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Excess antisense RNA from infectious recombinant SV40 fails to inhibit expression of a transfected, interferon-inducible gene

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SV40-based infectious virus constructs were used to produce a high copy number of full-length antisense RNA in essentially every cell in a population. Chloramphenicol acetyltransferase (CAT) cDNA was placed in either the sense or antisense orientation relative to the SV40 early promoter in helper-free recombinant virus. RNA synthesised at high levels from the antisense virus was without effect on the expression of a stably-transfected CAT mini-gene controlled by an interferon-inducible promoter in monkey CV1 and large T antigen-expressing tsCOS cells. In double infection experiments the antisense RNA was similarly without effect on expression from CAT cDNA placed in the sense orientation in a second virus vector. No activation of the ppp(A2′p)$_n$A($n \geq 2$) system was observed after interferon treatment in either type of experiment. There was no evidence, therefore, for the formation of double-stranded (ds)RNA. It can be concluded that a large excess of a full-length antisense RNA is not necessarily sufficient to cause inhibition of gene expression even when interferon treatment is used to enhance any effect of dsRNA.

The ability of complementary strands of RNA to form a stable duplex provides a potentially powerful method of regulating RNA function. Complementary (antisense) RNA can inhibit specific gene expression in both eukaryotes (e.g. [1, 2]) and prokaryotes (e.g. [3, 4]). This use of antisense RNA as a molecular tool for genetic analysis has been extensively reviewed [5–7].

Antisense RNA has been introduced into intact cells in a variety of ways. The precise mechanisms of the observed inhibition of gene expression are not, however, fully understood. In mammalian cells successful approaches have used plasmid constructs stably transfected into chromosomal DNA to synthesise the antisense RNA [2, 8–11]. Antisense oligodeoxynucleotides can also inhibit viral or cellular gene expression (e.g. [12, 13]). No single approach, however, has been consistently effective in mammalian cells. In an optimal system every cell in the population should contain antisense transcripts in considerable excess over the target sense RNA. It is likely that a strong promoter will be required and, ideally, the level of the antisense RNA should be controllable. Here, a system using infectious (but defective) recombinant SV40 constructs was developed to fulfil these conditions. High and regulatable levels of an antisense RNA complementary to chloramphenicol acetyltransferase (CAT) mRNA were obtained from the recombinant SV40 by supplying large T antigen (TAg) in *trans* in tsCOS cells. The effect of this antisense RNA was assayed on the expression of a stably-transfected, interferon-inducible CAT mini-gene construct. The approach was also used to ask whether in the intact cell the sense and antisense RNAs form double-stranded RNA (dsRNA) capable of activating the interferon-modulated ppp(A2′p)$_n$A ($n \geq 2$) system [14, 15]. This was with a view to determining whether interferon could be used to enhance any inhibitory effect of antisense RNA through the localised activation [16–18] of this system or the similarly modulated dsRNA-dependent protein kinase system [14, 15].

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Abbreviations. ppp(A2′p)$_n$A, (for $n = 2$), 5′-triphosphoadenylyl-(2′-5′)adenylyl(2′-5′)adenosine; CAT, chloramphenicol acetyltransferase; dsRNA, double-stranded RNA; p.i., post-infection; SV40, simian virus 40; TAg, large T antigen.

Enzyme. Chloramphenicol acetyltransferase (EC 2.3.1.28).

METHODS

Growth of cells: interferon treatment and virus infection

Monkey CV1, CV1-P (from Dr P. Berg, Stanford University, CA, USA) COS [19] and tsCOS [20] cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum. The tsCOS cells were maintained at 37 °C but shifted to 32 °C in 10% CO_2 for 14–24 h before interferon treatment or virus infection. Wellferon, a highly purified mixture of human α-interferons (> 10^8 IU/mg protein [21]), was generously supplied by Dr K. Fantes of Wellcome Research Laboratories. Virus adsorption (90 min at 32 °C) and growth was in medium containing 2% foetal calf serum. Zero time was taken as the time of addition of the virus.

Transfection procedures

Cells were transfected with viral or plasmid DNA by a calcium phosphate procedure [22]. Cells were seeded at 10^6/90-mm-diameter dish and incubated overnight. Plasmid DNA (20 μg) or viral DNA (up to 5 μg mixed with carrier salmon sperm DNA from Sigma at 50 μg/ml) was added as a calcium phosphate precipitate. After 8–16 h the cells were treated with glycerol (10% by vol. [22]). Cells transfected with viral DNA were maintained in medium containing 2% foetal calf serum. CV1 and tsCOS cells were stably transfected with an interferon-inducible CAT gene construct (p6-16cat, Fig. 2) [23] by co-transfection to hygromycin resistance using a molar

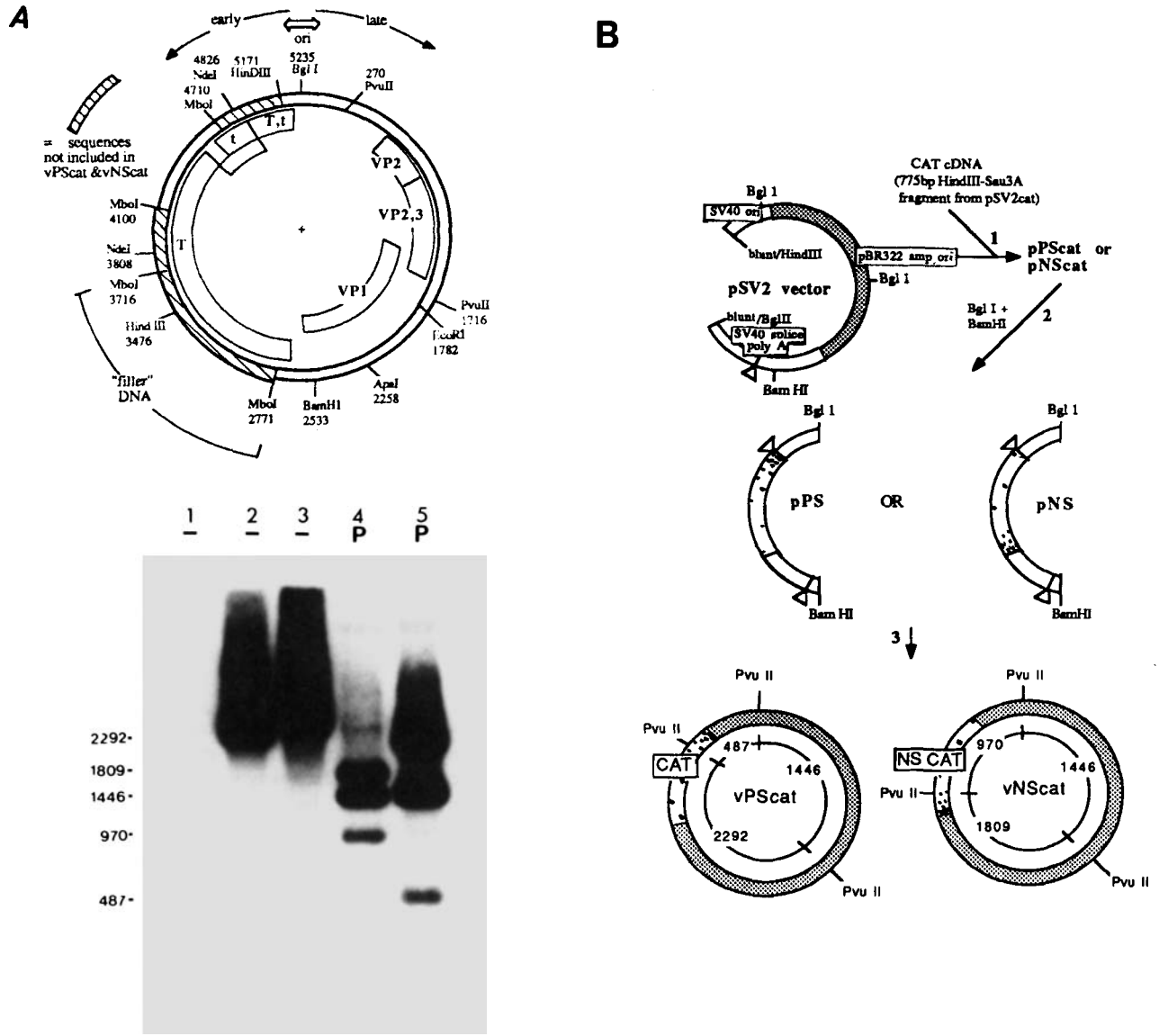

Fig. 1. *Construction of the viral vectors vPScat and vNScat and characterisation of the DNA in virus stocks.* (A) Map of SV40 DNA showing the location of restriction enzyme sites relevant to the construction of the plasmid and viral vectors. The numbering of the nucleotide sequence of the DNA begins with the unique *Bgl*I site at the origin [46]. The directions of early and late transcription are indicated and the position of the replication origin is shown together with the protein-coding regions. The position of the 'filler' DNA is also indicated. (B) Vector construction. The pSV2 vector was obtained by *Hin*dIII and *Bgl*II digestion of the plasmid pSV2*β*-globin. The regions derived from SV40 are shown unshaded, those from pBR322 are shaded. Step 1: a CAT cDNA with blunt ends isolated from pSV2cat (Methods) was ligated to end-filled, phosphatase-treated pSV2 DNA and introduced into *Escherichia coli* HB101 by transformation, resulting in plasmids designated pPScat and pNScat, for proper sense and antisense respectively. Step 2: early region DNA containing the CAT insert was isolated from pPScat and pNScat after digestion with *Bgl*I and *Bam*HI. The two fragments are shown schematically with the insert in either orientation and triangles representing the positions of deletions with respect to the wild-type SV40 early region. Step 3: wild-type SV40 DNA was cut with *Bgl*I, *Bam*HI and *Nde*I and the *Bgl*I – *Bam*HI fragment from the late region was ligated to the early region DNA from pPScat and pNScat, resulting in the viral vectors vPScat and vNScat. (C) Chracterisation of the viral construct DNA. DNA from COS cells infected with vNScat (lanes 2 and 4), vPScat (lanes 3 and 5) or mock-infected (lane 1) was extracted by Hirt lysis (Methods) four days after infection with primary virus stocks harvested eight days after transfection of COS cells with the construct DNA. The viral DNA was either digested with *Pvu*II (P) or left intact (–) and analysed by electrophoresis through an agarose gel, transfer to a nylon membrane and hybridisation with a nick-translated SV40 DNA probe. The positions of the *Pvu*II sites and the sizes of the expected DNA fragments are indicated in B

ratio of p6-16cat to pY3 [24] of 10:1. Stable transfectants were maintained in 100 μg/ml hygromycin B throughout.

Virus growth

Recombinant SV40 stocks were prepared from COS cells transfected with viral DNA. Primary virus was harvested when a cytopathic effect developed, usually after eight days. Phosphate-buffered saline (0.1 vol.) was added and the culture (cells plus medium) put through three freeze (−20 °C)/thaw cycles prior to sonication for 10 min in a Decon FS100 ultrasonic bath and storage at −20 °C. Secondary and tertiary stocks were prepared by infecting subconfluent COS cells with 0.1 vol. of the appropriate stock and harvesting as above

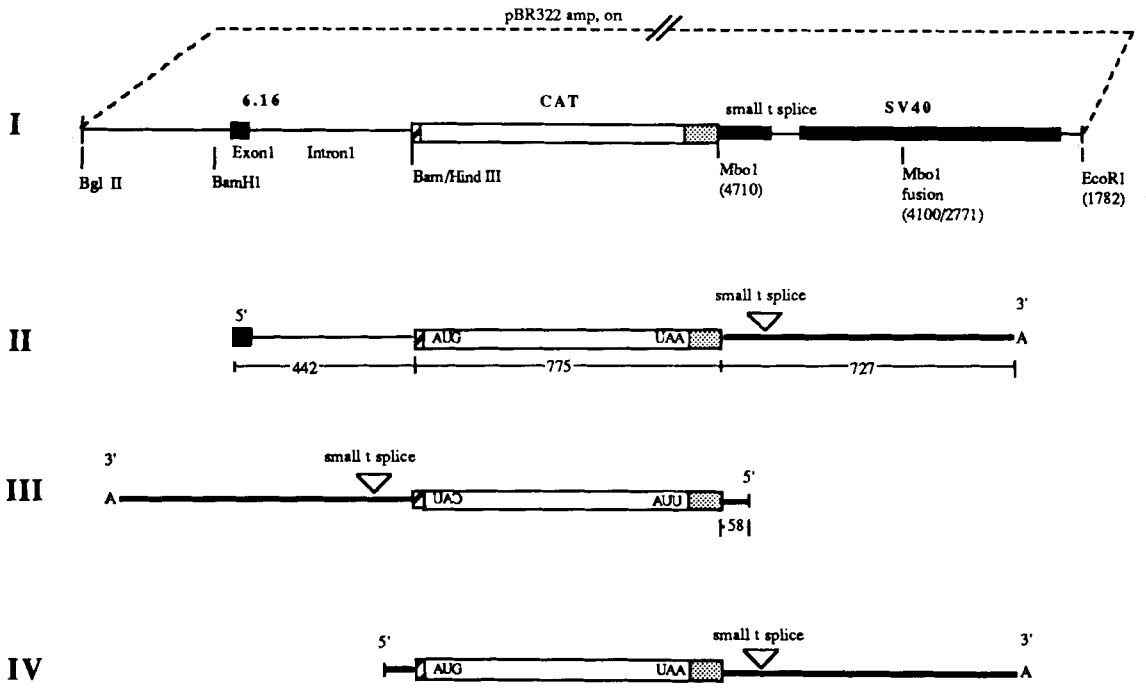

Fig. 2. *Structure of p6-16cat DNA, 6-16cat mRNA, vNScat antisense RNA and vPScat sense RNA.* (I) Schematic representation of the p6-16cat plasmid, with the DNA from the 5′ end of the 6-16 gene, the CAT cDNA and SV40 3′ end sequences drawn to scale. The positions of the restriction enzyme sites used in the construction of the plasmid are numbered with respect to wild-type SV40 DNA (Fig. 1A). (II) Predicted 6-16cat mRNA from p6-16cat DNA. The RNA is shown starting at the normal site for 6-16 and containing a portion of the first 6-16 intron followed by the CAT sequences. At the 3′ end it is assumed that the small t antigen intron has been correctly spliced and that termination and polyadenylation have occurred normally within the SV40 DNA. The observed size of the major (longer) transcript (Fig. 5B) is consistent with the structure shown. (III) The predicted antisense RNA from vNScat is shown in the 3′ to 5′ direction, to illustrate the region of complementarity. The 58-nucleotide 5′ sequence is derived from the SV40 early promoter and is followed by the antisense CAT sequence and sequences from the 3′ end of the SV40 early region spliced and polyadenylated as for II. (IV) Predicted sense CAT mRNA from vPScat. This is equivalent to the antisense RNA from vNScat (III), apart from the orientation of the CAT inset

after four days. Titres were measured by plaque assay on monolayers of CV-1 or CV1-P cells co-infected with helper virus tsB201 defective in the late region [25]. Viral DNA was isolated by the method of Hirt [26] and analysed by Southern transfer [27].

Construction of plasmid and viral vectors

The constructions are illustrated schematically in Fig. 1. Standard techniques were used for the treatment of DNA with restriction endonucleases, calf intestinal phosphatase, T4 DNA polymerase and the Klenow fragment of DNA polymerase I [28]. Plasmid DNA was prepared by alkaline lysis [29] and ultracentrifugation through caesium chloride.

Plasmid constructs. A 775-bp CAT mini-gene containing 29 bp upstream of the initiator ATG codon, the coding sequence and 86 bp downstream of the translation termination codon was obtained by digestion of pSV2cat [30] with *Hin*dIII and *Sau*3A. The ends of the fragment were filled by treatment with the Klenow fragment of DNA polymerase I and ligated into the vector pSV2 [31], formed by digestion of pSV2β-globin [31] with *Hin*dIII and *Bgl*II (Fig. 1B) [1]. The resulting plasmids containing CAT in either orientation were designated pPScat and pNScat for proper sense and antisense respectively.

Viral vectors. The above plasmids formed the basis for the recombinant SV40 viral vectors vPScat and vNScat, which were constructed by replacing pBR322 sequences with the late region of SV40 as described in Fig. 1. In addition, recombinant SV40 viruses containing 'filler' DNA to increase their size were constructed for sense and antisense CAT and β-globin by inserting part (Fig. 1A) of the open reading frame of the TAg (Fig. 1A) previously deleted in the construction of the pSV2 vector. For example, in the case of the globin constructs the *Sau*3A fragment 3716–2771 in wild-type SV40 was cloned into the unique *Bgl*II site of pSV2β-globin. The resulting plasmid was digested with *Hin*dIII and a β-globin cDNA insert (derived from pSV2β-globin) inserted in either orientation. The resulting sense and antisense plasmids pPSβ-globin and pNSβ-globin were converted into viral DNA using the procedure outlined above. The plasmid p6-16cat (Fig. 2, I) [23], kindly provided by Dr A. C. G. Porter, was constructed by ligating an end-filled *Bgl*II/*Sma*I fragment containing 1040 bp of 6–16 DNA into pSV0cat, linearised at the unique *Hin*dIII site [30].

Preparation of RNA from CV1 and tsCOS cells

Cells were harvested and lysed in NP40 buffer (0.65%, by vol., NP40, 0.15 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris/HCl pH 7.9) and nuclei pelleted by centrifugation. RNA was extracted from the cytoplasmic fraction with 4 M guanidinium thiocyanate, 0.5% sodium *N*-lauryl sarcosine, 25 mM sodium citrate, pH 7.0. The RNA was recovered by centrifugation through a 5.7 M CsCl gradient, precipitated with ethanol and stored in 10 mM Tris/HCl pH 7.2, 1 mM EDTA at −80°C.

Northern analysis of RNA

Aliquots of RNA (5–20 μg) were glyoxalated, fractionated through 1–1.4% agarose gels and transferred to nylon membranes (Pall Corp.) [32]. These were baked at 80°C for 2 h, pre-hybridised for 8–24 h and hybridised with ^{32}P-labelled single-stranded M13 or double-stranded DNA probes in 50% formamide, 0.9 M NaCl, 5 mM EDTA, 50 mM sodium phosphate pH 7.4, 5 × Denhardt's solution, 0.2% SDS and 100 μg/ml denatured salmon sperm DNA for 18–36 h at 42°C. The 775-bp CAT mini-gene labelled to $>10^8$ cpm/μg by the random priming technique [33] provided a double-stranded probe. The same CAT fragment was cloned into the *Sma*I site of the double-stranded DNA replicative form of the single-stranded DNA bacterophage M13mp18 [34] and strand-specific probes were prepared by labelling purified single-stranded recombinant M13 DNA [35]. For slot blot analysis the RNA samples were denatured in 6 × SSC (SSC = 0.15 M NaCl, 0.15 M sodium citrate, pH 7.5), 7.4% formaldehyde prior to transfer to nylon membranes and hybridisation as above. The membranes were washed for 1 h in 2 × SSC, 0.1% SDS at 60°C, then for 1 h in 0.2 × SSC, 0.1% SDS at 60°C. Kodak XAR-5 film was used for autoradiography. Levels of RNA were compared by scanning the appropriate bands in the linear range of response on autoradiographs. Filters were re-hybridised with different probes after removing the bound probe by incubation in 10 mM Tris/HCl, pH 7.5, 1 mM EDTA in a shaking water bath at 75°C for 30 min.

Assay of CAT activity

Cells were pelleted and lysed in NP40 buffer as described above for the preparation of RNA. A tenth of the volume of each extract was assayed for CAT activity by a rapid nonchromatographic method [36]. Where necessary, lysates were diluted to ensure that the assay was in the linear range. In all experiments the interferon-induced level of activity in the CAT assay was at least 2000 cpm above the background (zero-time) value. The protein content of each extract was measured spectrophotometrically using the Bio-Rad dye reagent [37].

RESULTS

Construction and characterisation of recombinant SV40 vectors

Infective SV40 constructs were designed (Fig. 1, Methods) to introduce antisense RNA into every cell in a given population. Propagation in COS cells [19] resulted in helper-free virus stocks in which there was no detectable rearrangement of the viral DNA (Fig. 1C). As expected for recombinant viruses defective in the early region, infection of monkey CV1 cells, even at high multiplicity, did not cause any cytopathic effect over a period of at least 10 days.

Bacterial CAT and rabbit β-globin cDNAs were inserted into the vectors in both possible orientations yielding DNA of approximately 80% of wild-type genome size (Fig. 1). Titres of between 5×10^6 and 1×10^7 pfu/ml were obtained for viruses containing inserts in either the sense (vPScat, vPSβ-globin) or antisense (vNScat, vNSβ-globin) orientations. This is low compared with wild-type SV40, but is in the upper range of the values expected for a recombinant SV40 system (Dr Y. Gluzman, Cold Spring Harbor Laboratories, NY, USA, personal communication). Addition of 'filler' DNA (see Methods) to increase the size to approximately that of wild-type virus DNA did not increase the titre. The titres were, however, sufficiently high to allow infection of every cell with up to 50 pfu of virus in small-scale experiments.

A transcript corresponding to the entire CAT protein-coding sequence plus 29 nucleotides of 5′- and 86 nucleotides of 3′-flanking sequence in the antisense orientation would be expected to by synthesised from the SV40 early promoter in the antisense virus vNScat (Fig. 2, III). This antisense RNA was targeted against a CAT mRNA (Fig. 2, II) produced from an interferon-inducible CAT cDNA construct (p6-16cat, Fig. 2, I) [23] stably transfected into the genomic DNA of CV1 and TAg-expressing tsCOS cells. Expression of CAT from this construct is driven by transcriptional control sequences from the 5′ end of the human interferon-inducible gene 6-16 [23, 27] permitting a high degree of induction of gene expression from a low basal level in response to interferon. Together these components provided a model system to investigate the feasibility of inhibiting the expression of an endogenous interferon-inducible gene with antisense RNA.

Interferon-inducible expression of CAT in CV1 and tsCOS cells stably transfected with an interferon-inducible CAT plasmid

The structure of the p6-16cat plasmid, stably-transfected into CV1 and tsCOS cells (Methods), is schematically illustrated together with the predicted 6-16cat mRNA in Fig. 2, I and II. Individual clones of transfectants were expanded into cell lines in which the structure and copy number of the integrated 6-16cat DNA should be the same in each cell. Several of these clones were analysed for the kinetics and dose response of induction of CAT expression in response to IFN treatment. Similar results were obtained for the CV1 and tsCOS clones at both 32°C and 37°C. CAT activity was inducible up to at least 50-fold from a level close to background. A small increase was observed with 1 IU/ml. A nearly maximal response was obtained at 1000 IU/ml, the concentration chosen for use in the antisense experiments. At this concentration CAT activity in, for example, tsCOS.6-16cat clone 1 cells increased for at least 20 h after treatment with interferon (Fig. 3).

Analysis of the sense 6-16cat transcripts in the stably transfected CV1 and tsCOS cell clones revealed low levels of two interferon-inducible RNAs (for example, Fig. 5B). One was of the expected size (Fig. 2, II), the other smaller, perhaps reflecting the use of a cryptic splice site. Induction of the sense 6-16cat RNAs paralleled CAT activity in the CV1.6-16cat and tsCOS.6-16cat cells. Both transcripts were found to be relatively stable (half-life > 8 h) in the presence of actinomycin D in tsCOS.6-16cat cells (data not shown).

Accumulation of antisense RNA from vNScat in tsCOS cells

Three strategies were used to maximise the level of antisense RNA synthesised from vNScat relative to the inducible sense CAT mRNA, in order to increase the probability of success in inhibiting CAT expression. Firstly, as early gene expression from the recombinant viruses was at least 10-fold higher in monkey CV1 cells than in several human and mouse cell lines (data not shown), high multiplicity infection of monkey cells was used. Secondly, TAg was supplied in *trans* to allow the viral DNA to replicate, increasing the number of templates for transcription of antisense RNA. Thirdly, the experiments were designed to allow high levels of antisense

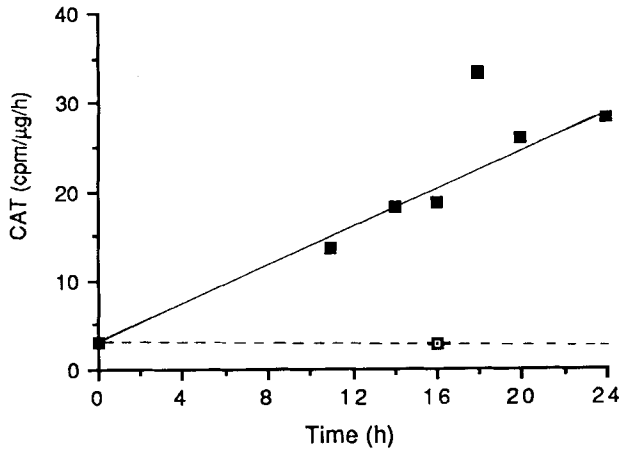

Fig. 3. *Kinetics of interferon-inducible CAT expression in stably transfected cells.* tsCOS cells stably transfected with p6-16cat (tsCOS.6-16cat, clone 1) were incubated at 37°C with (——) or without (– – –) 1000 IU/ml interferon for the times indicated and assayed for CAT activity (Methods). Specific activities are given (ordinate); the observed value for the induced enzyme was >2000 cpm above background

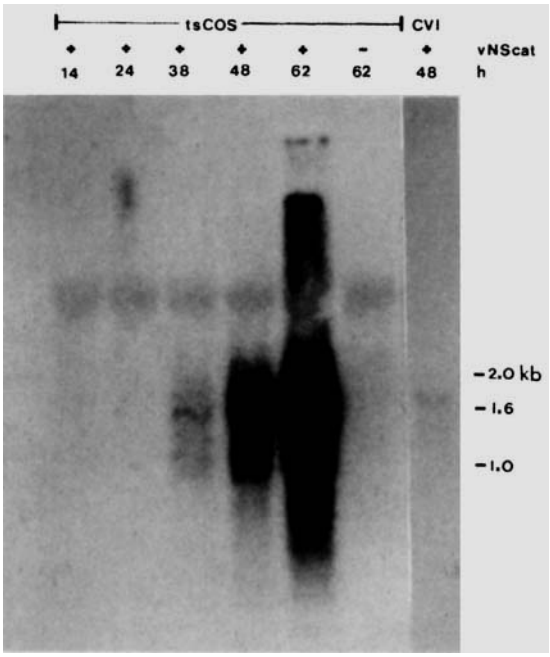

Fig. 4. *Accumulation of antisense RNA from vNScat in tsCOS.6-16cat cells: comparison with CV1 cells.* tsCOS.6-16cat clone 1 and CV1 cells were either infected with vNScat (20 pfu/cell) (+), or mock-infected (−), incubated at 32°C (tsCOS) or 37°C (CV1), harvested at the indicated times p.i., frozen and lysed in NP40 buffer. The cytoplasmic RNA was extracted in guanidinium thiocyanate and centrifuged through caesium chloride gradients (Methods). RNA samples (11 μg CV1, 15 μg tsCOS) were electrophoresed in a 1.4% (mass/vol.) agarose gel, blotted onto nylon membranes and hybridised to a double-stranded ^{32}P-labelled M13cat DNA probe. Size markers (in kb) are indicated to the right

RNA to accumulate before induction of the sense 6-16cat RNA by interferon.

Individual COS cells express variable amounts of TAg [19]. In contrast, for both tsCOS and tsCOS.6-16cat clone 1 cells immunological staining showed a high and uniform level of TAg expression on shift down to 32°C (data not presented). Accordingly uniform expression of antisense RNA from vNScat would be expected in these cells. Antisense RNA from vNScat of the predicted size (Fig. 2, III) accumulated to very high levels, with a strong signal 48 h post-infection (p.i.) and a further increase up to 62 h p.i. (Fig. 4). By comparison, CV1 cells infected with vNScat accumulate substantially lower levels of antisense RNA (Fig. 4). Allowing for the fact that the antisense RNA accumulates more rapidly at 37°C in the CV1 cells than at 32°C in the tsCOS cells, densitometric scanning of autoradiographs indicated that there was an approximately 40-fold differential. Similar results were obtained with a second independent clone of tsCOS.6-16cat cells.

It would be disadvantageous if synthesis of RNA from vNScat were decreased by interferon treatment. Interferon, however, exerts its antiviral action on SV40 before the onset of early transcription, possibly at the uncoating stage and is only antiviral if added prior to virus uptake [38, 39]. As expected, control experiments showed that treatment with interferon 2–3 days p.i., as in the present antisense experiments in CV1 and tsCOS cells, had no effect on early gene expression from vNScat (data not shown).

Antisense RNA from vNScat fails to inhibit inducible CAT expression

Lack of inhibition of interferon-inducible CAT in stably-transfected tsCOS and CV1 cells. A series of antisense experiments in clones of tsCOS.6-16cat and CV1.6-16cat cells showed a uniform lack of inhibition of interferon-inducible CAT activity by vNScat (e.g. Table 1). The experimental strategy was based on the kinetics of accumulation of antisense RNA and induction of CAT expression by interferon described above. The interferon treatment was kept relatively short to ensure that at the time of harvest new CAT protein was being synthesised in small amounts in the linear range of response in the presence of a large excess of preformed antisense RNA. The vNSβ-globin virus provided a control for possible non-specific effects caused by infection with recombinant virus (Table 1).

The lack of inhibition of CAT activity made it particularly important to analyse sense and antisense RNA extracted from the same cell lysates. A Northern blot of RNA from the tsCOS.6-16cat clone 1 cells, assayed for CAT activity in experiment 2 of Table 1, was probed first with double-stranded CAT DNA to detect both sense and antisense RNA, then stripped and re-hybridised with a strand-specific probe to CAT mRNA (Fig. 5, A and B respectively). It is clear that there is a large excess of antisense RNA over 6-16cat sense RNA. There was a similar lack of inhibition of CAT activity by lower levels of antisense RNA in CV1 cells (data not shown). In several experiments in tsCOS.6-16cat cells the excess of antisense RNA from vNScat to the induced sense RNA was estimated to be at least 300-fold. For example, in experiment 1 of Table 1, the excess was >1000-fold (Fig. 5C). In agreement with this, CAT expression from the analogous sense virus, vPScat, was approximately 350-fold higher than that from the stably transfected p6-16cat gene induced with IFN (Table 1, expt 1).

If dsRNA is formed in interferon-treated cells, activation of the ppp(A2′p)$_n$A system with cleavage of mRNA by the ppp(A2′p)$_n$A-dependent ribonuclease would be predicted (reviewed in [14, 15]). Strand-specific probing revealed no decrease in the size or abundance of 6-16cat mRNA in samples

Table 1. *Absence of an effect of antisense RNA from vNScat on expression of interferon-inducible CAT in tsCOS and CV1 cells*
Clones of tsCOS and CV1 cells stably transfected with p6-16cat were mock-infected or infected with vPScat, vNScat or vNSβ-globin at the multiplicities shown. In expt 1 the cells were incubated at 32 °C throughout. In expt 2 they were shifted from 32 °C to 40 °C 47 h p.i. Interferon treatment (1000 IU/ml) was at 58 h (expt 1), 62 h (expt 2) or 48 h (expt 3) p.i. Cells were harvested 72 h (expts 1 and 2) or 60 h (expt 3) p.i. Extracts were assayed and the specific activity of the CAT was calculated (Methods)

Expt	Cell line	Interferon	Virus			CAT activity
			vPScat	vNScat	vNSβ-globin	
			pfu/cell			cpm μg^{-1} h^{-1}
1.	tsCOS.6-16cat	–	–	–	–	5
	clone I	+	–	–	–	42
		+	5	–		17500
		+	–	20	–	49
2.	tsCOS.6-16cat	–	–	–	–	5
	clone II	+	–	–	–	28
		+	–	20	–	25
3.	CV1.6-16cat	–	–	–	–	2
	clone V	+	–	–	–	30
		+	–	–	30	29
		+	–	–	30	29

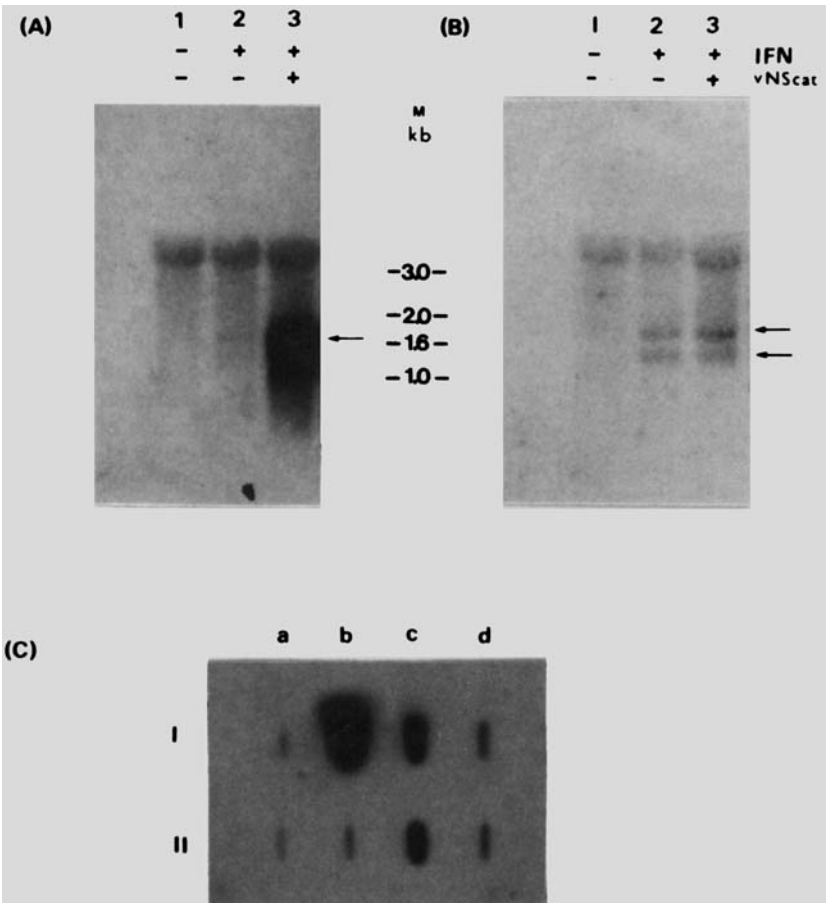

Fig. 5. *Analysis and quantification of sense and antisense RNA in tsCOS.6-16 cat cells infected with vNScat and treated with interferon.* tsCOS.6-16cat clone 1 cells were (1 and 2) mock-infected or (3) infected at 32 °C with vNScat (20 pfu/cell) shifted to 40 °C at 47 h p.i. and (2 and 3) treated with IFN (1000 IU/ml), at 62 h (expt 2, Table 1). All cells were harvested 72 h p.i. and cytoplasmic RNA was extracted as in Fig. 4. Samples (8 μg) were electrophoresed in a 1.4% (mass/vol.) agarose gel-transferred onto nylon membranes and hybridised (A) to a nick-translated double-stranded M13cat DNA probe and (B), after stripping, re-hybridised to a strand-specific ^{32}P-labelled M13 probe to CAT mRNA. The arrows show the position of the antisense (A) and sense (B) RNAs and size markers (in kb) are shown. The signal from the material migrating more slowly arose from spurious hybridisation with 28S rRNA. (C) Slot blot analysis of RNA prepared as above but from the same cells as were used in expt 1 in Table 1. RNA (10 μg) was from tsCOS.6-16cat clone 1 cells without (slots a I and II) or with (b II) interferon (1000 IU/ml) treatment from 58 h to 72 h. The remainder of the RNA samples were from cells infected with vNScat (20 pfu/cell) and treated with interferon (1000 IU/ml) from 58 h to 72 h: b I, 1 μg RNA; c I and II and d I and II, 0.1 and 0.01 μg RNA, respectively. Hybridisation was to a double-stranded ^{32}P-labelled M13 CAT probe (Methods)

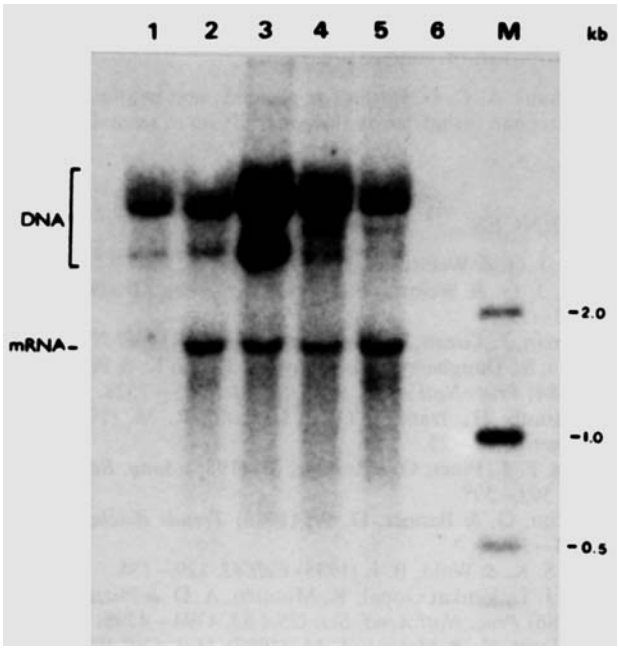

Fig. 6. *Analysis of CAT mRNA in cells co-infected with viruses coding sense and antisense RNA.* RNA was from CV1 cells (lane 6) or CV1 cells infected with (1) vNScat (1 pfu/cell); (2) vPScat (1 pfu/cell); (3) vPScat and vNScat (1 and 10 pfu/cell, respectively); (4) vPScat and vNScat (1 and 10 pfu/cell) respectively and treated with 1000 IU/ml interferon 21–48 h p.i.; (5) vPScat (1 pfu/cell) and treated with interferon 21–48 h p.i. All cells were harvested at 48 h and cytoplasmic RNA was prepared from NP40 lysates by phenol/chloroform extraction (Methods). Samples (20 μg) were denatured by glycoxalation, electrophoresed in a 1.8% (mass/vol.) agarose gel and transferred to a nylon membrane. Hybridisation was with a strand-specific ^{32}P-labelled M13 DNA probe to CAT mRNA (Methods). The positions of single-stranded DNA markers are indicated in kb to the right and viral DNA (Methods) and CAT mRNA to the left of the autoradiograph

Table 2. *Absence of an effect of antisense RNA from vNScat on expression of CAT from vPScat*
tsCOS cells incubated at 32 °C for 24 h were infected with vNScat or vPSβ-globin or mock-infected. After a further 48 h at 32 °C they were infected with vPScat and harvested 17 h later. Extracts were assayed and the specific activity of the CAT calculated (Methods). Averages from duplicate plates of cells are presented

Virus			CAT activity
vNScat	vPScat	vPSβ-globin	
pfu/cell			cpm μg^{-1} h^{-1}
–	10	–	595
60	10	–	645
–	10	60	750

in which there was an excess of antisense CAT RNA (Fig. 5B), nor was cleavage of rRNA to characteristic products, a highly sensitive assay for activation of the ppp(A2′p)$_n$A-dependent RNase [40], observed. No evidence was, therefore, obtained for dsRNA formation and activation of the ppp(A2′p)$_n$A system in these experiments.

It can be concluded that, in this system, a large excess of antisense RNA is not in itself sufficient to cause inhibition of CAT activity or, in the presence of interferon, degradation of RNA. In case this somewhat surprising result reflected a problem unique to the 6-16cat mRNA (Fig. 2, II), it was decided to use another approach in which a different sense CAT transcript (Fig. 2, IV) was synthesised from a second recombinant virus. In addition, double infection experiments have the advantage that it is unlikely that separate compartmentalisation of the sense and antisense transcripts within the cell could occur.

Double infection experiments. The sense virus vPScat expresses a single early region transcript (Fig. 2, IV) which is equivalent in structure to the antisense RNA from vNScat (Fig. 2, III), apart from the orientation of the CAT insert. Initial double infection experiments involved simultaneous co-infection of CV1 cells with vPScat and vNScat. Once again, RNA analysis did not reveal cleavage of sense mRNA (Fig. 6) or rRNA (data not shown) in the doubly-infected cells despite interferon treatment from 21 h to 48 h p.i. Similar results were obtained with or without interferon treatment. In this system the excess of antisense to sense RNA was 3–10-fold. A substantially greater excess was obtained by infecting tsCOS cells with vNScat and allowing replication of the viral DNA before infection with vPScat. Despite this no inhibition of the expression of CAT activity from the sense virus occurred (Table 2).

DISCUSSION

It is extremely probable that the recombinant SV40 system used here achieved the objective of introducing high levels of antisense RNA into virtually every cell in a population. Sufficient virus was used to infect every cell, the tsCOS cells expressed a uniformly high level of TAg and a massive overall excess of antisense to sense RNA expression was achieved. Despite this, the antisense CAT RNA was without effect on the expression of CAT either from an interferon-inducible construct (Table 1, Fig. 5) or a second recombinant virus (Table 2, Fig. 6). Comparison with other systems provides no obvious explanation for this. Several examples of inhibition of expression of stably-integrated or endogenous chromosomal genes by antisense RNA synthesised from stably-transfected plasmid DNA have been reported for mammalian cells [2, 8–11]. In one case no inhibitory effect was detectable until the copy number of the antisense plasmid was increased by gene amplification [8]. In another a 10–20-fold excess of antisense RNA had no detectable effect [42]. In contrast, however, a very small excess can be effective (e.g. [10]). Here the ratio of antisense to sense RNA observed in the tsCOS cells was routinely in excess of 300 (e.g. >1000, Fig. 5C). It seems unlikely that it was insufficiently high.

Nor is there any obvious reason why the antisense RNA from vNScat should be ineffective. The vNScat was constructed to yield RNA complementary to the entire CAT insert consisting of the protein coding sequence, 29 nucleotides of 5′ and 86 nucleotides of 3′ untranslated sequence (Fig. 2, III). Targeting to intron sequences and intron-exon boundaris (e.g. [10]) may be more effective than targeting to only mature (exon) sequence RNA. However, the TK gene, which has been used extensively in antisense experiments [1, 8], lacks introns;

antisense RNAs directed solely against mature mRNA sequences have also proved effective (e.g. [9]). The particular structure of the vNScat antisense RNA could be unsuitable for causing inhibition of gene expression. However, the viral vector vNScat is analogous to the SV40-based plasmid vector pNSlacZ with which a potent antisense effect was obtained in transient expression experiments [41]. This latter effect was not obviously potentiated by treatment with interferon (S.M.K., John Rubenstein, G.R.S. and I.M.K., unpublished data). Furthermore, an RNA very similar in structure to that made by the vNScat used here, but synthesised from a plasmid, effectively inhibited CAT activity in transient expression transfections in mouse cells [2]. Also microinjection of antisense CAT RNA synthesised *in vitro* inhibited the translation of sense CAT RNA in *Xenopus oocytes* [43]. There is no reason, therefore, to suppose that antisense CAT RNA should be in any way intrinsically unsuited to antisense experiments. Similarly, the fact that negative results were obtained here in both stable transfection and double infection experiments, involving different structures for the sense CAT mRNAs (Fig. 2, II and IV), suggests that it is unlikely that any peculiarity in the sense CAT transcripts was responsible for the lack of inhibition.

Although inhibition in the reports quoted above was not of an integrated CAT gene, the results imply that when both sense and antisense CAT RNAs are produced in the same cell hybridisation to form dsRNA occurs. No evidence for formation of dsRNA was, however, obtained here, despite the use of interferon to potentiate any inhibitory effect. Generalised activation of the interferon-mediated, dsRNA-dependent ppp(A2′p)$_n$A system did not occur, nor was nucleolytic cleavage of the sense CAT RNA observed (Fig. 5B). The latter would have been expected if the localised activation hypothesis for the ppp(A2′p)$_n$A system [14] is correct and if the partially dsRNA structures predicted for the hybridisation of the sense and antisense CAT RNAs (cf. II, III and IV, Fig. 2) were indeed formed in the intact cell. It seems unlikely that failure to activate the ppp(A2′p)$_n$A system reflects any intrinsic defect in that system. We know that the CV1 cells have a fully activatable ppp(A2′p)$_n$A system, induction of synthetase mRNA was monitored in some of the experiments and we have no reason to believe that the tsCOS cells (derived from CV1s) are in any way defective in this regard. Compartmentalisation separating the sense and antisense transcripts in the cell could have occurred, but seems extremely improbable in the double infection experiments. A dsRNA unwinding activity capable of preventing microinjected antisense RNA from inhibiting gene expression in *Xenopus* eggs has recently been described [44, 45]. It cannot be excluded that an analogous activity is present at unusually high levels in the tsCOS cells used here and the CV1 cells from which they were derived. For the moment, however, the reason for the apparent failure of dsRNA to form or accumulate remains unclear. Equally, it remains possible that with alternative systems interferon treatment of cells could be useful in potentiating a specific antisense effect. This might be particularly true for antisense constructs corresponding to the 3′ ends of mRNAs, which are generally less effective in inhibiting gene expression but could form partially dsRNA capable of activating the interferon-mediated dsRNA-dependent pathways.

In conclusion, in these model systems, antisense RNA synthesised by recombinant SV40 did not inhibit CAT expression from either a virally-introduced or a stably-transfected, interferon-inducible CAT construct. Accordingly, although a large excess of an appropriate antisense RNA over sense RNA may be desirable, it is not necessarily sufficient to cause inhibition of gene expression.

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