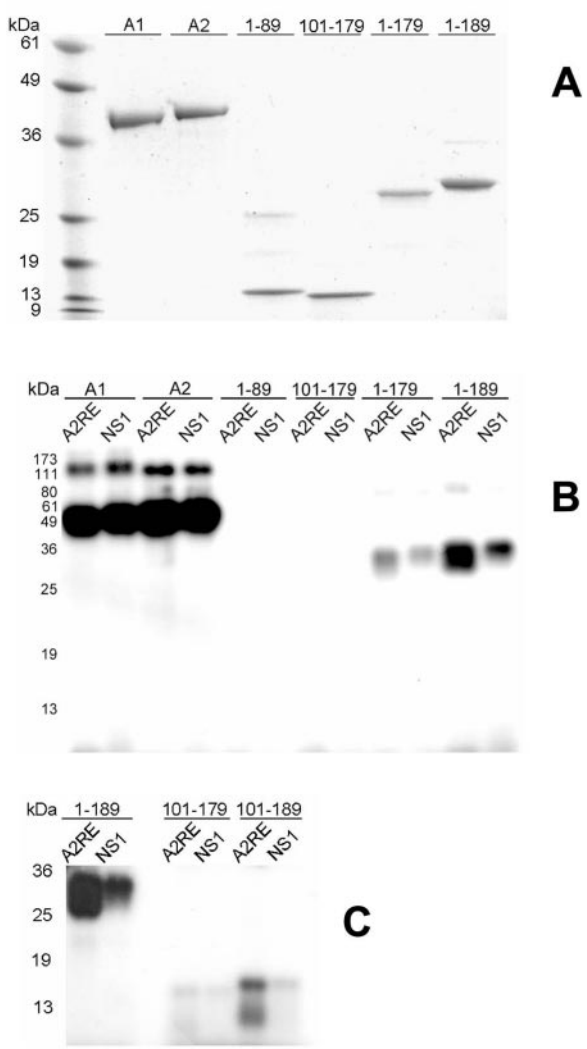

FIG. 10. **UV cross-linking analysis of nucleotide binding to hnRNP A2 fragments.** *A*, Coomassie Blue-stained SDS-polyacrylamide gel of approximately equal weights of hnRNPs A1 and A2 and A2 fragments used in biosensor (Fig. 9), and in the UV cross-linking experiments, performed in the absence of heparin, shown in *B*. Equimolar amounts (25 pmol) of the polypeptides were loaded into each lane in *B*. No binding of A2RE (1.4 nmol) or NS1 (1.4 nmol) to RRM1 (residues 1–89) or RRM2 (residues 101–179) was detected in any of several replicate experiments. The concatenated RRMs (residues 1–179 and 1–189) showed nonspecific and stronger specific binding. The upper bands visible in most lanes were not obtained consistently and may arise from dimer formation (see Fig. 3). *C*, UV cross-linking with equimolar amounts (25 pmol) of protein. This longer exposure shows weak binding of RRM2 (residues 101–179), which is increased a little by the additional 10 C-terminal residues of 101–189, but is still markedly weaker than the binding of residues 1–189. This reinforces the view that residues 180–189 do contribute significantly to A2RE binding.

however, some of the 2′-OH groups interact with the proteins, whereas the similar affinity displayed for A2RE and its 2′-deoxy counterpart by hnRNP A2 suggests that the 2′-OH groups of A2RE do not play such a role.

The small number of structural studies of RNA binding to RRM-containing proteins has uncovered considerable diversity in the modes of interaction, including the binding of single (48) or multiple RRMs (45), binding of single- and double-stranded RNA, and monomeric or self-associated proteins. UP1, the two tandem RRMs of hnRNP A1, for example, binds nucleic acid homopolymers through only one of its RRMs (48). UP1 can also form antiparallel dimers in which two single-stranded DNA segments straddle both protein molecules, binding RRM1 of one molecule and RRM2 of the other (49). The RRMs lie side-by-side in the crystal structure of UP1 (13). It is possible that UP1 is not dimeric in solution and has an altered mode of oligonucleotide binding (49).

TABLE III
Binding of hnRNP A2 fragments to oligonucleotides

Biosensor data obtained with purified fragments of hnRNP A2 and immobilized oligonucleotides (Fig. 9, *D–F*) were fitted to Equation 2 (for dNS1) or its two-site equivalent (for dA2RE). Values are for K_d (nM).

	Residues		
	1–179	1–189	hnRNP A2
dA2RE[a]	127 ± 17	63 ± 11	49 ± 6
dNS1	235 ± 35	225 ± 33	219 ± 34

[a] Values given are for the A2RE-specific site.

A2RE appears to bind to monomers of hnRNP A2; equivalent results were obtained in the experiments reported here with the A2RE or hnRNP A2 immobilized on the biosensor surface. As the protein in these experiments was immobilized from a solution in which it was shown by sedimentation equilibrium experiments to be monomeric, it is unlikely that the observed A2RE binding is to a dimer of the protein. In addition, hnRNP A2 (or A1) added to a biosensor bearing immobilized hnRNP A2 resulted in no increase in response, again indicating that, under the conditions used, hnRNP A2 does not self-associate or bind hnRNP A1.

Although the glycine-rich region of hnRNP A1 has been implicated in other RNA-hnRNP interactions (11, 39), this region of hnRNP A2 does not appear to be involved in the interaction with A2RE. The expressed segment of hnRNP A2, including both RRMs and the short region C-terminal to the second RRM that has been shown previously to markedly enhance RNA binding to hnRNP A1 (38), bound A2RE almost as strongly as the full protein. Binding to the individual RRMs was weak or undetectable. Thus, the specific association appears to involve a 1:1 interaction of monomeric hnRNP A2 with a short single-stranded RNA, the binding site being formed by the two RRMs. Verification of this model will await more detailed structural studies, such as three-dimensional structure determination of the RNA-protein complex.

In summary, we have demonstrated that: (i) recombinant hnRNPs A1 and A2 possess a site capable of binding RNAs with diverse sequences; (ii) in addition, both possess a site that binds the 21-nucleotide A2RE but not equivalent oligonucleotides with scrambled sequences; (iii) A2RE binding to the specific site on hnRNP A2 is considerably stronger than to hnRNP A1; (iv) the 5′ 11 nucleotides of A2RE bind to hnRNP A2 with an affinity equal to that of the 21-mer, thus the sequence-specific binding involves at most 11 nucleotides; (v) several single base mutations within the A2RE abrogate specific binding to hnRNP A2; and (vi) the specific binding site is formed by the two tandem RRMs and the proximal C-terminal flanking region. It is hoped that the present demonstration of the existence on hnRNP A2 of a site exhibiting specificity for A2RE may prompt further investigations to identify more closely the oligoribonucleotide-binding sites on the heterogeneous nuclear ribonucleoproteins involved in mRNA transport.

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Jianguo Shan, Kim Moran-Jones, Trent P. Munro, Grahame J. Kidd, Donald J. Winzor, Keith S. Hoek and Ross Smith

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