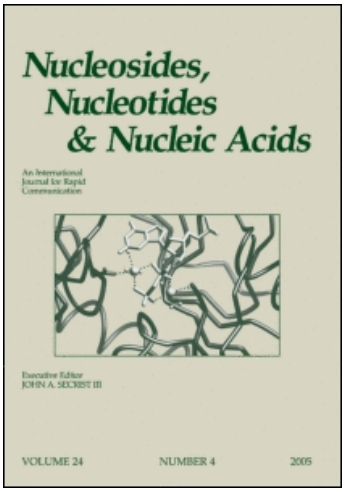

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EFFECT OF 5-FU SUBSTITUTION AND MUTATION ON Sm PROTEIN BINDING TO HUMAN U4 snRNA

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ABSTRACT

The effects of native and non-native nucleotide substitution on the binding of Sm proteins to human U4 snRNA were investigated to determine if the Sm site was a likely target for the RNA-mediated effects of the anticancer drug 5-FU, and other nucleoside analogues. The Sm binding site of human U4 snRNA was prepared by in vitro transcription, and Sm protein binding was assessed using gel mobility shift assays. The U4:Sm RNA:protein complex was identified by immunoprecipitation with the Sm-specific Y12 antibody. The effects of 5-FU substitution were assessed by including FUTP in the in vitro transcription reactions. The effects of native nucleotide substitution were assessed by mutagenesis. Deletion mutants were used to assess the relative importance of the two stem-loops that flank the Sm binding site for protein binding. Point mutation ($U \rightarrow G$) to the 5′-Urd in the Sm site reduced Sm protein binding while similar point mutation to the 3′-Urd had a lesser effect. Mutation ($U \rightarrow G$) of all Urd in the Sm site completely inhibited Sm protein binding. The central stem-loop contributed significantly to Sm protein complex formation but the 3′ stem-loop had little effect. Substitution of Urd by 5-fluorourdine (FUrd) did not inhibit Sm protein binding, but reduced the stability of the resulting complex. The results indicate that 5-FU, or other Uracil analogues, are unlikely to exert RNA-mediated effects through inhibition of Sm protein binding.

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Small ribonucleoprotein particles (snRNPs) are essential components of spliceosomes, dynamic macromolecular nuclear complexes that are sites for pre-mRNA splicing[1]. Each spliceosome requires five principal snRNAs (U1, U2, U4, U5 and U6) each of which binds to several snRNA-specific proteins[2,3]. All of these snRNAs, except U6, also bind to seven common core proteins (B/B′, D1, D2, D3, E, F, G) referred to as Sm proteins[4,5]. The snRNAs that bind the Sm proteins are transcribed in the nucleus by RNA polymerase II and are modified by an m^7G cap at the 5′-terminus (U snRNAs). The monomethyl cap, and the nuclear cap binding complex (CBC) that binds to the cap, are essential for nuclear export of the primary U snRNA transcripts[6–8]. Cytoplasmic export of capped U snRNAs from nuclei occurs after complexation with nuclear cap-binding protein complex (CBC) and importin-α. U snRNAs exported from the nucleus are displaced from the export complex by importin-β and reside transiently in the cytoplasm[9–11]. Morphogenesis of U snRNAs in the cytoplasm involves complexation of Sm proteins to the Sm binding site of the U snRNA and trimethylation of their m^7G caps to $m^{2,2,7}G$ (TMG) caps. Sm protein binding and TMG cap formation constitute a bipartite nuclear localization signal for the nuclear re-entry of these snRNAs. The nuclear import of appropriately modified U snRNPs is mediated by importin β, while importin α and transportin do not mediate U snRNP transport[11].

Spliceosome formation occurs in the nucleus and failure of U snRNAs to re-enter the nucleus following export to the cytoplasm is deleterious to the cell. The Sm binding sites from all U snRNAs are highly conserved with each binding site consisting of a Uracil-rich, single-stranded sequence that in mammalian U snRNAs is flanked by two stem-loops[12–14]. Mutational studies indicate Sm binding sites from some U snRNA are highly intolerant of variation, while limited variation is tolerated in other snRNAs. For instance, point mutation at any one of three Uracils in the Sm binding site of U4 snRNA was lethal in *Saccharomyces cerevisae*[15]. In contrast to the sensitivity of the yeast U4 snRNA to mutation in the Sm binding site, the binding site for U5 snRNA from the same organism was tolerant to point mutation[16]. These differing susceptibilities to mutations in the Sm binding site may arise from differences in the Sm recognition sequence between yeast U4 snRNA ($A_2U_5G_2$) and U5 snRNA (UAU_6G_2). Double mutations in the Sm binding site from yeast U5 snRNA did result in growth defects, although point mutations did not. These studies suggest that the additional Uracil in the yeast U5 snRNA Sm binding site rendered this sequence less vulnerable to mutation. Despite the apparent dependence of U snRNAs on Sm protein complexation for nuclear re-localization, Sm proteins are not required for active spliceosome formation in vitro[17].

The hypothesis that 5-fluorouracil (5-FU) is inhibitory to RNA processing has been the subject of several investigations since this drug was first observed to have anticancer activity nearly four decades ago. Chaudhuri et al.

observed that in cultured cells treated with 5-FU, 5-fluorouridine (FUrd) was incorporated into RNA at a level 6% that of the uridine (Urd) content[18]. Subsequent studies both in cell culture and in-vivo have implicated the RNA-mediated effects of 5-FU as contributing to the anticancer activity of this drug[19,20]. Cellular targets for the RNA-mediated effects of 5-FU include pre-mRNA splicing[21–23], rRNA assembly[24,25] and mRNA translation[26].

Over the last several years, our laboratory has demonstrated that 5-FU alters the structure, stability, and dynamics of RNA and DNA duplexes and RNA stem-loops. In particular, we have shown that the conformation of the 5′ stem-loop and the stem II region of the U4-U6 snRNA complex were altered by 5-FU substitution[27–30]. In addition, our laboratory demonstrated that RNA duplexes were stabilized by 5-FU substitution at an A-U base pair, but destabilized when substitution occurred at a G-U base pair[30].

The Urd-rich character of the Sm protein binding site and observations of deleterious consequences to organisms upon mutation of this site raises the possibility that substitution of 5-FU in the Sm binding site might disrupt Sm protein complex formation (Fig. 1). Failure of U4 snRNA to bind the Sm complex would reduce the efficiency of U4 snRNA nuclear import, and inhibit formation of spliceosomes. A recent study by Raker et al. showed that the two purines at the 5′ terminal portion of the Sm binding site and the adjacent Urd were essential for Sm protein binding in vitro[31]. In the present manuscript, we describe the effects of deletions, point mutations and substitution of non-native nucleotides (i.e. FUrd) on binding of Sm proteins to the Sm binding site of human U4 snRNA. In this regard, it is important to

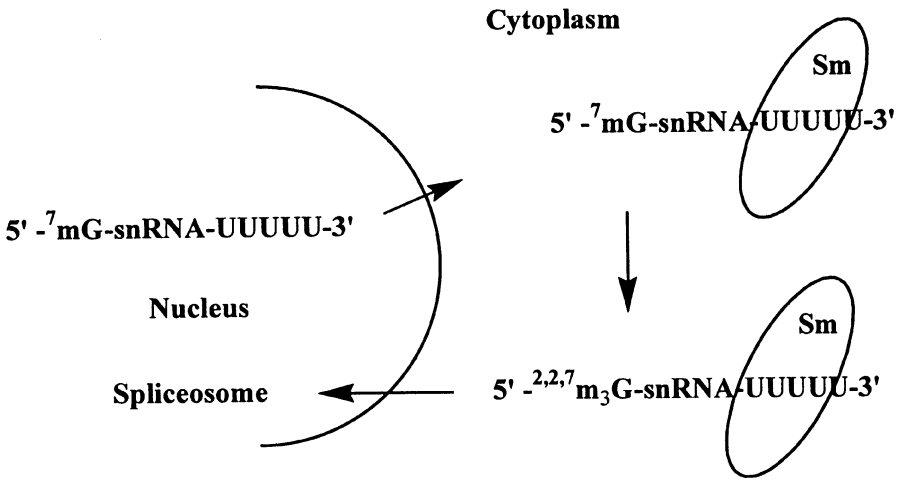

Figure 1. Post-transcriptional modification during U snRNA maturation. U snRNA are transcribed in the nucleus and exported transiently to the cytoplasm. Nuclear re-localization is signaled by hypermethylation of the m^7G cap and binding of the Sm proteins to the U-rich Sm binding site.

note that 5-FU substitution has been shown to perturb the native conformation of U4 and U6 snRNAs[32]. Point mutation (U → G) to the 5′-Urd in the Sm site reduced Sm protein binding while similar point mutation to the 3′-Urd had a lesser effect. Mutation (U → G) of all Urd in the Sm site completely inhibited Sm protein binding. Substitution of Urd by 5-fluorourdine (FUrd) did not inhibit Sm protein binding, but reduced the stability of the resulting complex. The results indicate that the structural determinants of Sm protein binding are complex and that 5-FU, or other Uracil analogues, are unlikely to exert RNA-mediated effects through inhibition of Sm protein binding.

EXPERIMENTAL METHODS

Preparation of RNAs by In Vitro Transcription

The cDNA for human U4 snRNA was obtained by RT-PCR amplification of RNA from human macrophage cells. The sequence was the same as previously reported[33]. The parent plasmid used as a template for transcription of the 3′-terminal 72 nucleotides of human U4 snRNA (from nucleotide 72 to 144 as numbered in[33]) was constructed by sub-cloning the human U4 snRNA cDNA into pSP72. This fragment contained the central stem loop, the Sm binding site and the 3′-stem loop of human U4 snRNA and is referred to as [rU4] (Fig. 2). Five other plasmids were derived from this construct by standard cloning techniques: (1) – A point mutant in which the Urd in the Sm binding site nearest the 5′ terminus was mutated to guanosine (Gua) [5′U-G]; (2) – A point mutant in which the Urd in the Sm binding site nearest the 3′ terminus was mutated to Gua [3′U-G]; (3) – A mutant in which all five of the Urd in the Sm binding site were mutated to Gua [5GSm]; (4) – A deletion mutant in which the five Urd in the Sm binding site were deleted [ΔSm]; and (5) – A deletion mutant in which the 3′ stem loop was deleted [Δ3′SL]. Templates were linearized with BamHI prior to in vitro transcription. In addition to these six RNA probes prepared by in vitro transcription, two RNA probes were prepared synthetically: (1) A deletion mutant lacking the central stem loop [ΔCSL]; and (2) A 20mer RNA probe with the Sm binding site in the center and the flanking regions of the central stem loop and the 3′stem-loop [Sm]. The sequences for [rU4] and the three deletion mutants are shown in Fig. 2.

Radiolabeled U4 snRNA probes were prepared using T7 RNA polymerase and [^{32}P]CTP, and were purified using a QIAquick nucleotide removal kit (Qiagen) before gel mobility shift assays. Non-radioactive transcripts used for competition studies were prepared in the same manner, except unlabeled CTP was used for the transcription reactions. Substitution of FUrd for Urd was accomplished by including FUTP (Sierra Bioresearch;

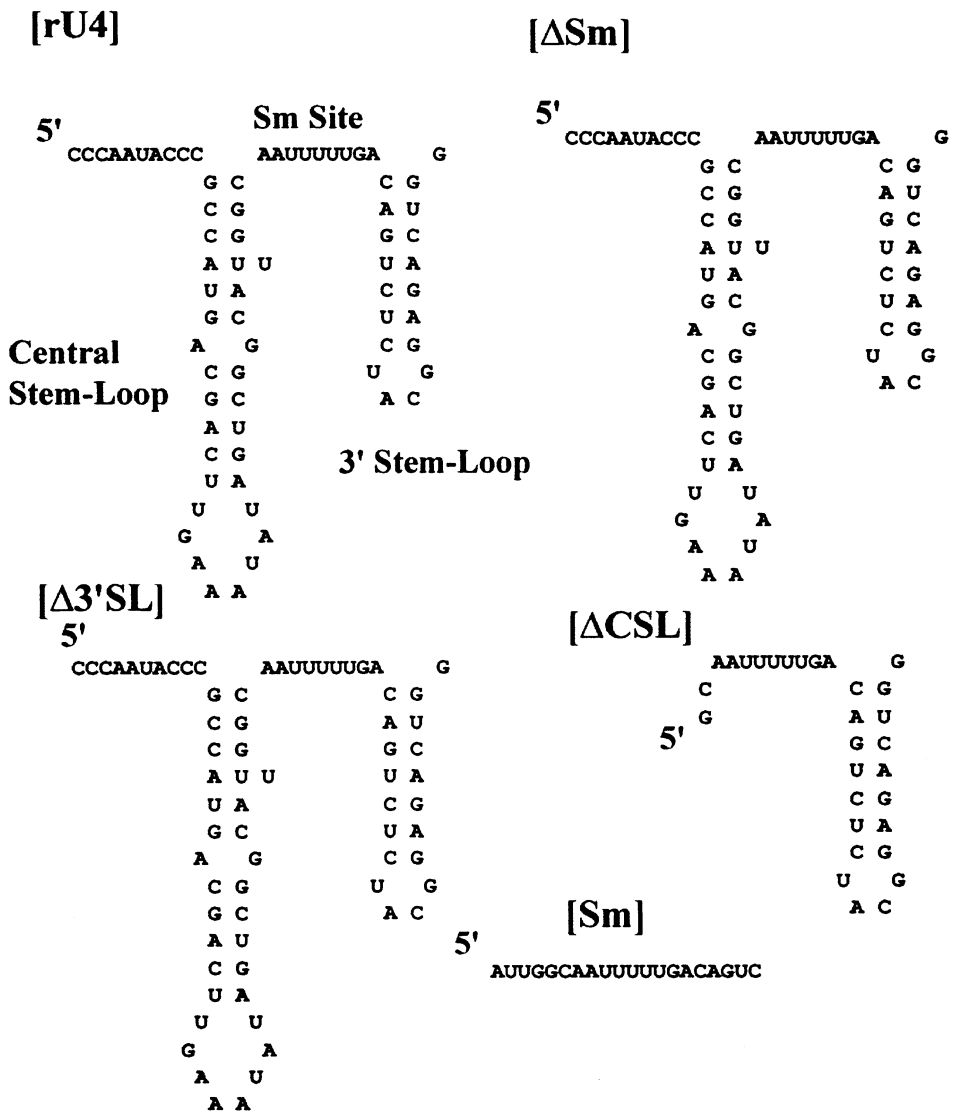

Figure 2. Secondary structures for the five RNA molecules considered in the present investigation. [rU4] (top left) consists of only native nucleotides and includes the Sm binding site and the two flanking stem loops. [ΔSm] (top right) differs from [rU4] in that the five Urd in the Sm binding site were deleted. [Δ3′SL] (bottom left) differs from [rU4] in that the 3′SL was deleted while in [ΔCSL] (bottom right) the central stem-loop was deleted. [Sm] (bottom center) consists of the Sm binding site and flanking nucleotides.

Tuscon, AZ) in the transcription reactions. All of the RNAs generated by in vitro transcription were treated identically. Probes were frozen at −70°C immediately after their preparation. The transcripts were thawed gradually at room temperature before use in gel mobility shift assays. The two

synthetic probes, [ΔCSL] and [Sm] were fluorescently labeled with 5-iodoacetamidofluorescein for detection using T4 polynucleotide kinase (Amersham). The fluorescently-labeled RNA was purified using SurePure columns (Amersham).

Gel Mobility Shift Assays

Gel mobility shift assays were performed in manner similar to that described previously[34]. Binding reactions of the ^{32}P-labeled RNA probes were performed using HeLa cell nuclear extract. The RNA probe was prepared in 10 mM HEPES pH 7.9, 10% glycerol, 1 mM DTT, 0.1 mg/mL tRNA, 0.5 mg/mL BSA and 200,000–300,000 cpm of the ^{32}P-labeled RNA probe. The probe mixture was heated at 85°C for five min, and then cooled to 0°C. The binding reaction mixture consisted of 20 mM HEPES-KOH pH 7.9, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTE and 3.6 μg/μL of HeLa cell nuclear extract (GIBCO). Binding reactions were initiated by mixing together 10 μL of the RNA probe solution with 10 μL of the binding reaction mixture and were incubated at 30°C for 30 min. For competition reactions, 200 pmol of an unlabeled RNA probe were included in the binding reaction mixture. Samples from the binding reaction were then subjected to electrophoresis through 5% non-denaturing polyacrylamide gels (acrylamide:bis, 29:1) and TBE (22.5 mM Tris-Borate, 0.5 mM Na_2EDTA) as a running buffer. The gels were pre-electrophoresed at 10 V/cm for 0.5 h. Samples were loaded and the gel was electrophoresed at 160 V for 5–6 h at 4°C. Gels were dried on a vacuum gel drier (BioRad) with heat for one hour. The gels were then visualized using a STORM phosphorimager (Molecular Dynamics). Reactions containing fluorescently-labeled RNA probes were electrophoresed at 120 V for 2 h at 4°C with inclusion of 1 pmol of the fluorescent probe. The gels for fluorescent probes were also analyzed using a STORM phosphorimager (Molecular Dynamics) with fluorescence scanning.

Radioimmunoprecipitation of [rU4]:Sm

Radioimmunoprecipitation reactions were run as previously described[34]. 10 μL of 200 μg/mL of Y12 (anti-Sm) antibody purified from ascites fluid by Protein A chromatography (NeoMarkers) was incubated with 20 μL of Protein G Sepharose beads (Pharmacia) and mixed overnight at 4°C in 10 mM PBS pH 7.4. The anti-Sm beads washed three times in IPP_{150} buffer (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.1% Nonidet P-40) to remove unbound antibody and then equilibrated in binding buffer (20 mM potassium phosphate, pH 8.0, 0.10 M NaCl). To this was added 60 μL of ^{32}P-labeled [rU4]:Sm snRNP complex (600,000–900,000 cpm) was incubated in an end-

over-end rotary stirrer (Fisher) at 4°C for 4 h. The antibody-bound snRNP complexes were then pelleted by centrifugation (1 min at 2,000 ×g), and then washed three times with five volumes IPP_{150} buffer. The beads were suspended in 150 μL of TE buffer and the RNA was displaced from the Sm protein bound to the anti-Sm beads by addition of 150 μL of phenol in Tris buffer pH 5.6. Following mixing, the aqueous solution containing the radio labeled [rU4] RNA probe was separated by centrifugation (10 min at 12,000 ×g). The RNA was recovered by precipitation with addition of 3 volumes of cold absolute ethanol and refrigeration at −20°C overnight. The RNA was analyzed by PAGE on a 6% denaturing (7 M urea) gel for 4–5 h at 200 volts. Control reactions using the mouse IgG2a antibody (Sigma) were done using an identical protocol except for addition of 2 μL of 1 mg/mL mouse IgG2a rather than Y12.

RESULTS

Sm Protein Binding to [rU4]

The mobility shift properties of [rU4] (Fig. 2), the RNA transcript that included the central stem loop, the 3′-stem loop and the Sm binding site of human U4 snRNA were compared to [ΔSm], a deletion mutant of [rU4] lacking the five Urd from the Sm Urd from the Sm binding site. Mobility shifted bands observed for [rU4], but not [ΔSm], may result from Sm protein binding. Subsequent immunoprecipiation experiments verified that this was the case. The sequences for four of the RNA transcripts included in the present study, as well as the secondary structure for [rU4], are shown in Fig. 2. The [rU4] and [ΔSm] RNA probes were incubated with HeLa cell nuclear extract as a source of Sm proteins. Incubation of [rU4] with HeLa cell nuclear extract resulted in one principal mobility shifted band observed in the RNA gel shift assay (Fig. 3, Lane 2; see also Fig. 6 Lane 2) which did not appear with the RNA alone (Fig. 3, Lane 1). In addition to the principal mobility shifted band (see arrows in Fig. 3 and Fig. 6) bands of slightly increased mobility occurred due to limited degradation of the RNA probe and bands of slower mobility also were observed, probably as a result of non-Sm protein binding. Similarly shifted bands were not apparent when [ΔSm], a deletion mutant of [rU4] lacking the Sm binding site (Fig. 2), was incubated with HeLa cell nuclear extract, consistent with Sm protein binding being required for the presence of all shifted bands (Fig. 3). The observed intensity of the principal mobility shifted band observed upon incubation of [rU4] with HeLa cell nuclear extract was proportional to the amount of the extract included up to 2 μL, while saturation of the RNA probe occurred for larger additions of extract. Detection of a mobility shifted band for the [rU4] RNA probe that includes the Sm binding site, but not for the Sm deletion mutant

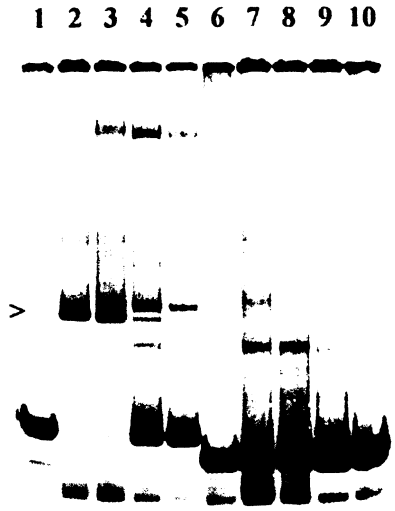

Figure 3. Electrophoretic mobility shift assays investigating the effects of the Sm binding site on Sm protein binding. Lanes 1–5 include the [rU4] RNA probe (Fig. 2) incubated with 0, 4, 2 and 1 μL of HeLa cell nuclear extract. The principal mobility shifted band due to Sm complex formation is indicated with > symbol. Lanes 6–10 are the analogous shifts with the [ΔSm] probe.

[ΔSm], is consistent with this mobility shifted species being the [rU4]:Sm RNA:protein complex.

Immunoprecipitation of [rU4]:Sm Complex

Characterization of the Sm protein complex as the protein bound to [rU4] following incubation of the RNA probe with HeLa cell nuclear extract was accomplished by immunoprecipitation of the radiolabeled probe with the Y12 (anti-Sm) antibody. The results of the radioimmunoprecipitation experiment are shown in Fig. 4. The [rU4]:Sm complex was not immunoprecipitated with the IgG2a antibody that served as a negative control (Fig. 4, Lane 2), but was immunoprecipitated with the Y12 antibody that is specific for the Sm protein complex (Fig. 4, Lane 3).

Effects of Mutation on Sm Binding

Single and multiple mutations were introduced into the Sm protein binding site to determine if mutations in this region affected Sm protein binding. A recent study by Raker et al. showed that the two purines at the 5′ end of the single-stranded Sm binding site were critical for Sm complex formation[31]. Despite the Urd-rich character of the Sm site, only the Urd

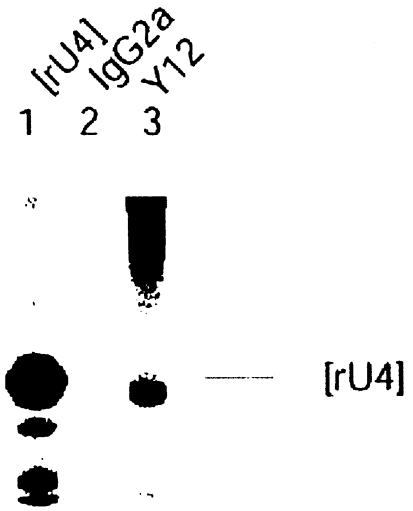

Figure 4. Radioimmunoprecipitation of the reconstituted [rU4]:Sm protein complex. Immunoprecipitation was performed using the Y12 (anti-Sm) antibody. The immunoprecipitated RNA was isolated and fractionated on a 6% polyacrylamide denaturing gel. Lane 1 shows the radiolabeled [rU4] RNA probe. Lane 2 shows the results of immunoprecipitation with IgG2a that serves as a negative control. Lane 3 shows the immunoprecipitation of radiolabeled [rU4] by the Y12 antibody. The results are consistent with the principal mobility shifted band in the gel shift assays as being due to the [rU4]:Sm complex.

adjacent to the 5′ purines significantly affected Sm protein binding. In the present study, single U → G point mutations were engineered both at the Urd adjacent to the 5′ purines, [5′U → G], as well as the Urd adjacent to the two purines at the 3′ end of the Sm binding site, [3′U → G]. The results of the mobility shift assays with these two RNA probes are shown in Fig. 5. Qualitatively, the mobility shift properties of both [5′U → G] (Fig. 5, lane 4) and [3′U → G] (Fig. 5, Lane 8) are similar to the native sequence [rU4]. One principal mobility shifted band is observed in each case, as well as at least four mobility-shifted bands of lesser intensity. The less intense bands may represent sub-complexes of the Sm protein complex, or may involve both non-Sm proteins and Sm proteins. The intensity of the principal mobility shifted band was decreased for [5′U → G] relative to [rU4], a result consistent with the observations of Raker et al. that this site is important for Sm protein complex formation[31]. The reduction in intensity is however, was not as substantial as was observed previously, perhaps indicating that the U → G mutation is better tolerated than the U → C mutation used in previous studies. Mutation of the 3′ Urd had a lesser effect than mutation of the 5′ Urd, although the intensity of the principal mobility shifted band was reduced relative to the native sequence. Mutation of all of the Urd in the Sm binding site to Gua, [5GSm], completely inhibited Sm protein complex formation

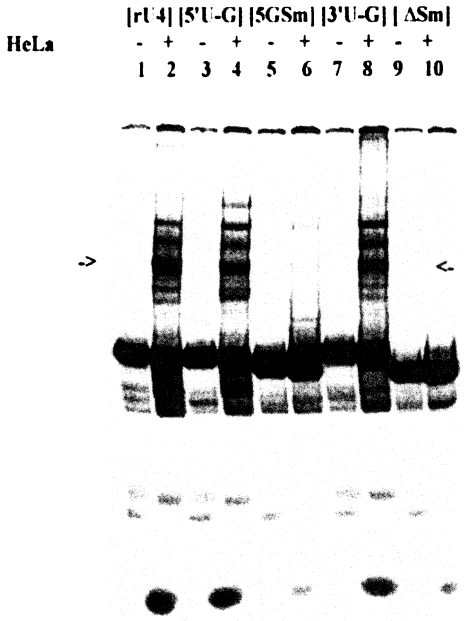

Figure 5. Effect of native nucleotide substitution in the Sm binding site on Sm protein binding. Complexes of [rU4] (lanes 1 and 2) and mutants (lanes 3 – 10) with Sm proteins were prepared by incubation of the RNA with HeLa cell nuclear extract. Lanes 1, 3, 5, 7 and 9 represent incubations in the absence of any HeLa cell nuclear extract. Lanes 2, 4, 6, 8 and 10 represent gel shifts following incubation with HeLa nuclear extract. The extract was pretreated with unlabeled [ΔSm] to reduce non-specific Sm protein binding. Lanes 1 and 2 included [rU4], the native nucleotide sequence. Lanes 3 and 4 included [5′U → G] with the 5′ Urd of the Sm site mutated to G. Lanes 5 and 6 included [5GSm] in which all five Urd were mutated to G. Lanes 7 and 8 included [3′U → G] with the 3′ Urd mutated to G, lanes 9 and 10 included [ΔSm] with the five Urd of the Sm site deleted.

(Fig. 5, Lane 6), and the results of the mobility shift assay for [5GSm] were the same as for the deletion mutant [ΔSm] (Fig. 5, Lane 10).

Stem-Loops Enhance Sm Binding

The Sm binding site in human U4 snRNA is flanked by the 3′- and central stem loops. The relative contributions to Sm protein binding by these two stem loops were assessed by analyzing the mobility shift properties for deletion mutants that lacked either the 3′ stem-loop [Δ3′SL] or the central stem loop, [ΔCSL]. The results from the 3′SL deletion experiments are shown in Fig. 6. Deletion of the 3′ stem-loop had little effect on Sm protein binding (Fig. 6) while deletion of the central stem-loop inhibited binding, as monitored by a direct gel shift (data not shown) and competition binding experiments (Fig. 7). The contribution of these stem-loops was also assessed

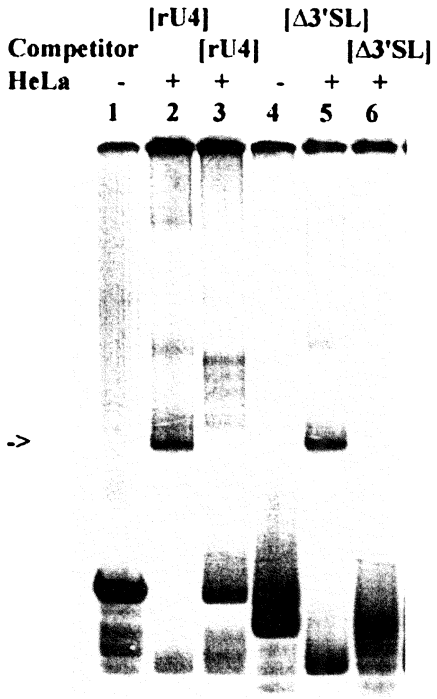

Figure 6. Deletion of the 3′ stem-loop does not affect complex formation with the Sm proteins. Lanes 1–3 show [rU4] while lanes 4–6 show [Δ3′SL]. Lanes 1 and 4 are the free probe while lanes 2 and 4 show the mobility shift for the RNA probe following incubation with HeLa cell nuclear extract. Lanes 3 and 6 are identical to lanes 2 and 4 except unlabeled RNA was included as a competitor for the radiolabeled probe.

by the effectiveness of these deletion mutants and a synthetic RNA containing the Sm binding site, [Sm], to compete for Sm protein binding by [rU4] (for RNA sequences, see Fig. 1). [Sm] was a poor competitor for binding of [rU4] by the Sm protein complex as was the deletion mutant lacking the central stem-loop. The probe lacking the 3′ stem-loop was, however, an effective competitor of Sm protein binding to [rU4]. The rank of each probe as a competitor for Sm protein binding was: [rU4] $\sim$ [Δ3′SL] $>$ [ΔCSL] $\sim$ [Sm] $>$ [ΔSm]. These results indicate that the central stem loop promotes Sm protein binding while the 3′ stem loop has a lesser effect. The deletion mutant lacking the five Urd of the Sm binding site, [ΔSm], was not effective in the competition binding assays.

FUrd Does Not Affect Sm Binding

The observed effects of point mutations at the 5′ Urd of the Sm binding site is consistent with the hypothesis that substitution of Urd-analogues, such

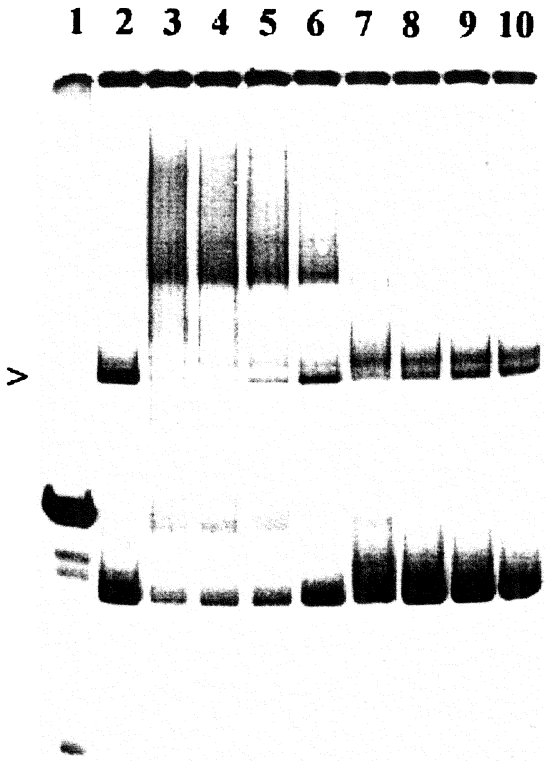

Figure 7. Competition of the deletion mutants [Δ3′SL] (lanes 3–6) and [ΔCSL] lanes 7–10 for binding of [rU4] by Sm proteins. The radiolabeled [rU4] probe was included in all lanes while HeLa cell nuclear extract was included in lanes 2–10. Competitors were included at 200 (lanes 3 and 7), 100, 50 and 25 pm.

as the anticancer drug 5-FU, might also inhibit Sm protein binding (Fig. 1). To investigate if FUrd substitution decreased affinity of the Sm binding site towards protein complex formation, in vitro transcription of [rU4] was undertaken with FUTP, rather than UTP, in the transcription reactions [rU4-FU]. The results are summarized in Fig. 8. Even with inclusion of 100% FUTP rather than UTP in the transcription reactions, the mobility-shifted band due to Sm protein complexation characteristic of the [rU4]:Sm complex was observed following incubation of the probe with HeLa cell nuclear extract. The time course for the appearance of this mobility-shifted band was similar for [rU4] and [rU4-FU] with maximal formation of the Sm protein complex after a 2 h incubation. The rate of decay for the [rU4-FU]:Sm complex was, however, greater than for the native sequence indicating FUrd substitution affected complex stability, but did not inhibit Sm complex formation. FUrd substitution also decreased the mobility of the free RNA under non-denaturing conditions, a result consistent with our previous studies with the 5′ stem-loop from human U4 snRNA (data not shown). Mobility shifts

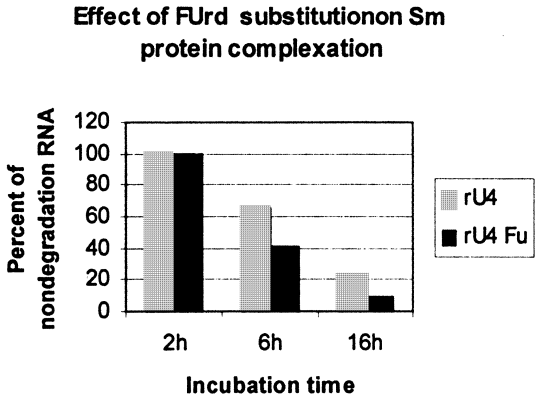

Figure 8. Time course of the Sm protein complex with [rU4] and with the [rU4] probe substituted with FUrd. FUrd did not inhibit Sm complex formation, but increased the rate at which the complex disassembled.

were most substantial when FUrd was substituted at very high levels in the RNA indicating that the effects of FUrd on RNA conformation in the Sm site region are probably not significant at physiologically relevant levels of FUrd incorporation (<10%).

DISCUSSION

Pre-mRNA splicing is an essential RNA-catalyzed process in eukaryotic cells. Most of the snRNA components of the spliceosome are exported to the cytoplasm following transcription, and their nuclear re-localization is essential for spliceosome formation. Failure of the snRNA to re-enter the nucleus will prevent spliceosome formation, and ultimately be deleterious to the cell. The lethal phenotype observed in *Saccharomyces cerevisae* that resulted from mutations in the Sm binding site of U4 snRNA presumably resulted from reduced affinity of the mutant U4 snRNA to bind Sm proteins[15]. Complexation of the Sm proteins to the Sm binding site, together with formation of the trimethylguanosine cap, constitute the bipartite nuclear localization signal for snRNA. In the present manuscript, we have shown that substitution of both native, as well as non-native nucleosides, in the Sm binding site affects the formation and/or the stability of Sm:snRNA complexes.

The conservation of Urd-rich sequences in the Sm binding sites of snRNAs from yeast to mammals makes it likely that Urd-specific contacts are essential for Sm protein complexation. Despite the high degree of conservation in Sm sequences, both binding data in vitro and viability data in vivo indicate that most of the Urd nucleotides in the Sm binding site are

not essential for Sm protein complexation[3,31]. Data from the present studies are consistent with these previous findings. For example, single U → G point mutations at either the 5′-Urd or the 3′-Urd in the Sm binding site from human U4 snRNA reduced, but did not inhibit Sm protein binding. The effects of point mutation were greater for the 5′-Urd than for the 3′-Urd, a result consistent with the observations of Raker et al. that U → C point mutation at the 5′-Urd substantially decreased Sm protein binding[31]. The U → G point mutation appears to be better tolerated than the U → C point mutation at this site, perhaps indicating that the placement of the imino hydrogen and carbonyl oxygen are important recognition motifs for Sm protein binding. Nonetheless, the results from the present study, as well as from previous studies, are somewhat paradoxical in light of the sequence conservation in the Sm binding site. The presence of Urd in the Sm binding site must confer advantages beyond strict recognition of the Sm proteins, although the nature of these advantages remains to be characterized.

The efficacy and toxicity of the anticancer drug 5-FU are both probably related to its metabolism to FUTP and incorporation into RNA. The mechanism by which FUrd substitution affects RNA function remains, however, incompletely understood[35,36]. FUrd substitution in snRNA inhibited pre-mRNA splicing[21,37], but there is no evidence that splicing inhibition is responsible for the observed correlation between FUrd substitution in RNA and patient response to 5-FU[19]. The highly conserved Urd-rich Sm site provides a rational RNA sequence that FUrd substitution is likely to perturb. The present studies indicate, however, that FUrd substitution does not affect the binding of Sm proteins, even at substitution levels much higher than could occur in vivo. The Sm:snRNA complex was less stable for the FUrd-substituted snRNA relative to the native sequence. FUrd-substitution may decrease the stability of RNA:protein interactions and increase the rate of RNA turnover in cells. Changes in RNA stability may thus be a principal source of the RNA-mediated effects of 5-FU.

The mechanism by which Sm proteins bind snRNA remains incompletely understood. Both the present studies, as well as previous reports, have shown that RNA structural elements outside the Sm binding site enhance the binding of Sm proteins to U4 snRNA. From the results of the present study, it is clear that the central stem-loop contains structural elements that enhance Sm protein binding. This is evident from the observation of a gel shift due to Sm protein binding for [Δ3′SL] but not [ΔCSL] and from the greater effectiveness of [Δ3′SL] as a competitor for binding of Sm protein to [rU4]. These results are consistent with previous studies showing that structural elements outside the Sm binding site enhanced the binding of Sm proteins to *Xenopus* U1 and U5 snRNAs[38]. In the case of *Xenopus* U5 snRNA, a six nucleotide inner loop on the stem-loop that is 5′ to the Sm binding site is the sole structural element that enhanced complexation of U5 snRNA to the Sm proteins. Additional studies are in progress to elucidate the structural

features of the central stem-loop that contribute to Sm protein binding in human U4 snRNA.

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