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Renaturation of complementary DNA strands mediated by purified mammalian heterogeneous nuclear ribonucleoprotein A1 protein: Implications for a mechanism for rapid molecular assembly

(annealing/nucleic acid)

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ABSTRACT **Purified heterogeneous nuclear ribonucleoprotein (hnRNP) A1 protein, which is found *in vivo* associated with heterogeneous nuclear RNA (hnRNA), promotes the rapid renaturation of nucleic acid strands. Maximal renaturation activity requires the glycine-rich carboxyl-terminal one-third of the protein, although the amino-terminal two-thirds also has activity. The A1-mediated reaction is second-order with respect to complementary DNA concentration, and the renaturation rate constant at 37°C with A1 is about 3000-fold greater than in the absence of the protein. At 60°C, the A1-mediated renaturation rate is even faster, and is about 300-fold greater than protein-free reactions carried out at 68°C in 1 M NaCl. Provided that sufficient A1 protein is present to coat all strands in solution, the presence of nonhomologous, single-stranded DNA does not significantly inhibit the reaction. Moreover, renaturation of short strands to their complement contained in very long strands is nearly as efficient as between two short strands. These results indicate that A1 may be useful for procedures that rely on nucleic acid renaturation. We propose that A1 promotes rapid renaturation primarily by reducing the entropic barrier of bimolecular strand association through relatively transient interactions between A1-coated strands. Such interactions, mediated by flexible repeating domains, may act generally to increase the association kinetics of highly specific molecular assemblies in processes such as RNA maturation, transcription, translation, and transport.**

Newly synthesized heterogeneous nuclear RNA (hnRNA) rapidly associates with a discrete set of proteins in the mammalian nucleus, forming what has been termed the heterogeneous nuclear ribonucleoprotein (hnRNP) complex (1–3). The role of these proteins in hnRNA biogenesis is unclear, although it has been suggested that they are involved in packaging, processing, and transport. Several hnRNA-associated proteins share similar structural features, including an RNA-binding motif (4–6). This binding motif has also been identified in a variety of other proteins that are found associated with RNA *in vivo* (7, 8). These include proteins of the small nuclear RNP (snRNP) complexes (9, 10), yeast and mammalian poly(A)-binding proteins (11–13), and gene products encoded by regulators of sex determination in *Drosophila* (14–16), which are thought to affect gene expression at the level of RNA splicing (17). A number of these proteins include additional domains, which are often rich in glycine, proline, or another predominant amino acid (8). The roles of these domains are unknown, but they are thought to be involved in protein–protein and protein–nucleic acid interactions.

The A1 hnRNP protein is a member of the core hnRNP complex, which is isolated after limited nuclease digestion of hnRNAs purified from mammalian cell nuclei (1). When core complexes are incubated in mild ionic strength, A1 protein dissociates from the complex (1). In addition, A1 is sensitive to mild protease treatment (18), suggesting that it is associated on the outside of the core. Besides being a core component, A1 has been shown to be associated in a specific complex at 3′ splice sites, suggesting a possible involvement in splicing at that junction (19).

Studies of the structural features and binding properties of A1 indicate that A1 contains two amino-terminal RNA-binding domains (4), as well as a glycine-rich carboxyl terminus (20, 21). The glycine-rich region, which comprises more than one-third of the protein, is thought to be further subdivided into a short repeating unit (21). A1 has been shown to bind both RNA and single-stranded DNA, with dissociation occurring at high ionic strength (20, 22, 23). Binding affinity to different RNA homopolymers is similar, suggesting a lack of sequence specificity (20, 24). A proteolytic fragment of A1, consisting of the amino-terminal RNA-binding domains, was initially purified from calf thymus (25). This fragment, called UP1, retains the ability to bind nucleic acid (25, 26). A multimer of the glycine-rich region repeat also binds nucleic acid *in vitro*, implicating it in making additional contacts (22). This may account for the larger binding site of A1 [15 nucleotides (nt)] when compared to UP1 (7 nt) (22). The glycine-rich region is also required for cooperative binding to nucleic acid (22), suggesting that it may participate in protein–protein interactions as well.

This paper describes an additional property of A1: the ability to promote the renaturation of DNA strands *in vitro*. A1-mediated renaturation is second-order, and its glycine-rich carboxyl terminus is required for maximal rates. Renaturation can be stimulated more than 3000-fold in the presence of A1 and, at elevated temperatures, is 300-fold faster than uncatalyzed reactions under standard optimal conditions (68°C; 1 M NaCl). These properties suggest that A1 may be useful for procedures that rely on the renaturation of nucleic acid strands. In addition, the properties of A1-promoted renaturation, coupled with the localization of A1 and related proteins to a wide variety of RNAs *in vivo*, support a model where these proteins function to facilitate the assembly of RNP complexes. We suggest a mechanism for A1-mediated renaturation that may apply to a wide range of rapid assembly processes *in vivo* and *in vitro*.

MATERIALS AND METHODS

DNA Substrates. Routine DNA manipulations were performed as described (27). The labeled probe used to monitor renaturation was prepared by digesting 20 μg of pSV2gpt (28)

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Abbreviations: hnRNP, heterogeneous nuclear ribonucleoprotein; hnRNA, heterogeneous nuclear RNA; snRNP, small nuclear ribonucleoprotein; nt, nucleotide(s).

with *Hin*dIII and *Bgl* II endonucleases. The digest was electrophoresed on a native polyacrylamide gel, and the 120-base-pair (bp) fragment was eluted from a crushed gel slice by incubation at 24°C for 16 hr in 500 μl of water. The eluate was extracted with butanol, evaporated to a small volume, and end-labeled by using [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I. This ^{32}P-labeled 124-bp fragment was electrophoresed a second time, reisolated, and stored in 10 mM Tris, pH 7.5/1 mM EDTA. These strands were used as the substrate in subsequent renaturation experiments. DNA concentrations were quantitated by comparison with known quantities of *Hin*dIII/*Bgl* II endonuclease-cut pSV2gpt DNA on ethidium-stained acrylamide gels. M13mp18 single-stranded DNA containing homology to one strand of the labeled probe was generated by cloning the 120-bp *Hin*dIII–*Bgl* II fragment into M13mp18 replicative form DNA and recovering single strands from phage as described (29).

Protein Preparation. Recombinant A1 protein was purified as described (22), and aliquots were stored in modified buffer E (1 M NaCl/50 mM Tris, pH 7.5/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride) at −80°C. A1 protein concentrations were determined by quantitative amino acid analysis. UP1 was provided by Ken Williams (Yale University).

Renaturation Reaction Conditions. Reactions were carried out in 20 μl of 10 mM potassium phosphate, pH 7.0/1 mM EDTA/80 mM NaCl containing the ^{32}P-labeled 124-base-pair (bp) probe. Single-stranded phage M13 DNA and A1 protein were included as indicated. All components with the exception of A1 protein were premixed, incubated at 95°C for 5 min, and then rapidly chilled in ice water to generate single strands. The mixtures were then preincubated at 37°C for 2 min before the addition of A1 protein. After incubation, the reactions were stopped as described in the figure legends and electrophoresed on 10% polyacrylamide gels under nondenaturing conditions. Gels were vacuum-dried and subjected to autoradiography. Results were quantitated by densitometry. When comparing A1 and protein-free renaturation reactions, an equal volume of buffer E was added, and NaCl from this source was included in the final concentration.

Reaction mixtures for renaturation at 68°C in 1 M NaCl were incubated in 10 mM buffer (pH 7.0; either Tris chloride as in Fig. 3 or potassium phosphate as in Fig. 5). These reaction mixtures were preincubated at 68°C for 1 min before the addition of NaCl to a final concentration of 1 M.

RESULTS

Fig. 1 shows renaturation of the 124-nt-long complementary DNA strands over time. In the presence of A1, detectable renaturation occurs within a few seconds, with no measurable lag after the addition of protein. About half of the single strands were converted to duplex DNA after 2 min, and renaturation was virtually complete within 32 min. In contrast, under the same conditions in the absence of protein, no renaturation was observed, even after 32 min.

Similar experiments were carried out comparing the annealing activities of A1 and UP1, the protein fragment corresponding to A1's RNA-binding domains (Fig. 2). The same experimental design was used, except that the reactions included increasing concentrations of protein and were terminated after 5 min at 37°C. Maximal A1-mediated renaturation occurred with 30–60 nM protein, and additional A1 was inhibitory. UP1 also promoted renaturation, but to a far lesser extent. Maximal renaturation with UP1 required 300 nM protein, and additional UP1 did not inhibit the reaction.

The orders of the reactions were determined by measuring the half-times of renaturation at different initial DNA concentrations. When the logarithms of the half-times of renaturation are plotted against the logarithms of the initial DNA

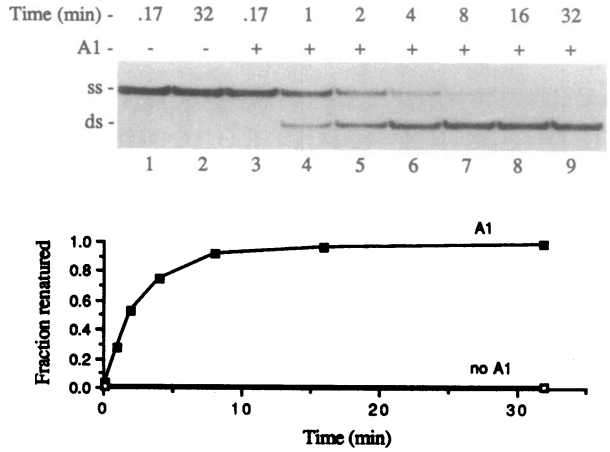


FIG. 1. Time course of A1 hnRNP protein-mediated renaturation of 124-nt-long DNA strands. Reactions were performed at 37°C with 4.5 nM single strands (ss; expressed in nt) as described. Lanes: 1 and 2, no A1; 3–9, A1 protein at a concentration of 32 nM. Reactions were stopped by the addition of 3 μl of buffer containing tRNA at 0.3 mg/ml, proteinase K at 0.3 mg/ml, 0.67% SDS, 30% glycerol, and 0.3% bromophenol blue and then were incubated for an additional 5 min at 37°C before electrophoresis.

concentrations, a straight line with a slope of −1 indicates second-order reaction kinetics. Fig. 3 shows that A1-mediated renaturation follows second-order kinetics. The kinetics of renaturation under the same conditions in the absence of protein and at 68°C in 1 M NaCl were also second-order as expected (data not shown). A comparison of the second-order rate constants for these reactions indicates that A1 increases the renaturation rate by more than 3000-fold. In addition, the A1-mediated reaction under these conditions is 50-fold faster than renaturation at 68°C in 1 M NaCl. In contrast, the kinetics of UP1-mediated renaturation does not appear to be second-order. Rates of renaturation with UP1 follow more closely the square root of the probe concentration (slope = −0.5). Therefore, although renaturation occurred more rapidly with A1 than with UP1 under all conditions tested, the extent of this difference is a function of the concentration of complementary strands.

The rate of renaturation was strongly influenced by the ratio of A1 protein to total DNA in solution. This is illustrated by comparing the extent of renaturation (for 5-min reactions) at two A1 concentrations in the presence of increasing amounts of noncomplementary single-stranded M13mp18 DNA (Fig. 4). In the absence of M13 DNA, renaturation occurred with similar efficiency at both A1 concentrations. As the amount of M13 DNA was increased, the extent of renaturation was relatively unaffected until a ratio of 12–15 nucleotides per A1 monomer was reached. When the M13 DNA concentration was increased an additional 2-fold, re-

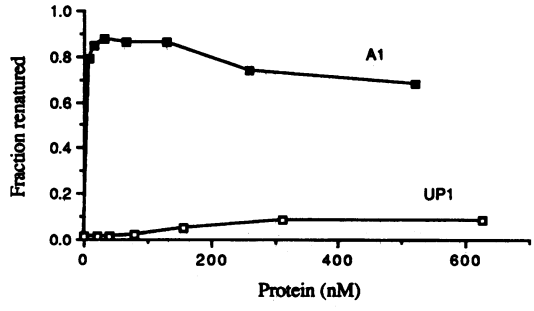
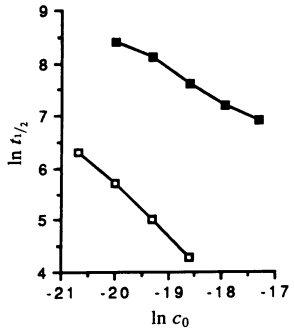


FIG. 2. Relative rates of A1- and UP1-mediated renaturation of DNA strands. The reactions were carried out as in Fig. 1, with 5-min incubations.



	Slope	k_2
37°C, + A1	−1.0	1.7×10^6
37°C, −A1	−1.0	5.0×10^2
68°C, 1 M NaCl	−1.0	3.5×10^4
37°C, UP1	−0.6	—

FIG. 3. The kinetics of uncatalyzed, A1 (□)-, and UP1 (■)-mediated renaturation reactions. A1- and UP1-mediated reactions were performed as described in Fig. 1. The uncatalyzed reactions were stopped by diluting at 0°C 1 μl of each reaction mixture with 9 μl of 10 mM Tris chloride buffer (pH 7.0) containing 5.5% glycerol and 0.06% bromophenol blue. The natural logarithm of the half-time ($t_{1/2}$) of renaturation in sec is plotted against the natural logarithm of the initial nucleotide molar concentration (c_0). Second-order association rate constants in $M^{-1}\cdot sec^{-1}$ were calculated by using the equation $k_2 = c_0^{-1}\cdot t_{1/2}^{-1}$.

naturation was sharply inhibited. The molar ratio of nucleotide to A1 at which this sharp reduction occurred agrees well with the known binding stoichiometry of 12 nucleotides per A1 monomer (22) and suggests that A1-mediated renaturation requires a protein-coated nucleic acid strand.

In the reaction with 1.1 μM A1 and a 14:1 molar ratio of nucleotide to protein, the M13 DNA is present in a 1000-fold excess over the probe strands. Rapid renaturation under these conditions showed that a large excess of heterologous single-stranded DNA is not strongly inhibitory. Similar reactions with the 124-nt probe strands and equimolar amounts of M13 DNA containing the sequence complementary to one of the probe strands showed that M13 DNA in solution is also competent for renaturation (Fig. 5). The products of the reactions were electrophoresed under conditions that separate both labeled single strands from each other and from the double-stranded products. The reactions proceeded to completion as indicated by the complete utilization of the probe strand complementary to the insert in the M13 DNA. Subjecting the reaction products to agarose gel electrophoresis demonstrated that the slowest migrating labeled species comigrated with M13 single-stranded DNA. In addition, it was shown that formation of the high molecular weight product required that a sequence in the M13 strand was complementary to the probe (data not shown).

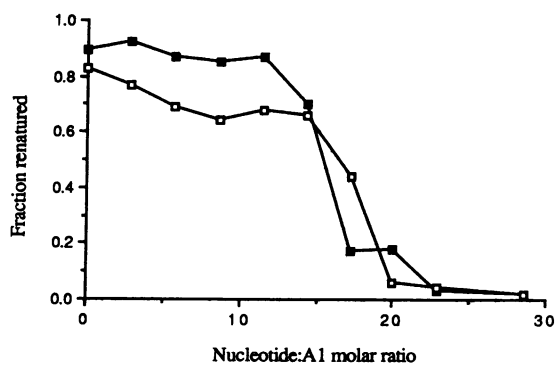


FIG. 4. A1-mediated renaturation as a function of the total nucleotide:A1 protein molar ratio. Five-minute renaturation reactions were performed as described in Fig. 1 with 0.26 (■) or 1.1 (□) μM A1 protein and increasing amounts of M13mp18 DNA expressed in nt. After proteinase K treatment, reaction mixtures were extracted with 1:1 (vol/vol) phenol/chloroform and subjected to electrophoresis as described.

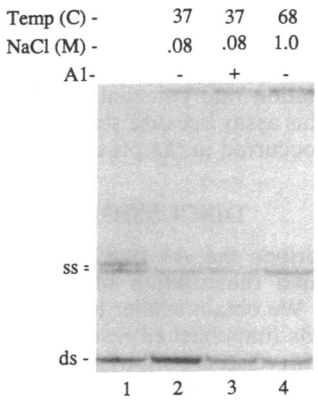


FIG. 5. Comparison of renaturation between short strands and M13 single-stranded DNA containing the sequence complementary to the short strand. Lanes: 1, 124-nt single-stranded and 124-nt double-stranded markers; 2, renaturation at 37°C in the absence of A1 hnRNP protein (Reactions were incubated for 20 hr with 780 pM of the two short strands and 780 pM M13 single-stranded DNA containing the sequence complementary to one of the short strands.); 3, renaturation at 37°C in the presence of 260 nM A1 protein (Reactions were incubated for 30 min with each strand present at 52 pM.); 4, renaturation at 68°C in 1 M NaCl in the absence of protein (Reactions were incubated for 30 min with each strand present at 780 pM.). A 2-fold greater signal for the short 124-bp product is a consequence of both short strands being end-labeled.

The efficiency of renaturation between the complementary probe strands and between a probe strand and the M13 DNA containing complementary sequence is a function of the reaction conditions. At 68°C in 1 M NaCl, the extent of renaturation between the two probe strands or a probe strand and the M13 strand was about equal. This result is consistent with previous work showing that the rate of renaturation under these conditions is a function of the length of the shortest complementary partner (30). When reactions were performed in the absence of protein at 37°C in 80 mM NaCl, the rate of renaturation between the short probe strands was greater by about 7-fold. This result is also expected, as the renaturation of long strands is inhibited by the formation of intrastrand secondary structures under these conditions (31–33). When A1 was included under the same conditions, renaturation between probe and M13 strands occurred with a 2-fold increase in relative efficiency. This result shows that, at 37°C in 80 mM NaCl, A1 protein increases the renaturation efficiency between short and long strands relative to the reaction between two short strands.

Thus far, we have shown that A1 markedly increases the renaturation rate between complementary DNA strands at 37°C. However, the rate of A1-mediated renaturation increased still further at higher temperatures (Fig. 6). The

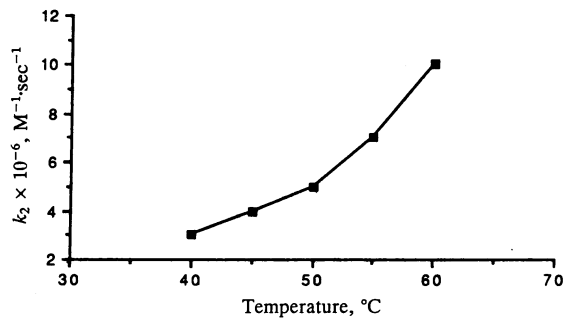


FIG. 6. A1-mediated renaturation as a function of temperature. Reactions were incubated as in Fig. 1 with 2 nM single strands (expressed in nucleotides) and 32 nM A1 protein. Reactions were stopped and analyzed as described in Fig. 1.

second-order rate constant for renaturation at 60°C was about 6-fold faster than at 37°C and 300-fold greater than reactions run in the absence of protein at 68°C in 1 M NaCl. Above 60°C, the association rate constants could not be directly measured with this assay because strand dissociation as well as renaturation occurred in the presence of A1.

DISCUSSION

This work describes the A1 hnRNP protein's ability to promote the rapid renaturation of complementary DNA strands *in vitro*. We obtain similar results with complementary RNA strands (unpublished results), and these findings are consistent with related work by A. Kumar and S. Wilson (unpublished data) and, independently, by X. Dong and S. Munroe (unpublished data).

A Mechanism for A1-Mediated Renaturation. A comparison between the characteristics of other renaturation reactions and those mediated by A1 helps in considering how A1 promotes the renaturation of complementary nucleic acid sequences. *Escherichia coli* single-stranded DNA-binding protein (SSB), for example, also promotes the renaturation of complementary strands in a second-order reaction (34). By coating the strands, SSB is thought to enhance renaturation by reducing the electrostatic repulsion of the negatively charged DNA molecules and by melting out intramolecular secondary structures (34). Because of this, SSB is most efficient in enhancing the renaturation of long DNA strands, where these problems are a greater barrier to renaturation. For strands about 200 nt long, SSB enhances renaturation only 6-fold. This is less efficient by a factor of 500 than the A1-mediated reactions, which use even shorter complementary strands. In addition, renaturation at 68°C in 1 M NaCl is not thought to be significantly inhibited by either electrostatic repulsion or the formation of intrastrand secondary structures (31–33). Because A1 is so effective in renaturing short strands and because rates of A1-mediated renaturation can be 300-fold greater than renaturation at 68°C in 1 M NaCl, it seems unlikely that A1 acts only to reduce these barriers to duplex formation.

Other studies indicate that renaturation can be enhanced by increasing the effective concentration of nucleic acids in solution, thereby reducing the entropic barrier of bimolecular strand association. The limit of this effect is presumably the rate of hairpin formation, which is extremely rapid (35). RecA protein, which renatures strands by decreasing the entropic barrier, causes the formation of large DNA–protein aggregates (36–39). These aggregates are thought to be intermediates in the first-order renaturation process, which is limited by the rate of association of complementary sequence within an aggregate. However, A1-mediated renaturation is clearly different, in that it is second-order and does not cause aggregates similar to those found in the RecA reactions (unpublished results). Renaturation has also been enhanced by high concentrations of polymers (40) and with certain phenol emulsions (41). Both of these are thought to increase the concentration of strands with respect to each other. However, A1 is effective at low concentration and does not produce an emulsion.

We suggest that A1 facilitates renaturation primarily by mediating frequent, but transient, associations between non-base-paired strands. This could occur if A1 proteins on one strand interact with either the DNA backbone or other A1 proteins on a complementary strand. A transient association, which held bases on complementary strands in close proximity, would lead to stable base pairing if the complex had sufficient flexibility so that rapid thermal fluctuations could lead to a nucleation event. This would make the initial bimolecular event a rather nonspecific, and therefore more probable and rapid, interaction between A1 and its corresponding partner and would make the nucleation event a relatively rapid monomolecular reaction. The overall kinetics remain second-order so long as the initial association between strands (which leads to nucleation) is rate-limiting. Our supposition that the initial complexes are transient stems from the finding that noncomplementary DNA strands do not greatly inhibit renaturation, which would not be expected if A1-coated strands bind tightly to noncomplementary sequences.

A1 could increase the rate of renaturation by either extending the duration of a given encounter or by increasing the frequency of encounters between strands. Any interaction that held strands in proximity would extend the duration of a given encounter, but this would not necessarily increase the rate of initial association. However, several features of A1's glycine-rich carboxyl terminus would also be expected to increase the frequency of encounters. This domain, which is composed of a short repeating unit containing multiple aromatic residues and charged groups, is thought to exist as a random coil (21). A binding interaction mediated by a random coil composed of repeating units would allow any part of one binding partner to provide binding energy when it comes in contact with any part of its corresponding binding partner. This will increase the likelihood of an initial contact providing binding energy. The initial binding event could then mature into higher affinity binding as more contacts between partners were made. Long, random coils would be particularly large targets for such intermolecular binding, thereby further increasing the probability of an interaction. A flexible coil could also provide binding energy from any orientation as it approached its complementary partner. Electrostatic and hydrophobic interactions, which do not require specific orientations to be effective, further increase the probability of an interaction. Such binding partners would be expected to greatly increase the likelihood of molecules interacting with each other, particularly in comparison to the formation of hydrogen bonds between complementary bases, which require close proximity and specific alignment to provide binding energy.

The Role of A1 *in Vivo*. The ability of A1 to promote the rapid renaturation of complementary sequence raises questions about its role *in vivo*. There is no evidence that A1 has a role in the formation of duplex DNA *in vivo*, but our results are consistent with such a possibility. For example, some models for DNA recombination (42) necessitate the formation of new duplexes through the association of single strands derived from two different but homologous strands. Such an association could be facilitated by A1 or proteins with similar characteristics.

A more likely role for A1 derives from its association with hnRNAs and from the association of similar proteins with snRNPs (3, 8). This suggests that A1, and possibly other members of the RNA-binding family, are involved in the rapid association of various RNA species during processes such as splicing, 3′-end cleavage, the processing of pre-rRNAs, and antisense interactions. It is known that the rate at which U1 snRNPs bind to the 5′ splice sites of RNA substrates *in vitro* is drastically reduced by protease treatment prior to binding (43). Examination of this data indicates that renaturation between the U1 snRNPs and 5′ splice sites is much faster than would be expected for the unaided association of complementary base pairs in solution and is kinetically similar to the reactions described in this paper. Similar interactions between A1-like proteins could facilitate the binding of snRNPs within the intron and at the 3′ splice site.

Relationships to Other Molecular Assembly Processes. Domains analogous to those in A1 may participate more generally in the formation of specific assemblies *in vivo*. Multicomponent complexes, in particular, face daunting entropic barriers

(44), and those that must form rapidly might require a mechanism for assembly. Our model suggests that high-probability transient interactions mediated by proteins resembling A1 could drive the association of highly specific, and therefore entropically unfavorable and kinetically slow, binding interactions elsewhere on the interacting molecules. Differences in the repeating units of these flexible domains could permit additional selectivity between interacting partners.

It has been pointed out that RNP proteins share some of their structural features with enhancer-binding proteins (8). These proteins possess DNA-binding domains and additional, acidic "activating" domains that are presumed to interact with other proteins of the transcription complex (see, for example, refs. 45–48). Using the analogy of the way we propose that A1-like proteins overcome the entropic barrier of spliceosomal complex formation, we suggest that the activating regions of DNA-bound enhancer-binding proteins interact with corresponding domains on factors, free in solution, that are required for each round of transcription. This results in a kinetically fast, specific association of such factors at the promoter. In the absence of enhancer-binding proteins, the kinetics of specific complex formation at the promoter would be reduced. Several domains, including repeating units of transcription-initiating factor TFIID (49, 50) and the carboxyl-terminal heptamer repeat of RNA polymerase itself (51, 52), are candidates for interactions of this type. In our model, these domains are not meant to stabilize or otherwise activate the promoter complex; rather, they would function to increase the association kinetics of a separate and more specific interaction. While it has been suggested that these domains may be important for the association or activation of the transcriptional apparatus, the mechanism of such interactions and their relationship to other processes have not been well established.

The broader implications of this model are speculative, but we believe that these ideas are consistent with the principles that govern macromolecular interactions (53). Our observations suggest that flexible repeating domains, such as those described, could be used to increase the association kinetics of highly specific molecular assemblies under a wide variety of circumstances.

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