Mammalian Heterogeneous Nuclear Ribonucleoprotein Complex Protein A1

LARGE-SCALE OVERPRODUCTION IN ESCHERICHIA COLI AND COOPERATIVE BINDING TO SINGLE-STRANDED NUCLEIC ACIDS*

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Characterization of mammalian heterogeneous nuclear ribonucleoprotein complex protein A1 is reported after large-scale overproduction of the protein in Escherichia coli and purification to homogeneity. A1 is a single-stranded nucleic acid binding protein of 320 amino acids and 34,214 Da. The protein has two domains. The NH2-terminal domain is globular, whereas the COOH-terminal domain of about 120 amino acids has low probability of α-helix structure and is glycine-rich. Nucleic acid binding properties of recombinant A1 were compared with those of recombinant and natural proteins corresponding to the NH2-terminal domain. A1 bound to single-stranded DNA-cellulose with higher affinity than the NH2-terminal domain peptides. Protein-induced fluorescence enhancement was used to measure equilibrium binding properties of the proteins. A1 binding to poly(ethenoadenylate) was cooperative with the intrinsic association constant of \( 1.5 \times 10^6 \) M\(^{-1}\) at 0.4 M NaCl and a cooperativity parameter of 30. The NH2-terminal domain peptides bound non-cooperatively and with a much lower association constant. With these peptides and with intact A1, binding was fully reversed by increasing [NaCl]; yet, A1 binding was much less salt-sensitive than binding by the NH2-terminal domain peptides. A synthetic polypeptide analog of the COOH-terminal domain was prepared and was found to bind tightly to poly(ethenoadenylate). The results are consistent with the idea that the COOH-terminal domain contributes to A1 binding through both cooperative protein-protein interaction and direct interaction with the nucleic acid.

Single-stranded DNA binding proteins have been purified from a variety of mammalian sources and characterized extensively (1–3). These proteins bind to single-stranded con-

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†† The abbreviations used are: HDN, helix-stabilizing protein; hhnRNP, heterogeneous nuclear ribonucleoprotein; ssDNA, single-stranded DNA; poly(εA), poly(ethenoadenylate); Lys-C, endopeptidase Lys-C.

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fied recombinant protein revealed interesting details about the mechanism of its strong binding to single-stranded nucleic acids.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

We chose to overexpress the A1 protein from the open reading frame of a full-length cDNA (13) using the *Amp* promoter-based bacterial expression system pRC23 (20). Placement of the cDNA in the vector positioned the ribosome binding site of the vector 8 bases upstream of the initiation codon in the cDNA. Cells transformed with the expression plasmid had large quantities of a new 34-kDa protein, and immunoblotting experiments with anti-A1 antibody revealed that the 34-kDa protein contained a reactive epitope. Using the purification procedure described here we routinely obtained 50 mg of 34-kDa A1 protein from the soluble extract of 10 g of pelleted cells in 10-12 h. The final preparation of A1 is homogeneous by amino acid sequencing and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and as expected, the purified protein itself is immunoreactive with anti-A1 antibody.

The amino acid composition of purified recombinant A1 is in agreement with that predicted from the nucleotide sequence of the cDNA, and sequencing of the protein demonstrated that residues 1-25 are in exact agreement with residues 2-26 of the sequence predicted from the cDNA; the purified protein does not contain the cDNA-predicted NH2-terminal methionine (13). Enzymatic cleavage of the protein was conducted with endoproteinase Lys-C (EC 3.4.99.30). The sequence at the COOH-terminal end of the protein was determined by sequencing of the only endoproteinase Lys-C peptide that did not contain lysine. The sequence matches the COOH-terminal sequence predicted from the cDNA nucleotide sequence. Several additional endoproteinase Lys-C peptides also were sequenced. These peptides, together with the NH2- and COOH-terminal sequences described above, account for ~40% of the complete A1 sequence. Finally, the amino acid compositions of the other Lys-C peptides were determined and were found to match peptides predicted from the cDNA sequence. Taken together, the results indicate a perfect match between the recombinant A1 protein sequence and the sequence predicted from the cDNA at 90% of the residues (Table I).

**STRUCTURE-FUNCTION STUDIES**

**A1 Domain Structure and ssDNA-Cellulose Binding**—A1 is a two-domain protein exhibiting a sharp transition at residues 195-200 between the globular NH2-terminal domain and the randomly structured COOH-terminal domain (13). We obtained three preparations of the NH2-terminal domain peptide for comparison with intact A1. These preparations were 1) the NH2-terminal domain isolated from calf thymus, termed UP1; 2) the NH2-terminal domain obtained by *in vitro* trypsinization of a recombinant A1, termed P24*; and 3) a recombinant UP1 obtained by subcloning a truncated A1 cDNA into an appropriate expression vector.

Our initial structure-function experiments with recombinant A1 involved examination of the ssDNA-cellulose column chromatographic behavior of the intact protein and of products of limited proteolysis. We found that light trypsinization converted the 34-kDa A1 protein to species of 32, 30, 27, and 24 kDa. When a mixture of the 34-kDa protein and all four of these truncated species was subjected to NH1-terminal sequence analysis, only one NH1-terminal sequence was found corresponding to that of the intact species (data not shown). This indicated that conversion to the smaller species was due to truncation at the COOH-terminal end only. This mixture of A1 and truncated species then was chromatographed on an ssDNA-cellulose column. Elution was with a linear gradient of buffer C containing 0.4-1 mM NaCl (Fig. 1). The two smaller species, 24 and 27 kDa, emerged from the column just after the start of the gradient. The 30-, 32-, and 34-kDa species emerged successively as the concentration of NaCl in the gradient increased.

In additional experiments, the 24-kDa species was produced by trypsin digestion of recombinant A1 as described by Kumar *et al.* (14) for the HeLa A1 protein. This truncated protein, termed P24*, lacked the COOH-terminal 124 amino acids of A1 and was analogous to UP1 isolated from calf thymus. The behavior of P24* during ssDNA-cellulose chromatography was identical to that of the 24-kDa species shown in Fig. 1. Finally, similar results also were obtained with the recombinant UP1. These results indicated that A1 binds much tighter to ssDNA than truncated species lacking either all or part of the COOH-terminal domain.

**Quantitative Aspects of A1-Nucleic Acid Binding—A1-nucleic acid binding was evaluated spectrofluorimetrically using the fluorescent receptor poly(ethenoadeny1ate) (poly(A)). Binding to proteins enhances poly(A) fluorescence such that the amount of fluorescence increase is directly proportional to the amount of protein bound. Hence, titration curves of A1 with a fixed level of poly(A) can reveal equilibrium concentrations in a binding mixture of both free A1 and A1-poly(A) complex.**

Under conditions of low ionic strength, 0.01 mM NaCl, the overall association constant is sufficiently high so that all of the A1 added to the mixture is complexed with poly(A) at saturating ratios of protein to polynucleotide (Fig. 2). Saturation of the polynucleotide with protein corresponded to 12 nucleotide residues/protein monomer (n). In order to determine the intrinsic association constant and degree of binding cooperativity, a titration was conducted in the presence of 0.4 mM NaCl, conditions where each binding mixture contained equilibrium concentrations of both A1-poly(A) complex and free A1. The shape of the titration curve suggested positive cooperativity, and this was confirmed using a modified Scatchard analysis, as described by McGhee and
Panel b, calibration curves (29) are shown, each with a monomer. This value was 12, since 0.1 M NaCl presence of 0.01 M NaCl between 5 and 7 nucleotide residues/protein monomer. Curve was examined with two curves (not shown) where n and K were fixed at 12 and 1.45 M. The cooperativity parameter, K, was estimated as in Fig. 2b. With n fixed at 12, a nonlinear least squares curve fitting procedure gave a best fit with the cooperative parameter, K, of 1.45 M. A plot of logKw versus log[Na+] was linear for both A1 and UP1 (Fig. 4b). The slopes of the plots for A1 and UP1 were ~1 and 4, respectively, illustrating that A1 binding was much less sensitive to salt reversal that UP1 binding. In additional experiments not shown, P24* was examined. We obtained results virtually identical to those with calf thymus UP1 shown in Figs. 3 and 4. The results of these poly(A) binding comparisons are summarized in Table II. In the presence of 0.4 M NaCl, A1 binding was about 10,000 times stronger than UP1 binding. This difference was not fully accounted for by the cooperatively parameter.

Cooperative Binding of Recombinant A1 to ssDNA—To further document cooperative binding by A1, gel retardation analysis (31) using fd DNA as the single-stranded nucleic acid...
ligand was conducted. In this analysis binding mixtures were incubated at 25 °C and then subjected to agarose gel electrophoresis where nucleoprotein complexes migrate slower than free fd DNA. Typical results are shown in Fig. 5a. About 0.4 nmol of A1 (lane 7) was sufficient to complex all of the fd DNA in the mixture (3.2 nmol of nucleotide residues), and the nucleoprotein complexes in this mixture migrated in a relatively sharp band. This complex probably corresponded to fd DNA molecules fully saturated with A1. Protease digestion of the complex released free fd DNA molecules with the same mobility as normal fd DNA (lanes 8 and 1). With binding mixtures containing less A1, fd DNA migrated either as a nucleoprotein complex or free molecules; the complex at each protein level migrated to approximately the same position as observed with the saturating level of protein. For example, with the lowest level of protein, 0.17 nmol (lane 3), some of the fd DNA molecules remained free whereas other molecules were complexed. Thus, even though the protein amount was insufficient to fully saturate all of the fd DNA in the binding mixture, those complexes formed appeared to represent many A1 monomers/DNA molecules. These results agree well with the cooperativity and estimate of nucleotides covered (n = 12) in the poly(cA) binding studies.

The fd DNA gel retardation pattern observed with recombinant UP1 (Fig. 5b) was different from that with A1. At each level of UP1, all of the fd DNA was complexed, and the extent of retardation of the nucleoprotein complex was proportional to the amount of protein in the binding mixture. This is consistent with noncooperative binding and agrees with our results on UP1 binding to poly(cA).

**Synthetic Analog of the COOH-terminal Domain Peptide and Poly(tA) Binding**—The results described above indicated that the presence of the COOH-terminal domain in A1 causes the protein to bind to poly(cA) or fd DNA with higher affinity and positive cooperativity. To gain insight into the mechanism of the COOH-terminal domain effect, we prepared a synthetic peptide resembling the native domain and studied its binding to poly(cA). Efforts to express the COOH-terminal domain peptide itself in *E. coli* from the appropriate cDNA segment were not successful, and similarly, attempts to isolate the domain peptide after mild trypsin digestion failed. The ~120-amino acid native COOH-terminal domain is composed of repeated units of about eight amino acids with consensus sequence of \( \text{GNYGGGRG} \). We prepared a 16-
in most cases, it remains a formidable experiment achievement to obtain appropriate modification and subcloning of a coding sequence, overexpression, and purification of large amounts of undergraded protein. In the present case, with the full-length A1 cDNA, we obtained desirable subcloning and overexpression in a straightforward fashion. However, the complete purification of the recombinant A1 protein in undergraded form proved difficult. Initially, we found that protein samples purified by standard methods were partially degraded, and upon storage at 4 or 25 °C, there was progressive degradation due to contaminating protease activity. This protease activity could be removed by the method described here, in which a key step is washing the ssDNA-cellulose column with 5 column volumes of 0.4 M NaCl (in buffer D) prior to gradient elution. The A1 protein in the final fraction is stable during long term storage at either 4 or 25 °C. The purification procedure described also has been adapted to the preparation of pure recombinant protein from a large amount (50 g) of cell paste.

The recombinant rat A1 protein may be identical to the A1 proteins isolated from calf thymus or HeLa cells, except that the latter two proteins have blocked NH₂ termini and a methylated arginine at position 194; the corresponding arginine in the recombinant rat A1 protein is not methylated (Table V). The complete sequence of the A1 proteins is known only for the rat protein, but the sequence of the first 195 residues of the calf thymus protein (32, 33) and the last 196 residues of the human protein (15) are known. In these overlapping regions, the sequences of the three proteins are identical.

Various applications of controlled proteolysis have been used to probe structure-function relationships of single-stranded nucleic acid binding proteins (34). These proteins generally have one relatively large trypsin-resistant domain that retains partial nucleic acid binding activity, and this is also true for A1 (14). HeLa A1, for example, yields a fragment of Mᵣ = 24,000 corresponding to the NH₂-terminal domain that binds specifically and tightly to single-stranded polynucleotides; this trypsin-resistant fragment is identical to the UP1 protein isolated directly from cells (14). The data presented here show that trypsinization of recombinant A1 also yields a Mᵣ = 24,000 fragment equivalent to UP1 and that with lighter proteolytic digestion three other fragments of somewhat higher Mᵣ are obtained. Sequencing revealed that all of these fragments had the same NH₂ terminus end, and thus, the fragments contained different amounts of the glycine-rich COOH-terminal domain. We found that each of these fragments bound to an ssDNA-cellulose column in 0.4 M NaCl but was eluted at lower NaCl concentrations than the intact protein in the order 24 < 30 < 32 kDa (Fig. 1). Hence, we concluded that the COOH-terminal domain exerts an effect on binding affinity of A1 to ssDNA.

Next, we made use of fluorescence properties of poly(ethenoadenylate) to obtain equilibrium binding data for A1 and NH₂-terminal domain peptides. The binding is expressed as Kᵊ, where K is the intrinsic association constant and ω is the cooperativity parameter. We found that A1 covers 12 nucleotide residues upon binding to poly(A) and that in 0.4 M NaCl, K and ω are 1.5 × 10¹⁰ M⁻¹ and 30, respectively. In contrast, UP1 covers 5–7 nucleotides, is noncooperative, and K is 0.6 × 10⁴ M⁻¹. This finding of positive cooperativity for A1 binding and noncooperativity for UP1 binding was corroborated, in a qualitative sense, by gel retardation analysis (Fig. 5) using fd DNA as the single-stranded nucleic acid.

The association constants for A1 and UP1 binding to poly(A) over a range of [NaCl] were obtained from salt-
reversal (Fig. 4). Plots of logKw versus log[Na+] illustrated that A1 binding is much less salt-sensitive than UP1 binding. The slope of the logKw versus log[Na+] plot is interpreted (30) in terms of the number of ion pairs involved in the binding, and this can be assigned as ±1 and 3 for A1 and UP1, respectively. Further, the extrapolated value of Kw at 1 M NaCl represents the affinity due to nonelectrostatic interactions (30), and this is equal to 1 × 10⁶ and 30 for A1 and UP1, respectively. The plot also facilitates comparison of A1 binding with that of other single-stranded nucleic acid binding properties (35-37). The intrinsic association constant for T4 gene 32 protein binding to poly(A) is 1.2 × 10⁶ M⁻¹ in 0.2 M NaCl, and the interpolated value for A1 binding to poly(A) is 3 × 10⁵ M⁻¹. In 0.02 M NaCl, the mouse helix-stabilizing protein has noncooperative affinity of 4 × 10⁴ M⁻¹ for single-stranded DNA (2); the interpolated value for the A1-poly(A) interaction is 4.5 × 10⁵ M⁻¹.

Taken together our comparisons of intact A1 with the NH₂-terminal domain peptides reveal that the COOH-terminal domain strongly influences nucleic acid binding by A1. The mechanism of this effect involves nonelectrostactic interactions and positive cooperativity. But, even without the cooperativity parameter, A1 binding to poly(A) is at least 100-fold stronger than UP1 binding. This could be due to direct interaction between the COOH-terminal domain and the polynucleotide or to an allosteric effect of the COOH-terminal domain upon binding by the NH₂-terminal domain. Our studies with the synthetic COOH-terminal domain analog demonstrate that a peptide with features similar to those of the native domain is indeed capable of very strong nucleic acid binding, and structural modeling of the COOH-terminal domain of A1 (38) indicates that the domain is capable of direct interfacing with nucleic acids. This modeling was based upon theoretical considerations of protein secondary structure, the regular spacing of most of the phenylalanine or tyrosine residues, and hydrophobic interaction of these residues with nucleic acid base residues. Therefore, in addition to providing protein-protein contact, we suggest that the mechanism of A1 binding involves direct interaction of the COOH-terminal domain and the nucleic acid. Clearly, much more study is required to understand the mechanism of A1 binding, and the fact that large amounts of the recombinant protein can now be obtained points to particularly powerful approaches, such as photochemical cross-linking, NMR, and crystallography. These studies of the A1 protein binding mechanism will have implications for other eukaryotic RNA binding proteins, since these proteins share certain structural features. The COOH-terminal domain motif of regularly spaced aromatic residues separated by α-helix-free regions of high flexibility is present in nucleolin, hnRNP complex protein A2, Drosophila P9, and RNA polymerase large subunit (38-46). The NH₂-terminal domain of A1 contains four oligopeptide sequences conserved among several RNA binding proteins (44-46). Based upon the results presented here and the photochemical cross-linking result of Merrill et al., it seems clear that all of these converted regions are involved in binding.

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![Diagram](image)

**Fig. 9. Analysis of A1 purified from E. coli MB1 carrying pEB1.**

(A) Photograph of Commassie blue stained gel after 5% polyacrylamide gel electrophoresis. Temperature induction, sample preparation, and gel electrophoresis are the same as described previously. Protein markers (10 μg each) in the left hand lane were from top to bottom phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme. The faint band at the level of bovine serum albumin in lanes 1, 2, and 5 was not contributed by the A1 sample.

(B) Western blotting of purified A1. Bands were visualized using horseradish peroxidase conjugated goat anti-rabbit IgG. Lanes 1, 2, and 3 contain 2, 4, and 8 μg of purified A1, respectively. Anti-A1 serum was diluted 1:10000.

**Primary structure of expressed A1.** As shown in Table III, the amino acid composition of the purified protein was in good agreement with that predicted from the nucleotide sequence of the cDNA. The average deviation between the observed and expected amino acid compositions was 0.01, which is close to the 0.05 deviation found for a more extensive series of analyses done on several standard proteins [28].

**Table III**

| Amino Acid | Resident/Molecular Protein |
|------------|----------------------------|
| Arg        | 35.8 (15)                  |
| Thr        | 12.2 (12)                  |
| Ser        | 32.4 (24)                  |
| Glu        | 29.4 (28)                  |
| Pro        | 9.1 (9)                    |
| His        | 60.3 (64)                  |
| Asp        | 17.1 (17)                  |
| Lys        | 3.8 (3)                    |
| Ile        | 7.9 (8)                    |
| Leu        | 10.3 (10)                  |
| Tyr        | 11.6 (12)                  |
| Phe        | 31.0 (32)                  |
| Met        | 8.5 (8)                    |
| Val        | 10.0 (10)                  |
| Cys        | 9.4 (9)                    |

Values were the average of two independent hydrolyses and were calculated on the basis of a molecular weight of 33,692. This latter number was based on the predicted A1 sequence [23] after correcting for cysteine and tryptophan that were not determined. Values in parentheses were derived from the sequence deduced from the cDNA (13).

**Table IV**

| Cycle | Amino acid | Yield |
|-------|------------|-------|
| 1     | Ser        | 67    |
| 2     | Lys        | 220   |
| 3     | Ser        | 51    |
| 4     | Gln        | 170   |
| 5     | Ser        | 25    |
| 6     | Pro        | 110   |
| 7     | Gln        | 115   |
| 8     | Arg        | 68    |
| 9     | Gln        | 86    |
| 10    | Gln        | 64    |
| 11    | Leu        | 70    |
| 12    | Lys        | 30    |
| 13    | Gln        | 68    |
| 14    | Gln        | 85    |
| 15    | Lys        | 64    |
| 16    | Gln        | 35    |
| 17    | Gln        | 125   |
| 18    | Gln        | 160   |
| 19    | Gln        | 71    |
| 20    | Ser        | 12    |
| 21    | Gln        | 88    |
| 22    | Ser        | 55    |
| 23    | Gln        | 25    |

Values following trichloroacetic acid precipitation (70 μl) of the A1 protein were dissolved in 0.5 ml trifluoroacetic acid and then subjected to gas phase sequencing as described in Experimental Procedures. The yields given were not corrected for either the increasing background level of nonspecific sequencing amino acid derivatives as the run progressed or for carryovers.

**Fig. 10.** Reverse-phase HPLC chromatogram of peptides derived from 10 pmol of the A1 protein. The digest (10 μg) was lyophilized and then dissolved in a 0.1 M column equilibrated with 0.05% trifluoroacetic acid. Elution was with increasing concentrations of acetonitrile.
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Table VI

| Residue Number | Amino Acid Composition of Peptide | Value as Residue per Molecule |
|----------------|---------------------------------|------------------------------|
| 6-15           | 148.16                          | 0.714                        |
| 24-33          | 148.16                          | 0.714                        |
| 52-61          | 148.16                          | 0.714                        |
| 107-126        | 148.16                          | 0.714                        |
| 167-179        | 148.16                          | 0.714                        |
| 219-231        | 148.16                          | 0.714                        |
| 261-270        | 148.16                          | 0.714                        |
| 311-320        | 148.16                          | 0.714                        |

Fig. 11. Peptide retention HPLC chromatogram of residues 1-5 (left) and 14-31 (right) of the gels shown begins at 2.0 min after the MAb nonretained open peptide that spans residues 179-230. The peptide was isolated at 4°C in 10% methanol and then derivatized with molybdate as described in reference 5. The two chromatograms at the bottom correspond to 100 pmol of each peptide labeled with 125I and dimer, respectively. The peaks labeled P and Q in columns 1 and 3 are the glycerophosphate derivatives of P and Q, respectively.

Fig. 12. Construction of expression plasmid pE775 for overproduction of b1. A partial BglII fragment of pGFP containing the b1 coding region was inserted in pGFP, or similarly with the desired sequence was named pE775. pE775 was ascribed in pRCAS vector. The final plasmid, pE785.

The presence of mammalian hnRNP complex in the CRNP coding region on the basis of a partially cleaved fragment not detected in CRNP and CRNP only. This fragment then was obtained from CRNP, along with a partial region of CRNP (500, 180, 180, 200, 200) at the CRNP end and of the coding region. This translated coding region was then sequenced using the dideoxynucleotide method, as shown in Fig. 12, which indicates that the CRNP coding region has been sequenced (17) and is the full-length open reading frame for CRNP. The expression of recombinant CRNP was detected by Coomassie blue staining of SDS-polyacrylamide gel and by Western blot analysis. The purification of recombinant CRNP was as described in reference 11, except that the total yield was divided from the DEAE-chromatography column at about 0.15 M NaCl, using a linear gradient of 0 to 0.4 M NaCl in Buffer D. The yield of over-expressing protein was 15 mg per 10 g cells.

Slicing studies with purified recombinant A1. Samples of freshly prepared A1 were used in slicing studies. Samples of purified recombinant A1 had a tendency to lose their cooperative properties during storage, which made it impossible to perform the experiments. There was also a consistent decrease in solubility. Consequently, these changes were the result of proteolytic degradation occurring during storage, which was further evidenced upon interaction with gelatinase (which would occur with a cooperative binding assay) was provided.