Modified U1 snRNA suppresses expression of a targeted endogenous RNA by inhibiting polyadenylation of the transcript

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ABSTRACT

We have previously demonstrated that a modified U1 snRNA inhibits expression of a number of targeted transgenes. Here we exploit the ability of the modified U1 snRNA to inhibit endogenous gene expression and define the mechanism responsible for this inhibitory action. MC3T3-E1 cells stably transfected with U1 anti-Cbfa1 show a change of morphology from polygonal to fibroblast-like cells. This visual observation was supported by an 80% reduction of Cbfa1 expression and suppression of downstream genes associated with osteoblast differentiation. In rat ROS 17/2.8 cells, osteocalcin and Col1a1 gene expression was reduced up to 90% by the U1 anti-osteocalcin or U1 anti-Col1a1 construct, respectively. The length of mature osteocalcin mRNA poly(A) tail was significantly shortened in the targeted mRNA by transcript-specific poly(A) test (PAT)-PCR. These data demonstrate that the modified U1 snRNA is able to reduce endogenous gene expression by limiting the polyadenylation of the targeted pre-mRNA transcript.

INTRODUCTION

Reducing expression of a specific gene plays a central role in studying gene function and molecular therapy. We have previously demonstrated that modified U1 snRNA is able to suppress expression of a number of transgenes by targeting unique sequences located within the terminal exon in both transient and stable transfection protocols (1,2) with constructs which include the SV40 promoter driving β-gal, CAT, GFP and luciferase. Modified U1 snRNA mediated gene inhibition is dependent on hybrid formation between a specific targeted sequence and the 5'-end of the modified U1 snRNA (1,2). Earlier work by Gunderson et al. (3) demonstrated that an interaction of a U1 70K protein with poly(A) polymerase (PAP) inhibits polyadenylation, suggesting that such a mechanism might explain the inhibitory effect of the modified U1 snRNA on its target sequence. More recently this group has shown that the ‘AAUAAA’ type of polyadenylation was critical for the action of the modified U1 snRNA (4), providing indirect evidence of reduced polyadenylation of a reporter transcript.

The work presented here was designed to directly test if the U1 anti-target construct acts to reduce the length of the poly(A) tail of an endogenous gene. Furthermore, we felt it was important to demonstrate that the modified U1 snRNA vector could induce a phenotypic change in cultured cells as a result of targeted reduction of a specific gene transcript. We engineered the 5' end of U1 snRNA to target a unique sequence at the terminal exon of the mouse Core-binding factor α1 (Cbfa1) gene, rat osteocalcin (OC) or rat Col1a1. Cbfa1 is a member of the Runx2/Cbfa family of transcription factors and is a key regulator of osteoblast differentiation in vivo (5). Reduction in its level of expression would be predicted to inhibit the osteogenic potential of an osteoblastic cell line. OC is the most osteoblast-specific gene expressed in mature osteoblasts at the late stage of differentiation and is intermediate in expression level between Cbfa1 and Col1a1. Because Col1a1 is highly expressed in osteoblasts, it provides a severe test of the capacity of the U1 anti-target construct to inhibit expression of an endogenous gene. In addition, the OC transcript served as an excellent template to assess the length of the poly(A) tail before and after downregulation by a U1 anti-OC construct.

MATERIALS AND METHODS

Construction of U1 anti-target genes

The parental U1 snRNA consists of the five snRNA-specific enhancer elements in the 315 bp promoter, the U1 coding sequence and a unique 3'-termination sequence (Fig. 1A and B) (6). The 10 bp sequence on the 5' end of U1 snRNA that binds to the 5' splice site was replaced with the sequence of a targeted gene located at the terminal exon or 3'-UTR region (Fig. 1B). The targeted regions were selected from the terminal exon or 3'-UTR region that was not homologous to other RNA expressed in a particular cell type by BLAST search of the mouse and rat EST databases. We have previously demonstrated the ability of the modified U1 snRNA to discriminate two RNA targets differing by a 1 or 2 bp mismatch (2).
The modification of U1 snRNA was carried out as described previously (1) (Fig. 1B). Briefly, PCR-directed mutagenesis of the 5′ end of U1 snRNA was made between bases +1 and +10, which normally complements the 5′ splice donor site. The 5′ (mutagenic) primers (Fig. 1B and Table 1) contain a proximal BglII restriction site (underlined) for insertion into position −8 bp in the U1 promoter. The 3′ (selection) primer (5′-AGTGCCAAGCTTGCCAGGTC-3′) extends through the U1 termination sequence and into the pUC18 polylinker, terminating with a HindIII site. A base change was made to destroy a PstI site (underlined) proximal to the HindIII site to allow selection against plasmids containing the original gene. The PCR was digested with a combination of BglII, HindIII and PstI. The wild-type U1 will be referred to as U1 snRNA; the modified U1 snRNA constructs will be identified as U1 anti-target(xxx), in which xxx indicates the 5′-most base of the complementary RNA transcript sequence (Table 1). The modified constructs were verified by sequencing.

**Cultures and transfection of osteoblast cells**

Rat osteosarcoma ROS17/2.8 cells (7) were cultured in DMEM/F12 and mouse calvarial MC3T3 cells (8) in αMEM supplemented with 10% FCS with 2 mM glutamine, 1% non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin. Transfection was carried out the day after passage at 80–90% confluency in 100-mm plates using a calcium phosphate precipitation protocol.

After antibiotic selection with G418 at concentration of 200 μg/ml for 4 weeks, MC3T3-E1 cells were examined for morphologic change under an Olympus IX50 inverted microscope (Olympus Optical Co. Ltd, Tokyo, Japan). Sister plates were fixed with 4% paraformaldehyde and washed with 1× PBS. The fixed MC3T3-E1 cells were stained for alkaline phosphatase (ALP), an early marker of osteoblast differentiation, using an ALP staining kit (Sigma) according to the manufacturer’s instruction as previously described (9).

Total RNA was extracted from both pooled populations and individuals for northern analysis of Cbfa1, OC and Col1a1 expression using previously described protocols (2).

**Immunocytochemistry**

To examine the targeted osteocalcin expression at protein level, immunocytochemical staining was performed in stably transfected ROS 17/2.8 cells with U1 snRNA and U1 anti-OC plasmids as described above. These cells were cultured on coverslips in F12/DMEM containing 10% FBS. At the end of culture, these cells were fixed with 4% paraformaldehyde and washed with PBS. The first antibody (goat anti-rat osteocalcin antibody; Diagnostic Systems Laboratories Inc., Webster, TX) was applied to the cells and incubated at 4°C overnight. After washing with PBS three times, the second antibody (rhodamine (TRITC)-conjugated AffiniPure donkey anti-goat IgG (H+L), (Jackson ImmunoResearch Laboratories Inc., West Grove, PA)) was added. The cells were examined for OC expression by fluorescent microscopy.

Western blot analysis was performed as described previously (10). Polyclonal anti-Cbfa1 [Pebp2aA (C19)] was purchased from Oncogene Research Products (Cambridge, MA).

**RT–PCR and the poly(A) test (PAT)–PCR analysis**

Total RNA was extracted from pooled populations of cells transfected with either U1 snRNA or U1 anti-OC constructs. cDNA was synthesized using Superscriptase (Invitrogen, Carlsbad, CA). To confirm OC specificity of the OC F-1896 primer used for PAT analysis, RT–PCR was carried out using OC primers (forward OC F-1896 5′-GGCCCAAGATCCCATCCATGTGCCAG-3′). The modified constructs were confirmed by sequencing.

**Table 1. U1 snRNA constructs used to reduce Coll1a1, OC and Cbfa1 gene expression**

<table>
<thead>
<tr>
<th>Construct names</th>
<th>DDBJ/EMBL/GenBank access no.</th>
<th>Target sequences</th>
<th>Mutagenic oligonucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>U1 snRNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U1 anti-Cbfa11758</td>
<td>NM_009820</td>
<td>CAGGTAAGTIA</td>
<td>5′-GGCCCAAGATCCCATCCATGTGCCAGG-3′</td>
</tr>
<tr>
<td>U1 anti-OC1817</td>
<td>J04500</td>
<td>CAGATGTGAT</td>
<td>5′-GGCCCAAGATCCCATCCATGTGCCAGG-3′</td>
</tr>
<tr>
<td>U1 anti-Cbfa14390</td>
<td>XM_213440</td>
<td>CAGGAGCTG</td>
<td>5′-GGCCCAAGATCCCATCCATGTGCCAGG-3′</td>
</tr>
</tbody>
</table>

Underlined bases indicate the BglII restriction site used to insert the mutagenized DNA into the context of the U1 snRNA expression vectors.
CCA CCG TTT AG; reverse OC R-2086 5'-ACC ACC ACC ACA ATG GAC AGA C), which amplified the 3'-most region of the terminal exon just before the poly(A) tail. PCR products were examined on a 2% agarose gel. The measurement of poly(A) tail lengths was performed as previously described (11). In brief, total RNA was extracted from U1 snRNA control cells and U1 anti-OC targeted cells using Trizol reagent (Invitrogen, Carlsbad, CA). Five micrograms of total RNA was used for cDNA synthesis using an anchored 3'-primer (5'-GCG AGC TCC GGC GCCGCG-T12). The synthesized cDNA was diluted as indicated and PCR was carried out using oligo(dT)12 anchor and OC message-specific primer (5'-GCA TCT ATG GCA CCA CCG TTT AG) under the conditions described previously (11). PCR products were visualized by electrophoresis on ethidium bromide stained 10% PAGE.

RESULTS

Reduction of Cbfa1 expression and its downstream gene expression in MC3T3-E1 cells

To test for inhibition of an endogenous gene by the modified U1 snRNA, we targeted a region located at 1758 to 1768 nt at the terminal exon of the mouse Cbfa1 gene, designed as U1 anti-Cbfa1. Either the U1 snRNA or U1 anti-Cbfa1 construct was cotransfected with an SV2Neo selection gene into mouse osteoblastic MC3T3-E1 cells. After 4 weeks of selection with 200 μg/ml of G418, the transfected cells were examined by microscopy. Cells with SV2 Neo or SV2 Neo plus U1 snRNA retained their osteoblastic morphology. However, cells with the U1 anti-Cbfa1 changed their morphology to fi broblast-like cells (Fig. 2A). The expression of Cbfa1 in cells transfected with U1 anti-Cbfa1 was reduced to 80% of the control by northern blot analysis (Fig. 2B). The expression of osteogenic genes downstream of Cbfa1, such as OC and ALP, was also examined in cells transfected with U1 snRNA or U1 anti-Cbfa1. Compared with cells transfected with U1 snRNA, OC mRNA expression decreased by up to 90% in cells with U1 anti-Cbfa1 (Fig. 2B). As shown in Figure 2C, ALP was strongly expressed in cells transfected with U1 snRNA and was markedly reduced in cells transfected with U1 anti-Cbfa1. The reduction of Cbfa1 expression was further confirmed by western blot analysis as shown in Figure 2D. U1 anti-Cbfa1 significantly suppressed the Cbfa1 protein in comparison with that of cells transfected with Neo or Neo plus U1 snRNA. These results indicate that U1 anti-Cbfa1 reduces Cbfa1 expression and secondarily its downstream genes.

Reduction of OC and Col1a1 expression

To assess the effectiveness of the modified U1 snRNA to suppress the output of a highly expressed endogenous gene, we targeted OC and Col1a1 in ROS 17/2.8 cells. The U1 anti-OC construct was stably transfected into ROS 17/2.8 cells. Total RNA was extracted from the pooled cells after 4 weeks selection with 200 μg/ml of neomycin. Northern blot was performed to analyze expression of OC and Col1a1 mRNA. As shown in Figure 3A, the reduction of OC expression was observed in cells transfected with U1 anti-OC by 88–90% as quantitated by the PhosphorImager™ (Molecular Dynamics, Sunnyvale, CA) in comparison with cells with the Neo gene or both Neo and U1 snRNA. Although U1 anti-OC caused reduction of OC expression, it had no inhibitory effect on Col1a1 expression (Fig. 3A).

To confirm the results obtained from stable transfection experiments, we also transiently introduced U1 snRNA or U1 anti-OC into ROS 17/2.8 cells. Total RNA was extracted after 2 days of transfection. Results from the transient experiments were similar to those of the stable experiments (Fig. 3B). Compared with the U1 snRNA, the U1 anti-OC construct greatly reduced osteocalcin mRNA expression in a dose-dependent manner (inhibition of osteocalcin by 64% at 15 μg/plate, 80% by 30 μg/plate and 60% by 60 μg/plate, respectively). We also examined the expression of Col1a1 in the transfectants. As shown in Figure 3B, in contrast to reduction of OC, there was no difference in Col1a1 expression between U1 snRNA and U1 anti-OC. These data suggest that U1 anti-OC specifically downregulated OC mRNA expression. The expression of Col1a1 in cells with a high concentration of the U1 anti-OC construct is equivalent to that in other groups. Furthermore, a reduction in OC protein was also observed in the cells transfected with U1 anti-OC construct by immunocytochemistry, compared with that of the control as seen in Figure 3C.
The modified U1 anti-Col1a1 construct was used to target Col1a1 expression in ROS17/2.8 cells. The cultures were stably cotransfected with U1 anti-Col1a1 and SV2 Neo, selected with 200 μg/ml of G418 for 4 weeks. The expression of Col1a1 mRNA was analyzed by northern blot using total RNA extracted from individual clones except that the control RNA was from a pooled population. Variable but significant reduction of Col1a1 expression was observed in all clones (Fig. 4).

Assessing the status of polyadenylation of targeted osteocalcin mRNA

In previous work directed at the possible mechanisms of modified U1 snRNA mediated inhibition of gene expression, we were unable to demonstrate accumulation of the targeted transgene mRNA in the nucleus or cytoplasm, suggesting that the modified U1 snRNA does not block the export of mature mRNA from the nucleus into cytoplasm (1,2). We also found that the U1 70K protein and the RNA hybrid formed between the modified U1 and its target sequence are required components for U1 antiRNA mediated inhibitory action (1,2). Based on these observations and other studies (3,12), we hypothesized that the modified U1 snRNA brings its U1 70K protein into proximity with PAP and exerts its known inhibitory action on PAP. This inhibitory interaction leads to shortening of a poly(A) tail length, reducing the stability of the targeted transcript. To test this hypothesis, we performed PAT–PCR analysis to examine the poly(A) status of targeted OC mRNA. To ensure that the 5'-oligo used for PAT–PCR was OC-specific, RT–PCR was designed to amplify the 3'-most region of the terminal exon just before the poly(A) tail using 5'-oligo OC F-1896 and 3'-oligo OC R-2086 (Fig. 5A). Figure 5B shows that the U1 anti-OC markedly reduced expression of OC to the same degree as shown by northern blot analysis as described above in Figure 3B. This indicates that the 5'-OC F-1896 oligo is OC-specific. Combined with an oligo(dT) anchor, this 5'-OC F-1896 oligo can specifically amplify the poly(A) tail of targeted OC mRNA. To compare the size of the poly(A) tail, the control cDNA was diluted (1:20 to 1:40) prior to PAT–PCR analysis to ensure that relatively equal amounts of OC cDNA from the control and targeted mRNA were amplified. The PAT–PCR analysis showed that the poly(A) tail of target OC mRNA is significantly shortened compared with that of the control (Fig. 5C), indicating that the modified U1 snRNA limits the polyadenylation of the targeted mRNA.

DISCUSSION

The present study demonstrates that the modified U1 snRNA specifically downregulates endogenous genes in mammalian cells. For example, the reduction of Cbfa1 expression causes a change from an osteoblastic phenotype and affects downstream gene expression. Alkaline phosphatase is an early marker for osteoblast differentiation, followed by collagen and OC as a later marker for differentiation (13). The inhibition of Cbfa1 expression by the modified U1 anti-Cbfa1 construct precludes the expected onset of osteoblast gene expression, including ALP activity and OC expression. These changes indicate that osteoblast differentiation is arrested due to the downregulation of Cbfa1 gene expression. Our in vitro results are consistent with the in vivo observation that a defect of Cbfa1 gene expression has a haploid-insufficient phenotype in membraneous bone resulting in the clinical disorder cleidocranial dysplasia (CCD) (14). In vitro studies of Cbfa1-deficient calvarial cells show a complete lack of the ability to differentiate into osteoblasts (15). Because the MC3T3 cell line was derived from calvarial osteoblasts, an 80% inhibition of Cbfa1 would appear to be able to block the initiation of osteoblast differentiation. However, Cbfa1 action is also dependent on its phosphorylation status (16,17), which is not reflected by its mRNA level, and this could have been affected.
by our experimental manipulations. The modified U1 anti-OC construct successfully reduced OC expression, but had no effects on Col11a1 gene expression. This suggests that the modified U1 snRNA mediated downregulation of gene expression is gene specific.

The poly(A) tail is formed in the nucleus in conjunction with RNA splicing and 3' end cleavage. Incompletely polyadenylated transcripts are rapidly degraded. The only exceptions are the replication-dependent histone genes which utilize the U7 snRNA complex for forming 3' ends (18,19). Controlling polyadenylation represents a distinct level of regulation of pre-mRNA 3' processing (20). Polyadenylation is inhibited by the proximity of a 5' splice site when a poly(A) site recognition is positioned within the body of a gene (20).

The interaction between U1 70K protein and PAP is believed to be responsible for this inhibition (3). Examples of this kind of regulation are demonstrated in HIV-1 (12,21,22) and bovine papilloma virus (BPV) (23).

Based on this inhibitory regulation machinery, we speculated that the modified U1 snRNA may also reduce PAP activity by placing the U1 70K protein in proximity to the poly(A) site, leading to a shortened poly(A) tail and instability of the targeted mRNA. Previously, we found that U1 70K protein is essential for repression by the modified U1 snRNA construct (1). In addition, when the 3'-UTR, cleavage and poly(A) site of a pSV targeted transcript was replaced with a fragment containing the histone 3' end processing signals, it was resistant to the inhibitory action of a U1 anti-SV construct. When an artificially synthetic cleavage and polyadenylation sequence was added back, it became a target for the U1 anti-SV construct (4). In the present study, we performed PAT±PCR to directly assess the length of poly(A) tail of the targeted endogenous mRNA and control. Because the poly(A) tail length was significantly reduced by the U1 anti-OC, we conclude that the modified U1 snRNA associated snRNP complex, especially the 70K protein, inhibits PAP activity. This leads to deficient polyadenylation of the pre-mRNA, instability of mature mRNA and a decrease in the expression of a targeted gene (Fig. 6).

Although the modified 10 bp at the 5' end of U1 snRNA recognizes the sequence of a targeted pre-mRNA in a base-pairing manner, the modified U1 snRNA is believed to function differently from classic antisense mechanisms. We have demonstrated that the modified U1 snRNA no longer preserves its inhibitory action when directed to regions other than the terminal exon or 3'-UTR (1), when the U1 70K protein binding site within the modified U1 snRNA is inactivated (2) or when the 3'-UTR and cleavage and polyadenylation sequence in a targeted gene is replaced with sequence containing the histone 3' end processing signals (4).

All the evidence points to a mechanism affecting the stability of a targeted transcript as a consequence of a shortened poly(A) tail length.

Modified U1 antiRNA has several properties that may make it an attractive effector for molecular therapy. It functions in the nucleus through a mechanism that inhibits polyadenylation.
of pre-mRNA and can target a unique sequence located at the untranslated region of the terminal exon of a given gene, which is a site not employed by other antiRNA strategies. A synergistic inhibition of gene expression may be possible by a combination of U1 antiRNA with a ribozyme, antisense oligonucleotide or siRNA, since each agent functions through a different mechanism and cellular localization. This complementary action may be required for inhibition of a strong dominant-negative acting gene transcript.

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