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Influence of Capping and Polyadenylation on mRNA Expression and on Antisense RNA Mediated Inhibition of Gene Expression

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In order to investigate the influence of CAP and poly(A)-tail on mRNA expression and on antisense mediated inhibition of gene expression, coinjections of different expression vectors coding for Chloramphenicol-Acetyltransferase (CAT) sense- or antisense RNAs, respectively, were performed. Different *in vitro* transcribed and modified sense and antisense RNAs were injected into nucleus or cytoplasm of COS7-cells. It can be concluded that the combination of capping and polyadenylation ensures efficient gene expression and is required for transport of mRNA from the nucleus to the cytoplasm. In contrast, antisense experiments suggest that the length of antisense RNAs play an important role for the inhibitory capability of antisense molecules and expression reduction occurs independently of CAP and poly(A) tail. A model for intermolecular hybridization and suppression of gene expression based on the secondary structures of the CAT mRNA and its corresponding antisense RNA is presented.

A promising tool for selective regulation of single genes on pre- or post-transcriptional level in mammalian cells is the use of antisense-DNA and -RNA molecules to provide a more detailed insight into the mechanism of gene expression [1, 2]. Gene expression is influenced by both, capping and polyadenylation. So far, not all mechanisms of increasing or decreasing effects of capping and polyadenylation are fully understood. It is also not known how antisense mediated inhibition of expression could be influenced by these RNA modifications.

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Polymerase II transcripts usually are capped cotranscriptionally with the $m^7G(5')pppN^{(m)}pN^{(m)}p$ structure [3] by at least the guanylyltransferase and a methyltransferase. mRNA capping and methylation play an important role in initiation of transcription and in protection against 5′-exonucleolytic degradation [4, 5]. It also supports initiation of protein biosynthesis, pre-mRNA processing and export of polymerase II transcripts from the nucleus to the cytoplasm [6-9]. It can cooperate synergistically with the 3′-poly(A) tail [10]. Another modification of mRNA is the synthesis of a 3′-poly(A) tail [11]. Several functions for the poly(A) tail were discribed. A correlation between polyadenylation and stability of mRNA was shown [12]. mRNA polyadenylation has also been described as a regulatory determinant for efficient translation [12-14]. Very little is known about the compartment association of the poly(A) synthesis. Most mRNAs are polyadenylated posttranscriptionally in the nucleus [15], but there also exist cytoplasmic polyadenylation elements (CPEs) [16].

The mechanism(s) of antisense mediated inhibition of gene expression is still not fully understood. The inhibition of thymidine kinase (TK) gene activity provided first evidence for the suppression of fullength antisense DNA- and RNA molecules [17, 18]. In contrast to these results large antisense DNA- or RNA molecules did not influence early or late SV40 gene expression, whereas short antisense RNA- and DNA molecules strongly suppress SV40 gene activity [19, 20]. So far there is no clear explanation for the stronger inhibition of gene expression by short antisense molecules (for review see [21]. Antisense molecules cannot always suppress genetic activity, even in high excess [22]. Whereas the procaryotic Chloramphenicol Acetyltransferase gene (CAT) could be inhibited efficiently in a transient expression system in mouse cells [18] as well as in *Xenopus laevis* oocytes

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[23], CAT activity could not be blocked with interferon inducible infectious recombinant SV40 constructs that generated antisense CAT RNA in high copy number [24].

In the experiments described here compartmented microinjection of *in vitro* transcribed CAT sense and antisense RNAs that were 5′- and/or 3′-*in vitro* modified to discriminate between each modification and its influence onto gene expression were performed. Since direct microinjection allow to place defined amounts of DNA or RNA molecules into the nucleus or the cytoplasm of single cells, CAP structure and poly(A)-tail on gene expression as well as on antisense RNA-mediated reduction of gene expression will be presented. Furthermore sequence analysis and computer modelling [25] suggest that the sizes of the sense- and antisense RNAs and their resulting secondary structures are compatible with a putative intermolecular double strand formation and inhibition of gene expression as a consequence.

MATERIALS AND METHODS

Materials. Chemicals and reagents were obtained from Merck, Serva and Sigma, Germany. COS 7 cells were a kind gift of Y. Gluzman, Cold Spring Harbor Loboratory, New York. Media and sera for cell culture and DNA- and RNA-modifiing enzymes were obtained from Life Technologies, Detroit, USA and from Pharmacia, Uppsala, Sweden.

DNA analysis, RNA-analysis and RT-PCR were done according to standard procedures.

Construction of vectors for in vitro transcription. A 800 bp Bgl II-Xho II fragment containing the coding region and some flanking sequences was isolated from pBLCAT2 [26]. This segment was ligated into the BamHI site of pSPT18 (Pharmacia) giving the construct p800-18(2). From this plasmid it was cut with Kpn I and Pst I and ligated under the control of the T7 promoter sequence into pSPT19 giving the construct p800-19 that was used for *in vitro* transcription. For construction of a plasmid containing the CAT coding region and an addional 800 bp segment with the polyadenylation site of SV40, the corresponding fragment from pBLCAT2 was cut with Bgl II and Sma I and ligated it into the Bam HI and Sma I sites of pSPT19, resulting in the vector for *in vitro* transcription p1600-19.

Construction of vectors for CAT activity in mammalian cells. Construction of the antisense expression vector without the SV40 polyadenylation signal was performed by isolating the SV40 promoter sequence without origin of replication by Hind III cut of pSVPUC-18(b) (M. Gräßmann, unpublished), fill-in reaction and following Sst I cut. This fragment was ligated into the Sma I and Sst I sites of p800-18(2) as described above, giving the final plasmid p800-Pr-a. Construction of the antisense expression vector with the SV40 polyadenylation signal was performed by cutting the 800 nt pSV40 polyadenylation signal containing fragment via Xho II and Sma I. The fragment was ligated blunt into the EcoRV site of pBluescriptSK$^-$ (Stratagene), giving the construct pBlueSK$^-$/SVpoly(A). It was cut again by Xho II and Sma I and ligated blunt into the filled-in Hind III site of p800-Pr-a, giving the final plasmid p1600-Pr-a.

In vitro trancription, in vitro polyadenylation and in vitro capping of RNA. *In vitro* synthesis of cRNA was done by the bacteriophage T7 RNA polymerase [27]. As a template the above described CAT gene containing plasmids for *in vitro* transcription were used. Reaction was carried out at 37°C for 30 min and stopped by the addition of RNase-free DNaseI. *In vitro* transcribed RNA was subsequently purified. Quality of RNA was tested by denaturing gel electrophoresis, subsequent Northern blotting and by the labelling with α^{32}P(UTP) and subsequent gel electrophoresis. Synthesis and addition of a poly(A) tail to the 3′-terminus of RNA was done with the poly(A) polymerase from *E.coli* [28] (BRL, Life Technologies). Reaction was carried out with 1 μg RNA for 10 min at 37°C and *in vitro* polyadenylated RNA was purified subsequently. *In vitro* polyadenylation was controlled by RT-PCR with an oligo(dT) primer. Synthesis and addition of a CAP structure to the 5′-terminus of RNA was done with the Guanylyltransferase (Life Technologies). Reaction was carried out with 1 μg RNA for 45 min at 37°C and *in vitro* capped RNA was purified.

Microinjection of in vitro transcribed RNA molecules. Cells were cultured on 3×3 mm glass slides in DMEM containing 10% fetal calf serum. Microinjection of DNA or RNA molecules was done as described [29]. Various concentrations of RNA or DNA per cell were injected, either into the nucleus or into the cytoplasm. 150 COS 7 cells were injected in each experiment.

Determination of CAT activity [*30*]. Cells were were lysed 20 h post injection (p.i.) and after addition of AcetylCoA and 0.2 μCi ^{14}C-chloramphenicol, enzyme reaction was done at 37°C overnight. Acetylated forms of chloramphenicol were extracted with ethyl acetate and separated by thin layer chromatography. CAT activity was quantified by determination of cpm of the monoacetylated forms of chloramphenicol. Mean values of 3 experiments and significance were calculated by Students t-test. Background level was determined as 600 cpm and subtracted.

Computer modelling of RNA sequences. Secondary RNA structure analysis was generated by the GENMON program [25].

RESULTS

To investigate the effect of capping and polyadenylation of sense and antisense RNAs on gene expression microinjection into nucleus and cytoplasm of COS 7-cells was performed. For this reason CAT gene was cloned under the control of the T7-promoter, either with or without the polyadenylation signal of the SV40. The resulting *in vitro* transcribed RNAs without polyadenylation signal consisted of 800 nucleotides and the template with the additional segment containing the SV40 polyadenylation signal leads to a 1600 nt RNA in length.

After injection of *in vitro* transcribed SV40(A) CAT-RNA containing the SV40 polyadenylation signal into the nucleus as well as after injection into the cytoplasm a 2-fold higher CAT activity in comparison to non-modified RNA was detected (Fig. 1A). Cytoplasmic injection of the SV40(A) RNA lead to an average of 2-fold higher CAT activity compared with nuclear injection. To examine the influence of the CAP structure on CAT expression, *in vitro* capped CAT RNA, either lacking or harboring the SV40 polyadenylation signal was microinjected into nucleus and cytoplasm (Fig. 1B). Capping increased the level of CAT expression up to 2-fold. The average values of double modified molecules showed the highest expression with about 12000 cpm

as compared to 2000-6000 cpm of non modified RNA or 3000-8000 cpm of one side-modified RNAs. The enzyme activity was not significantly different after nuclear or cytoplasmic injection.

To exclude an effect of the additional 800 nts of the SV40 poly(A) signal-containing segment, in the above experiments capped RNA without this segment was *in vitro* polyadenylated and controlled by RT-PCR with an oligo(dT) primer (data not shown). *In vitro* capped and *in vitro* polyadenylated RNA was injected into nucleus and cytoplasm. Highest CAT activity was 12000 cpm, when the RNA was 5′- and 3′-modified. CAT activities in the nucleus and in the cytoplasm were similar and no difference between *in vitro* polyadenylated RNA or RNA in the presence of the SV40 poly(A) signal could be detected after injection of double-modified RNA. The quantitative data of all the different CAT-RNAs are summarized in Fig 2.

The effect of various antisense molecules to inhibit the Chloramphenicol Acetyltransferase was analysed by compartmented coinjections of sense- and antisense-RNA expressing plasmids, as well as *in vitro* transcribed sense- and antisense-RNAs. The plasmids encoding sense RNA contained the CAT coding region including the SV40 polyadenylation signal. Antisense RNAs carried or lacked the SV40 polyadenylation signal and carried or lacked the CAP.

To investigate inhibition of CAT activity by full-length RNA transiently, pBLCAT2 with the antisense vector p800-Pr-a, that encodes antisense CAT without the SV40 polyadenylation signal sequence were coinjected. Equimolar amounts of sense and antisense plasmids reduced CAT activity to 46% of the control. A 10-20-fold excess of coinjected antisense expressing plasmids reduced CAT activity to 7% of the control. A 1:0.1 sense to antisense DNA ratio did not inhibit CAT activity. These results indicate that full-length antisense RNA significantly inhibits CAT activity (Fig. 3A). Next, the effect of RNA stabilizing SV40 polyadenylation signal in an expressed antisense RNA on CAT activity was analysed. FIG. 3B shows that CAT activity was also reduced by RNA containing the SV40 poly(A) signal, and a molar sense : antisense ratio of 1:5 decreased CAT activity to 13% of the control. Titration of the above antisense RNA from molar ratios of 1:0.05 to 1:10 resulted in a steady decrease of CAT activity to $<$10% of the control.

In order to differentiate inhibition of CAT activity by antisense expression vectors in the two compartments, *in vitro* transcribed antisense RNAs were coinjected with *in vitro* transcribed CAT sense RNAs into the nucleus and the cytoplasm of COS 7 cells. The RNAs were used with and without CAP and with and without the polyadenylation signal of SV40. Nuclear coinjection of *in vitro* transcribed and capped CAT-sense RNA with the polyadenylation signal of SV40 in an additional segment of 800 bases with fullength, unmodified CAT antisense RNA of about 800 nt, in a sense:antisense

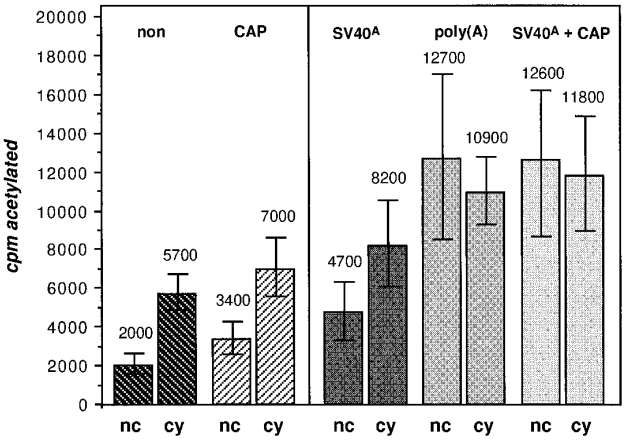

FIG. 2. Quantitative summary of CAT activities after compartmented injection of nonmodified, *in vitro* transcribed, *in vitro* capped and *in vitro* polyadenylated CAT RNA. 2-4 $\times$ 10^4 RNA molecules per cell were injected, either into nucleus (nc) or into cytoplasm (cy), and 150 COS 7 cells were injected per experiment. The cpm of monoacetylated forms of chloramphenicol were evaluated. The data show means+/− linear deviation (Students' t-test). Error bars give the linear deviation of the arithmetic mean values. Following RNAs were used: non modified RNA (non), capped RNA (CAP), *in vitro* polyadenylated RNA (poly(A) and SV40 poly(A) signal containing RNA ($SV40^A$).

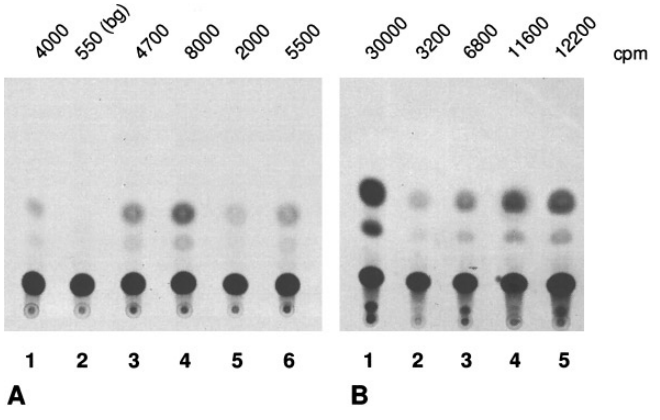

FIG. 1. CAT activities after compartmented injection of *in vitro* transcribed CAT RNA with or without the SV40 polyadenylation signal, with or without CAP. **A,** control injection with 20–40 molecules of pBLCAT2-DNA into 150 cells (lane 1); Background (bg) CAT activity of non injected cells (lane 2); injection of RNA with the SV40 polyadenylation signal into the nucleus (lane 3) or into the cytoplasm (lane 4); injection of RNA without the SV40 polyadenylation signal into the nucleus (lane 5) or into the cytoplasm (lane 6). **B,** CAT activity of 40 μg total protein of a stably transfected cell line (control, lane 1); injection of capped RNA without the SV40 polyadenylation signal into the nucleus (lane 2) or into the cytoplasm (lane 3); injection of capped RNA with the SV40 polyadenylation signal into the nucleus (lane 4) or into the cytoplasm (lane 5).

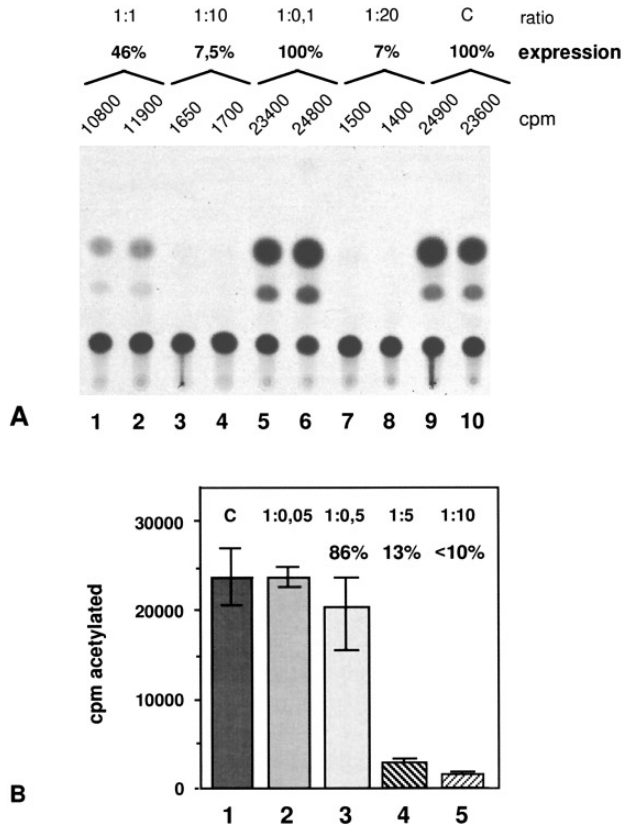

FIG. 3. CAT activity after nuclear injection of sense- and antisense CAT expression vectors. **A.** Injected expression vectors for sense RNA (pBLCAT2) and antisense RNA without the SV40 polyadenylation signal (800-Pr-a) in a molar ratio of 1:1 (lanes 1–2), 1:10 (lanes 3–4), 1: 0.1 (lanes 5–6) and 1: 20 (lanes 7–8). In lanes 9–10, 200-400 molecules/cell pBLCAT2 were injected as a control. **B.** Injected expression vectors for sense RNA (pBLCAT2) and antisense RNA containing the SV40 polyadenylation signal (1600-Pr-a) in a molar ratio of 1:0.05 (2), 1:0.5 (3), 1:5 (4) and 1:10 (5). 200-400 molecules/cell pBLCAT2-DNA were injected as a control (1).

ratio of 1:0.5 reduced CAT activity to 5% of the control. Using a sense:antisense ratio of 1:1 CAT activity was reduced to 3% of the control. In contrast coinjection of the same molecules at the same ratios into the cytoplasm reduced CAT activity to 35 %, or to 28% of the control, respectively (Fig.4).

To analyse the effect of antisense RNA-capping, *in vitro* capped CAT antisense RNA without the SV40 poly(A) signal was coinjected into the nuclei. *In vitro* transcribed capped sense RNA molecules were used as controls. Nuclear coinjection with unmodified antisense RNA in a sense:antisense ratio of and 1:1 reduced CAT activity to less than 10% of the control (Fig.5A). To determine the role of RNA polyadenylation on antisense RNA mediated reduction of gene expression a construct for *in vitro* transcription of antisense CAT RNA containing the polyadenylation signal of SV40 within an 800 bp segment was used. Coinjection of this CAT antisense RNA with *in vitro*-transcribed CAT sense RNA including the SV40 polyadenylation signal at a ratio of 1:0.5 into the nuclei of the cells reduced CAT activity to 43% of the control. Using a 1:1 ratio, CAT activity was further reduced to 28 % (FIG. 5B). Coinjection of CAT sense RNA with the poly(A) signal together with in vitro polyadenylated antisense RNA without the SV40 polyadenylation signal at ratios of 1:0.5 and 1:1 reduced CAT actvities to similar levels (data not shown).

DISCUSSION

To identify the role of elements that are involved in gene expression the microinjection method that allows the transfer of exactly defined amounts of molecules into the nuclear and cytoplasmic compartment of single cells was used [29]. In dependence on the template and using different enzymatic reactions, it is possible to produce *in vitro* transcribed RNAs of different sequences and modifications. For this reason this is an appropriate method to examine involvement of RNA modifications in gene expression as well as in inhibition of gene expression by antisense molecules.

The presented data indicate that RNA without any modification can be translated in both nucleus and cytoplasm. RNA with one side-modification can be more

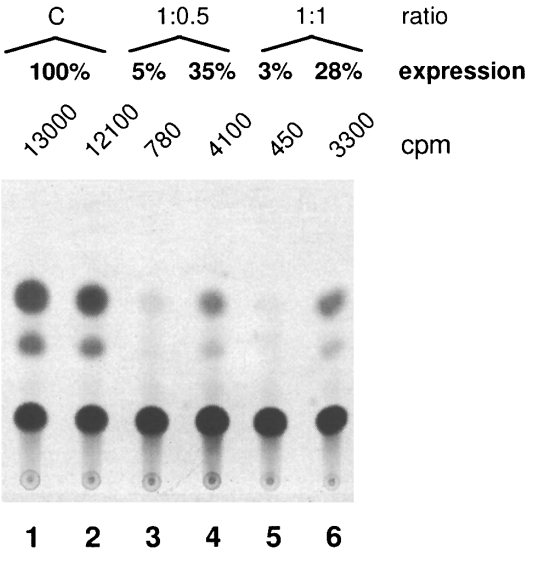

FIG. 4. CAT activities after coinjection of *in vitro* transcribed sense- and antisense-RNA: Sense-RNA with the polyadenylation signal of SV40, CAP and a size of 1600 nt coinjected with unmodified antisense-RNA with a size of 800 nt. Lane 1, $2–4 \times 10^4$ RNA molecules per cell, injected into the nucleus; lane 2, into the cytoplasm; lane 3, sense:antisense ratio of 1:0.5 nuclear injection; lane 4, 1:0.5 cytoplasmic injection; lane 5, 1:1 nuclear injection; lane 6, 1:1 cytoplasmic injection. Figures represent means+/− SD of 3 parallel experiments.

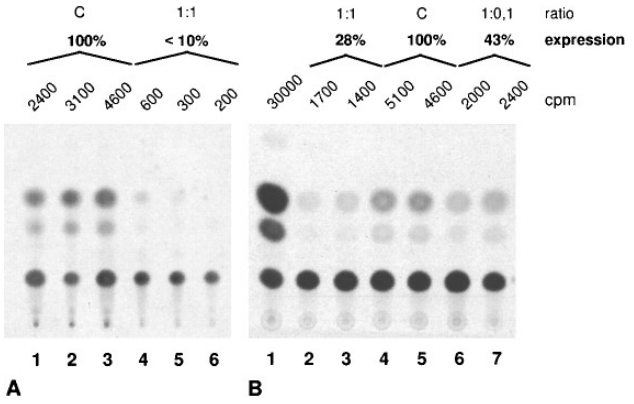

FIG. 5. CAT activities after nuclear injection of *in vitro* transcribed sense- and antisense-RNAs: Sense-RNA. **A:** With CAP and without polyadenylation signal of SV40 (size of 800 nt), antisense RNA with CAP and without polyadenylation signal of SV40 and a size of 800 nt. Lanes 1–3, injection of $2–4 \times 10^4$ RNA molecules per cell, lanes 4–6, coinjections of sense- and antisense RNA in a molar ratio of 1:1. **B:** Both with the polyadenylation signal of SV40 and a size of 1600 nt. Lane 1,. CAT-activity of 40 μg of total protein of a stably transfected cell line (A1, unpubl. results); lanes 4–5, $2–4 \times 10^4$ RNA molecules per cell, nuclear injection; lanes 2–3, coinjections of sense- and antisense RNA in a molar ratio of 1:1; lanes 6–7, same as lanes 5–6, but at a molar ratio of 1:0.1.

efficiently expressed. Poly(A) tail and CAP increase the level of CAT expression up to 1,5-2-fold. With non modified as well as with single modified RNAs the CAT activity is 2-3-fold higher after cytoplasmic injection compared with nucleus injection. One reason for the discrepancy in the levels of expression might be that 2-fold less molecules are degraded when injected directly into cytoplasm avoiding the way from nucleus to cytoplasm. Other reasons for the lower CAT activity after nucleus injection might be either the nuclear membrane which is known to act as a diffusion barrier or the lack of CAP and poly(A) tail [31]. The increased expression of single modified RNA could be probably explained with the protecting role of the CAP and/or polyadenylation against exonucleolytic degradation and the resulting stabilizing effect that is widely discussed [4-6]. Capping and polyadenylation serves as an inititiation signal for protein biosynthesis [7, 12-14]. If mRNA modifications additionally support the transport of RNAs from nucleus to the cytoplasm, this could lead to a shorter time until translation, resulting in fewer degraded molecules [32]. In the described experiments double modified RNAs show the highest level of expression. In contrast to all previous performed microinjection experiments with non modified or single modified RNAs there was no more difference between the expression after microinjection into nucleus or cytoplasm indicating that CAP and poly(A) tail probably cooperate synergistically in translational control. This is supported by investigations in other cell systems [10, 33]. To rule out an increasing effect of additional SV40-containing 800 nt on CAT expression, CAT RNA molecules which only contain the CAT coding region were *in vitro* polyadenylated. The same increase of expression after injection of these *in vitro* polyadenylated molecules compared to SV40 signal containing RNA could be detected assuming that not the additional 800 nt, but the poly(A) tail is responsible for the CAT activity-increasing effect. It is suggested that there probably exists a mechanism that recognizes the poly(A) signal in the cytoplasm and a poly(A) tail can be added in this compartment. This is supported by investigations of *Xenopus laevis* oocyte maturation and embryogenesis, where a poly(A) prolongation could be shown in the cytoplasm resulting in enhanced translational activation [16].

To analyse inhibition of gene expression and influence of antisense RNA modifcations on the inhibitory mechanism microinjection method was used again. Directly used *in vitro* transcribed non modified or in vitro capped and /or in vitro polyadenylated antisense CAT RNA were microinjected into nucleus or cytoplasm of COS7 cells. With antisense vectors with and without polyadenylation signal of SV40 a significant reduction of gene expression with large antisense RNA molecules could be shown. Even when CAT expression was driven by the strong SV40 promoter with the origin of replication CAT activity was reduced significantly (data not shown). For this reason it can be concluded that full-length antisense RNA molecules of the CAT reporter gene function as powerful inhibitors in a transient expression system in mammalian cells.

To differentiate between the influence of CAP and/or poly(A) tail to an expression reducing effect *in vitro* transcribed sense- and antisense RNA molecules that were *in vitro* capped, without CAP, with or without poly adenylation signal of SV40 or *in vitro* polyadenylated RNAs, respectively were injected. With all differently modified antisense RNAs gene expression was efficiently reduced, either after injection into the nucleus or after injection into the cytoplasm. No difference in reduction efficiency caused by CAP or poly(A) tail could be detected, but a smaller inhibitory effect was seen when using RNA molecules with a length of 1600 nt than after using the coding region with 800 nt. This might be caused by a more complex secondary structure of the longer antisense RNA.

Based on the assumption that the secondary structures of the molecules play an important role for hybridization between sense and antisense RNAs intramolecular folding structures of the CAT mRNA and its corresponding antisense RNA were calculated in order to get information about the distribution of stem/loop positions of each molecule. FIG. 6A shows the intramolecular folding of the CAT mRNA (s) and the corresponding fullength antisense RNA (a). In the CAT

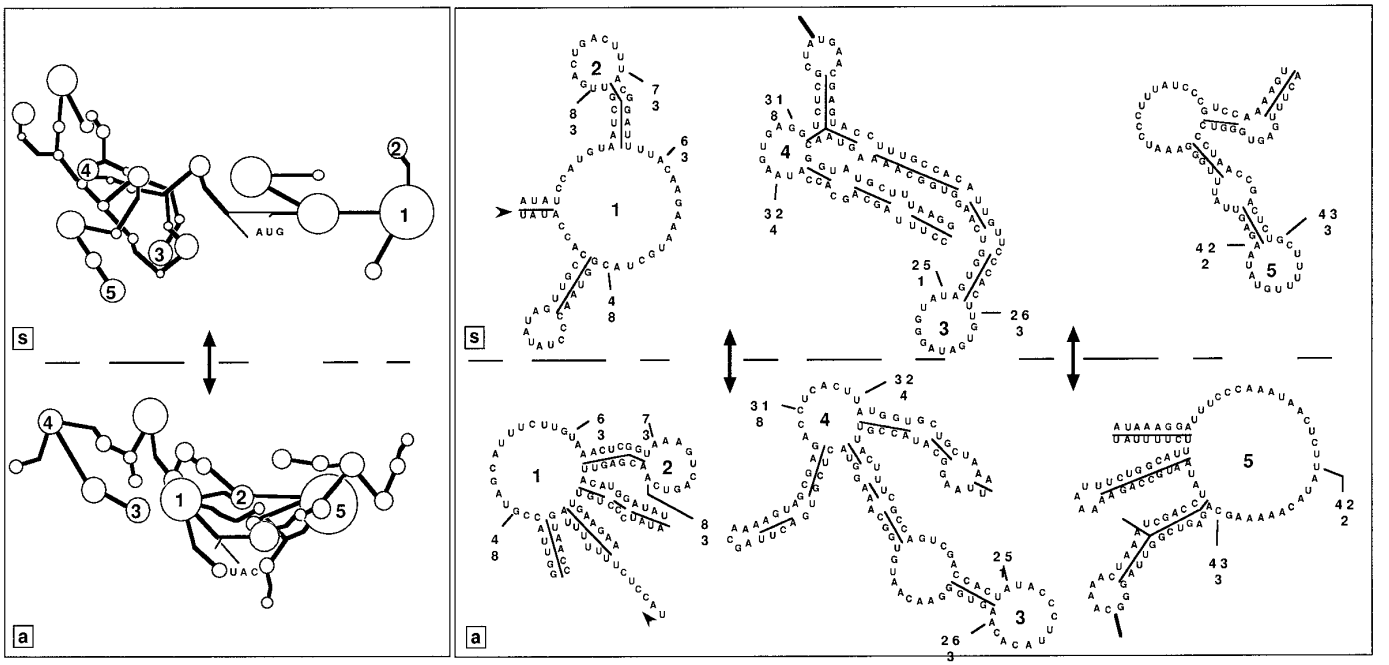

FIG. 6. **A:** Secondary structure of the CAT sense- and antisense RNA; the free energy was calculatedby the GENMON computer folding program [25]. The numbered loops show single stranded segments, at corresponding positions relative to the AUG start codon, that are found within the CAT sense-RNA and CAT antisense-RNA. s: sense RNA; the free energy was calculated as ΔG −109,7 Kcal/mol. a: antisense RNA; the free energy was calculated as ΔG −121,6 Kcal/mol. **B:** Sequences of the loop structures of sense RNA (s, shown on in the upper part) and the corresponding antisense segments (a, shown on the lower part). The figure shows single stranded regions as potential candidate sequences of hybridization: loop 1, nts 48–64, loop 2; nts 73–83; loop 3, nts 251–263; loop 4, nts 318–326; loop 5, nts 422–433.

sense and in the CAT antisense RNA several large single stranded sequences exist that can possibly interact with each other. The numbered loops show single stranded segments that were found in identical positions within the mRNA and its antisense RNA relative to the AUG start codon. It was shown that such loop structures are potential regions for double strand formation [20, 34, 35]. In FIG. 6B possible hybrid formation of CAT mRNA and the corresponding antisense RNA are suggested. It is shown that corresponding sequences, identical in the nucleotide positions may hybridize as a start point for a complete sense-antisense RNA hybrid complex. A possible function of these double stranded RNAs could result in a catalysed degradation of the CAT messenger RNA via nucleases [36, 37].

Taken together it can be concluded, that capping and polyadenylation of mRNA leads to an increase in gene expression but capping and polyadenylation of antisense RNA can not increase inhibition of gene expression. Antisense RNA-mediated inhibition depends on the length of the sequence, the primary structure and the resulting secondary structure and the interaction of the sense- and antisense-RNAs with other macromolecules. For efficient reduction of gene expression it could be assumed that mRNA and the corresponding antisense RNA should have several single stranded regions for hybrid formation.

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