Recombinant hnRNP protein A1 and its N-terminal domain show preferential affinity for oligodeoxynucleotides homologous to intron/exon acceptor sites

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ABSTRACT

The reported binding preference of human hnRNP protein A1 for the 3'-splice site of some introns (Swanson and Dreyfuss (1988) EMBO J. 7, 3519 – 3529; Mayrand and Pederson (1990) Nucleic Acids Res. 18, 3307 – 3318) was tested by assaying *in vitro* the binding of purified recombinant A1 protein (expressed in bacteria) to synthetic oligodeoxynucleotides (21-mers) of suitable sequence. In such a minimal system we find preferential binding of protein A1 to oligodeoxynucleotide sequences corresponding to the 3'-splice site of IVS1 of human β-globin pre-mRNA and of IVS1 of Adenovirus type 2 major late transcript. Mutation studies demonstrate that the binding specificity is dependent on the known critical domains of this intron region, the AG splice site dinucleotide and polypyrimidine tract, and resides entirely in the short oligonucleotide sequence. Moreover specific binding does not require the presence of other hnRNP proteins or of snRNP particles. Studies with a truncated recombinant protein demonstrated that the minimal protein sequence determinants for A1 recognition of 3'-splice acceptor site reside entirely in the N-terminal 195 aa of the unmodified protein.

INTRODUCTION

hnRNP protein A1 is a prominent member of a family of RNA binding proteins composed of more than twenty individual components in the molecular weight range of 34 to 120 Kilo daltons (Kd). These proteins associate with hnRNAs in the nucleus of eukaryotes to form heterogeneous nuclear ribonucleoprotein (hnRNP) particles generally appearing in the form of a linear array of globular structures of 20 nanometers in diameter (monoparticles) (1, 2). Attempts to correlate the positioning of hnRNP particles with the general structural features of transcripts have not given unambiguous results (3, 4). In one instance however the location of the particle at splice junction sequences was reported (5) suggesting a specific role for these complexes in pre-mRNA processing. However what the role is for any individual protein component is incompletely understood. In fact only recently has an involvement in the splicing process been demonstrated for some of these proteins, the C proteins (6, 7).

HnRNP proteins were initially thought to bind single stranded RNA or DNA without any sequence specificity.(8, 9). More recently, however, another lab showed that several hnRNP proteins do exhibit a binding preference for RNA homopolymers (10) and that hnRNP proteins A1, C and D, both in nuclear extracts and as immunopurified preparations, preferentially bind to the 3' end of introns (11). On the other hand it has also been claimed that crosslinking of hnRNP A1 and C to specific intron sequences requires the presence of U1 and U2 snRNPs (12). It therefore remains unclear whether A1 protein alone is capable of specific binding.

Among human hnRNPs, protein A1 is the best characterized. Its cDNA and gene have been isolated (13, 14) and an intrinsic two-domain structure revealed (15). Moreover the cDNA has been overexpressed in *E.coli* allowing both the purification of recombinant A1 protein in large amounts and physico-chemical characterization (16, 17). With a homogeneously pure preparation of recombinant A1 protein available, an understanding of the protein requirements for binding specificity is possible. Additionally, an understanding of the aminoacids determinants for sequence specific A1 association can begin.

We have approached these questions by performing minimal *in vitro* binding studies between only recombinant A1 protein and oligodeoxynucleotides (19–21 oligomers) of suitable sequence. We find that the reported specificity of protein A1 for the 3' end of introns is precisely mimicked in single-stranded oligodeoxynucleotides and resides entirely in the unmodified protein and in the nucleic acid primary structure alone. We also show that a truncated protein (N-terminal 195 aa) containing the two ribonucleoprotein consensus sequences (RNP-CS) previously identified as directly involved in nucleic acid binding (17, 18, 19) has the same specificity with nearly the same affinity for oligodeoxynucleotides containing the 3' acceptor splice site as the complete protein.

MATERIALS AND METHODS

Oligonucleotide synthesis and purification

Oligodeoxynucleotides were synthesized on a Beckman system 1 Plus DNA synthesizer, treated for 24 hours with NH_4OH at

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55°C, and purified by gel electrophoresis. The DNA concentration was determined by absorbance at 260 nm. Oligonucleotides were labeled with T4 polynucleotide kinase to a specific activity of 2×10^6 cpm/μg as described(20).

Protein purification

Recombinant protein A1 and its truncated version were purified as described (16).

DNA-protein crosslinking

^{32}P radiolabeled oligonucleotides and purified protein in the amounts indicated in the text were mixed and incubated at 22°C for 10 min in a 10 μl reaction mixture containing 20 mM Tris-Cl pH 7.5, 2mM EDTA, 50 μg/ml BSA and 5% Glycerol to allow complex formation. The reaction mixtures were then transferred to ice and irradiated in open polypropilene Eppendorf tubes (0.7ml) with a standard VIS FL15E germicidal lamp (USA) at a dose of 10 mJ/mm^2. After the addition of sample buffer (50mM TRIS-CL pH6.8, 2% w/v SDS, 10% v/v Glycerol, 5% w/v 2-mercaptoethanol, 0.01% w/v bromophenol blue final concentrations) the mixtures were boiled for 10 min and then analyzed on a 10% SDS-polyacrylamide gel. The gels were then dried and autoradiographed with intensifying screen for 16 hours. Competition experiments were performed by first preincubating the protein (10 min at 22°C) with increasing amounts of unlabeled oligonucleotides before adding a fixed amount of the labeled oligonucleotide.

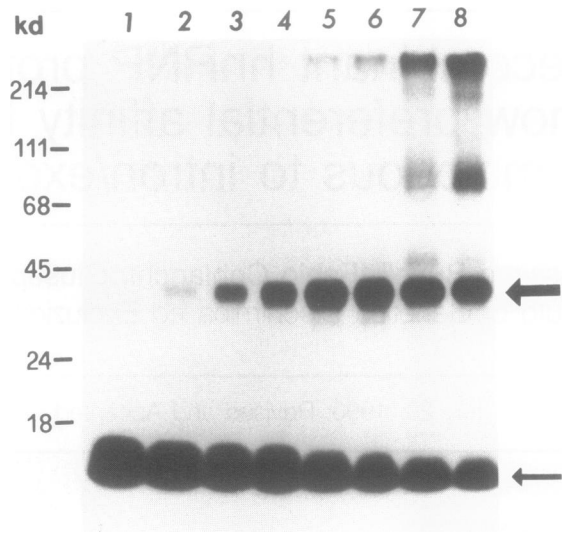

Fig. 1. UV induced crosslinking of protein A1 to oligonucleotides. Increasing amounts of purified A1 protein were incubated with 4 ng (0.2 μM) of ^{32}P labeled β globin wild type oligonucleotide (oligo 1, Fig. 2) and processed as described in Materials and Methods. Lanes 1 to 8 correspond to 0, 0.1, 0.2, 0.4, 0.86, 1.44, 1.88, and 5.2 μM protein A1 respectively. The thick arrow represents the single protein-oligonucleotide photocrosslinked product. The thin arrow indicates the unbound oligonucleotide. The position of the molecular weight standards is shown on the left.

RESULTS

Protein A1 binding to oligodeoxynucleotides

Protein-oligonucleotide complex formation could be detected both by electrophoretic mobility shift assay after UV-crosslinking of the complex and by a filter binding assay without UV irradiation. The first mrthod was chosen for the experiments described below since it permitted a more precise quantification of competition experiments. In a typical mobility shift experiment protein-DNA complexes (radioactive by virtue of the bound labeled oligonucleotide) were visualized by autoradiography of dried gels. One example of such an assay is shown in Fig.1 as a function of protein concentration. At low protein/DNA concentrations (molar ratios between 0.4 and 4) irradiation of protein A1-oligonucleotide complexes resulted in the formation of a single photocrosslinked product whose SDS-polyacrylamide gel electrophoresis mobility is consistent with a molecular mass of 40 Kd (thick arrow). This mass roughly corresponds to that of one molecule of A1 (34Kd) bound to one molecule of oligonucleotide (6Kd). At a higher protein concentration additional, more slowly migrating bands appeared. These signals probably correspond to crosslinked products containing multiple A1 molecules and oligonucleotides. The material that did not enter the gel is probably a high molecular weight aggregation of protein-DNA complexes; we have not investigated these effects further. A similar band shift was observed with oligonucleotides of unrelated sequence depicted in Fig. 2. Irradiation of uncomplexed A1 protein did not modify significantly its SDS-polyacrylamide gel electrophoretic migration. Heating protein A1 solution at 90°C for 10 min before the addition of the components of the binding assay resulted in complete abolition of binding activity; the addition of 2mM $MgCl_2$ in the incubation mixture reduced by a factor of 50% the signal of the 40 Kd band (data not shown). Thus the apparently weak association between A1

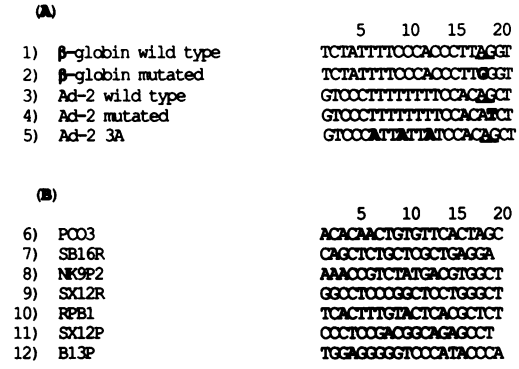

Fig. 2. Oligonucleotide sequences used for in vitro binding experiments. All sequences are written 5′ to 3′. **A**: wild type and mutated sequences analogous to 3′ end of IVS1 of human β-globin pre-mRNA (oligos 1 and 2) and of IVS1 of Ad-2 major late transcript (oligos 3, 4 and 5). **B**: unrelated sequences (oligos 6 to 12). Bold letters represent mutated nucleotides. The dinucleotide AG at the 3′ splice site is underlined.

protein and single-stranded DNA can be stabilized by UV crosslinking allowing any sequence preference of this association to be studied.

Sequence specificity of A1 binding to single-stranded oligodeoxynucleotides

Using the UV crosslinking assay we studied the binding of A1 protein to the oligonucleotides described in Fig. 2. Two well studied intron/exon junctions were chosen corresponding to the IVS1-E2 acceptor site of human β-globin pre-mRNA (21) and to the IVS1-E2 acceptor site of Adenovirus type 2 major late transcript (22) (oligos 1 and 3, Fig. 2A). The sequence of each of these two oligonucleotides were also changed in the AG

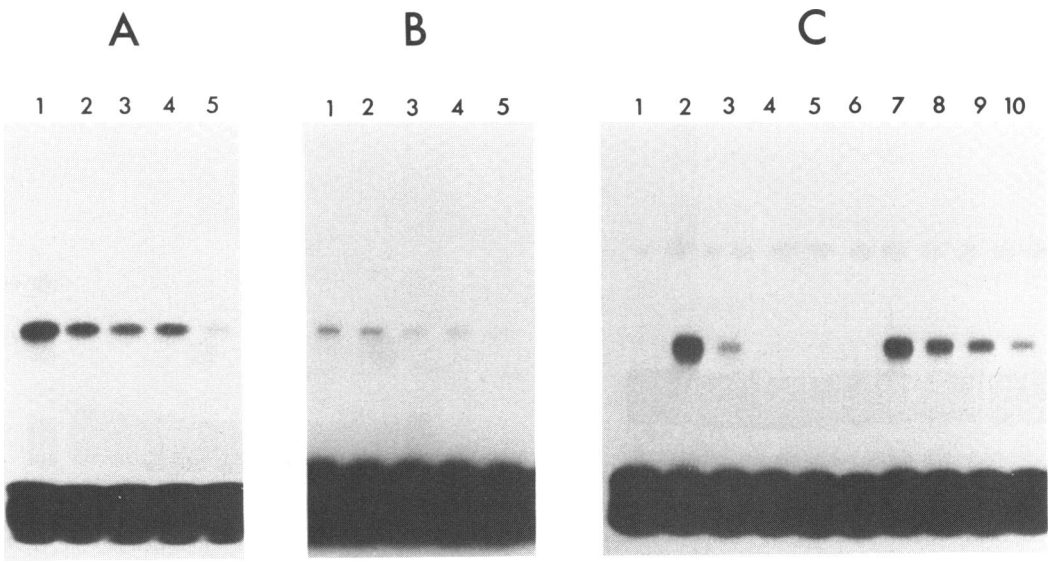

Fig. 3. Binding of protein A1 to oligonucleotides representing the 3′ end of introns. **A, B**: Salt dependent binding of A1 to oligonucleotides. 4 ng (0.2 μM) ^{32}P labeled (**A**): β-globin wild type oligonucleotide (oligo 1) or (**B**): β-globin mutated oligonucleotide (oligo 2) and 150 ng (0.4 μM) of A1 were incubated in reaction mixtures with 0.1, 0.2, 0.3, 0.4, and 0.5 M NaCl respectively (lanes 1−5). (**C**): Competition experiment between β-globin wild type and β-globin mutated oligonucleotides. 4 ng (0.2 μM) ^{32}P labeled β-globin wild type oligonucleotide were incubated with 0 (lane 1) and 150 ng (0.4 μM) of protein A1 in presence of 0 (lane 2) and 10, 50, 100, 200 fold excess of β-globin wild type (lanes 3, 4, 5 and 6) or of β-globin mutated (lanes 7, 8, 9 and 10) oligonucleotides respectively.

consensus dinucleotide (oligos 2 and 4). In addition, the Ad-2 wild type oligonucleotide was altered in the polypirimidine stretch by insertion of 3 As in place of 3 Ts (oligo 5). These mutations were chosen since in a previous study (23) they were shown to reduce the binding of hnRNP protein C and of U2 snRNP to the 3′ splice site of the first intron of Adenovirus type 10 RNA. Seven more oligonucleotides with unrelated sequences (Fig. 2B) were also tested.

The first binding experiment (Fig. 3) was performed as a function of NaCl concentration using the wild type and mutated β-globin oligonucleotides (oligos 1 and 2), labeled at exactly the same specific activity. As can be seen, substantially more complex formation is observed with the wild type oligonucleotide (Fig. 3A) compared to the mutated one (Fig. 3B). It should be observed that the unretarded band of the mutated oligonucleotide (Fig. 3B), although broader than that of wild type, contains the same number of counts. On the other hand both complexes are equally sensitive to salt concentration suggesting that specific base-protein interaction, not the stronger general electrostatic interaction between A1 and oligonucleotides, defines binding preference.

To confirm this preference for 3′ intron/exon junction sequence, competition binding assays were performed in which we measured the capacity of a small fixed amount of labeled β-globin wild type oligonucleotide to displace the previously bound unlabeled oligonucleotide (see Materials and Methods). As can be seen in Fig. 3C the complex formed with the wild type oligonucleotide (oligo 1) was competed completely with a 50 fold excess of the same unlabeled oligonucleotide (Fig. 3C, lane 4) while a 200 fold excess of mutated oligonucleotide (oligo 2) was not sufficient to completely prevent wild type complex formation (Fig. 3C, lane 10). Ten fold excess of the wild type oligonucleotide as unlabeled competitor (Fig. 3C, lane 3) was as efficient as 200 fold excess of mutated oligonucleotide (Fig. 3C, lane 10) suggesting an apparently 20 fold preference for the wild type sequence. In a reverse competition experiment where the mutated oligonucleotide (oligo 2, in Fig. 2) is labeled a similar preference is seen for the wild type sequence (oligo 1, in Fig. 2).

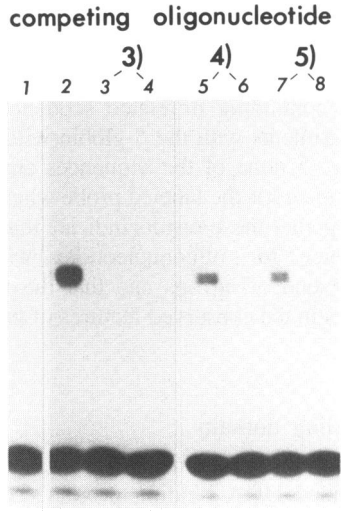

Fig. 4. Binding experiments with Ad-2 wild type and competitions with Ad-2 mutated or Ad-2 3A oligonucleotides. 0.2 μM of ^{32}P labeled Ad-2 wild type oligonucleotide were incubated with 0.4 μM of protein A1. Lane 1: no protein, lane 2: no competitor; lanes 3, 5, 7 and 4, 6, 8 contain 10 fold and 50 fold excess respectively of the indicated unlabeled oligonucleotides.

To further investigate the specific interaction of A1 with sequences analogous to the 3′ end of introns, we performed another set of direct binding and competition experiments with oligonucleotides representing the 3′ end of the first intron of Ad-2 major late transcript. As shown in Fig. 4 binding of A1 to the labeled Ad-2 wild type sequence is abolished by a 10 fold excess of the same unlabeled sequence (Fig. 4, lane 3) while a 50 fold excess is required in order to obtain a similar effect with the mutated Ad-2 oligonucleotide carrying a point mutation changing AG to AT at the 3′ splice site (Fig. 4, lane 6). Furthermore,

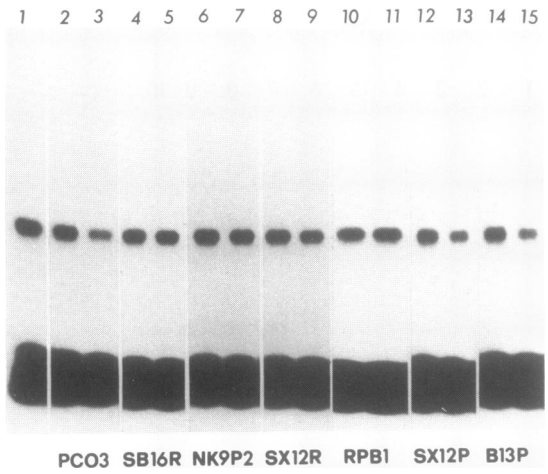

Fig. 5. Competition experiment with oligonucleotides of unrelated sequence. Binding of A1 to β-globin wild type oligonucleotide in the standard UV induced crosslinking assay (lane 1) was challenged in a competition experiment with 10 fold excess (even lanes) and 50 fold excess (odd lanes) of the indicated oligonucleotides (see Fig. 2).

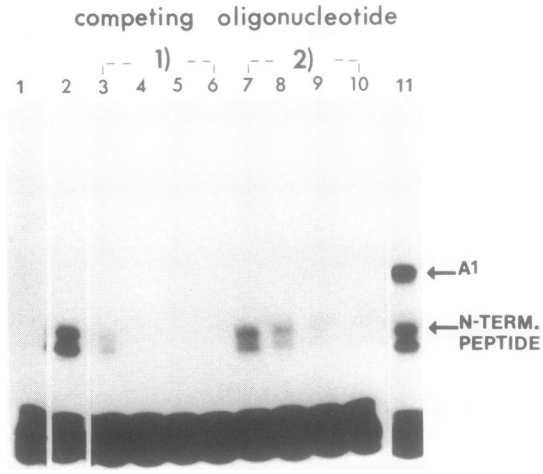

Fig. 6. Binding specificity of the truncated protein A1. 4 ng (O.2 μM) of β-globin wild type ^{32}P labeled oligonucleotide were incubated with 0 (lane 1) and 96 ng (0.4 μM) N-terminal peptide (lane 2). Lanes 3−6 and 7−10: competitions with 10, 50, 100, and 200 fold excess of the unlabeled indicated oligonucleotides. Lane 11: incubation with 150 ng (0.4 μM) of A1 and 96 ng (0.4 μM) of N-terminal peptide.

an oligonucleotide analogous to the same region of the intervening sequence of Ad-2 in which the natural polypyrimidine stretch has been altered with the introduction of a triple mutation (oligo 5, in Fig. 2) behaves very much as the mutated Ad-2 oligonucleotide (Fig. 4, lane 8).

Finally, we tested the binding activity of A1 with a series of oligonucleotides containing unrelated sequences (Fig. 2B) in competition experiments with the β-globin wild type sequence. As shown in Fig. 5 none of the sequences analyzed was able to efficiently compete for the labeled probe when used at 50 fold excess. Taken together these results indicate that protein A1 has binding preference for oligonucleotides whose sequences correspond to 3′ end of introns and that the majority of that preference resides in the conserved features of the 3′ intron/exon junction.

Protein A1 binding domain

The N-terminal portion of A1 (first 195 aa) was initially described as a single-stranded DNA binding protein (UP1)(24, 25). In previous work it was demonstrated that this portion of the protein can be readily crosslinked by UV light to thymine oligodeoxynucleotides (oligo(dT)$_8$) (26) and that defined phenylalanine residues are the sites of covalent adduct formation (17). These data suggested but did not prove that the single-stranded DNA binding domain of A1 resides in its N-terminal 195 residues.

In order to further define the structural determinants for DNA-binding specificity we conducted direct binding and binding competition experiments using a recombinant N-terminal A1 peptide purified from overexpressing cultures of *E.coli* as described (16). In this analysis the protein was UV crosslinked to ^{32}P β-globin wild type oligonucleotide in our standard binding assay. In direct binding experiments we observed DNA-protein complexes migrating with the expected molecular mass of 30 Kd (24 Kd contributed by the protein and 6 Kd by the oligonucleotide) (Fig. 6, lane 2) and with a protein concentration dependence very similar to that of the intact protein A1 (not shown). In competition experiments with the N-terminal peptide alone (Fig. 6, lanes 3 to 10) the binding to β-globin wild type probe was efficiently prevented by the addition in the reaction mixture of 10 fold excess of unlabeled wild type oligo (Fig. 6, lane 3), while an excess of 50−100 fold of the mutated sequence was required in order to obtain the same effect (Fig. 6, lanes 8 and 9). These results indicated that the N-terminal domain of protein A1, containing the two RNP consensus sequences (RNP-CS) previously identified in several RNA binding proteins (19, 27), is sufficient for specific DNA binding. The shifted band just below the N-terminal peptide is due to a 22 Kd proteolysis product of the 24 Kd protein generated during storage. Because this species also binds specifically, the size of the protein region conferring sequence specificity is further limited.

When A1 and N-terminal peptide were mixed in equimolar amounts in direct binding experiments both proteins were crosslinked with nearly identical efficiency to the oligonucleotide probes (Fig. 6, lane 11).

To eliminate the possibility that the observed protein A1 binding preference is a general property of proteins having affinity for single-stranded nucleic acids and to rule out an intrinsic bias for protein binding in the set of oligonucleotides tested, a prototypical ssDNA binding protein, the product of T4 gene 32 (P32 protein), was used as a control in similar binding experiments. It was previously shown (28, 29) that P32 protein binds with the same affinity to both DNA and RNA oligomers independent of their base composition. When UV crosslinking experiments were performed on our set of oligonucleotides with this purified protein the results shown in Fig. 7 were obtained. The binding of P32 protein to labeled β-globin wild type oligonucleotide (Fig. 7, lane 1) was competed to the same extent with 10 and 50 fold excess of unlabeled β-globin wild type (Fig. 7, lanes 2, 3) or β-globin mutated oligonucleotide (Fig. 7, lanes 4, 5). The band just below the arrow visible in Fig. 7 probably corresponds to a well described degradation product of gene 32 protein (30). The same results were observed in reverse competition experiments (Fig. 7, lanes 6 to 10), thus indicating no binding specificity for this protein in our assay. This result further confirms the view that preference for the conserved AG

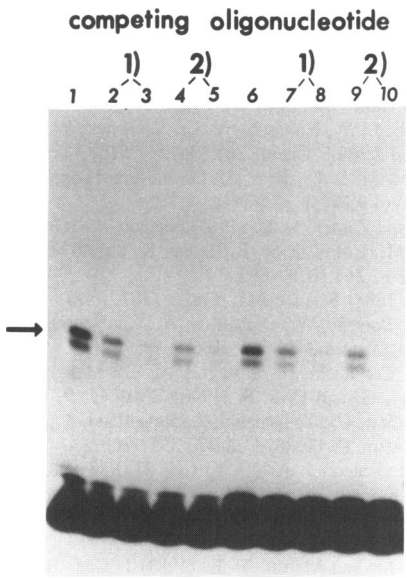

Fig. 7. Interaction of T4 P32 protein with oligonucleotides. 1.1 μg (3 μM) of P32 protein were mixed with 0.2 μM ^{32}P labeled β- globin wild type oligonucleotide (lane 1) or with 0.2 μM ^{32}P labeled β globin mutated oligonucleotide (lane 6). Lanes 2–5: competition experiments; lanes 7–10: reverse competition experiments. Lanes 2, 4, 7, and 9 contain 10 fold excess of indicated unlabeled oligonucleotide; lanes 3, 5, 8, and 10 contain 50 fold excess of indicated unlabeled oligonucleotide.

dinucleotide and polypyrimidine tract is a specific property of the A1 protein sequence.

DISCUSSION

The binding specificity of some hnRNP proteins for pre-mRNA sequences has been previously documented (11, 12). In particular, protein A1 was shown to bind preferentially to the 3′ end of some introns and to be dependent on the conserved dinucleotide AG and polypyrimidine tract at the splice site. Because this finding contradicted the prevailing notion that hnRNP proteins bind indiscriminately to single-stranded nucleic acids (RNA or DNA) without sequence specificity, it is important to expand our understanding of A1-nucleic acid interaction since it also occurs at a site with an obvious significance in the processing of pre m-RNA. Given the known properties of protein A1, it is to be expected (11) that the difference between specific and non-specific binding constants is too small to permit the detection of a binding specificity in experiments where large nucleic acid molecules can simultaneusly bind many protein molecules. A method by which the protein can bind only to a single site and the competition between different sites can be evaluated was therefore devised. Toward this end we report the binding of pure recombinant A1 protein to synthetic oligodeoxynucleotides. We synthesized a set of 21 nucleotide long oligomers corresponding to the 3′ terminal region of two well characterized introns as well as several corresponding mutated sequences and a number of unrelated sequences of comparable length (see Fig. 2). A general criticism to this approach could be that we have studied A1 association with deoxy rather than ribo-oligonucleotides. However in view of the results clearly demonstrating that preferential binding to the 3′ splice site is dependent on the identical sequence determinants (AG splice site dinucleotide and polypyrimidine tract) as RNA binding, our system seems vindicated and its extension to begin defining A1 protein domains involved in nucleic acid association is justified. Moreover several studies support the contention of an overall equivalence between single-stranded DNA and RNA in protein recognition and binding (4, 8, 17, 31).

Although the absolute binding affinity of A1 for any oligonucleotide is apparently low as judged by our inability to obtain conventional electrophoretic band shifts, UV crosslinking has allowed analysis of relative affinities toward each oligonucleotide. It is important to note that the described binding preference of A1 can be reproduced in a filter binding assay i. e. in the absence of UV crosslinking, (data not shown) thus ruling out any effect of UV photochemistry on the described specificity and validating UV-crosslinking assays. However filter binding assay was not suitable for competition experiments. Qualitatively the relative affinity of A1 for each class of target sequences is: wild type sequences > mutated sequences > unrelated sequences.

Such a marked sequence preference by A1 in this simple *in vitro* system has a number of implications for hnRNPs association with pre-mRNA. First, the target sequences are too short to allow significant secondary structure. Therefore the minimal sequence determinants for A1 recognition of 3′ splice acceptor site do not require association with more distant regions. However we cannot rule out the possibility that local nucleic acid structure would not substantially enhance A1 affinity *in vivo*. Second, correct A1 binding preference was obtained with purified protein synthesized in *E. coli*. Hence minimal splice site recognition does not require eukaryotic post-translational modifications, although we cannot rule out a role for such modifications in achieving full *in vivo* affinity. Finally specific binding was achieved in the absence of all other hnRNP proteins or other associated components. Thus minimal A1 binding requires no accessory proteins or nucleic acids, a conclusion that could not be drawn from the initial experiments (11). Again, however, association of A1 with other factors such as snRNPs may well augment and/or expand this minimal binding preference. Experiments in this direction have been recently reported (12).

Our observation that the N-terminal 195 residues of A1 expressed in *E. coli* retain the identical binding specificity with comparable affinity clearly limits the 3′ splice junction binding function to this region of the protein. This portion of A1 is itself composed of two similar subdomains (residues 3 to 93 and 94 to 184) (32). It will be interesting to further dissect binding functions to determine whether this two-fold structure is required for nucleic acids association as has been found for other nucleic acid binding proteins (33, 34). By elimination, the C-terminal 120 aa (glycine-rich domain) of A1 do not participate in the minimal sequence recognition deduced here. Although they may yet play a role in the complete *in vivo* sequence recognition more likely they are involved in the complex interaction accompanying hnRNP particle assembly as already suggested by us and others (11, 16). In accordance with this interpretation is the observation that the A1^{B} protein, a variant of the A1 protein with an expanded C-terminal domain (35) shows exactly the same binding preference as protein A1 (data not shown).

In recent years, a considerable assortment of proteins in addition to hnRNPs have been found to interact with the 3′ end of introns: some with polypyrimidine tract preference (23) and some with polypyrimidine tract as well as 3′ splice site specificity (36, 37, 38). Most of these proteins appear to be directly or indirectly involved in maturation of pre-mRNA at this site.

Clearly not all proteins and RNP's recognizing overlapping domains containing or near the 3′ splice site can simultaneously associate with pre-mRNA; some temporal order or hierarchy and subsequent displacement seems likely. HnRNP's are well documented to associate with nascent RNA (39, 40) significantly before other components more directly involved in the splicing event are thought to bind (2). Thus a potential role for A1 (and perhaps for other hnRNP proteins) could be to guide the particle to this critical region of a pre-mRNA, possibly sequestering this domain for later spliceosome-mediated processing, or, perhaps more directly, as an early step in spliceosome assembly at the 3′ splice junction. Whatever the precise role of A1 in hnRNA-mediated transactions of pre-mRNA's, its recognition of a limited sequence domain near the 3′ splice site seems critical to a complete understanding of RNA maturation.

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