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RESEARCH ARTICLE

Constitutive and Conditional Suppression of Exogenous and Endogenous Genes by Anti-Sense RNA

Jonathan G. Izant and Harold Weintraub

In order to study the biological functions associated with cloned gene sequences, we previously designed a strategy for specifically inhibiting their expression in vivo (*1*). We used, as a test system, the herpes simplex virus (HSV) thymidine kinase (TK) gene in plasmid DNA constructions designed to transcribe the anti-sense (noncoding) DNA strand. The anti-sense transcript has a sequence complementary to the target messenger RNA (mRNA) and can presumably anneal with the mRNA and disrupt normal processing or translation. The anti-sense TK plasmid is constructed in vitro by inverting the TK protein-coding sequence with respect to its promoter. Such a plasmid will specifically inhibit expression of the cognate sense TK plasmid after both plasmids are microinjected into LTK^- cells (*1*). The promising results with the HSV-TK model system suggest that anti-sense RNA can provide an additional methodology for genetic analysis in eukaryotic systems that are not readily amenable to standard mutational analysis. Inhibition of function by anti-sense RNA is a regulatory strategy in prokaryotes where it has been found to control translation (*2*) as well as the activity of RNA primers for initiating episome DNA replication (*3*). Similar mechanisms have not yet been described in eukaryotes.

We have now extended the use of anti-sense inhibition to both transient and stable DNA-mediated transformation systems. We show that a fragment as short as 52 bases of 5′ untranslated anti-sense TK RNA inhibits TK activity. The inhibition is sequence specific. Anti-sense herpes TK inhibits sense herpes TK, but not expression from the non–cross-hybridizing chicken TK gene, while anti-sense chicken TK inhibits expression from a sense chicken TK plasmid, but not from a sense herpes TK plasmid. Conditional, dexamethasone-inducible, anti-sense inhibition is demonstrated by the use of the long terminal repeat (LTR) of the murine mammary tumor virus (MMTV) to direct the synthesis of anti-sense TK RNA. We show that a stably introduced TK gene is also inhibited by anti-sense TK, and finally that expression of the normal endogenous cytoplasmic actin gene can be inhibited by anti-sense actin expression plasmid constructions. The actin inhibition is detected as a diminution of the actin microfilament array and as a decrease in cell viability.

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Table 1. Frequency of TK expression in cells co-injected with sense and anti-sense plasmid DNA. Sense TK plasmid (2 μg/ml) plus a 200-fold excess of anti-sense or control plasmid DNA's were microinjected into TK⁻ L-cell nuclei. After a 20-hour incubation period in [^{3}H]thymidine, TK activity was assessed by determining the number of cells incorporating labeled thymidine by autoradiography as a proportion of the cells that survived microinjection.

Plasmids injected	TK cells (%)	Cells injected (No.)
HSV TK plus:		
Anti-sense HSV TK	7.5*	223
Anti-sense 5′ TK	8.3*	216
Anti-sense chicken TK	34.0	190
Control plasmid	41.0	96
Chicken TK fusion gene (Fig. 1b) plus:		
Anti-sense HSV TK	38.0	170
Anti-sense 5′ TK	0.5*	188
Anti-sense chicken TK	17.0*	230
Control plasmid	35.0	203

*All cells with silver grains (detectable enzyme activity) were scored as TK$^+$. As in Fig. 3, however, analysis of autoradiographic grain density reveals a four- to fivefold suppression of TK activity by anti-sense plasmid DNA so that the actual inhibition is greater than that suggested by merely assaying the proportion of TK$^+$ cells (*1*).

Inhibition of TK expression. A DNA plasmid designed to transcribe a 1364-base anti-sense TK RNA complementary in sequence to the normal sense TK mRNA from base +51 to +1415 (relative to the transcription initiation site) specifically inhibits expression of a sense TK plasmid in mouse L cells (*1*). To determine the amount of sequence required for anti-sense inhibition of herpes TK, we flipped a TK gene fragment spanning the region between −80 (an Eco RI site) and +343 (an Acc I site) and inserted this sequence between the MSV LTR promoter and the SV40 polyadenylation site in inverse orientation (Fig. 1c). Co-injection of this DNA (termed the 5′ TK anti-sense plasmid) with the normal sense herpes TK plasmid (Fig. 1a) at a ratio of 200:1 (anti-sense to sense) gave marked inhibition of TK activity as determined by incorporation of ^{3}H-labeled thymidine (Table 1).

To test whether this 5′ anti-sense plasmid could inhibit a gene with even less complementarity, we used an HSV-TK fragment containing the promoter sequences and 52 bp of 5′ untranslated RNA ligated to the chicken TK structural gene. This fusion gene construction (Fig. 1b) gives normal levels of TK activity after microinjection into the nucleus. Since the herpes and chicken TK genes do not cross-hybridize, RNA transcripts from the 5′ TK anti-sense plasmid are complementary to only the first 52 bases of 5′ untranslated HSV-TK sequence present in the chimeric mRNA (black boxes adjacent to Fig. 1, b and c). The 5′ TK anti-sense plasmid transcript does not overlap the initiator AUG (A, adenine; U, uracil; G, guanine) codon of the

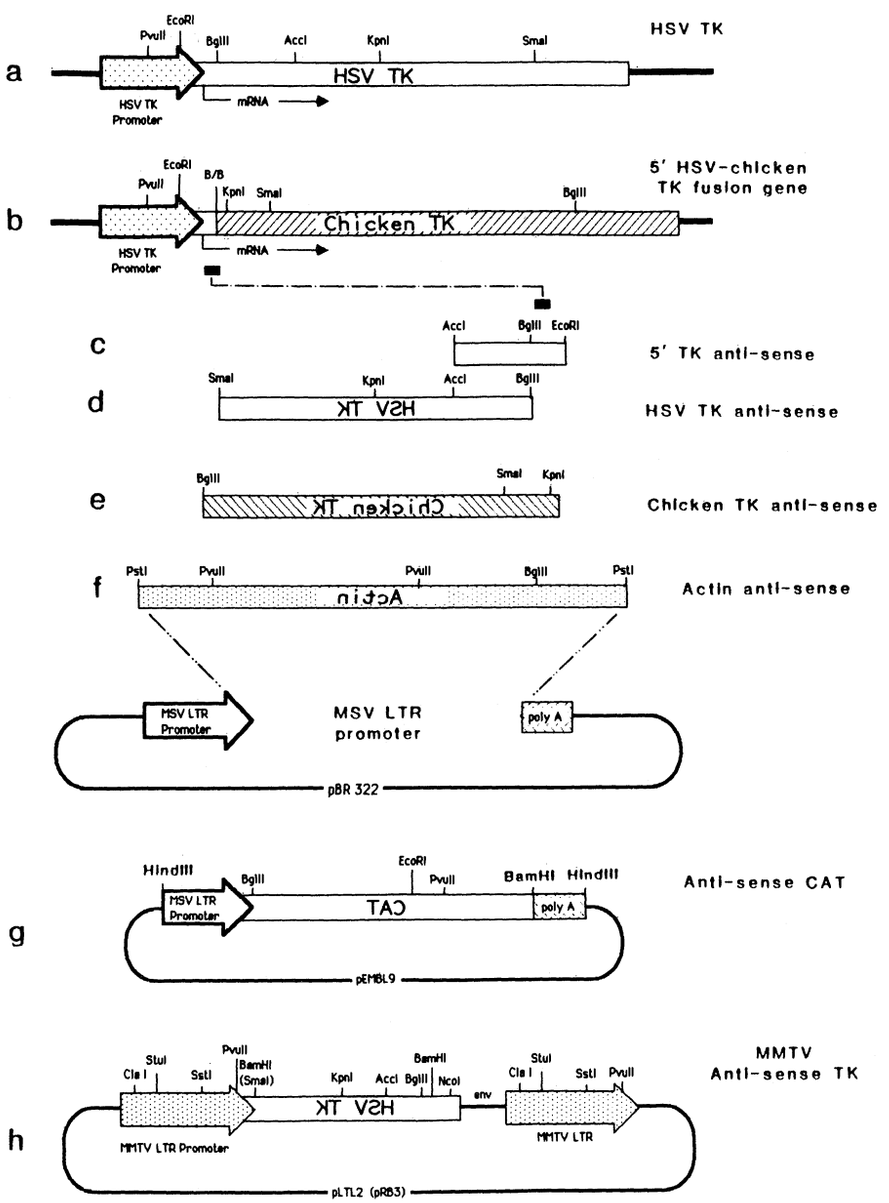

Fig. 1. Plasmid DNA's used in this study (*16*). (a) The wild-type HSV-TK plasmid, which uses its own promoter. (b) The chicken TK structural gene was fused in the sense orientation to the HSV-TK promoter sequence corresponding to 52 bp of 5′ untranslated DNA. (c to f) Various gene restriction fragments were cloned in flipped orientation into a pBR322 derived vector containing the MSV LTR promoter and an SV40 early transcript polyadenylation signal. The black box represents the 52 bases in common between the 5′ TK anti-sense (c) and the 5′ region of HSV-TK chicken TK fusion gene (b). B/B is a Bam HI, Bgl II ligation site. (g) Anti-sense CAT gene plasmid. This plasmid contains the CAT gene coding sequence inserted between an MSV-LTR promoter and an SV40 poly A site which has been cloned in the EMBL9 vector (*14*). (h) Murine mammary tumor virus LTR promoter. This hormone inducible anti-sense TK plasmid is derived from pLTL-2 (*6*) and contains an anti-sense HSV-TK coding sequence inverted next to the left-hand MMTV LTR promoter. Plasmid DNA's were constructed, produced, and purified as described (*1*).

fusion gene transcript and is not complementary to the protein coding domain. When the two plasmids were co-injected into LTK$^-$ cells at a ratio of 200:1 (anti-sense to sense), complete suppression of TK activity was observed (Table 1 and Fig. 2). Inhibition was significantly greater than that observed in the initial anti-sense experiments (*1*) which used an anti-sense vector covering the protein-coding domain of HSV-TK downstream of the Bgl II site. Hence anti-sense RNA complementary to the region upstream of the Bgl II site as well as regions downstream of Bgl II will inhibit TK activity.

The 5′ anti-sense herpes TK plasmid failed to inhibit expression from the wild-type chicken TK gene as would be expected. However, an anti-sense chicken TK construction (Fig. 1e) does give a four- to fivefold reduction of TK activity as assayed by autoradiographic grain density after injection of the sense chicken TK plasmid (Fig. 3). Thus, chicken TK is inhibited by anti-sense chicken TK, but not anti-sense herpes TK while herpes TK is inhibited in a reciprocal fashion by anti-sense herpes TK, but not anti-sense chicken TK plasmids. This provides additional evidence for the sequence specificity of anti-sense plasmid inhibition of gene activity.

Inhibition of TK colony formation by anti-sense DNA transfection. In the aforementioned studies as well as our previous report (*1*) anti-sense DNA was microinjected directly with glass micropipettes into cell nuclei. For many purposes, less technically demanding methods, such as DNA mediated anti-sense transformation, would be useful. To test whether anti-sense DNA vectors would work in transfection systems, 100-mm plates of LTK$^-$ cells were transfected with 50 ng of herpes TK plasmid, 20 μg of carrier calf thymus DNA, and from 0 to 5 μg of anti-sense or control DNA. The number of TK-positive colonies was then assayed (Table 2). Control cultures yielded from 250 to 500 colonies per dish, while cultures that received 5 μg of anti-sense TK DNA (a 100-fold excess of anti-sense to sense DNA) had 10 to 20 times fewer colonies (Fig. 4). In some experiments, as little as a tenfold excess of anti-sense TK gave similar degrees of inhibition. Reproducible inhibition was not apparent at a 1:1 ratio. We suspect that the residual TK-positive colonies that emerge at high levels of anti-sense TK represent the small proportion of transfected cells that fail to take up or express the co-transforming anti-sense DNA (*4*). When pBR322 (5 μg per dish) was used as a control, there was a no-

Abstract. *Plasmid DNA directing transcription of the noncoding (anti-sense) DNA strand can specifically inhibit the expression of several test genes as well as normal, endogenous genes. The anti-sense plasmid constructions can be introduced into eukaryotic cells by transfection or microinjection and function in both transient and stable transformation assays. Anti-sense transcripts complementary to as little as 52 bases of 5′ untranslated target gene mRNA specifically suppress gene activity as well as, or more efficiently than, anti-sense transcripts directed against the protein coding domain alone. Conditional anti-sense inhibition is accomplished with the use of hormone-inducible promoter sequences. Suppression of endogenous actin gene activity by anti-sense RNA is detected as a decrease in growth rate and as a reduction in the number of actin microfilament cables. These observations suggest that anti-sense RNA may be generally useful for suppressing the expression of specific genes in vivo and may be a potential molecular alternative to classical genetic analysis.*

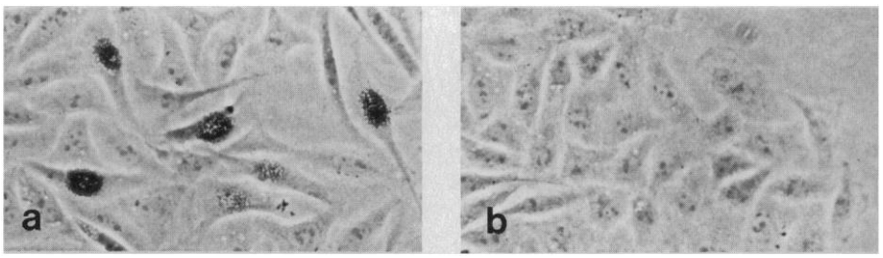

Fig. 2. Autoradiography of cells co-injected with TK genes and 5′ TK anti-sense DNA. Cells were injected with the chimeric HSV promoter chicken TK fusion gene (Fig. 1b) and (a) a control anti-sense β-galactoside plasmid or (b) a 200-fold excess of 5′ TK anti-sense plasmid (Fig. 1c). Thymidine labeling and autoradiography were performed as described (*1*). No significant signal is detectable in the cells co-injected with the anti-sense 5′ TK (×500).

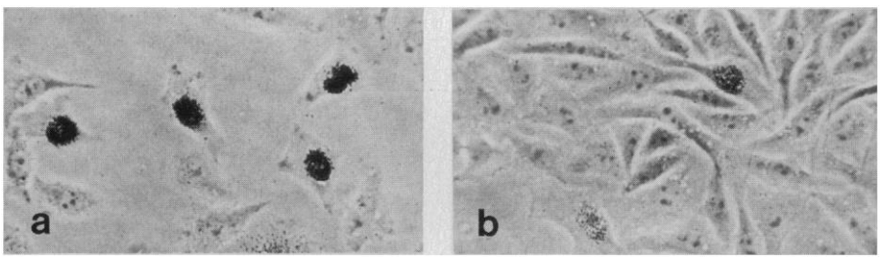

Fig. 3. Anti-sense chicken TK inhibits chicken TK. Representative micrographs of cells co-injected with a chicken TK plasmid and a 200-fold excess of a control herpes TK anti-sense plasmid (a) or a similar excess of anti-sense chicken TK plasmid DNA (b) (×500).

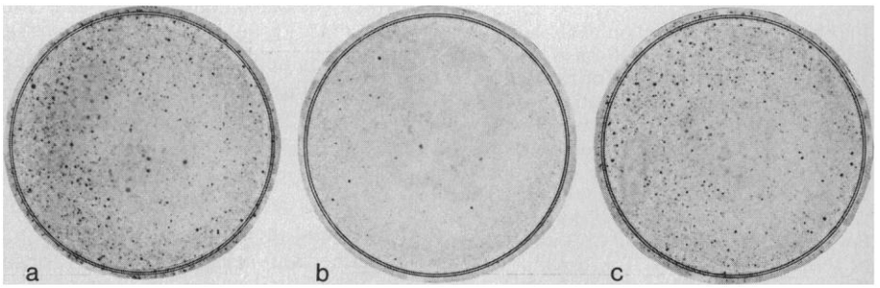

Fig. 4. Inhibition of herpes TK colony formation. LTK$^-$ cells were co-transformed with herpes TK (50 ng) and either 5 μg of anti-sense chicken TK (a) or 5 μg of anti-sense herpes TK (b) (*4*). Colonies were stained after 2 weeks in HAT medium. The inhibition by anti-herpes TK could be overcome by including a sense chicken TK plasmid (50 ng) in the transformation mix (c). All transformations contained 20 μg of carrier calf-thymus DNA.

ticeable decrease in transformation frequency, but this was marginal compared to the reproducible and more dramatic decreases observed with the anti-sense TK DNA. Controls with 5 μg of anti-sense chicken TK, anti-sense β-galactosidase DNA, and anti-sense chloramphenicol acetyltransferase (CAT) DNA (Fig. 1g) all gave marginal and variable levels of either stimulation or inhibition.

As with the microinjection studies described above, the 5′ untranslated anti-sense herpes TK DNA results in a greater degree of inhibition (Table 2) of TK transformation with the herpes-chicken fusion TK gene. At a 20-fold excess of anti-sense 5′ TK to sense TK fusion gene, the inhibition was characteristically two to three times greater than that seen with herpes TK and the standard herpes anti-sense TK vector. In further parallel experiments with chicken TK and the anti-sense chicken TK expression vector, we observed a similar 5- to 10-fold decrease in chicken TK colony formation compared to controls (Table 2).

We considered the possibility that the observed inhibition may have been the result of a nonspecific effect of the anti-sense expression plasmids on cell viability, rather than a direct inhibition of TK expression by anti-sense TK RNA. Cell cultures were transfected with sense and anti-sense herpes TK DNA under conditions where transformation would have been inhibited tenfold and each culture received in addition a "complementing" sense chicken TK plasmid. The cultures that received the additional chicken TK plasmid displayed normal transformation frequencies in HAT (hypoxanthine, aminopterin, thymidine) medium, suggesting that cells containing sense and anti-sense HSV-TK RNA are still viable (Fig. 4). As a control for nonspecific effects resulting from formation of double-stranded RNA and possible interferon production, Pestka (*5*) has directly assayed interferon production in our cultures that were transfected with a variety of sense and anti-sense combinations either by calcium phosphate or by DEAE-dextran methods. The very sensitive assay detected no interferon activity in these cultures.

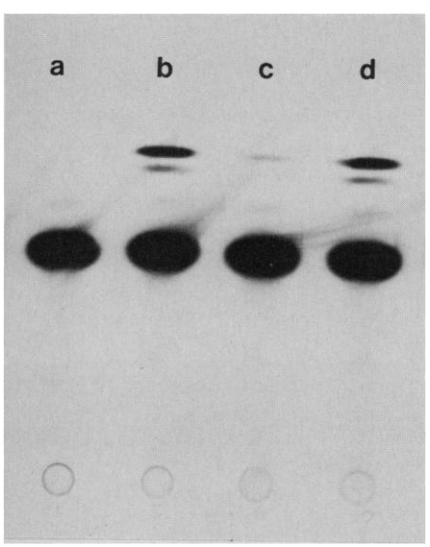

Fig. 5. Anti-sense inhibition of CAT activity. L cells were stably transformed with anti-sense CAT DNA by means of calcium phosphate–mediated cotransformation with TK. These cells each contained about 20 copies of anti-sense CAT gene and produced several hundred copies of anti-sense CAT mRNA. Then, 1 μg of sense CAT was transfected into either the anti-sense CAT polyclone line (a) or the parental LTK$^-$ cells (b). CAT assays were performed after 2 days. The parental LTK$^-$ cells were also transfected with 1 μg of sense CAT plus 5 μg of anti-sense CAT (c) by the DEAE-dextran procedure (*17*). Control cultures (d) received 1 μg of sense CAT and 5 μg of anti-sense herpes TK. CAT activity (*17*) was measured after 2 days.

The results with calcium phosphate–mediated TK transformation parallel the data from microinjection experiments. Anti-sense inhibition of herpes TK and chicken TK transformation is sequence specific and occurs with as little as a 10- to 20-fold excess of anti-sense DNA. Inhibition is dramatic, ranging up to 20-fold at high levels of anti-sense DNA (10- to 100-fold excess) and can be observed with as little as 52 bp of complementary 5′ untranslated RNA that does not cover the initiator AUG codon. At least two different fragments of complementary sequence (one from the protein coding region; the other from the 5′ noncoding region) can inhibit TK expression.

Transient anti-sense inhibition of the CAT gene. For transient expression assays, we utilized sense and anti-sense CAT (chloramphenicol acetyltransferase) expression plasmids (Fig. 1g). LTK$^-$ cells were transfected (with the use of DEAE dextran) with a 5:1 ratio of anti-sense to sense CAT DNA; after 2 days, CAT enzyme assays were performed on cell homogenates. The anti-sense CAT plasmid caused a 5- to 20-fold decrease in activity as compared to control experiments with anti-sense herpes or chicken TK plasmids (Fig. 5). LTK$^-$ cells were also stably cotransformed by calcium phosphate–mediated gene transfer with herpes TK (50 ng) as a selectable marker and including sense CAT (2 μg) and either anti-sense CAT (10 μg) or, as a control, anti-sense chicken TK (10 μg). TK-positive colonies (about 200 for each protocol) were collected after 2 weeks, and CAT activity was measured. Under these conditions, inhibition of CAT activity by anti-sense CAT DNA was sequence specific and ranged from 4- to 9-fold.

In a related type of analysis, an anti-sense CAT-producing cell line was constructed by cotransformation with herpes TK. The line contained about 20 copies of anti-sense CAT gene per cell and produced about 500 copies of anti-sense CAT RNA. Constitutive expression of such an anti-sense plasmid would be predicted to provide a measure of "molecular immunity" to the subsequent expression of that gene from a sense CAT plasmid. The sense CAT plasmid DNA was introduced into the anti-sense CAT cell line and into parental LTK$^-$ cells by transfection; CAT activity was assayed after transient expression. Expression of CAT activity was reduced in the anti-sense CAT cells (Fig. 5). This is not a nonspecific manifestation of inefficient DNA uptake or expression because stable transformation frequency to an APRT-positive phenotype (APRT, adenine phosphoribosyl transferase) was normal in the anti-sense CAT cell line.

These experiments extend the list of

Table 2. Inhibition of TK colony formation by cotransformation with anti-sense DNA plasmids. The transforming plasmids were used at 50 ng per plate.

*Cotransformed plasmid	Cotransformed DNA per plate (μg)	TK$^+$ colonies (% control)
HSV TK		
Alone	0	100
Anti-sense ChTK†	5	123
Anti-sense β-galactosidase	5	108
pBR322	5	91
Anti-sense CAT	5	143
Anti-sense TK	1	96
	2.5	63
	5	12
HSV-chicken TK fusion		
Alone	0	100
Anti-sense 5′ TK	1	62
Anti-sense 5′ TK	2.5	17
Anti-sense 5′ TK	5	8
PBR	5	86
Chicken TK		
Alone	0	100
Anti-sense chicken TK†	5	18
pBR322	5	89
Anti-sense CAT	5	130
Anti-sense TK	5	109
Anti-sense 5′ TK	5	122

*In each experimental series there were two to ten independent transformations. Results from a typical series are shown. †Anti-sense chicken TK has chicken TK activity, probably as a result of transcription from a cryptic pBR322 promoter. This value, which is about 10 percent of the sense chicken TK activity, has been subtracted from the tabulated frequency.

genes inhibited by anti-sense expression DNA vectors to the bacterial CAT gene, suggesting that the inhibition is not limited to eukaryotic genes. In addition, the inhibition occurs during transient expression assays, and an anti-sense CAT-producing cell line is resistant or "immune" to expression from subsequently introduced CAT genes. This observation reduces the probability that the inhibition is the result of targeted recombination or conversion events that might destroy the activity of the sense CAT DNA since it is highly unlikely that homologous recombination or gene conversion could occur between the bulk of the incoming sense CAT DNA and the integrated anti-sense CAT DNA in the majority of cells in the population (*1*).

Inhibition of an endogenous herpes TK gene. Using cells previously transformed to a herpes TK-positive phenotype as a convenient model system, we have investigated whether anti-sense RNA will inhibit an endogenous gene. A collection of about 1000 TK-positive transformants was removed from HAT selection and transfected with the APRT gene and cotransforming anti-sense herpes TK DNA or as a control with APRT and cotransforming anti-sense chicken TK. APRT-positive transformants were collected (the frequency of such clones was the same for both transfections), and TK enzyme activity was measured for each culture (Fig. 6). The TK enzyme activity in cells transformed with anti-sense herpes TK was six times lower than control cultures transformed with anti-sense chicken TK. As with the CAT results described above, these data were obtained in the absence of selection for the TK gene being assayed.

To see whether the anti-sense TK transformants display any growth abnormalities in culture, the cells were grown in conventional HAT medium. Under these conditions, both anti-sense herpes TK and anti-sense chicken TK lines proliferated at comparable rates. Initially this was surprising given the fact that the TK enzyme level was significantly lower in the anti-sense TK cells. The residual level of TK activity was not zero, however, and we suspected that this reduced activity might still be sufficient for growth. If this were true, cells with lower enzyme activity might have more difficulty growing in HAT medium containing a reduced thymidine concentration. Indeed, decreasing thymidine in the medium tenfold results in growth inhibition for the anti-sense herpes TK cell line, whereas there is no detectable effect on the anti-sense chicken TK control cell line growth even at 1/100 the normal thymidine concentration (Fig. 7).

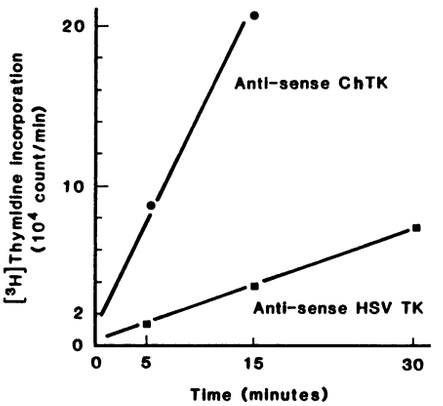

Fig. 6. Anti-sense inhibition of endogenous herpes TK activity. LTK$^-$ cells were transformed to TK$^+$ with HSV-TK. Cultures were then cotransformed with 5 μg (per dish) of anti-sense herpes TK or anti-sense chicken TK (ChTK) and an APRT plasmid. APRT-positive cells were selected, and TK enzyme activity was determined (*18*).

Conditional expression of anti-sense TK RNA. As with any mutational analysis, it will be difficult to discern the function of a crucial gene by anti-sense inhibition when the suppression of that gene has lethal consequences. One way to deal with this problem is to make the expression of the anti-sense RNA conditional, for example, by using an inducible promoter. In order to test whether conditional anti-sense inhibition is possible, we used the dexamethasone-inducible MMTV LTR to drive the synthesis of anti-sense herpes TK (Fig. 1h). Collections of about 500 herpes TK-positive colonies were first selected in HAT medium after calcium phosphate–mediated DNA transformation of TK$^-$, APRT$^-$ L cells with wild-type HSV-TK gene. Cultures were then divided in two parts, and one was cotransformed with APRT plus a control anti-sense chicken TK plasmid and the second was cotransformed with APRT and the MMTV anti-sense HSV-TK plasmid. The frequency of APRT-positive colonies was similar for the two cultures, and polyclones (about 50) from each culture were grown. Since the basal and induced levels of activity of the MMTV promoter vary from clone to clone (*6*), the polyclones were taken through two subsequent selections in HAT medium to ensure that the resident sense TK gene was still functioning efficiently. Cells with a high basal level of inhibitory anti-sense TK would also be selected against because they would presumably have lower TK activity. Then the cells were again passaged in APRT selection medium to ensure that they had retained the APRT gene and the cotransfected anti-sense TK gene. Finally, the cells were grown in varying concentrations of dexamethasone for 4 to 8 days (to allow for enzyme turnover), and then TK enzyme levels were determined (Fig. 8). Control cultures containing the anti-sense chicken TK gene had TK enzyme levels that were usually unaffected by dexamethasone. In contrast, after treatment with 10^{-6} or $10^{-7}M$ dexamethasone, a dose-dependent decrease (five-

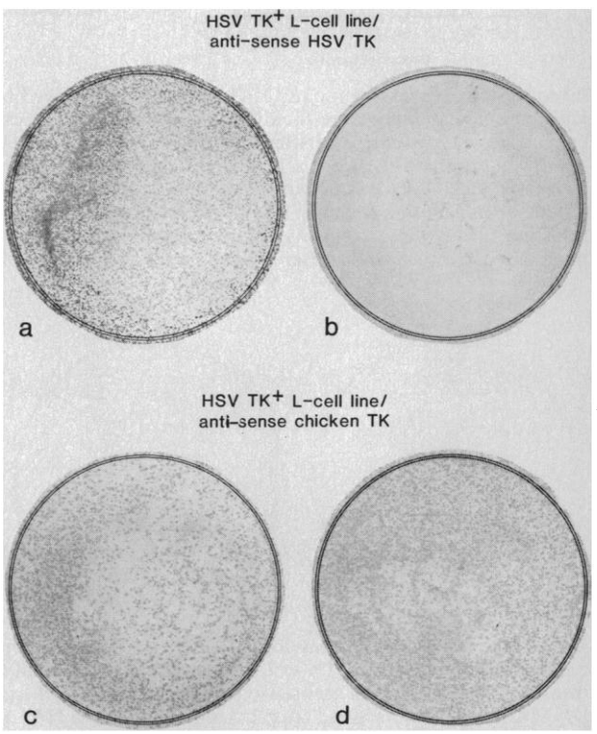

Fig. 7. Anti-sense inhibition of endogenous herpes TK phenotype. The anti-sense HSV-TK (a and b) or the anti-sense chicken TK (c and d) lines described in Fig. 6 were grown in normal HAT medium ($1.5 \times 10^{-5}M$ thymidine) or in HAT medium with one-tenth ($10^{-6}M$) or one hundredth ($10^{-7}M$) the usual amount of thymidine and stained after 1 week in culture. Both cell lines proliferated in normal HAT medium (a and c). The anti-sense HSV-TK line was unable to grow in HAT medium with one-tenth the normal concentrations of thymidine (b), whereas anti-sense chicken TK line grew well at one-hundredth the normal level of thymidine (d).

to tenfold) in TK activity was consistently found. While normal TK^+ cells grew almost as well in the presence of or absence of these concentrations of dexamethasone, cells that also contained the inducible anti-sense TK gene grew more slowly in the presence of $10^{-7}M$ dexamethasone (Fig. 8, inset) when put under HAT selection. When put under APRT selection, however, these cells grew normally in the presence of these concentrations of dexamethasone. This model system suggests that dexamethasone can be used to provide anti-sense transcription that can be regulated and, as a consequence, conditional inhibition of gene activity. Recent work suggests that a heatshock promoter can also be used to produce a conditional anti-sense phenotype (*7*).

Inhibition of endogenous actin gene expression. To test whether a normal cellular gene could be inhibited by anti-sense RNA, we constructed an anti-sense DNA expression vector that would produce anti-sense cytoplasmic β-actin (Fig. 1f). We anticipated that cotransformation of TK and anti-sense actin DNA would result in cell death for those cells that have taken up both genes because the phenotype of actin mutations in yeast is cell lethality (*8*). This can be assayed by monitoring the frequency of TK-positive transformed colonies. The results show that an anti-sense actin DNA plasmid dramatically decreases the frequency of normal TK-positive colonies as compared to parallel control cotransformations with insertless plasmids and anti-sense chicken TK plasmids (Fig. 9). Interestingly, the frequency of small colonies (four to ten cells) during the first several days after transfection was similar in control and anti-sense actin-transformed cultures. Subsequently, cell proliferation diminished in the cultures transformed with anti-sense actin. This probably indicates that the initial transformation of these cells is normal, but that the anti-sense actin DNA causes a sufficient decrease in actin production so that the cells can sustain normal growth for only two to three divisions.

To test whether cells containing anti-sense actin RNA show a structural phenotype, BSC-1 cells were injected with the anti-sense actin DNA, and 4 days later the cells were stained with *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phallacidin (*9*) to visualize actin cables. The cells were counterstained with propidium iodide, to stain the nuclear DNA and the perinuclear RNA. While BSC-1 cells display typically dramatic actin microfilament bundles after injection with anti-sense TK DNA, only about half of the cells show normal actin cables after injection of anti-sense actin DNA. This corresponds to the fraction of cells expected to express anti-sense actin RNA efficiently after microinjection. As was observed in the transfection experiments, cells with anti-sense actin plasmid show no obvious change in cell morphology in the first several days after microinjection, and the distribution of cytoplasmic RNA as assayed by propidium staining is unaltered. In contrast, the quantity and length of actin microfilament bundles in the affected cells was markedly reduced although the actin staining along the cell periphery, especially in the areas of active ruffling, appeared to be undiminished (overexposed in Fig. 10 to emphasize the diminution of the stress fiber array). This suggests that when actin levels are decreased the peripheral actin filaments are preferentially retained.

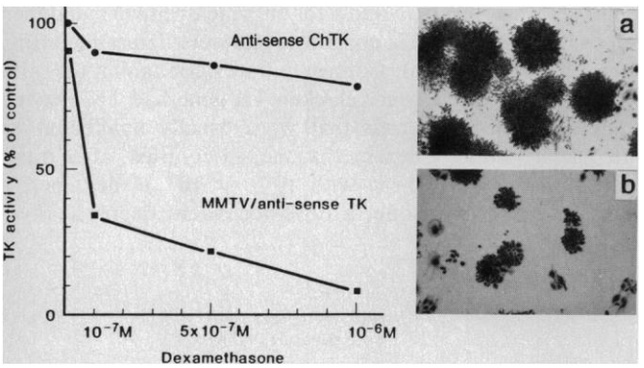

Fig. 8. Dexamethasone induction of MMTV anti-sense TK. Herpes TK-positive L cells were cotransformed with the inducible MMTV anti-sense TK plasmid (Fig. 1h) under APRT selection; control cultures were cotransformed with anti-sense chicken TK plasmid DNA. Transformed colonies were grown up and taken through successive rounds of HAT selection followed by APRT selection. Control and anti-sense TK cultures were then induced with increasing levels of dexamethasone, and TK enzyme activity was assayed after 5 days. The insets show representative colonies (a) from a control culture and (b) from a dexamethasone-treated culture, illustrating the difference in growth induced by dexamethasone in the MMTV anti-sense TK-containing cells. These differences are most dramatic at $10^{-7}M$ dexamethasone and do not occur in the absence of HAT selection. However, at slightly higher concentrations of dexamethasone ($5 \times 10^{-7}M$) an inhibition of cell growth is observed that is independent of HAT selection. [×13]

The decrease in actin cables is also observed 24 to 36 hours after capped anti-sense actin RNA is microinjected directly into BSC-1 cells (Fig. 10, b and c). Microinjection of anti-sense TK RNA has no obvious effect on the actin microfilament distribution. This RNA was made in vitro by means of an SP6 promoter ligated to anti-sense actin DNA encoding a 500-bp fragment of 5′ actin cDNA. Introduction of anti-sense actin RNA results in the disruption of actin cables, presumably because of a lower actin monomer concentration in injected cells.

Implications of anti-sense gene inhibition. The cytoplasmic manifestations of anti-sense actin inhibition and the subsequent inhibition of cell proliferation are striking. This analysis has yielded the interesting and unanticipated observation that actin microfilament cables are more labile to changes in actin monomer dynamics than the actin filaments present in the cell periphery. While we cannot readily assess the extent of actin synthesis inhibition in microinjected cells, the observed phenotypic changes suggest that any feedback mechanisms that may serve to regulate actin pool size are unable to compensate for the anti-sense actin perturbation.

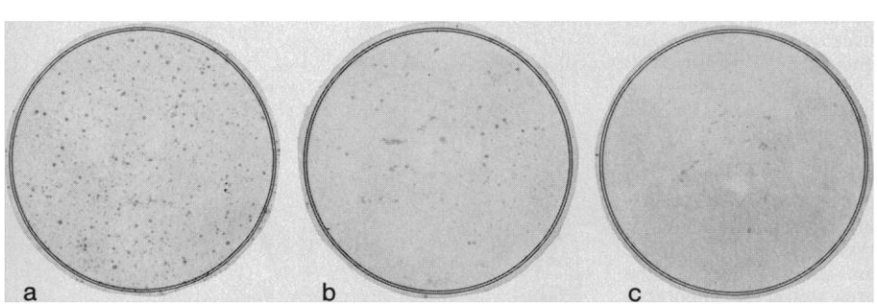

Fig. 9. Inhibition of TK colony formation by anti-sense actin plasmid DNA. LTK^- cells were cotransformed with herpes TK DNA (50 ng per dish), with calf thymus (carrier) DNA (10 μg per dish) and with (a) control anti-sense chicken DNA or (b) 1 μg or (c) 10 μg of anti-sense actin DNA. Colonies were stained after 2 weeks selection in HAT medium.

This study extends the number of genes whose expression has successfully been suppressed by anti-sense RNA and suggests that the technique may provide a general methodology for genetic analysis. In addition to the original work with herpes TK, we have shown that chicken TK, CAT, and cytoplasmic actin can be suppressed and others have shown that β-globin (*10*), β-galactosidase (*11*), the *Drosophila* krüppel gene (*12*), and a *Xenopus* gap junction gene (*13*) can be inhibited by anti-sense RNA. The cell types that have been used in these experiments include L cells, 3T3 cells, *Xenopus* oocytes, *Drosophila* embryos, and frog embryos. The method of delivery ranges from microinjection to calcium phosphate–mediated stable transformation, to DEAE–dextran mediated transient expression; the reagents include "flipped" plasmid DNA vectors designed to produce anti-sense RNA, synthetic anti-sense RNA, and synthetic oligonucleotides. While most work has been done inhibiting experimentally introduced genes, several endogenous genes (integrated herpes TK, actin, krüppel, and gap junction) have also been inhibited. The inhibition can be accomplished efficiently with as little as 52 bp of homology to the 5′ untranslated region of the sense mRNA and does not have to include the initiator AUG. When the anti-sense vector is linked to the dexamethasone-inducible MMTV promoter, dexamethasone-inducible inhibition of TK activity is observed. Conditional anti-sense inhibition has also been achieved with heatshock gene promoter sequences (*7*).

Many questions remain with regard to the mechanism of inhibition, and some of the available data is fragmentary. In addition to the anti-sense actin experiment (Fig. 10), anti-sense inhibition by anti-sense RNA synthesized in vitro has been demonstrated in frog oocytes by separately microinjecting sense and anti-sense RNA's for *Xenopus* β-globin (*10*),

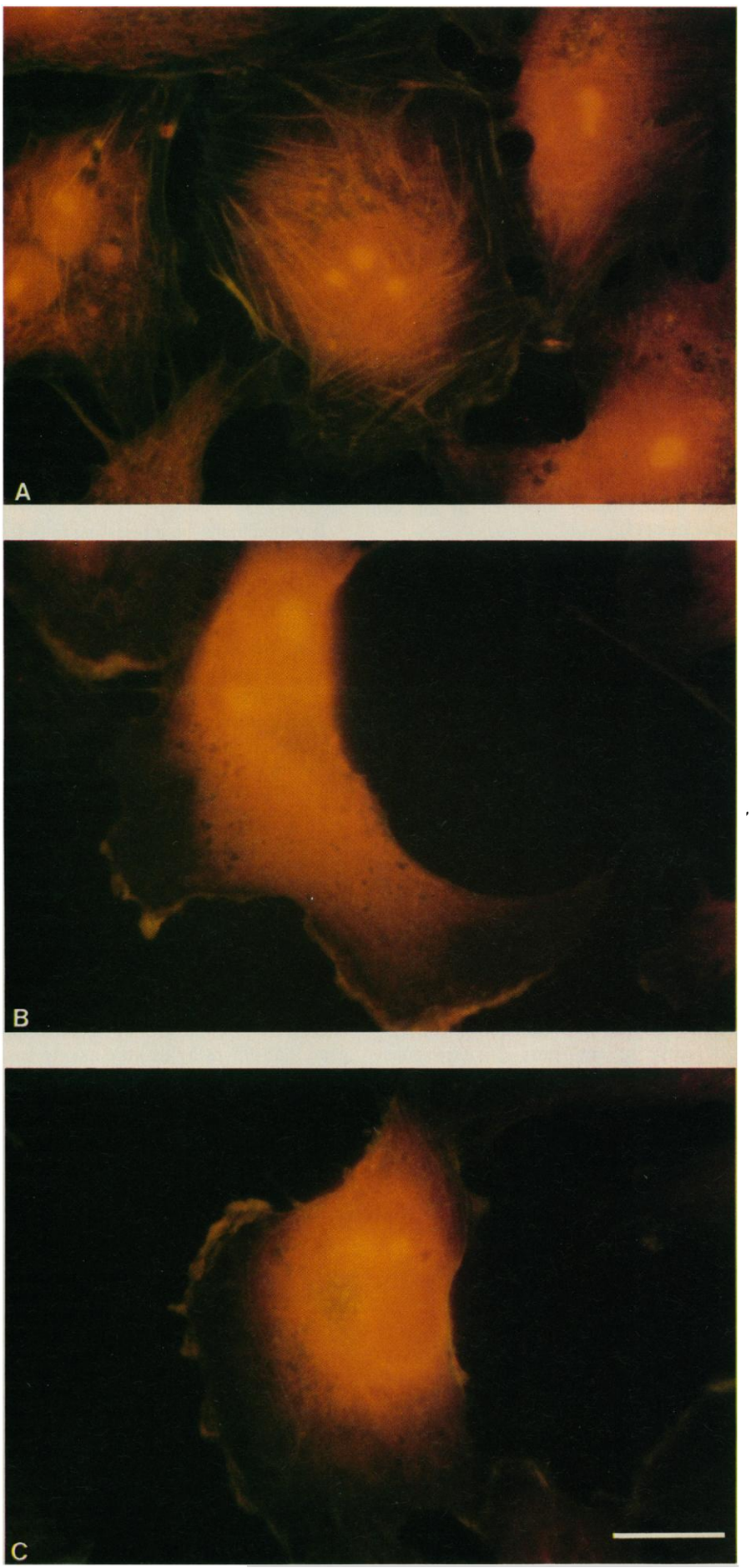

Fig. 10. Inhibition of actin cable formation by anti-sense actin RNA. Capped RNA was synthesized in vitro with SP6 RNA polymerase and plasmids that direct the synthesis of either (a) sense or (b and c) anti-sense actin RNA. In vitro capping was achieved by including 5 m*M* diguanosine triphosphate $G(5')p_3(5')G$ in the reaction (*14*). 0.1 pl of RNA at 0.5 μg/μl was injected into BSC-1 cells and after 30 hours the cells were fixed and stained with fluorescent phalloidin (green) (*9*) to stain filamentous actin and propidium iodide (red-orange) to stain DNA and RNA. Similar results are also obtained with the anti-sense actin plasmid DNA shown in Fig. 1f.

for herpes TK (*14*), and for CAT (*14*). This is an attractive system for studying the mechanics of anti-sense inhibition since it is possible to monitor the levels and integrity of sense and anti-sense transcripts during the course of an experiment. By means of this approach, double-stranded RNA hybrid molecules have been detected in oocytes as the putative intermediate in the inhibition process (*10*). Moreover, *Xenopus* β-globin mRNA's already loaded onto oocyte polysomes are susceptible to anti-sense inhibition, indicating that inhibition can be cytoplasmic and thus probably translational. These results, however, do not exclude the possibility of a nuclear anti-sense inhibition process as well. Indeed, Kim and Wold have shown that anti-sense TK RNA forms duplexes with sense TK RNA, and that the duplexes are preferentially localized in the cell nucleus (*15*).

How stable are anti-sense RNA's? In L cells anti-sense TK RNA may be less stable than sense RNA (*1*), whereas in frog oocytes, both are extremely stable (*14*). Sense and anti-sense CAT gene transcripts are stable in cultured cells as well as in oocytes (*14*). Can the inhibition be described by simple solution-type hybridization kinetics and how much of an excess of anti-sense RNA is usually necessary? In frog oocytes the kinetics of inhibition are generally consistent with hybridization theory predictions (*14*); however, the various amounts of anti-sense RNA needed to achieve significant inhibition in different systems is puzzling. For example, in *Xenopus* oocytes, a 5- to 50-fold excess of anti-sense over sense RNA results in a 10- to 20-fold inhibition of gene activity. When cultured somatic cells are used, a 1:1 ratio of sense to anti-sense β-galactosidase DNA yielded a 95 percent inhibition of activity (*11*); we found that a minimum ratio of 5:1 anti-sense to sense DNA is required for herpes TK inhibition in transfection studies and a ratio of 50:1 is required in microinjection studies to achieve comparable levels of inhibition.

We do not know how secondary structure and ribonucleoprotein (RNP) packaging of both the sense and anti-sense RNA's affect inhibition nor do we have unambiguous information on which regions of an RNA transcript are most susceptible to anti-sense inhibition. In frog oocytes β-globin is not inhibited by a 3′ anti-sense RNA (*10*), but CAT mRNA is (*14*), although in both cases the 5′ anti-sense probe is more effective. Our results also suggest that 5′ anti-sense may be a more efficient inhibitor in cultured cells than protein coding sequences alone. Further, this may explain why naturally occurring prokaryotic anti-sense paradigms utilize anti-sense transcripts complementary to the 5′ regions of their respective target transcripts (*2*, *3*).

It will actually be interesting to determine if anti-sense transcription functions as a normal cellular control mechanism in higher eukaryotes as it does in prokaryotes.

The observations on anti-sense 5′ TK also present a potential strategy for experimental gene switching manipulations and even gene therapy protocols wherein the gene of interest, either a normal or a deleterious gene, is inhibited by anti-sense RNA complementary to a nontranslated (possibly even an intronic) domain of the RNA transcript. A sense orientation RNA or DNA coding for the desired gene product could be introduced concomitantly; however, this gene would be designed so that it is missing the nontranslated sequences complementary to the anti-sense inhibitor. In this fashion a gene could be effectively "replaced" by a substitute gene that differs by as little as one base pair without having to alter the genomic copy of that gene.

Finally, it is important to know the general applicability of the anti-sense phenomenon and the efficiency of the available mechanical and viral methods for introducing anti-sense DNA and RNA into cells if this procedure is to be used as an alternative to genetic analysis in higher organisms. Our own experience promotes optimism although the number of examples is still limited and the details of the molecular mechanism of anti-sense inhibition are not known. A better understanding of the various facets of anti-sense inhibition will contribute to studies with the use of cell type–specific anti-sense RNA's to identify and characterize regulatory genes that are involved in cellular morphogenesis and embryogenesis.

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