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# A New Vector, Based on the PolII Promoter of the U1 snRNA Gene, for the Expression of siRNAs in Mammalian Cells

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**Several vectors for the induction of RNA interference in mammalian cells have been described, based mainly on pol*III*-dependent promoters. They transcribe short hairpin RNAs (shRNA) that, after being processed into short interfering RNAs (siRNAs), mediate the degradation of the target mRNA. Here, we describe the construction of a new siRNA-expressing vector (psiUx) based on the strong and ubiquitous pol*II*-dependent promoter of the human U1 small nuclear RNA (snRNA) gene. In psiUx, the only constraint for the shRNA sequence is a purine at position +1, since specific 3′-end formation is achieved by a box element located downstream of the transcribed region. Several constructs were designed against the lamin A/C target. Depending on the structure of the shRNA transcribed, a preferential or exclusive accumulation of the antisense strand is obtained, thus avoiding possible nonspecific targeting by the sense strand. In all cases tested, very effective siRNAs were produced, thus providing a proof-of-principle that a snRNA-type polII promoter can be used for the expression of siRNAs. We show that psiUx ensures high levels of expression and efficient knock down of the target gene also in stable cell lines.**

**Key Words: RNA interference, siRNAs, posttranscriptional gene silencing, miRNAs, U1 snRNA promoter, RNA polymerase II, miRNA biogenesis**

## INTRODUCTION

RNA interference is a process of sequence-specific post-transcriptional gene silencing highly conserved in evolution. The mediators of sequence-specific messenger RNA degradation are 21- to 23-nucleotide small interfering RNAs (siRNAs) generated by cleavage of a longer double-stranded RNA by Dicer, a ribonuclease III-like enzyme. Such a process, initially described in *Caenorhabditis elegans* [1], and subsequently reported in insects, plants, and fungi, has been recently reproduced in mammalian cells [2]. Since the degradation of target RNA takes place with very high efficiency and sequence specificity, RNA interference represents a useful tool for targeted gene inactivation in human cells, and its employment may lead to the development of therapeutic molecules against viral or genetic diseases.

In a process related to RNA interference, small (~22-nt-long) single-stranded RNAs named micro RNAs (miRNAs) are produced by the cleavage of an ~60- to 70-nt stem-loop precursor (pre-miRNA) operated by Dicer in the cytoplasm [3]. In mammalian cells, the pre-miRNA is generated by the nuclear cleavage of a longer primary transcript performed by the nuclear RNase III-like enzyme Drosha [3]. The pre-miRNA is then actively transported from the nucleus to the cytoplasm by Ran-GTP and the export receptor Exportin V [4,5].

Initially, RNA interference was induced in mammalian cells through the transient transfection of short double-stranded RNA oligonucleotides (21- to 23-bp siRNAs) [2,6]. Since the use of artificial siRNAs does not lead to a long-term effect, many groups have concentrated their efforts on producing plasmids encoding short hairpin RNAs (shRNAs), which are processed in the cell to generate siRNAs.

So far, several types of shRNA vectors have been conceived, most of them relying on polIII-dependent promoters; they include the utilization of the U6 small nuclear RNA (snRNA) [7–10] or the H1 RNA [11] genes and more recently of tRNA-expressing cassettes [12,13]. A modified cytomegalovirus polII promoter in conjunction



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with a minimal polyadenylation signal has also been used for the expression of shRNAs [14].

The rationale behind the use of polIII promoters for the expression of shRNAs lies in the fact that these promoters have a compact and simple organization and transcribe small, highly structured nonpolyadenylated RNAs that are abundant in mammalian cells [15]. However, much indirect evidence indicates that miRNA genes are transcribed by RNA polymerase II even though they do not have signals for polyadenylation [3].

Here, we describe the use of a new vector for RNA interference in mammalian cells, based on the polII-dependent regulatory regions of the U1 snRNA gene. The U1 snRNA promoter is among the strongest polII-promoters, is active in all cell types, and so far has not shown silencing in stable cellular clones [16]. In addition, the presence of a 3′ element, responsible for the correct formation of the U1 snRNA 3′ end ensures efficient 3′-end formation of the transcript [17,18]. We provide a proof-of-principle that a snRNA-type polII promoter can be used in mammalian cells to generate siRNAs.

## RESULTS AND DISCUSSION

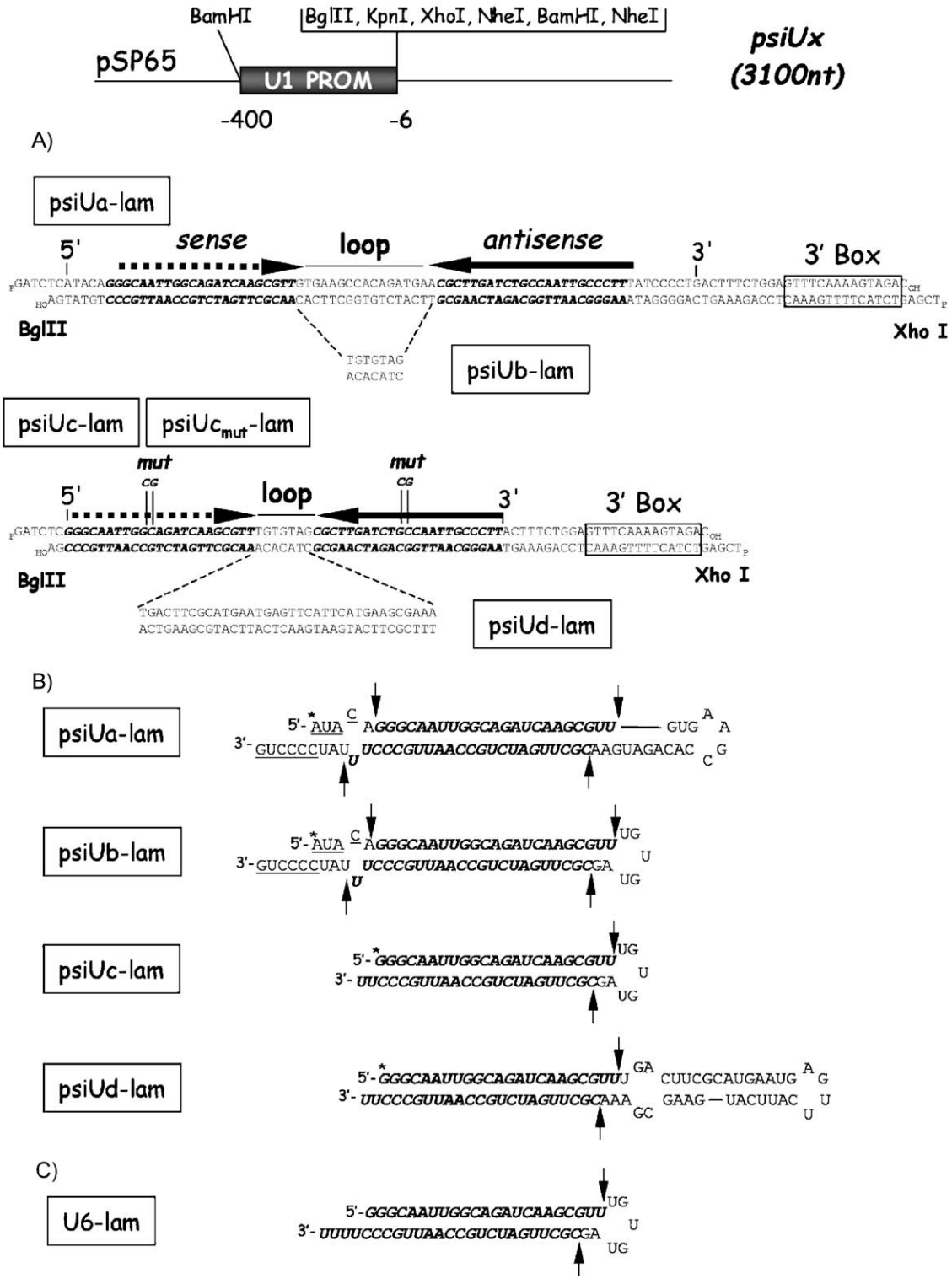
### Design of U1 snRNA Promoter-Driven Constructs

We cloned the region containing the U1 snRNA gene promoter extending from the *Bam*HI site, at position −400, to the *Bgl*II site, at position −6, with respect to the initiation site, into the *Bam*HI/*Nhe*I sites of the pSP65 plasmid with the addition of a synthetic polylinker (Fig. 1). This construct (psiUx) provides the promoter region but lacks both the transcription initiation site and the 3′ element. According to our design, these elements should be provided by the sequence to be inserted. The initiation sequence is 5′-GATCTC′A-3′, in which the last residue corresponds to the +1 nucleotide of the U1 snRNA (also a G is accepted in this position) [19]. The 3′ element is 5′-**CCCCTG**′ACTTTCTGGAGTTTCAAAAGTAGAC-3′, in which the underlined sequence is the so-called 3′ box, located 10 nucleotides downstream of the 3′ end of the transcript. The CCCCTG sequence corresponds to the last 6 nucleotides of the U1 snRNA, which were previously shown to contribute to an efficient and site-specific 3′ end formation [20]. Cloning of siRNA precursors can be easily performed by inserting into psiUx either an amplified fragment or annealed synthetic oligonucleotides with ends compatible with the selected site. While a 5′ *Bgl*II terminus is obligatory since it is required to restore the initiation site, any of the sites contained in the polylinker (*Kpn*I, *Xho*I, *Nhe*I, and *Bam*HI) can be utilized at the 3′ end of the insert (top of Fig. 1).

As a target sequence for testing the effectiveness of our vector we selected a region in the lamin A/C mRNA that has been previously demonstrated to be vulnerable to siRNAs [7]. We cloned a hairpin made of 21-nucleotide sense and antisense sequences, based on the lamin A/C cDNA, in different contexts to select the most appropriate design for efficient siRNA expression (psiU-lam constructs, Fig. 1). The resulting constructs differ not only in the type of loop sequence inserted but also in the 5′ and 3′ termini of the transcribed region. The sequences of the different inserted fragments are indicated in Fig. 1A and the structures of the primary transcripts are schematically represented in Fig. 1B. The loops utilized in psiUa-lam, psiUb-lam, and psiUc-lam are deduced from precursors of miRNAs [21,22], while that present in psiUd-lam is deduced from the canonical substrate of the yeast Rnt1p endonuclease, an RNase III-like enzyme [23]. Constructs psiUa-lam and psiUb-lam have 5′- and 3′-terminal nucleotides deduced from the corresponding regions of the U1 snRNA transcript (underlined in Fig. 1B). We inserted additional nucleotides into the 3′ region so as to produce a 3-bp-long stem and a single mismatch before the lamin-specific sequences (see Fig. 1B). This should mimic a Drosha substrate able to produce a pre-miRNA competent for export [3]. The psiUc-lam and psiUd-lam constructs differ from the previous ones in that the terminal nucleotides of U1 snRNA were removed, thus producing 5′ and 3′ ends of the primary transcript perfectly matching the lamin A/C target sequence. In this case, the +1 nucleotide is a G that was previously shown to be compatible with efficient transcription [19]. We also derived a control construct with two mismatches in the central part of the lamin A/C pairing region from psiUc-lam (psiUc$_{mut}$-lam). According to several reports [8,11,24], this mutated siRNA should not mediate interference on the target lamin A/C mRNA.

To be able to compare the activity of these vector systems with others previously utilized, we cloned into the U6 vector the same anti-lamin A/C hairpin sequence contained in psiUc-lam (plasmid U6-lam, Fig. 1C) as described by Castanotto *et al.* [22].

**FIG. 1.** A schematic representation of the psiUx vector, including the sites present in the polylinker downstream of the U1 snRNA promoter, is shown at the top. (A) Sequences of the different psiUx derivatives. The termini of the transcribed regions are indicated by 5′ and 3′. The sense and antisense sequences are deduced from the lamin A/C mRNA and are represented by convergent dotted and continuous arrows, respectively. The mutations introduced in the psiUc$_{mut}$-lam construct are indicated above the parental sequence. The 3′ element of the U1 gene is indicated as 3′ Box. The variant loop sequences present in psiUb-lam and psiUd-lam are indicated below the parental constructs. (B) Predicted structure of the four U1-derived anti-lamin primary transcripts. The arrows indicate the presumptive sites of processing by the Dicer enzyme. siRNA sequences are shown in bold. Underlined nucleotides identify the sequences derived from the 5′ and 3′ transcribed regions of U1 snRNA. The asterisk represents the monomethyl cap structure. (C) Predicted structure of the U6-derived anti-lamin primary transcript.



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### Expression of siRNAs from U1 snRNA Promoter in Human Cells

We tested the different constructs for expression and activity by transfection into HeLa cells. We cotransfected all constructs with a modified U7 snRNA gene (U7-3′) [25] as an internal control. At 48 h, we extracted RNA and analyzed it by Northern blot. Fig. 2A shows hybridizations with a probe specific for the antisense strand (probe a): all the U1-derived constructs produce the accumulation of the antisense strand with size ranging between 21 and 23 nucleotides. The same type of molecules accumulate in the case of the U6-derived construct (lane U6). The siRNAs produced by the mutated psiUc$_{mut}$-lam derivative are visible only with the **a$_{mut}$** probe, which has perfect complementarity to the mutation (Fig. 2C). Since these RNAs accumulate *in vivo* after such prolonged times, it must be

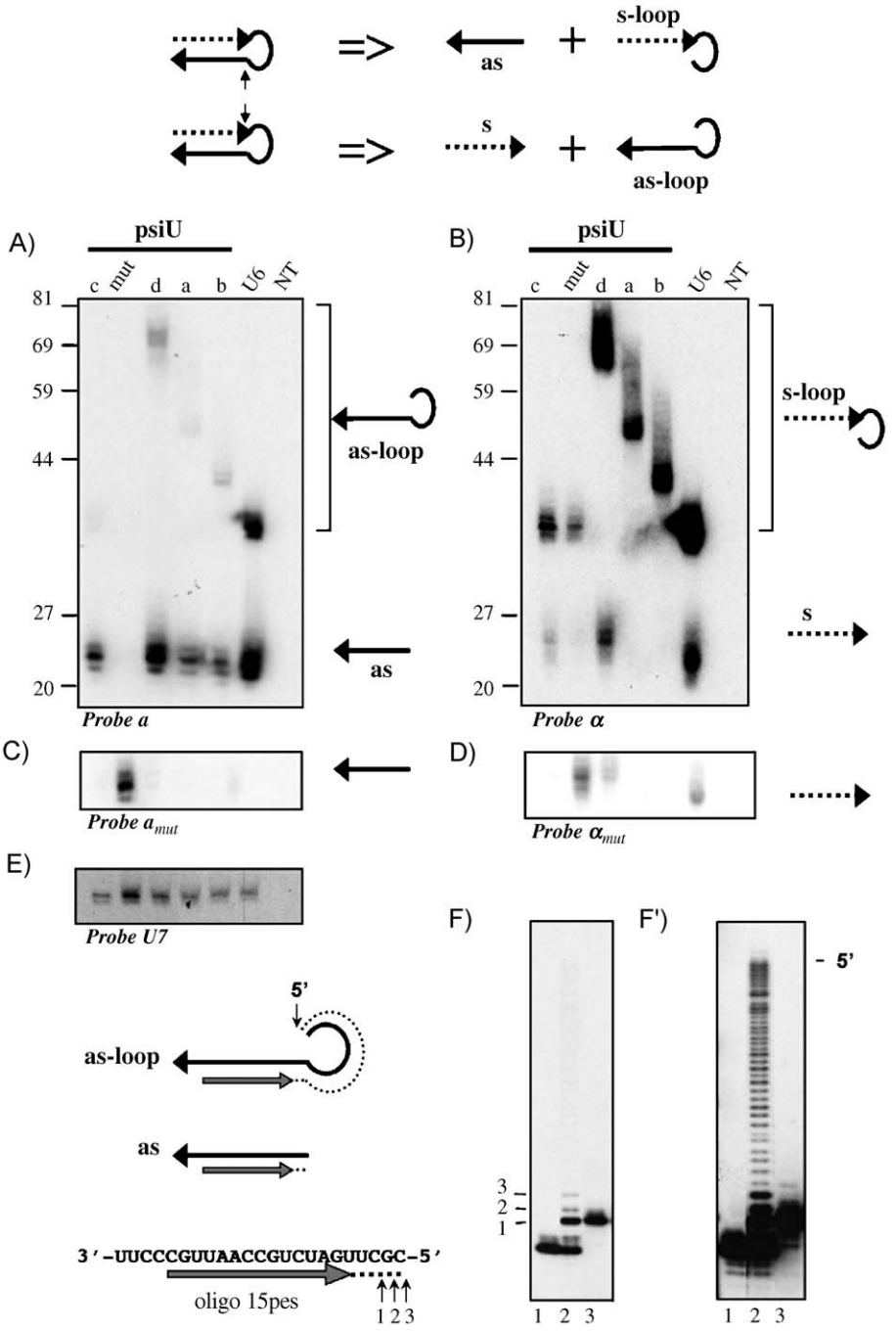


**FIG. 2.** Analysis of siRNA expression from the different anti-lamin constructs. HeLa cells were transfected with 6 μg of the different psiU-lam constructs (lanes c, psiUc-lam; lanes mut, psiUc$_{mut}$-lam; lanes d, psiUd-lam; lanes a, psiUa-lam; lanes b, psiUb-lam) or with 6 μg of U6-lam (lanes U6). 2 μg of a control U7 construct was cotransfected in all cases. After 48 h, total RNA was extracted and 15 μg were analyzed by Northern blot on a 10% polyacrylamide–urea gel. Lanes NT, RNA extracted from untransfected cells. (A) Hybridization with probe **a**, which recognizes the antisense strand (indicated by a continuous arrow) of the anti-lamin constructs. (C) Hybridization with probe a$_{mut}$. (E) Control hybridization with a probe specific for the cotransfected U7 construct. (B) After being stripped, the same filter was hybridized with probe α, which recognizes the sense strand (indicated by a dotted arrow). (D) Hybridization with probe **α$_{mut}$**. RNA size markers are indicated on the sides of the gels. The filters were always hybridized with 500,000 cpm/ml of probe ($10^6$ cpm/pmol) and exposed for 5 h. The schematic drawings on the sides of A and B depict the observed intermediates. (F) Primer extension analysis. Lane 1, $^{32}$P-labeled 15-mer oligo 15pes; lane 2, oligo 15pes was annealed with RNA from cells transfected with construct psiUd-lam and the products of reverse transcription were analyzed on a 15% acrylamide-urea gel; lane 3, $^{32}$P-labeled 17-mer control oligo. (F′) Overexposure of F; this allows the visualization of the extended products on the as-loop molecules, which are underrepresented in comparison to the mature siRNAs. A schematic representation of the primer and of the extended products is shown on the side.

argued that they are present in stable complexes. Similar findings have been interpreted in other cases as diagnostic for the association with an interference-competent complex. We performed primer extension analysis to confirm the nature of these molecules: an oligo complementary to 15 nucleotides of the antisense strand was annealed with RNA from cells transfected with construct psiUd-lam and extended with reverse transcriptase. Lane 2 of Fig. 2F shows that the primer is extended by 2, 3, and 4 nucleotides, consistent with cleavage occurring at the indicated positions (see schematic representation). Densitometric scanning of the hybridization signals of Fig. 2A and normalization with the hybridization signals of a cotransfected U7 snRNA derivative (Fig. 2E) indicate that the levels of psiU-lam siRNAs are lower than those of U6-lam siRNAs (psiUc-lam yields 25% siRNAs compared to U6-lam; psiUa-lam and psiUb-lam 30%, and psiUd-lam 45%).

The expression analysis of the psiUc-lam construct indicates that it is possible to eliminate most of the conserved nucleotides at the 3′ end of the U1 transcripts and still obtain efficient termination and processing. Indeed, other constructs raised in the lab have proven that the U1 snRNA terminal nucleotides are totally dispensable for efficient 3′ -end formation (not shown). Therefore, it can be concluded that cloning into the psiUx vector has no sequence constraints, other than the purine at position +1.

We performed hybridization on the same filter, with a probe specific for the sense strand (Fig. 2B, probe α). Within the detection limits of our system, no signal corresponding to the siRNA sense strand is obtained with the psiUa-lam and psiUb-lam derivatives and only tiny amounts with construct psiUc-lam. In contrast, U6, and to a minor extent psiUd-lam, shows the accumulation of appreciable amounts of the sense strand. Considering that the α probe had the same specific activity of the **a** probe and that the filters in Figs. 2A and 2B were exposed for the same time, it can be concluded that there is quite a strong asymmetric strand selection in U1-derived transcripts and in particular in constructs psiUa-lam and psiUb-lam.

The sense strands produced by the psiUc-lam and psiUd-lam constructs appear 1 or 2 nucleotides longer than the ones derived from the U6 construct (Fig. 2B, compare lanes c and U6). This is likely due to the presence of a cap structure as expected for U1 promoter-driven transcripts; in contrast a 5′ -pppG end should be present in the U6-derived transcripts [26]. Capped and uncapped RNAs transcribed *in vitro* were indeed shown to migrate with a 1- or 2-nucleotide difference (not shown).

Figs. 2A and 2B also show the presence of slower migrating products with sizes matching those expected for two-third processing intermediates originating from the cleavage of only one of the two strands of the shRNA (see schematic representation). Cleavage in the upper strand would produce the two-third molecules visible with probe **a** in Fig. 2A (''as-loop'' molecules) and their complementary 5′ cut-off products, the mature sense strand, visible with probe α in Fig. 2B (''s'' molecules). In contrast, cleavage in the lower strand gives rise to the two-third molecules of Fig. 2B (''s-loop'' molecules, visible with probe α) and their complementary 3′ cut-off products, the mature antisense strand (''as'' molecules of Fig. 2A, visible with probe **a**). The 5′ and 3′ cut-off products originating from a single cleavage on the upper or lower strand appear to accumulate in stoichiometric amounts. We confirmed the structure of the two-third molecules by primer extension analysis; Fig. 2F′ (lane 2) indicates that the 5′ end of as-loop molecules, tested on construct d, maps on the upper strand at positions opposite to the cleavages on the lower strand, as expected for Dicer cleavage (see schematic representation).

In agreement with what is shown for the mature siRNAs, the abundance of the two-third products also confirms the asymmetry of strand cleavage. We performed densitometric analysis on various exposures of the filters: constructs psiUa-lam and psiUb-lam produce high amounts of s-loop molecules and almost undetectable levels of as-loop species, in an approximate ratio of 20:1, while constructs psiUc-lam and psiUd-lam show a lower ratio (approximately 12:1). The U6-lam shows the lowest degree of asymmetry, with a ratio between the s-loop and as-loop molecules of 4:1. The mechanism underlying such strong asymmetric strand selection for constructs a and b is at the moment under study even though it reflects what occurs for the majority of miRNAs [3,6].

Khvorova [27] and Schwarz [28] have proposed that differential accumulation of sense and antisense siRNAs could be due to the differential stability of the two ends of the duplex after Dicer cleavage. In our case both ends of the duplex have the same G/C composition. Nevertheless, to exclude any sequence bias in strand selection we generated two types of constructs: in psiUb-swap the two strands of psiUb-lam were swapped, while in psiUb-5a the first nucleotides of the upper strand were changed from GG to AA and a reciprocal change was made at the 3′ end of the lower strand (Fig. 3A). According to the rule drawn by Khvorova and colleagues [27], the strand that accumulates is the one whose 5′ end pairs less tightly. If this rule, which was found with short synthetic duplexes in extracts, applies also in the context of a shRNA precursor, one would predict that for construct psiUb-5a the upper strand should accumulate.

We transfected constructs psiUb-lam, psiUb-swap, and psiUb-5a into HeLa cells, together with construct U7-3′ [25] as an internal control. After 48 h, we extracted RNA and analyzed it by Northern blot. Figs. 3B and 3C show hybridizations with probes **a** and α, which detect, respectively, the antisense and the sense strands. Comparing the two panels, it can be clearly observed that with all the constructs, only the siRNA corresponding to the lower strand is accumulated. Therefore, it can be concluded that

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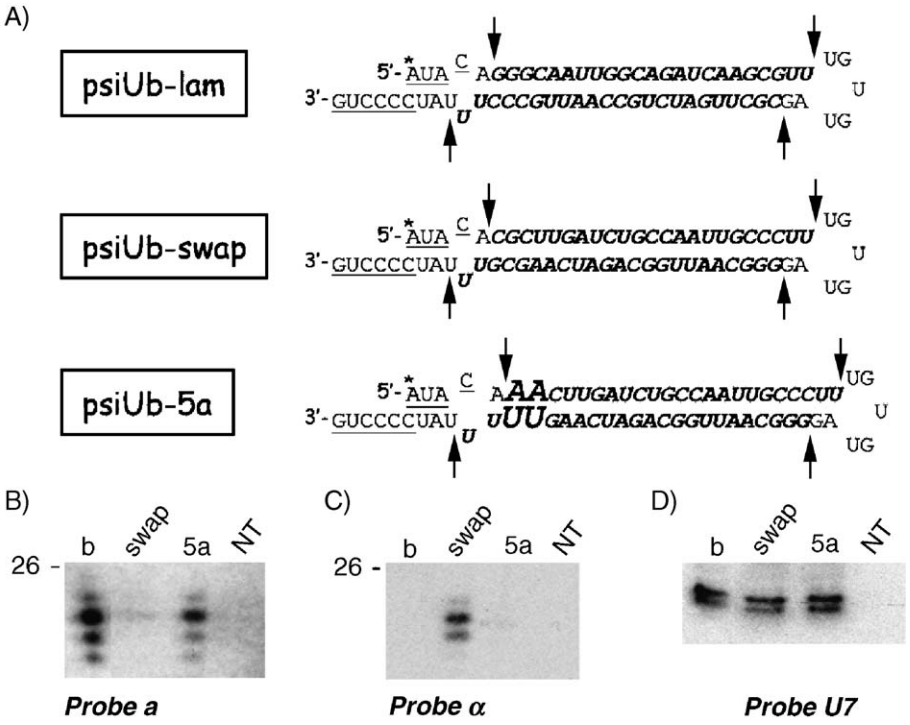


**FIG. 3.** (A) Predicted structures of psiUb-lam, psiUb-swap, and psiUb-5a. The arrows indicate the presumptive sites of processing by the Dicer enzyme. siRNA sequences are shown in bold. Underlined nucleotides identify the sequences derived from the 5′ and 3′ transcribed regions of U1 snRNA. The asterisk represents the monomethyl cap structure. (B) Northern blot analysis of siRNA expression from psiUb-lam construct (lane b), psiUb-swap (lane swap), and psiUb-5a (lane 5a). Lane NT, RNA extracted from untransfected cells. HeLa cells were transfected with 6 μg of psiUb-lam, psiUb-swap, or psiUb-5a constructs. 2 μg of a control U7 construct was cotransfected in each case. After 48 h, total RNA was extracted and 15 μg were analyzed by Northern blot on a 10% polyacrylamide–urea gel. Hybridization was performed with probe **a**, which recognizes the antisense strand of the anti-lamin constructs. (C) After being stripped, the same filter as in B was hybridized with probe α, which recognizes the sense strand. The filters were always hybridized with 500,000 cpm/ml of probe ($10^6$ cpm/pmol) and exposed for 5 h. (D) Control hybridization with a probe detecting the cotransfected U7 construct.

the nature of the ends of the duplex does not influence asymmetric strand accumulation.

In conclusion, these data indicate that, within the limits of our detection system, the terminal structure present in constructs psiUa-lam and psiUb-lam leads to a very strong asymmetry in siRNA strand selection. The generation of the sole antisense strand is of great importance in view of the therapeutic and applicative uses of these vectors.

### Effectiveness of U1 snRNA Promoter-Driven siRNAs

We tested the same cells analyzed in Fig. 2 for siRNA production also for RNAi activity. We analyzed 20 μg of total proteins extracted from HeLa cells transiently transfected with U1- and U6-derived constructs by Western analysis with anti-lamin A/C antibodies. As shown in Fig. 4 all siRNA vectors produce good levels of interference considering that the efficiency of transfection, in this type of experiments, is on the order of 80%. The U1 constructs give approximately 80% reduction of lamin levels. These depletion values are similar to those obtained with the U6 expression cassette. It is noteworthy that the U1 constructs, which accumulate three to four times fewer siRNAs than the polIII construct, show the same RNAi activity as U6-lam. This demonstrates that to have an effective RNAi response it is not necessary to reach the typically high levels of polIII-dependent transcription, in line with recent observations suggesting that siRNAs work in a catalytic fashion. We tested the specificity of the interference response by transfecting HeLa cells with the construct containing two mismatches in the central part of the 21-nucleotide-long pairing region (psiU$c_{mut}$-lam). Despite the fact that the mutated siRNAs accumulate at levels similar to the others (Fig. 2B), they mediate interference at a much lower extent; this is indicated by the amount of lamin A/C (Fig. 4, lane $c_{mut}$), which is 80% of the control (Fig. 4, lane NT).

### U1 snRNA Promoter-Driven siRNA Expression and RNAi Activity are Maintained in Stable Cellular Clones

To analyze long-term expression of siRNAs under the U1 promoter, we subcloned psiUc-lam and psiU$c_{mut}$-lam constructs into the pBabe-puro vector [29], which provides puromycin resistance (see Materials and Methods), yielding constructs pBabe-Uc-lam and pBabe-U$c_{mut}$-lam, respectively. We transfected HeLa cells with pBabe-Uc-lam and pBabe-U$c_{mut}$-lam and obtained stable cellular clones by puromycin selection. We detected efficient accumulation of siRNAs after 1 month of continuous growth (Fig. 5A) analogous to what was previously reported for other RNAs transcribed under the U1 promoter [16,25]. The persisting ability of these siRNAs to knock down the expression of lamin A/C is shown by the Western blot of Fig. 5B (lanes pBabe-Uc). This experiment also shows that the control mutated siRNAs do not display any RNAi activity (Fig. 5B, lanes pBabe-U$c_{mut}$), indicating that specificity is maintained also after such prolonged times.

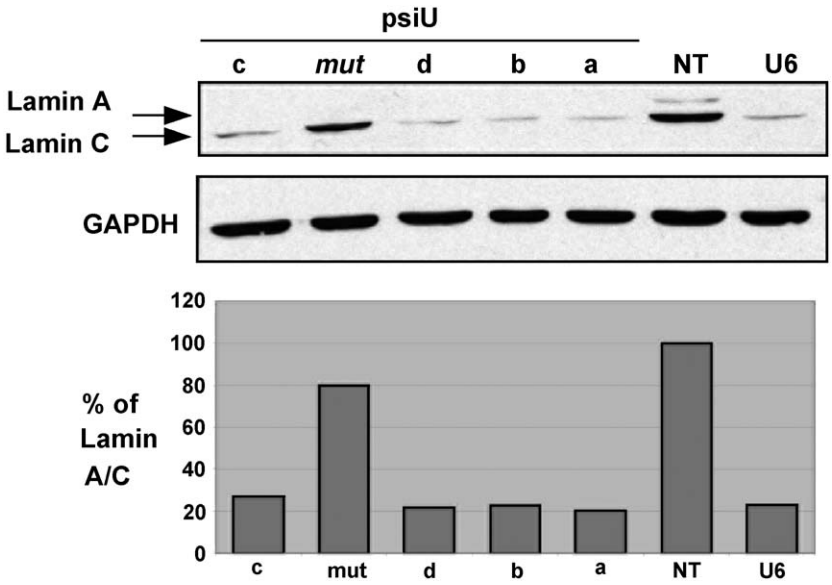


**FIG. 4.** Activity of the siRNAs transcribed from the anti-lamin constructs. 20 μg of total cellular proteins extracted 70 h after transfection was analyzed by Western blot with anti-lamin A/C monoclonal antibodies. Lane c, psiUc-lam; lane mut, psiUc$_{mut}$-lam; lane d, psiUd-lam; lane a, psiUa-lam; lane b, psiUb-lam; lane NT, proteins extracted from untransfected cells; lane U6, U6-lam. The arrows point to the two isoforms of lamin (A and C). In the middle, a control Western with an anti-GAPDH antibody is shown. The histogram at the bottom represents the amounts of residual lamin after normalization for the GAPDH signals.

Based on these observations, we raised in our laboratory U1-derived siRNAs against other substrates (necdin and msx2 mRNAs). These constructs were shown to knock down effectively and specifically the expression of their target mRNAs in mammalian cells [30].

Altogether, these data indicate that the U1-based vector here described has several interesting features: (1) the transcribed region has no sequence constraints other than a purine at position +1; the 3′ terminus is efficiently produced by a conserved box element located 10 nucleotides downstream of the mature 3′ end; (2) cloning is very easy and allows selection among different cloning sites; (3) specific elements have been identified that allow the exclusive accumulation of one siRNA strand, avoiding possible nonspecific targeting by the other; and (4) the U1-driven expression and RNAi activity are maintained in stable cellular clones.

## MATERIALS AND METHODS

***Construction of psiUx vector.*** The U1 snRNA gene-based vector was derived from plasmid pHU1-ID, containing the entire human gene [25].

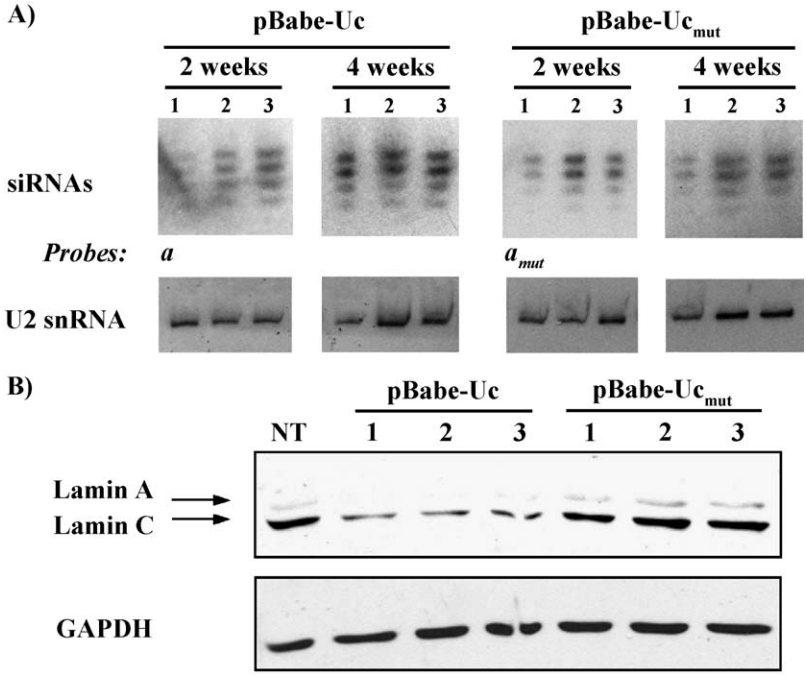


**FIG. 5.** Long-term expression and efficacy of the U1-derived siRNAs in HeLa cells. (A) Northern blot analysis of 15 μg of total RNA extracted from three mixed cell populations (lanes 1, 2, and 3) transfected with the pBabe-Uc or the pBabe-Uc$_{mut}$ construct and then kept in puromycin selection medium for 2 or 4 weeks. Hybridizations were performed with probe a and probe a$_{mut}$. The control hybridization with the U2R probe, which detects the endogenous U2 snRNA, is shown below. (B) Western blot analysis of the three mixed cell populations (lanes 1, 2, and 3) transfected with the pBabe-Uc or the pBabe-Uc$_{mut}$ construct, after 4 weeks of growth in selection medium. Lane NT, proteins extracted from untransfected cells. The arrows point to the two isoforms of lamin (A and C). A control Western with anti GAPDH antibodies is shown below.

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This plasmid carries the 600-bp *Bam*HI fragment containing the transcriptional unit of the human U1 snRNA gene inserted into the *Bam*HI site of the pSP65 vector (Promega), in the direction opposite to the SP6 promoter. Plasmid psiUx was derived from the latter by double digestion with *Bgl*II and *Nhe*I and religation in the presence of a polylinker containing the 5′ -*Bgl*II, *Kpn*I, *Xho*I, *Nhe*I, *Bam*HI, and *Nhe*I-3′ sites. The *Bgl*II site maps in the U1 snRNA gene, at position −6 with respect to the transcription initiation site, while the *Nhe*I site is in the vector, 300 nucleotides upstream of the SP6 promoter. The linker was made by annealing the two oligonucleotides *linkup,* 5′ -GATCTGGTACCCTCGAGGCTAGCGGATCCG-3′ , and *linkdn,* 5′ -CTAGCGGATCCGCTAGCCTCGAGGGTACCA-3′ .

***Construction of psiU derivatives expressing siRNAs for the lamin A/C protein.*** The selected target sequence on the lamin A/C was derived from Sui *et al.* [7] and covers nucleotides 1602–1622 of X03444 of the NCBI database. The sequences utilized in the different constructs are reported in Fig. 1 and were generated by annealing synthetic corresponding complementary oligos. To generate pBabe-Uc-lam and pBabe-$Uc_{mut}$-lam, the *Bam*HI–*Nhe*I fragments of psiUc-lam and psi$Uc_{mut}$-lam, respectively, were cloned into the *Bgl*II–*Nhe*I sites of the modified pBabe puro vector [25].

***Cell culture and transfection.*** Subconfluent HeLa cells were transfected in 60-mm plates by using Lipofectamine 2000 (Life Technologies, Gibco BRL) according to the manufacturer's instructions. Six micrograms of psiUx plasmid derivatives were cotransfected with 2 μg of plasmid U7-3′ [25]. To obtain selected pools, 48 h after transfection puromycin was added to the medium at the final concentration of 1 μg/ml (Sigma).

***RNA analysis.*** Total RNA from transiently transfected HeLa cells or from selected cellular pools was extracted using the Ultraspec RNA isolation system (Biotech Laboratories, Houston, TX, USA) according to the manufacturer's instructions. To detect siRNAs, 15 μg of total RNA was electrophoresed in a 10% polyacrylamide–8 M urea gel and transferred by electroblotting onto Hybond-$N^+$ membrane (Amersham Pharmacia Biotech.). The hybridization was carried out at 37°C in 5× SSPE, 5× Denhardt's solution, 0.5% SDS, 25 μg/ml salmon sperm DNA (Invitrogen). Washes were performed at 37°C in 6× SSPE and 2× SSPE and 0.2× SSPE. Probes used were terminally $^{32}$P-radiolabeled DNA oligos: probe a, 5′ -GGCAATTGGCAGATCAAGCG-3′ ; probe $a_{mut}$, 5′ -GGCAATTGCGAGATCAAGCG-3′ ; probe α, 5′ -CGCTTGATCTGCCAATTGCC-3′ . U7-3′ transcript was detected with probe U7a and the endogenous U2 snRNA was detected with probe U2R [25]. Filters were exposed on BioMax MS films (Kodak) for 5 h at 180°C with a BioMax MS intensifying screen (Kodak). Primer extension analysis was performed with oligo 15Pes (5′ -GCAATTGGCAGATCA-3′) on 15 μg of total RNA as described by Morlando [31].

***Immunoblotting.*** Protein extracts (20 μg) were separated on 10% polyacrylamide–SDS gels and transferred to nitrocellulose (ProTran; Schleicher and Schuell). The membranes were blocked with 3% skim milk in TBST. Mouse monoclonal anti-lamin A/C antibody (sc-7292; Santa Cruz Biotechnology) diluted 1:200 in TBST/3% milk was used as primary antibody. For the normalization a mouse monoclonal anti-GAPDH antibody (ab8245; Abcam, Cambridge, UK) was used, diluted 1:4000 in TBST/3% milk. The immunostaining was carried out using the ECL Western blotting detection system (Amersham UK).

***Densitometric analysis.*** Films were scanned with a GS-670 imaging densitometer (Bio-Rad, Hercules, CA, USA) and the signals were analyzed with the Molecular Analyst Software package (Bio-Rad).

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